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Colloidal stability of nano-sized particles in the peritoneal fluid: towards optimizing drug delivery systems for intraperitoneal therapy

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Abstract

Intraperitoneal (IP) administration of nano-sized delivery vehicles containing small interfering RNA (siRNA) is recently gaining attention as an alternative route for the efficient treatment of peritoneal carcinomatosis. The colloidal stability of nanomatter following IP administration has, however, not been thoroughly investigated yet. Here, enabled by advanced microscopy methods such as Single Particle Tracking (SPT) and Fluorescence Correlation Spectroscopy (FCS), we follow the aggregation and cargo release of nano-scaled systems directly in peritoneal fluids from healthy mice and ascites fluid from a patient diagnosed with peritoneal carcinomatosis. The colloidal stability in the peritoneal fluids was systematically studied in function of the charge (positive or negative) and Poly-Ethylene Glycol (PEG) degree of liposomes and polystyrene nanoparticles, and compared to human serum. Our data demonstrate strong aggregation of cationic and anionic nanoparticles in the peritoneal fluids, while only slight aggregation was observed for the PEGylated ones. PEGylated liposomes, however, lead to a fast and premature release of siRNA cargo in the peritoneal fluids. Based on our observations, we reflect on how to tailor improved delivery systems for IP therapy.

Keywords: Drug delivery, Aggregation, Peritoneal Carcinomatosis, Intraperitoneal administration, Release, siRNA

1. Introduction

Peritoneal metastases are one of the major causes of death in patients diagnosed with ovarian cancer [1]. Also in colorectal cancer, cancer cells often migrate to the abdomen where they spread and form peritoneal carcinomatosis [2]. The often late stage of discovery of peritoneal metastases, which can spread over the entire surface of the peritoneum ($\sim 2 \text{ m}^2$), make the treatment very difficult. This fact is well-demonstrated from clinical trials indicating the low median survival of patients diagnosed with peritoneal carcinomatosis [3].

Current treatment of peritoneal carcinomatosis involves removing the majority of peritoneal metastases (cytoreductive surgery) followed by intravenous (IV) administration of chemotherapeutic agents such as oxaliplatin in combination with 5-fluorouracil or leucovorin [4, 5] to kill remaining tumor cells. Also platinum-based (i.e. oxaliplatin, cisplatin) chemotherapeutics in combination with paclitaxel [6, 7] are used. Unfortunately, the majority of the patients develop disease recurrence [8, 9]. Therefore, more efficient post-surgical strategies to kill remaining tumor cells are needed [10]. In this context, intraperitoneal (IP) administration of chemotherapeutics has shown to be superior over the intravenous route [11, 12], particularly due to the ability to maintain high concentrations of cytotoxic agents in the peritoneal cavity [13]. Also, promising data have resulted from clinical trials evaluating hyperthermic intraperitoneal chemoperfusion (HIPEC) immediately after cytoreductive surgery [14, 15]. HIPEC involves flushing the peritoneal cavity with chemotherapeutic agents at an elevated temperature of 41-42°C. It is hypothesized that HIPEC is more efficacious compared to conventional intraperitoneal therapy since it not only takes advantage of the hyperthermic effect, but also enables distribution of the drug in all parts of the peritoneal cavity [16]. Nevertheless, the efficacy of HIPEC is still controversial as several studies claim that no synergistic effect exists between the anti-cancer agent and the hyperthermia [15, 17].

One strategy to improve the anticancer effect upon cytoreductive surgery is to use specialized drug delivery systems (DDSs) with the ability to reside in the peritoneal cavity for a prolonged period of time. Interestingly, recent *in vivo* data suggest that the intraperitoneal administration of DDSs that release chemotherapeutics results in an enhanced body distribution in general, and on the intratumoral level in particular [18]. Also the delivery of small interfering RNA (siRNA) for the treatment of ovarian cancer and peritoneal carcinomatosis has recently attracted considerable attention [19]. siRNAs are small (20-21 nucleotides) double stranded RNA molecules that can downregulate specific protein production.. siRNA has the benefit that it can target genes which are specific for tumor cells, leaving healthy, non-tumor tissue unaffected. Interestingly, carriers for combinatorial therapy of (specific) siRNA and conventional (non-specific) anti-cancer drugs (e.g. paclitaxel (PTX) or doxorubicin (DOX)) have been reported to result in some benefits compared to each one alone [20].

