

**THE MOLECULAR BASIS OF COMPLEMENT
ACTIVATION BY MODEL NANOMEDICINES**

by

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ABSTRACT

Complement activation-related pseudoallergy (CARPA) is a term refers to those hypersensitivity reactions where the allergen can activate complement. This study examined the effect of nanomedicines and polyethylene glycol on complement activation. Single-walled carbon nanotubes, which have many medical applications as drug delivery systems, activated human complement independently of the C1q-dependent classical and the alternative pathways, as reflected by a significant rise in serum levels of S-protein bound form of terminal complex (SC5b-9) and C4d. This study also shows that polyethylene glycol (PEG) with different molecular weights was capable of activating complement. It was found that molecular weight of PEG plays an important role in activating both calcium sensitive and alternative pathways of the human complement system, with relatively higher molecular weight polymer resulting in more activation to a certain extent.

Methoxypoly(ethylene glycol), (mPEG),-grafted liposomes are known to exhibit prolonged circulation time in the blood, but these liposomes are believed to cause anaphylatoxin production through complement activation. Despite the general view that vesicle surface grafted with mPEG should suppress complement activation, this study shows that bilayer enrichment of non-complement activating liposomes (DPPC) vesicles with phospholipids-mPEG conjugate induce complement activation resulting in vesicle recognition by macrophage complement receptors. This work has successfully delineated the likely structural features of phospholipids-mPEG conjugate responsible for PEGylated liposome-induced complement activation in normal as well as C1q-deficient human sera, using DPPC vesicles bearing the classical as well as newly synthesized lipid-mPEG conjugates. With PEGylated DPPC vesicles, the net anionic charge on the phosphate moiety of phospholipids-mPEG conjugate played a key role in activation of both classical and alternative pathways of complement and anaphylatoxin production. Since methylation of the phosphate oxygen of phospholipids-mPEG conjugate, and hence the removal of the negative charge, totally prevented complement activation.

These findings provide a rational conceptual basis for development of safer vesicles for site-specific drug delivery and controlled release at pathological sites. This study has also examined liposome-mediated complement activation in human sera with elevated lipoprotein (LDL and HDL) levels, since abnormal or racial differences in serum lipid profiles seem to modulate the extent of complement activation and associated adverse responses. Cholesterol-rich (45 mol% cholesterol) liposomes activated human complement. However, liposome-induced rise of SC5b-9 was significantly suppressed when serum HDL cholesterol levels increased by 30%. Increase of serum LDL to levels similar to that observed in heterozygous familial hypercholesterolemia also suppressed liposome-mediated SC5b-9 generation considerably. While intravenous injection of cholesterol-rich liposomes into pigs was associated with an immediate circulatory collapse, the drop in systemic arterial pressure following injection of liposome preincubated with human lipoproteins was slow and extended. Therefore, surface-associated lipoprotein particles (or apolipoproteins) seem to lessen liposome-induced adverse haemodynamic changes, possibly as a consequence of suppressed complement activation *in vivo*. The presence of projected methoxypoly(ethylene glycol) chains did not interfere with generation of C3 opsonic fragments. Moreover, this study shows that PEG is not responsible for PEGylated liposome-mediated complement activation.

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List of abbreviations

Cardiac Output	CO
Central Venous Pressure	CVP
Complement Activation-Related Pseudoallergy	CARPA
Dipalmitoylphosphatidylcholine	DPPC
Dipalmitoylphosphatidylethanolamine	DPPE
Dipalmitoylphosphatidylglycerol	DPPG
Distearoylphosphatidylethanolamine	DSPE
Enzyme-Linked Immunosorbent Assay	ELISA
Ethylenebis (oxyethylene-trinitrilo)tetraacetic acid	EGTA
Ethylenediaminetetraacetic Acid	EDTA
High-Density Lipoprotein	HDL
Hypersensitivity Reactions	HSR
Left Ventricular End-diastolic Pressure	LVEDP
Low-Density Lipoprotein	LDL
Mannan-associated Serine Protease-1	MASP-1
Mannan-associated Serine Protease-2	MASP-2
Mannan-associated Serine Protease-3	MASP-3
Mannan-Binding Lectin	MBL
Membrane Attack Complex	MAC
Methoxy(Polyethylene Glycol)	mPEG
Multilamellar vesicles	MLV
Nuclear Magnetic Resonance	NMR
Phosphate-buffered Saline	PBS
Polyethylene-Glycol	PEG
Pulmonary Artery Pressure	PAP
Pulmonary Vascular Resistance	PVR
Radiocontrast Media	RCM
Reticuloendothelial System	RES
Secretary Phospholipase A2	sPLA2
Single-walled Nanotubes	SWNT
Systemic Arterial Pressure	SAP
Systemic Vascular Resistance	SVR
Thromboxane-A2	TXA2
Thromboxane-B2	TXB2

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Dedication

For my beloved parents, who offered me unconditional love and support throughout the course of this thesis. I am infinitely in debt to you.

Declaration

This work has not previously been accepted in substance for any degree and is not being currently submitted in candidature for any degree

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Statement one:

This thesis is the result of my own work and investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references

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Chapter one:

Introduction

1.1 Nanomedicines: hypersensitivity reactions, causative factors and optimization of design parameters

Advances in nanomedicine are beginning to transform the detection/diagnosis and treatment of disease, and are turning promising molecular discoveries into benefits for patients. Examples of nanomedicines (ranging in size from a few to hundreds of nanometers) that have been administered into the body include liposomes, polymeric nanospheres, micelles, late-generation dendrimers, protein cages, vault nanocapsules, composite nanoshells, iron oxide nanocrystals and carbon nanotubes (Moghimi *et al.*, 2005; Moghimi & Kissel, 2006). Some of these entities (e.g., liposomes and polymeric nanospheres) can efficiently encapsulate or incorporate drug molecules, thus following administration affording protection against drug degradation or inactivation en-route to the target site (Moghimi & Hamad, 2008). This may even result in a reduction of the amount of active agent needed to obtain a beneficial effect, and may effectively reduce drug-induced toxicity and other side effects (Moghimi *et al.*, 2005).

Encapsulation capacity, drug release profiles (over a period of days or even weeks), and biological performance of nanomedicines vary with parameters such as chemical make-up, morphology and size (Moghimi *et al.*, 2005). These parameters can be tailored in relation to the type, developmental stage and location of given disease. For instance, intravenously injected polymeric nanoparticles and liposomes are rapidly intercepted by the hepatic and splenic macrophages (Moghimi *et al.*, 2005). This propensity of macrophages for the phagocytic/endocytic clearance of foreign entities provides a rational approach to macrophage-specific delivery of drugs, toxins, antigens and diagnostic agents (Moghimi *et al.*, 2005). Indeed, liposome-mediated macrophage suicide (ie, delivery of macrophage toxins) has proved to be a powerful approach in removing

unwanted macrophages during gene therapy, or in autoimmune blood disorders or spinal cord injury (Moghimi & Hamad, 2008). Furthermore, nanomedicines can be engineered to be pH-sensitive or fusion-competent; these properties aid the release of their entrapped cargo from endo-lysosomal compartment into the cytosol within minutes of internalization (Drummond *et al.*, 2000; Panyam *et al.*, 2002). Alternatively, pharmacokinetic profiles of nanoparticles can be altered by tailoring their size, shape and surface properties (e.g., by polymer grafting), thus providing opportunities for targeting to non-macrophage sites (Moghimi *et al.*, 2001). For example, “macrophage-evading” drug carriers of ≤ 150 nm in size exhibit prolonged circulation times in the blood and may escape from the vasculature at sites where capillaries have open fenestrations or when the integrity of the endothelial barrier is perturbed by inflammatory processes or tumour growth (Moghimi & Hamad, 2008). Indeed, there are many reported cases for successful delivery of drugs and contrast agents with different long-circulating entities to the underlying parenchyma of solid tumours and injured arteries (Moghimi *et al.*, and 2001).

The biggest commercial opportunities in the nanomedicine sector are envisaged to arise from the development of multifunctional entities capable of simultaneously detecting and treating diseases, as well as for follow-up monitoring of particular pathology. Here, advances in development of composite metal nanoshells, iron oxide nanocrystals and carbon nanotubes have been of paramount importance (Moghimi & Kissel, 2006). As a result of their nanosize (and hence different atomic arrangements), these materials exhibit a wide spectrum of unique physical and chemical properties (Moghimi *et al.*, 2005; Moghimi & Kissel, 2006). Composite metal nanoshells, for example, consist of a spherical dielectric core of 20-80 nm (e.g., made from silica) surrounded by a thin metal shell of 5-20 nm (e.g., made from gold). In such entities, incident light can couple to the

Plasmon excitation of the metal; a process that involves the light-induced motion of all valence electrons. Consequently, the type of Plasmon that exists on the surface of a metallic nanoparticle is directly related to the shape and curvature of the nanoparticle. Hence, by controlling the relative thickness of the core and shell layers, the Plasmon resonance and resultant optical absorption properties can be adjusted near UV to mid-infrared (Hirsch *et al.*, 2006). Drugs can be incorporated within the nanoshell core, and the surface of nanoshell is further amenable for decoration with biological ligands and polymers for site-specific targeting. Such intravenous are more precise than chemotherapy (being targeted) and exert fewer side effects; they are also potentially less expensive and therefore more affordable to poorer countries (Moghimi & Hamad, 2008). However, as a consequence of their small size, composition and altered pharmacokinetics, nanomedicines may induce inflammatory reactions and adverse immune toxicity (Moghimi *et al.*, 2005) for instance, acute allergic reactions with symptoms that fit in Coomb and Gell's Type I category (but, which are initiated or mediated by pre-existing IgE antibodies) have been reported to occur following administration of clinically available nanomedicines (e.g., liposomes, micelles and polymeric nanospheres) (Sculier *et al.*, 1986; Laing *et al.*, 1994; Lavine *et al.*, 1991; Richardson *et al.*, 1997; Uziely *et al.*, 1995; Kris *et al.*, 1986; Grosen *et al.*, 2000; Katten *et al.*, 1992). An understanding of the causative and molecular basis of these reactions is of great importance, and could lead to better design and nanoengineering strategies for preventing hypersensitivity to future nanomedicines.

1.2 Nanomedicine as drug delivery: applications and possible complement-mediated hypersensitivity

Most of us are familiar with the best known of all immunological diseases, the allergies, usually called “hypersensitivities” by physicians. When a person has been immunologically prepared or sensitized to an antigen, either naturally or deliberately (vaccinated), immune response will not necessarily occur in later further exposure to the antigen. Tissue damage reactions can also occur in some cases. A simple definition of hypersensitivity reaction is an allergic disorder in which the body becomes hypersensitive to particular antigens, which provoke characteristic symptoms whenever they are subsequently encountered. Some hypersensitivity reactions are immediate and dramatic, such as sensitivity to insect stings or to drugs such as penicillin. Hypersensitivity reactions can be divided into four types: type I, type II, type III and type IV, based on the mechanisms involved and time taken for the reaction. Frequently, a particular clinical condition may involve more than one type of reaction. Types I–III are antibody-mediated and are distinguished by the different types of antigens recognized and the different classes of antibody involved. Type I hypersensitivity is also known as immediate, atopic or anaphylactic hypersensitivity and the reaction may cause a range of symptoms from minor inconvenience to death (as in asthma). Immediate hypersensitivity is mediated by IgE and produced by the immediate release of histamine, tryptase, arachidonate and derivatives by basophils and mast cells. This causes an inflammatory response leading to an immediate (within seconds to minutes) reaction. This reaction could be local or systemic. Mild irritation to sudden death from anaphylactic shock could occur subsequently. Diagnostic tests for immediate hypersensitivity include skin (prick and intradermal) tests, measurement of total IgE and specific IgE antibodies against the

suspected allergens. Treatment usually involves antihistamines and corticosteroids. Some examples of type I hypersensitivity reactions include: allergic asthma and rhinitis, anaphylaxis and urticaria.

Type II hypersensitivity reactions (also known as cytotoxic hypersensitivity) involve immunoglobulin G or immunoglobulin M antibodies bound to cell surface antigens to form complexes that activate the classical pathway of complement activation for eliminating cells presenting foreign antigens (which are usually pathogens). These mediators of acute inflammation generated at the site and membrane attacking complex (MAC) cause cell lysis and death. The reaction time varies from minutes to hours up to a day. Diagnostic tests include detection of circulating antibody against the tissues involved and the presence of antibody and complement in the lesion (biopsy) by immunofluorescence. Treatment involves anti-inflammatory and immunosuppressive agents. Some examples of type II hypersensitivity reactions include: Autoimmune haemolytic anaemia, immune thrombocytopenia and transfusion reactions.

Type III reactions (also known as immune-complex reactions) involve circulating antigen-antibody soluble immune complexes. They are mostly of the IgG class, although IgM may also be involved. In this case, the antigen is soluble and not attached to the cells involved. Primary components are soluble immune complexes and complement (C3a, 4a and 5a). These soluble immune complexes may trigger an immune response according to the classical pathway of complement activation. The reaction may take 3 - 10 hours after exposure to the antigen (as in Arthus reaction). Diagnosis involves examination of tissue biopsies for deposits of Ig and complement by immunofluorescence. Treatment includes anti-inflammatory agents. Some examples of type III hypersensitivity reactions involve: rheumatoid arthritis, serum sickness, symptoms of malaria and Arthus reaction.

The fourth and last type IV of reactions (usually called delayed hypersensitivity reactions as the reaction takes two to three days to develop, cell-mediated immunity) are unlike the other types mediated by antibodies but are type of T cells-mediated responses. Damages in delayed hypersensitivity include T lymphocytes and monocytes and/or macrophages. CD8 cytotoxic T cells and CD4 helper T cells recognize antigen in a complex with either type 1 or 2 major histocompatibility complex. Macrophages here play the role of antigen-presenting cells which release a group of cytokines known as interleukin 1, which stimulates the proliferation of further CD4 cells. These cells release interleukin 2 and interferon gamma, further inducing the release of other Type 1 cytokines, thus mediating the immune response. Activated CD8 cells destroy target cells on contact while activated macrophages produce hydrolytic enzymes and, on presentation with certain intracellular pathogens, transform into multinucleated giant cells. *In vitro* tests for delayed hypersensitivity include mitogenic response, lympho-cytotoxicity and IL-2 production. While *in vivo* diagnosis includes delayed cutaneous reaction (*e.g.* Montoux test) and patch test (for contact dermatitis). Treatment in this type of hypersensitivity involves some immunosuppressive agents and corticosteroids.

The agents released from mast cells include histamine, tryptase, proteoglycans, other chemotactic factors and leukotrienes. Mast cells may be triggered by other stimuli such as exercise, emotional stress, chemicals (*e.g.*, photographic developing medium, calcium ionophores, codeine, *etc.*), anaphylotoxins (*e.g.*, C4a, C3a, C5a, *etc.*). These reactions, mediated by agents without IgE-allergen interaction, are not hypersensitivity reactions although they produce the same symptoms.

Some chemicals are known to cause an unusual hypersensitivity syndrome in certain population despite the intense premeditation and awareness being taken. These reactions are severe, life-threatening in up to 1-3% of patients with occasional deaths mostly due to cardiac arrest (Rowinsky *et al.*, 1992; 1993b; 1995; Kris *et al.*, 1986; Laher & karp, 1997; Tsavaris *et al.*, 1998; Bookman *et al.*, 1997; Hamad & Moghimi, 2008). Szebeni referred to these hypersensitivity reactions where the allergen can activate complement as complement activation-related pseudoallergy (CARPA) (Szebeni *et al.*, 1999 and 2000). This phenomenon is increasingly recognized as an immune toxicity issue that has particular significance in the modern field of pharmaceutical nanotechnology (Hunter & Moghimi, 2002). Known examples of pseudoallergy include the reactions caused by radiocontrast media (RCM), nonsteroidal anti-inflammatory drugs, analgetics, morphine and insect venoms, liposomes and micellar solvents, such as Cremophor EL (CrEL) in Taxol. While there is no known common underlying cause for most of these reactions, there is substantial evidence suggesting that the reactions caused by RCM, liposomes and CrEL have a common trigger mechanism: complement activation (Szebeni, 2005). Symptoms of CARPA are the same as seen in common allergy or classical type I Ig-E-mediated reactions, while others are unique to complement activation. The most important distinguishing feature of CARPA is the lack of presensitization and reinforcement, i.e., the reaction arises at the first exposure to the drug and then it may lessen or disappear, rather than increases upon later treatment (Szebeni, 2005). The increased awareness of (CARPA) is also reflected by the fact that testing for complement activation *in vitro* and/or *in vivo* has become one of the immuno-toxicology tests recommended by the US food and Drug Administration (FDA) that may be useful to identify the pseudoallergy potential of drugs, when needed (Szebeni, 2005).

Perhaps a good example of CARPA is paclitaxel (Taxol), one of the most widely used anticancer drugs today. Taxol is a lipophilic drug which is dissolved in cremophor EL to induce solubility upon administration. Taxol is a natural product that shows clinical antitumour activity against some otherwise nonresponsive solid tumours (Slichenmyer & Von Hoff, 1991; Gregory & Delisa, 1993). With its unique mechanism of action as a stabilizer of tubulin polymerization, Taxol has become an important agent in chemotherapy (Szebeni *et al.*, 1998). Taxol formulation is known to cause an unusual hypersensitivity reaction in about 2-7% of patients, characterized by dyspnea, flushing, rash, chest pain, tachycardia, hypotension, angiodema, and generalized urticaria (Rowinsky *et al.*, 1992; 1993a; 1993b; 1993c; 1994; Sharma *et al.*, 1993). The mechanism of induction of hypersensitivity reactions in response to Taxol administration has been linked to complement activation caused by its vehicle, i.e. CrEL (Szebeni *et al.*, 1998; Kessel *et al.*, 1995). Complement activation by Taxol or its vehicle may provide an explanation for the above anomaly in as much as it results in the production of anaphylatoxins (C3a and C5a), which, directly and through various secondary mediators can cause almost all symptoms of immediate hypersensitivity reactions (Hugli; 1984, Marceau *et al.*, 1987; Mousli *et al.*, 1992). In addition, complement activation explains the symptoms, which are uncommon with IgE-mediated allergy, such as the pulmonary infiltration (Ramanathan *et al.*, 1996), hypertension (Nannan Panday *et al.*, 1997) and severe cardiac arrhythmias (Laher & Karp, 1997).

1.2.1 Non-ionic polymers

In recent years the administration of several types of ethylene oxide (EO)-based nonionic surfactants, such as cremophor EL (CrEL) (as mentioned earlier) and poloxamers, has been generally used in parenteral drug delivery (Wang & Hanson, 1987; Eisenman & Bauer, 1995; Botempo, 1997; Nema *et al.*, 1997; Soderlind *et al.*, 2003). Present studies have demonstrated that cremophor EL (Szebeni *et al.*, 1998) and block copolymers of poloxamer 188 (Moghimi *et al.*, 2004) can induce hypersensitivity reactions. Those reactions are believed to arise as a result of complement activation-related pseudoallergy (CARPA).

Poloxamers are a family of non-ionic surfactants composed of a central polypropylene oxide chain flanked at either side by a polyethylene oxide segment, thus forming an ABA block co-polymer structure (Moghimi & Hunter, 2000). Among the members of the poloxamer family, poloxamer 188 and 407 have found many applications in experimental and therapeutic medicine. For example, poloxamer 188 has been used in the development of fluorocarbon-based artificial blood substitutes (e.g., Fluosol-DA) (Vercellotti *et al.*, 1982). Poloxamer 407 at a concentration of 250 mg/ml can form a thermoreversible gel; the polymer is in aqueous solution at temperatures below 5 °C, but will gel at higher temperatures due to micellization and micelle entanglements (Cabana *et al.*, 1997). As a result of its thermo-gelation properties, poloxamer 407 was recently used for reversible occlusions during percutaneous endovascular procedures (Poloxamer 407 forms a gel in concentrations above 200 mg/ml at 37 °C) (Raymond *et al.*, 2004). Adverse reactions to poloxamers have also been noted, which again may be linked to complement activation. For example, infusion of Fluosol-DA into rabbits and dogs has been associated with transient, profound haemodynamic collapse accompanied by leukopenia and

thrombocytopenia (Vercellotti *et al.*, 1982; Faithfull & Cain, 1988). And the effects were reproducible following administration of relevant quantities of poloxamer 188 (Vercellotti *et al.*, 1982). In addition, poloxamer 188 was capable of activating complement in rabbit plasma via the alternative pathway (Vercellotti *et al.*, 1982). In a clinical study, symptoms of complement activation related pseudoallergy were also reported in up to 30% of patients receiving Fluosol-DA as an adjunct to radiation treatment for advanced squamous tumours of the head and neck (Lustig *et al.*, 1989).

Recently, Moghimi *et al.* demonstrated that both poloxamer 188 (at sub-micellar concentrations) and 407 (in micellar form) can activate human complement via calcium-sensitive as well as alternative pathways (CMC of poloxamer 188 at 37 °C was 24-32 mg/ml) (Moghimi *et al.*, 2004). Poloxamer-mediated complement activation, however, is an intrinsic property of the polymer, and is independent of the degree of sample polydispersity or the presence of trace organic volatile (Moghimi *et al.*, 2004). The mechanisms of poloxamer-mediated complement activation are still poorly understood, but activation of the alternative pathway may be related to inhibition of factors H and I. However, direct interaction between poloxamer and C3 or C4, which could lead to generation of products resembling activated C3 and C4 is unlikely, since complement activation by poloxamer was totally abolished in the absence of sCR1, an inhibitor of C3/C5 convertases (Moghimi *et al.*, 2004).

Quasi-elastic light scattering studies have recently confirmed poloxamers favourably interact with both HDL and LDL (Moghimi *et al.*, 2004). For example, poloxamer 188 caused a slight but significant increase in the hydrodynamic size of human HDL particles. The hydrodynamic size distribution of HDL and LDL were in agreement with their expected size (8-11 nm for HDL and 20-30 nm for LDL). Remarkably, the presence of

poloxamer 188 generated two distinct populations of particles from LDL; one population of particles of 10-20 nm in size and a second population with particles in the range of 30-50 nm (Moghimi *et al.*, 2004). It is not clear whether these structures can activate complement, but elevation of serum HDL and LDL levels significantly reduced poloxamer 188-mediated rises of SC5b-9 (Moghimi *et al.*, 2004). These observations are in line with the reported low incidence of poloxamer-mediated hypersensitivity reactions in subjects with abnormal or elevated lipid profiles (Vercellotti *et al.*, 1982; Kent *et al.*, 1990). On basis of these studies it is imperative to examine possible regulatory and modulatory roles of lipoproteins and apolipoproteins on complement system during health and chronic inflammatory conditions.

1.2.2 Polyethylene Glycol

Poly(ethylene glycol) or PEG is a neutral, hydrophilic polyether which exhibits little reactivity unless modified with functional groups. PEG has a simple molecular structure, $\text{OH}-(\text{CH}_2-\text{CH}_2-\text{O})_n-\text{H}$, it is made from ethylene oxide and is similar to some non-ionic detergents. PEG and polyethylene oxide (PEO) are polymers that have an identical structure except for chain length and end groups, and are the most commercially important polyethers. PEG refers to the polymer with molecular weight less than 50,000 (a condensation polymer made from ethylene glycol), while polyethylene oxide is used for higher molecular weights (an addition polymer). PEG is a water-soluble polymer used to solubilize proteins and is biologically inert and considered not toxic (Borgens & Shi, 2000; Jumaa *et al.*, 2002).

PEG has many uses and applications including those related to its use as a non-toxic, non-immunogenic lubricant or carrier in pharmaceutical formulations. PEG has been

widely used in parenteral drug delivery. Jumaa *et al.*, (2002) have used PEG in parenteral lipid emulsions as targeting systems for poorly water-soluble drugs. Choe *et al.* (2004) have found that branching of the PEG allowed greater loading of the active drug when administered parenterally. Also, PEG has been used in long-circulating parenteral emulsions (Reddy & Venkateswarlu, 2005). PEGylation of therapeutic proteins reduces renal clearance rates and protects from proteolytic and other degradation often resulting in enhanced medical efficacy. Other benefits of PEGylation may include improved physical and thermal stability, as well as solubility. The latter is particularly important with regard to biopharmaceutical formulation and delivery (Fee & Alstine, 2006). PEG has been used to fuse the membrane of numerous cells in culture, producing giant multinucleated ones (Pontecorvo, 1975). Moreover, PEG precipitation is the basis for several techniques for the detection and measurement of circulating immune complexes and considered widely safe (Hyun *et al.*, 1992; Soltis *et al.*, 1983; Rayner *et al.*, 1981). Recently, Borgens and Shi, (2000) reported that a 2-min application of PEG parenterally led to immediate recovery from spinal cord injury through molecular repair of nerve membranes in animals. To date, there are no studies exploring the margins of safety of PEG after parenteral administration. This work will examine the toxicity of different grades of PEG and their possible mechanism of activating human immune responses.

