Chapter 11

Complement Activation

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The complement system is the most important biochemical cascade in the blood for the recognition, opsonization, and elimination of foreign materials. To date, the leading causes of death in the United States include cancer, cardiovascular and neurodegenerative diseases, and diabetes. New treatments are urgently needed to treat these devastating diseases and nanotechnology potentially provides new avenues to fight such illnesses. These avenues include the development of novel nanocarriers that deliver drugs in a specific and controlled manner, while minimizing secondary effects. The success of bioengineering effective nanocarriers for drug delivery purposes requires a deep understanding of the interaction between the complement system and the nanocarriers. This review focuses on reporting the current state of complement activation by different nanomaterials. Here, we assess various important parameters that influence the activation of the complement system, which include the physicochemical characteristics of both nanocarriers and complement proteins. We next evaluate the most recent engineering approaches to prevent or reduce complement activation. Finally, we discuss different *in vitro* and *in vivo* procedures to assess complement activation.

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1. The Complement System

The complement system is a group of about 35 soluble and cell surface proteins in blood and other body fluids which interact to recognize, opsonize, and clear or kill invading microorganisms, altered host cells, and other foreign materials, including many synthetic materials.[1] A simplified representation of the complement system is shown in Figure 1. The activation of the complement system can occur by any of three pathways, termed the classical, lectin, and alternative pathways.

1.1. *Pathways of complement activation*

1.1.1. *The classical pathway*

In the classical pathway (Figure 1), the recognition protein C1q binds to targets (activators), and the binding causes two proteases, C1r and C1s, attached to C1q, to become active. When C1s is activated, it cleaves and activates the next two proteins of the system, which are called C4 and C2. Domains of these proteins then form a complex called C4b2a, which is itself a protease that cleaves and activates the most abundant complement protein in blood, C3. C3 is activated to form C3b, which binds back on to the surface

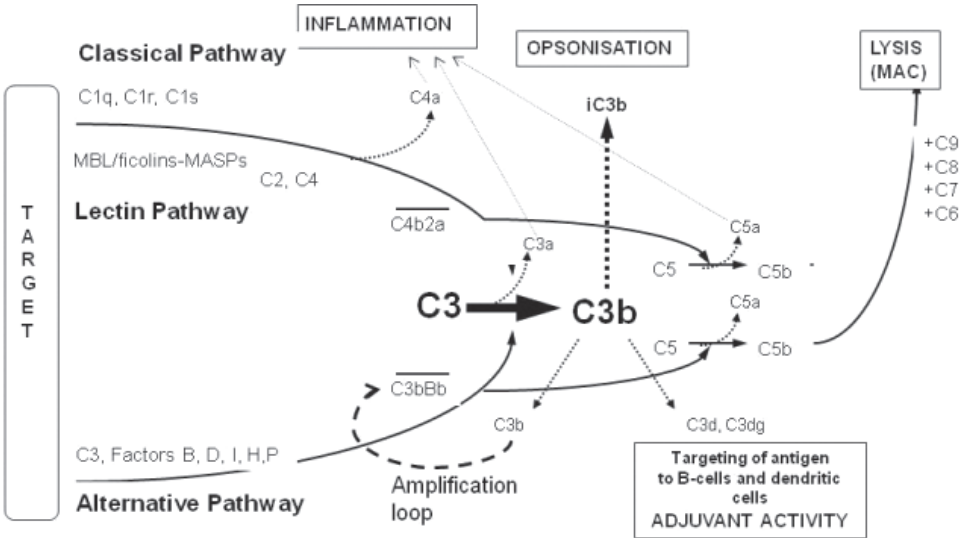

Figure 1. The pathways of the complement system. The complement system can be activated via three pathways, namely the classical, lectin, and alternative pathways.

of the target. The target becomes coated with clusters of hundreds of C3b molecules, which gradually are cleaved by other proteases to forms of C3 called iC3b or C3d/C3dg.

C3b is recognised by CR1 (complement receptor 1) which is on red blood cells and some white blood cells, and this causes the target to bind to the cells. A C3b-coated target, bound to red blood cells, can circulate in the blood, with the C3b gradually being converted to iC3b. As the red blood cell and target pass through the liver or spleen, they come into contact with macrophages, which have on their surface receptors for iC3b (named CR3 and CR4). The target, with its attached iC3b, is stripped off of the red blood cell and transferred to the macrophage, which ingests the target and destroys it intracellularly. This phenomenon, by which targets are coated with C3b or iC3b that promote their uptake and destruction by macrophages, is called opsonization (Figure 1). When iC3b is further broken down to C3d/C3dg, it interacts with another receptor, CR2, which is present on B lymphocytes. This interaction can stimulate the synthesis of antibodies against the target. Dendritic cells, which capture foreign materials and present them to the adaptive immune system as antigens, also have complement receptors, thus coating of the target with C3 fragments also helps to develop the adaptive immune response (antibodies and cytotoxic T cells) against the target. These activities that promote the antibody or T cell response are called the adjuvant activities of complement (Figure 1).

Once C3 has been activated, the protease C4b2a can then activate the next complement protein C5, forming the fragments C5a and C5b. C5b binds to C6, C7, C8, and C9, and this large protein complex called the MAC (membrane attack complex) can insert itself into lipid bilayers (cell membranes). If the target has a cell membrane, the MAC will effectively make holes in the membrane, killing the target ("lysis") (Figure 1).

During the activation of C4, C3, and C5, small peptides C4a, C3a, and C5a, collectively called anaphylotoxins, are released, and these potentially have inflammatory effects. They affect the smooth muscle of blood vessels and cause fluid leakage from the blood into the tissue spaces. They have effects on cytokine and chemokine release, and C5a is also a chemotactic factor, i.e., it attracts cells (i.e., granulocytes). At the site of a wound, these activities would cause the leakage of fluid into the wound, releasing more complement proteins from the blood into the site, to opsonize infectious microorganisms and also cause granulocytes to migrate to the site, where they ingest and kill bacteria.

1.1.2. *The lectin pathway*

The lectin pathway is initiated by the binding of mannan-binding lectin (MBL) or ficolins to carbohydrate structures present on a wide range of microorganisms including bacteria, viruses, fungi, and parasites. MBL has also been reported to bind to IgA and to agalactosyl IgG (IgG-Go). MBL and ficolins circulate in serum complexed with serine protease proenzymes called MBL-associated serine proteases (MASPs) and a small 19-kDa related protein (Map 19). MASPs are structurally similar to C1r and C1s with identical domain composition. The mechanisms for MASP activation have yet to be fully determined but upon MBL of ficolin binding to targets, MASP-2 is auto-activated and cleaves C4 and C2 to form the C3 convertase C4b2a, similar to that in the classical pathway.

1.1.3. *The alternative pathway*

The alternative complement pathway is initiated differently from the classical and lectin pathways. To trigger this pathway, C3b has to be deposited on the surface of a target. C3b may be derived from the activation of the other pathways, arise from a non-enzymic slow turnover of C3, or it may arise because other non-complement proteases can activate C3 to a minor extent. Once one molecule of C3b has bound to the target surface, factor B can bind to it, and is then cleaved by factor D to form C3bBb, which is a protease complex homologous to C4b2a in the other pathways. Once this C3-cleaving enzyme has formed, events occur as per the classical and lectin pathways. Since C3bBb can form more molecules of C3b, the alternative pathway acts as an amplification loop (Figure 1), causing more C3b to be produced and deposited on the target surface.

The different pathways of complement respond to different targets. C1q in the classical pathway binds to a very wide range of targets. These include antibody–antigen complexes formed with IgG or IgM, Gram-negative bacteria, some viruses, polyanionic molecules like DNA/RNA, or anionic phospholipid micelles, altered host proteins such as clots or amyloids, and many synthetic materials such as carbon nanotubes (Perspex). C1q recognises mainly charged clusters, but probably also hydrophobic patches on surfaces.

1.2. *Physicochemical characteristics of key complement proteins*

Among 3,700 different proteins in human serum,[2] only a handful play a role in the activation of the complement system. C1q is the recognition molecule

for the classical pathway, MBL or the ficolins act for the lectin pathway, while C3 and C3b are key molecules for the alternative pathway. The direct or indirect binding of C1q[3] and C3b[3] to a carbon nanotube surface activates the complement system. The extent of the complement activation induced by a nanomaterial will depend on the physicochemical characteristics of the nanomaterial and its interaction with complement proteins. Here, we discuss the most important physicochemical characteristics of C1q, C3, and C3b that activate the complement system when interacting with foreign materials.