In the past few years, different DDSs were evaluated for IP administration [21, 22], Among them are targeted nanocarriers [23], nanoparticles for intraperitoneal gene delivery [24], micelles [25], microparticle [26, 27] and hydrogels for sustained release in the peritoneal cavity [28-30]. For nanosized drug carriers, the state of aggregation and the release profile following IP administration may play a crucial role in their delivery performance. Indeed, the colloidal stability of nanocarriers influences e.g. the internalization of the cargo into cancer cells, and thus may alter the expected anti-tumor efficacy. Following administration, nano-carriers tend to bind/interact with various components that are present in biofluids [31], including proteins and enzymes forming the so called ‘protein corona’ [32, 33]. For instance, recent reports suggest that the targeting capability of ligands conjugated to nanomaterials is lost by adsorption of a protein corona to their surface [34, 35]. Increasing our knowledge on the relation between the physicochemical

properties of delivery systems and their obtained therapeutic effect is crucial. Since the route of administration plays a major role in whether or not certain carriers will work, each carrier should be optimized for the *in vivo* situation where it is intended to be used, e.g. the intraperitoneal fluid in the case of IP delivery. Although several studies have addressed the colloidal stability of nanoparticles in biofluids like blood, plasma and serum [36, 37], the physicochemical behavior of delivery vehicles in terms of aggregation and release of cargo in peritoneal fluids has not been investigated yet.

The main objective of this study is to provide insight in the requirements for IP delivery systems in terms of charge and PEGylation degree, to be colloiddally stable and to have an optimal release profile in the peritoneal fluid. Herein, for the first time, we study the aggregation of polystyrene (PS) nanoparticles and liposomal formulations in peritoneal fluid from healthy mice (transsudate) and ascites fluid (exudate) from a patient diagnosed with peritoneal carcinomatosis. Additionally, we study the release profile of liposomal formulations carrying siRNA in the peritoneal fluids. For this purpose, we utilize state of the art fluorescence techniques that were previously developed in our laboratory, namely Single Particle Tracking (SPT) and Fluorescence Correlation Spectroscopy (FCS) to respectively gain information on the aggregation of nanoparticles and the release of siRNA in undiluted biofluids [36, 38]. The results are compared to measurements of the same nanoparticles dispersed in human serum.

2. Materials and Methods

2.1 Materials

(2,3-Dioleoyloxy-propyl)-trimethylammonium-chloride (DOTAP) and 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Corden Pharma LLC (Liestal, Switzerland).

1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Chloroform, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), N-hydroxysulfosuccinimide (Sulfo-NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), Dimethylethylenediamine (DMEDA) and sodium chloride (NaCl) were purchased from Sigma Aldrich (Bornem, Belgium). Yellow-green fluorescent ($\lambda_{\text{ex}}= 505 \text{ nm}$, $\lambda_{\text{em}}= 515 \text{ nm}$) carboxylated PS FluoSpheres (0.1 μm in size) and 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate (DID) ($\lambda_{\text{ex}}= 644 \text{ nm}$, $\lambda_{\text{em}}= 665 \text{ nm}$) were purchased from Invitrogen (Merelbeke, Belgium). Methoxy-polyethylene glycol-amine (mPEGa) 2 kDa was purchased from Creative PEGWorks (Winston Salem, USA). Alexa Fluor-488 Negative Control siRNA ((Eurogentec, Seraing, Belgium).

2.2 Animals

Mice, heterozygous for Foxn1 (nu/+) were purchased from Charles River (Sulzfeld, Germany) and maintained by the animal core facility. Animals were kept at 22 °C in a humidified atmosphere with food and water ad libidum.

2.3. Collection of biofluids

To collect samples containing mouse intraperitoneal fluid, a lavage of the peritoneal cavity was performed. To this end, mice were euthanized by an overdose of the inhalation anesthetic isoflurane followed by cervical dislocation. The abdominal wall was opened immediately and the peritoneal cavity was washed with 1 mL of water. The lavage was taken and stored frozen until use. The procedure was approved and carried out in compliance with the guidelines for animal experiments of Leipzig University.

Human serum was obtained from a healthy donor. Briefly, blood was collected at the Ghent University Hospital into Venosafe™ 6 mL tubes containing gel and clotting activator (Terumo Europe™, Leuven, Belgium). Then the tubes were centrifuged for 10 minutes at $4,000 \times g$ and 20°C . The supernatant (serum) was transferred into microvette® 500 Z-Gel (SARSTEDT, Numbrecht, Germany) and centrifuged for 5 minutes at $1,0000 \times g$ and 20°C . The serum was portioned into 50 μl aliquots (to avoid freezing-thawing cycles) in sterile polypropylene tubes and stored in -20°C until use. Human ascites fluid was obtained from a patient diagnosed with peritoneal carcinomatosis at the medical oncology department, Ghent University hospital. The experiments with the ascites fluid were approved by the ethics committee of the Ghent University hospital (# 2013/589).

2.4 Protein analysis and capillary electrophoresis

Total protein in human serum, human ascites fluid, and mice peritoneal fluid was assayed using a pyrogallol red-molybdate method on a Cobas 8000 analyzer (Roche, Mannheim, Germany) [39].

Human serum and human ascites fluid protein electrophoresis was performed using a Capillarys 2™ CE system (Sebia, Paris, France) that is routinely employed in clinical laboratories [40, 41]. Prior to the hydrodynamic injection (4''), 40 µl of serum is automatically diluted 5x in the running buffer (pH 10). Then, 7 kV is applied in the 8 silica-fused capillaries (effective length 15.5 cm; internal diameter 25 µm; optical cell 100 µm) for 4' at 35.5°C (Peltier device). Proteins are detected at the cathode (deuterium lamp; 200 nm) as 5 fractions (γ-globulins, β-globulins, α2-globulins, α1-globulins and albumin) that are automatically quantified as percentages of the total signal.