1.2.3 Stealth liposomes

Liposomes are vesicular structures that form on hydration of dry phospholipids above their transition temperature and that are classified on the basis of their size and number of bilayers (Moghimi *et al.*, 2005). Drug molecules can be either entrapped in the aqueous space or intercalated into the lipid bilayer of liposomes, depending on the physiochemical

characteristics of the drug. Recently, a lot of work has been focused on developing long circulating vehicles with size within the nanoscale range. Surface stabilization of nanoparticles and liposomes with a range of non-ionic surfactants or polymeric macromolecules has been one of the most successful approaches for keeping the particles in the blood for prolonged periods of time (Moghimi *et al.*, 2001; Moghimi & Hunter, 2000; Monfardini & Veronese, 1998). Surface enrichment of nanocarriers with non-ionic surfactants can be achieved by physical adsorption, incorporation during production of the carriers or by covalent attachment to any reactive surface groups (Moghimi & Szebeni, 2003). The presence of such surfactants on the surface of the particle strongly reduces interparticulate attractive Van der Waals forces while increasing the repulsive barrier between two approaching particles. This steric mechanism of stabilization involves an elastic as well as an osmotic contribution (discussed in details by Moghimi & Szebeni, 2003) (deGennes, 1987; Moghimi *et al.*, 1993; Lasic *et al.*, 1991).

It is generally known now that the macrophage-resistant property of polymer-grafted particles is due to suppression of surface opsonization by serum plasma proteins (Moghimi *et al.*, 2001 and 1993). Therefore, it is not surprising that polymer-grafted particles exhibit prolonged residency times in the blood. Contradictingly, studies have shown that opsonization of sterically protected particles can occur efficiently. One example is complement activation by long-circulating liposomes, which results in surface opsonization with C3b and iC3b (Szebeni, *et al.*, 2000; 2002; 2001).

Following intravenous injection, liposomes and nanoparticles are cleared rapidly from the blood by the RES, particularly the hepatic Kupffer cell (Moghimi *et al.*, 2001). Conversely, stealth liposomes circulate for prolonged hours; reported half-lives vary from 2 to 24 h in mice and rats and can be as high as 45 h in humans depending on the particle

size and the characteristics of the coating polymer (Moghimi *et al.*, 2001; Allen *et al.*, 1991; Gabizon & Muggia, 1998). However, a rapid hepatic and splenic deposition of a fraction of the administered long circulating liposomes has been reported. For example, stealth liposomes (e.g. PEGylated vesicles) have been administered in doses ranging from 0.1 to 400 $\mu\text{mol/kg}$ body weight with reported hepatic and splenic sequestration of 10-15 nmol/kg within the first hour of injection (Laverman *et al.*, 2000; 2001; Allen *et al.*, 1991). Recently, biophysical studies have established that the bilayer concentration of mPEG-lipid controls conformation of the surface exposed mPEG chains (Triosh *et al.*, 1998). For example, with incorporation of 5-7 mol% mPEG2000-phospholipid into liposomal bilayer, the exposed mPEG chains predominantly assume a “mushroom-brush transition” conformation, whereas at concentrations of up to 4 mol% mPEG projections are in a “non-overlapped mushroom” regime (Triosh *et al.*, 1998). It is also known that particles with surface exposed mPEG2000-5000 chains in predominantly “mushroom brush” conformation are highly resistant to clearance by quiescent macrophages, whereas particles with “non-overlapped mushroom” display remain susceptible to ingestion by phagocytic cells (Gbadamosi *et al.*, 2002; Moghimi & Szebeni, 2003).

1.2.4 Carbon nanotubes

The complement system is an important effector arm of both innate and acquired immunity and can be triggered by three separate pathways: the classical, the lectin and the alternative pathway (Lambris *et al.*, 2008). Classical pathway activation is triggered when the hexameric C1q subcomponent of C1 binds to an activator. This interaction induces a conformational change within the C1 complex resulting in conversion of the zymogen C1s subcomponent into an active proteolytic enzyme that cleaves C4 (Lambris

et al., 2008). The lectin pathway of complement activation is initiated by the binding of mannan-binding lectin (MBL) or ficolins to an activator leading to activation of MBL-associated serine proteases (MASPs) (MASP-1, -2, -3) and a smaller protein sMAP (Map19) (Matsushita *et al.*, 2000; Vorup-Jensen *et al.*, 2000). Among these proteases, MASP-2 has an established role in cleaving and activating C4 (Vorup-Jensen *et al.*, 2000). Conversely, the alternative pathway is stimulated by the spontaneous cleavage of thioester bond in C3 or when the internal thioester bond in the α -chain of C3b undergoes nucleophilic attack in the presence of a foreign surface structure rich in nucleophilic groups (particularly hydroxyl- and amino-rich surfaces (Lambris *et al.*, 2008; Toda *et al.*, 2008).

Carbon nanotubes have received substantial attention as promising materials for a wide range of experimental diagnostic and therapeutic applications following intravenous injection (Lacerda *et al.*, 2006; Liu *et al.*, 2007 and 2008). However, the interaction between carbon nanotubes and the complement system has not received much attention. An isolated study with non-functionalized high pressure carbon monoxide single-walled carbon nanotubes (HIPco SWNTs) and double-walled carbon nanotubes (DWNTs) has revealed nanotube-mediated complement activation in human serum (Andersson *et al.*, 2002). Remarkably, activation pathway was dependent on nanotube morphology; while both hydrophobic HIPco SWNTs and DWNTs activated human complement system via the classical pathway through exclusive surface adsorption of C1q, DWNTs further activated complement through the alternative pathway (Andersson *et al.*, 2002). In order to enhance nanotube stability in the blood and suppress their rapid clearance by kupffer cells and splenic macrophages following intravenous injection, SWNTs were surface modified with PEG phospholipids (Liu *et al.*, 2007). However, it has now emerged that

PEGylated nanoparticles are still capable of activating complement (Salvador-Morales, *et al.*, 2006). This study will attempt to focus more on the interaction of these carbon nanotubes with the complement system.

1.3 Fate of intravenously injected nanoparticles

Colloidal carriers in the form of emulsions, liposomes or nanoparticles can have utility for the delivery of drugs to the systemic circulation. Normally, a foreign particle upon intravenous injection will be coated by blood components (opsonisation) and rendered 'visible' to the defence system of the body; the reticuloendothelial system (RES) (Göppert & Müller, 2005), which is extremely efficient at removing particles recognised as foreign. As a consequence, these systems are very efficient in targeting of drugs or diagnostic agents to the RES and briefly reviewed below (Moghimi *et al.*, 2001 and 2005). These processes are however controlled by particle size and surface characteristics.

Macrophages are widely distributed and strategically placed in many tissues of the body to recognize and clear, altered and senescent cells, invading particulates, as well as macromolecular ligands via a multitude of specialized plasma membrane receptors (Gordon *et al.*, 1986).

This propensity of macrophages for endocytosis/phagocytosis of foreign particles in the past has provided an opportunity for the efficient delivery of therapeutic agents to these cells in the treatment of certain infectious diseases. This was achieved based on targeting pathogenesis or microbial diseases in macrophages (Agrawal & Gupta, 2000) with the aid of colloidal drug delivery systems usually in the form of liposomes, polymeric nanospheres, micelles, and oil-in-water emulsions (Moghimi *et al.*, 2001). Apart from

therapeutic goals, colloidal carriers have proved to be useful for diagnostic purposes, for example, to assess macrophage phagocytic and clearance functions. Similarly, particulate colloids tagged with a suitable radiopharmaceutical or contrast agent were shown to be helpful for imaging certain pathologies e.g., deep-seated tumors, not via targeting but through macrophage loading (Kostarelos & Emfietzoglou, 1999).

The rapid recognition and sequestration of intravenously injected colloidal carriers such as nanospheres from the blood by RES (hepatic midzonal and periportal kupffer cells) is problematic for efficient targeting of drug carriers or diagnostic agents to a desired macrophage population e.g., splenic red pulp macrophages as well as to nonmacrophage sites (Moghimi *et al.*, 2001; Göppert & Müller, 2005).

As a result, there has been a growing interest in the engineering of long-circulating colloidal carrier systems that upon intravenous injection avoid rapid recognition by RES and adequately remain in blood circulation.

1.4 The influence of surface PEGylation on complement activation

Liposomes are increasingly used in medicine for targeted or controlled release of various drugs and diagnostic agents. However, studies have shown that these liposomes can cause hypersensitivity reactions with symptoms corresponding to CARPA. The frequency of HSRs to liposomal drugs shows large variation between 3 and 45% (Szebeni *et al.*, 1998 and 2001). Complement activation by liposomes has been analysed in a great number of studies. The results are somehow complex, as variations in liposome structure and/or other experimental conditions might result in huge difference in the extent, kinetics and the pathway of activation (Szebeni *et al.*, 1998 and 2001).

Bradley *et al.*, (1998) have examined the ability of PEG-lipids to inhibit the *in vitro* activation of complement in human serum. 100 nm liposomes composed of egg phosphatidylcholine, cholesterol and cardiolipin (35:45:20 molar ratio) were shown to activate complement system resulting in 80% complement consumption at liposome concentrations above 1 mM. Conversely, these investigators suggested that complement activation can be virtually abolished by incorporating 5-7.5 mol% of PE-mPEG₂₀₀₀ (phosphatidylethanolamine methoxypoly(ethylene glycol 2000)) into the liposomal bilayer. However, Moghimi and Szebeni (2003) in their review suggested that a closer look at the above study reveals that the inhibitory effect of the mPEG-lipid on complement activation is highly dependent on the liposome concentration used in the assay. Complement consumption was significantly above the baseline at liposome concentrations above 4 mM (Bradley *et al.*, 1998). For instance, incubation of 4, 8 and 15 mM of liposomes (containing 5 mol% PE-mPEG₂₀₀₀) with human serum for 30 min led to 20, 35 and 40% complement consumption, respectively. This claim was supported by series of studies by Szebeni who proved that the inclusion of 5% PE-mPEG₂₀₀₀ into liposomal bilayer may not be sufficient to prevent complement activation. For example, incubation of Doxil[®] (a PEGylated liposome with encapsulated anticancer agent; doxorubicin with a clinical dose calculated on the basis of patient weight and surface area and infused over a few hours) with 10 different normal human sera led to significant activation of complement levels over control (phosphate buffer) in 7 sera (Szebeni *et al.*, 2000). Doxil[®] was effective at concentration as low as 0.4 mg lipid/ml in activating complement. Therefore, Doxil[®] can cause significant complement activation *in vitro*, although the extent of this activation may substantially vary in different individuals.

Recent study by Mosqueira *et al* (2001) has also demonstrated that surface modification on polymeric nanocapsules with PE.G., regardless of the PEG chain length or density, cannot totally prevent complement activation. However, longer chains PEG were more effective in suppressing complement activation (Mosqueira *et al.*, 2001).

1.5 Opsonization and phagocytosis

Opsonization is a process by which the surface of protein particles are altered so that they are more readily and more efficiently engulfed and removed by macrophages of the RES (Donald & Nicholas, 2006).

Thus, the clearance of opsonised particles by macrophages involves binding to various receptors such as Fc receptors, complement receptors, engulfment by lamellipodia that project from the cell surface, internalization by zipper process and subsequent delivery of the particles to acidic endosomes and finally to lysosomes for degradation (Moghimi *et al.*, 2001).

Examples of opsonic molecules includes various subclasses of immunoglobulins, complement proteins like C1q and C3 fragments (C3b and iC3b), apolipoprotein, von willebrand factor, thrombospondin, fibronectin and mannose-binding protein (Szebeni, 1998). There are five known types of complement receptors (CR1, CR2, CR3, CR4 and C1qR), expressed on macrophage, monocytes and leukocytes. Nanoparticles (e.g., liposomes) can be intentionally opsonised with non-specific and monoclonal antibodies in order to enhance their recognition and clearance by macrophages via their Fc receptors both *in vivo* and *in vitro* (Rogers & Basu, 2005).

Moghimi and Patel (1998) have also found that rat serum contains two proteins which suppress recognition of liposomes by kupffer cells. These proteins are classified as

dysopsonins. Dysopsonins act by decreasing opsonisation and/or inactivating liposome-bound opsonins. Therefore, a balance between opsonins and dysopsonins could regulate the rate and quantity of liposome clearance from the blood by kupffer cells. These dysopsonins and their mode of action need to be identified, as they can be used in designing long circulatory systems.

It is crucial to note that complement activation can lead to cleavage of C3 into C3b, iC3b and C3a. However, production of C3a is thought to cause anaphylactic reaction in some individuals (Hunter & Moghimi, 2002).

1.6 Over view of the complement system

When the “immune system” is mentioned, attention is focused on the antibodies and B- and T-lymphocytes which guard human body against pathogens. Complement system, on the other hand, is a very important system in both acquired and innate immunity. The complement system consists of approximately 30 molecules consisting nearly 10% of the total serum protein, and forming one of the major defence systems of the body. The functions of the system include control of inflammatory reactions, clearance of immune complexes, cellular activation and anti-microbial defence. The system also plays a role in the development of the immune response and is a major effector system in autoimmune and hypersensitivity reactions. The ultimate result of complement activation is the formation of membrane attacking complex (MAC). MAC is a large complex that induces a pore in the membrane of the invading microorganisms. This results in the loss of membrane integrity and destroys the pathogen ability to control the concentration of the intracellular metabolites (Woodle, 1998). Activation of the complement pathways occurs via two enzyme cascades in which active components enzymatically cleave the following

component(s), the trigger being either antigen-antibody complexes (classical and lectin pathways) or a variety of surfaces favouring alternative-pathway activation (Fig. 1.1).

1.6.1 The classical pathway

The classical pathway of activation of the complement system is a group of blood proteins that mediate the specific antibody response. It is triggered by antigen-bound antibody molecules. Binding of a specific part of the antibody molecule to the C1 component initiates this pathway. The classical pathway is triggered by activation of the C1-complex, which consists of one molecule C1q and two molecules C1r and C1s, either by C1q's binding to antibodies from classes M and G, complexed with antigens, or by the binding C1q to the surface of the pathogen. C1q possesses no intrinsic activity, but when any of several activators bind to the C1q subcomponent of C1, the homologous C1r and C1s subcomponents are converted into catalytically active species, namely C1r and C1s triggering the first step of the classical pathway of complement activation. Then they cleave C1s (another serine protease). The C1-complex now binds to and splits C2 and C4, producing C2a and C4b. C4b and C2a bind to form C3-convertase (C4b2a complex) (Fig. 1.1). production of C3-convertase signals the end of the classical pathway, but cleavage of C3 by the enzyme leads to the start of the alternative pathway.

1.6.2 The alternative pathway

The alternative pathway of the complement system is a humoral component of the immune system's natural defense against infections which can operate without antibody participation. The alternative pathway is triggered by C3 hydrolysis directly on the

surface of pathogen. In this pathway, C3 split into C3a and C3b. Some of the C3b is bound to the pathogen where it will bind to factor B, this complex will then be cleaved by factor D into Ba and the alternative pathway C3-convertase, Bb. After hydrolysis of C3, C3b complexes to become C3b2a3b, which cleaves C5 into C5a and C5b. C5a and C3a initiate inflammatory reactions because they are both chemotactic factors for phagocytes and anaphylatoxins that cause mast cells to degranulate. C5b with C6, C7, C8 and C9 (C5b6789) complex to form the membrane attack complex (MAC) which is inserted into the cell membrane (bunches a hole) to initiate cell lysis.

1.6.3 The lectin pathway

This pathway is activated by binding mannan-binding lectin to mannose residues on the pathogen surface, which activates the mannan-binding lectin (MBL)-associated serine proteases, MASP-1, MASP-2, MASP-3, which can then split C4 and C2 into C4b and C2b. C4b and C2b then bind together to form C3-convertase, as in the classical pathway.

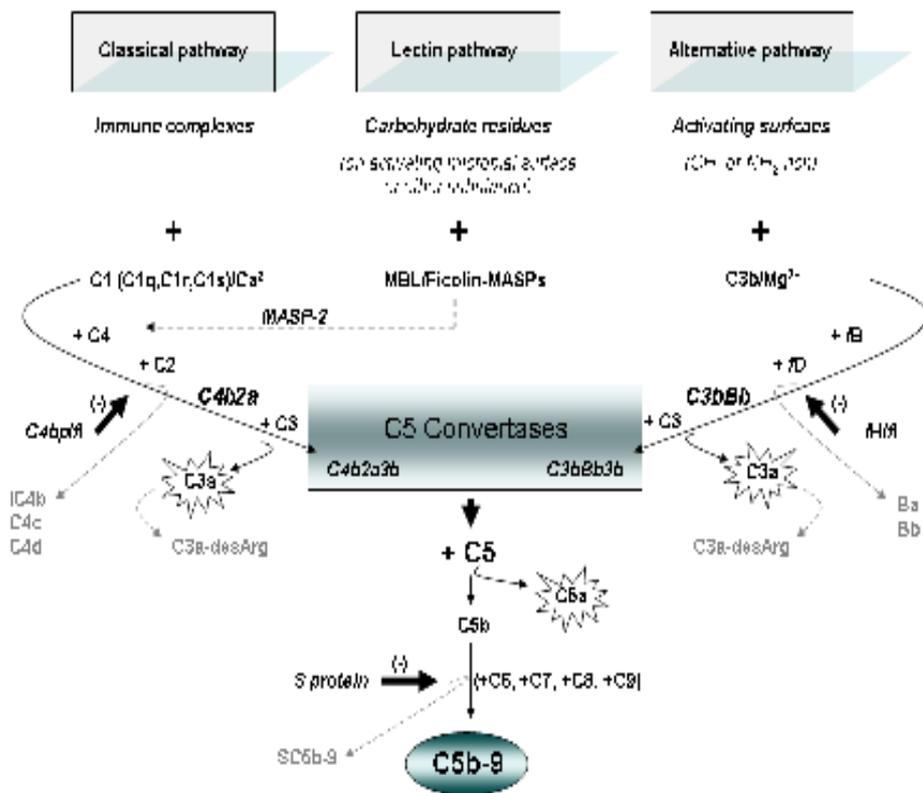


Fig. 1.1 Complement activation pathways. The complement system can be triggered by three separate pathways: the classical, the lectin and the alternative pathways. Classical pathway activation is triggered when the hexameric C1q subcomponent of C1 binds to an activator (e.g., immunocomplexes or a surface). This induces a conformational change within the C1 complex resulting in conversion of the zymogen C1s subcomponent into an active proteolytic enzyme that subsequently activates the C1s subcomponent. Activation of the lectin pathway proceeds following the binding of mannose binding lectin (MBL) or ficolins to an activator, leading to conversion of their MBL-associated serine proteases (MASPs)-1, -2, -3 zymogens to active proteolytic enzymes. Activated C1s of the classical pathway and the MASP-2 of the lectin pathway both cleave the C4 protein; this in turn activates C2 thus leading to subsequent generation of the classical/lectin pathway C3 convertase, C4b2a. Alternative pathway activation proceeds following spontaneous and induced C3 hydrolysis. This subsequently generates alternative pathway C3 convertases (C3bBb) through mediations of factors B and D. Three consequences subsequently arise from complement activation. These include priming of the activator surface by opsonic complement fragments (e.g., C3b, iC3b) for engulfment by phagocytes, generation of anaphylatoxins and chemoattractants (e.g., C3a, C5a) and formation of the membrane attack complex (C5b-9) through C5 convertases. Key negative regulators (C4bp, factor I, factor H, S protein) are also shown. Anaphylatoxin C3a is very short lived and in serum is cleaved rapidly to the more stable C3a-desArg.

1.7 Aims of the project

This study was undertaken to examine the hypersensitivity reactions caused by nanomedicines based on complement activation. The aims of the project were generally as follows:

- To Investigate and identify which structural features of phospholipid-mPEG conjugate are responsible for complement activation, and to design a novel conjugate that when incorporated to liposomes, suppress complement activation.
- To examine whether the elevation of human serum lipoproteins levels can exert a protective role against cholesterol-rich liposome-mediated complement activation both *in vitro* and *in vivo*.
- To study the safety of different molecular weights of PEG regarding hypersensitivity reactions. This included full understanding of possible activation pathways, effect of molecular weights, and different molar concentrations.
- To investigate the interaction of amino-PEG₅₀₀₀-distearoylphosphatidylethanolamine (amino-PEG₅₀₀₀-DSPE) and methoxy(MeO)PEG₅₀₀₀-DSPE functionalized SWNTs with the complement system both *in vitro* and *in vivo*.

Chapter two:

Methylation of the phosphate oxygen moiety of phospholipid-methoxy(polyethylene glycol) conjugate prevents PEGylated liposome-mediated complement activation and anaphylatoxin production

2.1 Introduction

The rapid sequestration of intravenously injected liposomes by macrophages in contact with blood is problematic for efficient targeting of particulate nanocarriers to non-macrophage cells at pathological sites (Moghimi *et al.*, 2001). Surface manipulation of liposomes with methoxy(polyethylene glycol), (mPEG), affords control over vesicle interaction and fate within biological systems (Moghimi *et al.*, 2001 and 2005). mPEG grafting suppresses liposome-macrophage interaction either directly or through reduced surface opsonization or both (Moghimi *et al.*, 2001). The extent of vesicle opsonization and vesicle-macrophage interactions are controlled by mPEG chain length and surface density. For example, vesicles bearing 5–7 mol% of phospholipid-mPEG2000 conjugate are usually resistant towards macrophage recognition. With shorter mPEG chain length (eg, 350-1000 Da), bilayer enrichment with 10–25 mol% phospholipid-mPEG is necessary in order to avoid rapid macrophage phagocytosis (Moghimi *et al.*, 2001). As a result of such surface manipulation strategies with mPEG the engineered vesicles exhibit prolonged residency time in the blood circulation and can escape from the vasculature. Vesicle extravasation, however, is restricted to capillaries with open fenestrations or sites where the endothelial barrier of blood capillaries is perturbed by inflammatory processes or by dysregulated angiogenesis (Moghimi *et al.*, 2001). Indeed, the first generation of long-circulating PEGylated liposomes with encapsulated doxorubicin are already on the USA and European markets for management and treatment of AIDS-related Kaposi's sarcoma, refractory ovarian cancer and metastatic breast cancer (Allen & Cullis, 2004).

One of the most frequently encountered clinical problems following infusion of PEGylated liposomes into certain subjects is initiation of non-IgE-mediated hypersensitivity reactions, which includes symptoms of cardiopulmonary distress, such as dyspnea, tachypnea, tachycardia, chest pain, hypertension, and hypotension (Uziely *et al.*, 1995; Alberts & Garcia, 1997; Chanan-Khan *et al.*, 2003). These pseudoallergic reactions are strongly believed to arise through rapid production of anaphylatoxins C3a and C5a via complement activation leading to the release of TXA2 and other anaphylatoxin-derived mediators (Chanan-Khan *et al.*, 2003; Szebeni *et al.*, 2000). In spite of the general view that surface camouflaging with mPEG should dramatically suppress blood opsonization processes, liposomes bearing phospholipid-mPEG conjugates in their bilayer surprisingly activate the complement system and fix complement proteins (Szebeni *et al.*, 2000; Moghimi & Szebeni, 2003). However, complement fixation seems to play a minor role in macrophage clearance of PEGylated vesicles via complement receptors since PEGylated liposomes remain intact in the blood pool for prolonged periods of time (Szebeni *et al.*, 2000; Moghimi & Szebeni, 2003). Interestingly, liposomes of the same size distribution and bilayer composition as their PEGylated counterparts but without the phospholipid-mPEG conjugates rarely activate human complement system, and hence do not generate anaphylatoxin. In view of numerous medical applications for PEGylated liposomes and the clinical importance of the observed complement-mediated hypersensitivity reactions that can lead to anaphylactoid shock and cardiac anaphylaxis (Szebeni *et al.*, 2006), this study sought to investigate and identify which structural features of the phospholipid-mPEG conjugate are responsible for PEGylated liposome-induced complement activation in human sera. The efforts were particularly focused on design

and synthesis of novel lipid-mPEG conjugates where the phospholipid moiety of the conjugates is made from pro-drug ether lipids, as a first critical step towards development of safer long circulating vesicles for drug release at pathological sites with elevated sPLA2 activity (Andresen *et al.*, 2005a and b). Accordingly, this study has designed PEGylated liposomes that do not activate complement both in vitro and in vivo.

