Chitosan nanoparticles for siRNA delivery: Optimizing formulation to increase stability and efficiency


Université Catholique de Louvain, Pharmaceutics and Drug Delivery Group, Louvain Drug Research Institute, 1200 Brussels, Belgium
University of Liege, Center for Education and Research on Macromolecules, 4000 Liège, Belgium
Ghent University, Laboratory of General Biochemistry and Physical Pharmacy, Faculty of Pharmaceutical Sciences, 9000 Ghent, Belgium
Université Catholique de Louvain, Institute of Condensed Matter and Nanosciences, 1348 Louvain-la-Neuve, Belgium
University of Liège, Center for Education and Research on Macromolecules, 4000 Liège, Belgium
Université Catholique de Louvain, Institute of Condensed Matter and Nanosciences, Bio & Soft Matter, 1348 Louvain-la-Neuve, Belgium

Corresponding author.
⁎ Corresponding author.
E-mail address: veronique.preat@uclouvain.be (V. Préat).

Article info
Article history:
Received 11 October 2013
Accepted 21 December 2013
Available online 21 December 2013

Keywords:
siRNA delivery
Chitosan
Nanoparticles
Stability in plasma
Gene silencing

Abstract
This study aims at developing chitosan-based nanoparticles suitable for an intravenous administration of small interfering RNA (siRNA) able to achieve (i) high gene silencing without cytotoxicity and (ii) stability in biological media including blood. Therefore, the influence of chitosan/tripolyphosphate ratio, chitosan physicochemical properties, PEGylation of chitosan as well as the addition of an endosomal disrupting agent and a negatively charged polymer was assessed. The gene silencing activity and cytotoxicity were evaluated on B16 melanoma cells expressing luciferase. We monitored the integrity and the size behavior of siRNA nanoparticles in human plasma using fluorescence fluctuation spectroscopy and single particle tracking respectively. The presence of PEGylated chitosan and poly(ethylene imine) was essential for high levels of gene silencing in vitro. Chitosan nanoparticles immediately released siRNA in plasma while the inclusion of hyaluronic acid and high amount of poly(ethylene glycol) in the formulation improved the stability of the particles. The developed formulations of PEGylated chitosan-based nanoparticles that achieve high gene silencing in vitro, low cytotoxicity and high stability in plasma could be promising for intravenous delivery of siRNA.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Over the past few decades, RNA interference (RNAi) has emerged as a promising strategy for the treatment of a wide range of diseases by silencing a gene and therefore by hindering the corresponding protein expression [1]. This mechanism constitutes a highly precise tool due to sequence specificity of the siRNA and the use of this nucleic acid in cancer therapy has promising potential as several oncologic targets have already been identified [2]. Clinical trials are currently being conducted on patients with solid tumors [3,4].

Since the instability and the lack of efficiency of naked siRNA have been demonstrated, many types of nanovectors have been investigated and some of them showed very good activity in vitro [5,6]. Nevertheless, the fact that a vector is efficient in vitro does not necessarily mean that it will be effective in vivo. Indeed, siRNA carriers are confronted with several barriers from their administration to their target site, i.e. the cell cytoplasm where the RNAi mechanism is located. If we consider the intravenous route which is one of the more direct ways to reach drug target (tumor tissues, endothelial tumor blood vessels), an ideal vector should gather the following characteristics: (i) its size must be below 200 nm, (ii) all the components have to be biocompatible, (iii) it must be stable enough in blood to protect the nucleic acid, (iv) opsonization and uptake by macrophages have to be avoided, and (v) after reaching the target site, endocytosis should occur in the cell but the nucleic acid must be able to further escape the endosome and be released in the cell cytoplasm [7]. All of these barriers need to be taken into account to develop powerful siRNA delivery systems.

Chitosan is one of the most commonly used polymers in non-viral gene therapy. Its positive charges under slightly acidic conditions allow its interaction with the siRNA and the formation of complexes or nanoparticles [8–13]. In addition, chitosan is biocompatible and biodegradable making it enable for in vivo use through intraperitoneal [14,15] or intravenous administration [16,17]. This polymer provides a good basis for nanoparticle construction but needs further developments in order to mediate high gene silencing, in particular after intravenous injection. One of the major limitations of this polymer is its low water solubility at physiological pH because of the partial protonation of the amino groups. This can influence nucleic acid binding capability [18] and therefore can affect the stability of the nanoparticles in the blood stream and thus their biological activity. To increase solubility and confer stability to the nanoparticles, poly(ethylene glycol) (PEG) grafting onto chitosan has been described [19–21].
The gene silencing efficiency is also dependent on the ability of the nanocarrier to escape the endosome and release siRNA in the cell cytoplasm. The endosomal rupture occurs in part thanks to the so-called proton sponge effect: the acidic plasm. The endosomal rupture occurs in part thanks to the so-called nanocarrier to escape the endosome and release siRNA in the cell cytoplasm.