For mice peritoneal fluid (characterized by low protein concentrations), agarose gel electrophoresis was carried out, followed by a sensitive staining using the Protur HiSi 100 system (Analis, Suarlée, Belgium).

2.5 Viscosity measurements

Viscosity measurements of human ascites fluid and human serum were performed using a micro-Ubbelohde viscosimeter (53610/I) (Schott-Geräte (Mainz, Germany)) at 22°C. 4 mL of each bio fluid were loaded on a capillary (ID number 100-002 with capillary constant $K = 0.009671 \text{ mm}^2/\text{s}^2$) and the flow time (in seconds) was measured 3 times for each biofluid. The averaged flow time was used to calculate the kinematic viscosity according to the following equation:

$$\nu = K \times (t - y)$$

where t is the averaged flow time, and y (in seconds) is the kinetic energy correction for the flow time provided by the manufacturer. For human serum, the calculated kinematic viscosity is $1.6 \text{ mm}^2/\text{s}$ and for the human ascites fluid is $1.39 \text{ mm}^2/\text{s}$. The dynamic viscosity η is defined using the following relation:

$$\eta = \rho \times v$$

Where η is the density of fluid (g/mL) and $1 \text{ cSt} = 1 \text{ mm}^2/\text{s}$. Since the density of human serum and human ascites fluid is very close to 1 g/mL [42], we assume all the densities of fluids used in this study are equal to 1, and thus in all Single Particle Tracking measurements the following values of dynamic viscosity were used: 1.39 cP for human ascites fluid, 1.6 cP for human serum, and 0.94 cP for mice IP fluid and HEPES buffer.

2.6 Functionalization of anionic polystyrene nanoparticles

Functionalization of 100 nm anionic nanoparticles to PEGylated and positively charged ones was performed according to previously published procedures [43]. The charge and size of the nanoparticles was measured using the Zetasizer Nano-ZS (Malvern, Worcestershire, UK). The average size of all the nanoparticles in HEPES buffer (pH 7.4) was around 110 nm , and zeta-potential around 30 mV for the positively charged nanoparticles, -13 mV for the PEGylated nanoparticles, and around -33 mV for the anionic nanoparticles.

2.7 Preparation of liposomes

DOTAP and DOPE lipids were dissolved in chloroform and mixed in a round bottomed flask. A lipid film was formed by rotary evaporation of the chloroform at 40°C . The dried lipid film was rehydrated with 20 mM HEPES buffer pH 7.4, resulting in a final concentration of 5 mM DOTAP and 5 mM DOPE. Thereafter, liposomes were sonicated using a probe sonicator (Branson Ultrasonics Digital Sonifier[®], Danbury, USA). For the preparation of PEGylated liposomes, the desired amounts of DSPE-PEG dissolved in chloroform (corresponding to $5 \text{ mol}\%$ or $10 \text{ mol}\%$ of the total lipids) were added to the lipids in the round bottomed flask before evaporation. For SPT experiments, liposomes were fluorescently labeled by incorporation of $1 \text{ mol}\%$ (of the total lipids)

with DID. The average size and zeta potential of the liposomes was measured using Zetasizer Nano-ZS (Malvern, Worcestershire, UK). The average diameter of the liposomes was around 90 nm for the cationic liposomes and 100 nm for the PEGylated ones. The zeta potential around + 45 mV for the cationic liposomes, 15 mV for the 5 mol% PEGylated liposomes and 7 mV for the 10 mol% liposomes.

2.8 Size and zeta-potential measurements

The average size and the zeta potential of the liposomes and the polystyrene nanoparticles were measured using the Nano-ZS Zetasizer (Malvern, Worcestershire, UK) in 4 different biofluids: HEPES buffer, mice IP fluid, human ascites fluid, and human serum. Equal volumes of respectively cationic, 5% PEGylated and 10% PEGylated liposomes and biofluids were mixed and incubated for 1 hour at 37°C. At the end of the incubation period, these mixtures were diluted with 20 mM HEPES buffer to a final concentration of 125 μ M DOTAP (\sim 2.5 vol% of biofluids). The size and zetapotential measurements were performed at 25°C. The size and the zeta potential of the PS nanoparticles were determined following the same procedure.

2.9 Fluorescence single particle tracking (SPT)

Single particle tracking (SPT) is a fluorescence microscopy technique that uses widefield laser illumination and a fast and sensitive CCD camera to record high speed movies of individual diffusing particles in biofluids. Thereafter, the movies are analyzed by a specific image processing algorithm to obtain the motion trajectories for all individual particles. The trajectories are then used to calculate the diffusion coefficient of each particle. After analyzing many particles, a distribution of diffusion coefficients is obtained which is transformed into size distribution using the Stokes-Einstein equation and refined by the maximal entropy method (MEM), as previously

described [36]. The conversion of diffusion coefficients to sizes requires knowledge of the viscosity of the biofluid and the temperature at which the experiment is performed.