2.2 Materials and methods

2.2.1 Materials

DPPE-mPEG350 and DPPE-mPEG2000 were from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

DPPC, DPPG, cholesterol, ELISA kits for plasma TXB2, and all other reagents were obtained from Sigma Chemical Company (Poole, UK).

C1q-depleted human serum, purified human C1q, and enzyme-linked immunosorbent assay kits (SC5b-9, C4d, Bb and C3a-desarg) were from Quidel Corporation (San Diego, CA, USA).

Mouse anti rat CD11b (azide free, clone MRC OX-42) and polyclonal mouse IgG were from Serotec (Oxford, UK).

Mouse monoclonal antibody against human complement factor B was obtained from Antibody Shop A/S (Gentofte, Denmark).

Near monodisperse polyethylene glycols (PEGs), Mw = 400 and 1960 Da, were from Fluka (UK).

2.2.2 Methods

2.2.2.1 Synthesis of phospholipid-mPEG conjugates

The synthesis of 1-O-DPPE- (Me)mPEG350 (compound 5) and 1-O-DPPE-mPEG350 (compound 6) was carried out using (*R*)-O-benzyl glycidol as a convenient starting material as described by (Andresen *et al.*, 2004), Fig. 2.1. The synthetic approach to the two ether lipids involves the synthesis of compound 3 (Fig. 2.1a), which is readily formed from compound 1 on multi-gram scale (Andresen *et al.*, 2004). Preparation of the protected phosphatidylethanolamine 4 was carried out using methyl dichlorophosphate and TMP as base. Deprotection followed by coupling with mPEG350 gave the desired 1-O-DPPE-(Me)mPEG350 (5, termed Conj-B). Conj-B was converted to 1-O-DPPE-mPEG350 (6, termed Conj-A). The two conjugates were analyzed by ¹H-NMR (300 MHz), ¹³C-NMR (75 MHz), and HPLC using evaporative light scattering detector (Andresen *et al.*, 2004).

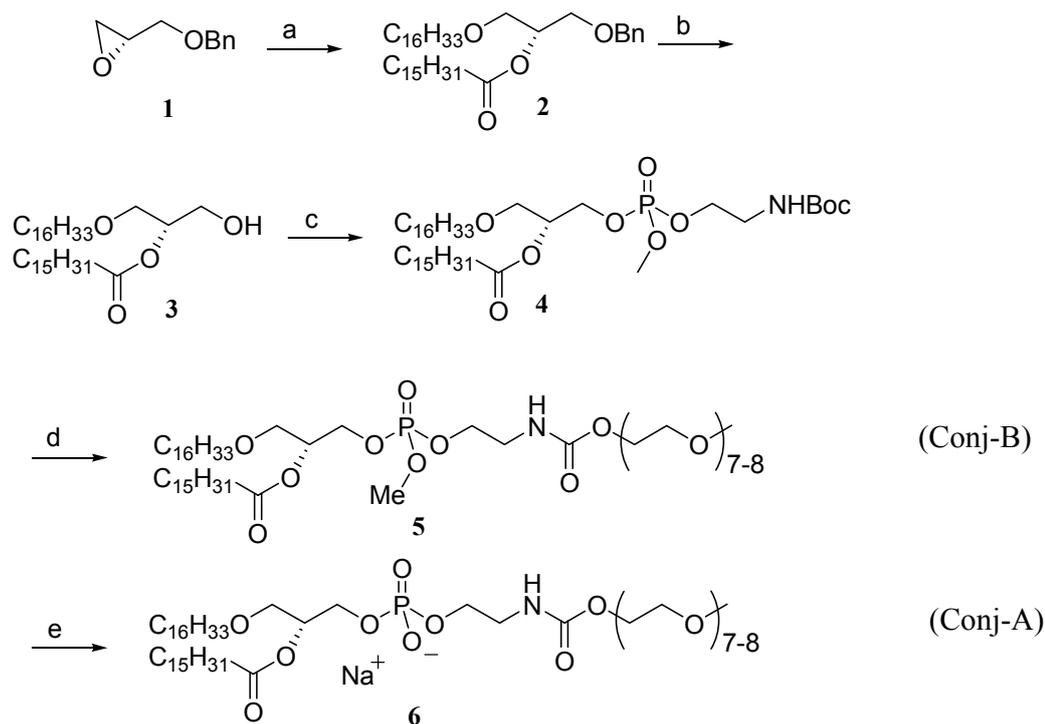


Fig. 2.1. Synthetic pathway and chemical structures of pro-drug ether lipid-mPEG conjugates (Conj-A and Conj-B). Synthetic steps are as follows: (step a): hexadecanol (C₁₆H₃₃OH), NaH, tetrahydrofuran (THF), dimethylformamide (DMF), 16h, 80°C, then palmitoyl chloride, pyridine, hexane, 16h, room temperature; (step b): H₂, Pd/C, ethyl acetate, 1.5h, room temperature; (step c): methyl dichlorophosphate, TMP, toluene, 16h, room temperature, then *N*-BOC-ethanolamine, TMP, 24h, room temperature; (step d): TFA, MeOH, CH₂Cl₂, 0.5h, 0°C, then mPEG350 *N*-succinimidecarbonate, Et₃N, CHCl₃, 2h, 40°C; (step e): NaI, 2-butanone, 2h, 75°C (Andresen *et al.*, 2005b).

2.2.2.2 Preparation and characterization of liposomes and phospholipids-mPEG micelles

Liposomes were composed of either DPPC or 1-O-DPPC with or without various amounts of different phospholipid-mPEG conjugates. Some DPPC preparations contained 5–20 mol% DPPG. Generally, liposomes were prepared by hydrating the dried lipid film with 10 mM PBS, pH 7.2, then extruded through polycarbonate Nuclepore filters with pore diameters of 200 nm using a high-pressure extruder.

The critical micelle concentration of selected phospholipids-mPEG conjugates was determined by measuring pyrene solubilization. The formation of micelles is associated with pyrene solubilization, which is monitored by measuring the solution fluorescence.

Liposome and phospholipids-mPEG micelle size distribution was determined by laser light scattering using a Malvern Zetasizer 3000 (Malvern Instruments, Malvern, UK) at 25 °C (Moghimi & Patel, 2002).

2.2.2.3 Collection and treatment of serum specimens

Blood was drawn from healthy male volunteers according to approved local protocols. Blood was allowed to clot at room temperature and serum was prepared, aliquoted and stored at –80 °C. Serum samples were thawed and kept at 4 °C before incubation with test reagents.

Commercially available C1q-depleted human serum was further depleted from Factor B. This was achieved by incubating serum with mouse monoclonal anti-complement factor B antibodies coupled to activated CNBr-sepharose (Klint *et al.*, 2000). Depletion of factor B was monitored by measuring zymosan-induced generation of serum complement activation product SC5b-9 (see below).

2.2.2.4 Assay of *in vitro* complement activation

To measure complement activation *in vitro*, we determined the liposome- micelle-, and PEG-induced rise of serum complement activation product SC5b-9, C4d, Bb and C3a-desarg formation, using respective Quidel's enzyme-linked immunosorbent assay kits according to the manufacturer's protocol (Szebeni *et al.*, 2000; Savay *et al.*, 2002; Szebeni *et al.*, 1997). Because of substantial biological variation in serum levels of complement proteins and the large number of positive and negative feedback interactions, this study monitored generation of complement activation products in sera of 5 healthy individuals separately. The reaction was started by adding the required quantity of liposomes, micelles, PEGs, and other required additives to undiluted serum (liposome to serum volume ratio of 1:4) in Eppendorf tubes (in triplicate) in a shaking water bath at 37 °C for 30 min, unless stated. The final incubation volume was 150 µl. Reactions were terminated by addition of 'sample diluent' provided with assay kit. SC5b-9 generation was also monitored in C1q-depleted human serum prior and after addition of physiological concentration of C1q (180 µg/ml). In some experiments, factor B was immunochemically depleted from C1q-depleted serum. Control incubations contained 10 mM PBS (pH 7.2) for assessing background levels of SC5b-9, C4d, Bb, and C3a-desarg. Zymosan (5 mg/ml) was used as a positive control for complement activation.

The efficacy of liposome treatments was established by comparison with baseline levels using paired *t*-test; correlations between two variables were analyzed by linear regression, and differences between groups (when necessary) were examined using ANOVA followed by multiple comparisons with Student-Newmann-Keuls test.

2.2.2.5 Determination of complement hemolytic activity and TXB2 level in rat blood

Prior to liposome injection 1–1.5 ml blood was taken from the tail vein of male Wistar rats (310 ± 30 g) for both plasma and serum preparation to obtain the required baseline parameters. For plasma preparation, blood was collected in EDTA/0.25 mM indomethacin-containing tubes to prevent prostaglandin metabolism. Appropriate liposome formulations were injected intravenously (80 mg/Kg body weight in 0.5–1.0 ml), bolus injection within 5 s, via the opposite tail vein. Further blood samples were taken at 8 and 60 min post liposome injection to obtain plasma and serum. Plasma TXB2 levels were determined by following the procedures supplied with the ELISA kit. Undiluted serum was used to measure CH_{50} using sheep red blood cell hemolysis assay as discussed by Szebeni *et al.*, (1994).

2.3 Results and discussion

2.3.1 Conjugate synthesis and rationale for liposome design

One of the key structural features in mPEG-phospholipid conjugate is the presence of a net anionic charge localized on the phosphate oxygen moiety of the mPEG-phospholipid conjugate, which could be responsible for complement activation. This hypothesis is based on previous observations that small unilamellar neutral non-PEGylated liposomes (liposomes of similar composition to clinical formulation of PEGylated vesicles but without the phospholipid-mPEG conjugate) causes no or minimal activation of the human and pig complement system (Szebeni *et al.*, 2000 and 2002). On the contrary, bilayer enrichment of non-PEGylated liposomes with dicetylphosphate or a variety of anionic phospholipids, regardless of their headgroup structure, induces complement activation in

both human and animal sera (Szebeni *et al.*, 2000; Moghimi & Szebeni 2003; Chonn *et al.*, 1991; Devine *et al.*, 1994; Bradley *et al.*, 1998; Moghimi & Hunter, 2001). Therefore, this study synthesized two types of phospholipid-mPEG conjugates bearing different charges as demonstrated in Fig. 2.1. The key structural features of the first conjugate (Conj-A) mirrors PE-mPEG; a conjugate with a proven capability of prolonging the circulatory half-life of liposomes in the vasculature. Thus, the phosphate moiety of Conj-A is anionic and its phosphodiester and amide linkage groups are the same as PE-mPEG. In the second conjugate (Conj-B), the phosphate oxygen is methylated to eliminate the net negative charge, thus yielding a nonionic species (Fig. 2.1). The mPEG segment of both Conj-A and -B are purposely short in length (7 ethylene glycol units, Mw = 350 kDa) for better exposure of phosphodiester linkage and liposome surface to complement proteins, thus testing the validity of the proposed complement activation hypothesis.

In contrast to classical phospholipid-mPEG conjugates, the phospholipid component of Conj-A and Conj-B bear a non-hydrolyzable ether bond in the 1-position (1-O-phospholipid). This feature is not directly applicable to complement activation hypothesis but is essential for designing long-circulating multi-functional 1-O-phospholipid-based vesicles for efficient drug delivery and trigger release at selected non-macrophage pathological sites, which has an active interest. 1-O-phospholipids form highly stable liposomes with no hemotoxicity as demonstrated recently (Andresen *et al.*, 2004; Moghimi & Patel, 2002). These vesicles also function as prodrugs; sPLA2 and its subtypes being the activating enzymes, and their levels are dramatically elevated in the interstitium of solid tumors as well as at various inflammatory sites (Andresen *et al.*, 2005a and b). sPLA2 not only act as a trigger resulting in the release of encapsulated cytotoxic drugs from pro-drug ether lipid liposomes, but also generates highly cytotoxic

lysolipids that destabilizes plasma membrane of cancer cells as shown recently (Andresen *et al.*, 2005a).

This study incorporated 1-O-DPPE-mPEG conjugates (Conj-A and Conj-B) into DPPC liposomes, since the resultant vesicles are highly stable in serum, and in addition DPPC vesicles do not activate the human complement system (see below). Also, the bilayer of constructed vesicles is not enriched with cholesterol, since incorporation of cholesterol, particularly at above 30 mol%, is known to activate the human complement system via the classical pathway following binding of anti-cholesterol antibodies, which are abundant in most human sera (Alving *et al.*, 1991). Indeed, cholesterol-rich liposomes have been shown to cause massive hemodynamic changes in pigs via complement activation (Szebeni *et al.*, 2000).

2.3.2 Liposome-mediated complement activation *in vitro*

This study demonstrated complement activation in sera of a number of healthy subjects with liposomes containing an anionic phospholipid (DPPC:DPPG, mole ratio 9.5:0.5) by measuring the generation of the complement activation product SC5b-9, which is a sensitive and established measure of the activation of the whole complement cascade (Szebeni *et al.*, 2003), Fig. 2.2a. These observations are in line with previous reports (Szebeni *et al.*, 2000; Chonn *et al.*, 1991; Devine *et al.*, 1994) suggesting that vesicles exhibiting a net negative charge, but not neutral DPPC vesicles, activate the human complement system. PEGylated liposomes also caused complement activation with significant rises of SC5b-9 levels over baseline, comparable to non-PEGylated DPPC:DPPG vesicles of the same size distribution in all tested sera (Fig. 2.2a). With Conj-A-containing (5 mol%) liposomes SC5b-9 generation proceeded on a time scale of

minutes, also identical to DPPE-mPEG350-incorporated vesicles and reached plateau at around 15–20 min. Further, Conj-A-containing liposomes were effective in causing significant rises of SC5b-9 levels in serum at a final lipid concentration of 1 mg/ml, with a trend of reaching maximal efficacy at lipid concentrations of 3–4 mg/ml. As a control experiment, this study assessed SC5b-9 generation in serum following incorporation of 1-O-DPPC (5 mol%) into DPPC vesicles as well as by pure 1-O-DPPC vesicles (Fig. 2.2b). Indeed, 1-O-DPPC was ineffective in raising serum SC5b-9 levels above the baseline, but bilayer enrichment of 1-O-DPPC vesicles with anionic phospholipid-mPEG conjugates raised serum SC5b-9 levels in a similar manner to PEGylated DPPC vesicles (Fig. 2.2b). Thus, complement activation, as reflected by increase in serum levels of SC5b-9, proceeds with anionic lipid-mPEG conjugate-incorporated liposomes independent of mPEG molecular weight (350 and 2000 kDa) or its conjugated lipid structure (DPPC or 1-O-DPPC).

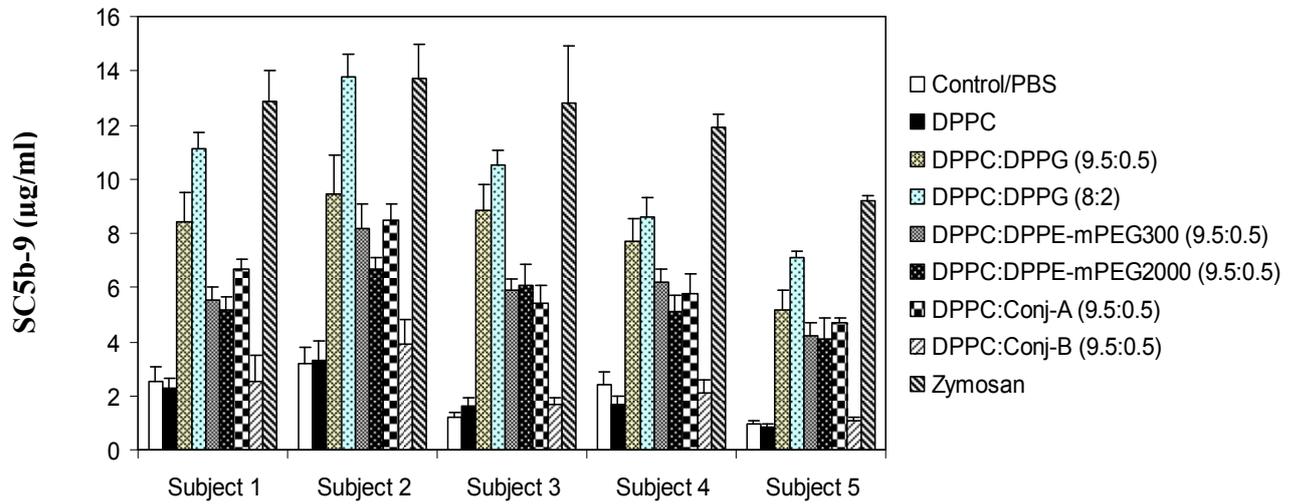


Fig. 2.2.a. Effect of liposomes on complement activation: SC5b-9 levels in sera of 5 healthy subjects 30 min after liposome treatment (3 mg lipid/ml) at 37 °C. The values in the brackets represent lipid mole ratios. With the exception of neutral liposomes and vesicles containing Conj-B, generated SC5b-9 levels were significantly higher than the background. $P < 0.05$. (n= 3).

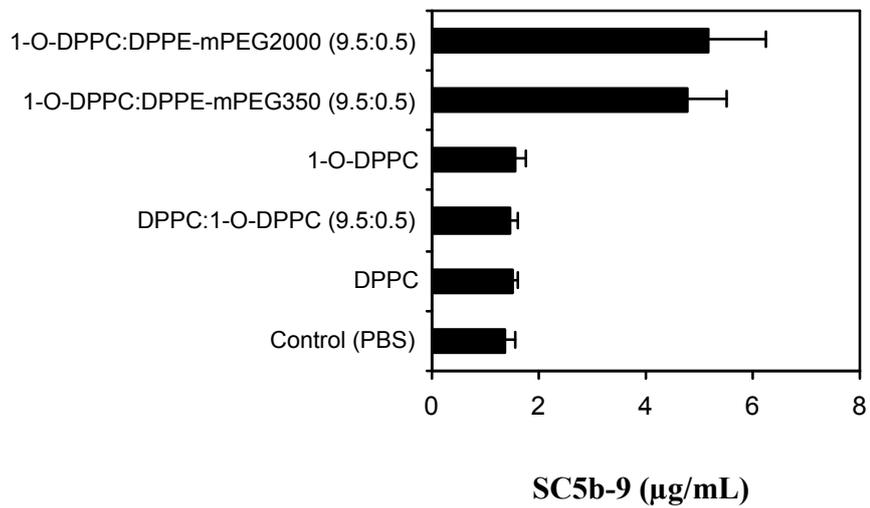


Fig. 2.2.b. Effect of liposomes on complement activation: the effect of 1-O-DPPC bearing liposomes (70–110 nm) on SC5b-9 generation in serum of subject 5.

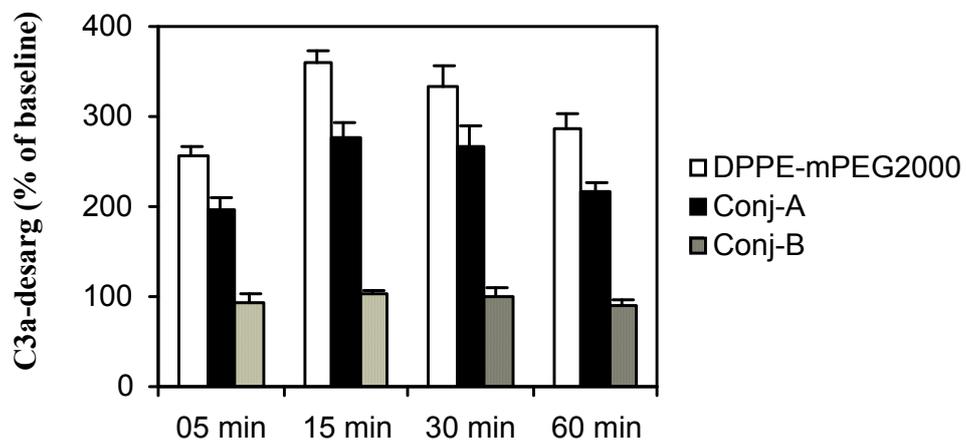


Fig. 2.2.c. Effect of liposomes on complement activation: the influence of lipid-mPEG conjugates (5 mol%) on DPPC liposome-mediated anaphylatoxin generation (C3a-desarg) over time in serum of subject 1. Results are expressed in percentage of control baseline. With the exception of Conj-B, results were significantly different relative to control ($P < 0.05$). All liposomes were in 70-110 nm size range.

To further confirm the role of the anionic charge of the phosphate oxygen moiety in complement activation this study demonstrated rises in C3a-desarg levels in human serum over time (Fig. 2.2c). The rise of SC5b-9 levels in human sera remarkably correlates with C3a production, providing further evidence that the anionic phosphate-induced rises of SC5b-9 levels is a reflection of complement activation rather than modulation of the terminal pathway only.

The PEG moiety of liposomes, regardless of their molecular weight, plays no role in complement activation as demonstrated in experiments with endotoxin-free near monodisperse PEG1960 (polydispersity index = 1.03) and PEG400 (polydispersity index = 1.07). Neither PEGs (2.5 mg/ml, final concentration in serum) were able to raise serum levels of C4d, Bb, and SC5b-9 levels above the background (Fig. 2.3).

2.3.4 The effect of surface mPEG-density and vesicle size

Increasing the bilayer concentration of anionic Conj-A above 2.5 mol% elevated serum SC5b-9 levels, and the effect was more dramatic with vesicles bearing 10–15 mol% Conj-A (Table 2.1). Similar observations were made with DPPC vesicles containing the corresponding levels of DPPE-mPEG350. These experiments indicated the importance of surface charge density in complement activation. This is a surprising observation, since the high density of surface projected mPEG chains, which are believed to assume a mushroom-brush conformation (Tirosh *et al.*, 1998; Chiu *et al.*, 2001; Moghimi, 2006; Kenworthy *et al.*, 1995), should sterically shield the liposome surface against protein adsorption.

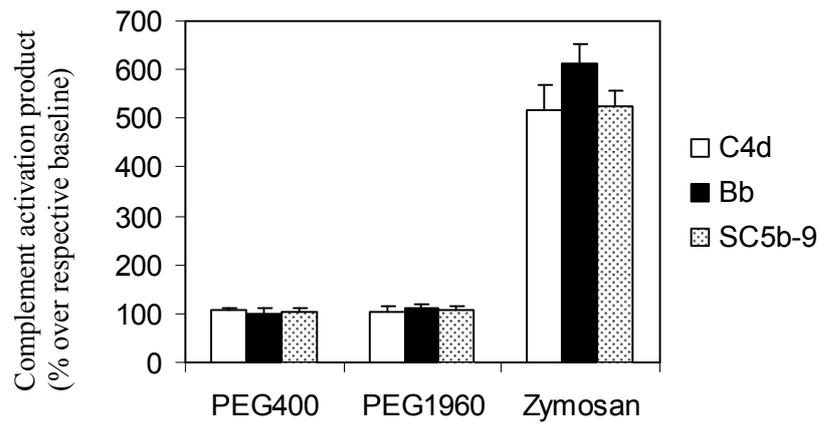


Fig. 2.3. The effect of near monodisperse PEG1960 and PEG400 on complement activation.

Table 2.1. The effect of liposome size and bilayer composition on SC5b-9 generation in human serum

Liposome composition (mole ratio)	SC5b-9 ($\mu\text{g/ml}$)		
	85 nm liposomes	140 nm liposomes	260 nm liposomes
DPPC	2.3 ± 0.4	2.5 ± 0.1	1.9 ± 0.4
DPPC:Conj-A (99:1)	2.6 ± 0.5	not done	not done
DPPC:Conj-A (97.5:2.5)	$3.8 \pm 0.8^*$	not done	not done
DPPC:Conj-A (95:5)	$6.3 \pm 0.4^*$	$7.6 \pm 1.1^{**}$	$10.1 \pm 1.0^{**}$
DPPC:Conj-A (90:10)	$8.1 \pm 0.9^{**}$	not done	not done
DPPC:Conj-A (85:15)	$8.5 \pm 0.6^{**}$	$9.2 \pm 0.6^{**}$	$12.2 \pm 0.9^{**}$
DPPC: Conj-B (95:5)	2.5 ± 1.0	3.0 ± 0.8	3.4 ± 0.4
DPPC:Conj-B (85:15)	3.1 ± 0.6	2.9 ± 0.1	2.9 ± 0.2

The baseline SC5b-9 level (control/PBS) was $2.5 \pm 0.5 \mu\text{g/ml}$. Liposomes were incubated with serum of subject 1 for 30 min at 37°C prior to SC5b-9 measurement. Significant differences with respect to control/PBS: $^* p < 0.05$, $^{**} p < 0.01$.