1.2.1. *Morphology of C1q*

C1q is a 460-kDa protein composed of six heterotrimeric collagen-like triple helices that converge in their N-terminal half to form a stalk, then diverge to form individual stems, each terminating in a C-terminal heterotrimeric globular domain[4] (Figure 2).

C1q binds to target ligands via the globular domains, or heads, triggering the activation of C1r and C1s, the proteases associated with C1q.[5] One of the key features of C1q morphology in initiating the complement system via the classical pathway is that its hexameric structure allows binding by multiple heads. Each head has three lobes, made up of homologous domains (called A, B, and C lobes). Each has distinct but overlapping binding specificity. C1q binds to targets by multiple weak binding interactions. Recognition of one motif (a charge cluster or hydrophobic patch) by one lobe of a head is a weak interaction, but since there are six heads and 18 lobes, multiple interactions

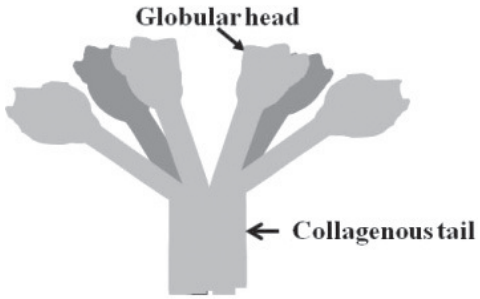

Figure 2. The structure of C1q. C1q is made up of three types of polypeptide chains — A, B, and C — which are homologous to each other. One of each type of chain interact with one another and intertwine to form a collagen triple-helical stalk and a three-lobed globular head. Six of these subunits assemble to form the umbel shape shown here. MBL and the ficolins have similar subunit structures, and form final assemblies with three–six subunits (i.e., three–six heads).

can form, and this results in high avidity binding. A requirement for high avidity binding is that the motifs recognized are in a certain form of regularly spaced array on the target surface. The spacing between motifs must correspond to the spacing between lobes (about 5 nm) or between heads (variable because of the flexibility of the whole molecule) of about 10–40 nm. C1q globular heads are approximately 6 nm wide and have multivalent charged groups for target recognition.[6] The whole C1q molecule measures approximately 40 nm in diameter. C1q morphology and its dimensions are relevant features in understanding its interaction with invaders and foreign materials. We have earlier reported the direct binding of C1q to double-walled carbon nanotubes (DWNTs).[3] C1q binds to DWNTs presumably through its globular heads, activating the complement system via the classical pathway. High-pressure monoxide single-walled carbon nanotubes (HIPco SWNTs) and multi-walled carbon nanotubes (MWNTs) also activate the complement system via the classical pathway. Although the direct binding of C1q to HIPco SWNTs and MWNTs has not been shown, it is likely that C1q binds to them since these carbon nanotubes are not chemically modified, and therefore have a large hydrophobic surface area. Ling *et al.*[7] in a series of transmission electron microscope studies, reported the binding of C1q and C1s–C1r–C1r–C1s to MWNTs, although no binding of C1q to either DWNTs or SWNTs was observed in their study. They observed binding of C1s–C1r–C1r–C1s to DWNTs, but not to SWNTs. In summary, Ling's team did not observe binding of C1q to SWNTs and DWNTs in contrast to our previous studies.[3] Although these two separate findings disagree with each other, we provide strong evidence of the binding of C1q to DWNTs.[3] For example, we measured the direct binding of C1q to DWNTs in the presence and absence of human serum proteins. C1q binds to DWNTs in both conditions. This result addressed the argument given by Ling and co-workers to explain the discrepancies in the findings. Ling's team believes that the SWNT and DWNT studies reported by us activated the complement system via the classical pathway because of the formation of a serum protein layer on DWNTs. We, however, clearly showed that C1q binds to DWNTs even in the absence of serum proteins. We believe that these differences are mainly due to the surface chemistry of these carbon nanotubes. A careful characterization of the SWNTs and DWNTs used in Ling's study would be extremely useful to further explain the disparate results.

There is a vacuum of knowledge with respect to the direct binding of C1 to core-shell nanoparticles — the preferred choice of nanoparticle design for drug delivery purposes. These particles are often coated with a layer of polyethylene glycol (PEG), which is an uncharged hydrophilic polymer that helps

reduce protein adsorption. The dimensions and the morphology of C1q become relevant for its interaction with core-shell nanoparticles since the whole C1q molecule is about 40 nm wide (Figure 2). Core-shell nanoparticles are mainly covered with PEG units in which the distance between each unit can be around 5 nm. Polymeric nanoparticles synthesized with poly(lactic-co-glycolic acid) (PLGA) and polylactic acid (PLA) are usually coated with PEG. Given these dimensions, it will be difficult for the C1 complex or C1q to reach the core even when PEG units are flexible. Nevertheless, if the surface of the core-shell particles is partially covered with PEG, C1 and C1q will most likely interact with the core via hydrophobic interactions and will therefore initiate the complement system activation via the classical pathway. C3 is another protein of the complement system that plays a key role in the complement activation cascade and will be discussed in detail in the next section.

1.2.2. *Morphology and chemistry of C3 and C4*

C3 is the central and most abundant protein of the complement system,[8] and it is present in the plasma at a concentration of 1.3 g/L.[8] It is a protein of 185 kDa with dimensions 15.2 nm × 9 nm × 8.4 nm[9] and contains an internal thioester bond. When C3 is activated to C3b, the thioester is exposed and is highly reactive, and can bind covalently to surfaces (e.g., the target) bearing NH_2 and OH groups, forming amide and ester bonds, respectively. It is generally agreed that this covalent binding reaction is the main route by which C3b becomes attached to biological targets. The same mechanism applies to C4b, which is a homolog of C3b.[10] For synthetic materials, however, there may not be suitable surface groups to which C3b and C4b can form covalent bonds. It is possible that other plasma proteins which bind to synthetic materials would provide such chemical groups. However, for carbon nanotubes, C3 and C4 fragments do bind to them, but we found no evidence that they were covalently bound to other proteins; instead, it appears that C3b and C4b bind by hydrophobic, non-covalent interactions.[11]

Recent findings report that although C3 is a large protein, it is able to interact with the diffuse shell of core-diffuse shell-structured nanoparticles.[12] In this case, complement activation takes place by the complex formed between C3 and bovine serum albumin (BSA), where the latter is able to reach the core of the particle. Other studies report that if C3b binds to or becomes trapped between surface PEG "brushes," C3 hydration and conformational changes (C3 tickover) may become accelerated, leading to the assembly of fluid phase C3 convertase.[13]

The assessment of the complement system via the alternative pathway might be more relevant from a clinical point of view than the assessment of the classical pathway since there are more reports which discuss the initiation of the alternative pathway via the binding of C3b and C3 convertase on the surface of artificial materials.[14–16] Surface plasmon resonance (SPR) is a quantitative technique that can measure the amount of C3b bound to the nanomaterials as reported by Toda *et al.*[17]

2. Physicochemical Characteristics of Nanoparticles

The physicochemical characteristics of different nanocarriers influence the adsorption of human serum and plasma proteins which in turn, might include those which activate the complement system cascade. The coating of different nanomaterials such as liposomes, carbon nanotubes, and polymeric nanoparticles with PEG is very well known as an effective way to prevent protein adsorption. The success of this technical strategy depends on the density of the coating (e.g., maximizing the surface coverage of the core), the length, and the configuration of the PEG (e.g., mushroom, brush, or mushroom–brush). These parameters will be examined carefully in the next section.

2.1. *PEG chain density*

As mentioned above, the decoration of a particle's surface by covalently grafting, entrapping, or adsorbing PEG chains diminishes protein adsorption. Each of these methods has its advantages and disadvantages. For instance, one of the biggest advantages of the covalent binding of the PEG chain to the nanoparticle surface is that it prolongs the nanoparticle's circulation half-life. However, the biggest drawback of this method is that it does not ensure complete surface coverage. In contrast, the adsorption method overcomes this disadvantage but poses new challenges which include the desorption of PEG chains from the nanoparticle's surface. The desorption of PEG will eventually cause the precipitation of the nanoparticles, preventing further use of these particles.