SPT measurements on different PS nanoparticles (Anionic, PEGylated, Cationic) and DID labeled liposomes dispersed in biofluids were performed as follows. First, formulations were diluted 400 times in HEPES buffer. Then 5 μl was added to 45 μl of biofluid (e.g. 90 vol% of mice IP fluid, human ascites fluid and human serum), and incubated for 1 hr at 37°C in a 96-well plate (Greiner bio-one, Frickenhausen, Germany). At the end of the incubation time, the sample was placed on the custom-built SPT set-up [36] and movies were recorded focused at about 5 μm above the bottom of the glassbottom 96-well plate. Videos were recorded at room temperature (22.5°C) with the NIS Elements software (Nikon) driving the EMCCD camera (Cascade II:512, Roper Scientific, AZ, USA) and a TE2000 inverted microscope equipped with a 100 \times NA1.4 oil immersion lens (Nikon). Analysis of the videos was performed using in-house developed software. During the incubation and measurements, the well plate was covered with Adhesive Plates Seals (Thermo Scientific, UK) to avoid evaporation of the sample and allow diffusion only.

2.10 FCS on siRNA containing liposomes (lipoplexes)

FCS is a microscopy-based technique that monitors the fluorescence intensity fluctuations of molecules diffusing in and out of the focal volume of a confocal microscope. Shortly, when free siRNA molecules are present in the focal volume, a fluorescence signal (baseline) is obtained which is proportional to the local siRNA concentration. When the siRNA is complexed with nanoparticles, the concentration of free siRNA (e.g. the baseline) drops and peaks of high

fluorescence intensity appear each time a nanocomplex containing many fluorescent siRNA molecules passes the detection volume. Vice versa, when siRNA dissociates from the complexes, the concentration of free siRNA increases again, resulting in an increase of the baseline. The drop/increase in the intensity of the baseline can be used to calculate the percentage of the complexed/released siRNA as was previously described by Buyens et al. [38]. Single color FCS measurements were performed on lipoplexes containing Alexa-488 siRNA Negative Control siRNA ((Eurogentec, Seraing, Belgium). To follow the release of the complexed siRNA in function of time, lipoplexes with +/- charge ratio of 8 were prepared by adding appropriate amounts DOTAP DOPE liposomes to siRNA. The mixture was then incubated at room temperature for 30 minutes to allow formation of the lipoplexes. The size of the lipoplexes was measured using dynamic light scattering and was about 100 nm for cationic ones and 110 nm for the PEGylated. For FCS measurements, 5 μ l of the lipoplexes were diluted in 45 μ l of each biofluid (resulting in 90 vol% of mice IP fluid, human ascites fluid and human serum in the final samples) and the fluorescent signal was measured respectively immediately after mixing the lipoplexes with the biofluids and after one hour of incubation at 37°C. During the incubation and the FCS measurements, the well plate was covered with Adhesive Plates Seals (Thermo Scientific, UK) to avoid evaporation of the sample and to minimize flow.

FCS measurements were performed on C1si laser scanning confocal microscope (Nikon, Japan), equipped with a Time-Correlated Single Photon Counting (TCSPC) Data Acquisition module (Picoquant, Berlin, Germany). The laser beam was held stationary and was focused through a water immersion objective lens (Plan Apo 60 \times , NA 1.2, collar rim correction, Nikon, Japan), at about 50 μ m above the bottom of the glassbottom 96-well plate (Grainer Bio-one, Frickenhausen, Germany), which contained the fluorescent samples (free Alexa488-siRNA and Alexa488-siRNA

complexed to non-PEGylated, 5% PEGylated or 10% PEGylated liposomes (5 nM Alexa488-siRNA in all samples)). The 488 nm laser beam of a krypton–argon laser (Bio-Rad, Cheshire, UK) was used and the green fluorescence intensity fluctuations were recorded using Symphotime (Picoquant, Berlin, Germany) during at least 60 seconds.

3. Results

3.1 Protein content of the biofluids

The data in table 1 depicts the total protein content in each biofluid. The highest protein content was determined in the human serum samples obtained from a healthy donor. Ascites fluid from the peritoneal cavity of a patient diagnosed with peritoneal carcinomatosis contained almost half the amount of proteins when compared to human serum. Peritoneal fluid extracted from mice was found to contain a rather low protein concentration. This most likely can be attributed to the collection procedure in which the peritoneal fluid of mouse is diluted between 10-50 times at least, unlike the human serum and ascites fluid, where the collection procedure did not involve any dilution. With regard to the type of proteins found in each sample, capillary electrophoresis of the human serum and ascites fluid reveals a very similar composition, with a major fraction of albumin (Fig. 1A, Fig. 1B). Also mouse peritoneal fluid contains a major albumin fraction (68 KDa) and a prominent transferrin fraction (80 KDa) (Fig. 1C).