Since the described liposomes can activate complement, the discrepancies in cell uptake studies may be rationalized by the steric hindrance of mPEG chains to binding of opsonized vesicles to CD11b. Hence, these results are in agreement with the reported prolonged circulation times of PEGylated vesicles (Moghimi *et al.*, 2001). In contrast to anionic conjugates, increasing the bilayer concentration of nonionic mPEG-lipid (Conj-B) in DPPC vesicles to 15 mol% failed to elevate serum SC5b-9 levels above the baseline, and the results were comparable to non-complement activating DPPC vesicles (Table 2.1). This again confirms a role for the anionic phosphate oxygen in activating complement.

Vesicle size have been demonstrated to play a critical role in complement activation; smaller vesicles (sub 100 nm) are less effective complement activators than their larger counterparts for equivalent concentrations (Szebeni *et al.*, 2000; Devine *et al.*, 1994; Bradley *et al.*, 1998). This reflects the importance of geometric factors and surface dynamics on the initial assembly of proteins involved in complement activation. In addition, surface curvature affects projected mPEG chain conformation; this in turn may exert some control over complement consumption and activation (Moghimi & Szebeni, 2003; Gbadamosi *et al.*, 2002). The results in Table 2.1 further confirm the non-complement activating nature of larger-sized Conj-B bearing liposomes when tested with bilayer concentrations up to 15 mol%. However, increasing size causes more complement activation with vesicles containing Conj-A.

2.3.4 Liposome-mediated complement activation in rats

Next, this study examined complement activation *in vivo*. Although a correlation between *in vitro-in vivo* dose responses is difficult, complement activation occurs at the point of injection with low doses of activator (Szebeni *et al.*, 1994). Intravenous injection of liposomes containing 5 mol% of anionic phospholipid-mPEG conjugates (regardless of mPEG chain length) was associated with a significant reduction in serum total hemolytic activity on a time scale of minutes with a parallel rise of TXB₂ (Fig. 2.4). TXB₂ is an established and direct marker for TXA₂ production (Szebeni *et al.*, 1994). Anaphylatoxins arising from complement activation (C3a and C5a) induce TXA₂ release from blood cells, thus demonstration of increased serum TXB₂ levels provide further evidence for *in vivo* PEGylated liposome-mediated complement activation (Szebeni *et al.*, 1994). On the contrary, neither *in vivo* TXB₂ levels nor *in vitro* complement activity in serum (CH₅₀ measurements) was affected with liposomes bearing nonionic lipid-mPEG conjugate (Fig. 2.4), thus confirming a role for the negative charge of phosphate oxygen in complement activation.

2.3.5 The involvement of both classical and alternative pathways

Having established a critical role for anionic phospholipid-mPEG conjugates in liposome-mediated complement activation, this study next sought to investigate through which pathway(s) complement activation proceeds. Calcium is essential for operation of both classical and MBL pathways, whereas operation of the alternative pathway is fully maintained in EGTA-chelated (Mg²⁺ supplemented) serum (Szebeni *et al.*, 1994 and 1997).

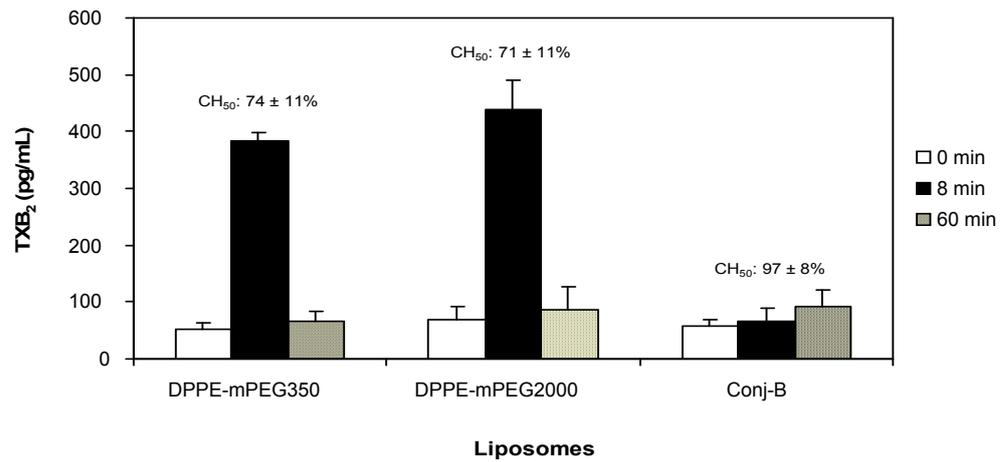


Fig. 2.4. Plasma TXB₂ levels in rats following bolus intravenous injection of liposomes (80 mg/Kg in 0.5 ml). Liposomes were composed of DPPC and 5 mol% of designated conjugates (size range 80-130 nm). With the exception of Conj-B, CH₅₀ values were significantly different from baseline. ($P < 0.05$).

Studies in EGTA-chelated sera of the five individuals demonstrated significant but variable reduction (15–60%, depending on serum) in SC5b-9 levels following challenge with liposomes bearing anionic phospholipid-mPEG conjugates. This confirmed a direct role for the alternative pathway, rather than amplification of C3 convertase initially triggered through calcium-sensitive pathways. To address the role of calcium-sensitive pathways, this study also followed the liposome-induced changes in C4d levels in sera, and these were elevated by 1.8- to 3-fold above the background level as determined by ELISA, depending on serum source.

To further elucidate the mechanism of complement activation by anionic phospholipids-mPEG conjugates C1q-depleted serum was used, which enables clarification of the role of C1q-dependent classical pathway activation. The results in Fig. 2.5 show a significant reduction in SC5b-9 generation in C1q-depleted serum with liposomes bearing anionic phospholipid-mPEG conjugates, when compared to incubations with restored physiological levels of the deficient factor (C1q), thus confirming the involvement of C1q-dependent classical pathway. As a positive control, zymosan-mediated complement activation proceeded efficiently in C1q deficient serum and the process was unaffected following the addition of C1q, as zymosan activates complement via all three pathways. Notably, liposome-mediated elevation of SC5b-9 in C1q-depleted serum was significantly above the baseline level (Fig. 2.5). This was due to direct involvement of the alternative pathway, since SC5b-9 levels in EGTA-chelated (Mg^{2+} supplemented) C1q-depleted serum remained unchanged. Furthermore, immunochemical depletion of factor B in C1q-depleted serum totally abolished SC5b-9 generation by both liposomes and zymosan (control experiment) providing further evidence for direct involvement of the alternative pathway.

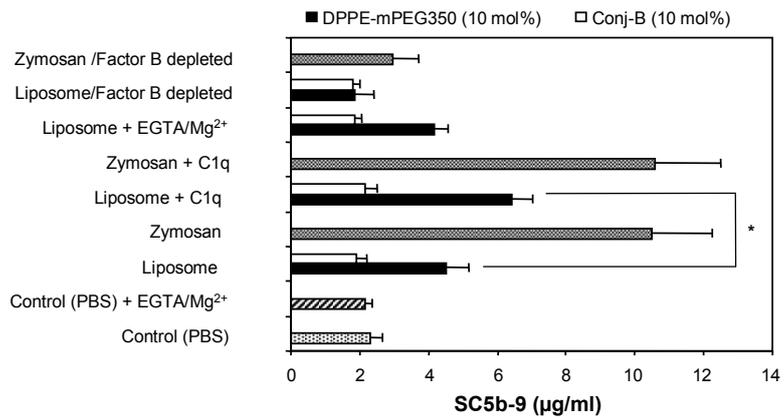


Fig. 2.5. Liposome-mediated SC5b-9 generation in C1q-depleted human serum. DPPC liposomes were used and contained 10 mol% of designated conjugates in their bilayer (size range 80-130 nm). (* $P < 0.05$).

This study did not investigate contribution of the MBL pathway, since MBL selectively recognizes glucans, lipophosphoglycans and glycoinositol phospholipids that contain mannose, glucose, fucose, or *N*-acetylglucosamine as their terminal hexose, and none of which were presented on the tested liposomes (Moghimi & Hunter, 2001; Gulati *et al.*, 2002).

2.3.6 The role of phospholipid head-group—A hypothesis

In contrast to PEGylated liposomes, anionic phospholipid-mPEG350 and -mPEG2000 conjugates in micellar form were incapable of rising serum SC5b-9, C4d, Bb and C3a-desarg levels above the respective baseline. Thermodynamically, micelle solution is at equilibrium; the concentration of monomers being equal to the critical micelle concentration. Hence, neither anionic phospholipid-mPEG monomers nor micelles are capable of activating the human complement system. However, lack of complement activation by these micelles may be due to their small hydrodynamic size, which was in the region of 25–32 nm as determined by photon correlation spectroscopy. On the contrary, related nonionic micelles (2.5–10 mg/ml) of similar sizes such as those assembled from polyoxyethene/polyoxypropylene block copolymers (eg, poloxamer 407 and poloxamine 908) activate complement in human serum (2- to 4-fold rise in serum Bb and SC5b-9 levels above the respective background). On the basis of these observations, a possible role for liposomal zwitterionic phospholipid head-groups as an additional (or prerequisite) factor contributing to complement activation can not be excluded. Recent studies have demonstrated that following incubation in serum, the surface of PEGylated liposomes becomes coated with apolipoproteins, antibodies and complement proteins

(Szebeni *et al.*, 2000; Price *et al.*, 2001; Moghimi & Szebeni, 2003). It is therefore proposed that for binding to PEGylated liposomes, complement activating natural anti-phospholipid antibodies (IgG and IgM) may require the presence of both DPPC head-groups and the anionic moiety of phospholipid-mPEG in a spatial relationship that orients the antibody into a complement activating posture. Indeed, structural modelling studies have demonstrated that the Fab/Fc orientation of IgG might be a key factor in controlling access of the C1q globular B module to the C_H2 domain of human IgG1 (Gaboriaud *et al.*, 2003 & 2004). Likewise, the globular internal A and/or C modules of C1q show preferential binding to oligomeric IgM (Gaboriaud *et al.*, 2004; Kishore *et al.*, 2003). It is also known that antibodies can activate the alternative pathway via their F(ab) portion (Moore *et al.*, 1982; Fries *et al.*, 1984), whose binding of C3b is also likely to depend on a two antigenic subsite-fixed orientation of IgG. Thus, on the basis of this study's observations, it could be further suggested that the steric arrangement imposed by the bulky methyl group in Conj-B either prevents simultaneous binding of anti-phospholipid antibodies to the phosphate oxygen moiety of both phospholipid head-groups and phospholipid-mPEG conjugates and/or interfere with spatial organization of surface-bound antibodies for correct recognition by all three modules of globular C1q domain.

It is also appropriate to stress here that the presence or absence of additional epitopes for anti-phospholipid antibody binding, such as apolipoprotein-H, (Alving & Swartz 1991; Szebeni *et al.*, 1996; McNiel *et al.*, 1990) and direct binding of C1q (Bradley *et al.*, 1999a and b) to the anionic bilayer surface are further factors for consideration. As for the latter process, the top of the C1q head is predominantly basic and as a result C1q can function as a charge pattern recognition molecule. Indeed, the highly cationic region of

C1q A chain, comprising residues 14–26, binds to cardiolipin-containing liposomes (Bradley *et al.*, 1999a). Binding of C1q to phosphatidylglycerol-containing liposomes has also been demonstrated (Bradley *et al.*, 1999b) where following binding C1q and phosphatidylglycerol colocalize into domains with characteristic radii of curvature higher than that of the surrounding bilayer, which eventually bud into small vesicles. Hydrophobic interactions and/or hydrogen bonding have also been suggested to participate in the binding of C1q to cardiolipin-containing liposomes (Bradley *et al.*, 1999b); hydrogen bonding is presumably mediated by the central 2'-hydroxyl moiety of the glycerol backbone of cardiolipin. With respect to these observations and the results in Fig. 2.5, a direct role for small numbers of C1q molecules in initiating complement activation by liposomes bearing anionic phospholipid-mPEG conjugates can not be excluded. Thus, in addition to electrostatic interactions between C1q and the anionic phosphate oxygen, the ether oxygen groups in the mobile mPEG moiety may play a role in accommodating C1q on the liposome surface via hydrogen bonding.

2.4 Conclusion

Methoxypoly(ethylene glycol), (mPEG),-grafted liposomes are known to exhibit prolonged circulation time in the blood, but their infusion into a substantial percentage of human subjects triggers immediate non-IgE-mediated hypersensitivity reactions. These reactions are strongly believed to arise from anaphylatoxin production through complement activation. Despite the general view that vesicle surface camouflaging with mPEG should dramatically suppress complement activation, here this study shows that bilayer enrichment of non-complement activating liposomes (dipalmitoylphosphatidylcholine, DPPC, vesicles) with phospholipid-mPEG conjugate

induces complement activation resulting in vesicle recognition by macrophage complement receptors. The extent of vesicle uptake, however, is dependent on surface mPEG density. This study has delineated the likely structural features of phospholipid-mPEG conjugate responsible for PEGylated liposome-induced complement activation in normal as well as C1q-deficient human sera, using DPPC vesicles bearing the classical as well as newly synthesized lipid-mPEG conjugates. With PEGylated DPPC vesicles, the net anionic charge on the phosphate moiety of phospholipid-mPEG conjugate played a key role in activation of both classical and alternative pathways of complement and anaphylatoxin production (reflected in significant rises in SC5b-9, C4d and C3a-desarg levels in normal human sera as well as SC5b-9 in EGTA-chelated/Mg²⁺ supplemented serum), since methylation of the phosphate oxygen of phospholipid-mPEG conjugate, and hence the removal of the negative charge, totally prevented complement activation. To further corroborate on the role of the negative charge in complement activation, vesicles bearing anionic phospholipid-mPEG conjugates, but not the methylated phospholipid-mPEG, were shown to significantly decrease serum hemolytic activity and increase plasma thromboxane B2 levels in rats. In contrast to liposomes, phospholipid-mPEG micelles had no effect on complement activation, thus suggesting a possible role for vesicular zwitterionic phospholipid head-groups as an additional factor contributing to PEGylated liposome-mediated complement activation. These findings provide a rational conceptual basis for development of safer vesicles for site-specific drug delivery and controlled release at pathological sites.

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Chapter three

Complement Activation by PEGylated Single-Walled Carbon Nanotubes is Independent of the C1q- Dependent Classical and the Alternative Pathways

3.1 Introduction

Carbon nanotubes have received considerable attention as promising materials for a wide range of experimental diagnostic and therapeutic applications following intravenous injection (Lacerda *et al.*, 2006; Liu *et al.*, 2007; Liu *et al.*, 2008). However, the interaction between carbon nanotubes and the complement system, which is an important effector arm of both innate and acquired immunity (Lambris *et al.*, 2008), has not received much attention. The complement system can be triggered by three separate pathways: the classical, the lectin and the alternative pathways (Lambris *et al.*, 2008). Three consequences arise from complement activation (Lambris *et al.*, 2008). These include priming of the activator surface by opsonic complement fragments (e.g., C3b, iC3b) for engulfment by phagocytes, generation of anaphylatoxins and chemoattractants (eg, C3a, C5a) and formation of the membrane attack complex (C5b-9) (Fig. 1.1). To date, an isolated study with non-functionalized high pressure carbon monoxide single-walled carbon nanotubes (HIPco SWNTs) and double-walled carbon nanotubes (DWNTs) has revealed nanotube-mediated complement activation in human serum (Salvador-Morales, *et al.*, 2006). Recently, Salvador-Morales *et al.*, suggested that HIPco SWNTs and DWNTs activate complement through classical pathway which was mediated by surface adsorption of the C1q component of complement, DWNTs, on the other hand, activated complement through the alternative pathway (Salvador-Morales, *et al.*, 2006).

Nanotube stability in the blood can be enhanced by surface functionalization with poly(ethyleneglycol)₅₀₀₀-phospholipid (PEG₅₀₀₀-PL) conjugates (Liu *et al.*, 2007). Such surface modification procedures also confer longevity to SWNTs in the systemic circulation (SWNTs reported half life is 12h); this is most likely arises from the steric hindrance of the projected long PEG chains to nanotube–macrophage interaction

(Moghimi *et al.*, 2001; Moghimi & Szebeni, 2003; Moghimi *et al.*, 2006). Indeed, PEGylated SWNTs, through prolonged circulation times in the blood, can ultimately target elements of tumour vasculature following conjugation of targeting ligands to the distal end of the projected PEG chains (Liu *et al.*, 2007). Recent studies with PEGylated liposomes such as Doxil[®] (a clinically approved PEGylated liposome with encapsulated doxorubicin) as well as PEGylated polymeric nanoparticles have demonstrated that the steric hindrance of PEG may not necessarily prevent complement activation and fixation, and their administration into human subjects may initiate untoward reactions (Gbadamosi *et al.*, 2002; Chanan-Khan *et al.*, 2003; Moghimi & Szebeni, 2003; Szebeni, 2005). For example, infusion of Doxil[®] into a substantial percentage of human subjects has triggered immediate acute pseudoallergic reactions with symptoms of cardiopulmonary distress such as dyspnoea, tachypnoea, hypertension/hypotension and chest and back pain (Szebeni, 2005). These reactions are strongly believed to arise through the generation of complement anaphylatoxins C3a and C5a, leading to the subsequent release of thromboxane A₂ (TXA₂) and other inflammatory mediators from immune cells (Szebeni, 2005; Chanan-Khan *et al.*, 2003). Furthermore, the terminal half of the complement pathway generates multiprotein C5b-9 complexes (Lambris *et al.*, 2008) and these have the capacity to elicit non-lytic stimulatory responses from vascular endothelial cells and, therefore, modulate endothelial regulation of haemostasis and inflammatory cell recruitment (Hamilton *et al.*, 1993). In light of these observations, we sought to investigate the interaction of amino-PEG₅₀₀₀-distearoylphosphatidylethanolamine (amino-PEG₅₀₀₀-DSPE) and methoxy(MeO)PEG₅₀₀₀-DSPE functionalized SWNTs with the complement system both *in vitro* and *in vivo*.

3.2 Materials and methods

3.2.1 Materials

amino-poly(ethylenglycol)₅₀₀₀-(1,2-distearoyl-sn-glycero-3-phosphoethanolamine) (amino-PEG₅₀₀₀-DSPE) and methoxy (MeO)-PEG₅₀₀₀-DSPE were purchased from NOF Corporation, Japan.

Dipalmitoylphosphatidylcholine (DPPC) and Fluka standard PEGs were obtained from Sigma-Aldrich (UK).

Commercially available human C1q-depleted serum was obtained from Quidel (distributed by Technoclone, UK).

MBL-C4 complex ELISA kit was purchased from HyCult Biotechnology, The Netherlands.

Wielisa[®]-Total Complement Screen kit was obtained from Lund, Sweden.

Sephacryl S-200-HR gel was obtained from Sigma-Aldrich, UK.

Futhan, Mouse monoclonal antibodies to human C1s (IgG1, clone M81), MBL, L-ficolin (clone GN5) and H-ficolin (clone 4H5) were from HyCult Biotechnology (The Netherlands).

3.2.2 Methods

3.2.2.1 Preparation of PEGylated SWNTs

As-grown SWNTs were sonicated in the presence of either 1 mg/mL amino-poly(ethyleneglycol)₅₀₀₀-(1,2-distearoyl-sn-glycero-3-phosphoethanolamine) (amino-PEG₅₀₀₀-DSPE) or methoxy (MeO)-PEG₅₀₀₀-DSPE for 1 h followed by centrifugation at 24,000 X g for 6 h to remove large bundles, aggregates and impurities (Lacerda *et al.*, 2006). Excess PEG-phospholipid conjugates were removed by filtration through 100-kDa Millipore filters.

3.2.2.2 PEGylate liposomes

Liposomes were composed of dipalmitoylphosphatidylcholine (DPPC) and MeOPEG₂₀₀₀-DPPC (mole ratio 9.5:0.5) and prepared by hydrating the dried lipid film with 10 mM phosphate-buffered saline (pH 7.2) and then extruded through polycarbonate Nuclepore filters with pore diameters of 200 nm using a high-pressure extruder. The prepared liposomes were 118 ± 12 nm in size (polydispersity index = 0.07), determined by laser light scattering (Liu *et al.*, 2008).

3.2.2.3 Preparation of human serum

Blood was drawn from healthy male volunteers according to approved local protocols. Blood was allowed to clot at room temperature and serum was prepared, aliquoted and stored at -80°C. Serum samples were thawed and kept at 4°C before incubation with test reagents. Commercially available human C1q-depleted serum was obtained from Quidel.

3.2.2.4 Assays of *in vitro* complement activation

To measure complement activation *in vitro*, this study determined SWNT-induced rise of serum complement activation product SC5b-9, Bb and C4d, using respective Quidel's ELISA kits according to the manufacturer's protocols as described previously (Liu *et al.*, 2008; Lambris *et al.*, 2008). As a result of substantial biological variation in serum levels of complement proteins and the large number of positive and negative feedback interactions, this study monitored generation of complement activation products in sera of 4 healthy individuals separately (Liu *et al.*, 2008). The concentration of mannan binding lectin (MBL) in both healthy and C1q-depleted sera was determined by using the MBL-C4 complex ELISA kit. Only sera with physiological concentrations of MBL, in the range of 3000–5000 ng/mL, were selected for subsequent complement activation assays. The functional activity of classical, lectin and the alternative pathways of complement were confirmed in all sera with Wielisa[®]-Total Complement Screen kit (Lund, Sweden).

For measurement of complement activation, the reaction was started by adding the required quantity of SWNTs to undiluted serum (SWNT to-serum volume ratio 1:4) in Eppendorf tubes (in triplicate) in a shaking water bath at 37°C for 30 min, unless stated otherwise. Reactions were terminated by addition of “sample diluent” provided with assay kit. SWNT-induced rises of serum SC5b-9, Bb and C4d were then measured following nanotube removal (to minimize interference in ELISA tests) by carefully layering 200 μ L of Sephacryl S-200-HR gel and subsequent centrifugation. The extent of nanotube-trapping in the gel was followed by measuring the absorbance of the supernatant at 808 nm (e.g., ~70–75% of amino-PEG₅₀₀₀-DSPE coated nanotubes were trapped by these procedures). Subsequently, appropriate controls were also made by adding sufficient quantities of SWNTs to saline for background

correction in ELISA experiments. The gel-trapping procedures had no effect in removing fluid phase complement activation products (as will be shown in results). Control serum incubations contained saline (the same volume as nanotubes and other additions) for assessing background levels of SC5b-9, Bb and C4d. Zymosan was used as a positive control for complement activation (Liu *et al.*, 2008; Lambris *et al.*, 2008). To monitor the possible binding of complement activation products to the nanotube surface, SWNTs were incubated with standard samples of activation products. The level of the standard activation products in the supernatant was then measured by the respective ELISA test and compared with control incubations in the absence of nanotubes. In some experiments, SWNT-induced complement activation was monitored following pretreatment of serum with EDTA (20 mM final concentration), Futhan (150 $\mu\text{g}/\text{mL}$), and monoclonal antibodies (against C1s, MBL, H- and L-ficolins, or an irrelevant antibody). The anti-C1s monoclonal antibody, but not an irrelevant murine IgG antibody, blocked activation of the classical pathway in human serum as confirmed by complement activation experiments (C4d measurements) in the presence of cholesterol-rich liposomes (these liposomes activate complement through the classical pathway) (Moghimi *et al.*, 2001; Moghimi & Szebeni, 2003).

For quantification of complement activation products, standard curves were constructed using the assigned concentration of each respective standard supplied by the manufacturer and validated. The slope, intercept and correlation coefficient of the derived best-fit line for Sc5b-9, Bb and C4d standard curves were within the manufacturer's specified range.

