Title: Interactions of siRNA loaded dextran nanogel with blood cells

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Abstract

Although the behavior of nanoscopic delivery systems in blood is an important parameter when contemplating their intravenous injection, this aspect is often poorly investigated when advancing from *in vitro* to *in vivo* experiments. In this paper, the behavior of siRNA loaded dextran nanogels in human plasma and blood is examined using fluorescence fluctuation spectroscopy, platelet aggregometry, flow cytometry and single particle tracking. Our results show that, in contrast to their negatively charged counterparts, positively charged siRNA loaded dextran nanogels cause platelet aggregation and show increased binding to human blood cells. Although PEGylating the nanogels did not have a significant effect on their interaction with blood cells, single particle tracking revealed that it is necessary to prevent their aggregation in human plasma. We therefore conclude that PEGylated negatively charged dextran nanogels are the most suited for further *in vivo* studies as they do not aggregate in human plasma and exhibit minimal interactions with blood cells.

1. Introduction

Although RNA interference (RNAi) is a promising strategy for treating various disorders [1-3], detailed knowledge and new scientific advancements are needed before this form of nucleic acid therapy can be applied clinically. Insufficient insights in endogenous gene regulation [4, 5] and especially difficulties regarding safe and efficient delivery of RNAi mediators are currently responsible for the inability of RNAi to live up to its full potential [6, 7]. Among these mediators, small interfering RNA (siRNA) is used the most for down regulation of mRNA in both clinical and research environments. Several routes of administration are currently investigated for siRNA [8] and although the advantages of local non invasive delivery are clear, many applications will require intravenous injection of (nanoscopic) siRNA carriers [9]. As this entails direct contact between these nanocarriers and blood components, profound knowledge on their behavior in blood is pivotal for the development of formulations which are not only efficient but also safe. Although of fundamental importance, safety is often only studied in a later stage of development.

Hemocompatible formulations can be defined as formulations which do not result in any form of toxicity and remain efficacious after being exposed to blood. Currently, complement activation, blood coagulation and hemolysis assays are the only tests regularly conducted when evaluating the hemocompatibility of new nanoformulations [10-13]. However, in addition to blood clotting, activation of the complement system and erythrocyte lysis, many other phenomena can occur. High salt and protein concentrations in blood, for instance, can destabilize nucleic acid loaded nanoparticles and seriously limit their efficacy, as illustrated recently by the disassembly of siRNA polyplexes upon contact with serum [14]. The latter can logically be explained by negatively charged serum components displacing the (negatively charged) siRNA, hereby exposing it to serum nucleases and fast glomerular filtration [15]. It is also common knowledge that nanosized particles in blood can be cleared efficiently by the mononuclear phagocyte system (MPS), thereby seriously limiting their blood circulation time and extravasation into target tissues [16-18]. Decreasing the susceptibility of nanocarriers to recognition by the MPS through coverage of their surface with hydrophilic polymers, such as polyethylene glycol (PEG), is an established strategy to prolong the residence time of a nanoformulation in the systemic circulation [16]. Aggregation of nanoparticles upon intravenous injection can also occur and is considered very important as it influences their clearance, bio-distribution and toxicity [19-21]. Commonly used methods which study the size and aggregation of nanoparticles, e.g. dynamic light scattering (DLS), are however in most cases incompatible with complex media such as blood or serum. A recently developed technique based on fluorescence Single Particle Tracking (fSPT) allows size measurements of nanosized matter in undiluted human plasma [20] and is used in the current study to investigate the aggregation of nanoparticles, in this case dextran nanogels, in human plasma.

Dextran nanogels have been proposed earlier as a nanocarrier system and have already been shown to successfully deliver siRNA *in vitro* [22, 23]. To further explore the potential of dextran nanogels for the intravenous delivery of siRNA, the first aim of this study is to evaluate to which extent siRNA loaded dextran nanogels are hemocompatible using several advanced methods that recently became available. Secondly, several new approaches which can provide additional information on the hemocompatibility of nanoformulations are introduced. In this regard we evaluate what aggregometry and flow cytometry can teach us about the interactions between nanogels and blood cells, under conditions closely resembling the *in vivo* situation.