2. Material and methods

2.1. Material

Several types of chitosan provided by Sigma-Aldrich (Belgium) and Kitozyme (Belgium) were tested. Their characteristics are summarized in Table 1. Branched poly(ethylene imine) (PEI, 25 kDa), sodium tripolyphosphate (TPP) and poly(L-arginine) (PLR) were purchased from Sigma-Aldrich. The methacrylic acid copolymer Eudragit S 100® was purchased from Evonik (Germany). Hyaluronic acid (234,400 Da) was supplied by Eurogentec (USA). SiRNA labeled with Alexa488 was bought from Eurogentec (Belgium). Oligonucleotides 3′-GCAAGCUGACCCUGAAGUUCAU-3′ and 5′-CUUACGCUGAGUACUUCGATT-3′ were carefully applied. RNase free materials and conditions were mentioned, the solutions were made in RNAse-free water (Gibco, Belgium) and/or PEI (1 mg/mL) and then vortexed for 30 s. The mix was left for 1 h and centrifuged for 30 min at 17,860 × g (15,000 rpm Biofuge 15R, Heraeus Sepatech). The composition of the formulations is summarized in Fig. 2. For example, CPEG/PEI 1 NPs contained 10 μg siRNA, 250 μg CPEG, 83 μg TPP and 50 μg PEI; C/CPEG/PEI NPs contained 10 μg siRNA, 250 μg CPEG, 125 μg TPP and 50 μg PEI.

2.4. Preparation of chitosan–siRNA nanoparticles

To avoid RNase contamination, chitosan and PEI solutions were treated with the reagent RNAsafe (Ambion, Belgium) as described by the manufacturer, except for the chitosan–PEG solution. If not mentioned, the solutions were made in RNase-free water (Gibco, Belgium) and filtered through 0.22 μm filters (VWR, Belgium). Throughout all experiments, RNase free materials and conditions were carefully applied.

The method used to formulate the nanoparticles is the ionic gelation [9]. The negatively charged components i.e. siRNA (50 μM), tripolyphosphate (1 mg/mL) and when stated hyaluronic acid (1 mg/mL) were added to the positively charged components i.e. chitosan (1 mg/mL in sodium acetate buffer 0.2 M, pH 5.5), PEIylated chitosan (1 mg/mL) and/or PEI (1 mg/mL) and then vortexed for 30 s. The mix was left for 1 h and centrifugated for 30 min at 17,860 × g (15,000 rpm Biofuge 15R, Heraeus Sepatech). The composition of the formulations is summarized in Fig. 2. For example, CPEG/PEI 1 NPs contained 10 μg siRNA, 250 μg CPEG, 83 μg TPP and 50 μg PEI; C/CPEG/PEI NPs contained 10 μg siRNA, 250 μg CPEG, 125 μg TPP and 50 μg PEI.

2.4.1. Size and zeta potential

The size and the zeta potential of the nanoparticles were determined using the Nanosizer NanoZS (Malvern Instruments Ltd., Malvern, UK) by photon correlation spectroscopy and electrophoretic mobility respectively. All samples were measured in triplicate in RNAse-free water.

2.4.2. Encapsulation efficiency

The percentage of encapsulation was calculated using Oligreen® reagent (Invitrogen, Belgium) according to the manufacturer guidelines: 100 μL of each sample was transferred in a 96-well plate and mixed with 100 μL of a diluted solution of Oligreen® (1:200 in buffer TE 1 ×). The plate was incubated at room temperature covered from light for 5 min. The fluorescence measured corresponds to the free (i.e. non-encapsulated) siRNA. The wavelengths of excitation and emission were 480 and 520 nm respectively (Vicor Perkin Elmer, Belgium).

Table 1

<table>
<thead>
<tr>
<th>Characteristic of the different chitosans used.</th>
<th>Molecular weight (kDa)</th>
<th>Deacetylation degree DD (mol%)</th>
<th>Origin</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>90°</td>
<td>79.7</td>
<td>Fungi (Agaricus hirsutus)</td>
<td>Kitozyme</td>
</tr>
<tr>
<td>C2</td>
<td>50–190°</td>
<td>75–85</td>
<td>Animal (shrimp and crab shells)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>C3</td>
<td>190–310°/250°</td>
<td>75–85</td>
<td>Animal (shrimp and crab shells)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>C4</td>
<td>173°</td>
<td>65.0</td>
<td>Fungi (Agaricus hirsutus)</td>
<td>Kitozyme</td>
</tr>
<tr>
<td>C5</td>
<td>36°</td>
<td>83.0</td>
<td>Fungi (Agaricus hirsutus)</td>
<td>Kitozyme</td>
</tr>
<tr>
<td>C6</td>
<td>82°</td>
<td>77.6</td>
<td>Fungi (Agaricus hirsutus)</td>
<td>Kitozyme</td>
</tr>
<tr>
<td>C120</td>
<td>120°</td>
<td>80.7</td>
<td>Fungi (Agaricus hirsutus)</td>
<td>Kitozyme</td>
</tr>
</tbody>
</table>

* Based on intrinsic viscosity ([η] in 0.3 M acetic acid and 0.2 M sodium acetate, Marck Houwing relation [η] = 4(Mw/α)° where Mw is the viscosity average molecular weight of polymer, α and κ are constants (α = 0.77 and κ = 0.074) and [η] is the limiting viscosity number or intrinsic viscosity [30]).