The efficacy of SWNT treatments was established by comparison with baseline levels using paired *t* test; correlations between two variables were analysed by linear

regression, and differences between groups (when necessary) were examined using ANOVA followed by multiple comparisons with Student-Newmann-keuls test. Similar patterns were observed in all tested sera; the result of a typical experiment is presented.

3.2.2.5 Determination of thromboxane B2 (TXB2) level in rat blood

Prior to intravenous injection of SWNT, PEG-Phospholipids and liposome, 1.0 mL blood was taken from the tail vein of male Wistar rats (250–280 g) for plasma preparation to obtain the required baseline parameters. For plasma preparations, blood was collected in EDTA/0.25 mM indomethacin-containing tubes to prevent prostaglandin metabolism. Bolus intravenous injections (SWNT, 1.2 mg/kg, PEG-PL conjugates, up to 10 mg/kg; PEGylated liposomes, 80 mg/kg) were made through the opposite tail vein. Further blood samples were taken at 6 and 60 min post-injection to obtain plasma. Plasma TXB2 levels were determined in triplicate samples (Liu *et al.*, 2008; Moghimi & Szebeni, 2003) by following the procedures supplied with the ELISA kit.

3.3 Results and Discussion

The diameters and lengths of functionalized nanotubes were 1–5 nm and 50–300 nm, respectively, as determined by AFM (Fig. 3.1). The binding of PEG-PL conjugates represents a minimum available surface area, where the projected PEG chains are expected to assume a ‘brush-like’ or laterally compressed elongated random coil configuration, which is a necessary prerequisite for minimizing and/or combating

protein adsorption (Moghimi & Szebeni, 2003; Gbadamosi *et al.*, 2002). The prolonged stability of PEGylated SWNTs in both buffer and fresh serum was confirmed earlier (Liu *et al.*, 2007).

During complement activation, C5b-9 complexes formed in the absence of a target membrane bind to a naturally occurring regulatory serum protein, the S protein; the generated SC5b-9 complex is the soluble, non-lytic form of the terminal attack complex (Lambris *et al.*, 2008). Therefore, this study monitored nanotube-mediated complement activation in sera of healthy subjects by measuring SC5b-9 generation by enzyme-linked immunosorbent assay (ELISA) (Chanan-Khan *et al.*, 2003; Szebeni, 2005). PEGylated nanotubes caused complement activation, irrespective of the terminal end moiety of the projected PEG chains, as evident with significant rises of serum SC5b-9 levels over baseline at a final nanotube concentration of 40 $\mu\text{g/mL}$ (Figure 3.2a), with a trend of reaching maximal efficacy at 60–80 $\mu\text{g/mL}$. Nanotube-mediated SC5b-9 generation also proceeded on a time scale of minutes and reached plateau at around 10 min. In contrast to PEGylated SWNTs, PEG-PL conjugates, even at concentrations as high as 2 mg/mL (twice above the PEG-PL conjugates initially used for SWNT stabilization), did not elevate serum SC5b-9 levels; this was also in accordance with the previous studies (Moghimi *et al.*, 2006).

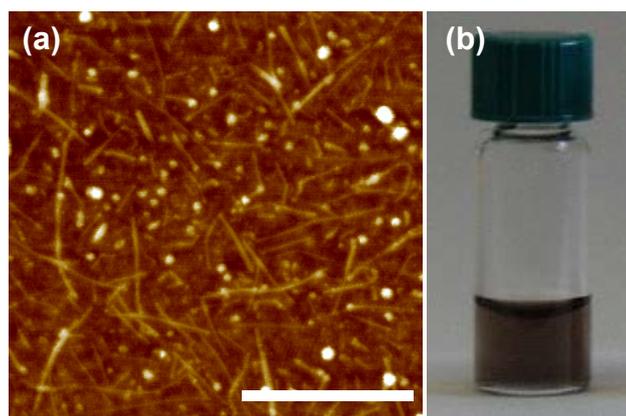


Fig. 3.1 An atomic force microscope image of amino-PEG₅₀₀₀-DSPE functionalized SWNTs over a SiO₂ substrate (a) and a photograph of MeO-PEG₅₀₀₀-DSPE stabilized SWNT suspension (b). Similar AFM images were also obtained with MeO-PEG₅₀₀₀-DSPE stabilized SWNTs. Scale bar = 250 nm. Photon correlation spectroscopy further revealed a mean particle size (equivalent spherical size) of 259 nm and a modal particle size of 214 nm for amino-PEG₅₀₀₀-DSPE functionalized SWNTs. The corresponding values for MeO-PEG₅₀₀₀-DSPE functionalized SWNTs were 175 nm and 117 nm, respectively.

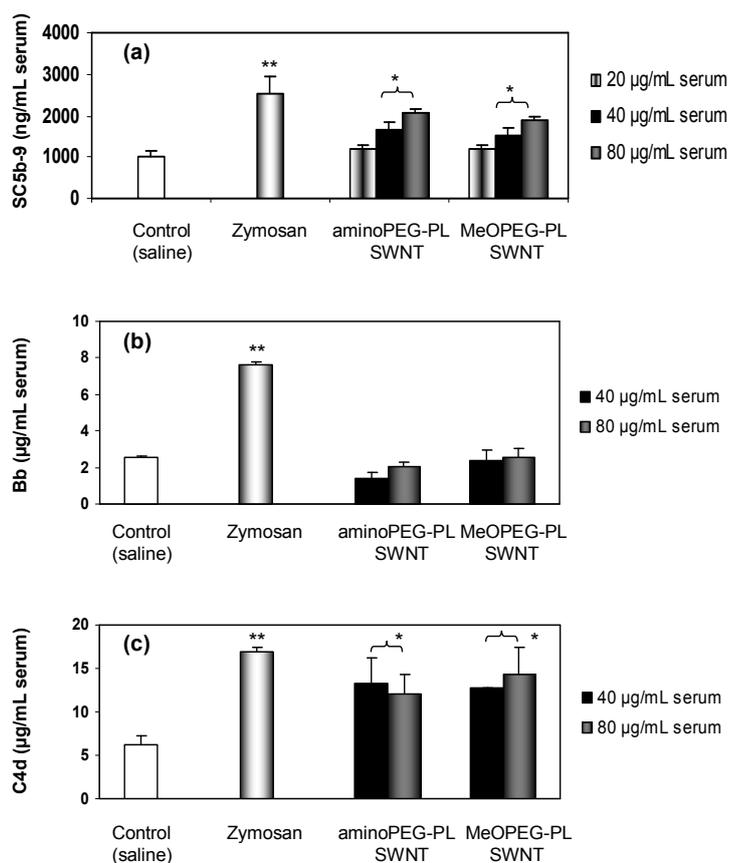


Fig. 3.2 PEGylated SWNT-mediated complement activation in human serum. SC5b-9, Bb and C4d levels in human serum. In (a) SC5b-9 levels reached maximum at a final nanotube concentration of 60 µg/mL of serum (not shown); the results are similar to those at 80 µg/mL. Zymosan was used as a positive control. No complement activation was achieved by the coating materials in the absence of nanotubes, even at concentrations as high as 2 mg/mL in serum. Significant difference with respect to control (saline): * $p < 0.05$, ** $p < 0.01$.

This study next sought to investigate through which pathway(s) nanotube-mediated complement activation proceeds, starting with the alternative pathway. The alternative pathway is stimulated by the spontaneous cleavage of thioester bond in C3 or when the internal thioester bond in the α -chain of nascent C3b undergoes nucleophilic attack in the presence of a foreign surface structure rich in nucleophilic groups (particularly hydroxyl- and amino-rich surfaces) (Szebeni *et al.*, 1998; Lambris *et al.*, 2008; Toda *et al.*, 2008). These structural modifications in C3 firstly allow the binding of complement Factor B, and secondly promote its cleavage by factor D thus initiating the formation of the C3 convertase C3bBb. Subsequently, through spontaneous decay and/or mediation of complement factor H, the 60 kD Bb fragment becomes dissociated from the C3 convertase (Lambris *et al.*, 2008). This split-product (Bb) is indeed a specific marker of complement activation through the alternative pathway and can be measured by ELISA in the fluid phase (Szebeni *et al.*, 1998). There was no effect of PEGylated SWNTs on serum Bb levels even at concentrations as high as 80 μg nanotube/mL (Fig. 3.2b); this was rather surprising since the projected surfaces are rich in nucleophilic groups (Toda *et al.*, 2008). Control experiments, with standard Bb samples, further confirmed that Bb has no affinity for the nanotube surface. The lack of factor B cleavage also exclude a role for direct C3 adsorption to SWNTs with subsequent formation of the C3Bb convertase through altered C3 conformational changes that resembles C3b; such mode of activation was recently reported for the polystyrene surface (Andersson *et al.*, 2002). Nevertheless, these results are in-line with the recent suggestion of Salvador-Morales *et al.* (2006), where non-functionalized HIPco SWNTs also failed to activate human complement through the alternative pathway.

Classical pathway activation is triggered when the hexameric C1q subcomponent of C1 binds to an activator. This induces a conformational change within the C1 complex resulting in conversion of the zymogen C1s subcomponent into an active proteolytic enzyme that subsequently activates the C1s subcomponent (Lambris *et al.*, 2008). Activation of the lectin pathway proceeds following the binding of mannose binding lectin (MBL) or ficolins to an activator. Both MBL and ficolins are complexed with MBL-associated serine proteases (MASPs)-1, -2, -3 and the sMAp19 protein in their proenzyme forms; it is the binding of MBL and ficolin to an activator that induces a conformational change in MASPs and convert them into active proteolytic enzymes (Vorup-Jensen *et al.*, 2000; Matsushita *et al.*, 2000; Fujita, 2002). The activated form of the serine protease C1s of the classical pathway and the MASP-2 of the lectin pathway, both cleave the C4 protein; this in turn activates C2 thus leading to subsequent generation of the classical/lectin pathway C3 convertase, C4b2a (Lambris *et al.*, 2008, Vorup-Jensen *et al.*, 2000; Fujita, 2002). One of the final fluid phase degradation products of C4 cleavage is the C4d fragment, and this is an established marker of complement activation initially triggered through both classical and lectin pathways (Moghimi *et al.*, 2006; Szebeni *et al.*, 1997). In this study, SWNTs elevated fluid phase C4d levels in all tested human sera by 2–3 fold above the background level as determined by ELISA (Fig. 3.2c), thus indicating the role of these two pathways in nanotube-mediated complement activation. In agreement with SC5b-9 measurements, PEG-PL conjugates were shown not to elevate serum C4d levels (Moghimi *et al.*, 2006). Next, a C2-depleted human serum was used to confirm that the observed SWNT-mediated elevation of SC5b-9 (in Fig. 3.2a) was dependent on the generation of C4b2a convertases, as these convertases trigger downstream

reactions. Indeed, in C2-depleted serum SWNTs failed to dramatically elevate SC5b-9 levels (872 ± 54 ng/mL serum) above the background (811 ± 28 ng/mL serum).

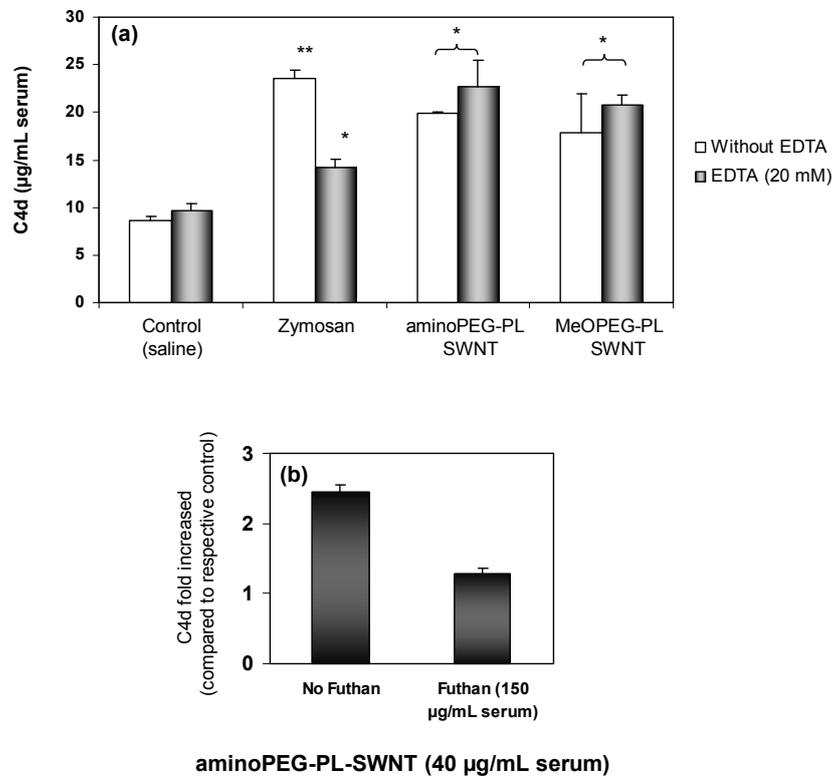


Fig. 3.3 PEGylated SWNT-mediated rises of the fluid phase C4d in C1q-depleted human serum. In (a) the final nanotube concentration in serum was 40 µg/mL. Zymosan was used a positive control for monitoring activation of the lectin pathway. Significant difference is compared with the respective control (saline or EDTA): * $p < 0.05$, ** $p < 0.01$.

To further distinguish between the calcium-sensitive C1q-dependent classical pathway and the lectin pathway mode of activation, a C1q-depleted human serum was used (with physiological MBL and C3 levels). Remarkably, nanotubes induced significant elevation of C4d levels in the C1q-depleted serum both in the absence and the presence of EDTA (Fig. 3.3a); this is in contrast to non-functionalized carbon nanotubes where complement activation was reported to occur directly via C1q binding (Salvador-Morales *et al.*, 2006). To further eliminate a direct role for the serine protease C1s in PEGylated SWNT-mediated complement activation, a C1q-depleted serum was pretreated with a monoclonal antibody against C1s (the antibody recognizes the binding site of C1s for C4 and reacts with both active and inactive C1s). Again, nanotubes significantly elevated C4d and SC5b-9 levels in serum above the respective baselines (incubations in the presence of an irrelevant antibody), thus eliminating the role of the classical pathway in SWNT-mediated complement activation. PEGylated SWNTs, therefore, most likely trigger complement activation through the lectin pathway (Fig. 3.5); further indication for the involvement of lectin pathway in nanotube-mediated complement activation was derived from experiments with Futhan (Fig. 3.3b), a serine protease inhibitor with broad specificity, and which is known to prevent complement activation through all three pathways (Pfeifer *et al.*, 1999). Elevation of nanotube-mediated rises of serum SC5b-9 was also halted in the presence of Futhan in the C1q-depleted serum and was comparable to the background level (not shown). Throughout these experiments zymosan was used as a positive control; zymosan-mediated C4d elevation proceeded in C1q-depleted serum (an established mode of activation through the lectin pathway) but was partly suppressed in the presence of EDTA (a calcium-binding-mediated role for MBL) (Moghimi *et al.*, 2006).

To establish whether PEGylated SWNTs are also capable of inducing complement activation in vivo, this study monitored plasma thromboxane B2 (TXB2) levels following intravenous nanotube injection into rats. TXB2 is a direct marker for TXA2. When complement is activated in vivo, then generated anaphylatoxins C3a and C5a usually induce TXA2 release from blood cells, but due to its short half-life (~30 seconds) TXA2 is hydrolyzed rapidly to TXB2. Thus, TXA2 formation can be monitored by quantifying TXB2 and demonstration of increased serum TXB2 levels provides evidence for in vivo SWNT-mediated complement activation (Moghimi *et al.*, 2006; Szebeni *et al.*, 1997). Erratic responses were observed in rats; while in some animals nanotube administration (1.2 mg/kg) was associated with a significant rise in plasma TXB2 level on a timescale of minutes with return to background levels at 1 h (a feature consistent with complement activation), other rats showed no response (Fig. 3.4). In contrast to nanotubes, intravenous administration of PEG-PL conjugates (1.2 mg/kg) did not alter plasma TXB2 levels, which was also in agreement with previous studies (Moghimi *et al.*, 2006). As a positive control, PEGylated liposome administration induced rises in plasma TXB2 levels in all tested rats. The lack of response to SWNT injection in some rats could be due to generation of insufficient quantities of anaphylatoxins C3a and C5a necessary for initiating immune cell degranulation either directly or through the release of a secondary co-stimulus, such as those arising from the kallikrein-kinin system (Szebeni, 2005; Hamad & Moghimi, 2008). It is also plausible that, in contrast to human serum, SWNT-mediated complement activation could proceed through other pathways in the rat model. However, if nanotube-mediated complement activation in rats is through the lectin pathway, then the observed variations may arise as a result of different levels of plasma MBL- or ficolin-/MASPs and/or MASP activity among individual animals.

Liposome-mediated complement activation, however, occurs through both antibody-mediated C1q-dependent and alternative pathways in the described model, which presumably raises sufficient quantities of anaphylatoxins necessary for subsequent immune cell degranulation (Moghimi *et al.*, 2006; Szabeni *et al.*, 1997).

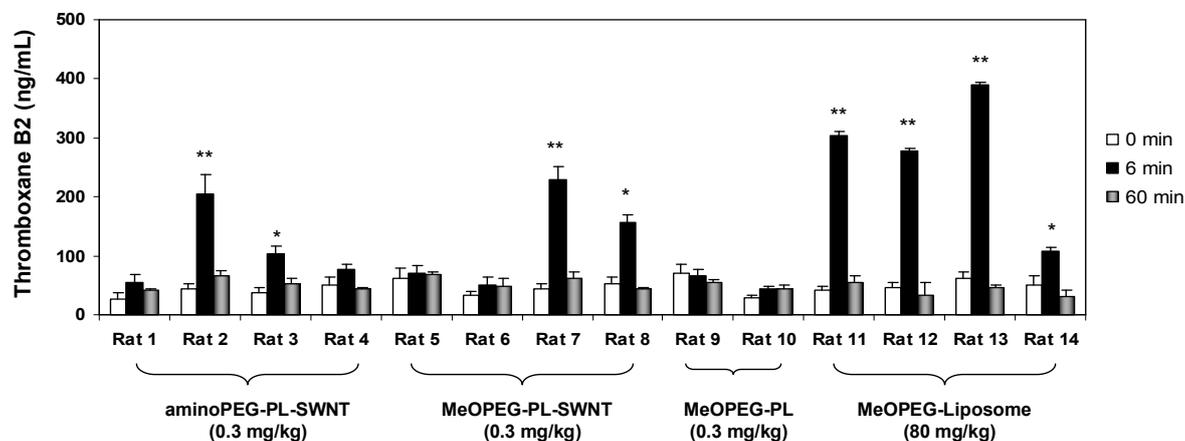


Fig. 3.4 Plasma TXB2 levels in rats following bolus intravenous injection of PEGylated SWNTs, PEG-PL conjugates and PEGylated liposomes. Data from each individual animal is shown. For MeOPEG-PL, 4 rats were also used and no responses were observed; only two representative examples are shown. Even at higher concentrations of MeOPEG-PL, (tested up to 10 mg/kg), TXB2 levels were comparable to the background (not shown). Significant difference is compared with the 0 min (baseline, TXB2 level in plasma prior to nanotube, MeOPEG-PL and liposome injection): * $p < 0.05$, ** $p < 0.01$.

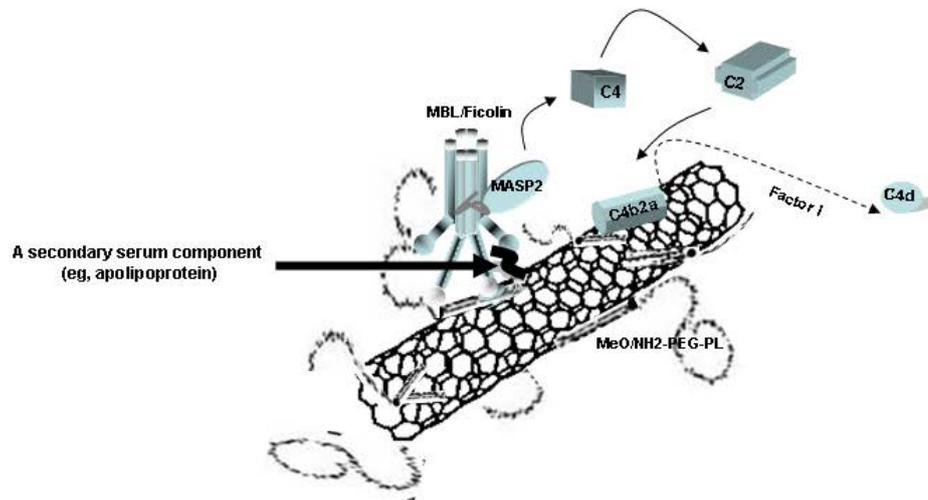


Fig. 3.5 Proposed schematic representation of SWNT-mediated complement activation via the lectin pathway. Plasma MBL and/or ficolin presumably bind in a simultaneous manner to some structural determinants of both the projected PEG chains and the nanotube surface. However, MBL and ficolins express specificity for sugars with N-acetylated groups, but these structures are absent on the surface of the native PEGylated SWNTs. It is most likely that the recognition domain for MBL and ficolin binding is provided via some other adsorbed serum components, such as apolipoproteins. Hydrophobic interactions may also play some role in MBL/ficolin binding to PEG chains/nanotube surface as well as electrostatic interactions between MBL/ficolin and the anionic phosphate oxygen moiety of the PEG-PL conjugate. Nevertheless, this binding activates MASP-2, a serine protease that is initially associated in a proenzyme form with both MBL and ficolin. Activated MASP-2 cleave complement component C4, and this in turn cleave the next complement protein (C2) to form the C4b2a convertase, which subsequently triggers downstream reactions (C3 conversion, anaphylatoxin generation, and formation of the membrane attack complex C5b-9).

3.4 Conclusion

Aminopoly(ethyleneglycol)₅₀₀₀-distearoylphosphatidylethanolamine (aminoPEG₅₀₀₀-DSPE) and methoxyPEG₅₀₀₀-DSPE coated as-grown HIPco single-walled carbon nanotubes activate complement in undiluted human serum. Complement activation was independent of both the classical (C1q-dependent) and the alternative pathways, but the data from C1q-depleted sera strongly suggest a likely role for the involvement of the lectin pathway. Moreover, intravenous injection of nanotubes in some rats was associated with a significant rise in plasma thromboxane B2 levels, indicative of in vivo nanotube-mediated complement activation.

Acknowledgment

I would like to thank the department of Chemistry at Stanford University, Stanford, CA, USA for the preparation of carbon nanotubes, carrying out the *in vivo* studies and the AFM in Fig. 3.1.

Chapter four:

Poly(ethylene glycol)s trigger complement activation in human serum through alternative and lectin pathways: a plausible cause for anaphylaxis

4.1 Introduction

Poly(ethylene glycol) (PEG) is a linear polyether diol. This nonionic surfactant, which is commonly used in a wide range of human and veterinary pharmaceutical agents, has so far been perceived to be immunologically inert and safe, and is eliminated from the body intact by kidneys (for PEGs $> 20000 \text{ g mol}^{-1}$) following intravenous injection (Yamaoka *et al.*, 1994; Harris & Chess, 2003). However, manufacturers data sheet on some high PEG content intravenous medicines (eg, as in veterinary settings) warns about unexplained acute adverse reactions such as ataxia, restlessness and trembling, respiratory abnormalities, frothing at the mouth, collapse and even death in cattle, sheep and swine receiving such formulations (Bio-Mycin[®] 200 Data Sheet, 2008). Interestingly, adverse non-IgE-mediated hypersensitivity reactions, which are also associated with cardiac anaphylaxis and rapid haemodynamic collapse, are known to occur in some humans and animals who have received intravenous formulations containing macromolecular nonionic surfactants that are structurally similar to PEG. Examples include polyethylene oxide-polypropylene oxide based block copolymers, such as poloxamer 188 (Tremper *et al.*, 1984; Police *et al.*, 1985; Kent *et al.*, 1990). Block copolymers like poloxamer 188 can trigger complement activation both at sub-micellar concentrations as well as in micellar forms (Moghimi *et al.*, 2004), and the observed adverse responses following administration of poloxamer-based medicines are strongly believed to be secondary to complement activation through the generation of anaphylatoxins C3a and C5a, leading to the subsequent release of thromboxane A2 and other inflammatory mediators from immune cells (Moghimi *et al.*, 2004; Vercellotti *et al.*, 1982; Ingram *et al.*, 1993; Szebeni, 2005). Complement activation further generates the multiprotein terminal complex C5b-9, which has the capacity to elicit non-lytic stimulatory responses from vascular endothelial cells and,

therefore, modulate endothelial regulation of haemostasis and inflammatory cell recruitment (Hattori *et al.*, 2004).