2. Materials and methods

2.1 Preparation of PEGylated dextran nanogels loaded with siRNA

Cationic dextran nanogels were prepared from dextran methacrylate (dex-MA), [2-(methacryloyloxy)ethyl]trimethylammonium chloride (TMAEMA) and 2-aminoethyl methacrylate hydrochloride (AEMA) using inverse mini-emulsion photopolymerization as described earlier [22, 23]. The resulting cationic nanogels of about 200 nm were lyophilized and stored in a desiccator at room temperature. A weighed amount of the lyophilizate was dispersed in a known volume of 20 mM HEPES buffer at pH 7.4 and exposed to a short burst of ultrasound to disintegrate loose particle aggregates formed during lyophilization (Branson Tip Sonifier, 10 s, amplitude 10%). The nanogels were loaded by adding increasing amounts of Dicer substrate 25/27-mer siRNA (DsiRNA) targeting EGFP (IDT, Leuven Belgium) to the initially positively charged nanogels. Increasing the siRNA/nanogel ratio resulted in neutral or negatively charged nanogels as described earlier [22, 23] (Figure 1). The nanogels were consecutively PEGylated by adding 0, 0.25, 0.5, 1, 2.5 and 5 mg NHS-PEG/mg nanogels N-hydroxysuccinimidyl activated methoxypolyethylene glycol 5000 propionic acid (NHS-PEG, Sigma, Belgium) for 30 min [23]. The influence of siRNA loading and PEGylation on the zeta potential of the nanogels is depicted in Figure S1 (supplementary information).

2.2 Hemolysis assay

The hemolytic activity of dextran nanogels was investigated using an established method based on the release of hemoglobin from damaged erythrocytes. Briefly, human erythrocytes were isolated from 5 mL of fresh citrated blood by centrifugation (600 g, 10 min). The erythrocytes were washed in PBS until the supernatant was clear and colorless and then diluted to the original volume of 5 mL. 50 µL aliquots of the erythrocyte suspension were incubated with 50 µL of a nanogel dispersion for 30 min at 37°C under constant shaking. For this experiment, both positively and negatively charged nanogels, with or without a PEG coating, were added in order to achieve final concentrations of 0.05 to 0.5 mg nanogels/ml. After centrifugation (600 g, 10 min) to pellet the erythrocytes, the hemoglobin concentration in the supernatant was quantified using a UV-1800 UV-VIS Spectrophotometer (Shimadzu, Belgium) at 541 nm. A 1% triton X-100 solution and pure PBS were used as the positive and negative controls respectively and all samples were prepared in triplicate.

2.3 Platelet aggregometry

The use of blood or blood plasma inadvertently implies the presence of an anticoagulant to prevent rapid blood clotting. As high concentrations of negatively charged heparin can have a destabilizing effect on the siRNA loaded nanogels and EDTA is known to cause structural, biochemical and functional damage to platelets [24], trisodium citrate was used as an anticoagulant in accordance to clinical laboratory protocols.

Fresh citrated blood was drawn from healthy volunteers and subsequently centrifuged at 280 g for 15 min and 3200 g for 10 min to obtain platelet rich plasma (PRP) and platelet poor plasma (PPP) respectively. Using PPP, the platelet concentration of the PRP was adjusted to $3x10^6$ cells per µL before pre-incubating 200 µL of this suspension at 37°C. 50 µL of a nanogel dispersion was added to the 200 µL of platelet dispersion to investigate nanogel induced platelet aggregation. The turbidity of the samples was measured as a function of time using a Chrono-log aggregometer (Kordia, Leiden, The Netherlands).

2.4 Fluorescence fluctuation spectroscopy

The complexation degree of fluorescently labeled siRNA by dextran nanogels with different PEGylation degrees was determined in 20 mM HEPES buffer (pH 7.4) using fluorescence fluctuation spectroscopy (FFS) as reported before [23, 25]. FFS is a microscopy based technique that monitors the fluorescence intensity fluctuations in the focal volume (~1 fL) of a confocal microscope. When a solution of uncomplexed (i.e. free) fluorescently labeled siRNA is measured via FFS, a fluorescence signal proportional to the concentration of siRNA is obtained. Fluctuations in the fluorescence signal are caused by siRNA molecules diffusing in and out of the focal volume and result in a baseline signal. When the siRNA is complexed in nanoparticles, this baseline drops and highly fluorescent peaks (originating from nanoparticles containing large amounts of siRNA) occur. The shift of this baseline can be used to calculate the percentage of unbound siRNA as explained in detail by Buyens et al. [25].