* Determined by Gel Permeation Chromatography (in acetic acid/sodium acetate buffer, pH = 4.5 at 30 °C with a Waters 1717 liquid chromatograph equipped with a Waters 2414 refractive index detector. Columns Waters Ultrahydrogel 250, 500 and 1000 were calibrated with poly(ethylene oxide) standards).
2.4.3. $^1$H NMR spectroscopy of nanoparticles

NMR spectroscopy (NMR) data were recorded on a Bruker Avance spectrometer operating at 500 MHz and 202 MHz for $^1$H and $^3$P, respectively. A nanoparticle solution was prepared as described above (II.3) but using deuterated solvents and reagents, i.e. acetic acid-d$_4$ (99.5 atom% D, Aldrich, Belgium), trimethyl silyl-3-propionic acid-d$_4$ (99.5 atom% D, Aldrich, Belgium), trimethylsilyl-phosphate (98 atom% D, Euriso-Top, Belgium) and D$_2$O (99.9 atom% D, Euriso-Top, Belgium). 12 μg siRNA (81 μM) and 99.6 μg tripropyolphosphate (1 mg/ml in D$_2$O) were added to 300 μg PEGylated chitosan (1 mg/ml in D$_2$O) and 60 μg PEI (1 mg/ml in D$_2$O adjusted to pH 6.5–7 with acetic acid-d$_4$). The mixture was left to rest for 1 h and then centrifuged for 30 min at 17,860 × g (15,000 rpm). The small amount of material available, a Shigemi tube was used, which, by requiring only a volume of about 250 μL, leads a higher concentration sample, which in turn gave a better signal to noise ratio. The glass bottom of the Shigemi was matched to the magnetic susceptibility of D$_2$O, the solvent in which the samples of CPEG PEI nanoparticles were prepared. Thus, the residue was suspended in 250 μL of a 1 mM solution of TMSP in D$_2$O and introduced in a 5 mm Shigemi microtube. The $^1$H NMR analyses were acquired at 20 °C with the following experimental settings: delay between scans (D1) of 20 s and number of scans (ns) between 3000 (17 h) and 17,000 (4 days). The HDO signal was suppressed using presaturation prior to acquisition of the $^1$H signal. The TMSP signal was used as reference (0 ppm).
was added to each well. The absorbance was read at 560 nm using a microplate photometer (Thermo Scientific, Belgium).

The NPs were compared to PEI/siRNA complexes (PEI:siRNA weight ratio of 5:1).

### 2.6.2. Transfection efficiency

B16F10 luc cells were plated on 96-well plates (8000 cells/well) in 100 μL of medium containing 10% FBS 24 h prior to the transfection. Cells were incubated with the nanoparticles or the positive control INTERFERin® (Polyplus Transfection, France), containing luc siRNA or control siRNA duplex at 100 or 200 nM siRNA/well (i.e. 10 or 20 pmol/well). After 4 h incubation, the medium was replaced with fresh medium. At 48 h, 100 μL ONE-Glo™ reagent (Promega, Netherlands) was added to the cells grown in 100 μL of medium.

The luminescence (Relative Light Units, RLU) was measured within 30 min using Victor Perkin Elmer luminometer. The percentage of luciferase inhibition is expressed by the equation: %inhibition = 100 − ((100 × RLUluc) / RLUctl) where RLUluc is the mean of RLU for luc siRNA and RLUctl is the mean of RLU for control siRNA.

Using the same protocol, the transfection efficiency was measured in SiHa cells (human cervical tumor cells) expressing green fluorescence protein (GFP) using EGFP22 siRNA duplex (sense 5'−GCAAGCUGACCC UGAAGULUCAU-3'; [32]) supplied from Eurogentec. The GFP SiHa cells were observed using an inverted fluorescent microscope (Axiovert S100, Zeiss) equipped with a objective 20× LD Plan-Neofluar.

#### 2.6.3. Confocal microscopy

B16F10 luc cells were seeded in 2 chamber Labtek® (1.5 × 10^5 cells/chamber) and transfected with nanoparticles for 4 h at a concentration of 200 nM Alexa488–siRNA. Following transfection, cells were subjected to Hoechst 342 and Lysotracker Red DND 99 staining (Invitrogen, Belgium) to visualize nuclei and acidic endosomal vesicles respectively.