The spatial distribution of PEG on a nanoparticle surface is also relevant to protein adsorption and consequently important to diminishing or preventing complement activation. Research has demonstrated that PEG chains can take on two main different spatial arrangements on a nanoparticle surface, referred to as the mushroom and brush configurations.[18] In the mushroom configuration, particles present very low surface coverage of PEG chains. On the contrary, particles holding a brush configuration have a high

surface coverage of PEG chains. The prevention or diminution of protein adsorption will not only depend on balancing the PEG chain density on the particle surface, but also on other factors. For instance, high PEG chain density ensures the complete coverage of the nanoparticle surface, but decreases the mobility of the PEG chains, thus diminishing the steric hindrance properties of the PEG layer.[19] To date, there is no rule of thumb for obtaining this delicate balance. Some scientists believe that the optimal thickness of hydrodynamic layer effectively shielding the particle surface from protein adsorption is 5% of the particle's hydrodynamic size. According to another view, the optimal thickness of hydrodynamic layer should be at least twofold greater than the radius of the polymer coil at the polymer conformation in its diluted solution.[19]

The physicochemical characteristics of the proteins are factors that also influence protein adsorption. New studies report that the shell of core-diffuse shell structured nanoparticles synthesized with PIBCA [poly (isobutylcyanoacrylate)]–dextran block copolymers by a self-assembly process[12] do not prevent the adsorption of small flexible proteins such as BSA, even when the density of chains within the diffuse shell is quite high. PIBCA is a bioerodible and bioeliminable polymer. Large proteins such as fibrinogen (340 kDa) and C3 (185 kDa) interact with these nanoparticles in a specific manner. Fibrinogen not only reaches the diffuse shell of the nanoparticles, but also reaches their hydrophobic core. On the other hand, C3b penetrates the diffuse shell, but does not reach the core of the nanoparticle.[20] The authors observed complement activation on nanoparticles that trapped C3b in their diffuse shell. They hypothesized that complement activation in this case is due to a complex formed between C3b and BSA.

2.2. *PEG chain length*

PEG chain length is another important parameter that plays a crucial role in both protein adsorption and complement activation. Mosqueira *et al.*[21] demonstrated that polymeric particles covered with a high density of 20-kDa PEG chains show the lowest protein adsorption and lowest complement activation among the materials they tested. This is presumably due to steric barriers surrounding these particles. Mosqueira's team hypothesized that the long PEG chains act through creating a PEG-hydrated cloud "shielding" negatively charged groups located underneath it. In addition, they reported that particles coated with 5-kDa PEG with high density are more effective in reducing complement activation than at low density.

Recent studies on PEG–PLGA nanoparticles further corroborate that long chain lengths reduce protein adsorption and complement activation.[22] Research on the *in vivo* effects of short and long chains of monolayer-protected gold nanoparticles indicated that the former presented short circulation half-life, whereas longer half-life was observed with long PEG chains.[23]

2.3. *Morphology of the nanocarrier*

Activation of the complement system has been assessed on nanomaterials that are either spherical or tubular, including liposomes, micelles, lipid-polymer nanoparticles, and carbon nanotubes. Although these studies are signs of progress on this topic, there still lacks a deep understanding of the influence of the nanocarrier's shape on complement activation. To the best of our knowledge, the only insight on this topic comes from a study that suggested that the higher the curvature, the lower the human plasma protein surface coverage, thus rendering activation of the complement system less likely.[24] For spherical nanocarriers, the curvature is directly related to the size of the particles.[24] Small nanoparticles (hydrodynamic diameter of ~70 nm) have higher curvature than large nanoparticles (hydrodynamic diameter of ~300 nm). Despite this finding, the morphology of the nanocarrier itself seems to be irrelevant for the activation of the complement system; rather, it is the surface chemistry of the nanocarrier that is the key to complement activation. As a matter of fact, it has been demonstrated that lipid PEGylated carbon nanotubes activate the complement system regardless of the terminal functional group and the PEG brush-like surface structure on HIPco SWNTs.[25] The activation of the complement system by lipid PEGylated HIPco SWNTs might be due to the incomplete coverage of PEG on their surface. Since the un-PEGylated surface area of HIPco SWNTs is hydrophobic, it is likely that C1q molecules bind to that surface via hydrophobic interactions. Previous studies on non-chemically modified HIPCo SWNTs showed the activation of the complement system via the classical pathway, which is initiated by the binding of C1q to the activator surface.[3]

In the field of polymeric nanoparticles, Gref *et al.*[26] reported the influence of the core composition on protein adsorption. Protein binding studies were conducted with nanoparticles synthesized with three different polymeric cores made of PLGA, PLA, and poly(ε-caprolactone) (PCL) polymers. During this synthesis, the length and density of the PEG were kept constant. The results showed slight differences in the pattern of protein binding which

suggested that the core has a part in determining the proteins that bind to the nanoparticle surface.

3. Recent Engineering Approaches to Avoid or Reduce Complement Activation

Understanding the factors that activate the complement system is very important to engineering biocompatible nanocarriers that can be used for a wide range of applications in medicine. In the last two decades, there has been great progress in understanding the complement system cascade and its interaction with different organic and inorganic materials. The complement system views liposomes, carbon nanotubes, polymeric nanoparticles, and micelles as invaders. Synthetic strategies such as the use of PEG to coat the surface of these nanocarriers to evade the complement system cascade have been employed.[18,25,27] This chemical approach has worked only to a certain extent for immune evasion. For instance, studies have demonstrated that PEGylation does not necessarily suppress complement opsonization.[27] Complement activation induces the opsonization of PEGylated liposomes because of the covalent deposition of C3b on the liposomal surfaces.

In previous sections, we mentioned that PEG can present two different spatial arrangements known as the mushroom- and brush-like configurations. These configurations influence protein adsorption which might or might not lead to complement activation. Lately, it has been found that the transition from one configuration to another influences the activation of the complement system.[28]

The reduction or prevention of complement activation is relevant for several reasons. Preventing the activation of the complement system can help to extend the circulation half-life of the nanocarrier in the bloodstream, improve the nanocarrier's biodistribution profile, and avoid a set of allergies known as CARPA (complement activation-related pseudoallergy).[29] For all these reasons, it is urgent to develop new synthetic strategies for diminishing or preventing complement system activation. We next discuss the most recent advances in this area.

3.1. *Influence of PEG mushroom- and brush-like configurations on complement activation*

Surface PEGylation not only helps diminish protein adsorption, but can also cause alterations of the pathway for complement activation. Moghimi *et al.*[28] reported that the transition of copolymer architecture on nanoparticles with

polyethylene oxide (PEO) chains from the mushroom–brush to brush-like configuration not only switches complement activation from the C1q-dependent classical to lectin pathway, but also reduces the level of generated complement activation products C4d, Bb, C5a, and SC5b-9.

According to the authors, changes in adsorbed polymer configuration trigger alternative pathway activation differently and through different initiators. This was the first study to demonstrate the importance of configurational mobility of surface-projected PEG chains in the modulation of complement activation with a spectrum of model nanoparticles that exhibited different pharmacokinetic profiles.

The mushroom–brush and brush-like PEG configurations are observed on the surface of different nanocarriers, including liposomes,[29] polymeric nanoparticles,[26] gold nanoshells[30] and carbon nanotubes.[25] In all these cases, the brush-like configuration has been identified as the surface structure that reduces protein adsorption, though it does not necessarily diminish complement activation. PEGylated liposomes are a good example of this case. Bradley *et al.*[31] found that the incorporation of PE–mPEG [phosphatidylethanolamine–monomethoxylpoly(ethylene glycol)2000] from 5–7.5 mol% into the liposomal bilayer was not enough to prevent complement activation. Their results revealed that the inhibitory effect of mPEG–lipid on complement activation is highly dependent on the liposomal concentration used in the complement assay.[31] Other studies have reported that the concentration of PEG in liposomes causes the transition from the mushroom- to brush-like regime.[32] Liposomes prepared with up to 4 mol% of grafted PEG exhibited the mushroom configuration because the neighboring coils did not interact laterally. On the other hand, liposomes synthesized with PEG concentration above 4 mol% showed a brush regime since the neighboring PEG chains pushed against each other, extending farther out from the surface on which they were grafted.[32] This study did not report on complement system activation under these circumstances.