To determine the extent of siRNA displacement from different nanogels by plasma constituents, FFS measurements were performed after incubating the siRNA loaded nanogels for 1h in human plasma (volume nanogels/plasma = 20/80). The final concentration of the nanogels was 0.068 mg/mL as this would be the theoretical concentration needed to deliver 1 mg/kg siRNA to a mouse. The experiments were conducted with both positively charged and negatively charged nanogels loaded with Alexa Fluor 488-labeled 25/27-mer siRNA (AF488-DsiRNA, IDT, Leuven, Belgium) targeting enhanced green fluorescent protein (EGFP).

2.5 Fluorescence single particle tracking (fSPT)

Single particle tracking (SPT) is a fluorescence microscopy-based method where the movement of individual fluorescently labeled particles or molecules is monitored in time and space. Using an epi-fluorescence microscope adjusted for widefield laser illumination equipped with a fast and sensitive CCD camera, the movement of individual particles in biological fluids can be recorded under the form

of movies. Particle trajectories subsequently calculated from these movies by specialized algorithms can then be used to measure both the size and number concentration of the particles, as recently shown by Braeckmans and colleagues [20].

2.5.1 **Aggregation of nanogels in human plasma using fSPT**

Aggregation of negatively charged nanogels was followed as a function of time in both HEPES buffer and human plasma. Nanogel dispersions were prepared as described previously and were loaded with fluorescently labeled DsiRNA; the zeta potential of the non-PEGylated nanogels was approximately -10 mV. 5 µL of a nanogel dispersion was then incubated with 45 µL HEPES buffer or PPP at 37°C (0.068 mg nanogels/mL) and diluted 1:10 with buffer and PPP respectively immediately before the measurements. This particular concentration was selected as this would be the initial nanogel concentration in the blood of a mouse upon intravenous injection of a formulation containing 20 µg of siRNA. At each time point, 20 movies of 10 s each were recorded and consecutively analyzed to determine the size distribution of the nanogels. Using the nearest neighbor algorithm, trajectories were calculated taking into account the maximum distance a particle could reasonably have travelled in two consecutive frames. Using an algorithm based on the mean square displacement analysis, a mean diffusion coefficient could then be calculated for each trajectory. The distribution of these diffusion coefficients could be transformed in a size distribution using the Stokes-Einstein equation, provided that both the viscosity and the temperature of the sample were known. Both the objective and the sample were kept at 37°C during the measurement using an objective heater (Bioptechs, Butler, USA) and a sample heater (Linkam, Surrey, U.K.). During the calculations, the viscosity of human plasma was set to 1.35 cP at 37°C in accordance to Braeckmans et al [20]. Due to the stochastic nature of Brownian motion, the acquired size distribution was convoluted by a gamma distribution. Using a deconvolution algorithm known as the maximal entropy method (MEM), the actual size distribution was obtained [20].

2.5.2 **Measuring the concentration of nanogels in human plasma using fSPT**

Although the number of calculated trajectories under 2.5.1 is not equal to the actual number of nanogels in a sample, a certain correlation exists between both values. Using additional information such as the detection volume, the track length and the diffusion coefficient of each particle, a maximum likelihood estimation could be used to determine the particle concentration in a dispersion. In this paper, this newly developed method is applied for the first time to estimate the number concentration of free nanogels in blood (i.e. non bound to cells).

The experiments were performed as follows: PEGylated negatively charged nanogels were incubated with fresh citrated blood in different concentrations at 37°C for 1 hour prior to a short centrifugation step (600 g, 60 s) to remove the cell fraction. After the centrifugation step, 5 µL of the supernatant was placed on a heated microscopy slide for the collection of at least 20 movies of 10 seconds at approximately 22 fps. In order to calculate the fraction interacting with blood cells, the measurements were also performed in human plasma instead of full blood. Because the volume occupied by cells is not available for diffusing nanogels, the hematocrit value was used to correct the total volume of plasma.