Uptake of duplex siRNA was monitored by a Zeiss confocal microscope Cell Observer Spinning Disk equipped with a 100× oil objective.

#### 2.6.4. Flow cytometry

Cellular uptake of nanoparticles was quantified by flow cytometry instrument. B16F10 luc cells were seeded in 12-well plates (2 × 10^5 cells/well) 24 h before experiment. Cells were incubated with nanoparticles for 4 h at a concentration of 100 nM Alexa488–siRNA. Following transfection, cells were subject-d to Hoechst 342 and Lysotracker Red DND 99 staining (Invitrogen, Belgium) to visualize nuclei and acidic endosomal vesicles respectively.

To quench extracellular trytophanes, cells were incubated with tryptan blue (0.2% v/v) for 7 min. Cells were rinsed 2 times with PBS, trypsinized (0.25% trypsin) and diluted with medium.

After centrifugation (250 × 5 min, 4 °C), the cell pellet was resuspended in 100 μL PBS. The measurements were done on a FACSscan cytometer and the data were analyzed using Flowjo software. 10^4 cells were analyzed in each measurement.

### 2.7. Statistics

The experiments were performed in triplicate, unless otherwise stated. Values are given as means ± standard deviations (SD). For the statistics and plotting, PRISM (GraphPad, USA) was used. Statistically significant differences were assessed by an analysis of variance (ANOVA) at a 0.05 significance level and followed by Tukey's post test.

### 3. Results

#### 3.1. Synthesis of PEGylated chitosan CPEG

In order to increase the solubility of chitosan into water and provide stealth properties to the nanoparticles, PEG chains were selectively grafted onto the hydroxyl groups of chitosan. For this purpose, the coupling of PEG chains functionalized at one chain-end by an isocyanate group with a chitosan chain, whose primary amines were protected by phthalimide groups was investigated. A percentage of grafting of 35 mol% was targeted, in order to achieve a complete solubility of the polymer CPEG in water [35].

The 1H NMR analysis of the purified CPEG (Fig. 1) allowed the determination of the number of PEG graft by chitosan chain by comparison of the intensity of the integrals of the signal of the two CH2 of PEG (3.6 ppm) with the signal of the CH of chitosan (3.0 ppm). The molecular weight of the PEG graft was also calculated by comparison of the signal of the two CH2 of PEG (3.3 ppm). The percentage of grafting measured by NMR was equal to 30 mol%, which was lower than the expected value of 35 mol%. This observation could be explained by a partial deactivation of the terminal isocyanate of PEG, highly sensitive to hydrolysis. As expected, CPEG was soluble into water at neutral pH. After the synthesis,
the characteristics of CPEG were measured: the deacetylation degree was found to be 84% and the Mw 37 kDa due to a supposed degradation during the deprotection step. CPEG (12 mol% grafting, Mw 20 kDa, DD 84%) was synthesized and characterized using the same methods.

3.2. Formulation and characterization of siRNA loaded chitosan-based nanoparticles

3.2.1. Formulation of the nanoparticles

The rationale for the selection of the excipients to formulate chitosan-based nanoparticles loaded with siRNA was the following. First, as the optimal properties of chitosan are still controversial, chitosan of different origins and molecular weights was tested [36]. Secondly, TPP was used for ionic gelation method, which ensured a good entrapment of the nucleic acid. In preliminary experiments, the optimum C:TPP weight ratio for nanoparticle formation was determined. Depending on the composition of the particles, this ratio was ranged between 4:1 and 2:1. Third, PEGylated chitosan was used to enhance nanoparticle solubility at physiological pH and to provide stealth properties in blood and endosomal disrupting agents were added to the formulation [37]. Finally, hyaluronic acid was tested to strengthen nanoparticle stability [38]. The amount of siRNA was fixed at 4% of chitosan weight.

Fig. 2 shows the composition of several formulations that were tested.

3.2.2. Physicochemical characteristics of the nanoparticles

The characteristics of the siRNA loaded chitosan-based nanoparticles are summarized in Table 2. The nanoparticles had homogenous sizes, using dynamic light scattering (DLS). Indeed, the mean diameter was between 120 and 290 nm and the size distribution was relatively narrow, as shown by the polydispersity index (from 0.18 to 0.29) irrespective of the formulation composition. The molecular weight and the origin of chitosan did not influence the mean diameter or the PDI of the particles (p < 0.05). However, based on the zeta potential, 3 types of nanoparticles were distinguished. The nanoparticles containing a majority of non-PEGylated chitosan (nanoparticles C, C/CPEG, and C/CPEG/PEI) were positively charged. The CPEG/PEI and CPEG/PEI h nanoparticles showed a zeta potential close to neutrality. Finally, the inclusion of the negatively charged polymer hyaluronic acid (HA) led to the formation of negatively charged nanoparticles. The encapsulation efficiency was higher than 90% for all formulations using Oligreen assay. These results were correlated with those obtained with the FFS technique.