It should be noted that in the case of peritoneal carcinomatosis, the high protein content observed in the ascites fluid is attributed to the increased permeability of the peritoneal membrane induced mainly by vascular endothelial growth factor (VEGF) [44]. In patients with an earlier stage of peritoneal carcinomatosis, the total amount of proteins present in the peritoneal fluid is expected to be less. Nevertheless, a relative protein composition, similar as in figure 1B is expected.

3.2 Colloidal stability of PS nanoparticles and liposomes in diluted peritoneal fluids and serum

The data in Fig. 2. demonstrate the size and the zeta-potential of PS nanoparticles measured by DLS following 1 hr of incubation at 37°C in each of the studied biofluids. Samples were incubated in 50 vol% of biofluids and further diluted to 2.5% biofluids for the actual measurements. As illustrated in Fig. 2A. (note the broken axis), cationic nanoparticles (white bars) show pronounced aggregation in biofluids with a low protein concentration and less aggregation in biofluids with a high protein content. This aggregation is accompanied by a significant decrease in the zeta potential: the positively charged nanoparticles (+ 28 mV) in HEPES buffer turn negative upon dispersing them in the biofluids (Fig. 2B.). Interestingly, an “opposite” aggregation pattern was observed with the anionic nanoparticles (dark grey bars), whose state of aggregation seemed to be correlated with the protein content of the biofluids. Yet, when compared to cationic polystyrene nanoparticles, this aggregation was less pronounced. Notably, the zeta potential of the anionic nanoparticles increases from -33 mV in HEPES buffer to less negative nanoparticles in the rest of the biofluids. The size of the PEGylated nanoparticles (Fig. 2A. grey bars) did not change upon incubation in the biofluids, indicating that the PEG-chains effectively inhibit aggregation.

As liposomes are widely used in drug delivery and hold potential for siRNA delivery in the peritoneal cavity, it is of interest to have an insight on the aggregation profile of different liposomal formulations in IP fluid. Therefore, we investigated the colloidal stability upon incubating non-PEGylated, 5% PEGylated and 10% PEGylated liposomes in the biofluids. The data in Fig. 3. show the hydrodynamic diameter and the zeta potential of cationic and PEGylated liposomal formulations following 1 hr of incubation in the biofluids. It can be seen that the extent of

aggregation (Fig 3A, broken axis) is more severe in human serum > ascites fluid > mice IP fluid which correlates with the protein content of the biofluids (table 1). In particular, the aggregation is the most pronounced for the cationic liposomes (white bars), while PEGylation significantly diminishes aggregation of cationic liposomes, indicating that the PEG chains improve the colloidal stability of the cationic liposomes. As can be seen from Fig. 3B, a drop in the zeta potential was observed for all the liposomal formulations upon dispersing them in the biofluids (especially in human serum and ascites fluid).

3.3 Aggregation of PS nanoparticles and liposomes in undiluted biofluids

Dynamic light scattering (DLS) is the most common technique to measure the average size of nanoparticles in aqueous media. However, measuring the size of nanoparticles in biofluids by DLS is challenging as proteins in the biofluids can scatter the light and interfere with the measurements. As an example size distributions of the biological fluids only diluted in HEPES buffer are shown in Supplemental Fig. 1 A and Fig. 1 B. Therefore, the DLS measurements in the previous sections were performed on highly diluted samples (only 2.5 vol% of biofluids in Fig. 2 and Fig. 3).

We have previously shown that SPT is a powerful technique to measure the size of nanoparticles in undiluted biofluids such as serum and blood [36, 45, 46]. Here, we present for the first time the aggregation behavior of nanoparticles in undiluted intraperitoneal fluids, and compared the aggregation profile with the one obtained in buffer and human serum. A particular benefit of SPT is that size measurements are performed on a per particle basis so that, contrary to DLS, there is no bias towards larger sizes.

The size distributions of the PS nanoparticles, as obtained by SPT in undiluted biofluids, are depicted in Fig 4. In line with the DLS data (Fig 2A), on average 100 nm size particles are

observed in HEPES buffer. In the biological fluids, particles sizes increase to about 200 nm in human serum, 300 nm in ascites fluid and even μm sized aggregates in mice IP fluid, again confirming the DLS results. For the anionic nanoparticles (Fig 4B), a different aggregation pattern was observed: only a slight increase in size was noticed for the ascetic and mice IP fluid, while a broadened distribution was observed in human serum. In the case of PEGylated nanoparticles (Fig. 4C.), the particles remained stable in mice and ascites IP fluid. Also, human serum resulted only in a very minor increase in size compared to the buffer sample. These findings are all consistent with the trends observed by DLS (Fig. 2A).