2.6 Flow cytometry

Flow cytometry was used to investigate the adhesion and internalization of nanogels by blood cells. In order to compare various formulations, the fluorescence of every nanogel particle has to be similar. For this reason, a fixed amount of (green) fluorescently labeled siRNA (10 nmol per mg nanogels) was supplemented with unlabeled siRNA (0 to 20 nmol per mg nanogels) to achieve the desired siRNA loading and charge of the nanogels. The green fluorescence associated with a cell could be attributed to the sum of the adsorbed and internalized nanogels on/by this cell. By incubating the samples at 4°C instead of 37°C, active internalization of the nanogels by the cells can be inhibited and the green fluorescent signal associated with every cell can in this case be ascribed to

labeled nanogels adsorbed on the surface. By treating the samples with trypan blue (Sigma Aldrich, Belgium) prior to the antibody labeling (see below), fluorescent nanogels attached to the surface of the cells are quenched so that only the internalized fraction of the nanogels is measured. Trypan blue quenching was performed by adding 25 µL of a trypan blue solution (0.2 %) to 5 µL of sample. After 5 minutes incubation the trypan blue was subsequently removed by washing three times using PBS.

The expertiments were performed as follows: 5 µL of a nanogel dispersion containing AF488-DsiRNA was incubated with 45 µl fresh citrated blood from healthy volunteers and incubated for 30 min at 37°C. The final concentration of nanogels in blood was 0.068 mg nanogels/mL and all samples were prepared in triplicate. Antibodies against CD61 (platelets) and CD45 (leukocytes) conjugated to phycoerythrin (PE) and phycoerythrin-Cy5 (PE-Cy5) respectively were purchased from BD Pharmingen (Erembodegem, Belgium) and were used to resolve certain cell populations in blood. The scatter plot of the CD45 positive population was used to distinguish granulocytes, monocytes and lymphocytes. After 20 min at room temperature, unbound antibodies were washed away using PBS and a short centrifugation step (1100 g, 5 min). After discarding the supernatant, all samples were measured using a FACSCalibur flow cytometer (BD, Erembodegem, Belgium). The generated data was analyzed in SPSS using an ANOVA analysis ($p < 0.05$).

3. Results and discussion

3.1 Hemolysis

With over 4 million cells per microliter, erythrocytes are abundantly present in human blood. As damage to erythrocytes and the subsequent release of hemoglobin causes the first symptoms of toxicity (e.g. renal failure), hemolysis assays are generally considered valuable in testing the hemocompatibility of a drug formulation. A standard hemolysis assay was performed to assess if siRNA loaded dextran nanogels induce erythrocyte lysis. Both positively and negatively charged nanogels, with different PEGylation degrees, were tested. As shown in Figure 2, the nanogels did not cause substantial erythrocyte lysis (< 1%), even not at high concentrations. The osmotic activity of

nanocarrier dispersions is often neglected although it is known that especially erythrocytes are prone to osmotic disruption. Measurements using a freezing point depression osmometer indictated that the osmolarity of each nanogel dispersion (in PBS) studied in this paper was within the reference range of 275-299 mOsm per kg. A major disadvantage of a standard hemolysis assay is however the removal of the plasma components prior to the incubation of the erythrocytes with the nanoparticles as adsorption of plasma proteins on the nanoparticle surface can have an important influence on the interactions between cells and the nanoparticles [26].

3.2 Platelet aggregation

Light transmission aggregometry is currently the standard method in clinical practice to evaluate platelet functions. By adding platelet activators to whole blood or platelet-rich plasma (PRP) and measuring the turbidity as a function of time, specific information can be obtained on possible platelet defects. Platelet clumping will result in a *decreased* turbidity of the sample due to the formation of a single clot. This method was used here to investigate whether nanogels induce undesired platelet clumping. Figure 3 shows that the addition of positively charged nanogels to PRP causes rapid aggregation of platelets while negatively charged nanogels do not (light transmission is ~20% and similar to the transmission measured after adding buffer (negative control)). PEGylation of the nanogels did not influence the extent of platelet aggregation. While the hemolysis assay (Figure 2) indicates all nanogel dispersions to be safe for erythrocytes, light transmission aggregometry suggests that positively charged nanogels cause extensive platelet aggregation (Figure 3). Based on these results, experiments in the continuation of this paper were generally conducted with negatively charged nanogels.