To further characterize the formulations, CPEG/PEI nanoparticles were analyzed using atomic force microscopy (AFM). In Fig. 3, it can be observed that the particles have a spherical shape. Furthermore, we noticed the coexistence of very small particles with larger particles of around 200 nm in diameter, which might be aggregates of the smaller ones. The nanoparticles presented smaller size when using AFM than when measured by DLS. This can be explained by the fact that the PEG chains were not in a hydrated state, hence were not extended, as they are during the DLS measurements.

To further investigate the behavior and surface properties of nanoparticles, NMR spectroscopy of CPEG/PEI nanoparticles was performed in aqueous media at physiological pH (Fig. 4C). This characterization gives information on the soluble parts of the nanoparticles, i.e. the surface or the surrounding solution. The CH2 of PEG (3.7 ppm) gave a narrow signal which demonstrated the presence of PEG as a stabilizing corona. On the opposite, chitosan protons gave a broad peak centered at 3.5 ppm, suggesting that chitosan was organized in a rigid structure forming a solid core with only a small part in contact with water. Surprisingly, three groups of proton situated at 2.6, 2.8 and 3.1 ppm, corresponding to CH2 of PEI, appeared clearly after long measuring times and a sojourn of several days in water (Fig. 4C) whereas in a freshly prepared solution, those protons emerged from a broad peak (see Fig. 4B) corresponding to poorly soluble rigid PEI. One interpretation of these facts could be that nanoparticles suffered self-reorganization, as part of the PEI migrated inside of the solid core whereas some of it ended up close to the surface or in the surrounding media. Nanoparticles were also submitted to 31P NMR but no peak was detected. Given that TPP in D2O gave three signals in 31P NMR (−5.9, −7.1 and −21.2 ppm) at the same concentrations, it could be deduced that TPP, and therefore siRNA, were situated inside of the nanoparticles. Thus, NMR observations confirmed the expected structure of nanoparticles presenting a PEG corona, a solid chitosan core collapsed around TPP and siRNA and PEI segments situated both inside and outside of the nanoparticles. This result is coherent with the zeta potential measurements exhibiting a slightly positive charge, certainly due to the shielding effect of PEG and a partial contact of water with chitosan and PEI.

3.3. Transfection efficiency of the nanoparticles

To evaluate the gene knockdown efficiency of the formulations, B16F10 luc cells were incubated with the different formulations loaded with luc siRNA. The specificity of knockdown was confirmed using scramble siRNA formulations.
3.3.1. Determination of the optimal dose of siRNA

To determine the optimal siRNA concentration for the transfection efficiency, several siRNA doses ranging from 1 nM to 400 nM siRNA/well were investigated, using CPEG/PEI l nanoparticles (Fig. 5). No effect was noticed at the lower concentrations (1 and 10 nM siRNA/well). At a dose of 400 nM, RLU signal of nanoparticles loaded with scramble siRNA decreased, suggesting toxicity of the nanoparticles or an off-target effect. Therefore, the gene silencing experiments were performed at 100 and 200 nM.

3.3.2. Gene silencing mediated by siRNA loaded chitosan-based nanoparticles

The basic formulations containing chitosan and TPP only demonstrated poor transfection efficacy regardless of the type of chitosan employed (Fig. 6A, p < 0.05). Based on the literature, we hypothesized that this low efficiency could be due to the poor buffering capacity of chitosan [39]. Consequently, nanoparticles with endosomal disruption properties were formulated. To promote endosomal escape, the amine rich polymers poly(ethylene imine) (PEI) or poly-l-arginine (PLR) were included in the formulations (data not shown). A third formulation was prepared, loading the nanoparticles with methacrylic acid copolymer Eudragit® which also demonstrated good buffering capacities [40]. The nanoparticles possessed suitable physico-chemical characteristics (data not shown). Among these approaches, better luciferase inhibition was obtained with the addition of PEI, therefore this polymer was chosen as additional component for the rest of the study.

Regardless of the formulation, higher level of luciferase inhibition was observed at 200 nM (54% to 71%) than at 100 nM (35% to 56%) (Fig. 6B). Moreover, no significant difference was noticed between the positive control INTERFERin (78% luciferase inhibition) and three of the formulations, i.e. C/CPEG/PEI l (71%), C/CPEG/PEI h (62%) and C3/CPEG/PEI (66%), on B16F10 luc cells.

Regarding C/CPEG/PEI nanoparticles, an influence of the chitosan molecular weight on the transfection efficiency was observed. Indeed, nanoparticles made of low molecular weight chitosan (C2) seemed to be less efficient than the same formulation composed of high molecular weight chitosan (C3 and C4). To fortify these results, a second assay was performed with several fungal chitosans of molecular weight ranging from 36 to 120 kDa and 12% mol PEGylated chitosan CPEG′. As shown in Fig. 6C, a strong correlation between molecular weight and luciferase inhibition was observed.