The spatial configuration of PEG on polymeric nanoparticles has been studied for more than a decade. It is well known that PEG can exhibit either the mushroom- or brush-like configuration on a polymeric nanoparticle surface. PEG with high molecular weight and at high density is expected to exhibit the brush-like configuration.[33] Mosqueira *et al.*[21] demonstrated that 20-kDa PEG chains are more effective in preventing C3 cleavage than 5-kDa PEG chains because of the steric barrier created by the PEG surrounding the particles.

Core-shell nanoparticles synthesized with di-block copolymer of methoxyPEG–PCL (MePEG–PCL) and tri-block copolymer of PCL–PEG–PCL present mushroom- and brush-like surface structures, respectively. The

mushroom-like structure was thought to be more efficient in preventing opsonization because it could form a more effective conformational cloud.[34] Recently, a quantitative and nondestructive assay based on surface enhanced Raman scattering (SERS) spectroscopy has been reported to determine the number of PEG molecules bound to gold nanoshell surfaces.[30] From this estimate, one can obtain the pack density of PEG units on nanoshell surfaces, which helps to infer their configuration.[30]

Heterogeneity is a phenomenon that sooner or later will have impact on complement activation, and therefore must be taken into account when designing new nanocarriers. The concept of surface heterogeneity in essence describes the incomplete coverage of the nanoparticle's surface with PEG.[35] Moghimi and Szebeni discussed the direct effect of the partial coverage of PEG on the nanoparticles' circulation half-life in the bloodstream.[35] The less the surface coverage, the poorer the steric shielding, and the shorter the circulation life, which is presumably caused by the binding of opsonic molecules to the unshielded area.[4] The higher the surface coverage, the greater the resistance to protein binding. For instance, microsphere populations covered with a high density of mPEG with the mushroom–brush intermediate and/or brush-like configurations were most resistant to phagocytosis and activated the complement system poorly.[36]

To date, there has been little discussion of the influence that factors such as temperature,[37] autoxidation catalyzed by transient metals,[38] and salt concentration[39] might have on the configuration and stability of PEG. Changes in the physicochemical characteristics of PEG might affect PEG configuration. There is evidence that the aforementioned parameters have a direct effect on PEG. As a matter of fact, it has been observed that increasing the salt concentration to 3 M and raising the temperature to 37°C resulted in the rapid aggregation of PEGylated nanoparticles.[40] This is due to the fact that high temperature causes the dissolution of the PEG hydration layer, leading to particle precipitation. Although the assessment of complement activation and protein binding studies were not conducted at such high salt concentrations, these parameters should be kept in mind until new studies report the temperature and salt concentration at which mushroom- and brush-like configurations are not only formed, but are also stable.

The identification of these parameters is very important for the efficient design of nanocarriers, especially when application is envisioned in the medical field. Depending on the application, the experimental conditions in which the nanocarriers will be immersed might be subject to high temperature and high salt concentration. Temperature is the one of the parameters that might have a strong influence in complement activation depending on the method

used. For example, hemolytic assays are conducted at 37°C and a great number of papers has described the assessment of complement activation using hemolytic assays (which can only be done between about 20°C and 37°C).

New synthetic approaches are already available as an alternative to PEG, which is traditionally the workhorse for preventing protein adsorption.[40] These novel polymers are not temperature-sensitive and the surface structure does not change at high salt concentration.[41] However, they are not as well studied and characterized as PEG since they have emerged only recently.

3.2. *Modulation of complement system activation via functional groups*

For more than 30 years, scientists have been trying to prevent or diminish complement activation to achieve blood biocompatibility. In the 1980s and 1990s, there were reports of various methods to diminish complement activation.[41–45] Carreno *et al.*[45] reported that the substitution of hydroxyl groups of Sephadex® (an activator of the alternative pathway) by carboxymethyl (CM), groups can reduce the activating capacity of the resulting polymer (CMSeph). Complement activation by CMSeph can be abolished when an average of one CM group is present per glycosyl unit. However, this method is a multistep procedure and involves complicated organic synthesis.

Another example of reducing activation of the complement system via direct chemical modification of the nanomaterial is in the field of carbon nanotubes. In previous studies, we reported that the chemical modification of pristine MWNTs reduces complement activation.[46] In this study, four different types of chemically modified MWNTs were tested for complement activation via the classical and alternative pathways using hemolytic assays. It was found that MWNTs functionalized with e-caprolactan or L-alanine showed, respectively, >90% and >75% reduction in classical pathway activation compared with unmodified MWNTs. The reduced level of complement activation via the classical pathway that is likely to increase biocompatibility is directly correlated with the amount of C1q protein bound to chemically modified carbon nanotubes. These results demonstrated for the first time that these types of chemical modifications are able to considerably alter the level of specific complement proteins bound by pristine MWNTs.

Lately, new synthetic methods have been developed not only to diminish complement system activation, but most importantly, to manipulate it at will. In this method, the manipulation of the complement system is due to the modulation of the nanoparticles' surface charge. For some nanocarriers, the surface charge dictates the activated pathway. This is the case with liposomes.

Negatively charged liposomes containing phosphatidyl glycerol, phosphatidic acid, cardiolipin, and/or phosphatidylinositol activate the complement system via the classical pathway.[47] Positively charged liposomes containing stearylamine activate the alternative pathway.[47] Neutral liposomes hardly activate the complement system.[47]

For other potential drug delivery systems such as PEGgylated lipid carbon nanotubes and polymeric nanoparticles, the role of surface charge is still relevant for complement activation, but differences among liposomes and in charges in carbon nanotubes and polymeric nanoparticles do not determine which pathway will be activated. Results reported by Hamad *et al.*[25] demonstrated that PEGylated lipid HIPco SWNTs activate the whole complement system independently of the terminal end moiety of the projected PEG chain. The authors hypothesized that HIPco SWNTs most likely activate the complement system via the lectin pathway. In addition, PEGylated lipid polymeric nanoparticles functionalized with NH_2, COOH, and CH_3 activated the alternative pathway, but not the classical pathway.[48] This scientific evidence suggested that the presence of functional groups in the PEG chain does not necessarily determine the pathway of the complement system that is activated.

So far, scientists have not yet determined the set of parameters that absolutely dictate the pathway activated. In an effort to shed light on this matter, Toda *et al.*[49] conducted studies assessing complement activation of PEGylated self-assembly monolayers (SAMs) functionalized with a mixture of NH_2–COOH and NH_2–CH_3. Briefly, functionalized SAMs were prepared with various molar ratios of a pair of NH_2,–COOH or NH_2–CH_3 on gold-coated glass plates. These coated glass plates were immersed in reaction mixtures for 24 h followed by several washes. Their results showed that the NH_2–COOH mixture activated the complement system, whereas the NH_2–CH_3 pair did not activate it. The authors believed that the NH_2–CH_3 mixture did not activate the complement system because of the numerous serum proteins adsorbed onto those SAMs, including albumin, that formed a protein layer which inhibited access of C1q or C3b to the surface. On the contrary, a high amount of C3b or C3 convertase was found to be deposited on the NH_2–COOH SAM.

We showed a direct correlation between the zeta potential, levels of complement activation, and the amount of C3b deposited on lipid–polymeric nanoparticles functionalized with a mixture of NH_2–COOH and NH_2–CH_3 and synthesized via the nanoprecipitation method.[48] We observed that as the presence of NH_2 on PEGylated lipid–polymer nanoparticles reduces, the level of complement activation diminishes and the zeta potential becomes more negative. The C3b binds to NH_2 and OCH_3 as well as the different molar ratio mixtures of these two functional groups. The molar ratio mixture of

COOH–NH_2 shows complement activation, which agrees with Toda's findings, even when those studies were conducted on SAMs and not in polymeric nanoparticles. This pair of functional groups mixtures also binds the C3 β chain, which might explain the mechanism involved in the activation of the complement system. The main difference between Toda's findings and our results is that we found that lipid–polymeric nanoparticles functionalized with several molar ratio mixtures of NH_2–CH_3 activate the complement system.[48] This could probably be due to the partial coverage of NH_2–CH_3 on the nanoparticle surface. Our findings clearly demonstrated a direct correlation of the surface charge of the particles and the activation of the complement system. In addition, our team's method was a simple, practical, and inexpensive way to modulate the complement system. Using this method, we can make these particles act as adjuvants by functionalizing their polymeric core with DSPE–PEG–NH_2 or make them function as biocompatible nanocarriers for drug delivery purposes by functionalizing their surfaces with CH_3.