In light of these observations, it is imperative to examine whether PEG can trigger complement activation, although it has long been considered to be a safe and a “biocompatible” macromolecule. Such studies are also important particularly with the notion that PEG has recently been identified as a therapeutic agent *per se* in a variety of experimental therapeutics and veterinary settings (Hattori *et al.*, 2004; Koob & Borgens, 2006). A remarkable example is the ability of intravenously injected PEG₃₅₀₀, particularly at high doses (600 mg/kg), to repair severe acute, naturally occurring paraplegia in dogs (Hansen type 1 lesions) (Hattori *et al.*, 2004). PEG is believed to target the spinal cord contusion and “anatomically” seal the membranes of damaged axons through membrane fusion and restore excitability. Indeed, earlier, direct application of PEG to exposed contusion injuries in guinea pigs was shown to rapidly restore variable levels of nerve impulsive conduction through the lesion, as documented by a rapid recovery of both somatosensory evoked potential conduction and cutaneous trunci muscle reflex (Borgens & Shi, 2000). Also, a recent study has further demonstrated that following traumatic axonal brain injury in rats, intravenously injected PEG can enter the brain parenchyma and repair cell membrane damage in corpus callosum and eliminate β -amyloid precursor protein accumulation in region of injury (Koob & Borgens, 2006).

For the first time, this study will examine whether that near-monodisperse endotoxin-free PEGs, at concentrations relevant to the above mentioned scenarios, can trigger complement activation in human sera. Depending on PEG concentration and M_w , and through which pathway/pathways this activation might proceed.

4.2 Materials and methods

4.2.1 Materials

Fluka standard PEGs were obtained from Sigma-Aldrich (UK). The weight average molecular weight (M_w) and the number average molecular weight (M_n) of PEG samples are presented in Table 4.1.

The following materials were bought from Sigma-Aldrich-UK: Analar grade ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) 99%, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol), cholesterol, zymosan, N-acetylglucosamine, D-galactose, D-mannose, pyridine, acetic anhydride, hydrochloric acid, ethyl acetate, copper sulfate, aqueous sodium hydrogen carbonate, sodium chloride and sodium sulfate. All solvents used in this research were 99% pure grades and used as received.

Complement activation product SC5b-9, C3a-desArg, Bb and C4d ELISA kits, commercially available human C1q-depleted serum and C2-depleted serum, and A commercially available factor H were obtained from Quidel (distributed by Technoclone, UK).

MBL-C4 complex ELISA kit and horseradish peroxidase-conjugated goat anti-mouse antibody were obtained from HyCult Biotechnology, The Netherlands.

Wielisa[®]-Total Complement Screen kit was purchased from Lund, Sweden.

4.2.2 Methods

4.2.2.1 PEG diacetylation

PEG₄₂₄₀ (100mg) in pyridine (40mL) and acetic anhydride (16mL) was stirred at room temperature overnight. The mixture was poured into dilute hydrochloric acid and the product extracted with ethyl acetate. The combined extracts were washed with copper sulfate solution, aqueous sodium hydrogen carbonate, water, saturated sodium chloride solution and dried over sodium sulfate. The solvent was removed *in vacuo* to afford diacetoxyPEG as a gum. Acetylation of the PEG₄₂₄₀ was confirmed by both ¹H and ¹³C NMR spectroscopy [(CDCl₃;360 MHz) ¹H NMR 2.08 ppm (-COCH₃) 3.64 ppm (-CH₂-); ¹³C NMR 19.81 ppm (-COCH₃) 70.55 ppm (-CH₂-) 169.89 ppm (-COCH₃)]. The ¹H NMR of the starting material contained a signal at 3.64 ppm consistent with CH₂ in the polymer backbone. In addition to this resonance the product contained a new peak at 2.08 ppm from the terminal methyl group of the acetate. Further structural support for the diacetylation was found in the ¹³C NMR spectrum of the product with new methyl (19.81 ppm) and quaternary carbon (169.89 ppm) resonances present for the acetate group, but was devoid of the CH₂OH signal in the starting material thus confirming acetylation had taken place.

4.2.2.2 Liposome preparation

Unilamellar vesicles of 204 ± 39 nm (polydispersity index = 0.065), and composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) and cholesterol in a mole ratio of 50:5:45 were prepared by hydrating the dried lipid film with 10 mM phosphate-buffered saline (pH 7.2) and subsequent extrusion through polycarbonate Nuclepore filters with pore diameters of 200 nm using a high-pressure extruder (Moghimi *et al.*, 2006). Liposome size was determined by laser light scattering using Malvern Zetasizer 300 (Malvern Instruments, Malvern, UK) at 25 °C (Moghimi *et al.*, 2006).

4.2.2.3 Preparation of human serum

Blood was drawn from healthy male volunteers according to approved local protocols. Blood was allowed to clot at room temperature and serum was prepared, aliquoted and stored at -80°C . Serum samples were thawed and kept at 4°C before incubation with test reagents. Commercially available human C1q-depleted serum and C2-depleted serum were used in this study.

Table 4.1. PEG characteristics

Designation	M_w (gmol⁻¹)	M_n (gmol⁻¹)	Polydispersity	(OCH₂CH₂)_n
PEG₁₉₆₀	1960	1900	1.032	$n \sim 44$
PEG₄₂₄₀	4240	4120	1.029	$n \sim 96$
PEG₈₃₅₀	8350	8100	1.031	$n \sim 189$
PEG₁₁₆₀₀	11600	10800	1.074	$n \sim 263$

4.2.2.4 Assays of *in vitro* complement activation

To measure complement activation *in vitro*, this study determined PEG-induced rise of serum complement activation product SC5b-9, C3a-desArg, Bb and C4d, using respective Quidel's ELISA kits according to the manufacturer's protocols as described previously (Moghimi *et al.*, 2004 and 2006). As a result of substantial biological variation in serum levels of complement proteins and the large number of positive and negative feedback interactions (Moghimi *et al.*, 2006), this experiment monitored generation of complement activation products in sera of 5 healthy individuals separately. The concentration of mannan binding lectin (MBL) in healthy and selected complement protein-depleted sera was determined by using the MBL-C4 complex ELISA kit. Only sera with physiological concentrations of MBL, in the range of 3000–5000 ng/mL, were selected for subsequent complement activation assays. The complement haemolytic activity of C1q-depleted serum and C2-depleted serum was restorable following the addition of C1q (180 µg/mL) and C2 (650 µg/mL), respectively (Szebeni *et al.*, 1994). The functional activity of classical, lectin and the alternative pathways of complement were confirmed in all sera with Wielisa[®] - Total Complement Screen kit.

For measurement of complement activation, the reaction was started by adding the required quantity of PEG solutions (in sterile physiological saline) or liposomes to undiluted serum (typically, 10 µl of PEG solution or liposomes were added to 40 µl of serum (in a 1:4 ratio)) in Eppendorf tubes (in triplicate) in a shaking water bath at 37°C for a set period of 30 min. when stated and prior to PEG or liposomes addition, other components were added to serum to assess the role of individual pathways. Reactions were terminated by addition of “sample diluent” provided with assay kit. Control serum incubations contained saline (the same volume as PEGs or liposomes)

for assessing background levels of complement activation products. Zymosan (5 mg/mL) was used as a positive control for complement activation. The level of the complement activation products was then measured by the respective ELISA kits and compared with control incubations in the absence of PEG. In some experiments, PEG-induced complement activation was monitored following pretreatment of serum with EGTA/Mg²⁺ (10 mM/2.5 mM), Futhan (150 µg/mL), N-acetylglucosamine (25 mM), D-galactose (25 mM) and D-mannose (25 mM). Control serum incubations contained the same quantity of the added compounds and PEG was replaced with the same volume of saline.

For quantification of complement activation products, standard curves were constructed using the assigned concentration of each respective standard supplied by the manufacturer and validated. The slope, intercept and correlation coefficient of the derived best-fit line for SC5b-9, C3a-desArg, Bb and C4d standard curves were within the manufacturer's specified range. The efficacy of PEG treatments was established by comparison with baseline levels using paired *t* test; correlations between two variables were analyzed by linear regression, and differences between groups (when necessary) were examined using ANOVA followed by multiple comparisons with Student-Newmann-keuls test. Similar patterns were observed in all tested sera; the result of a typical experiment is presented.

4.2.2.5 SDS-PAGE and Western blot analyses

Serum was treated with PEG₄₂₄₀ (5mM and 10 mM final concentration) or PEG₁₁₆₀₀ (1.5 mM final concentration) for 5 min at room temperature. Aggregated proteins were precipitated by centrifugation (16000 x *g*, 20 min) and re-suspended in physiological saline. Proteins were then subjected to SDS-PAGE using 10–12% gels,

immunoblotted with a murine monoclonal antibody against factor H (1:2000 v/v;) and bound factor H was detected using horseradish peroxidase-conjugated goat anti-mouse antibody (1:2000). A commercially available factor H was used as a positive control.

4.3 Results and discussion

4.3.1 Activation of the whole complement pathway

Activation of the terminal half of the complement pathway culminates in the formation of the C5b-9 complex, which in the absence of a target membrane binds to vitronectin (S protein) (Lambris *et al.*, 2008). Therefore, PEG-mediated complement activation in human serum was first monitored by measuring the generation of the stable, soluble, non-lytic fluid-phase SC5b-9 complex, which is a sensitive measure of the activation of the whole complement cascade (Moghimi *et al.*, 2004 and 2006). The results in Fig. 4.1 demonstrate that PEGs of varying M_{ws} can activate complement in a concentration-dependent manner, but on molar basis high M_w PEG species are more effective in activating complement than their low M_w counterparts. The results (Fig. 4.1e) also show a good correlation between SC5b-9 rises and PEG-mediated anaphylatoxin (C3a-desArg) generation, providing additional evidence that PEG-induced rises of SC5b-9 levels in serum is a reflection of complement activation rather than modulation of the terminal pathway only.

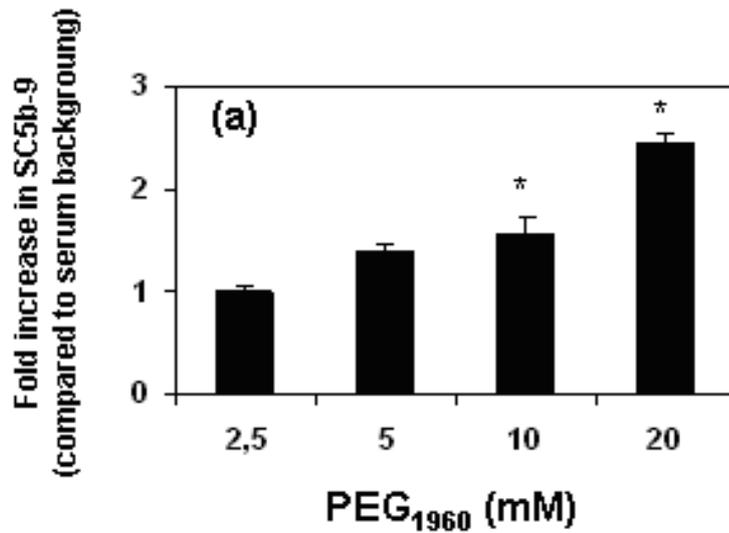


Fig. 4.1a. Complement activation as a function of PEG₁₉₆₀ concentration: PEG₁₉₆₀-mediated rises of SC5b-9 over background. The background SC5b-9 level in this particular serum (saline treated) was 873 ± 46 ng/mL. Zymosan (5 mg/mL) was used as a positive control and raised serum SC5b-9 levels by 3.32 fold. Similar patterns in SC5b-9 elevations were obtained in 4 other tested human sera. Significant difference is expressed with respect to the background (saline-treated serum): $*p < 0.05$.

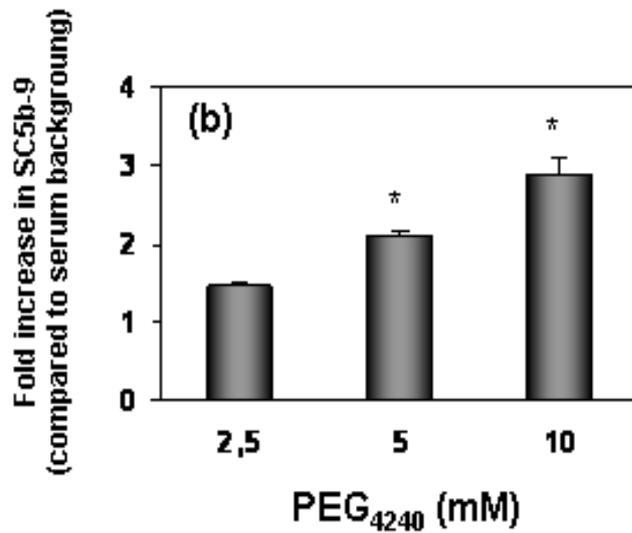


Fig. 4.1b. Complement activation as a function of PEG₄₂₄₀ concentration: PEG₄₂₄₀-mediated rises of SC5b-9 over background. The background SC5b-9 level in this particular serum (saline treated) was 873 ± 46 ng/mL. Zymosan (5 mg/mL) was used as a positive control and raised serum SC5b-9 levels by 3.32 fold. Similar patterns in SC5b-9 elevations were obtained in 4 other tested human sera. Significant difference is expressed with respect to the background (saline-treated serum): $*p < 0.05$.

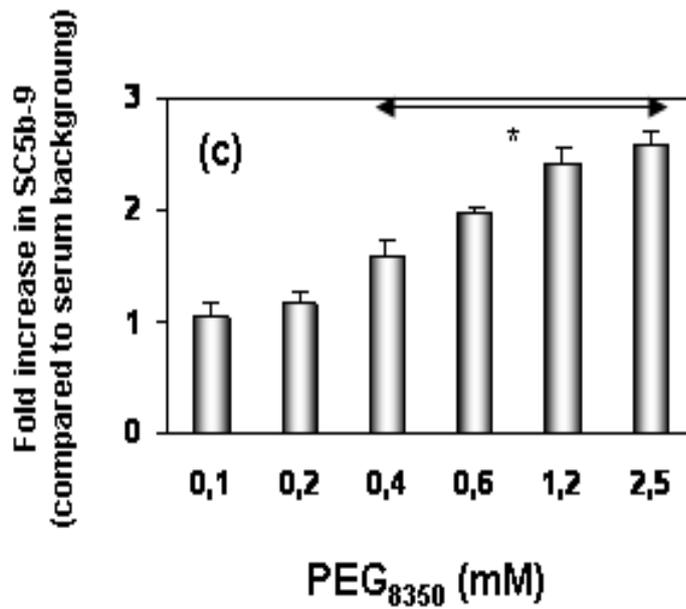


Fig. 4.1c. Complement activation as a function of PEG₈₃₅₀ concentration: PEG₈₃₅₀-mediated rises of SC5b-9 over background. The background SC5b-9 level in this particular serum (saline treated) was 873 ± 46 ng/mL. Zymosan (5 mg/mL) was used as a positive control and raised serum SC5b-9 levels by 3.32 fold. Similar patterns in SC5b-9 elevations were obtained in 4 other tested human sera. Significant difference is expressed with respect to the background (saline-treated serum): $*p < 0.05$.

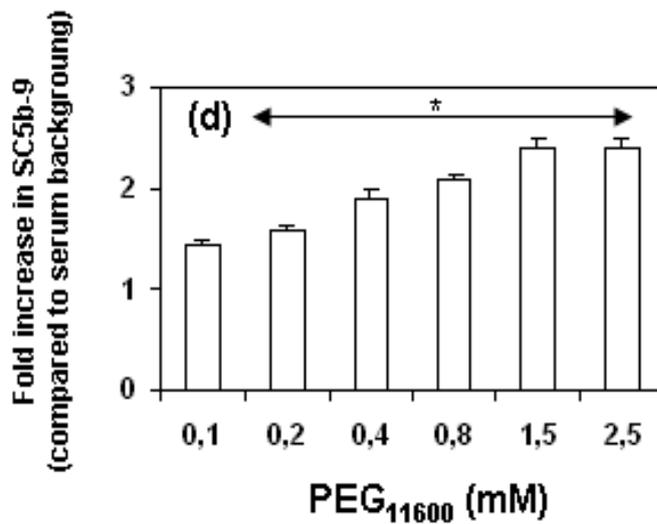


Fig. 4.1d. Complement activation as a function of PEG₁₁₆₀₀ concentration: PEG₁₁₆₀₀-mediated rises of SC5b-9 over background. The background SC5b-9 level in this particular serum (saline treated) was 873 ± 46 ng/mL. Zymosan (5 mg/mL) was used as a positive control and raised serum SC5b-9 levels by 3.32 fold. Similar patterns in SC5b-9 elevations were obtained in 4 other tested human sera. Significant difference is expressed with respect to the background (saline-treated serum): $*p < 0.05$.

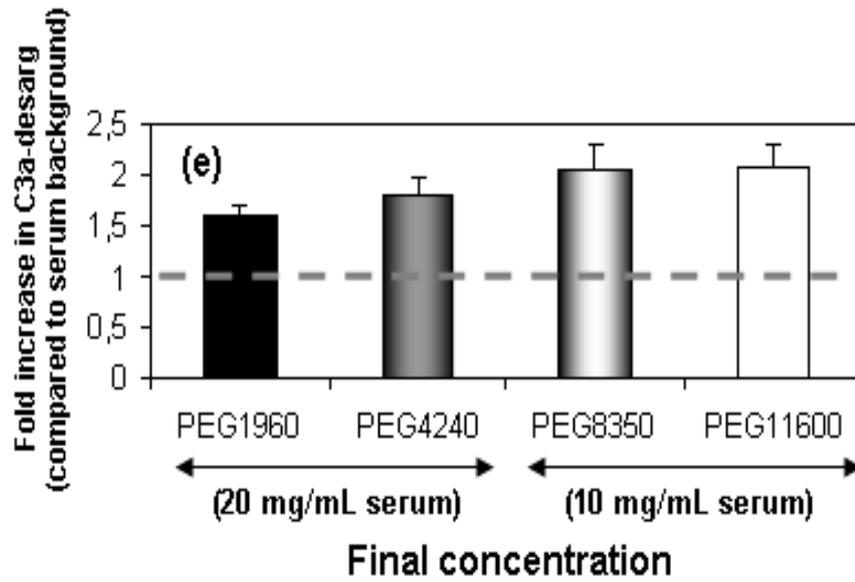


Fig. 4.1e. The influence of different molecular weights of PEG on anaphylatoxin C3a-desArg. All treatments elevated serum C3a-desArg levels significantly above baseline ($p < 0.05$ in all cases).

4.3.2 Alternative pathway mode of activation

Biophysical evidence suggests that in an aqueous environment, PEG chains, as a result of forming hydrogen bonds with water molecules, assume helical conformation (Kozielski, 2006; Tasaki, 1996; Crupi *et al.*, 1996). Statistically, more regions of “temporary ordering” or “structured water” are attainable with longer PEG chains compared with their shorter counterparts, and this may partly explain why higher M_w PEG species are more effective in triggering complement activating at far lower molar concentrations than low M_w PEGs. Here, complement activation most likely proceeds through the alternative pathway, where regions of “structured water” could act as a platform initiating hydrolysis of the thioester bond in C3 to form C3(H₂O), thus allowing for subsequent accommodation of factors B and D and assembly of fluid-phase C3bBb convertases. Therefore, this study monitored PEG-mediated rises of the split-product Bb in serum, which is a specific marker of complement activation through the alternative pathway (Moghimi *et al.*, 2004; Szebeni *et al.*, 1998; Moghimi *et al.*, 2006). In accordance with the above hypothesis, the results in Fig. 4.2a show that, on molar basis, the longer the PEG molecules the more effective they are in triggering alternative pathway activation. In the case of PEG₁₉₆₀, serum Bb levels were not elevated even at concentrations as high as 20 mM (40 mg/mL of serum), but the simultaneous increase in the serum levels of SC5b-9 and C3a-desArg (Fig. 4.1 a, e) strongly indicate that PEG₁₉₆₀-mediated complement activation proceeds through other pathways (classical and/or lectin pathway).

Activation of the alternative pathway by high M_w PEG species ($M_w = 4240 \text{ gmol}^{-1}$ or 8350 gmol^{-1}) may further represent amplification of C3 convertases initially triggered through C4-dependent pathway (Szebeni *et al.*, 1994). In order to demonstrate that PEG molecules can directly trigger activation of the alternative pathway this study

monitored Bb and SC5b-9 generation simultaneously in a C2-depleted human serum (Fig. 4.3a). With PEG₈₃₅₀ serum levels of both complement markers were significantly above background (Fig. 4.3a), thus showing the capability of these species to directly activate alternative pathway. On the contrary, PEG₄₂₄₀ only at 10 mM concentration was able to trigger Bb generation in C2-depleted serum (Fig. 4.3a). Although, at lower PEG₄₂₄₀ concentrations (5 mM) neither serum Bb nor SC5b-9 levels were elevated, significant rises in SC5b-9 levels above background in C2-depleted serum was only demonstrable following restoration of C2 at physiological concentrations; this observation strongly suggest a role for the C4-dependent pathway and involvement of C4b2a convertases in subsequent assembly of the terminal complement complex.

In a protein solution, PEGs, in a concentration-dependent manner, can favour the formation of protein crystals by decreasing the protein solubility through “depletion attraction”, but PEG can also induce other phase changes such as “liquid-liquid” phase separation, protein aggregation and the formation of gels (Asakura & Oosawa, 1954; Tardieu *et al.*, 2003). Results here therefore reasoned PEG-mediated triggering of the alternative pathway may in part arise from depletion of factor H, the major fluid-phase regulator of the alternative pathway (Pangburn *et al.*, 1977). Factor H attenuates alternative pathway activation by inhibiting the binding of factor B to C3b, accelerating the decay of preformed C3Bb convertases and acting as a cofactor for the serine protease factor I to cleave C3b (Walport, 2001a,b). Western blot analysis of PEG₄₂₄₀- and PEG₁₁₆₀₀-induced protein aggregates in human serum has confirmed the presence of factor H (Fig. 4.3b). With PEG₄₂₄₀ treatment, factor H was only detectable when surfactant concentration was at 10 mM or above. These observations are in-line with PEG-mediated Bb generation in the C2-depleted serum (Fig. 4.3a).

4.3.3 C4-Dependent complement activation

C4d is a fluid phase degradation product of C4 cleavage, mediated by complement control protein C4bp and factor I, and an established marker of classical and lectin pathway activation (Moghimi *et al.*, 2006; Scharfstein *et al.*, 1978; Fujita *et al.*, 1998). Accordingly, further indication for the involvement of C4-dependent pathway was obtained by showing PEG-mediated rises of C4d in both normal and C2-depleted human serum (Fig. 4.2b & 4.3a). Low M_w PEG molecules (eg, PEG₁₉₆₀) and PEG₄₂₄₀ (at concentrations below 10 mM), therefore, seem to activate complement exclusively via C4-dependent pathway, whereas PEG₄₂₄₀ (at 10 mM) and higher M_w PEG species trigger complement through both C4-dependent and alternative pathways.

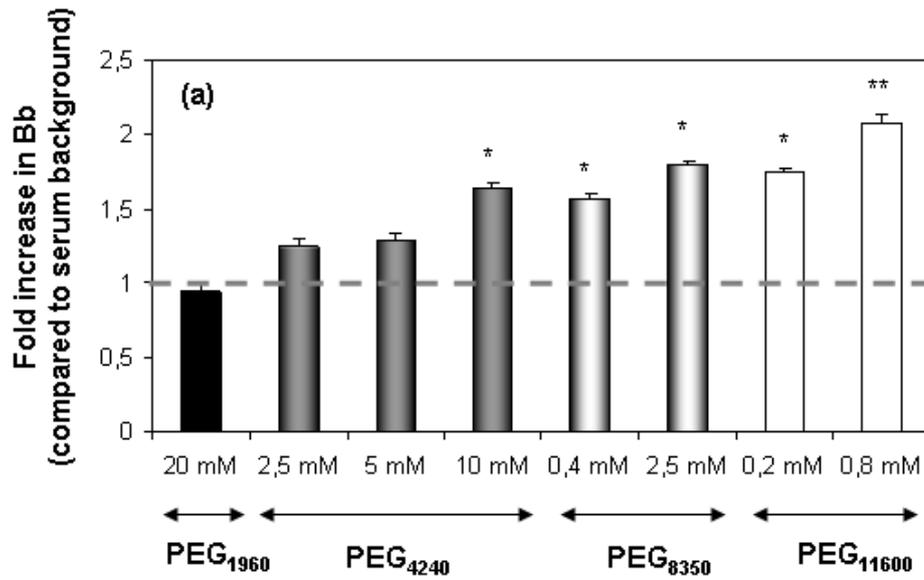


Fig. 4.2a. Elevation of complement split-product Bb as a function of PEG molar concentration and molecular mass. Serum source was the same as in Fig. 4.1. Serum Bb background level was $0.42 \pm 0.06 \mu\text{g/mL}$. Similar patterns were further obtained with 4 other tested sera from separate individuals. Significant difference with respect to background (saline-treated serum): * $p < 0.05$, ** $p < 0.01$.