To confirm the gene silencing observed on B16F10 luc cells, the biological activity of three of our formulations (CPEG/PEI/HA, CPEG/PEI l, C3/CPEG/PEI) was tested on a different cell model, using another protein reporter. SiHa cells expressing the green fluorescence protein (GFP) were transfected with nanoparticles loaded with EGFP siRNA. As shown in Fig. 7, CPEG/PEI/HA nanoparticles were able to decrease the GFP signal. The same effect was observed for CPEG/PEI l and C3/CPEG/PEI nanoparticles (data not shown). This suggested the high potential of these nanoparticles in different cell models.

3.4. Uptake profiles of the nanoparticles: confocal microscopy and flow cytometry studies

To better understand the mechanisms of intracellular trafficking of siRNA depending on the characteristics of its carrier, confocal microscopy

Fig. 6. Percentage of luciferase inhibition with different types of nanoparticles. Luminescence was measured 48 h after an incubation time of 4 h with the nanoparticles. Data represent mean ± SD. A: Chitosan-based nanoparticles. B: Chitosan and PEI PEGylated nanoparticles. The nanoparticles were compared to the positive control INTERFERin at 100 nM (#) or 200 nM (**). C: Influence of the chitosan molecular weight on the luciferase inhibition by C/CPEG/PEI nanoparticles at 200 nM siRNA/well. The nanoparticles were compared to those made with chitosan of 120 kDa (* p < 0.05, ** p < 0.01, *** p < 0.001) (N = 2–4, n = 8).

Fig. 7. SiHa GFP cells. A: medium and B: cells transfected with CPEG/PEI/HA at 200 nM. Observation of the cells 48 h after the initiation of transfection.
microscopy studies were performed. SiRNA molecules were labeled in green and acidic endosomal vesicles were stained in red. B16F10 luc cells did not present any intracellular siRNA signal after incubation with C2/CPEG/PEI nanoparticles (Fig. 8D) or C2 nanoparticles (data not shown). On the contrary, regarding CPEG/PEI, C3/CPEG/PEI or CPEG/PEI/HA particles, siRNA molecules were present in the acidic endosomes as revealed by the yellow dots. Moreover, the green dots suggested that a fraction of siRNA were localized in non-acidic vesicles or were released into the cytoplasm (Fig. 8A–C). Flow cytometry experiments confirmed the results that have been observed in the confocal microscopy study. Indeed, only few amounts of C2/CPEG/PEI nanoparticles were taken up by the cells (58% uptake; Fig. 9) contrary to the other types of particles (100% of the cells have taken up the particles).

3.5. Cytotoxicity

Toxicity of the formulations was assessed on B16F10 luc cells, at 200 nM siRNA, using MTT and LDH tests to quantify cell viability and cell mortality respectively (Fig. 10). The formulations were compared to siRNA/PEI complexes, containing the same amount of PEI. These complexes induced a toxic effect with more than 40% cell mortality. On the other hand, the nanoparticles did not exhibit any severe toxicity (10 to 20% cell mortality with LDH test). This suggested that the presence of chitosan and PEGylated chitosan enhanced the toxicity profile of the formulations. However, regarding more precisely MTT test results, CPEG/PEI and CPEG/PEI/HA nanoparticles showed higher cell viability (82% and 72% respectively) than type C/CPEG/PEI (60% viability). Moreover, no significant difference was noticed between CPEG/PEI nanoparticles and cells growing in medium.

3.6. Nanoparticle stability in plasma

Little knowledge is available about the stability of chitosan-based particles in plasma although most of them are designated to I.V. administration. Assisted by two innovative technologies i.e. fluorescence fluctuation spectroscopy and single particle tracking, we were able, both to determine the ability of our formulations to protect siRNA in plasma and to measure the size of our particles in plasma.

3.6.1. SiRNA release in plasma using fluorescence fluctuation spectroscopy (FFS)

The first consideration is an effective protection of the siRNA molecules in blood. Using FFS it is possible to quantify the siRNA release and therefore to determine the integrity of the formulations in plasma. The complexity efficiency of the formulations was monitored in 80% plasma as a function of time (Fig. 11). After 2 h incubation, approximately 100% complexation efficiency was maintained for the majority of the nanoparticles, suggesting that they did not dissociate in plasma. However, regarding the CPEG free formulation (C3 nanoparticles), a very fast release of siRNA occurred.

3.6.2. Sizing using single particle tracking (SPT)

Another crucial parameter of stability is size, since it affects the nanoparticle biodistribution in blood and the potential in vivo toxicity. Unlike dynamic light scattering (DLS), SPT allows the study of nanoparticle aggregation in real time in biological fluids such as plasma.