3.3 Dissociation of siRNA loaded nanogels in human plasma

Fluorescence fluctuation spectroscopy (FFS) was performed to measure the siRNA complexation efficiency by the nanogels upon contact with human plasma. As described before [25], FFS allows to

distinguish between free and complexed siRNA in a sample without the need to physically separate the free and complexed molecules. Figure 4 shows that approximately 50% of the siRNA is released from the nanogels when dispersed in human plasma. PEGylation of the nanogels did not prevent this. All FFS experiments were performed at a nanogel concentration of 0.068 mg/ml which is the nanogel concentration in blood needed to deliver 1 mg/kg siRNA to a mouse (see 2.4).

3.4 Aggregation of nanogels in human plasma

Using fluorescent single particle tracking (fSPT), aggregation of nanogels in human plasma was studied. The results for negatively charged nanogels are depicted in Figure 5. In HEPES buffer, both non-PEGylated and PEGylated negatively charged nanogels were shown to be colloidally stable for at least 3 hours (Figure 5 A-D). In contrast, dispersing these nanogels in human plasma causes a marked aggregation of the non-PEGylated nanogels (Figure 5 E-H). Their aggregation could however be slowed down and even prevented by sufficiently coating the nanogels with PEG. Even for the highest PEGylation degree (5 mg NHSPEG/mg nanogel), a low number of aggregates were still detected. This is probably caused by a fraction of nanogels which is not sufficiently PEGylated (inhomogeneous PEGylation). Preventing nanoparticles from aggregating in blood is important as this will not only have an effect on their clearance and biodistribution but also on their toxicity, as illustrated by the accumulation of aggregated nanoparticles in lung capillaries upon intravenous injection.

3.5 Adhesion and uptake of nanogels to/by blood cells

Using flow cytometry, interactions between green fluorescent siRNA loaded nanogels and different cell populations in blood were examined. It was possible to measure the amount of nanogels internalized by the cells by quenching non internalized nanogels using trypan blue (Supplementary information, Figure S2). As shown in Figure 6A, all blood cell populations except erythrocytes show an outspoken charge dependent interaction with nanogels. Cationic nanogels clearly bind much more

to cells than anionic ones. In contrast to surface charge, PEGylation of the nanogels did in most cases not influence the binding to the blood cells significantly ($p < 0.05$).

As far as internalization of the nanogels by blood cells concerns (Figure 6B), monocytes and to a limited extent granulocytes, appeared to be the only cell types which efficiently internalize nanogels in a charge dependent manner. Although monocytes and neutrophilic granulocytes are both considered to be specialized phagocytes, the internalization of nanogels by neutrophils was low. These observations were confirmed by investigating the internalization of PEGylated, negatively charged nanogels as a function of time (Supplementary information, Figure S3.). In analogy to the binding, PEGylation of the nanogels did not influence their internalization by monocytes although it did prevent nanogel aggregation in human plasma (Figure 5). This could imply that the stabilizing effect of PEG is the primary reason for the longer blood circulation time associated with PEGylated formulations.

Flow cytometry data in Figure 6 are in line with the outcome of the hemolysis (Figure 2) and aggregometry (Figure 3) assays. Figure 2 showed that nanogels do not cause erythrocytes lysis which can be expected as they do not bind to erythrocytes (Figure 6A). In addition, the binding of positively charged dextran nanogels to platelets (Figure 6B) support the results of the platelet aggregation assay in Figure 3. It seems that platelets do not internalize nanogels (Figure 6B) confirming that nanoparticles end up in the surface connected open canalicular system (OCS) of a platelet as suggested by Werb et al. [27].