Hydroxyl groups are another terminating group that has been used to functionalize SAMs to study their effects on complement activation. Sperling and co-workers reported that these surfaces strongly activate the complement system.[50] As the amount of surface OH increases, the amount of C5a generated also increases.[50] Other studies also reported the activation of the complement system induced by terminal hydroxyl group of tri(ethylene glycol)-terminated alkanethiol (HS-TEGOH).[51] Again, the cause of such activation was the deposition of C3b on these surfaces.

4. Methods for the *In Vitro* Study of Complement Activation by Different Nanomaterials

4.1. *Hemolytic assay (CH_{50})*

For several decades, hemolytic assays have been used as the standard procedure to assess complement activation. CH_{50}, or total hemolytic complement assay, measures the ability of the classical pathway-activated MAC to lyse sheep red blood cells (SRBCs) coated with an antibody.[52] The alternative pathway hemolytic assay (APH_{50}) measures the ability of the MAC generated by this pathway to lyse rabbit red blood cells. Both assays indicate a deficiency of a complement component by the absence of lysis. Hemolytic assays have several advantages over other complement tests. For example, the biggest advantage of the CH_{50} assay is the possibility of evaluating the activation of the complement system in most mammalian species' sera. Also, this method is inexpensive. However, limitations include their labor intensity (particularly

the pipetting of small volumes), the short shelf-life of SRBCs, the variable inter-lot performance of SRBCs, and the low sensitivity of the assay relative to measuring C scission products such as C5a and SC5b-9.

Complement hemolytic assays can vary widely in sensitivity from day to day, so it is possible to compare only results obtained in the same assay and not results obtained on different days. This is mainly because of metabolic changes in the cells, which make them more sensitive to lysis as they age. The most common assay for complement consumption is done in a buffer containing Ca^{2+} and Mg^{2+} ions. This measures classical pathway activation. If Ca^{2+} ions are removed by the chelator EGTA, the classical and lectin pathways are completely inhibited, and only the alternative pathway is measured. There are numerous versions of the hemolytic CH_{50} assay, which is arbitrarily standardized with respect to the concentration of sensitized SBRCs (10^8/1.5 mL), and the concentration and type of sensitizing antibody (heterophilic Forssmann, i.e., rabbit anti-RBCs).

Hemolytic assays have also been used to evaluate the activation of the complement system by different nanomaterials such as liposomes[47] and pristine and chemically modified carbon nanotubes.[3,46] It is worth noting that this simple complement consumption assay can only be used if the activator is particulate and can be separated from the serum by filtration or centrifugation before complement activity is assayed in the serum. If the activator is soluble and cannot be easily separated from the serum, more sophisticated assays are required to distinguish between complement consumption and inhibition of the complement assay.

4.2. *ELISA kits*

Lately, ELISA-like assays have been used to assess *in vitro* complement activation.[25,48–49] In these studies, complement activation was assessed using Quidel kit SC5b-9. This ELISA-based assay measures the cleavage of C5 and subsequent terminal pathway activation. Specifically, SC5b-9 is generated by the assembly of C5–C9 as a consequence of complement activation via all three pathways and subsequent binding to the naturally occurring regulatory serum protein, the S protein (vitronectin). SC5b-9 forms when the MAC (Figure 1) fails to insert into a lipid bilayer, but instead reacts with S protein. C9 within this complex expresses a neo-epitope (i.e., an epitope not present in C9 which is not incorporated in the SC5b-9 complex), so C9 itself does not interfere in the assay. ELISA-like assays have several advantages over hemolytic assays. For instance, the former is a faster and easier method than the latter. These methods do not involve the use of SRBCs so they eliminate

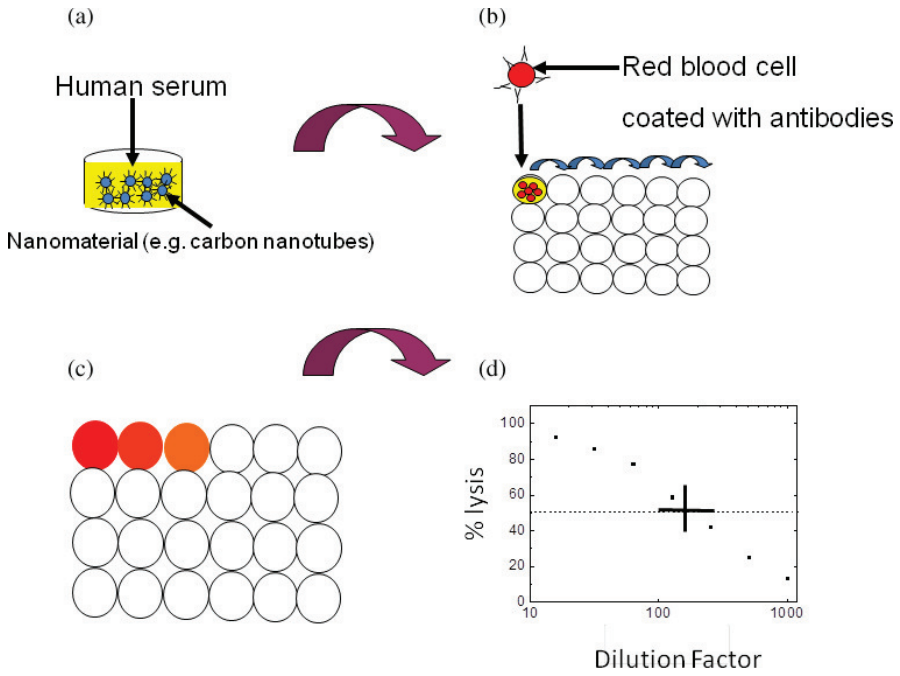

Figure 3. Schematic diagram showing the main steps in hemolytic assays. This diagram illustrates the assessment of the complement system via the classical pathway. This type of assay measures the ability of the classical pathway-activated membrane attack complex (MAC) to lyse sheep red blood cells (SRBCs) coated with an antibody. Likewise, the functional activity of the alternative pathway and the terminal components can be measured by the lysis of rabbit erythrocytes in human serum. (a) Assessing the activation of the complement system is done by simply incubating the nanomaterial (e.g., carbon nanotubes) with serum. Samples are incubated at 37°C for 1 h followed by centrifugation. (b) The supernatant of the sample is serially diluted and placed in a microtiter plate. One hundred microliters of each dilution are incubated with 100 µL of antibody-sensitized SRBCs (EA) (10^8 cells/mL in veronal buffer). SRBCs sensitized with an antibody (EA) are used as an immune complex to activate the classical pathway. When EA cells are added to serum or plasma, they are lysed as a result of their activation of the complement system and the intercalation of C5b-9 MAC into their cell membranes. (c) After incubation, cells are spun down (2500 rpm, 10 min, room temperature) and hemoglobin is measured at 405 nm in the supernatant. (d) Calculating complement activation is done by plotting the percent lysis against the dilution of human serum (logarithmic scale). (Adapted from Ref. 52.)

problems such as short shelf-life and inter-lot variability and sensitivity. For these reasons, the ELISA-like assay method is superior to hemolytic assays. In addition, this method can be adapted to assess complement activation in a high-throughput screening fashion. On the other hand, disadvantages of ELISA-based assays include the evaluation of the complement system only in human sera and not in other mammalian species (although a few reagents,

e.g., antibodies, are available for mice or rats). Another disadvantage is that this assay only indicates whether the complement system is activated or not. Further experiments are needed to identify the activated complement system pathway. For this purpose, there are similar kits that can be used. For instance, Quidel C4d and Bb ELISA kits are specific for classical/lectin and alternative pathway complement activators, respectively. Both analytes are by-products of complement activation; C4d is a scission derivative of C4b, whereas Bb rises in blood as a consequence of spontaneous dissociation of the alternative pathway C3 convertase. Other assay kits which measure C3a or C5a generation are available from Enzo Life Sciences, BD Biosciences, R& D systems, and others. All of these kits are relatively expensive and they expire in less than a year.