Initially, the measurements were performed in RNAse-free water at 37 °C. The size distributions are shown in Fig. 12A. At this temperature, the aggregation process is speeded up. Indeed, we noticed an instable behavior for some of the formulations (C3/CPEG/PEI, C4/CPEG/PEI and C2) which was expressed by an increase in their mean diameter. These formulations did not contain CPEG (C3/CPEG/PEI or C4/CPEG/PEI), which may not have been sufficient to prevent aggregation. On the other hand, CPEG/PEI, CPEG/PEI h and CPEG/PEI/HA nanoparticles, containing a high amount of CPEG, remained stable at 37 °C. Indeed, their size distributions at 37 °C were similar to those measured at 25 °C with DLS.

Afterwards, the experiments were carried in plasma at 37 °C: the formulations were incubated in 80% plasma and the measurements were done immediately after the incubation. As seen with FFS, C3 particles fell apart and released the majority of siRNA. C3/CPEG/PEI and C4/CPEG/PEI nanoparticles aggregated immediately after incubation in plasma. In the case of CPEG/PEI particles, the aggregation process occurred as well, even though it was more delayed. Compared to buffer, their size distribution was broadened and the mean diameter was increased (Fig. 12B). On the other hand, the size profile of CPEG/PEI/HA nanoparticles did not change in plasma (Fig. 12B).
4. Discussion

Numerous barriers have to be faced in order to deliver the siRNA molecules in the cell cytoplasm. First, the nanoparticles have to remain stable in the biological fluid (blood when I.V. is required), to be taken up by the cells and finally to escape the endosomes in order to deliver the siRNA in the cell cytoplasm, where the RNAi machinery is located. Chitosan possesses attractive properties, especially its biodegradability, biocompatibility and easy formulation into NPs. However, the major limitations of this polymer are first its low water solubility at physiological pH that can result in a lack of stability, and secondly its poor endosomal disrupting properties[41]. In this work, we aimed at elucidating the parameters influencing the formulation of efficient chitosan-based systems for I.V. siRNA delivery.

The first method of nanoparticle formulation described in the literature was the simple complexation, where the chitosan and the siRNA were simply mixed [8]. In our hands, this method did not lead to the formation of nanoparticles, probably because of the electrostatic interactions between siRNA and chitosan that were too weak. Another approach, called the ionic gelation, consisted in the addition of a crosslinker agent, mostly TPP to strengthen the interactions between siRNA and chitosan [9]. By using this method of formulation, nanoparticles with suitable physico-chemical characteristics were obtained (mean diameter < 300 nm, PDI < 0.29). However, these nanoparticles were not efficient to silence the luciferase gene in a murine B16F10 melanoma cell model stably expressing luciferase, regardless of the type of chitosan employed (Fig. 6A). It has been described that chitosan possesses inferior endosomal disrupting properties than the other cationic polymers such as PEI or PLR [24]. Recently, some authors hypothesized that a threshold concentration of chitosan in the complex and/or in the free form could be required to mediate an efficient endosomal escape. Moreover, free PEI seemed to be more efficient than free chitosan at disrupting the endosomal membrane, because of its higher charge density and lower intercharge spacing [42]. Thus, to promote the siRNA release from the endosomes, three types of nanoparticles, all of them containing a low fraction of PEI (1/5 w/w of the amount of chitosan) were formulated. The first kind of nanoparticles (C/CPEG/PEI) was prepared by mixing non-PEGylated chitosan and CPEG. The nanoparticles thus obtained were positively charged. The second type of nanoparticles contained CPEG only and presented a zeta potential close to the neutrality. In this case, the PEG chains of chitosan may hide the
positive charges on the surface of the nanoparticles. Finally, with the inclusion of the anionic polymer hyaluronic acid, negatively charged nanoparticles were obtained (CPEG/PEI/H). Three of these formulations (CPEG/PEI, C2/CPEG/PEI and CPEG/PEI/H) were found to be as efficient as the positively charged nanoparticles and INTERFERin and mediated up to 70% luciferase inhibition. Interestingly, the C2/CPEG/PEI nanoparticles exhibited a lower gene silencing efficiency (30%) than the same formulation made with a chitosan of higher MW (C3/CPEG/PEI or C4/CPEG/PEI) (66% and 60% luciferase silencing respectively, Fig. 6B). Furthermore, confocal microscopy and flow cytometry experiments revealed that the C2/CPEG/PEI nanoparticles were poorly taken up by the cells (Figs. 8D and 9), compared with the other formulations. Therefore, the poor luciferase inhibition obtained with C2/CPEG/PEI particles may have resulted from their instability in serum containing medium. Thus, C/CPEG/PEI particles made of low molecular chitosans were probably not densely packed enough to protect siRNA efficiently and released a large amount of siRNA before being endocytosed by the cells. As demonstrated for other cationic polymers [43], these results confirmed the importance of the chemical composition as well as the physical structure of the nanoparticles in their biological activity. Despite the low molecular weight of CPEG (35 kDa, due to a degradation of the chitosan chains during the synthesis), the CPEG/PEI and CPEG/PEI/H nanoparticles were efficiently taken up by the cells and mediated high gene silencing. In this case, the presence of PEG may compensate for the low molecular weight of the chitosan.

