



Anti-PEG antibodies compromise the integrity of PEGylated lipid-based nanoparticles *via* complement

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ABSTRACT

PEGylation of lipid-based nanoparticles and other nanocarriers is widely used to increase their stability and plasma half-life. However, either pre-existing or *de novo* formed anti-PEG antibodies can induce hypersensitivity reactions and accelerated blood clearance through binding to the nanoparticle surfaces, leading to activation of the complement system.

In this study, we investigated the consequences and mechanisms of complement activation by anti-PEG antibodies interacting with different types of PEGylated lipid-based nanoparticles. By using both liposomes loaded with different (model) drugs and LNPs loaded with mRNA, we demonstrate that complement activation triggered by anti-PEG antibodies can compromise the bilayer/surface integrity, leading to premature drug release or exposure of their mRNA contents to serum proteins. Anti-PEG antibodies also can induce deposition of complement fragments onto the surface of PEGylated lipid-based nanoparticles and induce the release of fluid phase complement activation products.

The role of the different complement pathways activated by lipid-based nanoparticles was studied using deficient sera and/or inhibitory antibodies. We identified a major role for the classical complement pathway in the early activation events leading to the activation of C3. Our data also confirm the essential role of amplification of C3 activation by alternative pathway components in the lysis of liposomes.

Finally, the levels of pre-existing anti-PEG IgM antibodies in plasma of healthy donors correlated with the degree of complement activation (fixation and lysis) induced upon exposure to PEGylated liposomes and mRNA-LNPs.

Taken together, anti-PEG antibodies trigger complement activation by PEGylated lipid-based nanoparticles, which can potentially compromise their integrity, leading to premature drug release or cargo exposure to serum proteins.

1. Introduction

An increasing number of therapeutic compounds encapsulated in liposomes are approved for clinical use. Recently developed lipid nanoparticles (LNPs), comprised of conventional phospholipids

combined with ionizable lipids, facilitate stable encapsulation and cellular delivery of RNA-based therapeutics.

Surface coating with polyethylene glycol (PEG) is widely used to increase the stability and half-life of intravenously administered therapeutics [1]. PEG is a hydrophilic, biocompatible polymer that has been

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acknowledged to significantly reduce recognition and clearance of nanoparticles by the reticuloendothelial system (RES) [1,2]. Currently, different PEGylated liposome-based therapeutics, such as Doxil® (PEGylated liposomal doxorubicin) and Onivyde® (PEGylated liposomal irinotecan), are used in the clinic [3–5]. PEGylation is also used in lipid nanoparticles (LNP) encapsulating nucleic acid-based drugs like the recently FDA-approved Onpattro® (patisiran), an siRNA-loaded LNP formulation for the treatment of polyneuropathies caused by hereditary transthyretin-mediated amyloidosis (hATTR) [6]. Furthermore, two of the COVID-19 vaccines authorized for emergency use by the FDA are based on mRNA-loaded LNP formulations [7]. Most liposomal formulations contain 5 mol% of DSPE-PEG₂₀₀₀ lipid, which, due to the anchoring by C18 lipid tails, stays associated to the surface of the liposomes upon intravenous administration. LNP formulations usually contain 1.5 mol% of the sheddable DMG-PEG₂₀₀₀. The C14 lipid tails causes temporal stabilization upon injection but the PEG-lipid is lost within hours from the particle surface and replaced by a biomolecular corona [7].

Despite the low immunogenicity of PEG [8–10], anti-PEG antibodies may appear after treatment with PEGylated pharmaceuticals [11–16]. Moreover, pre-existing anti-PEG antibodies have also been detected in individuals that have never been treated with PEGylated therapeutics, likely due to the use of PEG-containing cosmetics, pharmaceuticals and processed food products [17,18]. The presence of anti-PEG antibodies has been associated to a considerably faster clearance of PEGylated drugs and nanocarrier systems upon repeated administration, potentially hindering their therapeutic efficacy [19]. This phenomenon is known as accelerated blood clearance (ABC) [20,21]. The presence of anti-PEG antibodies has also been linked to pseudo-allergic reactions observed in patients upon intravenous administration of PEGylated drugs. These hypersensitivity-like reactions are thought to be caused by the activation of the complement system (complement activation-related pseudo-allergy, CARPA) [22,23].

The complement system can be activated *via* three different pathways: the classical pathway, the alternative pathway and the lectin pathway. The classical pathway is triggered by binding of C1q to an antibody-antigen complex, where the antibodies are either multimers of IgG or a single pentameric IgM antibody. By contrast, the alternative pathway is triggered by spontaneous C3 activation on permissive surfaces and also serves as amplification route once C3 is generated [24]. Complement activation can lead to diverse effector functions. C3 fixation can result in the formation of C5 convertase followed by the deposition of C5 and the other terminal complement factors into the lipid membrane of pathogens, forming the membrane attack complex (MAC) [24]. The MAC forms a small pore that leads to the release of cellular contents and eventually cell death [25,26]. Recently, it has been shown that the MAC can also form on the surface of doxorubicin-loaded PEGylated liposomes [27], what possibly results in premature, intravascular drug release. Moreover, deposited C3 fragments (C3b, iC3b) can serve as ligands for complement receptors (CRs) expressed on phagocytes, leading to clearance of complement-opsonized structures mainly in the liver and spleen. Additionally, the release of fluid phase complement activation products, such as C5a and C3a, serve as anaphylatoxins that swiftly attract macrophages and monocytes to the site of infection [24].

In this study, we investigated the mechanism and consequences of complement activation by anti-PEG antibodies on PEGylated lipid nanoparticles using newly developed assays. Calcein-loaded liposomes enabled analysis of the kinetics of complement-mediated lysis and the role of each complement pathway in the process. Furthermore, we investigated anti-PEG antibody-induced loss of the lipid nanoparticle integrity of doxorubicin-containing liposomes with the drug loaded in their aqueous interior as nanorod-crystals and mRNA-loaded LNPs. Finally, we investigated the correlation between anti-PEG antibody titers in sera of healthy donors and the strengths of complement effector functions.

2. Materials & methods

2.1. Preparation and characterization of the liposomes

Liposomes were prepared by the lipid film hydration method as described previously [28]. In brief, to prepare biotinylated 5 mol% PEG liposomes, appropriate amounts of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; Avanti Polar Lipids), cholesterol (Sigma-Aldrich), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (mPEG₂₀₀₀-DSPE; Avanti Polar Lipids) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (biotin-PEG₂₀₀₀-DSPE; Avanti Polar Lipids) were dissolved in a mixture of chloroform and methanol (3:1 v/v) in a molar ratio of 1.85:1:0.075:0.075, respectively. To prepare non-biotinylated 5 mol% PEG liposomes, DPPC, cholesterol and mPEG₂₀₀₀-DSPE were used in a molar ratio of 1.85:1:0.15. See Table 1 for an overview of all liposomes used in the present study.

Next, the organic solvent was removed using a rotary evaporator at 55 °C until a dry lipid film was formed. This film was hydrated either with HEPES-buffered saline, pH 7.4 (HBS), or a 161 mM calcein (Sigma-Aldrich) solution in HBS, pH 7.5. The liposome dispersion was then extruded 10 times through a high-pressure extruder equipped with a membrane filter of 100 nm pore size (Whatman). After extrusion, calcein-loaded liposomes were dialyzed against HBS in a 10 kD Slide-A-Lyzer G2 dialysis cassette (Thermo Fisher Scientific) for 72 h at 4 °C to completely remove unloaded calcein. Lipodox®, a generic version of Doxil®/Caelyx® (Sun Pharmaceutical Industries Ltd.), was used to study clinical-grade doxorubicin-loaded liposomes. Like Doxil®/Caelyx®, these liposomes are remotely loaded with doxorubicin hydrochloride forming nanorod crystals in the presence of intraliposomal ammonium sulfate [29]. Lipodox® contains 5 mol% PEG₂₀₀₀, particle size is approximately 100 nm. The liposome size and polydispersity index (PDI) were characterized by Dynamic Light Scattering (DLS) using a ZetaSizer NanoS (Malvern Panalytical). For these measurements, the liposomes were diluted in phosphate-buffered saline, pH 7.4 (PBS) and measured in 3 procedures, each of at least 10 runs at an angle of 173°. The zeta potential was measured using a ZetaSizer NanoS by diluting the liposomes in a 10 mM HEPES solution (pH 7.4). Phosphate content was determined according to Rouser et al. [30], to determine the final total lipid concentration.

2.2. Preparation and characterization of mRNA-LNPs

LNPs were prepared with Dlin-MC3-DMA (synthesized by GKV Bio), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC; Avanti Polar Lipids), cholesterol and mPEG₂₀₀₀-DSPE or 1,2-Dimyristoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG₂₀₀₀; NOF America Corporation). The lipids were dissolved in 100% ethanol at a final concentration of 10 mM and a molar ratio of either 50:10:35:5 (MC3: DSPC:cholesterol:mPEG₂₀₀₀-DSPE) or 50:10:38.5:1.5 (MC3: DSPC: cholesterol:DMG-PEG₂₀₀₀). CD40L mRNA (kindly provided by eTherNA Immunotherapies) was diluted in 100 mM sodium acetate buffer, pH 4, to yield a final molar ratio of ionizable lipid MC3 to mRNA of 6:1. The aqueous and organic phases were then combined using a Nano-Assemblr® Benchtop (Precision Nanosystems) at a ratio of 2:1 (aqueous: organic) and a speed of 9 mL/min. Formulations were dialyzed against Tris-buffered saline, pH 7.4 (TBS), in 20 kD Slide-A-Lyzer G2 dialysis cassettes (Thermo Fisher Scientific) for 24 h at room temperature (RT).

The size, polydispersity, encapsulation efficiency and lipid concentration of the formulations were determined. The LNP size and PDI were measured with Dynamic Light Scattering using a ZetaSizer NanoS as described in the previous section. The encapsulation efficiency was determined using the Quant-It™ Ribogreen® RNA Assay kit (Thermo Fisher Scientific) by comparing the fluorescence of intact LNPs (in TE buffer: 200 mM Tris-HCl, 20 mM EDTA, pH 7.5 in diethylpyrocarbonate (DEPC)-treated water, diluted 20× in RNase free water) to that of completely lysed LNPs (in 2% Triton-X-100 (v/v)). The fluorescence of

Table 1
Main characteristics of the lipid-based nanoparticles used in this study.

| | | Z-average (nm) | Std dev | PdI | Std dev | Zeta potential (mV) | Std dev | [enc. mRNA] (µg/mL) & EE (%) | Std dev |
|-----------|--|----------------|---------|-------|---------|---------------------|---------|------------------------------|------------|
| Liposomes | 5 mol% biotinPEG ₂₀₀₀ -DSPE | 142.2 | 5.3 | 0.213 | 0.011 | −13 | 0.563 | n.a. | n.a. |
| | 5 mol% PEG ₂₀₀₀ -DSPE | 153.8 | 5.0 | 0.241 | 0.007 | −11.1 | 0.153 | n.a. | n.a. |
| | 5 mol% biotinPEG ₂₀₀₀ -DSPE + calcein | 155.2 | 4.8 | 0.08 | 0.016 | −13.7 | 0.873 | n.a. | n.a. |
| LNPs | 5 mol% PEG ₂₀₀₀ -DSPE | 72.4 | 5.0 | 0.12 | 0.008 | −5.5 | 0.467 | 55.2 µg/mL 89.8% | 2.8 6.1 |
| | 1.5 mol% PEG ₂₀₀₀ -DMG | 92.4 | 3.9 | 0.143 | 0.012 | −2.9 | 0.632 | 53.2 µg/mL 92.3% | 2.3 4.4 |

lysed LNPs corresponds to the total mRNA (RNA_t), and the fluorescence from the non-lysed samples to the un-encapsulated mRNA (RNA_{free}). To calculate the mRNA concentration, a reference calibration curve in either 2% Triton-X-100 or TE buffer was used. The percentage of encapsulated RNA is then calculated as follows: (RNA_t-RNA_{free})/RNA_t × 100. Finally, the lipid concentration was determined by measuring total cholesterol with the LabAssay™ Cholesterol kit (Fujifilm).

2.3. Complement active sera and inhibitory antibodies

Complement active human serum from pooled donors was purchased from Innovative™ Research. Complement active sera from individual donors were collected from healthy volunteers at the UMC Utrecht after written consent. Blood was collected in serum tubes (Becton Dickinson), left to clot for 30 min at RT, and centrifuged at 1500 ×g for 5 min at 4 °C. Serum was collected in Eppendorf tubes, snap frozen in aliquots in liquid nitrogen and stored at −80 °C until use.

Factor B (FB)-depleted (A335), C2-depleted (A312), C1q-depleted (A300), Properdin (factor P)-depleted (A339) and factor D-depleted (A336) sera were obtained from Complement Technology. Mannose-binding lectin (MBL)-deficient serum was obtained from the mini-donor service from UMC Utrecht. Absence of lectin pathway activity was confirmed with a Wieslab complement assay [31].

To investigate the role of the different complement pathways, normal human complement active serum was pre-incubated with 10 mM EDTA (ethylenediaminetetraacetic acid, Riedel de Haen), 10 mM MgEGTA (Complement Tech) or inhibitory anti-factor B antibody for 15 min on ice. EDTA inhibits all complement activation whereas MgEGTA only inhibits the classical and the lectin pathways through depletion of Ca²⁺ ions, while leaving the alternative pathway active. To prevent lysis of the liposomes, the serum was pre-incubated with 25 µg/mL of the anti-C5 monoclonal antibody (mAb) eculizumab (Soliris®, Alexion Pharmaceuticals) for 15 min on ice.

An inhibitory monoclonal antibody against factor B (anti-FB mAb, clone 1E1.2, mouse IgG1) was generated in-house at the UMC Utrecht using standard hybridoma techniques. Anti-FB-1E1.2 inhibited C3 fixation to zymosan-coated plates in the presence of MgEGTA, as well as the spontaneous generation of C3b/c in serum incubated at 37 °C (unpublished data, CEH, PB).

2.4. Kinetic measurement of liposomal cargo release

Calcein-loaded liposomes, commercially available rabbit monoclonal anti-PEG IgM antibody against the PEG backbone (Abcam, ab133471) or rabbit monoclonal anti-PEG IgG (Abcam, ab190652), rat anti-PEG IgM antibody (Abcam, ab94764), a fully humanized anti-PEG IgG1 antibody (Hu-6.3 IgG, Institute of Biomedical Sciences at Academia Sinica [27]), chimeric anti-PEG IgM antibody (cAGP4, Institute of Biomedical Sciences at Academia Sinica [27]) and pooled complement active serum (normal human serum, NHS) were added into a black 96-well plate at final concentrations of 0.016 mM, 1.25 µg/mL (IgM) or 2.5 µg/mL (IgG), and serum 12 times diluted in veronal buffer, respectively, and at a final volume of 60 µL. The rat anti-PEG antibody

was tested at 30, 20, 10 and 3 µg/mL. The humanized anti-PEG IgG antibody and the chimeric anti-PEG IgM antibody were tested at 25, 10, 5 and 1 µg/mL. Mixtures were incubated for 50 min at 37 °C. Lysis of liposomes was assessed by measuring fluorescence released from the liposomes every minute at excitation and emission wavelengths of 470 nm and 509 nm, respectively with a SpectraMax® M2e (Molecular Devices). As controls, serum was pre-incubated on ice for 15 min with 10 mM EDTA, 10 mM MgEGTA, or 25 µg/mL anti-C5 monoclonal antibody eculizumab (Soliris®). Maximal fluorescence of calcein was assessed by adding 3% Triton X-100 in PBS to liposomes. The percentage of lysis induced by complement and anti-PEG antibodies was calculated relative to the Triton X-100 control set as 100% lysis and serum-EDTA set as 0%.

Doxorubicin-loaded liposomes (Lipodox®), commercially available rat monoclonal anti-PEG IgM antibody against the PEG backbone (Abcam, ab94764), humanized anti-PEG IgG1 antibody (Hu-6.3 IgG), chimeric anti-PEG IgM antibody (cAGP4) and pooled complement active NHS were added into a black 96-well plate at final concentrations of 0.037 mM (total lipid concentration), 30 µg/mL or 10 µg/mL, and 10 times diluted serum, respectively, and at a final volume of 60 µL. Mixtures were incubated for 90 min at 37 °C. The amount of doxorubicin released from the liposomes was recorded by measuring the doxorubicin-associated fluorescence (excitation at 480 nm, emission at 590 nm) every minute with a Spectramax® M2e (Molecular Devices). As a control, serum was pre-incubated on ice for 15 min with 10 mM EDTA. Maximal fluorescence of doxorubicin was assessed by adding 3% Triton X-100 in PBS to the liposomes. The percentage of lysis induced by complement and anti-PEG antibodies was calculated relative to the Triton X-100 control set as 100% lysis and serum-EDTA set as 0%.

2.5. Column-based assay to assess cargo release

Calcein-loaded and doxorubicin-loaded liposomes (Lipodox®) at a total lipid concentration of 0.04 mM in veronal buffered saline (VBS) were incubated with 1 µg/mL rabbit anti-PEG IgM (Abcam) and 10% complement-active serum, at a final volume of 270 µL and at 37 °C for 1 h. As a negative control, samples were incubated with serum-EDTA, and as a positive control, samples were treated with 1% Triton X-100 (without anti-PEG). After incubation, the samples were diluted 10× and loaded on Sephadex G-25 M PD-10 columns (GE Healthcare) with VBS as running buffer. Fractions of 1 mL were collected; the first fraction contained the liposomes, whereas the following fractions contained free calcein or doxorubicin. The amount of cargo released from the liposomes was recorded by measuring the fluorescence of each fraction (at excitation and emission wavelengths of 470 nm and 509 nm for calcein and 480 nm and 590 nm for doxorubicin) with a Spectramax® ID3, and expressed as area under the curve (AUC). The percentage of lysis was calculated by comparing the AUC of samples to be tested, with those of the Triton X-100 (100% lysis) and EDTA (0% lysis) controls.

2.6. Lysis of mRNA-loaded LNP leads to exposure of mRNA

mRNA-loaded LNPs, anti-PEG IgM antibody and complement active serum were added to a V-bottom 96-well plate at final total lipid

concentrations of 0.03 mM, 1.25 µg/mL and 12 times diluted serum, respectively, and a final volume of 60 µL. Both the anti-PEG antibody and the serum were previously pre-mixed with 5 units of a human ribonuclease inhibitor (Sigma-Aldrich, R2520). After 1 h incubation at 37 °C, the samples (50 µL) were transferred to a black 96-well plate and mixed with 50 µL of 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE) from the Quant-It™ Ribogreen® RNA Assay kit, or 50 µL 2% Triton X-100 (v/v) in TE. The plate was then incubated for 10 min on a plate shaker at 400 rpm. Eventually, 100 µL of 200 times diluted Quant-It™ Ribogreen® RNA Reagent was added, and the mixtures were incubated for 5 min in the dark on a plate shaker at 300 rpm. The fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm with a Spectramax® iD3 (Molecular Devices). Reference calibration curves of RNA in 12 times diluted serum were also included. Results were expressed as percentage (%) release by taking the Triton X-100 control as 100% lysis, and the sample with only LNPs and serum as 0% release. Rabbit IgM isotype antibodies were used as isotype controls. As another control, serum pre-incubated with eculizumab (Soliris®) for 15 min on ice, was also tested.

2.7. C3 fixation on liposomes

96-well microplates (Greiner Bio-One) were coated overnight at RT with 10 µg/mL streptavidin (SA; Sigma-Aldrich) in PBS, and blocked for 1 h at RT with 1% bovine serum albumin (BSA, VWR) in PBS. Subsequently, plates were incubated with biotinylated 5 mol% PEG liposomes (0.5 mM total lipid) for 1 h at RT. Next, the liposomes were incubated with 0.5 µg/mL anti-PEG IgM antibody for 1 h at RT in PBS. Then, normal human complement-active serum, diluted 20 times in VBS, was incubated in the plate for 5 or 30 min at 37 °C. The plate was washed with PBS and fixed C3 was detected using horseradish peroxidase (HRP)-conjugated anti-human C3 antibody (MyBioSource), 500 times diluted in PBS. Plates were incubated with a TMB chromogen solution (3,3',5,5'-tetramethylbenzidine, Invitrogen™, Thermo Fisher Scientific) for 3 min and the reaction was stopped with 1 M HCl (Fisher Scientific). Finally, the optical density (OD) at 450 nm was measured with an absorbance reader (iMark™, BioRad).

2.8. Detection of soluble complement activation products

Complement active NHS was pre-incubated with 10 mM MgEGTA, 10 mM EDTA, inhibitory anti-FB mAb clone 1E1.2 at 4:1 mAb:FB molar ratio, or a mouse IgG1 isotype control on ice for 15 min. Serum was then mixed with liposomes or LNPs, and anti-PEG antibody, to yield final total lipid concentrations of liposomes or LNPs of 0.012 mM, of rabbit anti-PEG antibody of 7.5 µg/mL (liposomes) or 2.5 µg/mL (LNPs), and 1 to 10 diluted serum in VBS, respectively. This mixture was then incubated for 30 min at 37 °C. The activation was stopped by adding 10 mM EDTA and samples were kept on ice. Rabbit IgM isotype antibodies were used as controls.

Activated C3 in the mixtures was measured with ELISA [32]. As this assay does not discriminate between C3b, C3bi or C3c, activated C3 detected with this assay is denoted as C3b/c. Normal aged serum (NAS) was prepared by incubating normal human serum at 37 °C for one week, and used to generate a standard curve to calculate the amount of C3b/c in arbitrary units.

Soluble C5b-9 was measured with a sandwich ELISA, developed in-house using a mouse monoclonal antibody against a human C5b-9 neoantigen (Quidel Corporation). Results were expressed as µg/mL using a standard curve of soluble C5b-9 complex (Complement technologies) diluted in serum.

2.9. ELISA for the detection of anti-PEG antibodies

To detect anti-PEG antibodies an ELISA method described previously was used [13]. Briefly, 96-well microplates (Greiner) were coated with

1 µg/mL PEGfilgrastim (Neulasta®, Amgen) in PBS overnight at 4 °C and then blocked with 2% BSA in PBS for 2 h at RT. Serum samples from different healthy donors, diluted 100-fold in PBS, were added to the wells. To examine the binding specificity, 1 mol% mPEG₅₀₀₀ (Sigma-Aldrich) was added as a competitor. Next, the plates were washed with PBS/CHAPS 0.05% (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, A.G. Scientific). The anti-PEG IgG antibodies were detected with HRP-conjugated goat anti-human IgG (Jackson ImmunoResearch), 10,000 times diluted in 2% BSA in PBS. IgM antibodies were detected with rabbit anti-human IgM (Dako) followed by HRP-conjugated swine anti-rabbit Ig (Dako), both diluted 1000 times in 2% BSA in PBS. The detection antibodies were incubated for 1 h at RT. Binding of anti-PEG antibodies was visualized by adding a TMB chromogen solution and the reaction was stopped with 1 M HCl. The OD was measured at 450 nm using an absorbance reader.

3. Results

3.1. Characterization of liposomes and LNPs

To model different types of lipid-based drug delivery systems we have studied three formulations: i) a liposomal formulation using fluorescent calcein as model drug for low molecular weight hydrophilic drugs being entrapped in the aqueous interior as free molecules; ii), a clinically used liposomal formulation in which doxorubicin is present in the internal aqueous compartment in a nanorod crystallized form; and finally, iii) a lipid nanoparticle formulation consisting of an ionizable lipid that facilitates mRNA complexation.

The main characteristics of the lipid formulations used in this study are summarized in Table 1. All lipid-based nanoparticles used in this study have exposed PEG₂₀₀₀ on their surface. However, the percentage of PEG is different; for the liposomes it was 5 mol%, whereas for the LNPs we tested both 5 and 1.5 mol% PEG. The three liposomes (5 mol% biotinPEG₂₀₀₀-DSPE, 5 mol% PEG₂₀₀₀-DSPE and calcein-loaded 5 mol% biotinPEG₂₀₀₀-DSPE) had comparable size (Z-average range from 142 to 155 nm) and charge (Zeta potential range from -13.7 to -11.1). Both LNPs (5 mol% PEG₂₀₀₀-DSPE and 1.5 mol% PEG₂₀₀₀-DMG) had comparable size (Z-average of 72.4 and 92.4 nm) and charge (Zeta potential of -5.5 -2.9).

3.2. Complement-mediated lysis of liposomes results in drug release

To investigate the lytic potential of complement towards drug-loaded PEGylated liposomes, we set up a kinetic assay using calcein-loaded liposomes. Liposome-encapsulated calcein has a greatly reduced fluorescence due to self-quenching. However, when released from the liposomes self-quenching is reduced and fluorescence increases, allowing longitudinal monitoring of the lysis (Fig. 1A). The incubation of liposomes with anti-PEG IgM or IgG and complement active normal human serum (NHS) led to an almost maximal release (~90%, as determined relative to triton-mediated lysis) of the entrapped calcein, suggesting complement-mediated liposomal release (Fig. 1B and C). Similar findings were observed when using the rat anti-PEG IgM (ab94764) antibody, the humanized anti-PEG IgG₁ (Hu6.3 IgG) antibody and the chimeric anti-PEG IgM (cAGP4) antibody (Fig. S1). No release of calcein was observed when liposomes were incubated with either serum only or antibody isotype controls (rabbit IgM and IgG). Incubation of serum with EDTA inhibits all complement activation, whereas MgEGTA selectively inhibits the classical and lectin pathways while leaving the alternative pathway of complement intact. Serum with MgEGTA resulted in a delayed and partial anti-PEG IgM-induced calcein release compared to NHS. No release of calcein was detected with rabbit anti-PEG IgG during the observation period.

In order to investigate the role of the different complement pathways, this assay was also performed with sera depleted (DP) for different complement factors. Lysis was delayed and reduced with C2- or C1q-

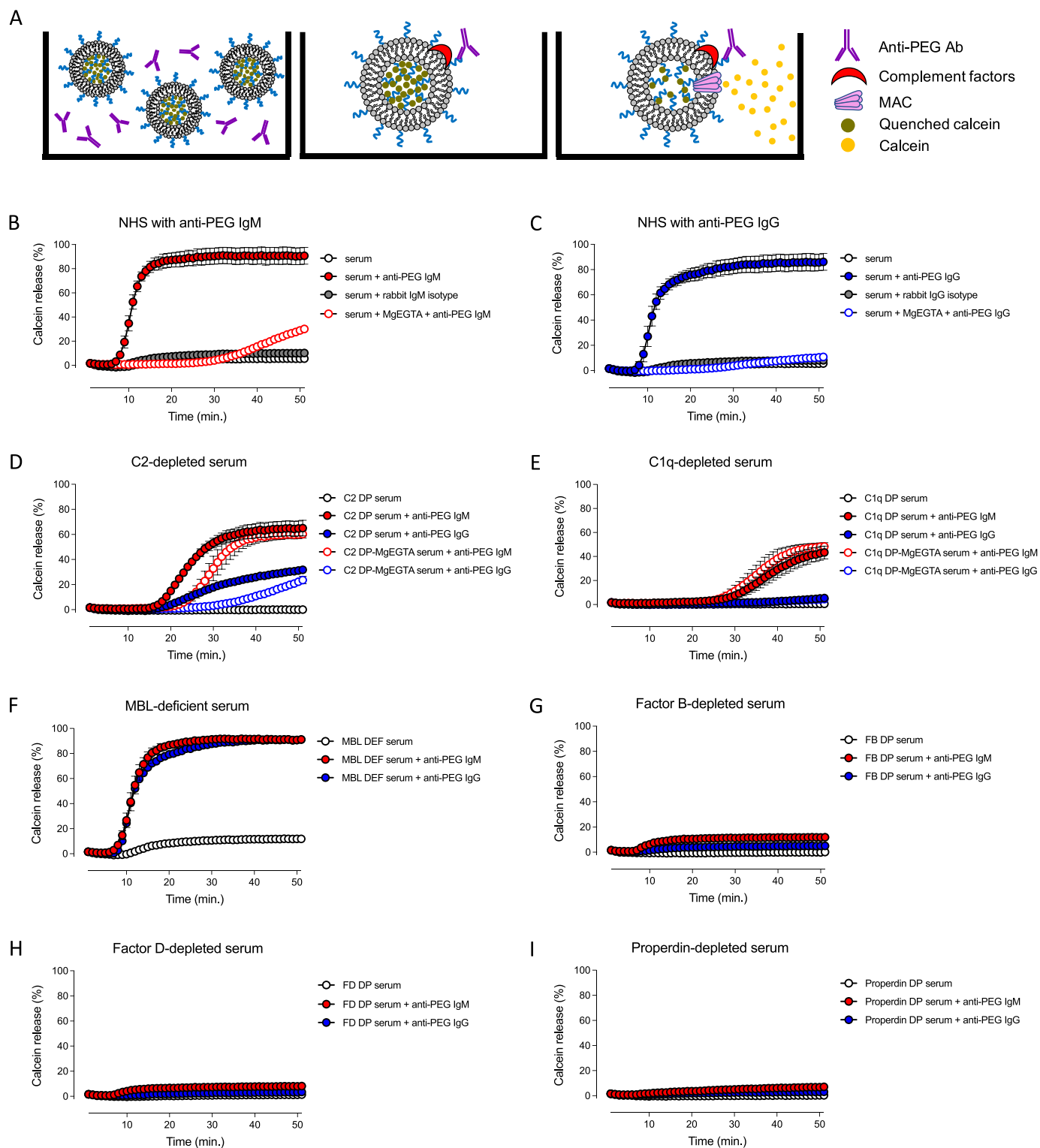


Fig. 1. Complement activation compromises the integrity of PEGylated liposomes. (A) Cartoon of the kinetic assay to monitor anti-PEG antibody and complement mediated lysis of calcein-loaded 5 mol% PEGylated liposomes by different types of sera (for simplicity only one MAC is shown but multiple MACs per nanoparticle are plausible). Lysis induced by complement active pooled NHS incubated with rabbit anti-PEG IgM (B) and rabbit anti-PEG IgG (C), or serum depleted (DP) or deficient (DEF) for specific complement factors (D to I) incubated with rabbit anti-PEG IgM or IgG antibodies. Percentage of lysis was calculated by setting serum-EDTA at 0% and Triton X-100-mediated lysis at 100%. Representative results of three independent experiments (mean of triplicates \pm SD). Abbreviations: MAC; membrane attack complex, NHS; complement active normal human serum, DP; depleted, MBL; mannose-binding lectin, FB; factor B, FD; factor D.

depleted sera, which are two components of the classical pathway (Fig. 1D and E). With C2-depleted serum, the calcein release induced by anti-PEG IgG was lower than that induced by anti-PEG IgM. With C1q-depleted serum, no calcein release was detected when liposomes were exposed to anti-PEG IgG. Additionally, the incubation of these sera with MgEGTA only moderately affected lysis. The mannose-binding lectin (MBL)-deficient serum shows the same kinetics and percentage of calcein release as NHS, suggesting no role for the lectin pathway in this process (Fig. 1F). Using sera with an impaired alternative pathway due to depletion of Factor B (FB), Factor D (FD), or properdin, no calcein release was observed (Fig. 1G to I).

These results indicate that inhibition of the classical pathway reduces and delays the observed lysis, and that the alternative pathway is essential for anti-PEG antibody-induced lysis of liposomes.

3.3. Release of calcein and doxorubicin from liposomes detected by column assay

To confirm that the actual release of liposomal cargo from the model calcein-loaded liposomes is also observed with clinical-grade liposomes, we assessed the release of cargo from doxorubicin-loaded liposomes (Lipodox®) in comparison to that of calcein-loaded liposomes. Both calcein-loaded and doxorubicin-loaded liposomes were incubated with 1 µg/mL rabbit anti-PEG IgM and complement active NHS. Subsequently, 'free' cargo (*i.e.* molecules released by the liposomes) was separated from liposomes with size exclusion chromatography (Fig. 2A and B). Remarkably, free calcein eluted from the column much faster than free doxorubicin. Some calcein was present in the first fraction, implicating incomplete separation of free calcein from the liposomes. Despite this, the area under curve (AUC) can still be determined. Notably, the AUC of released calcein is considerably higher than that of doxorubicin at the same antibody concentration. The relative amount of

released drug upon exposure to anti-PEG IgM in serum for the calcein-loaded liposomes 62% and, for doxorubicin-loaded liposomes 22%. Maximum release was reached after 10 and 12 min for Lipodox® and calcein, respectively. To directly confirm release of encapsulated drug, we assessed drug release from Lipodox® taking advantage of the fact that the self-quenched doxorubicin loses this property when dissolved (Fig. 2C and Fig. S2).

3.4. Lysis of mRNA-LNP leads to exposure of encapsulated RNA

To further extend our observations, we tested whether anti-PEG IgM antibodies on the surface of mRNA-LNPs could activate complement and induce the release of the encapsulated mRNA. Upon incubation of either 5 mol% PEG₂₀₀₀-DSPE or 1.5 mol% PEG₂₀₀₀-DMG mRNA-LNPs with rabbit anti-PEG IgM and serum, part of the encapsulated mRNA was released: with 5 mol% PEG₂₀₀₀-DSPE LNPs 50.5% of the encapsulated mRNA was released, (Fig. 3A), whereas with 1.5 mol% PEG₂₀₀₀-DMG this was 34% (Fig. 3B). Upon incubation of LNPs with either serum only or antibody isotype controls (rabbit IgM), mRNA was not released. Also, when C5 activation in the serum was prevented with anti-C5 monoclonal antibody eculizumab, no mRNA was released, indicating that the cargo release is complement-mediated.

3.5. C3 fixation on the surface of the liposome

Fixation of C3 onto the liposome surface is a key event during complement activation. Fixed C3 may serve as a ligand for various C3-receptors on phagocytic and other cells, which may enhance clearance of the nanoparticles from the circulation. To study complement fixation on the surface of liposomes, we used biotinylated liposomes immobilized on SA-coated surfaces (Fig. 4A). Background fixation of C3 was evaluated by addition of EDTA to the serum, because EDTA inhibits all

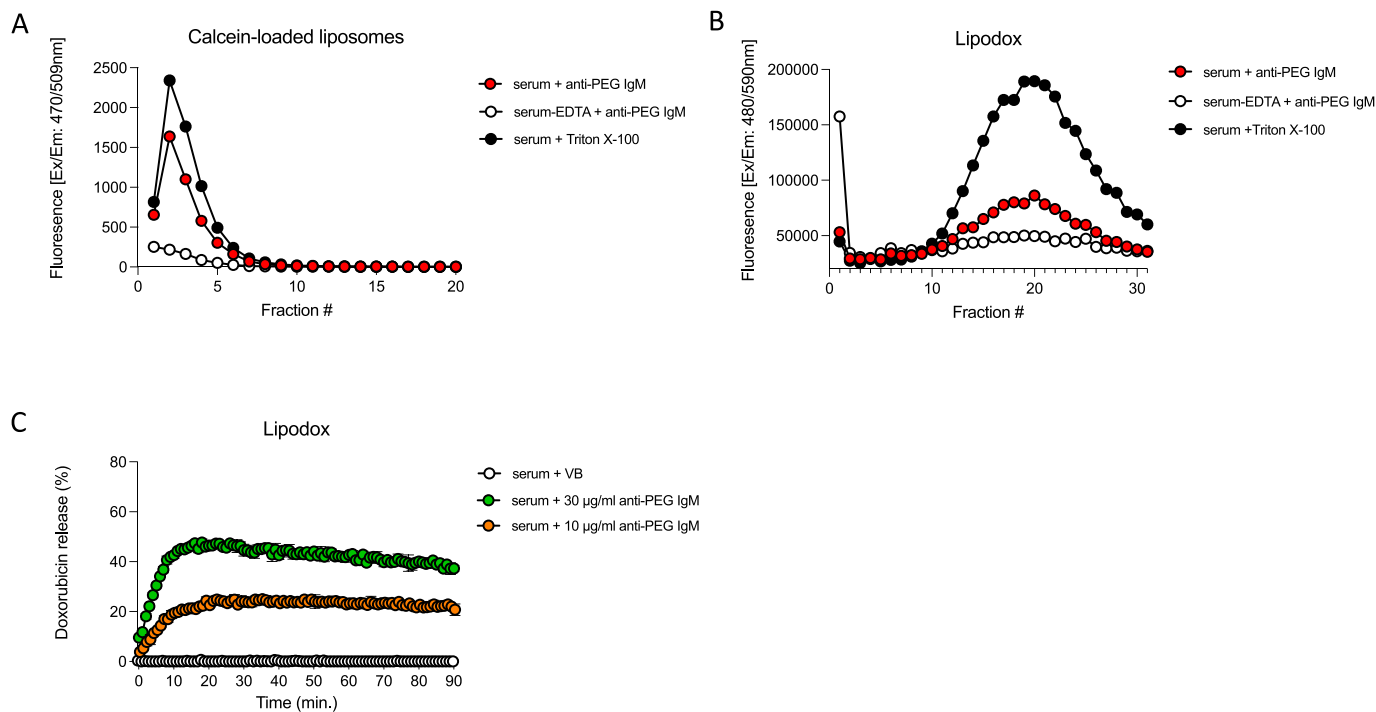


Fig. 2. Complement activation leads to release of calcein from PEGylated liposomes and doxorubicin from Lipodox®. PEGylated liposomes loaded with calcein (A) or Lipodox® (B) were incubated with 1 µg/mL rabbit anti-PEG IgM in complement active serum for 1 h. The reaction mix was subjected to column-based separation to separate released cargo and unlysed liposomes. The amount of lysis was calculated by comparing the AUC of 100% lysed liposomes (by Triton X-100) and liposomes incubated with antibody and serum, which resulted in 22% drug release for Lipodox® and 62% drug release for calcein-loaded liposomes. Both are representative results of at least two experiments. The high signal in the serum+EDTA condition is likely caused by a buffer effect. (C) Kinetic assay to monitor complement-mediated release of doxorubicin from Lipodox® by rabbit anti-PEG IgM in the presence of complement active pooled NHS (mean of triplicates ± SD).

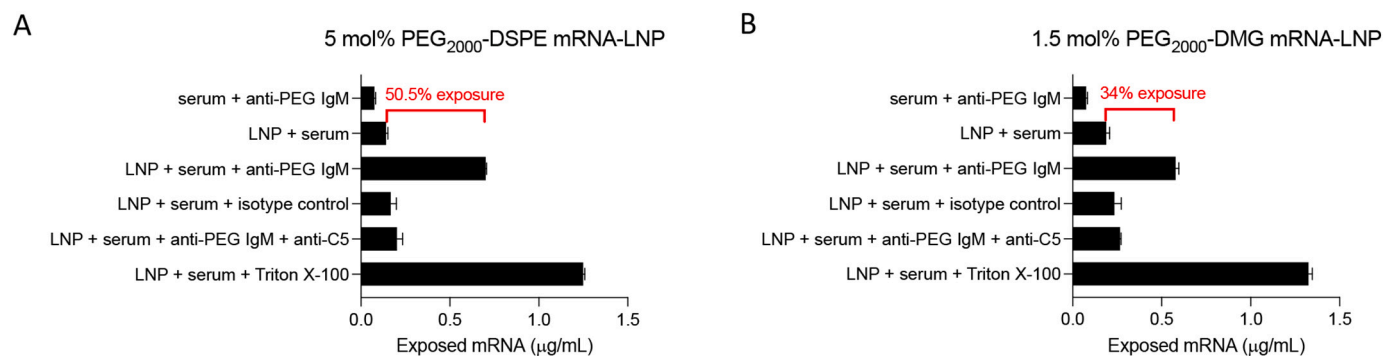


Fig. 3. Exposure of mRNA after complement activation by LNP-mRNA. 5 mol% PEG mRNA-LNP (A) and 1.5 mol% PEG mRNA-LNP (B) were incubated with rabbit anti-PEG IgM in complement active NHS. Exposed mRNA as a result of compromised LNP integrity was revealed by staining with RiboGreen. The amount of exposed mRNA was calculated using a standard curve prepared in the presence of Triton X-100. (Mean of duplicates ± SD). Representative results of 2–3 experiments.

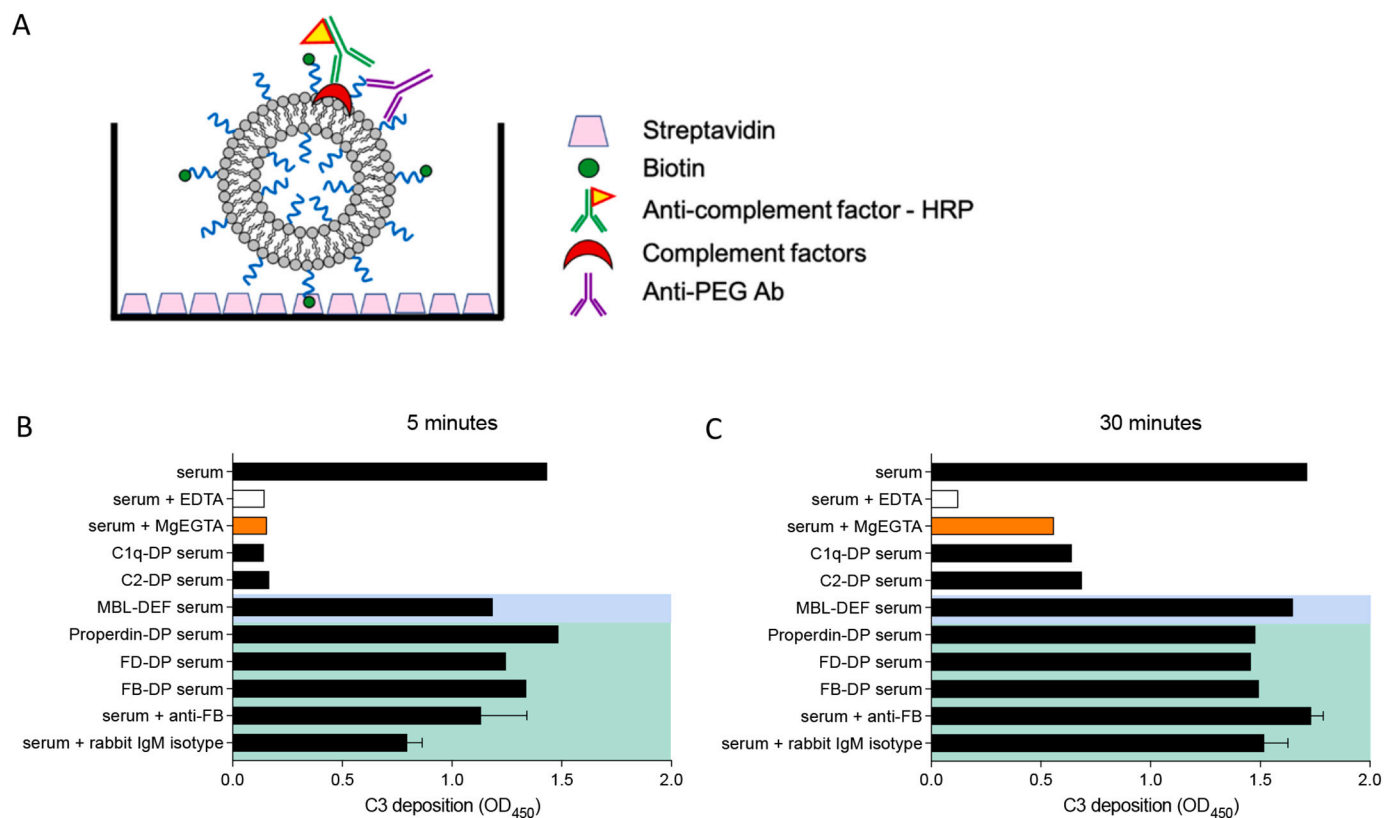


Fig. 4. The classical complement pathway plays a dominant role in C3 fixation on the surface of the liposomes. (A) To study fixation of complement fragment on PEGylated liposomes, we used biotin-labeled PEGylated liposomes and these were immobilized to streptavidin-coated plates. After incubation of the liposomes with rabbit anti-PEG IgM antibodies, complement active NHS or sera depleted for various complement factors were added. Complement active NHS was also pre-incubated with EDTA, or MgEGTA or inhibitory anti-factor B mAb, or isotype control before added to the wells. Complement activation was allowed to proceed for 5 (B) or 30 min (C). Deposited C3 fragments were revealed using an HRP-labeled anti-C3 antibody. Representative results of two independent experiments (single data points or mean of duplicates ± SD). Abbreviations: NHS; normal human serum, DP; depleted, DEF; deficient, MBL; mannose binding lectin, FB; factor B, FD; factor D.

complement pathways. Inhibition of the classical pathway, with either C1q-, C2-depleted (DP) sera, or MgEGTA supplementation, led to abrogated C3 fixation after 5 min. Contrarily, the inhibition of either the alternative pathway (Factor B-, Factor D- and properdin-depleted sera) or the lectin pathway (MBL deficient serum) did not affect the C3 fixation on the liposomal surface (Fig. 4B). After 30 min, the effect of the classical pathway inhibition (C1q-DP, C2-DP or MgEGTA) on the C3 fixation levels was less pronounced than after 5 min (Fig. 4C).

These results indicate that the initial fixation of C3 by anti-PEG IgM on the surface of PEGylated liposomes is mediated by the classical pathway. After 30 min, partial restoration of C3 fixation occurred

mainly propagated by the alternative pathway.

3.6. Fluid phase complement activation

To further corroborate that anti-PEG antibodies bound to PEGylated nanoparticles can activate complement and that the fluid phase complement fragments can indirectly influence nanoparticle clearance by effects on phagocytic cells, we assessed fluid phase C3b/c and sC5b-9 formation in serum induced by anti-PEG IgM antibodies on both PEGylated liposomes (5 mol% PEG₂₀₀₀-DSPE) and LNPs (1.5 mol% PEG₂₀₀₀-DMG). Only a fraction of cleaved C3 is fixed to the activator

upon activation by a C3 convertase, the majority stays in the fluid phase and can be detected with specific ELISA. SC5b-9 is the nonfunctional soluble form of the C5b-9 (or membrane attach complex, MAC).

5 mol% PEGylated liposomes, in the presence of anti-PEG IgM, led to increased C3b/c and sC5b-9 concentrations compared to the serum only control (Fig. 5A and B). When incubating liposomes with either only serum or serum and an antibody isotype control (rabbit IgM), C3b/c and sC5b-9 levels were comparable to background levels. These background levels presumably reflect spontaneous activation of C3 due to the so-called tick-over of C3, as in presence of EDTA, C3b/c was lower in spite of the presence of anti-PEG IgM (Fig. 5A). In MgEGTA supplemented serum, lower levels of C3b/c and sC5b-9 were generated. Moreover, adding an inhibitory anti-Factor B mAb to the serum had no effect on the generated C3b/c levels. MgEGTA did not fully block the C3b/c and sC5b-9 generation, probably reflecting spontaneous activation of complement, or direct activation of the alternative pathway by IgM as described earlier [33].

Like liposomes, 1.5 mol% PEGylated LNPs, in the presence of anti-PEG IgM, led to an increased C3b/c concentration compared to the serum only control (Fig. 5C). Incubating the nanoparticles with either only serum or antibody isotype control (rabbit IgM), led to a C3b/c formation comparable to background.

3.7. Pre-existing anti-PEG antibodies induce complement effector functions upon interaction with PEGylated lipid nanoparticles

3.7.1. Pre-existing anti-PEG IgM and IgG antibodies in healthy donors

Twenty-four healthy donor serum samples from the UMC Utrecht

minidonor service were screened for the presence of pre-existing endogenous anti-PEG IgG and IgM antibodies (Fig. 6). We have identified donors with high and low anti-PEG IgG and IgM antibody titers. For all donors, the binding of anti-PEG IgG antibodies to the PEG coating was completely competed with 1% of free mPEG₅₀₀₀. However, this was not the case for the IgM isotype. Presumably, this reflects a lower affinity of anti-PEG IgM antibodies than the IgGs and the tendency of IgM to bind aspecifically to the plate surface.

3.7.2. Pre-existing anti-PEG antibodies mediate lysis of calcein-loaded PEGylated liposomes

We used individual complement active serum to test the ability of pre-existing anti-PEG antibodies to induce complement effector functions by interaction with PEGylated liposomes. The endogenous anti-PEG IgM antibodies present in sera of donor 4, previously identified as anti-PEG IgM positive, were able to induce calcein release from PEGylated liposomes (Fig. 7A). Pre-existing anti-PEG antibodies from donor serum 4 led to a 13% of calcein release. In contrast, when PEGylated liposomes were incubated with the serum of donor 2, which tested negative for anti-PEG IgM antibodies, no calcein release was detected (Fig. 7B). Fig. 7A and B show representative results of the calcein-loaded liposome lysis mediated by pre-existing anti-PEG antibodies. The control with anti-C5 antibody (eculizumab) supplemented serum showed no lysis, indicating that the calcein release was complement-dependent.

3.7.3. Pre-existing anti-PEG antibodies induce C3 fixation on PEGylated liposomes

The ability of pre-existing anti-PEG Abs from human sera to induce

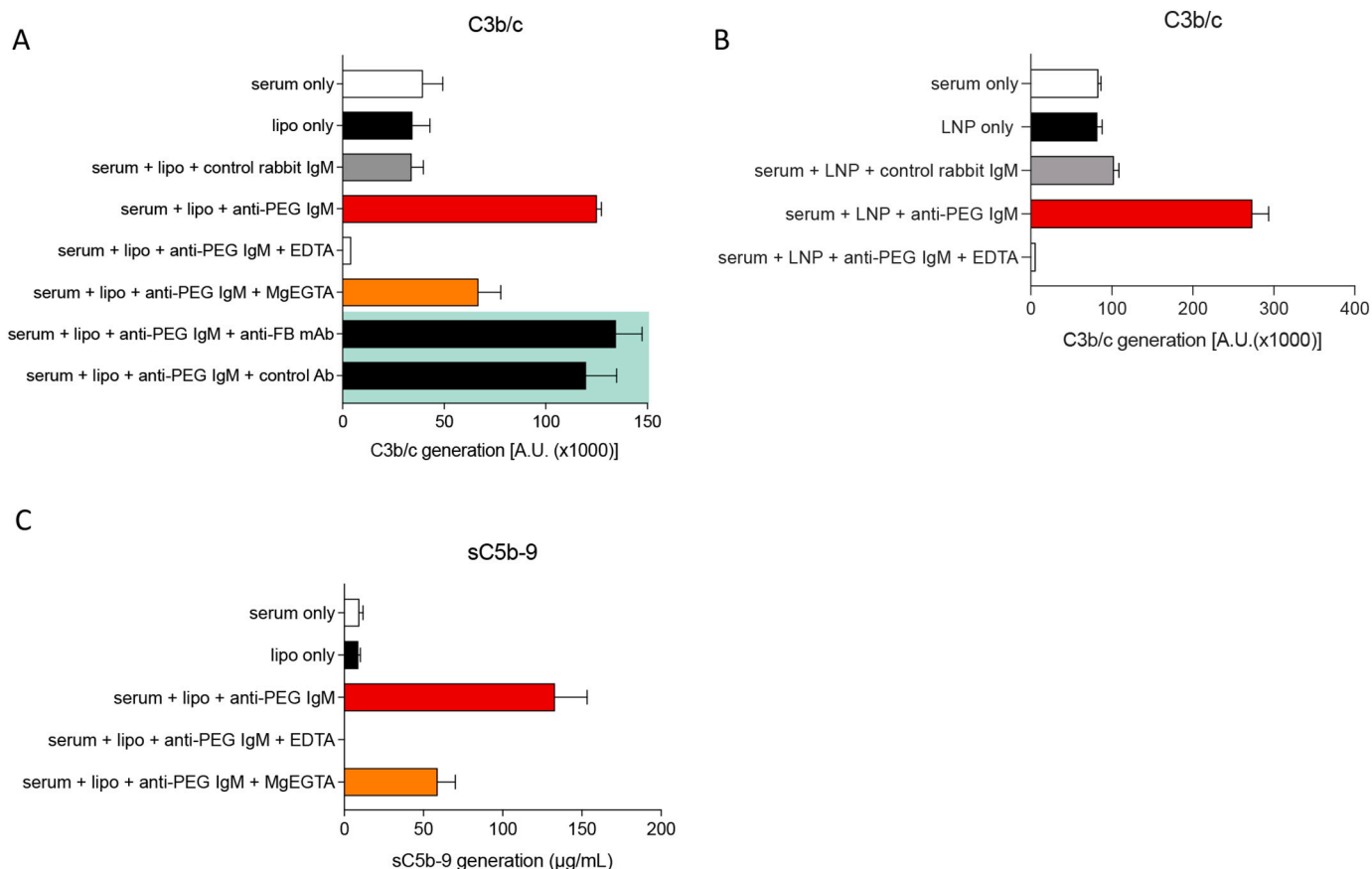


Fig. 5. PEGylated liposomes and mRNA-LNP induce the generation of fluid phase complement activation products with anti-PEG IgM. The generation C3b/c (A and C) and sC5b-9 (B) by 7.5 µg/mL rabbit anti-PEG IgM with PEGylated liposomes (A and B) or PEGylated 1.5 mol% mRNA-LNP (C) was assessed. Complement active NHS was incubated with PEGylated liposomes or mRNA-LNPs for 45 min and the levels of complement activation products were measured by ELISA. During the assay time low levels of C3b/c were spontaneously generated, which could be inhibited by addition of EDTA. Serum was either pre-incubated with EDTA, MgEGTA or inhibitory mAbs against Factor B. (mean ± SD of one to four data points in the ELISA assay).

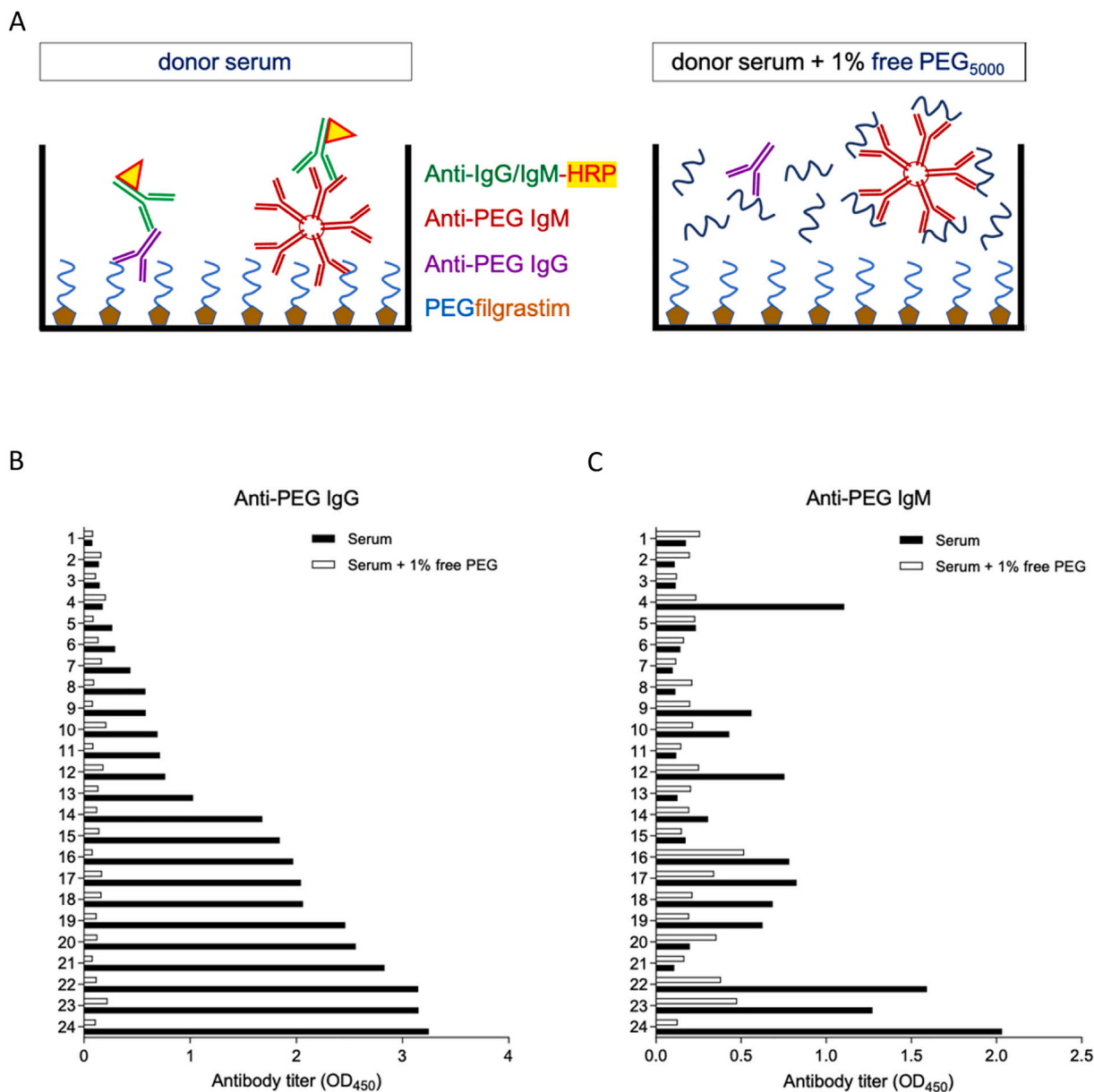


Fig. 6. Serum from healthy donors contain a range of anti-PEG IgM and IgG titers. The levels of anti-PEG IgM and IgG antibodies in sera from healthy donors was determined by ELISA (A). PEGylated G-CSF (pegfilgrastim) was coated on ELISA plates, and incubated with serial dilutions of the collected human serum samples. Bound human anti-PEG IgG (B) and IgM (C) antibodies were revealed by staining using a directly labeled secondary antibody. Specificity of the mAbs was confirmed by competition of 1% free PEG₅₀₀₀ during the incubation step. In total we performed four independent ELISA assay with similar results.

complement fixation was also assessed. Because each individual serum has different complement activity, the minimum and maximum complement fixation had to be identified for each serum. Serum incubation with EDTA was used to determine 0% fixation and supplementation with anti-PEG IgM antibody was employed to set the 100% fixation. These values were used to calculate the C3 fixation induced by pre-formed anti-PEG Abs. IgM positive serum from donor 4 induced 77% C3 fixation, whereas IgM negative serum from donor 2 induced only 43% C3 fixation (Fig. 7D and E). Fig. 7D and E are representative results of the C3 fixation induced by pre-existing anti-PEG on the surface of PEGylated liposomes.

3.7.4. Levels of pre-existing anti-PEG IgM correlate with complement effector functions

We assessed the relationship between the levels of pre-existing anti-PEG IgM and IgG antibodies in the sera of healthy donors and their

ability to induce complement effector functions (lysis and C3 fixation) in the *in vitro* assays. Our data, based on a limited set of donor sera, suggests that the anti-PEG IgM titers in serum correlate with complement effector mechanisms in all the drug delivery systems that we studied, even though in case of mRNA-LNP the correlation was not significant (Fig. 7C, F, G and H). Interestingly, we did not find correlation with anti-PEG IgG titers, except for the calcein release assay (Fig. S3 and data not shown).

4. Discussion

In this study we present the following key observations: 1) complement activation by anti-PEG antibodies can compromise the integrity of various types of PEGylated lipid nanoparticles, leading to release or exposure of the cargo; 2) during activation C3 is fixed onto the surface of PEGylated liposomes and LNPs upon interaction with anti-PEG