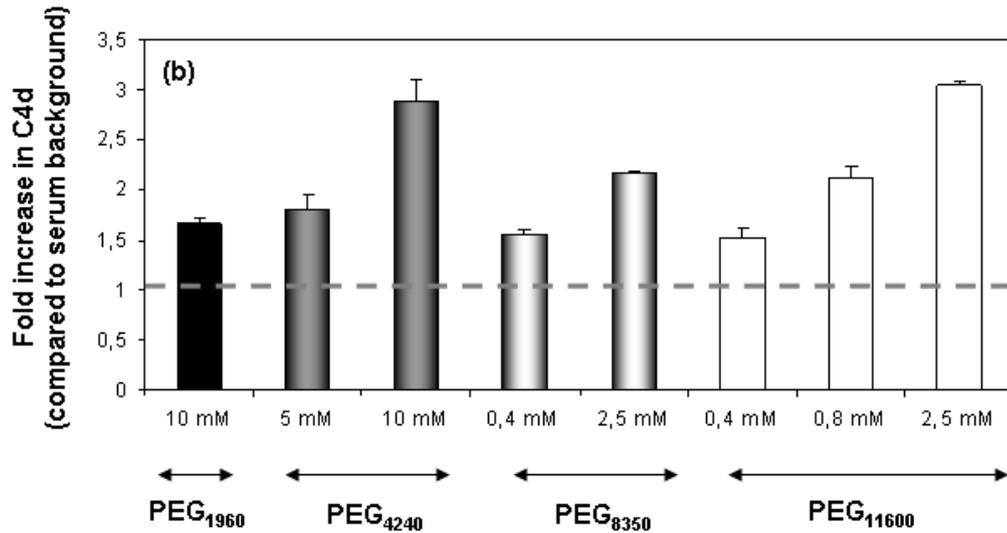


Fig. 4.2b. Elevation of complement split-product C4d as a function of PEG molar concentration and molecular mass. Serum source was the same as in Figure 4.1. Serum C4d background level was $2.24 \pm 0.16 \mu\text{g/mL}$. Similar patterns were further obtained with 4 other tested sera from separate individuals. Significant difference with respect to background (saline-treated serum): all treatments elevated serum C4d levels significantly ($p < 0.05$ in all cases).

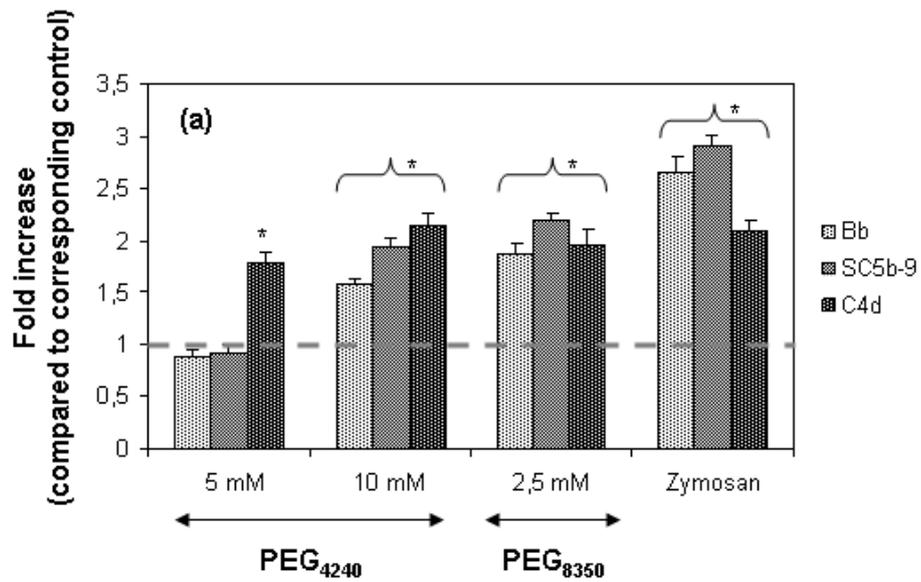


Fig. 4.3a. PEG-mediated elevation of complement activation products SC5b-9, Bb and C4d in C2-depleted human serum. SC5b-9, Bb and C4d background levels (saline-treated serum) were $1.83 \pm 0.15 \mu\text{g/mL}$, $0.79 \pm 0.07 \mu\text{g/mL}$ and $3.88 \pm 0.17 \mu\text{g/mL}$, respectively. Significant difference with respect to background (saline-treated serum): $*p < 0.05$.

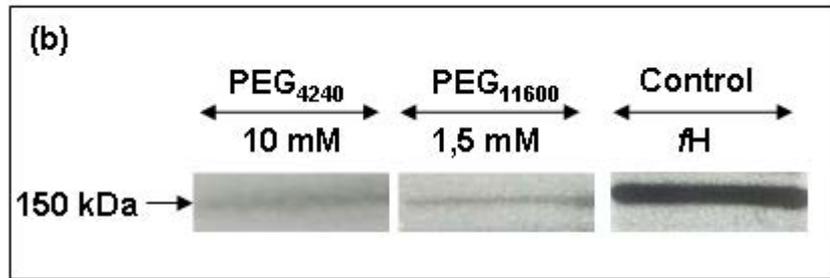


Fig. 4.3b. Western blot analysis of PEG-induced protein aggregates for factor H in normal serum. A commercially purified factor H was included as a positive control with immunoblots.

Next, this experiment examined through which pathway(s) PEGs could initiate C4 cleavage. Complement activation through the classical pathway may be initiated by binding of naturally occurring antibodies against PEGs. Since Ca^{2+} is essential for the operation of the classical pathway (Moghimi *et al.*, 2004 and 2006; Szebeni *et al.*, 1994 and 1998) first this experiment measured PEG₁₉₆₀-mediated rises of both C4d and SC5b-9 in sera of 5 healthy individuals by excluding Ca^{2+} from the assay (PEG₁₉₆₀ was used since it activated complement only through C4-dependent pathway). Remarkably, in all EGTA/ Mg^{2+} supplemented sera, PEG₁₉₆₀ treatment significantly elevated both C4d and SC5b-9 levels above background (Fig. 4.4a), thus eliminating the role of classical pathway. With longer PEG chains, C4d levels in EGTA-chelated sera were also elevated (not shown). As a control, Ca^{2+} chelation in serum halted cholesterol-rich liposome-mediated rises of C4d but not SC5b-9 level (Fig. 4.4b), as these vesicles activate complement through both antibody-mediated classical pathway as well as alternative pathway (Szebeni *et al.*, 1994 and 1998). However, PEG-mediated complement activation through the classical pathway in a serum with high titer of anti-PEG antibodies, notably of IgM class still cannot be disregarded. To further eliminate the role of classical pathway, this study examined PEG-mediated C4d rises in a C1q-depleted serum and the results in Fig. 4.5a corroborate these findings with EGTA-chelated sera. In addition, these results further exclude a direct role for C1q in PEG-mediated complement activation.

Exclusion of the C1q-dependent pathway in PEG-mediated complement activation raises the question as to whether PEGs are capable of triggering complement via the lectin pathway. The lectin pathway initiator complex consists of either mannan-binding lectin (MBL) or ficolin and three MBL-associated serine proteases 1–3 (MASP-1, -2, -3) and the smaller non-enzymatic component sMAP (Fujita, 2002;

Wallis, 2002). MBL bind to monosaccharides such as mannose, fucose and N-acetylglucosamine with affinities typically in mM range, where the sugar binding site is localized around one of two Ca^{2+} sites of the carbohydrate-recognition domain (CRD) (Wallis, 2002; Lee *et al.*, 1991; Jack *et al.*, 2001; Weis *et al.*, 1992). Equatorial hydroxyl groups at the 3- and 4-OH positions of the sugar residue serve as coordination ligands for the Ca^{2+} (Weis *et al.*, 1992). Additional coordination ligands are further provided by asparagine and glutamic acid residues in the CRD that form hydrogen bonds with the equatorial 3-OH and the 4-OH groups. Ficolins, on the other hand, express specificity only for sugars with N-acetylated groups (Fujita, 2002) as well as acetylated compounds, relatively independent of the structure of the acetylated molecule (Krarup *et al.*, 2004).

Next this experiment eliminated the possible involvement of MBL in PEG-mediated complement activation, since PEG treatment of C1q-depleted serum in the presence of 25 mM D-mannose also elevated C4d levels but not when mannose was replaced with N-acetylglucosamine (Fig. 4.5a). D-galactose was used as a nonantagonist (negative control). In addition, the OH groups at PEG termini seem to play a minor role in complement activation since acetylated PEGs also raised C4d levels equally in C1q-depleted serum (Fig. 4.5a). These observations therefore suggest a likely role for ficolins in PEG-mediated complement activation. PEG-mediated C4d elevation in C1q-depleted serum was further inhibited by Futhan (a broad-spectrum serine protease inhibitor) (Pfeifer *et al.*, 1999), thus indicating a role for activation of a serine protease (presumably MASP-2) for subsequent C4 cleavage.

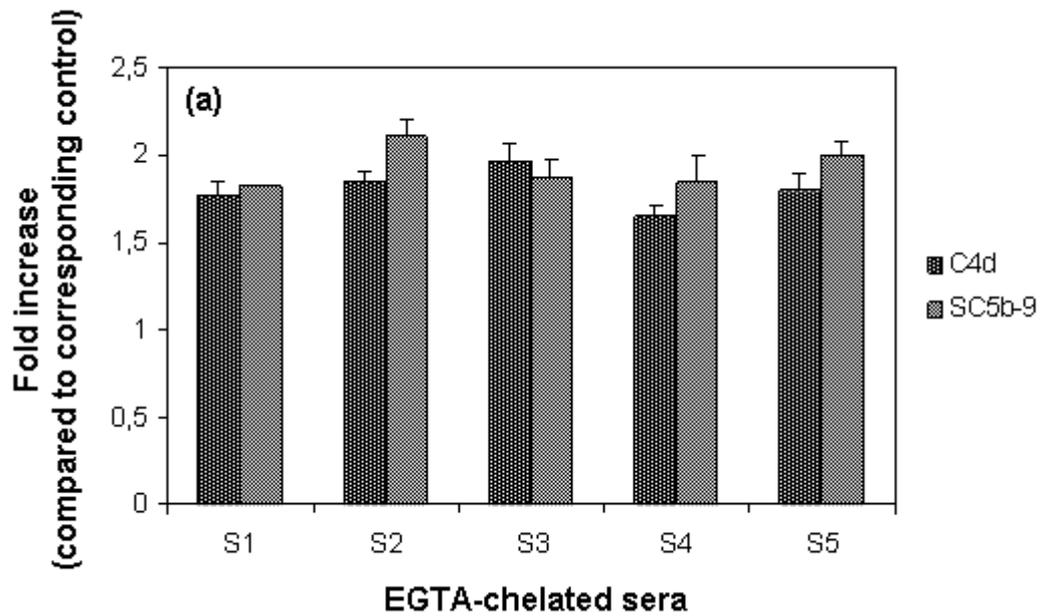


Fig. 4.4a. Comparison of PEG- and cholesterol-rich liposome-mediated complement activation in EGTA/Mg²⁺-treated sera. Sera from 5 healthy male subjects were used (designated as S1–S5): PEG-mediated elevation of complement activation products. In all cases $p < 0.05$.

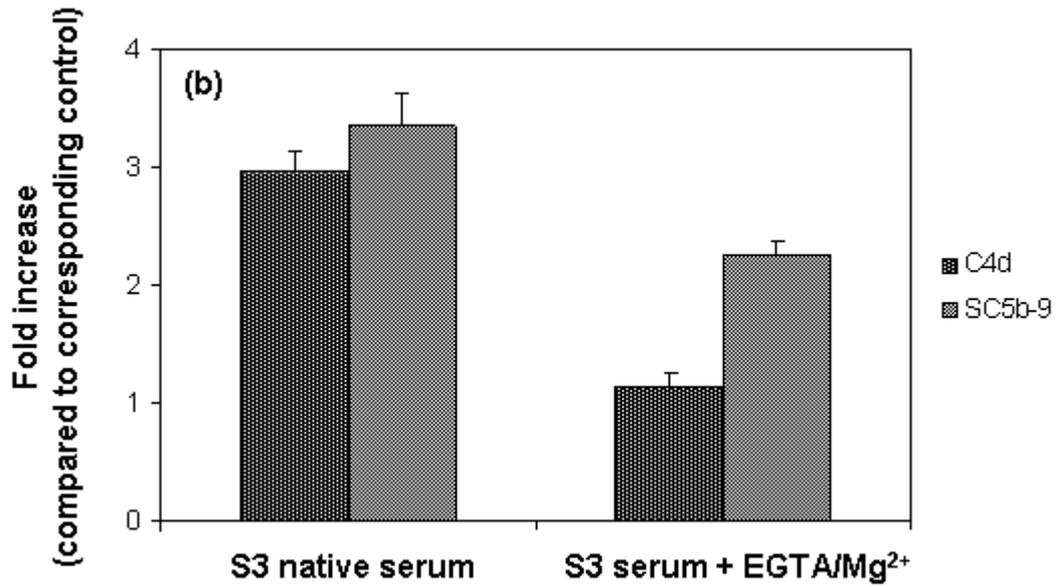


Fig. 4.4b. Comparison of PEG- and cholesterol-rich liposome-mediated complement activation in EGTA/Mg²⁺-treated sera: The effect of liposomes (3 mg lipid/mL) on serum SC5b-9 and C4d levels of subject 3 (S3) in the absence and presence of EGTA/Mg²⁺. In all cases $p < 0.05$ with the exception of liposome-mediated C4d level in S3 + EGTA/Mg²⁺ (not significant) when compared with corresponding background levels of complement activation products.

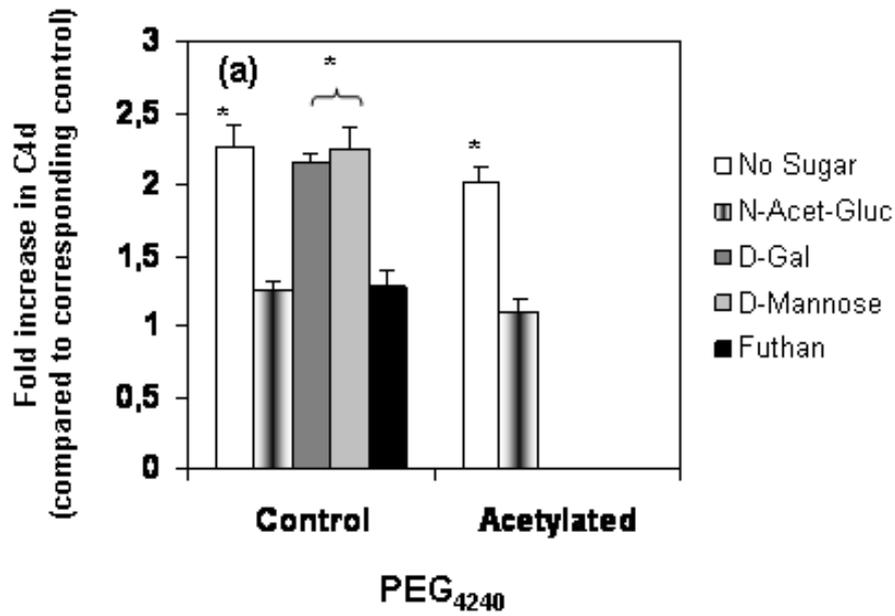


Fig. 4.5a. The effect of native and acetylated PEG₄₂₄₀ on C4d generation in C1q-depleted human serum. The C4d background level was $3.64 \pm 0.37 \mu\text{g/mL}$. PEG-mediated C4d levels were also measured by prior treatment of serum with sugars (25 mM final concentration) or Futhan (150 $\mu\text{g/mL}$ final concentration). Sugar or Futhan addition had no significant effect on C4d background levels. Significant difference to the respective background: $*p < 0.05$.

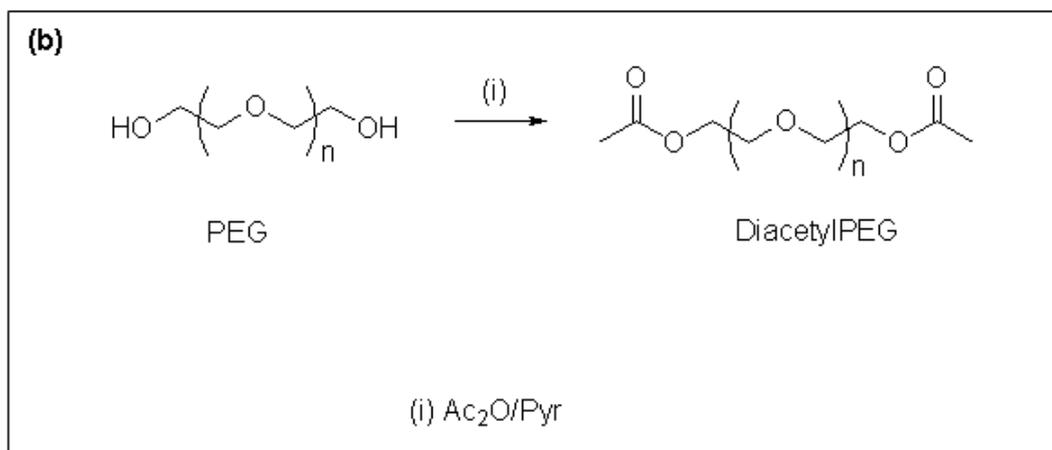


Fig. 4.5b. The chemical structure of acetylated PEG. For reaction chemistry see Materials and Methods.

A recent study has shown that after precipitation of serum proteins with 4% w/v PEG₆₀₀₀, approximately 80% of both L- and H-ficolins remained in the supernatant while the amounts of MBL and C1q were reduced dramatically (Krarup *et al.*, 2004). With lower PEG concentrations (2% w/v) virtually all ficolins were recoverable in the supernatant (Krarup *et al.*, 2004). Since we have observed C4d elevations in serum with both PEG₄₂₄₀ and PEG₈₃₅₀ at concentrations even below 2%, then it is rather unlikely that the apparent PEG-mediated lectin pathway activation may predominantly arise from binding of ficolin-MASPs to exposed substrates from aggregated proteins. Thus, these observations raise an intriguing question as to whether PEGs can directly interact with ficolins and activate MASP-2. Detailed investigations are currently in progress to address this issue.

4.3.4 Kinetics of PEG-mediated complement activation

The results in Fig. 4.6 show the kinetics of PEG-mediated complement activation. PEG₁₁₆₀₀-mediated elevation of serum SC5b-9 and Bb levels proceeded on a time scale of minutes and reached plateau at about 5 min (Fig. 4.6). Similar profiles were also observed with PEG₄₂₄₀ (10 mM) and PEG₈₃₅₀ (not shown). Remarkably, such complement activation time scales are in line with the rapidity of the observed acute adverse reactions or cardiovascular collapse in animals receiving intravenous injections of PEG-containing medicines.

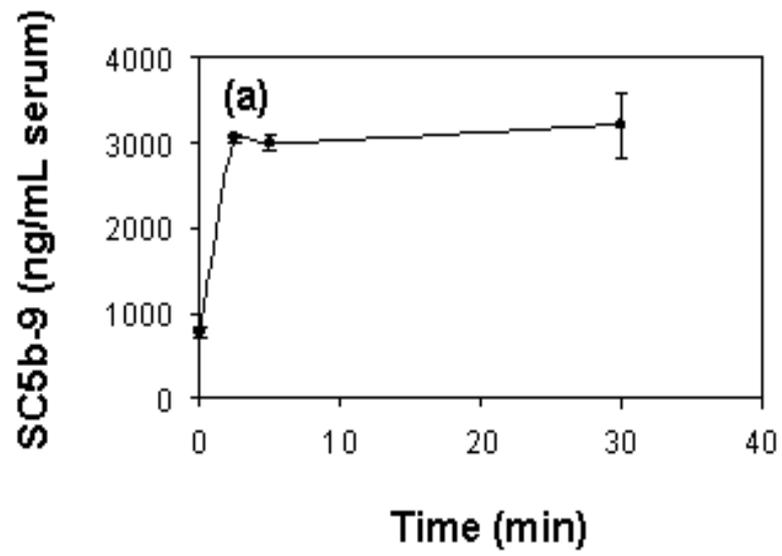


Fig. 4.6a. PEG₁₁₆₀₀-mediated SC5b-9 generation in a normal serum over time. PEG concentration was 1.5 mM.

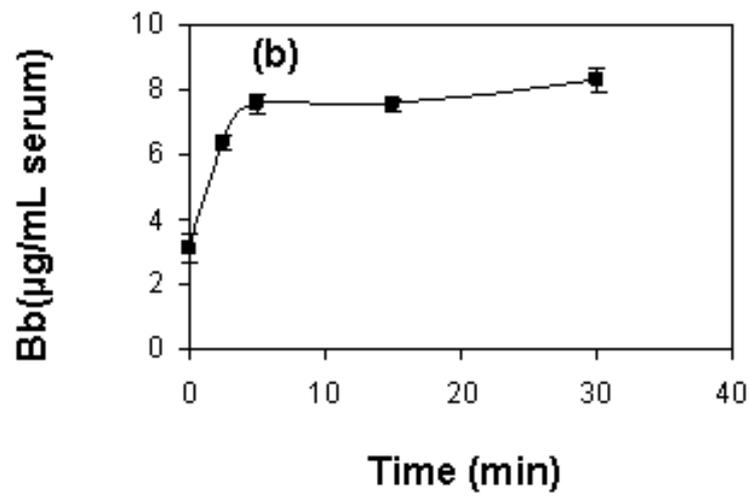


Fig. 4.6b. PEG₁₁₆₀₀-mediated Bb generation in a normal serum over time. PEG concentration was 1.5 mM.

4.4 Conclusion

Adverse non-IgE-mediated hypersensitivity reactions, which are associated with cardiac anaphylaxis and rapid haemodynamic collapse, are known to occur in some humans and animals who have received intravenous formulations of radiocontrast media, nonionic drug solubilizers (eg, cremophor EL, poloxamers) and particulate nanomedicines. These reactions arise at first treatment without prior sensitization (and may lessen or disappear on later treatments) and are strongly believed to be secondary to complement activation through the generation of anaphylatoxins C3a and C5a. These reactions have been reported to occur in some patients within 5-10 minutes of infusion in up to 45% of the individuals receiving nanomedicines (Szebeni, 2005).

Unexplained, but similar acute adverse reactions also occur to intravenous medicines with high contents of poly(ethylene glycol) (PEG) as solubilizer. Although PEG is often perceived to be immunologically inert and safe, here this study demonstrates that near-monodisperse endotoxin-free PEGs, at clinically relevant concentrations, can trigger complement activation in human sera on a time scale of minutes (reflected in significant rises in SC5b-9, C4d, Bb and C3a-desArg levels). With the aid of sera deficient in either C2 or C1q, results further demonstrate that, depending on PEG concentration and M_w , complement activation proceeds either exclusively through lectin pathway or through both lectin and alternative pathways. Although the highly hydrated PEG backbone could as act as a platform initiating hydrolysis of the thioester bond in C3 to form C3(H₂O), activation of the alternative pathway was also linked to PEG-mediated partial depletion of the fluid-phase regulator factor H. Competitive studies in C1q-depleted serum with D-mannose and N-acetylglucosamine further suggested a likely role for ficolins in PEG/diacetylatedPEG-mediated C4d elevation. Complement activation may therefore

provide a plausible explanation to the previously reported unexplained anaphylaxis or the referred cardiovascular collapse in species that have received medicines containing high levels of PEG as solubilizer/carrier. Results in this study are also relevant to potential therapeutic applications of PEGs (as in spinal cord injury and traumatic axonal brain injury) and warn about possible acute PEG infusion-related reactions.