One of the major concerns when designing a delivery system intended for I.V. administration is to maintain a good stability in blood. On one hand, the nanoparticles can be destabilized by interactions with the blood components, resulting in the dissociation of the nanoparticles. Therefore, the stability of the formulation was evaluated in human plasma. FFS is a microscopy-based technique, namely the fluorescence fluctuation spectroscopy (FFS) and the single particle tracking (SPT), the stability of the formulations was evaluated in human plasma. FFS is a microscopy-based technique that can monitor the integrity of the delivery system, while SPT allows the determination of the size distribution of the nanoparticles in human plasma [34,45]. First, the complexation efficiency of the nanoparticles was monitored by FFS. A rapid disassembly of the CPEG-free nanoparticles (C1) was observed (Fig. 11). Indeed, 70% of the initial amount of siRNA was released in 30 min, indicating that these nanoparticles may have fallen apart right after the incubation in plasma, rendering them insufficiently stable to deliver siRNA in vivo. These results were also confirmed by the SPT measurements, where no remaining CPEG-free nanoparticles were visible after incubation in plasma (data not shown). This instability might be explained by the deprotonation of some of the chitosan amino groups at physiological pH, rendering the binding to siRNA less effective. Moreover, competition between siRNA and anionic plasma proteins for chitosan binding might play a part in the dissociation of these nanoparticles. Therefore, nanoparticles made of TPP and chitosan are not suitable for I.V. administration of siRNA.

On the other hand, the PEGylated nanoparticles did not dissociate in human plasma: approximately 100% complexation efficiency was maintained after 2 h incubation, showing that the PEGylation of the nanoparticles contributes to their stabilization in plasma. The 1H NMR analysis of the surface of the CPEG/PEI particles confirmed that the nanoparticles displayed a PEG corona (Fig. 4).

By using SPT, CPEG/PEI particles appeared to be more stable than the nanoparticles containing a lower amount of PEG (C/CPEG/PEI and C/CPEG/PEI/H). Indeed, C/CPEG/PEI and C/CPEG/PEI particles aggregated rapidly in plasma. Plasma proteins, such as fibrinogen, may adsorb on these cationic particles creating a linkage between them and leading to aggregation. According to the previous FFS results, the complexation efficiency of these formulations was around 100%, indicating that the siRNA molecules may be encapsulated in the aggregates. The amount of PEG seemed thus to be an important factor of stability by reducing interparticular aggregation. Indeed, it has been demonstrated that the aggregation of liposomes in blood became more significant as the PEGylation decreased [34]. Moreover, by enhancing the proportion of CPEG into the nanoparticles, the cell viability was improved (Fig. 10). The PEG chains may hide the cationic charges of chitosan, responsible for interactions with cell membrane and thus toxicity [35]. Finally, the inclusion of hyaluronic acid seemed to increase the stability of the particles in plasma, as the size distribution of the CPEG/PEI/H nanoparticles did not change upon incubation in plasma (Fig. 12B). This could be attributed to the negative zeta potential of these nanoparticles, leading to repulsive forces between particles and anionic plasma components. Moreover, hyaluronic acid possesses a shielding effect that might have reinforced the stability of the nanoparticles [46,47].

5. Conclusions

We aimed at developing siRNA loaded chitosan-based nanoparticles suitable for I.V. administration and thereby to better understand the parameters governing the ability of a carrier to deliver siRNA efficiently. We showed that the inclusion in the formulations of the cationic polymer PEI led to high gene silencing. Moreover, we demonstrated that the stability of the nanocarrier is a crucial parameter for reaching its final goal. Combining TPP to chitosan, as described frequently in the literature, would not be a relevant strategy when I.V. route is required. The addition of PEG as well as the use of high molecular weight chitosan strengthened the structure of the nanoparticles, providing stability and subsequently great levels of in vitro gene silencing. Nevertheless, if I.V. administration is required, stability in plasma and in blood is crucial. We demonstrated that by increasing the amount of PEG and by including an anionic polymer (HA), it was possible to generate nanocarriers which were stable in plasma without altering their biological activity. These novel nanoparticles are promising for in vivo systemic delivery and will create new perspectives for future gene silencing treatments.

Acknowledgments

This work was supported by the Region Wallonne (BioWin project). The authors thank Dr Paolo Porporato who kindly provided the SiHa cells, Dr Patrick Van der Smissen for the help with the confocal microscopy and Min Suk Schauder.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.12.026.

References
