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Single stranded siRNA complexation through non-electrostatic interactions

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ABSTRACT

As double stranded, single stranded siRNA (ss-siRNA) has demonstrated gene silencing activity but still requires efficient carriers to reach its cytoplasmic target. To better understand the fundamental aspect driving the complexation of ss-siRNA with nanocarriers, the interactions between surfaces of various compositions across a ss-siRNA solution were investigated using the Surface Forces Apparatus. The results show that ss-siRNA can adsorb onto hydrophilic (positively and negatively charged) as well as on hydrophobic substrates suggesting that the complexation can occur through hydrophobic interactions and hydrogen bonding in addition to electrostatic interactions. Moreover, the binding strength and the conformation of ss-siRNA depend on the nature of the interactions between the ss-siRNA and the surfaces. The binding of ss-siRNA with nanocarriers, such as micelles or liposomes through non-electrostatic interactions was also evidenced by a SYBR® Gold cyanine dye. We evidenced the presence of interactions between the dye and oligonucleotides already complexed to non-cationic nanovectors biasing the quantification of the encapsulation. These results suggest that non-electrostatic interactions could be exploited to complement electrostatic interactions in the design of nanocarriers. In particular, the different highlighted interactions can be used to complex ss-siRNA with uncharged or anionic carriers which are related to lower toxicity compared to cationic carriers.

1. Introduction

Small interfering ribonucleic acids (siRNA) are small double strand RNA molecules of 20–30 base pairs [1] capable of inhibiting specific gene expression in plants [2] and animals [3] by causing cleavage of specific messenger RNA (mRNA) after transcription. The specific gene silencing activity of double stranded siRNA (ds-siRNA) is currently clinically assessed to treat large variety of diseases, such as genetic disorders [4], cancer [5] or infectious diseases [6]. Compared to other gene silencing strategies, such as single-stranded antisense RNA or steric-blocking oligonucleotides [7], siRNA require a lower dosage, since a single molecule can cleave a large number of target mRNA. Interestingly, several studies have shown that only one RNA strand is required to activate the RNAi pathway and that single stranded siRNA (ss-siRNA) can bypass the ds-siRNA form [8,9]. Lima et al. have recently optimized the ss-siRNA structure to silence protein expression with potency similar to the duplex [10]. Moreover, ds-siRNA are known to induce non-specific gene silencing [11], a phenomenon caused by the saturation of the endogenous RNAi machinery by sense and antisense siRNA strands [12], resulting in toxic phenotypes [13]. Therefore, delivering ss-siRNA prevents from administrating the sense strand of ds-siRNA, which would contribute to reduce the non-specific gene silencing [14] and other toxicity issues [7,15,16].

Because of its high negative charge and hydrophilic nature, siRNA does not cross biological membranes easily, which limits its bioavailability [17]. In some clinical applications, naked siRNA can be administered locally, such as in lung, eyes, and muscles [18,19]. However, many tissues can only be reached after systemic administration, such as the liver, a major target of siRNA therapeutics in clinics [18]. In physiological conditions, siRNA can be rapidly eliminated by endogenous nucleases degradation [20], by the reticuloendothelial system [21] or by glomerular filtration. Therefore, many recent efforts have focused on the careful design of drug delivery systems (DDS), able to overcome each of the biological

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barriers encountered until the cytoplasm of the cell, i.e. the siRNA's site of action [17,18,21].

Viral vectors are among the first vehicles studied for small oligonucleotides delivery but can induce unacceptable toxicity [21]. The complexation of ds-siRNA with synthetic vectors such as lipid nanoparticles [22–24], cyclodextrin based polymeric nanoparticles [25,26] or polymeric nanoparticles [27,28] have been extensively studied and some of them are currently under clinical trials [18,29]. Whatever the nanocarrier, they are generally positively charged and involve complexation of the negatively charged siRNA through electrostatic interactions [23,30,31]. However, this cationic feature has been related to higher toxicity compared to uncharged or anionic carriers. Such toxicity is associated with non-specific interactions and internalization with cells, a high rate of opsonisation and a short blood circulation half-life [32,33]. To optimize the nanocarrier design, extensive chemical diversity has been explored. High throughput screening of cationic lipids, for instance, has shown that too many or too strong (*e.g.* quaternary amines) cationic groups can significantly impede the cytosolic release of the siRNA payload, and limit the transfection efficiency [34]. Interestingly, several studies have demonstrated that ds-siRNA and ss-mRNA do not require the same carrier for optimized delivery [35]. However, structure-activity relationships are not deeply understood, and more in-depth knowledge about siRNA and the nanocarrier interactions would be a great advantage for the design of efficient and safe DDS.

Theoretical studies have shown that double and single stranded oligonucleotides can interact with hydrophilic and hydrophobic surfaces through different kind of interactions [36–39]. However, experimental investigation of single stranded oligonucleotides complexation with surfaces and more particularly with DDS has been less reported. Because of the short sequence, 3D structure of ss-siRNA is quite simple or inexistent [40] and most probably lost during adsorption onto the surface. Nevertheless, thanks to its variety of chemical groups, ss-siRNA can be a partner for hydrogen bonding, hydrophobic and/or electrostatic interactions, as illustrated in Fig. S1. Hydrophobic interactions are possible [41] and probably via the aromatic cycle, hydrogen bonding via the hydroxyl carried by ribose or amino groups of nucleobases and electrostatic interactions via the negatively charged phosphate groups of ribose or positively charged amino groups of the nucleobases. In this study, the binding mechanisms of ss-siRNA with surfaces was investigated by measuring the adsorption of ss-siRNA onto hydrophilic (positively and negatively charged) and hydrophobic surfaces using the Surface Forces Apparatus (SFA). The ability of charged and uncharged DDS to encapsulate ss-siRNA using electrostatic and non-electrostatic interactions was then demonstrated with micelles and liposomes of various chemical nature and surface charges using fluorescence assays.