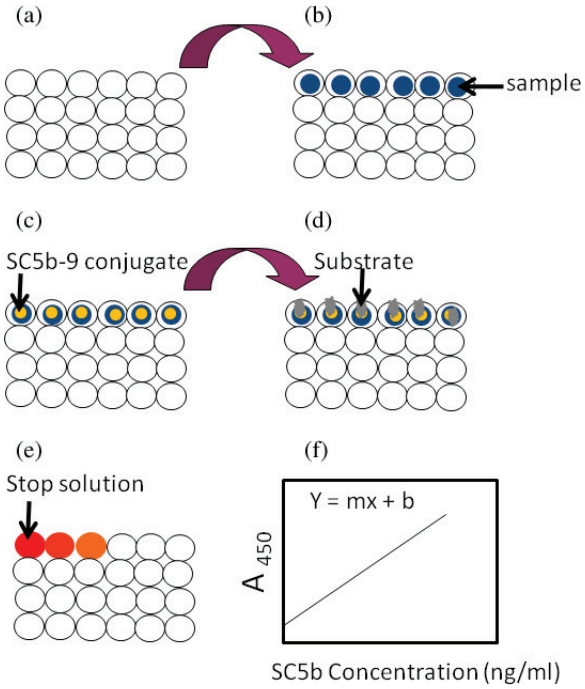

Figure 4. Schematic diagram of the Quidel-kit as an example of Elisa-like assays. (a) Washing assay wells with wash solution as indicated in Quidel kit SC5b-9. (b) Pipetting specimen diluents (black), standards, controls, and specimens into assay wells followed by incubation and several washes. (c) Pipetting SC5b-9 with the conjugate into assay wells followed by incubation and several washes. (d) Pipetting substrate into assay wells. (e) Adding stop solution into assay wells. (f) Reading the optical density at 450 nm and analyzing the assay results using a linear curve fit ($y = mX + b$). (Adapted from Ref. 48.)

4.3. *Wieslab diagnostics kits*

Wieslab diagnostics kits are also ELISA-like assays for the qualitative determination of functional classical, alternative, and lectin pathways in human serum.[53] Wieslab products are available via Eurodiagnostica (http://www.eurodiagnostica.com/). These complement assays combine the principles of the hemolytic assay for complement activation with the use of labeled antibodies specific for the SC5b-9 neoepitope produced as a result of complement activation. The amount of SC5b-9 generated is proportional to the functional activity of complement pathways. The wells of the microtiter strips are coated with specific activators of the classical, lectin (MBL-only), or the alternative pathway. Patient serum is diluted in diluents containing specific reagents to ensure that only the appropriate pathway is activated. During the incubation of the diluted patient serum in the wells, complement is activated by the specific coating. It is worth noting that the level of complement activity evaluated by functional assays such as the Wieslab complement kits takes into account the rate of synthesis, degradation, and consumption of the components, and provides a measure of the integrity of the pathways as opposed to immunochemical methods, which specifically measure the concentration of various complement components.

4.4. *2D immunoelectrophoresis method*

The 2D immunoelectrophoresis method is another technique that has been used to assess complement system activation.[12] This technique separates and characterizes proteins based on electrophoresis and reaction with antibodies. The cleavage of C3 into breakdown products C3b, iC3b, and C3c alters the electrophoretic mobility of C3. C3 is separated from its breakdown products by electrophoresis on agarose, and the proteins are electrophoresed at right angles into an agarose gel containing anti-C3 antibodies. The proteins react with antibodies and form a visible precipitate over an area proportional to the protein concentration. This is a time-consuming procedure, unsuitable for high-throughput screening, but for a one-time assessment, it could be a good option. Also, this technique is not as sensitive for complement activation assessment as ELISA-like assays. However, this technique could be less expensive than ELISA-Quidel Kits and hemolytic assays as the reagents for the test can be used for other research purposes and be frozen for future use. The long shelf-life of antibodies against complement proteins is one of the biggest advantages of the 2D

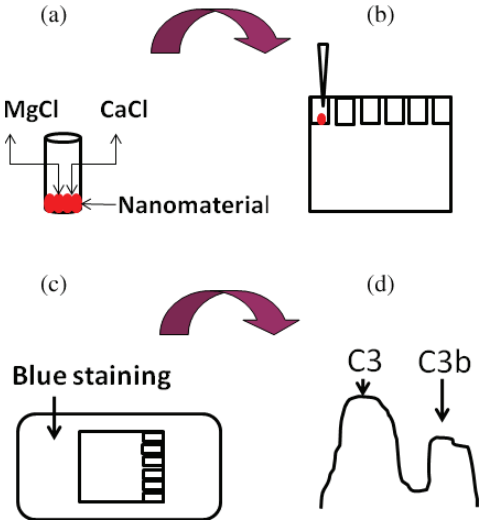

Figure 5. Schematic diagram of activation of the complement system evaluated from 2D immunoelectrophoresis method. (a) Incubation of the nanomaterial (e.g., nanoparticles) with human serum in veronal buffer supplemented with MgCl and CaCl. (b) 2D electrophoresis gel loaded with incubated samples as described in Ref. 12. (c) Coomassie blue staining of 2D electrophoresis gel. (d) Analysis of the immunoelectrophoregrams showing the presence of C3 and C3b proteins after incubation of the serum with the nanomaterial. (Adapted from Ref. 12.)

Table 1. Summary of *in vitro* studies of complement activation on different nanomaterials.

Assay method	Assay source/protocol
Hemolytic assays (CH_{50})	Salvador-Morales, *et al.*[3] Gbadamosi J K. *et al.*[4] Whaley K[54]
ELISA-like kits	Quidel Corp., San Diego, CA
Enzyme immunoassays	Ferraz N *et al.*[55]
Wieslab	http://www.eurodiagnostica.com/
2D immunoelectrophoresis	Vauthier C *et al.*[12] Bertholon I *et al.*[56]

immunoelectrophoresis method as both ELISA-like assays and CH_{50} have short shelf-lives. For example, reagents for hemolytic assays such as SRBCs only last for several days, whereas ELISA kits last for 12 months. Table 1 summarizes all the *in vitro* assays employed to assess complement activation described above.

5. *In Vivo* Studies of Complement Activation by Different Nanomaterials

To date, *in vivo* studies on complement system activation are still limited.[29,57–60] Szebeni and co-workers have made substantial contributions on this topic by assessing complement activation in different animal models including pigs, dogs, and rats.[29] Their findings showed that these animals present a set of allergy symptoms when they are injected with lipid-based nanomaterials. These allergy symptoms include tachypenia, tachycardia, hypotension, hypertension, chest pain, and back pain. These reactions, commonly known as infusion reactions, form the concept of CARPA.[60] Evidence from *in vitro*, *in vivo,* and clinical trials suggest that these reactions are derived from complement system activation and different mammalian species respond differently to the intravenous (i.v.) administration of liposomes. For example, rats are less sensitive than dogs and pigs; pigs are the most sensitive animal model for the i.v. injection of lipid-based materials.[60] Szebeni and co-workers' findings also show that the presence of cholesterol and phospholipids in liposomes are responsible for complement system activation.[61–62] In addition, it is well known that cationic and anionic liposomes activate the complement system via the alternative and classical pathways, respectively.[47] In this case, it is reported that the complement system is activated not only due to the presence of cholesterol and phospholipids, but also cationic and anionic molecules in liposomes.

Other lipid-based materials such as micelles cause infusion reactions in dogs and pigs.[60] However, other studies demonstrated that mPEG–phosholipid in micelles and liposomes coated with methoxyl functional groups did not activate the complement system. These results indicated that charge is the property of the liposomes that causes complement activation. PEGylated HIPco SWNTs with CH_3 and NH_2 terminal ends also induce *in vitro* and *in vivo* complement activation.[25] In this study, the *in vivo* complement activation assessment was measured indirectly by the levels of thromboxane B2 in rat blood.

The differences in complement system activation across mammals are based on species, dose dependence, and the influence of lipid composition. For instance, the mechanism involved in the activation of the complement system in pigs that are injected with Doxil® is the anaphylaxis phenomenon, which is basically the increment of C5a and C3a elements. This increment causes severe cardiac abnormalities in porcine models. On the other hand, a similar amount of undiluted Doxil® can be fatal for men and dogs. Rats are two to three orders of magnitude less sensitive to liposomes, at least to those containing <50% cholesterol,[42] although complement-dependent shock and

tissue damage induced by the i.v. injection of cholesterol-enriched liposomes in rats have been observed.