Chapter five

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

5.1 Introduction

A number of studies have revealed that immediately after infusion of liposomes non-IgE-mediated hypersensitivity reactions occur in some individuals (Levine *et al.*, 1991; Ringdén *et al.*, 1994). The symptoms include cardiopulmonary distress such as dyspnea, tachypnea, hypertension/hypotension, chest pain, and back pain. Unlike Type I allergy, the response to liposomes arises at the first exposure without prior sensitization, and the symptoms may lessen or disappear on later treatment. Such pseudoallergic reactions are also common following infusion of stealth PEGylated liposomes (Alberts *et al.*, 1997), for instance the frequency of pseudoallergic responses among 705 patients treated with Doxil[®] (PEGylated liposomes with entrapped doxorubicin) was 6.8% (Dezube, 1996). Recent *in vitro* and *in vivo* studies have indicated that these reactions could arise through rapid production of anaphylatoxins (C3a, C5a) via complement activation, and the individual sensitivity to anaphylatoxin-derived mediators (Szebeni *et al.*, 2000; Chanan-Khan *et al.*, 2003). Liposome-encapsulated haemoglobin has potential application as a red cell substitute in transfusion medicine. Because of complement activation, the potential use of liposomes (with encapsulated haemoglobin) as a substitute for shed blood in trauma patients, who are prone to develop adult respiratory distress syndrome partly as a consequence of injury-related complement activation, could be problematic (Moore, 1994). Thus, further complement activation might aggravate the clinical state of trauma patients.

Adverse non-IgE-mediated hypersensitivity reactions also occur following infusion of Fluosol-DA (perfluorochemicals emulsified with the nonionic polyoxyethylene/polyoxypropylene block copolymer poloxamer 188), which are secondary to complement activation by poloxamer 188 (Temper *et al.*, 1984;

Police *et al.*, 1985; Lustig *et al.*, 1989). Interestingly, a low incidence of complement activation and complement-mediated adverse events has been observed following intravenous infusion of Fluosol-DA in subjects with abnormal or elevated LDL and HDL profiles (Vercellotti *et al.*, 1982; Kent *et al.*, 1990). On the basis of these reports, it has recently been demonstrated that the elevation of serum concentrations of LDL and HDL cholesterol relevant to two clinical situations exerted a protective role against poloxamer-mediated complement activation (Moghimi *et al.*, 2004).

Therefore, this study examined whether the elevation of human serum LDL and HDL cholesterol levels can exert a protective role against cholesterol-rich (45 mol% cholesterol) liposome-mediated complement activation *in vitro*, since cholesterol-rich liposomes can interact with lipoproteins. The effect of lipoproteins was also studied on liposome-induced (and complement-mediated) cardiovascular distress in the established porcine model.

5.2 Materials and Methods

5.2.1 Materials

Dimyristoylphosphatidylcholine, Dimyristoylphosphatidylglycerol, Cholesterol and Zymosan were all purchased from Sigma, UK.

Complement enzyme-linked immunosorbent assay kit was purchased from Quidel Co., San Diego, CA, USA.

HDL and LDL were purchased from Calbiochem, Germany.

Chloroform was purchased from Sigma-Aldrich-UK.

5.2.2 Methods

5.2.2.1 Preparation of liposomes

In accordance with earlier observations, large multilamellar liposomes consisting of dimyristoylphosphatidylcholine, dimyristoylphosphatidylglycerol, and cholesterol (50:5:45 mole ratios) were used, which are very potent at inducing pulmonary hypertension in pigs, resulting in circulatory collapse and even death (Szebeni *et al.*, 2000). Liposomes were prepared by hydrating the dried lipid film with chloroform, and then the organic solvent was removed using a rotary evaporator. The LML liposomes were then suspended in 10 mM PBS.

5.2.2.2 Subject and collection of serum specimen

Blood was drawn from healthy volunteers according to protocols approved by the school of pharmacy and the clinical centre at University of Brighton. Blood was allowed to clot at room temperature and serum was prepared, and then stored at -80 °C. Serum samples were thawed and kept at 4 °C before incubation with test agents.

5.2.2.3 Assay of *in vitro* complement activation

Liposome-mediated complement activation through the classical and alternative pathways in undiluted healthy human serum was monitored by measuring the production of the S-protein-bound form of the terminal complex, SC5b-9 using an enzyme-linked immunosorbent assay kit according to the manufacturer's protocol (Moghimi *et al.*, 2004).

SC5b-9 is the soluble, S-protein-bound form of the terminal complex, a sensitive measure of C5a formation through both the classical and the alternative pathways,

while Bb is the proteolytically active fragment of factor B, a specific marker of complement activation through the alternative pathway. The test agents were incubated with serum in a shaking water bath (80 rpm cycle) for 30-60 minutes at 37 °C at an activator to serum ratio of 1:5. Typically, 5-10 µL of the testing agent stock solution was added to 40 µL of serum placed in Eppendorf tubes. The reaction was stopped by adding 20 volumes of PBS that contained 2 mM EDTA, 25 mg/mL bovine serum albumin, 0.05% Tween 20 and 0.01% thiomersal (pH 7.4). The final concentration of liposomes in serum was 5 mg/mL. Zymosan (5 mg/mL) was used as a positive control to confirm the presence of a functional complement system in human serum (Szebeni *et al.*, 1998). Levels of produced SC5b-9, Bb, C4d and C3a-desarg were measured with the respective ELISA kits.

5.2.2.4 *In vivo* Closed-Chest Instrumented Pig

For *in vivo* experiments female Yorkshire swine (25–40 kg) were sedated with *i.m.* ketamine (500 mg), anesthetized with 1% halotane, and instrumented as described in detail by (McLoughlin, *et al.*, 1996; Szebeni *et al.*, 1999). In brief, a catheter was advanced via the right internal jugular vein into the pulmonary artery to measure pulmonary artery pressure (PAP), central venous pressure (CVP), and cardiac output (CO); another was advanced through the right femoral artery into the proximal aorta to measure systemic arterial pressure (SAP) and for blood sampling; and a third catheter was placed into the left ventricle through the left femoral artery to monitor left ventricular end-diastolic pressure (LVEDP). Systemic vascular resistance (SVR) and pulmonary vascular resistance (PVR) were calculated from SAP, PAP, CO, CVP, and LVEDP by standard formulas (McLoughlin *et al.*, 1996). All procedures on pigs were performed in accordance with the guidelines of the Committee on Animal Care

of the Uniformed Services University of the Health Sciences. Indeed, the porcine model is well established and provides highly sensitive detection of cardiopulmonary side effects of liposomes resulting from complement activation (Szebeni *et al.*, 1999). Blood pressure and leads II and V5 of the ECG were recorded continually.

5.3 Results and Discussion

The results in Fig. 5.1 show a dramatic rise in serum SC5b-9 level after 30 min incubation with liposomes, compared to the control incubation (serum supplemented with phosphate buffered saline, pH 7.2, in place of liposomes), thus confirming the complement activating nature of these vesicles. The cholesterol and triglyceride levels of the serum were normal, and approximately 180 mg/dL and 200 mg/dL, respectively. The additive effect of purified serum HDL and LDL on liposome-mediated complement activation was monitored next. The addition of purified HDL to serum increased cholesterol levels by 25–30%, which is considered beneficial by the National Cholesterol Education Program Expert Panel (NIH, USA), and represents a target for many therapies. The addition of purified LDL doubled serum cholesterol levels; a situation similar to that encountered in heterozygous familial hypercholesterolemia (Moghimi *et al.*, 2004). Elevation of both serum HDL and LDL levels had no effect on SC5b-9 production and the results were comparable to that of the control incubation. Remarkably, elevated serum HDL and LDL levels suppressed liposome-mediated SC5b-9 production significantly ($p < 0.01$). This suppression was more effective with LDL than HDL, which may be due to preferred fusion of cholesterol-rich liposomes with LDL particles (Zakharovats *et al.*, 1994). Manipulation of serum lipoprotein levels had no effect on zymosan-mediated complement activation.

When injected intravenously as a bolus in pigs via the jugular vein, liposomes (5 mg/mL) induced an immediate circulatory collapse (Fig. 5.2), which was intervened with epinephrine administration (0.5 mg/kg) and resuscitation. These observations were in accord with previous studies (Szebeni *et al.*, 1999 and 2000). After normalization of all haemodynamic parameters, animals received an injection of liposomes preincubated with human lipoproteins for 10 min at room temperature. For example, following treatment with HDL (1.4 mg/mL total protein, 0.37 mg/mL cholesterol, 0.12 mg/mL triglyceride), liposome-induced drop of systemic arterial pressure was slow and extended (Fig. 5.2), suggesting that surface-associated lipoprotein particles (or apolipoproteins) lessened the adverse haemodynamic changes, possibly as a consequence of suppressed complement activation *in vivo* (although other important physiological changes on the system might play a role, *e.g.*, the renin angiotensin system and corticosteroid release). Since liposome-mediated haemodynamic changes are not tachyphylactic (Szebeni *et al.*, 1999), without lipoprotein pre-treatment, the second injection would have caused very similar anaphylactic shock as observed after the first liposomal injection. In contrast to liposomes and liposome-lipoprotein preparations, injection of HDL and LDL particles alone was harmless and no haemodynamic changes were observed. Based on earlier studies using fluorescence-activated cell sorting analysis, this study suggested that the binding of naturally occurring IgG and IgM antibodies to liposomes could be the rate-limiting factor to both complement activation (predominantly via the classical pathway) and subsequent haemodynamic changes in the porcine model (Szebeni *et al.*, 2000). Therefore, adherence and/or fusion of HDL and LDL particles, or a mixture of apolipoproteins such as apoB-100, apoA-I, apoA-II, and apoA-IV to liposomes

may offer some protection against antibody and complement protein binding, and hence suppressing complement activation. As mentioned earlier, other chapters have investigated the effect of liposomal lipid composition, cholesterol content, and size on complement activation. Nevertheless, considering the abundance of lipoproteins in the blood, these observations predict a yet unrecognized and presumably universal modulatory function of lipoproteins/apolipoproteins on complement activation.

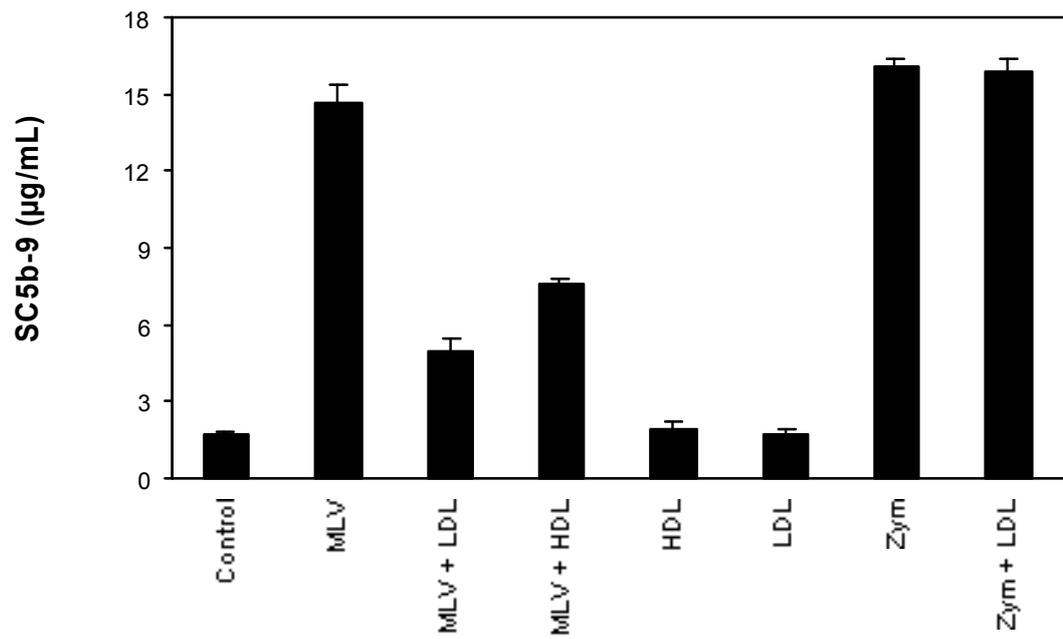


Fig. 5.1. The effect of human lipoproteins on liposome-mediated complement activation in human serum (liposomes mean size was 450 ± 80 nm).

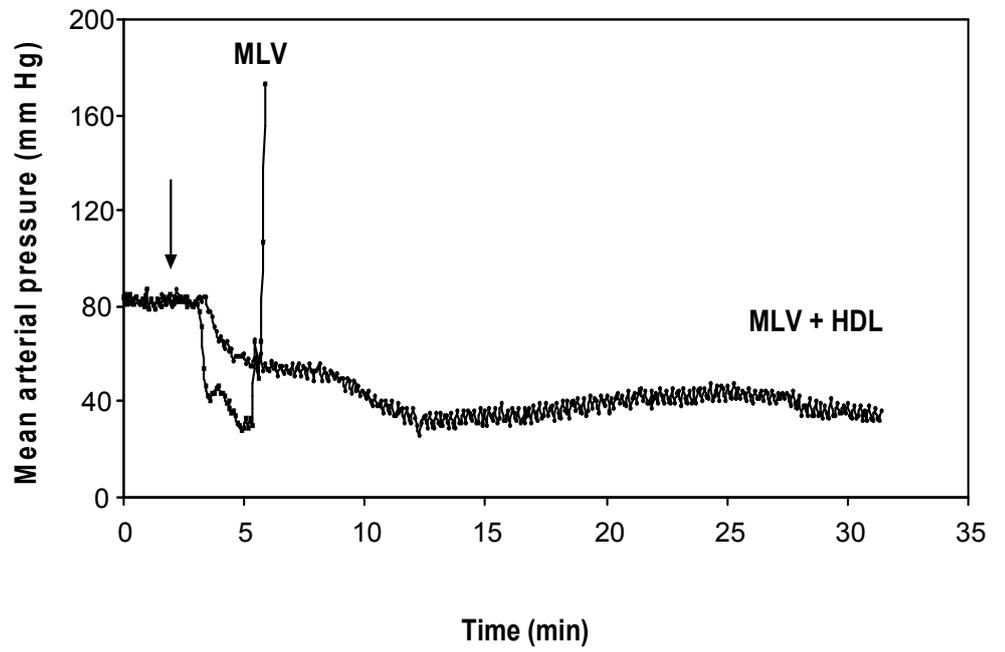


Fig. 5. 2. The effect of human lipoprotein on liposome-mediated complement activation: Mean systemic arterial blood pressure in a typical pig (out of 3) following injection of liposomes. Liposomes and liposome/HDL (5 mg liposomal lipid with a mean size of 450 ± 80 nm) were injected in a total volume of 1.0 ml. MLV liposomes were injected first (the arrow indicates the point of injection), the pigs circulatory collapse was intervened with epinephrine (0.5 mg/kg) and normalization of all haemodynamic parameters was achieved.

5.4 Conclusion

Intravenously infused liposomes may induce cardiopulmonary distress in some human subjects, which is a manifestation of “complement activation-related pseudoallergy”.

This study has now examined liposome-mediated complement activation in human sera with elevated lipoprotein (LDL and HDL) levels, since abnormal or racial differences in serum lipid profiles seem to modulate the extent of complement activation and associated adverse responses. In accordance with other earlier observations, cholesterol-rich (45 mol% cholesterol) liposomes activated human complement, as reflected by a significant rise in serum level of S-protein-bound form of the terminal complex (SC5b-9). However, liposome-induced rise of SC5b-9 was significantly suppressed when serum HDL cholesterol levels increased by 30%.

Increase of serum LDL to levels similar to that observed in heterozygous familial hypercholesterolemia also suppressed liposome-mediated SC5b-9 generation considerably. While intravenous injection of cholesterol-rich liposomes in to pigs was associated with an immediate circulatory collapse, the drop in systemic arterial pressure following injection of liposomes preincubated with human lipoproteins was slow and extended. Therefore, surface-associated lipoprotein particles (or apolipoproteins) seem to lessen liposome-induced adverse haemodynamic changes, possibly as a consequence of suppressed complement activation *in vivo*.

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Chapter six:

General conclusions and suggestions for future work

This study has elaborated on understanding of the molecular basis of complement activation by PEGylated nanoparticulate agents as well as by PEG in solution. Initially, the involvement of the anionic charge localized on the phosphate oxygen moiety of phospholipid-mPEG conjugates in PEGylated liposome-mediated complement activation and anaphylatoxin production was demonstrated. Subsequently, liposomes bearing a nonionic 1-O-phospholipid-mPEG conjugate that do not activate complement in human and rat sera were designed successfully. This is a critical step towards development of safer zwitterionic vesicles, which are temperature sensitive as well as susceptible to degradation by sPLA2. These observations and strategies not only provide a rational conceptual basis for design of safer PEGylated liposomes for site-specific drug delivery and targeting, but also highlight the importance of linkage chemistry in complement activation. The latter is of importance for surface engineering of implants and nanodevices with mPEG conjugates and related polymers for in vivo applications. It is still needed to fully understand how methylation of the phosphate oxygen can affect antibody and C1q accommodation on the liposome surface. Understanding of these events may eventually lead to prediction and elimination of subjects at risk, and even enhance the utility of PEGylated liposomes bearing anionic phospholipid-mPEG conjugates among low risk individuals.

Others have recently synthesized a range of neutral lipopolymers, such as distearoyl glycerol (carbamate-linked)mPEG and variations thereof for liposome engineering (Garbuzenko *et al.*, 2005). Remarkably, preliminary investigations have also demonstrated that such lipopolymer-incorporated liposomes are, indeed, poor activators of the human and porcine complement system when compared to vesicles bearing anionic phospholipid-mPEG conjugates (Zalipsky & Barenholz, 2004), thus

supporting the stated hypothesis. Other related uncharged lipid conjugates for construction of stealth liposomes includes mPEG-substituted synthetic ceramides (Webb *et al.*, 1998), but such conjugates generally exhibit poor packing into the phospholipid bilayer.

The work described in this thesis also is the first demonstration of PEGylated SWNT-mediated complement activation at both *in vitro* and *in vivo* levels. As to the molecular basis of PEGylated SWNT-mediated complement activation via the lectin pathway, at present I have only speculated a possible role for MBL and/or ficolin binding to some structural determinants of SWNTs, leading to subsequent MASP-2 activation, C4 cleavage and formation of C4a convertases. The structural features required for MBL and/or ficolin binding are presumably expressed in a simultaneous manner by both PEG chains and the nanotube surface, and therefore complement activation may have been triggered from inadequate surface protection of a certain population of SWNTs by PEG₅₀₀₀-PL conjugates. Surface heterogeneity (with some populations poorly protected by PEG chains) may also explain the observed rapid deposition of a significant fraction of intravenously injected PEGylated SWNT in liver and spleen macrophages through blood opsonization events, such as complement fixation (Liu *et al.*, 2007). Therefore, for better protection against complement activation, PEG-PL conjugates of longer PEG chains or those displaying branched PEG chains may be used. Indeed, such surface engineered SWNTs have recently provided improved blood pharmacokinetics (Liu *et al.*, 2008). Also, earlier work with PEGylated liposomes has identified a role for the net anionic charge on the phosphate moiety of the PEG-PL conjugate, which in concert with specific structural moiety of the lipid bilayer orchestrate vesicular-mediated complement activation through antibody binding (Szebeni, 2005). A similar mode of action could also operate with

PEGylated SWNTs, where the surface may activate MASP-2 directly or partly through electrostatic interaction with MBP and/or ficolins. Non-ionic PEG-lipid conjugates may be used for SWNT stabilization to establish whether the anionic phosphate-oxygen is of central importance in complement activation. Finally, experiments in pigs and dogs would be prudent to establish whether PEGylated SWNT can induce complement activation-related pseudoallergy. The pig and dog are established models for assessing such acute reactions to nanomedicines, although the nature of responses is different (Andersson *et al.*, 2002; Moghimi & Szebeni, 2003). For example, minute amounts of liposome administration into pigs induces rapid haemodynamic changes such as a massive rise in pulmonary arterial pressure, and a decline in systemic arterial pressure, cardiac output and left ventricular end-diastolic pressure. In dogs, haemodynamic responses are less dramatic, but remarkably dogs display considerable vegetative neural dysfunction, presumably indicating a set of unique interactions between the immune and the neural systems in this species (Moghimi & Szebeni, 2003).

Perhaps the most intriguing observation reported in this thesis was the ability of PEGs, in soluble form but at clinically relevant concentration, to trigger complement activation through both alternative and lectin pathways. These results are relevant to potential therapeutic applications of PEGs, as in spinal cord injury and traumatic axonal brain injury, and warn about possible acute PEG infusion-related reactions. Similar to binding of allergens to IgE on the surface of mast cells and basophils, complement anaphylatoxins can trigger immediate release of various proinflammatory mediators (prostaglandins, leukotrienes, etc.) from these cells as well as macrophages in contact with the blood. This cascade of secondary mediators substantially amplifies effector immune responses and may induce anaphylaxis in sensitive individuals.

Indeed, recent studies in pigs have demonstrated that systemic complement activation (eg, induced following intravenous injection of PEGylated liposomes) can underlie cardiac anaphylaxis where C5a played a causal role. Cardiac mast cells express high-affinity receptors for complement anaphylatoxins and their triggering induces the release of a variety of inflammatory mediators and vasoactive molecules. C5a was also shown to intensify the allergen-induced anaphylactic crises in isolated, perfused guinea pig hearts. Additionally, complement activation may therefore provide a plausible explanation to the previously reported unexplained anaphylaxis or the referred cardiovascular collapse in species that have received medicines containing high levels of PEG as solubilizer/carrier. In light of these observations, future experiments in relevant animal models and with anti-C5a antibodies are necessary to establish whether PEG-mediated peripheral complement activation can explain cardiac anaphylaxis. Finally, as a spin-off, PEGs may provide an additional platform for elucidation of ligand topology/structure for the pattern recognition molecules L- and H-ficolins, and subsequently illuminate the role of ficolins in defense as well as in endogenous homeostatic mechanisms.

In summary, hypersensitivity reactions caused by nanomedicines and their polymeric constituents may be strongly linked to complement activation and generation of anaphylatoxins. Hypersensitivity reactions arising as a consequence of complement activation could be life-threatening. Therefore, monitoring the formation of SC5b-9 in patients prior to nanomedicine infusion could be a significant factor, but since SC5b-9 depends completely on C9 polymerization, and hence this might be influenced during the assay by a number of biological parameters, this might not give the best indication for patient preselection. However, simultaneous monitoring of both SC5b-9 and anaphylatoxins (C3a and C5a) generation could provide a better rationale. General

precautions may also be followed to tackle nanomedicine-related complement hypersensitivity reactions. Some can be non specific (slow infusion of nanomedicines and pre-medication with steroids as anti histamines) or specific (Cox inhibitors as indomethacin, or complement inhibitors).

Finally, this study has examined the effect of elevated lipoprotein (LDL and HDL) levels on liposome-mediated complement activation in human serum. Cholesterol rich liposomes activated human complement. However, liposome-induced complement activation was significantly suppressed when serum HDL or LDL levels increased. Remarkably, in-vivo studies showed that surface-associated lipoprotein particles (or apolipoproteins) seem to lessen liposome-induced adverse haemodynamic changes, possibly as a consequence of suppressed complement activation in vivo.

It would be worthwhile to extend the complement activation studies to other nano particles or non-ionic surfactants. For instance, Cremophor EL has shown hypersensitivity reactions which were related strongly to complement activation. Detailed study might be useful in revealing the exact mechanism in which Cremophor EL-induced complement activation operates, consequently, this will help in developing new attempts towards controlling this side effect.

As noticed in PEG-induced complement activation, strong evidence suggested the involvement of the lectin pathway. Further studies needed to explore the possibility of PEG-ficolin interaction as a causative factor of lectin pathway activation. The results obtained from in vitro biological performance (complement activation) were found to correlate closely with *in vivo* studies in rats. It would be beneficial if more in vivo studies are carried out using bigger animals (dogs and pigs) as different reactions are expected with bigger animals. This would give a better idea about the adverse

haemodynamic changes in vivo and a good chance towards development of relatively safer drug-carriers.

Chapter Seven:

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