The aggregation behavior of the liposomes dispersed in the undiluted biofluids is shown in Fig. 5 and Fig. 6. For the cationic liposomes, large non-diffusing aggregates (up to 2-3 μm) in size were observed at the bottom of the well following 1 hr of incubation in mice IP fluid, ascites fluid and human serum (Fig. 5). As these aggregates did not show Brownian motion, SPT data could not be obtained. The size distributions of the 5% PEGylated liposomes (Fig. 6A) confirm the outcome of the DLS data in Fig. 3A, and show the profound aggregation of these liposomes in human serum. In the mice IP fluid and ascetic fluid, this aggregation is less prominent. Finally, the 10% PEGylated liposomes hardly aggregate in the peritoneal fluids. In human serum, at least a part of the liposomes did not aggregate as can be seen from the bimodal behavior. Note that the size distribution for the 10% PEGylated liposomes in HEPES buffer seems broader than the one for the 5% PEGylated liposomes. Possibly, this is due to the structure the liposomes adopt with higher degrees of PEGylation, including small disks and large aggregates which contribute to the polydispersity of the sample [47].

3.4 Release of siRNA from liposomes in undiluted biofluids

siRNA has the potential to treat peritoneal metastasis by preventing the growth and spread of circulating tumor cells. For siRNA to be biologically active, it needs to reach the cytoplasm of the tumor cells. Therefore, it is generally ‘complexed’ with cationic carriers such as polymers or liposomes, as ‘naked’ siRNA is not taken up by cells. In the next set of experiments, we aimed to determine the stability of siRNA-liposome complexes in peritoneal fluids, with respect to siRNA release from the formulations. When siRNA is prematurely released from the complexes in the biofluids, the biological activity will be lost. The percentage of free siRNA in HEPES buffer at the zero hour time point (Fig 7A, grey bars), shows the amount of siRNA that remained free (e.g. uncomplexed) when the siRNA/liposome complexes were formed. About 2%, 6% and 15% of siRNA is not encapsulated in respectively the cationic, 5% PEGylated and 10% PEGylated liposomes. This indicates that a higher PEGylation degree lowers the siRNA encapsulation efficiency. Following 1 hr of incubation, the effect of PEGylation becomes even more pronounced: only the non-pegylated liposomes retain the complexed siRNA. For the 5% and 10% PEGylated liposomes, respectively 30% and even 85% of siRNA is released into HEPES buffer. Next, the siRNA containing complexes were incubated with the undiluted biofluids. When compared to HEPES buffer (Fig 7A, grey bars), we observe an immediate release of siRNA at the zero time point upon dispersing the complexes in the biofluids (Fig 7, B-D, grey bars). This immediate release most likely corresponds to the release of surface-bound siRNA and increases with PEGylation degree. Further incubating the complexes in the biofluids for 1 hour did not result in a substantial additional release of siRNA (Fig 7B-D, white bars), except for the 5% PEGylated complexes in human serum (Fig 7D, white bars). Overall, the siRNA release was limited to maximally 30% for the cationic liposomes, while for the 5% and 10% PEGylated liposomes,

between 65-80% of siRNA was released into the peritoneal fluids and even close to 100% siRNA was released in human serum after 1 hour.

4. Discussion

Designing new delivery systems for targeting peritoneal cancer cells is a major challenge. Apart from the IV route of administration, intraperitoneal (IP) delivery of nanoparticles that target cancer cells over a prolonged period of time is being explored. Upon IP delivery, nanoparticles are directly administered at the target site. Hence, interactions of the nanoparticles with blood components that potentially induce immune responses or stability issues could be avoided [48]. The stability of nanoparticles in the IP fluid is a major determinant for their efficacy. Indeed, both particle aggregation or premature release of cargo in the IP fluid could diminish the biological effect. The colloidal stability of nanoparticles in IP fluids has, however, not been studied in detail before. In this study, we employed advanced microscopy techniques to directly assess stability of nanoparticles in terms of aggregation and cargo release, in undiluted biofluids such as mice IP fluid and ascites fluid from a patient diagnosed with peritoneal carcinomatosis and compared it to human serum.

4.1 Aggregation of model PS nanoparticles and liposomes in peritoneal fluids

Aggregation of different PS nanoparticles and liposomal formulations in the peritoneal fluids was tested using DLS and SPT. Two main questions were addressed, namely: (1) does the physicochemical properties of the material (e.g. charge and PEGylation degree) influence aggregation and (2) does the concentration of the proteins in each of the biofluids correlate with the aggregation profiles?

PS nanoparticles were used in this study as an inert hydrophobic model system. Liposomes, on the other hand, are frequently used to deliver therapeutic agents to target cells. A summary of their aggregation profiles can be found in Table 2. It was already demonstrated before that serum induces quick aggregation of positively charged nanoparticles [36]. This was confirmed in our study, especially for the positively charged liposomes. Also in mice and human IP fluid, severe aggregation of positively charged particles was observed. As seen in Fig 1., albumin is the major protein fraction in both mice and human IP fluid, as well as in human serum (~60%). Under physiological conditions (pH 7-7.4) albumin and other negatively charged proteins are capable of binding to cationic nanoparticles, inducing the formation of micrometer sized protein-nanoparticles complexes [31]. It is thus most likely that albumin is the most abundant component in the protein corona around the positively charged nanoparticles, as was observed before [49]. The formation of protein-nanoparticle complexes also explains the drop observed in the zeta potential in Fig. 2A. and Fig. 2B. It should be noted that negatively charged nanoparticles tend to bind proteins with an isoelectric point greater than 5.5, such as IgG [50]. This explains why the zeta potential of the negatively charged polystyrene beads becomes less negative upon incubation with the biofluids.