Lipid composition plays an important role in complement activation. Szebeni and co-workers reported that the cholesterol content of liposomes may be an important determinant of high sensitivity reactions (HSR). Pulmonary hypertensive effects of multilayer vesicles in pigs were found to be proportional to the amount of cholesterol in the vesicle in the 20–71% range,[63] and rat murine leukemic virus (MLV) containing 71% cholesterol were significantly more reactogenic compared to liposomes with 45% cholesterol.[42]

Recently, Szebeni and co-workers have reported the activation of the complement system in porcine models because of the presence of polyethylenimine polymers.[64] This type of polymer triggers anaphylactic reactions in low percentages of hypersensitive individuals regardless of PEGylation. The results should be kept in mind when engineering new drug delivery systems, as transient and mild reactions can be fatal for patients who suffer from allergies and heart diseases.[65] More studies are urgently needed to be conducted on this topic to fully elucidate the mechanisms involved in *in vivo* complement activation.

6. Concluding Remarks

Complement system activation can be triggered by the direct binding of key complement proteins such as C1q, MBL, ficolins, and C3b to different nanoparticle surfaces. The binding of other plasma proteins such as fibrinogen and BSA can serve as a bridge for the binding of proteins such as C3b, which in turn activates the complement system. Thus, protein adsorption undoubtedly plays a significant role in complement activation. Therefore, there is a great need to reduce protein adsorption by understanding the parameters of the nanosurfaces that enhance or avoid protein binding.

In this chapter, we have highlighted the physicochemical characteristics of both complement proteins and nanomaterials that cause complement activation. Parameters such as the PEG chain length, density, and conformation strongly influence complement activation. The process of engineering nanocarriers with a long circulation half-life and a good pharmacokinetic profile involves making several trade-offs.

After more than three decades of research in this area, we still have not produced a recipe that guarantees the synthesis of ideal nanocarriers. The existing literature on this topic undoubtedly has helped us to much better understand the parameters that we need to consider to engineer the ideal nanocarrier. We have remarked that future decisions and trade-offs will depend on the severity of the disease and the willingness of patients to take

the risk. As shown above, lipid-based nanocarriers activate the complement system *in vitro* and *in vivo*. Most importantly, clinical trials have demonstrated the activation of the complement system. These studies should not be overlooked, but should be seriously considered when Doxil® and other lipid-based drugs are consumed by patients who have heart diseases. Future developments in the area of biomaterials await to abrogate complement activation, minimizing secondary effects. Bacteria have evolved ways of avoiding attack by complement, e.g., by binding to their surface a complement downregulatory protein, factor h, from the host. It has recently been suggested that mimicking bacteria such as by attaching factor h-binding peptides to nanomaterial surfaces, may be a strategy worth exploring.[66–67]

References

1. Carroll MV, Sim RB. Complement in health and disease. *Adv Drug Rev* 2011; 63:965–975.
2. Muthusamy BHG, Suresh S, Rekha B, Srinivas D, Karthick L, Vrushabendra BM, Sharma S, Mishra G, Chatterje P. Plasma proteome database as a resource for proteomics research. *Proteomics* 2005;3531–3536.
3. Salvador-Morales C, Flahaut E, Sim E, Sloan J, Green ML, Sim RB. Complement activation and protein adsorption by carbon nanotubes. *Mol Immunol* 2006;43:193–201.
4. Gaboriaud C, Juanhuix J, Gruez A, *et al.* The crystal structure of the globular head of complement protein C1q provides a basis for its versatile recognition properties. *J Biol Chem* 2003;278:46974–46982.
5. Arlaud GJ, Gaboriaud C, Thielens NM, Budayova-Spano M, Rossi V, Fontecilla-Camps JC. Structural biology of the Cl complex of complement unveils the mechanisms of its activation and proteolytic activity. *Mol Immunol* 2002;39:383–394.
6. Kishore U, Gaboriaud C, Water P, *et al.* C1q and tumor necrosis factor superfamily: Modularity and versatility. *Trends Immunol* 2004;25:551–561.
7. Ling WL, Biro A, Bally I, *et al.* Proteins of the innate immune system crystallize on carbon nanotubes but are not activated. *ACS Nano* 2011; 5: 730–737.
8. Law SKA, Reid KBM. *Complement*. IRL Press, 1995.
9. Sim RB, Perkins SJ. Molecular modelling of C3 and its ligands. *Curr Top Microbiol Immunol* 1990;153:209–222.
10. Sim RB, Sim E. Autolytic fragmentation of complement components C3 and C4 and its relationship to covalent binding activity. *Ann N Y Acad Sci* 1983;421:259–276.
11. Malgorzata J, Rybak-Smith MJ, Pondman KM, Flahaut E, Salvador-Morales C, Sim RB. Recognition of carbon nanotubes by the human innate immune system. In: Klingeler R, Sim RB, eds. *Carbon Nanotubes for Biomedical Applications.* Springer-Verlag, 2011, pp. 183–210.
12. Vauthier C, Persson B, Lindner P, Cabane B. Protein adsorption and complement activation for di-block copolymer nanoparticles. *Biomaterials* 2011;32:1646–1656.
13. Moghimi SM, Andersen AJ, Hashemi SH, *et al.* Complement activation cascade triggered by PEG-PL engineered nanomedicines and carbon nanotubes: The challenges ahead. *J Controlled Release* 2010;146:175–181.

14. Hed J, Johansson M, Lindroth M. Complement activation according to the alternative pathway by glass and plastic surfaces and its role in neutrophile adhesion. *Immunol Lett* 1984;8:295–299.
15. Kazatchkine MD, Carreno MP. Activation of the complement system at the interface between blood and artificial surfaces. *Biomaterials* 1988;9:30–35.
16. Chenoweth DE. Complement activation in extracorporeal circuits. *Ann N Y Acad Sci* 1987;516:306–313.
17. Toda M, Arima Y, Iwata H. Complement activation on degraded polyethylene glycol-covered surface. *Acta Biomater* 2010;6:2642–2649.
18. Peracchia MT, Harnisch S, Pinto-Alphandary H, *et al.* Visualization of *in vitro* protein-rejecting properties of PEGylated stealth (R) polycyanoacrylate nanoparticles. *Biomaterials* 1999;20:1269–1275.
19. Stolnik S, Illum L, Davis SS. Long circulation microparticle drug carriers. *Adv Drug Delivery Rev* 1995;16:195–214.
20. Thomas SN, van der vlies AJ, O' Neil CP, *et al.* Engineering complement activation on polypropylene sulfide vaccine nanoparticles. *Biomaterials* 2011;32:2194–2203.
21. Mosqueira VC, Legrand P, Gulik A, *et al.* Relationship between complement activation, cellular uptake and surface physicochemical aspects of novel PEG modified nanocapsules. *Biomaterials* 2001;22:2967–2979.
22. Yang A, Liu W, Li Z, Jiang L, Xu H, Yang X. Influence of polyethyleneglycol modification on phagocytic uptake of polymeric nanoparticles mediated by immunoglobulin G and complement activation. *J Nanosci Nanotechnol* 2010;10:622–628.
23. Simpson CA, Agrawal AC, Balinski A, Harkness KM, Cliffel DE. Short-chain PEG mixed monolayer protected gold clusters increase clearance and red blood cell counts. *ACS Nano* 2011;5:3577–3584.
24. Cedervall T, Lynch I, Lindman S, *et al.* Understanding the nanoparticle–protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. *Proc Natl Acad Sci* 2007;104:2050–2055.
25. Hamad I, Hunter C, Rutt KJ, Liu Z, Dai H. Complement activation by PEGylated single-walled carbon nanotubes is independent of C1q and alternative pathway turnover. *Mol Immunol* 2008;45:3797–3803.
26. Gref R, Luck M, Quellec P, *et al.* Stealth corona-core nanoparticles surface modified by polyethylene glycol (PEG): Influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. *Colloids Surf B Biointerfaces* 2000;18:301–313.
27. Laverman P, Brouwers AH, Dams ET, Oyen WJ, Storm G, van Rooijen N. Preclinical and clinical evidence for disappearance of long-circulating characteristics of polyethylene glycol liposomes at low lipid dose. *J Pharmcol Exp Ther* 2000;293:996–1001.
28. Hamad I, Al-Hanbali O, Hunter AC, Rutt KJ, Andresen TL, Moghimi SM. Distinct polymer architecture mediates switching of complement activation pathways at the nanosphere-serum interface: Implications for stealth nanoparticle engineering. *ACS Nano* 2010;4:6629–6638.
29. Szebeni J, Alving CR, Rosivall L, *et al.* Animal models of complement-mediated hypersensitivity reactions to liposomes and other lipid-based nanoparticles. *J Liposome Res* 2007;17:107–117.
30. Levin CS, Bishnoi SW, Grady NK, Halas NJ. Determining the conformation of thiolated poly (ethylene glycol) on Au nanoshells by surface-enhanced raman scattering spectroscopy assay. *Anal Chem* 2006;78:3277–3281.