2. Materials and methods

Ruby mica sheets were purchased from S & J Trading, Inc. (Glen Oaks, NY, USA). Plasma Prep II from SPI Supplies was used to activate freshly cleaved mica surfaces using argon (5.0 grade). Milli-Q quality water was obtained from a Millipore Gradient. Aminoundecyltriethoxysilane (AUTES) was purchased from Gelest, Inc, (Morrisville, PA, USA). Dodecyltrichlorosilane was purchased from Sigma-Aldrich (Oakville, ON, Canada). Single stranded siRNA (5′-UAAGGCUAUGAAGAGAUAC-3′) was purchased from Fidelity Systems, Inc., (Gaithersburg, MD, USA). Cholesterol, 1-2-dioleoyl-sn-glycerol (DAG), 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) and 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1-rac-glycerol) (sodium salt) (DMPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Poly(styrene-block-N-methyl-4-vinyl pyridinium iodide, ($PS_{3.5}$-b-$PM4VPI_{11.6}$), poly(styrene-block-acrylic acid) ($PS_{3.2}$-b-$PAA_{12.5}$) and poly(styrene-block-ethylene glycol) ($PS_{3.2}$-b-$PEG_{12.5}$), the indexed values correspond to the molecular weight in kDa, were purchased from Polymer Source Inc. (Montréal, QC, Canada). SYBR® Gold fluorescent cyanine dye was purchased from Thermo Fisher Scientific (Ottawa, ON, Canada).

2.1. Liposome preparation

Stock solutions (10–20 mg/mL) of commercial lipids and cholesterol were prepared in chloroform. The lipid stock solutions were combined in a 5 mL round-bottom flask at a 50/50 M ratio (5 μmol of lipid and 5 μmol of cholesterol). The chloroform was removed under reduced pressure and the lipid film was further dried for 1 h under high vacuum to remove any residual solvent. The dried lipid film thus obtained was hydrated with 1 mL of Milli-Q water. This solution was heated to 65 °C and vortexed 2 min to obtain a homogenous suspension of large multilamellar liposomes. The solution was then extruded 11 times through a 200 nm polycarbonate membrane using a LiposoFast manual extruder (Avestin Inc., Ottawa, ON, Canada). Liposome preparations were stored in darkness at 4 °C in microcentrifuge tubes. Cholesterol, DOTAP, DMPG and DAG successfully incorporated in the liposomal formulations were quantified by HPLC-UV/MS using an Agilent 1260 Infinity HPLC equipped with a UV detector and a 6120 single-quad mass spectrometer (Mississauga, ON, Canada). Detailed procedures are summarized in Table S1.

2.2. Micelle preparation

Cationic, anionic and uncharged micelles were prepared in water by direct dissolution using $PS_{3.5}$-b-$PM4VPI_{11.6}$, $PS_{3.2}$-b-$PAA_{12.5}$ and $PS_{3.2}$-b-$PEG_{12.5}$, respectively. Block copolymers were dissolved in Milli-Q water (0.2 g/L) at pH 10 for $PS_{3.2}$-b-$PAA_{12.5}$ and pH 5.8 for $PS_{3.5}$-b-$PM4VPI_{11.6}$ and $PS_{3.2}$-b-$PEG_{12.5}$. The resulting micellar suspensions were adjusted to pH 5.8 when required and sonicated for 1 h prior to use.

2.3. DDS characterization

The hydrodynamic diameter and zeta potential of suspended liposomes and micelles in Milli-Q water before and after co-incubation with ss-siRNA were determined using a dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instrument Ltd., Malvern, Worcestershire, UK) at 20 °C in back-scattering mode. Mean values of triplicates are reported in the result section.

2.4. ss-siRNA complexation with DDS and encapsulation efficiency

A SYBR® Gold assay was used to quantify the ss-siRNA encapsulation efficiency of the formulations [42–44]. Stock solutions of DDS (liposomes or micelles) and ss-siRNA were diluted in Milli-Q water at appropriate concentrations, for given lipid/ss-siRNA or copolymer/ss-siRNA ratio. DDS suspensions and ss-siRNA solutions were all prepared for a final ss-siRNA concentration of 250 nM. ss-siRNA solutions were added to the DDS suspension followed by brief vortexing. Complexation was allowed to occur for 30 min at room temperature. After complexation, the solutions were centrifuged at 20,000 *g* for 30 min. ss-siRNA in the supernatant was quantified against a calibration curve of ss-siRNA (20–250 nM), using an excess of SYBR® Gold fluorescent cyanine dye ($\lambda_{ex/em} = 495/537$ nm) and a Safire microplate reader (Tecan, Seestrasse, Switzerland). ss-siRNA encapsulation efficiency (%) was calculated assuming that measured fluorescence intensity at 537 nm corresponds to the unencapsulated ss-siRNA as follow: