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RESEARCH PAPER

Hemocompatibility Assessment of two siRNA Nanocarrier Formulations

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ABSTRACT

Purpose Since the discovery of RNAi and its therapeutic potential, carrier systems have been developed to deliver small RNAs (particularly siRNA) for modulation of gene expression at the post-transcriptional level. An important factor determining the fate and usability of these systems *in vivo* is interaction with blood components, blood cells, and the immune system. In this study, a lipid-based and a polymer-based carrier system containing siRNA have been investigated *in vitro* in terms of their hemocompatibility.

Methods The nanocomplexes studied were Angiplex, a targeted lipid-based system, and pHPMA-MPPM polyplex, a formulation based on a cationic polymer. siVEGFR-2 was encapsulated in both carriers and activation of platelets, coagulation, and complement cascade as well as induction of platelet aggregation were evaluated *in vitro*.

Results Both systems had been shown before to cause significant silencing *in vitro*. Our findings indicated that pHPMA-MPPM polyplex triggered high platelet activation and aggregation although it did not stimulate coagulation substantially. Angiplex, on the other hand, provoked insignificant activation and aggregation of platelets and activated coagulation minimally. Complement system activation by Angiplex was in general low but stronger than pHPMA-MPPM polyplex.

Conclusions Taken together, these *in vitro* assays may help the selection of suitable carriers for systemic delivery of siRNA in early preclinical investigations and reduce the use of laboratory animals significantly.

KEY WORDS Coagulation · Complement activation · Hemocompatibility · Platelet activation · siRNA delivery system

ABBREVIATIONS

CHEMS	Cholesteryl hemisuccinate
C system	Complement system
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DSPE-PEG2000	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)] with PEG molecular weight of 2,000 $g.mol^{-1}$
DSPE-PEG2000-Mal	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]
HBG	20 mM HEPES buffer containing 5% glucose at pH 7.4
HBS	20 mM HEPES and 150 mM NaCl
MPV	Mean platelet volume

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PC	1,2-dioleyl-sn-glycero-3-phosphocholine
PE	1,2-dioleyl-sn-glycero-3 phosphoethanolamine
PGI_2	Prostacyclin
pHPMA-MPPM	Poly((2-hydroxypropyl) methacrylamide 1-methyl-2-piperidine methanol
PS	1,2-dioleyl-sn-glycero-3-phospho-L-serine
HT	HEPES-Tyrode
SATA	N-succinimidyl S-acetylthioacetate
siVEGFR-2	Human siRNA against VEGFR2
TF	Tissue factor
TRAP-6	Thrombin receptor activator peptide 6
VEGFR2	Vascular endothelial growth factor receptor-2
vWF	Von Willebrand factor

INTRODUCTION

Unmodified siRNA is prone to fast degradation by nucleases in the blood after systemic administration. In addition, large size and negative charge of siRNA hampers internalization of these molecules into the cells. Therefore, delivery systems are being investigated to protect siRNA in the circulation and increase their cellular uptake. Two main categories of synthetic carriers used for siRNA delivery are polyplexes and lipoplexes where siRNA is complexed with a polymer or with lipids, respectively. We have recently observed the successful delivery of a targeted lipoplex (Angiplex) containing siRNA against VEGFR-2 to HUVECs [1] with no toxicity and long circulation time *in vivo*. Also, we have reported on prominent gene silencing efficiency of pHPMA-MPPM complexes with siRNA against firefly luciferase in H1299 human lung cancer cells expressing firefly luciferase [2]. However, these polyplexes have resulted in complications *in vivo* (personal communication) which refers to the fact that translating *in vitro* results to the *in vivo* situation remains a challenge in the field as it has been shown that efficient delivery systems *in vitro* might not lead to optimal *in vivo* results [3–5]. Many factors need to come together to obtain successful transfection, such as stability in the circulation, lack of toxicity, extravasation to the target tissue, target cell entry, and endosomal escape [6, 7]. In this paper, we are focusing on the initial interactions with the blood when nanocarriers enter the systemic circulation as well as the immune response to the presence of these particles. After intravenous administration, nanoparticles first come into contact with blood components (such as albumin, coagulation factors and proteins of the complement system (C system)), blood cells (for example platelets), and the cells of the immune system. Since these interactions could negatively affect their delivery to the target site or even worse, trigger systemic toxicity, it is vital to evaluate behavior of such particles in the blood before proceeding to *in vivo* studies.

Regarding platelets, in addition to being involved in inflammation and hypersensitivity, these cells play an important role in primary hemostasis, which is the blocking of hemorrhage proceeding vascular injury. Aside from trauma, platelets can become activated upon encountering foreign particles (bacteria, viruses, and nanomedicine) which could also lead to thrombotic occlusion of the lumen of vessels and subsequently tissue damage. Secondary hemostasis is regulated by the coagulation cascade which occurs through extrinsic (or tissue factor (TF) coagulation) pathway and intrinsic (or contact activation) pathway. Studying the influence of drug carriers on activation of platelets and the contact system could assist in predicting *in vivo* fate of these nanoparticles.

A key player in host defense is the C system, the activation of which has been reported to be a cause of low transfection efficiency of non-viral nucleic acid delivery systems [8]. C system can be activated by nano medicine and lead to opsonization and thus rapid elimination of these particles. In this paper, a set of hemocompatibility assays have been investigated to be used as a first *in vitro* screen to identify which siRNA formulations would be suitable for systemic application. Angiplex and pHPMA-MPPM polyplex have been chosen to be tested in these assays as examples because of previous *in vitro* and *in vivo* results obtained for them in our laboratory. We have investigated thrombogenicity, effect on coagulation, and complement activation and how physicochemical characteristics of these particles determine these interactions.

EXPERIMENTAL SECTION

Materials

Human siRNA against VEGFR2 (siVEGFR-2) with the following sequence: sense 5′-GGA-AAU-CUC-UUG-CAA-GCU-AUU-3′ and anti-sense 5′-UAG-CUU-GCU-AGA-GAU-UUC-CUU-3′ was purchased from Eurogentec Nederland B.V. (Maastricht, the Netherlands). Poly((2-hydroxypropyl) methacrylamide 1-methyl-2-piperidine methanol (pHPMA-MPPM) 240 kDa was synthesized and purified in our group as described previously [9]. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)] with PEG M_w of 2,000 $g.mol^{-1}$ was obtained from Lipoid (Ludwigshafen, Germany). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-Mal) were supplied by Avanti® Polar Lipids Inc. (Alabaster, USA). Cholesteryl hemisuccinate (CHEMS) and protamine sulphate salt from salmon (Grade X) were obtained from Sigma-Aldrich (St. Louise, USA). Protected N-terminal SATA-modified anginex peptide was

synthesized by China Peptides Co., Ltd. (Shanghai, China) with the following sequence:

SATA-ANIKLSVQMKLFKRHLKWKIIVKL NDGRELSLD.

JetPEI was obtained from Polyplus Transfection (Illkirch, France) and Lipofectamine 2000 was purchased from Invitrogen (Breda, the Netherlands). Thrombin Receptor Activator Peptide 6 (TRAP-6) and Chromogenic substrate L-2120 (H-D-Pro-Phe-Arg-pNA), were obtained from Bachem (Bubendorf, Switzerland). PE-conjugated anti P-selectin was acquired from BD Biosciences (Breda, the Netherlands). FITC-conjugated polyclonal rabbit anti human fibrinogen was purchased from DAKO (Glusdorp, Denmark). Blood was obtained from the Mini Donor Dienst (UMC, Utrecht, the Netherlands). Kaolin (Light) was purchased from BDH Ltd. (Poole, UK). Citrated human normal pooled plasma was prepared from the whole blood of approximately 170 healthy volunteers, according to standardized procedures [10]. 1,2-Dioleyl-sn-glycero-3-phospho-L-serine (PS), 1,2-dioleyl-sn-glycero-3-phosphocholine (PC), and 1,2-dioleyl-sn-glycero-3 phosphoethanolamine (PE) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Prostacyclin (PGI_2) (100 μg/ml) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Microvue SC5b-9 Plus ELISA kits were provided by Quidel Co. (CA, USA). All the other reagents were of analytical grade.

Methods

Preparation and Characterisation of Angiplex and pHPMA-MPPM Polyplex

Angiplex was prepared from DOPE, CHEMS, DSPE-PEG2000-Mal and DSPE-PEG2000 at molar ratios of 6:4:0.3:0.3, respectively. Lipids were dissolved in ethanol: chloroform (4:3*v*/*v*) on a rotavapor (Buchi, Switzerland) at 40°C after which a lipid film was made by evaporation of the solvents under vacuum. Meanwhile, an siRNA-containing core was prepared from siVEGFR-2 and protamine at ratio of 1:1.2 (*w*/*w*) in 20 mM HEPES buffer containing 5% glucose at pH 7.4 (HBG). After incubating this mixture for 20 min at room temperature, the complex was added to the lipid film. Total lipid concentration of lipoplexes was 10 mM and siRNA concentration was 1 μM. The lipoplex suspension was extruded repeatedly through polycarbonate membranes (Nuclepore, Pleasanton, CA, USA) by a Lipex^{TM} Extruder (Northern Lipids, Burnaby, BC, Canada) until a monodisperse sample was obtained with an approximate size of 100 nm. Then anginex-coupling was performed by firstly removing the protective group (SATA) of the peptide with a deprotecting solution (0.05 M HEPES, 0.05 M hydroxylamine and 0.03 mM EDTA at pH 7.0) at a peptide to deprotecting solution ratio of 1:10 (*v*/*v*). The reaction was completed by incubation for 45 min at room temperature. Secondly, the unprotected anginex was mixed with lipoplexes and incubated over night at 4°C. Uncoupled peptide was removed by ultracentrifugation at 200,000 g for 1 h at 4°C. Anginex concentration was calculated as 10 μg per μmol phospholipid. Untargeted lipoplexes were prepared in the same manner excluding the peptide-coupling step.

Polyplexes consisted of siVEGFR-2 complexed with pHPMA-MPPM at N/P ratio of seven (where N represents the moles of cationic nitrogens in the polymer and P represents the moles of phosphate groups in siRNA). siVEGFR-2 (2.1 μM) was added to the polymer in buffer containing 20 mM HEPES and 150 mM NaCl (HBS) at pH 5, vortexed for 5 sec, and incubated for 30 min at room temperature.

Hydrodynamic diameter of lipoplexes and polyplexes was determined by dynamic light scattering on a Malvern 4,700 system at 25°C using an argon-ion laser (488 nm) operating at 10.4 mW (Uniphase) and PCS (photon correlation spectrometry) software for Windows version 1.34 (Malvern Instruments Ltd., UK). Measurement angle was 90° and viscosity and refractory index of water at 25°C were applied. The apparatus was validated by standard polystyrene beads with a size of 200 nm (Thermo scientific, DE, USA). Polydispersity index (PDI) obtained from the measurements, represents size distribution of particles which can vary between 0 and 1 where 0 indicates single sized particles and one a heterogeneous mixture of particles. Charge of particles was characterised by Zeta sizer Nano-Z (Malvern Instruments Ltd., UK) which measures the ζ-potential. The apparatus was calibrated by Zeta Potential Transfer Standard (Malvern Instruments Ltd., Worcestershire, UK) with a known ζ-potential.

Platelet Activation Assay of Angiplex and pHPMA-MPPM Polyplex in Whole Blood and in Isolated Platelets

In this assay, PE-anti-P-selectin and FITC-anti-fibrinogen were added to Angiplex, pHPMA-MPPM polyplex, untargeted lipoplex, and jetPEI polyplex with fluorescent marker conjugate to particle solution ratio of 1:25 and 1:100 (*v*/*v*), respectively. The buffer used was HBS buffer containing 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$ and 5 mM KCl at pH 7.4 and siRNA concentration was 1.1 μM. Platelets were isolated according to the method introduced by Korporaal *et al.* [11]. In brief, blood was first centrifuged at 160 g in citrate anticoagulation tubes for 15 min at room temperature to obtain platelet-rich plasma. The number of platelets and the mean platelet volume (MPV) of the supernatant were determined by the Abbott Cell Dyne 1,800 (Abbott diagnostic division, USA) and then the supernatant was treated with acid citrate dextrose solution (2.5% tri-

sodium citrate, 1.5% citric acid and 2% D-glucose) at a ratio of 1:10 (*v*/*v*). After another centrifugation step at 340 g for 15 min at room temperature, the pellet was resuspended in HEPES-Tyrode (HT) buffer (1.45 M NaCl, 50 mM KCl, 5 mM Na_2PO_4, 10 mM $MgSO_4$, and 100 mM HEPES) at pH 6.5. Prostacyclin was added to this solution at a ratio of 1:1000 (*v*/*v*). Then the solution was centrifuged at 340 g for 15 min at room temperature and the pellet was resuspended in 1 ml HT buffer at pH 7.2. The number of platelets and the MPV were measured to ensure that the blood platelets were not excessively activated and that the concentration was approximately the same as in the body. The MPV may only differ 1.5 fL and the blood platelet count was adjusted to 200,000 platelets per µl by adding more HT buffer at pH 7.2. Isolated platelets were left to rest for another 30 min before further use. Whole blood or isolated platelets were gently mixed with the particles at a ratio of 1:10 (*v*/*v*) and were incubated for 1, 10, or 20 min at room temperature. TRAP-6 which is a strong platelet activator was used as positive control of the assay. Thereafter, a fixative solution (154 mM NaCl and 0.2% formaldehyde) was added to each sample with particle sample to fixative solution ratio of 1:20 (*v*/*v*). Fluorescence intensity of P-selectin (PE) and fibrinogen (FITC) was measured by flow cytometry with a FACS Canto II apparatus (BD Biosciences, San Jose, CA, USA) and analysed with FACSDiva^{TM} software (BD Biosciences, San Jose, CA, USA). 10,000 events were recorded per sample.

Light Transmission Aggregometry in Isolated Platelets

Isolated platelets were prepared as described in the previous section. 2 µl fibrinogen (25 mg/ml) was added to 500 µl isolated platelets and then different formulations (pHPMA-MPPM polyplex, jetPEI polyplex, Angiplex) were added to the platelet-fibrinogen mixture at the same volume. The final siVEGFR-2 concentration was 1.1 µM which was selected according to the highest clinical dose. JetPEI was used as a positive control particle and the complexes were prepared according to the manufacturer's protocol. Resting isolated platelets and HT buffer at pH 7.4 were used as negative controls. Then light transmission was measured by Model 700 Whole Blood/Optical Lumi-Aggregometer (Chronolog, UK).

Evaluation of Contact System Activation in the Coagulation Cascade

Particle solutions (Angiplex, pHPMA-MPPM polyplex, untargeted lipoplex, and jetPEI polyplex), pooled plasma and L-2120 were pipetted in 96-well plates with volume ratios of 4:3:2, respectively, and optical density was measured in Spectra max 340 microplate reader (Molecular Devices, USA) at 405 nm. Kaolin (0.5 mg/ml) was used as control.

Complement Activation Assay

Whole blood samples of 6 healthy volunteers were collected in BD Vacutainer® silicon coated glass serum tubes with no additives (BD Biosciences, NJ, USA). Blood samples were allowed to clot at room temperature and centrifuged at 3,000 rpm for 5 min. Serum was collected and stored at −80°C which for usage was rapidly thawed at 37°C and placed on ice during the experiments. Complement activation was measured by Microvue SC5b-9 Plus ELISA kits. Sera were incubated with different lipoplex and polyplex formulations (4:1 *v*/*v*) for 30 min at 37°C in a shaking water bath. All tested formulations contained 1 µM siRNA. Zymosan (5 mg/ml) was used as positive control. After incubation, ELISA assays were performed according to the manufacturer's protocol. Absorbance was measured using BMG Labtech SpectroStarNano (BMG Labtech GmbH, Germany) at 450 nm. SC5b-9 concentrations were calculated using a linear curve fit. The percentage of increase in SC5b9 complex formation compared to the buffer indicated activation of the C system. Experiments were repeated twice. Statistical analysis was performed with Graph Pad Prism four and two-tailed Student's *t*-test was used to calculate statistical significance.

RESULTS AND DISCUSSION

Preparation and Characterisation of Angiplex and pHPMA-MPPM Polyplex

Angiplex is a PEGylated lipoplex which has been used for targeted delivery of siRNA to tumor endothelial cells in our group [1]. The formulation consists of siRNA-protamine core encapsulated within a liposome composed of DOPE, CHEMS, DSPE-PEG2000-Mal, DSPE-PEG2000 at molar ratios of 6:4:0.3:0.3, respectively. Anginex peptide is coupled to the surface of the liposome to target galectin-1 receptors on endothelial cells of tumor vasculature. Angiplex has shown promising results in delivering siVEGFR-2 and has led to significant gene knock-down *in vitro* [1]. Another nanoparticle investigated in the current study was based on pHPMA-MPPM polymer which is a cationic and water-soluble polymer with a biodegradable linker that is stable in the endosome at pH 5 but is degraded at pH 7 in the cytosol [9]. pHPMA-MPPM siRNA polyplex has been reported to successfully silence firefly luciferase gene in H1299 human lung cancer cells expressing firefly luciferase *in vitro* [2]. Luten *et al.* administered complexes of pHPMA-MPPM with DNA i.p. in a Neuro2A tumor model in A/J mice. They observed poor gene expression in the tumor while the particles had mainly accumulated in the spleen [4]. These results suggest high clearance of these particles by the mononuclear phagocyte system which

Table 1 Mean size (based on number intensity) and mean zeta potential of pHPMA-MPPM polyplex, Angiplex and untargeted lipoplex ($n = 3$)

Sample name	Size (nm) ± SD	PDI	Zeta potential (mV) ± SD
siVEGFR-2 pHPMA-MPPM polyplex	198 ± 12	0.3	25 ± 4
siVEGFR-2 protamine lipoplex (untargeted lipoplex)	121 ± 5	0.2	−5 ± 1
siVEGFR-2 protamine anginex lipoplex (Angiplex)	150 ± 2	0.2	−5 ± 1

could also occur upon i.v. administration. Therefore, it seems crucial to evaluate nanoparticles in terms of potential interactions with blood components before continuing further with *in vivo* studies. In this study, the carriers were loaded with siRNA as therapeutic payload.

To evaluate the effect of anginex peptide on the toxicity of lipoplexes, a untargeted lipoplex was prepared as control with the same composition as Angiplex excluding the peptide. As demonstrated in Table 1, pHPMA-MPPM polyplex had a size of around 200 nm with a net positive charge. Angiplex and untargeted lipoplex were approximately 150 and 120 nm, respectively, with a net negative charge.

Activation of Platelets by Angiplex and pHPMA-MPPM Polyplex Both in Isolated Platelets and in Whole Blood

Activation of platelets induced by Angiplex and pHPMA-MPPM polyplex was investigated by flow cytometry with PE-conjugated anti P-selectin monoclonal antibody and FITC-conjugated anti-fibrinogen polyclonal antibody. As a consequence of vascular injury, adhesive membrane proteins such as P-selectin (CD62p) relocate to the cell surface to further aid accumulation of platelets and aggregation [12]. Moreover, Von Willebrand Factor (vWF) and fibrinogen are released from secretion granules of platelets to form the crosslink between platelets in the aggregation phase [13]. Therefore, the level of these proteins on the surface of platelets can be used as indicators of platelet activation.

Assessing platelet behaviour towards nanoparticles in isolated platelets provides information about the direct effect of contact with these cells. Nevertheless, it could be more clinically relevant to evaluate interaction of nanoparticles with platelets in whole blood which is more similar to physiological conditions. It has been reported in the literature that blood components, red blood cells and white blood cells can have an impact on platelet activity [14]. Therefore, we investigated platelet activation in isolated platelets and in whole blood.

It has already been established that particles with a size between 100 and 300 nm can be recovered from the open canalicular system of platelets [15]. Since both particles investigated in this paper have a mean size around or less than 200 nm, their interaction with platelets is expected to be through this channel system. These interactions could induce signaling from the open canalicular system to the platelet membrane which could result in platelet activation.

Neither Angiplex nor untargeted lipoplexes induced significant expression of both types of activation markers be it in isolated platelets (Fig. 1) or in whole blood (Fig. 2). Levels of both fibrinogen (Figs. 1, 2a) or P-selectin (Figs. 1, 2b) remained low on the platelet surface over the 20 min incubation time. Untargeted lipoplex induced similar platelet activation as that of Angiplex which could indicate that the presence of anginex peptide did not have a significant impact on platelet activation. A general low platelet activation was concluded for Angiplex which can be attributed to the PEG molecules present in this formulation. PEG creates a hydrophilic shield around the particles. It has already been reported that more hydrophobic particles create higher platelet activation and aggregation [16].

In contrast, activation by pHPMA-MPPM polyplexes was prominent even compared to the positive control activator TRAP-6. Both for fibrinogen and P-selectin, surface expression was between one and over two orders of magnitude higher both in isolated platelets and whole blood. It is known that the effect of particles on the hemostatic system is mainly dependent on charge interactions. Since the surface of platelets is negatively charged by virtue of their sialic acid groups, positively charged particles such as pHPMA-MPPM polyplex are expected to interact with them via charge interactions. The mechanism behind this excessive activation could be through neutralizing the surface charge of platelets and consequently providing bridges between different platelets [17]. Therefore, we compared the activation pattern of pHPMA-MPPM to another cationic polymer, jet-PEI. The high levels of platelet activation, with a similar profile as for pHPMA-MPPM that were observed for jetPEI indicates that the positive charge could be responsible for the activation. There was a marginal effect of particle concentration on platelet activation in the siRNA concentration range from 0.5 to 10 μg/ml at different incubation times in both isolated platelets and whole blood (see Supporting Information Fig. 1, 2).

Of note is the observation that platelet activation was higher in general when the experiments were performed in isolated platelets than in whole blood. This could indicate that the particles interact directly with platelets rather than being dependent on opsonization by blood components. Another hypothesis, particularly in case of positive particles (such as pHPMA-MPPM polyplex), would be that interactions and surface adsorption of nanoparticles with those blood proteins

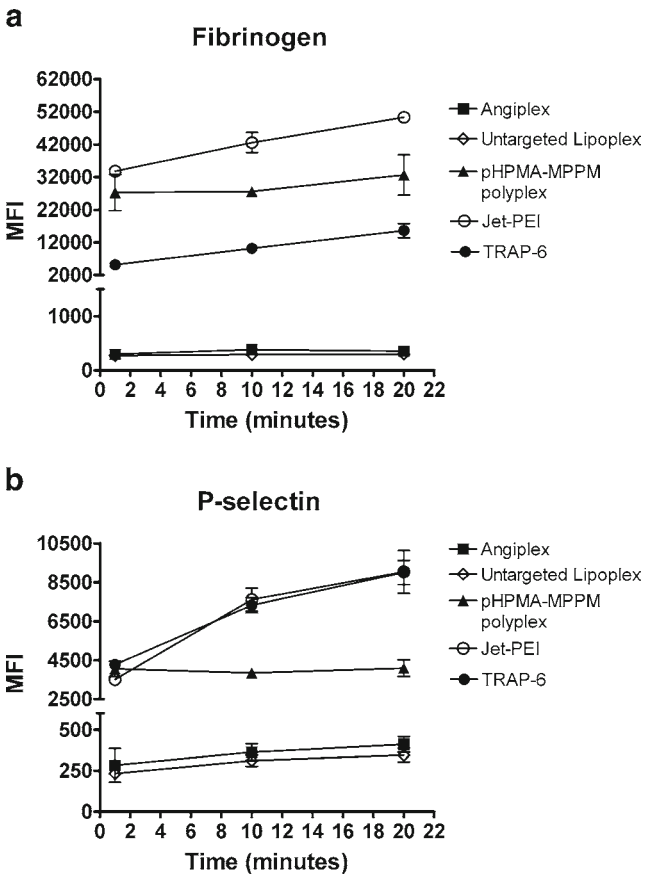

Fig. 1 Platelet activation assay of Angiplex, untargeted lipoplex, pHPMA-MPPM polyplex, jetPEI polyplex, and TRAP-6 after incubation for 1, 10, and 20 min in isolated platelets represented by mean fluorescence intensity (MFI) of (**a**) FITC-conjugated anti-fibrinogen and (**b**) PE-conjugated anti P-selectin ($n = 3$). All values are subtracted from blank.

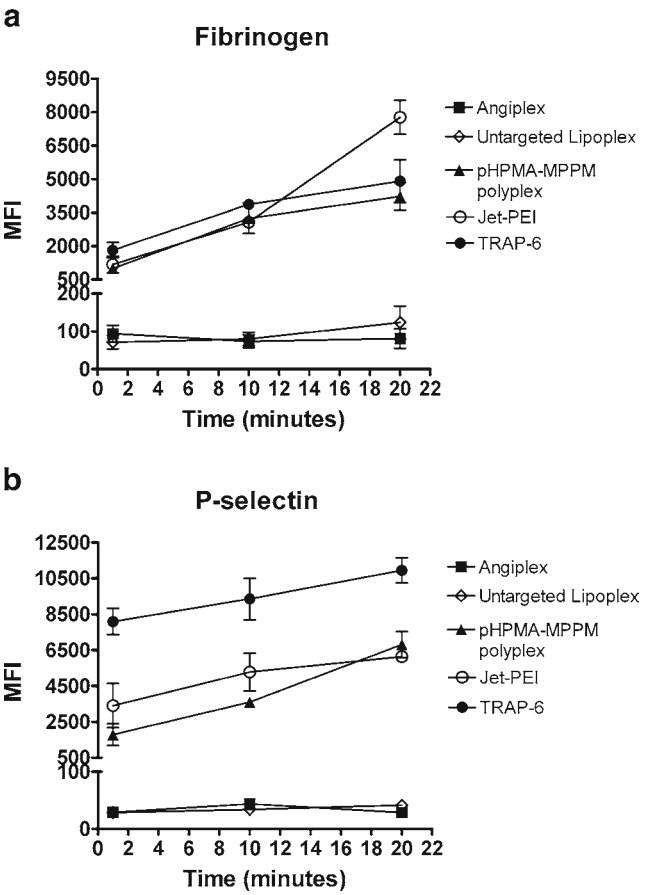

Fig. 2 Platelet activation assay of Angiplex, pHPMA-MPPM polyplex, jetPEI polyplex, and TRAP-6 after incubation for 1, 10, and 20 min in whole blood represented by mean fluorescence intensity (MFI) of (**a**) FITC-conjugated anti-fibrinogen and (**b**) PE-conjugated anti-P-selectin ($n = 3$). All values are subtracted from blank.

which have a high negative charge density on their surface (such as albumin) have led to reduced platelet interaction and subsequent activation. Jones *et al.* have observed that due to such mechanism positively charged dendrimers, which activate isolated platelets *in vitro*, do not elicit platelet activation *in vivo* [18]. Another possible reason for the observed effect could be that the steps taken for isolating platelets have already primed these cells for activation [19]. Taken together, these results suggest that Angiplex is likely to evoke less platelet activation than pHPMA-MPPM polyplex *in vivo*.

Platelet Aggregation Induced by Angiplex and pHPMA-MPPM Polyplex

In order to assess the effect of Angiplex and pHPMA-MPPM polyplex on platelet aggregation, an aggregometry assay was performed. Lower turbidity indicates more aggregation, since aggregation results in sedimentation of platelets and thus clearing up the sample within the light pathway. In line, with the FACS results on platelet activation, platelet aggregation was higher for pHPMA-MPPM polyplex (60%) than for Angiplex (30%) (Fig. 3). JetPEI, which had shown a similar effect on platelet activation in FACS showed an intermediate aggregation 45%. The net positive charge of pHPMA-MPPM polyplex could have resulted in more interactions with platelets and therefore a rise in aggregation as it has already been reported in the literature that positively charged particles interact more strongly with platelets and thereby induce aggregation [20, 21].

Contact System Activation by Angiplex and pHPMA-MPPM Polyplex

Impact of Angiplex and pHPMA-MPPM polyplex on initiating the intrinsic pathway of coagulation compared to a negatively charged particle, kaolin, which is employed as positive control was tested by contact system activation assay. Upon activation of the intrinsic pathway by nanoparticles, factor XII is auto activated (denoted by XIIa). Subsequently,

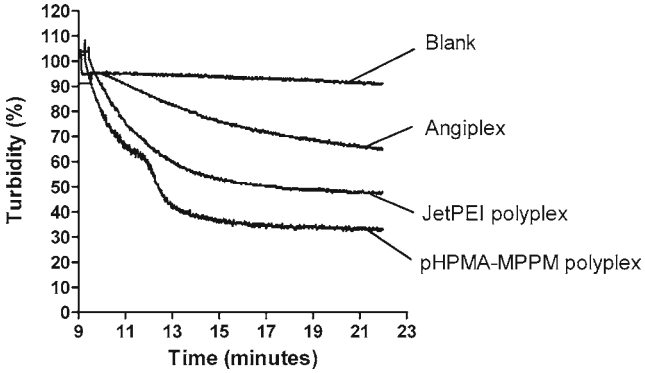

Fig. 3 Platelet aggregometry assay of pHPMA-MPPM polyplex, jetPEI polyplex, and Angiplex. % Turbidity is reversely correlated to the level of aggregation.

prekallikrein is activated which leads to the formation of kallikrein. In pooled plasma, the pathway stops at this stage because of the absence of calcium and phospholipids. In our assay, L-2120 was used as a substrate for kallikrein which recognizes L-2120 sequence due to the analogy with its

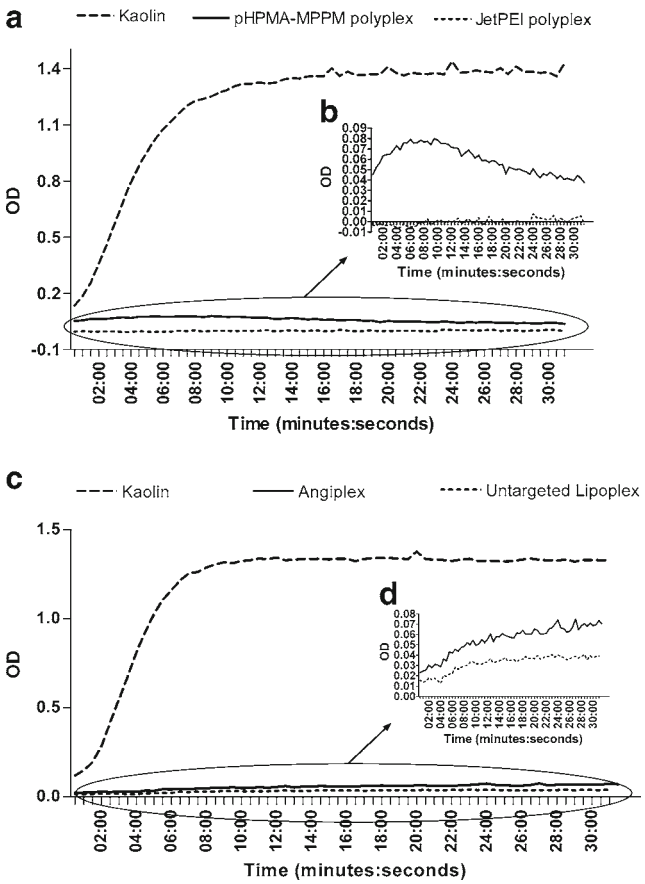

Fig. 4 Contact system activation by (**a**, **b**) pHPMA-MPPM polyplex and jetPEI polyplex and (**c**, **d**) Angiplex and untargeted lipoplex expressed as the amount of L-2120 absorbance at 405 nm (*OD: optical density*). Graphs (**b**) and (**d**) are zoomed versions of graphs (**a**) and (**c**), respectively. In graphs (**a**) and (**b**), dash-dotted line represents jetPEI polyplex, solid line pHPMA-MPPM polyplex, and dashed line kaolin. In graphs (**c**) and (**d**), solid line represents Angiplex, dotted line untargeted lipoplex, and dashed line kaolin.

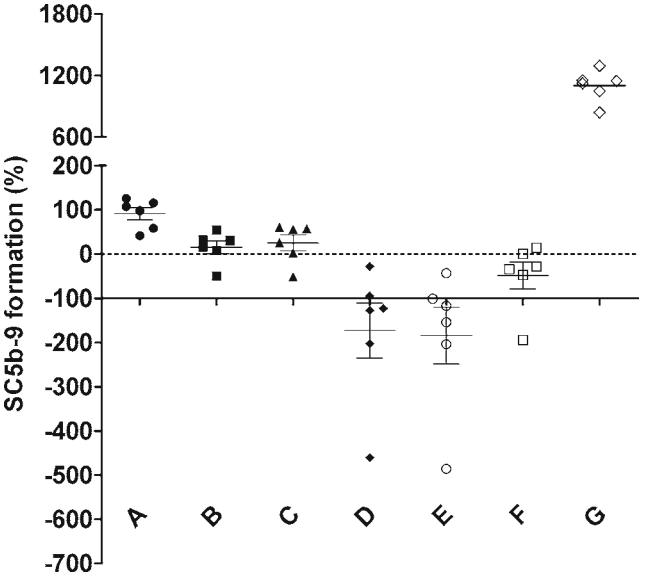

Fig. 5 Formation of SC5b-9 after incubation of human serum with lipoplexes and polyplexes. *A*: Untargeted lipoplex, *B*: Angiplex, *C*: Untargeted lipoplex without maleimide, *D*: Jet-PEI, E: pHPMA-MPPM polyplex, *F*: Lipofectamine-2,000, *G*: Zymosan. Results are given as % of SC5b-9 formation normalized to 20 mM HEPES buffer (*non-activator*). Dots indicate the mean of two measurements in an individual serum and error bars represent SEM.

natural substrate. Thereafter, factor XIIa and kallikrein cleave the peptide between arg-pNa and pNa becomes a free molecule with a yellow colour. The colour is measured at 405 nm by a spectrophotometer.

Intrinsic pathway is activated by negatively charged surfaces such as kaolin [22]. As evidenced in Fig. 4 (a, b), pHPMA-MPPM polyplex and jet-PEI had marginal effect on contact system activation compared to kaolin. This confirms previous findings that positively charged particles do not activate the contact system [14]. Miyamoto *et al.* have hypothesized that these particles are readily adsorbed to albumin (with a negatively charged surface). Another characteristic of particles contributing to coagulation is hydrophobicity [14]. This was in line with our observation that neither Angiplex nor untargeted lipoplex showed significant contact system activation (Fig. 4c, d). The absence of activation could be mediated by the PEG molecules on the surface of these particles which increase hydrophilicity. Moreover, although negatively charged liposomes are reported to trigger coagulation [23], PEG could have prevented this by shielding the negative charge.

Complement Activation Assay

Activation of the C system was assessed by determining the amount of SC5b-9 complex formation in sera of 6 individuals. It has been well established that anionic liposomes can activate the C system via the classical pathway [24]. However, our results showed that Angiplex did not trigger complement

activation to a significant extent (Fig. 5) which could be due to the very low overall negative charge of these particles, PEG shielding effect and the presence of the neutral lipid DOPE in the formulation which has been reported in other studies to reduce activation of the C system once included in DNA-lipid complexes [25]. Untargeted lipoplexes caused higher activation of the C system than Angiplex (Fig. 5). As this effect was absent when untargeted lipoplexes were prepared with DSPE-PEG2000 instead of DSPE-PEG2000-Mal or when sera were incubated with Angiplex (which has little reactive maleimide groups left after the coupling), the result was concluded to be related to free maleimide groups. Maleimide groups are quite reactive and could have reacted with complement proteins. Low activation was observed with pHPMA-MPPM polyplex and jetPEI polyplex which was consistent with the study of Plank *et al.* who showed that although positively charged polymers have the potential to activate the C system, complexation with nucleic acids decreases activation because less positive charges would be accessible for interaction with the complement proteins [26]. Moreover, complement activation by polyplexes was lower than lipoplexes. This is plausibly a consequence of including DSPE-PEG2000 in the formulation of lipoplexes. DSPE-PEG2000 in the formulation of liposomes has a dual effect; on the one hand it shields the charge and on the other hand it has been shown in the literature to increase C activation by liposomes likely due to the negatively charged phosphate moieties [27].

CONCLUSION

In this study, we investigated the behavior of a lipoplex (Angiplex) and a polyplex (prepared with pHPMA-MPPM) in terms of their thrombogenicity, plasma coagulation, and complement activation. We found that interactions depend on physicochemical properties of the nanoparticle and that each nanoparticle is affected differently by various components and cells in the blood. Angiplex did not stimulate platelet activation and aggregation, while pHPMA-MPPM and jetPEI polyplex had a strong stimulatory effect on platelets likely due to their net positive surface charge. Both formulations showed minimal activation of the C system and did not activate the contact system of coagulation significantly. Overall, these results suggest that Angiplex is expected to cause minimal adverse effects in the blood stream, whereas pHPMA-MPPM is likely to lead to complications due to stimulating platelets. Taken together, the assays performed in this study allow us to obtain a comprehensive overview of nanoparticle interactions with the blood from different aspects. This can be an essential step preceding *in vivo* administration in order to decrease animal usage and suffering.

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REFERENCES

1. Yousefi A, Bourajjaj M, Babae N, van Noort PI, Schaapveld RQJ, van Beijnum JR, Griffioen AW, Storm G, Schiffelers RM, Mastrobattista E (unpublished results). Dual action lipoplexes for delivery of anti-angiogenic siRNA. 2013.
2. Varkouhi AK, Lammers T, Schiffelers RM, van Steenbergen MJ, Hennink WE, Storm G. Gene silencing activity of siRNA polyplexes based on biodegradable polymers. Eur J Pharm Biopharm. 2011;77(3):450–7.
3. Verbaan FJ, Oussoren C, van Dam IM, Takakura Y, Hashida M, Crommelin DJ, *et al.* The fate of poly(2-dimethyl amino ethyl)methacrylate-based polyplexes after intravenous administration. Int J Pharm. 2001;214(1–2):99–101.
4. Luten J. Biodegradable cationic polymers as gene delivery carriers: from synthesis to in vivo application. Thesis: Utrecht University; 2007.
5. Meyer M, Dohmen C, Philipp A, Kiener D, Maiwald G, Scheu C, *et al.* Synthesis and biological evaluation of a bioresponsive and endosomolytic siRNA-polymer conjugate. Mol Pharm. 2009;6(3):752–62.
6. Pecot CV, Calin GA, Coleman RL, Lopez-Berestein G, Sood AK. RNA interference in the clinic: challenges and future directions. Nat Rev Cancer. 2011;11(1):59–67.
7. Yousefi A, Storm G, Schiffelers RM, Mastrobattista E. Trends in polymeric delivery of nucleic acids to tumors. J Control Release. 2013;170(2):209–18.
8. Wagner E, Curiel D, Cotten M. Delivery of drugs, proteins and genes into cells using transferrin as a ligand for receptor-mediated endocytosis. Adv Drug Deliv Rev. 1994;14(1):113–35.
9. Luten J, Akeroyd N, Funhoff A, Lok MC, Talsma H, Hennink WE. Methacrylamide polymers with hydrolysis-sensitive cationic side groups as degradable gene carriers. Bioconjug Chem. 2006;17(4):1077–84.
10. de Maat S, van Dooremalen S, de Groot PG, Maas C. A nanobody-based method for tracking factor XII activation in plasma. *Thromb Haemost. 2013, 109* (4).
11. Korporaal SJ, Van Eck M, Adelmeijer J, Ijsseldijk M, Out R, Lisman T, *et al.* Platelet activation by oxidized low density lipoprotein is mediated by CD36 and scavenger receptor-A. Arterioscler Thromb Vasc Biol. 2007;27(11):2476–83.
12. Merten M, Thiagarajan P. P-selectin expression on platelets determines size and stability of platelet aggregates. Circulation. 2000;102(16):1931–6.
13. Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, Deckmyn H. Platelets at work in primary hemostasis. Blood Rev. 2011;25(4):155–67.
14. Miyamoto M, Sasakawa S, Ozawa T, Kawaguchi H, Ohtsuka Y. Mechanisms of blood coagulation induced by latex particles and the roles of blood cells. Biomaterials. 1990;11(6):385–8.
15. White JG, Clawson CC. Effects of large latex particle uptake of the surface connected canalicular system of blood platelets: a freeze-fracture and cytochemical study. Ultrastruct Pathol. 1981;2(3):277–87.
16. Miyamoto M, Sasakawa S, Ozawa T, Kawaguchi H, Ohtsuka Y. Platelet aggregation induced by latex particles. I. effects of size, surface potential and hydrophobicity of particles. Biomaterials. 1989;10(4):251–7.

17. Taketomi Y, Kuramoto A. Ultrastructural studies on the surface coat of human platelet aggregated by polylysine and dextran. Thromb Haemost. 1978;40(1):11–23.
18. Jones CF, Campbell RA, Brooks AE, Assemi S, Tadjiki S, Thiagarajan G, *et al.* Cationic PAMAM dendrimers aggressively initiate blood clot formation. ACS Nano. 2012;6(11):9900–10.
19. Bagamery K, Kvell K, Barnet M, Landau R, Graham J. Are platelets activated after a rapid, one-step density gradient centrifugation? evidence from flow cytometric analysis. Clin Lab Haematol. 2005;27(1):75–7.
20. Naeye B, Deschout H, Roding M, Rudemo M, Delanghe J, Devreese K, *et al.* Hemocompatibility of siRNA loaded dextran nanogels. Biomaterials. 2011;32(34):9120–7.
21. Copley AL. Roles of platelets in physiological defense mechanisms and pathological conditions. Folia Haematol Int Mag Klin Morphol Blutforsch. 1979;106(5–6):732–64.
22. Jackson CM, Nemerson Y. Blood coagulation. Annu Rev Biochem. 1980;49:765–811.
23. Zbinden G, Wunderli-Allenspach H, Grimm L. Assessment of thrombogenic potential of liposomes. Toxicology. 1989;54(3):273–80.
24. Chonn A, Cullis PR, Devine DV. The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. J Immunol. 1991;146(12):4234–41.
25. Plank C, Mechtler K, Szoka Jr FC, Wagner E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. Hum Gene Ther. 1996;7(12):1437–46.
26. Plank C, Mechtler K, Wagner E, Szoka FC. Complement activation by polylysine-DNA complexes. In: Grigoriadis G, editor. In targeting of drugs: strategies for oligonucleotide and gene delivery in therapy. New York: Plenum Publishing Corp; 1995.
27. Moghimi SM, Andersen AJ, Ahmadvand D, Wibroe PP, Andresen TL, Hunter AC. Material properties in complement activation. Adv Drug Deliv Rev. 2011;63(12):1000–7.