The adsorption of proteins to the nanoparticles described above is drastically diminished when the surface of the PS nanoparticles and liposomes is decorated with PEG (table 2). Despite the role of PEG in avoiding aggregation, the data presented in Fig. 6B indicate that there is a limit to which extent the protein adsorption could be prevented. This finding is significant, suggesting that 10% PEGylated liposomes are very stable in the peritoneal fluids, but not in human serum where aggregation still takes place due to the high density of proteins bound on the surface of the PEG residues (Table 1). In general, it seems that PEGylation is necessary to avoid the formation

of aggregation in peritoneal fluids. Both positively and negatively charged non-PEGylated nanoparticles tend to form large aggregates upon incubation with peritoneal fluids or human serum.

Among all the studied formulations the aggregation tendency seemed proportional to the protein concentration of the incubation fluid, except for positively charged PS NPs, which show the greatest aggregation in mice IP fluid with lowest protein content. The exact reason for this is unclear. It should be noted, however, that the positively charged PS NPs become negatively charged upon incubation with the biofluids. The negatively charged PS NPs, on the other hand, become slightly less negative. Therefore, the actual proteins that bind to the NPs are expected to be different for cationic or anionic NPs, respectively, which might influence their aggregation behavior. Another observation is that the liposomes tend to aggregate more than PS NP in all biofluids. This most probably stems from the intrinsic properties of the building blocks. In particular, when collisions between two liposomes occur (especially non-PEGylated ones), hydrophobic interactions between lipids from both liposomes can take place, supporting the formation of aggregates. On the contrary, PS NP are 'inert' plastic particles that could 'bounce' upon collision and shown no additional attracting forces that would support the formation of aggregates.

4.2 Release of siRNA from liposomes in the peritoneal fluids

Apart from colloidal stability in terms of aggregation, the nanoparticles should be able to bring their cargo to the target site. The release of siRNA from liposomal formulations in the peritoneal fluids was evaluated using Fluorescence Correlation Spectroscopy (FCS). As we previously demonstrated, FCS is an elegant technique to follow the complexation behavior of small nucleic

acids to nanoparticles, both in buffer and in living cells [38, 51]. The data in Fig 7. clearly suggest better complexation and slower release of siRNA for the non-PEGylated lipoplexes over the PEGylated ones. The higher the PEGylation degree, the more rapid the siRNA molecules dissociate from the complexes, even in buffer conditions. This most likely stems from the proposed mechanism for lipoplex formation [52-54]. When cationic liposomes are added to negatively charged siRNA, strong electrostatic interactions occur, and the majority of the siRNA molecules attach to the surface of the liposomes. These siRNA-coated liposomes can subsequently fuse with other liposomes, so that siRNA is entrapped within the bilayer of the liposomes. In the case of PEGylated lipoplexes, the PEG chains prevent the fusion of different liposomes, resulting in lipoplexes in which siRNA is only bound to the outer surface and not “sandwiched” in between the multilayers of the liposomes. Also, the lower surface charge leads to less complexation, resulting in an increasing amount of free siRNA at zero time with higher degree of PEGylation as seen in Fig 7.

The release is also dependent on the composition of the biofluid in which the lipoplexes were incubated afterwards. For the PEGylated lipoplexes, the excess of albumin and other negatively charged proteins triggers the release of the surface bound siRNA from the complexes by competing for binding to the cationic lipids. In human serum, the release percentage of the siRNA is the highest when compared to other biofluids, as these samples contain the highest protein concentration. Interestingly, only small differences occur in the percentages of release of siRNA from non-PEGylated lipoplexes in between zero time and 1 hr of incubation in the different biofluids. This shows that negatively charged proteins in serum and IP fluids only compete with siRNA bound to the surface of the lipoplexes, leaving siRNA sandwiched in between the lipid bilayers unaffected.

4.3 Tailoring delivery systems for IP therapy

Nano-sized delivery vehicles for IP administration for the treatment of ovarian cancer and peritoneal carcinomatosis should meet several efficacy and safety requirements: (1) long retention time in the peritoneal cavity to ensure maximal therapeutic efficiency, (2) limited leakage into the systemic circulation to avoid toxic side effects, (3) a specific targeting of tumor cells and (4) limited immune and inflammation responses. All these are still major challenges in IP delivery systems [22].