As shown in Figure 7A, adding increasing amounts of nanogels to a fixed volume of blood revealed the saturation of the monocytes with nanogels at nanogel concentrations between 0.05 and 0.1 mg/mL (which corresponds to ~6 billion and ~11 billion nanogels/mL respectively). It was however striking to see that the internalization of nanogels by monocytes decreased significantly at nanogel

concentrations above 0.068 mg/mL (Figure 7B). A possible explanation is that phagocytosis is impaired above a critical nanoparticle concentration due to the large amount of nanogels adsorbed on the monocyte surface. This hypothesis is supported by the fact that the number of internalized nanogels (Figure 7B) decreases significantly at the concentration which corresponds to the saturation of the monocytes (Figure 7A). Interestingly, some early papers showed that monocytes can only engulf a finite quantity of particles or microorganism before entering a period of phagocytic inactivity [28, 29]. Visualization of the monocytes after incubation with the nanogels indeed revealed a high number of nanogels attached to the surface (Supplementary information, Video S1). To what extent saturation of the monocytes also influences their biological functions remains to be investigated.

3.6 Interactions between nanogels and blood cells measured by fSPT

To quantify the fraction of nanogels interacting with blood cells, the concentration of free (i.e. not adsorbed to cells) negatively charged PEGylated nanogels in blood was assessed by a method based on fSPT which calculates the concentration of fluorescent nanoparticles freely diffusing in a sample. As illustrated in Figure 8A, it is possible to calculate the percentage of nanogels bound to or internalized by blood cells by comparing the concentration of free nanogels measured in (platelet poor) plasma and blood. Figure 8B shows that increasing the number of nanogels added to a fixed volume of blood results in a larger fraction of free dextran nanogel suggesting that the blood cells are saturated with nanogels. At a nanogel concentration of 0.068 mg/mL (the concentration in blood needed to deliver 1 mg/kg siRNA to a mouse) approximately 69% of the nanogels remains free in blood while 31% is bound to/internalized by blood cells and is probably not able to extravasate into tissues. Flow cytometry data obtained under 3.5 (Figure 6A) were used to specify which of the different blood cell types are responsible for this 31% decrease in nanogel concentration (Figure 9).

4. Conclusion

In the context of developing a safe and efficient nanocarrier for drug delivery, the hemocompatibility of siRNA loaded dextran nanogels was investigated in this paper. Although none of the nanogel formulations caused significant erythrocyte lysis, positively charged nanogels induced platelet aggregation. Flow cytometry data confirmed that nanogels hardly bind to erythrocytes while a clear (charge dependent) interaction with platelets and leukocytes was observed. PEGylating the siRNA loaded dextran nanogels did not influence their interactions with cells significantly but was shown to be required to prevent their aggregation in human plasma. Based on these findings, PEGylated negatively charged siRNA loaded dextran nanogels are likely the safest formulation for *in vivo* siRNA delivery. However, the efficacy of this formulation will probably be influenced by the release of a significant fraction of the siRNA from the nanogels (approximately 50% *in vitro*) and their interactions with blood cells.

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[1] Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 1998;391:806-11.
[2] Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 2001;411:494-8.
[3] Davidson BL, McCray PB, Jr. Current prospects for RNA interference-based therapies. Nat Rev Genet. 2011;12:329-40.
[4] Morris KV. RNA-directed transcriptional gene silencing and activation in human cells. Oligonucleotides. 2009;19:299-306.
[5] Jackson AL, Linsley PS. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. Nat Rev Drug Discov. 2010;9:57-67.
[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;11:59-67.
[7] Raemdonck K, Vandenbroucke RE, Demeester J, Sanders NN, De Smedt SC. Maintaining the silence: reflections on long-term RNAi. Drug Discov Today. 2008;13:917-31.