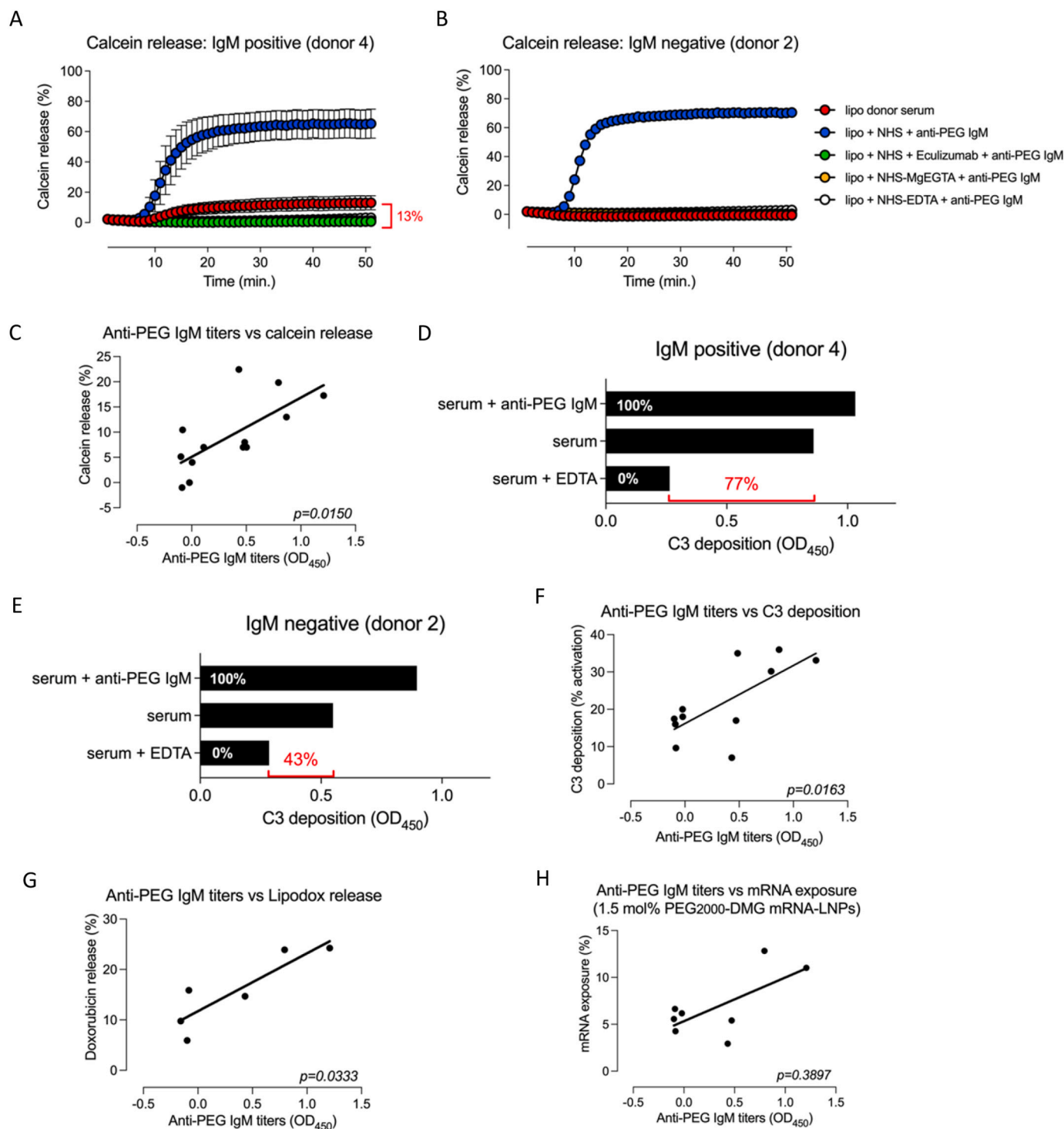


Fig. 7. Complement activation by pre-existing anti-PEG antibodies. Kinetic assay (see also Fig. 1) was performed using complement active individual sera collected from healthy donors. Blue circles indicate maximal lysis in the presence of additional rabbit anti-PEG IgM antibodies. Red circles depict lysis by serum only. Representative data obtained for two donors are shown, donor 4 considered IgM positive (A), and donor 2 considered IgM negative (B) for anti-PEG IgM. The final percentage of calcein release by donor 4 was estimated to be 13%. Fixation of complement C3 on the surface on PEGylated liposomes was assessed as described in Fig. 4. The capacity of individual sera to mediate C3 fixation was assessed relative to samples with EDTA (0%) or added anti-PEG IgM (100%). Relative to these values the fixation induced by donor serum alone was calculated. Representative example of an IgM positive (D) and IgM negative (E) donor is shown. Additionally, the correlation between titers of anti-PEG IgM was analyzed in relation to the ability of the serum to induce release of calcein (C), doxorubicin (G) and mRNA (H), as well as C3 fixation (F). Significance was assessed using the non-parametric Pearson Correlation Coefficient. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

antibodies; 3) the classical pathway seems dominant for the activation of complement until C3 is formed. For C5 activation amplification of C3 activation by alternative pathway factors is needed; 4) the titers of pre-existing anti-PEG IgM antibodies in sera correlate with their potency to induce lysis and complement fixation *in vitro*.

Exposure and release of encapsulated drugs due to loss of membrane integrity after MAC formation can severely compromise efficacy and safety of PEG nanoparticle formulations. We have demonstrated this phenomenon with two types of lipid-based nanoparticles with different cargoes. First, with PEGylated-liposomes loaded either with calcein (as model drug for encapsulated hydrophilic drugs) or with doxorubicin (in the form of nanorod crystals), and second, with mRNA-loaded LNPs. Rapid release of entrapped drugs (like topotecan) encapsulated in PEGylated liposomes has been suggested before but not in the context of complement [34]. Our findings are in line with a recent publication of Chen et al. showing release of doxorubicin from doxorubicin-liposomes induced by complement activation [27]. In addition to the results by Chen et al., we used columns to separate empty (lysed) liposomes from the cargo, demonstrating actual release of the drug. Both the kinetics assay as well as the column assay show that anti-PEG IgM and complement are capable of releasing drugs from liposomes present either in free form or as nanorod crystals.

LNPs encapsulating nucleic acids, such as mRNA or siRNA, like Onpatro® or the mRNA-based COVID-19 vaccines, are used in clinical practice [6,35]. For this type of drug delivery systems, loss of lipid packing integrity upon complement activation may lead to the premature release and/or exposure of the encapsulated nucleic acid to various plasma proteins, such as RNases. With mRNA-LNPs and by using a fluorescent RNA dye, we demonstrated a complement-mediated release of the encapsulated RNA. Despite mRNA-LNPs having a lower PEG density (1.5 mol% vs 5 mol%) and a sheddable PEG-lipid (DMG vs DSPE anchor lipid), we have observed comparable activation by anti-PEG antibodies, indicating that the process is active already at low PEG-densities.

The role that complement potentially plays in the therapeutic efficacy of LNP-encapsulated mRNA vaccines remains unresolved. Bednarczyk et al. suggested a positive effect of the complement system in enhancing the immune response induced by vaccines encapsulated in certain nanocarriers [36]. In the case of intramuscularly administered mRNA-LNP-based vaccines, complement opsonization in the lymph nodes could potentially lead to the enhanced uptake of vaccine particles by dendritic cells *via* complement receptors. Complement activation may also play a role in the increased occurrence of anaphylactic reactions to the mRNA vaccines [37].

Our findings suggest that drugs encapsulated in lipid-based PEGylated nanoparticles can be prematurely released into the circulation, or exposed to plasma proteins, thereby potentially reducing their efficacy and off-target effects and toxicity.

With the help of a novel assay that uses biotin-PEG to immobilize the nanoparticles on a SA-coated surface, we were able to detect complement factors deposited on the nanoparticle surface. Liposomes with and without biotin labeling showed comparable fluid phase complement activation with anti-PEG antibodies excluding interference of biotin labeling with binding of anti-PEG antibodies (data not shown).

Complement mediated clearance by anti-PEG IgM antibodies binding to PEGylated nanoparticles has been previously studied [20,38]. For instance, it has been reported that deposition of C3 can lead to rapid clearance of nanoparticles by Kupffer cells in the liver and splenic macrophages and marginal zone-B cells [19,39–41]. Additionally, it is known that the generation of soluble complement activation products, such as C3a and C5a by PEGylated liposomes and anti-PEG IgM antibodies can enhance ongoing inflammation [42]. Importantly, we have studied all three complement effector functions using the same experimental tool set.

In our study, we also investigated the relative roles that each complement pathway plays. Concerning PEGylated liposomes, inhibition of

the classical pathway resulted in delayed and attenuated lysis and C3 fixation. However, after 30–40 min, the processes continued to be driven only by the alternative pathway, possibly stimulated by the slow spontaneous hydrolysis of C3. Interestingly, even though the alternative pathway was completely dispensable for C3 fixation and generation of C3b/c, to achieve a full-blown activation and liposomal lysis, the amplification loop from the alternative pathway was found to be essential.

MBL-deficient serum was equally potent as normal serum in both the lysis and C3 fixation assay. Moreover, no additional effect was seen after addition of Mg-EGTA to C1q-DP serum in the lysis assay. C3 fixation by C2-DP serum was comparable to serum incubated with Mg-EGTA. Collectively, these results suggest no role for the Lectin Pathway in complement activation by PEGylated liposomes. Incubation of C2-DP serum with Mg-EGTA C2-DP results slight prolongation of lysis, this is likely caused by trace amounts of C2 still present in C2-DP serum, which is inhibited completely by Mg-EGTA. Contrary to our findings, mannan-binding lectin-associated serin protease-2 (MASP-2) was implicated in complement activation on PEGylated liposomes, however, in the absence of anti-PEG IgM antibodies [19].

The rabbit monoclonal antibodies used in our assays may have a higher affinity for PEG than naturally occurring antibodies. Moreover, the concentrations of naturally occurring anti-PEG antibodies in serum may be low. As expected, naturally occurring anti-PEG antibodies with unknown characteristics and specificity lead to less strong complement effector functions than the monoclonal anti-PEG antibodies. This is likely due to the fact that these individuals have probably never been treated with PEGylated (nano)medicines and their anti-PEG titers can likely be attributed to the use of certain cosmetic and pharmaceutical products that contain PEG as excipient [17]. In individuals treated with PEGylated drugs, these titers are likely to be higher. Despite the generally low anti-PEG antibody titers and the limited set of donors, we could still identify a correlation between anti-PEG IgM antibody titers and the complement activation. Interestingly, anti-PEG IgG titers only correlated with lysis of calcein-loaded liposomes.

This suggests that pre-existing anti-PEG IgM antibodies in healthy individuals could induce complement activation already upon the first dose of PEGylated nanoparticles [12,18]. In addition, *in vivo* studies in mice showed that the presence of pre-existing anti-PEG antibodies severely affects the pharmacokinetics of Lipodox® [43]. Together, this may explain why the ABC phenomenon also can occur in individuals that have not been treated with PEGylated drugs before [20]. Nevertheless, patients that were treated with PEGylated nanomedicines, and therefore have elevated anti-PEG antibody titers, are expected to induce stronger complement activation. The effects of intravascular cargo release in the case of fast (<10 min) clearance by the ABC phenomenon may be limited.

Naturally occurring anti-PEG IgM and IgG antibodies in healthy donors are polyclonal, composed of clones with different affinity and specificity for PEG, with likely varying ability to activate complement. As a result of this it is not straightforward to determine the minimal anti-PEG antibody concentration in serum that would induce complement activation.

Besides the results shown in this study, our work has a number of limitations. We only tested a limited set of healthy donors and these analyses should be repeated with a larger set of samples. We only demonstrate *in vitro* evidence and it would be important to extend these observations to *in vivo* models, examining complement-induced drug release and the effects of complement activation on the nanoparticle biodistribution and clearance.

In addition to providing independent confirmation of complement-induced drug release by lipid nanoparticles by Chen et al. [27], the results presented here extend these finding. We have used three types of lipid nanoparticles and have demonstrated for the first time that cargo release / accessibility can also happen for mRNA-LNP nanoparticles. We have systematically studied all three complement effector functions

induced by complement activation, not limiting ourselves to the complement mediated lysis. Finally, we have used depleted sera and have shown that both the classical and alternative pathway are important.

Taken together, our data provide important additional insights into complement activation by PEGylated lipid nanoparticles, which has important relevance for further improvement of the efficacy and safety of PEGylated nanomedicines.

Disclosure of conflicts of interest

PB and CEH are employees of Prothix B.V..

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jconrel.2021.11.042>.

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