Fig. 8. schematically represents the different steps and hurdles for nanoparticles administered to the IP cavity. In the case of siRNA, internalization of the carrier into the cells is needed, before knock down of proteins responsible for the proliferation of cancer cells can occur. The data presented in this study undoubtedly propose rapid aggregation of positively and negatively charged nanoparticles in the peritoneal fluids. Large size aggregates, however, are not efficiently taken up by cells anymore. Therefore, the siRNA activity of these aggregates will be lost (Fig. 8A., step 1). Also, premature release of siRNA from the carrier in the peritoneal fluid is not desired (Fig. 8A., step 2) as free siRNA is not able to penetrate into the cytosol of the cancer cells. While reduced aggregation is achieved by PEGylation of nanoparticles, the PEGylated liposomes suffered from a fast release of the complexed siRNA upon exposure to the IP fluids. Also, PEGylation has been associated with low transfection efficiency due to poor uptake and/or interaction with the endosomal membrane. Nevertheless, different PEGylation strategies can be exploited to prevent aggregation, while keeping the transfection efficiency [55]. An interesting strategy is the use of sheddable PEG-chains that protect the nanoparticles from aggregation in the extracellular environment, but dissociate once the nanoparticles enter certain intracellular compartments. Also, altering the formation procedure of liposomes is an option. By hydrating the

lipid film with a solution of siRNA at least part of the loaded siRNA is entrapped inside the liposomal core, even when PEGylated liposomes are used [56]. Also post PEGylation of preformed siRNA/liposome formulations (in which all siRNA is entrapped between lipid bilayers) is feasible. Apart from aggregation and premature cargo release, the clearance of nanoparticles from the peritoneal cavity is an important parameter. Ideally, nanoparticles should reside in the IP cavity as long as possible, without leakage into the systemic circulation (Fig. 8B., step 4). It has been suggested, however, that nanoparticles are cleared from the peritoneal cavity within 2 days [57]. Therefore, for developing RNAi-based therapy for the treatment of peritoneal cancer (which preferentially makes use of nano-sized particles for optimized cell internalization), a future strategy could be to load nanoparticles into a sustained release system. Taking into account the clearance rate of nanoparticles from the IP cavity, such a controlled release system can be tuned so that a constant amount of nanoparticles is present in the IP cavity. It should be noted that a good retention time in the peritoneal cavity was reported for 100 nm positively charged liposomes by Dadashzadeh et al. [58]. The size of the liposomes upon IP administration was however not continuously monitored. In our opinion, this slower clearance rate can be attributed to the aggregation of these nanoparticles to micrometer sized particles in the IP fluid. As has been suggested, these microparticles do indeed show a slower clearance rate from the IP cavity when compared to nanoparticles [59].

Unlike for siRNA, conventional anti-cancer agents such as paclitaxel and doxorubicin (DOX) do not need a carrier system to be taken up in cells. Therefore, the aggregation of nanoparticles or premature cargo release might not be a major problem for these type of formulations (Fig. 8B). Aggregation of nanoparticles would however lead to large size aggregates

which could lead to increasing difficulty of drug dissolution in the IP fluid (Fig 8B, step 1). Also, Kohane et al. concluded that microparticles do not appear to be good candidates for IP drug delivery due to the risk of adhesions [57]. Also, a disadvantage of the conventional cytostatics, is the non-specificity of the drugs which also affect healthy-non tumor cells and the fact that tumor cells can develop resistance after long-term treatment and exposure.

It should be noted that the environment of the peritoneal cavity seems to be less aggressive than the one of the systemic circulation: while 5% PEGylated liposomes remained stable in IP fluid, aggregation was still observed in the human serum. Therefore, one could argue that less colloidal stable nanoparticles could still be used for IP administration. We have the opinion, however, that IP administered nanoparticles should also be stable enough in the systemic circulation, since clearance of these particles from the peritoneal cavity to the systemic circulation is most likely inevitable (Fig, 8, step 4). When a colloidal stable nanoparticle, once it leaves the IP cavity, starts aggregating in the blood circulation, there is a risk of clogging blood capillaries, which should obviously be avoided.

Finally, it is important to stress out that the outcomes from the stability testing *in vitro* in biofluids with the techniques used in this study (FCS and SPT) represent a good prediction of the stability for the *in vivo* situation. Therefore, formulations that are not stable enough *in vitro* should not be considered for *in vivo* applications. Rather, further *in vitro* optimization should take place to enhance the stability of nanoparticles and to ensure that only stable formulations are used for further animal studies. It should be noted, however, that the *in vivo* situation is expected to be more complex than the *in vitro* one in biological fluids. Therefore, particles that showed good stability in the biofluids should always be further tested *in vivo* to determine their biological activity.

5. Conclusions

There is increasing interest from clinicians in treating carcinomatosis patients with some form of IP therapy. Unfortunately, none of the currently used drugs in this setting have been specifically designed or tested for IP application. In this study, for the first time, we investigate the aggregation and release of cargo from nanoparticles in peritoneal fluids. Our data indicate fast aggregation of positively and negatively charged nanoparticles in the peritoneal fluids, which can be prevented by decorating the surface with PEG. Conventional complexation of nucleic acids with our PEGylated liposomes results however in a rapid release of the nucleic acids in the peritoneal fluids, which is not preferred.

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Appendix A. Supplementary data:

Size distributions of biological fluids diluted in HEPES buffer.

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