31. Bradley AJ, Devine DV, Ansell AM, Janzen J, Brooks DE. Inhibition of liposome-induced complement activation by incorporated poly(ethylene glycol)-lipids. *Arch Biochem Biophys* 1998;357:185–194.
32. Garbuzenko O, Barenholz Y, Priev A. Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer. *Chem Phys Lipids* 2005;135:117–129.
33. Buscall R. The stability of polymer latices. In: Buscall R, Corner T, Stagema JF, eds. *Polymer Colloids*. Elsevier Applied Science, 1986, pp. 141–217.
34. Hu Y, Xie J, Tong YW, Wang CH. Effect of PEG conformation and particle size on the cellular uptake efficiency of nanoparticles with the HepG2 cells. *J Controlled Release* 2007;118:7–17.
35. Moghimi SM, Szebeni J. Stealth liposomes and long circulating nanoparticles: Critical issues in pharmacokinetics, opsonization and protein-binding properties. *Prog Lipid Res* 2003;42:463–478.
36. Gbadamosi JK, Hunter AC, Moghimi SM. PEGylation of microspheres generates a heterogeneous population of particles with differential surface characteristics and biological performance. *FEBS* 2002;532:338–344.
37. Efremova NV, Sheth SR, Leckband DE. Protein-induced changes in poly(ethylene glycol) brushes molecular weight and temperature dependence. *Langmuir* 2011;17: 7628–7636.
38. Luk YY, Kato M, Mrksich M. Langmuir. Self-assembled monolayer of alkane thiolates presenting mannitol groups are inert to protein adsorption and cell attachment. *Langmuir* 2000;16:9604–9608.
39. Ataman M. Properties of aqueous salt solutions of poly(ethylene oxide). Cloud points, θ temperatures. *Colloid Polym Sci* 1987;265:19–25.
40. Estephan ZG, Schlenoff PS, Schlenoff JB. Zwitteration as an alternative to PEGylation. *Langmuir* 2011;27:6794–6800.
41. Crepon B, Maillet F, Kazatchkine MD, Jozefonvicz J. Molecular weight dependency of the acquired anticomplementary and anticoagulant activities of specifically substituted dextrans. *Biomaterials* 1987;8:248–253.
42. Baranyi L, Szebeni J, Savay S, *et al.* Complement-dependent shock and tissue damage induced by intravenous injection of cholesterol-enriched liposome in rats. *J Appl Res Clin Exp Ther* 2003,3:3.
43. Montdargent B, Maillet F, Carreno MP, Jozefowicz M, Kazatchkine M, Labarre D. Regulation by sulphonate groups of complement activation induced by hydroxymethyl groups on polystyrene surfaces. *Biomaterials* 1993;14:203–208.
44. Montdargent B, Toufik J, Carreno MP, Labarre D, Jozefowicz M. Complement activation and adsorption of protein fragments by functionalized polymer surfaces in human serum. *Biomaterials* 1992;13:571–576.
45. Carreno MP, Labarre D, Jozefowicz M, Kazatchkine MD. The ability of Sephadex to activate human complement is suppressed in specifically substituted functional Sephadex derivatives. *Mol Immunol* 1988;25:165–171.
46. Salvador-Morales C, Basiuk EV, Basiuk VA, Green ML, Sim R. Effect of covalent functionalization on the biocompatibility characteristic of multi-walled carbon nanotubes. *J Nanosci Nanotechnol* 2007;8:2347–2356.
47. Chonn A, Cullis PR, Devine DV. The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. *J Immunol* 1991;146:4234–4241.

48. Salvador-Morales C, Zhang L, Langer R, Farokhzad OC. Immunocompatibility properties of lipid-polymer hybrid nanoparticles with heterogeneous surface functional groups. *Biomaterials* 2009;30:2231–2240.
49. Toda M, Hirata I. Effects of hydrophobicity and electrostatic charge on complement activation by amino groups. *ACS Appl Mater Interfaces* 2010;2:1107–1113.
50. Sperling C, Schweiss RB, Streller U, Werner C. *In vitro* hemocompatibility of self-assembled monolayers displaying various functional groups. *Biomaterials* 2005;26: 6547–6557.
51. Arima Y, Toda M, Iwata M. Complement activation on surfaces modified with ethylene glycol units. *Biomaterials* 2008;29:551–560.
52. Whaley K, North, J. Haemolytic assays for whole complement activity and individual components. In: Dodds AW, Sim RB (eds.), *Complement: A Practical Approach*, IRL Press, Oxford University Press, 1997, pp. 19–47.
53. Eelen MA, Roos A, Wieslander J, *et al.* Functional analysis of the classical, alternative, and MBL Pathways of the complement system: Standardization and validation of a simple ELISA. *J Immunol Methods* 2005;296:187–198.
54. Whaley K. Methods in complement for clinical immunologists. In: Whaley, D (ed.), *Measurement of Complement*, Churchill Livingstone, 1985, pp. 77–140.
55. Ferraz N, Karlsson-Ott M, Hong J. Time sequence of blood activation by nanoporous alumina: studies on platelets and complement activation. *Microsc Res Tech* 2010;73: 1101–1109.
56. Bertholon I, Vauthier C, Labarre C. Complement activation by core-shell poly (isobutylcyanoacrylate)-polysaccharide nanoparticles: Influences of surface morphology, length, and type of polysaccharide. *Pharm Res* 2006;23:1313–1323.
57 Szebeni J, Wassef NM, Spielberg H, Rudolph AS, Alving CR. Complement activation in rats by liposomes and liposome-encapsulated hemoglobin: Evidence for anti-lipid and antibodies and alternative pathway activation. *Biochem Biophys Res Commun* 1994;205:255–263.
58. Uetrecht J. Role of animal models in the study of drug-induced hypersensitivity reactions. *AAPS J* 2006;7:E914-E921.
59. Szebeni J, Fontana JL, Wassef NM, *et al.* Hemodynamic changes induced by liposomes and liposome- encapsulated hemoglobin in pigs: A model for pseudoallergic cardiopulmonary reactions to liposomes: Role of complement and inhibition by soluble CR1 and anti-C5a antibody. *Circulation* 1999;99:2302–2309.
60. Szebeni J. Complement activation-related pseudoallergy caused by amphiphilic drug carriers: the role of lipoproteins. *Curr Drug Delivery* 2005;2:443–449.
61. Moghimi MM, Hamad I, Bunger R, *et al.* Activation of the human complement system by cholesterol-rich and PEGylated liposomes-modulation of cholesterol-rich liposome-mediated complement activation by elevated serum LDL and HDL levels. *J Liposome Res* 2006;16:167–174.
62. Szebeni J, Wassef NM, Spielberg H, Rudolph AS, Alving CR. Complement activation in rats by liposomes and liposome-encapsulated hemoglobulin: Evidence for anti-lipid antibodies and alternative pathway activation. *Biochem Biophys Res Commun* 1994;205:255–263.
63. Szebeni J, Baranyi L, Savay S, *et al.* Complement activation-related cardiac anaphylaxis in pigs: Role of C5a anaphylatoxin and adenosine in liposome-induced abnormalities in ECG and heart function. *Am J Physiol* 2006;290:H1050-H1058.

64. Merkel OM, Urbanics R, Bedocs P, *et al. In vitro* and *in vivo* complement activation and related anaphylactic effects associated with polyethylenimine and polyethylenimine-graft-poly(ethylene glycol) block copolymers. *Biomaterials* 2011;32:4936–4942.
65. Szebeni J. Complement activation-related pseudoallergy: A new class of drug-induced immune toxicity. *Toxicology* 2005;216:106–121.
66. Sim RB, Willis R. Surface properties: Immune attack on nanoparticles. *Nat Nanotechnol* 2011;6:80–81.
67. Wu YQ, Qu H, Sfyroera G, *et al.* Protection of nonself surfaces from complement attack by factor H-binding peptides: Implications for therapeutic medicine. *J Immunol* 2011;186:4269–4277.