[8] Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discov. 2009;8:129-38.
[9] Tiemann K, Rossi JJ. RNAi-based therapeutics-current status, challenges and prospects. EMBO Mol Med. 2009;1:142-51.
[10] Sarma JV, Ward PA. The complement system. Cell Tissue Res. 2011;343:227-35.
[11] Oppermann M, Wurzner R. Modern determination of complement activation. Semin Thromb Hemost. 2010;36:611-9.
[12] Dobrovolskaia MA, Aggarwal P, Hall JB, McNeil SE. Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution. Mol Pharm. 2008;5:487-95.
[13] Radomski A, Jurasz P, Alonso-Escolano D, Drews M, Morandi M, Malinski T, et al. Nanoparticle-induced platelet aggregation and vascular thrombosis. Br J Pharmacol. 2005;146:882-93.
[14] Merkel O, Librizzi D, Pfestroff A, Schurrat T, Buyens K, Sanders N, et al. Stability of siRNA polyplexes from poly(ethylenimine) and poly(ethylenimine)-g-poly(ethylene glycol) under in vivo conditions: effects on pharmacokinetics and biodistribution measured by Fluorescence Fluctuation Spectroscopy and Single Photon Emission Computed Tomography (SPECT) imaging. J Control Release. 2009;138:148-59.
[15] van de Water FM, Boerman OC, Wouterse AC, Peters JGP, Russel FGM, Masereeuw R. Intravenously administered short interfering RNA accumulates in the kidney and selectively suppresses gene function in renal proximal tubules. Drug Metab Dispos. 2006;34:1393-7.
[16] Owens Dr, Peppas N. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. Int J Pharm. 2006;307:93-102.
[17] Roerdink F, Dijkstra J, Hartman G, Bolscher B, Scherphof G. The involvement of parenchymal, Kupffer and endothelial liver cells in the hepatic uptake of intravenously injected liposomes. Effects of lanthanum and gadolinium salts. Biochim Biophys Acta. 1981;677:79-89.
[18] Moghimi SM, Patel HM. Serum-mediated recognition of liposomes by phagocytic cells of the reticuloendothelial system - The concept of tissue specificity. Adv Drug Deliv Rev. 1998;32:45-60.
[19] Jiang JK, Oberdorster G, Biswas P. Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. Journal of Nanoparticle Research. 2009;11:77-89.
[20] Braeckmans K, Buyens K, Bouquet W, Vervaet C, Joye P, Vos FD, et al. Sizing Nanomatter in Biological Fluids by Fluorescence Single Particle Tracking. Nano Lett. 2010;10:4435-42.
[21] Brigger I, Morizet J, Laudani L, Aubert G, Appel M, Velasco V, et al. Negative preclinical results with stealth nanospheres-encapsulated Doxorubicin in an orthotopic murine brain tumor model. Journal of controlled release : official journal of the Controlled Release Society. 2004;100:29-40.
[22] Raemdonck K, Naeye B, Buyens K, Vandenbroucke RE, Hogset A, Demeester J, et al. Biodegradable Dextran Nanogels for RNA Interference: Focusing on Endosomal Escape and Intracellular siRNA Delivery. Adv Funct Mater. 2009;19:1406-15.
[23] Naeye B, Raemdonck K, Remaut K, Sproat B, Demeester J, De Smedt S. PEGylation of biodegradable dextran nanogels for siRNA delivery. Eur J Pharm Sci. 2010;40:342-51.
[24] White JG, Krumwiede MD, Escolar G. EDTA induced changes in platelet structure and function: influence on particle uptake. Platelets. 1999;10:327-37.
[25] Buyens K, Lucas B, Raemdonck K, Braeckmans K, Vercammen J, Hendrix J, et al. A fast and sensitive method for measuring the integrity of siRNA-carrier complexes in full human serum. J Control Release. 2008;126:67-76.
[26] Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T, Dawson K. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. Proc Natl Acad Sci U S A. 2008;105:14265-70.
[27] White JG. Platelets are covercytes, not phagocytes: Uptake of bacteria involves channels of the open canalicular system. Platelets. 2005;16:121-31.
[28] Werb Z, Cohn ZA. Plasma-membrane synthesis in macrophage following phagocytosis of polystyrene latex particles. Journal of Biological Chemistry. 1972;247:2439-&.

[29] Leijh PCJ, Vandenbarselaar MT, Vanzwet TL, Dubbeldemanrempt I, Vanfurth R. Kinetics of phagocytosis of Staphylococcus aureus and Escherichia coli by human granulocytes. Immunology. 1979;37:453-65.