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Fine-tuned PEGylation of chitosan to maintain optimal siRNA-nanoplex bioactivity

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ABSTRACT

Polyethylene glycol (PEG) is a widely used modification for drug delivery systems. It reduces undesired interaction with biological components, aggregation of complexes and serves as a hydrophilic linker of ligands for targeted drug delivery. However, PEGylation can also lead to undesired changes in physicochemical characteristics of chitosan/siRNA nanoplexes and hamper gene silencing.

To address this conflicting issue, PEG-chitosan copolymers were synthesized with stepwise increasing degrees of PEG substitution (1.5% to 8.0%). Subsequently formed PEG-chitosan/siRNA nanoplexes were characterized physicochemically and biologically. The results showed that small ratios of chitosan PEGylation did not affect nanoplex stability and density. However, higher PEGylation ratios reduced nanoplex size and charge, as well as cell uptake and final siRNA knockdown efficiency.

Therefore, we recommend fine-tuning of PEGylation ratios to generate PEG-chitosan/siRNA delivery systems with maximum bioactivity. The degree of PEGylation for chitosan/siRNA nanoplexes should be kept low in order to maintain optimal nanoplex efficiency.

1. Introduction

siRNA delivery is challenging due to the extremely fast degradation within biological fluids by endogenous nucleases. For a therapeutic application, siRNA has to cross the barrier of the cell surface and reach the cytoplasm, its site of action. However, the physicochemical properties of siRNAs (negative charge and size of ~13 kDa) hinder the permeation of the barrier of the cell surface which is equally negatively charged due to anionic proteoglycans (Deng et al., 2014; Zhang, Zhao, Jiang, Wang, & Ma, 2007). Unmodified siRNA can also be rapidly inactivated in blood and eliminated by the kidneys (Ragelle, Vandermeulen, & Préat, 2013). The success of RNAi therapy is therefore dependent on a safe, stable, specific, and efficient transport. Nanoparticle-based delivery is a promising approach to overcome the extracellular and intracellular barriers mentioned above, and to allow RNAi to function at therapeutically relevant levels (Howard et al., 2006).

In recent years, chitosan has become one of the most widely described non-viral delivery systems for nucleic acids. Chitosan has a randomly distributed ß-1-4 linked *D*-glucosamine (GlcN) and N-acetyl-*D*-glucosamine (GlcNAc) structure, with a 6.2–7.0 pKa of the D-glucosamine unit. This means that chitosan is protonated at pH values lower than ~6.5 and it can only be solubilized in slightly acidic solution (Singha, Namgung, & Kim, 2011). Its high positive charge density, versatility, biocompatibility, and biodegradability set chitosan apart from other polymeric nanocarriers (Liu et al., 2007; Mao, Sun, & Kissel, 2010). At an appropriate nitrogen to phosphate (N/P) ratio, chitosan can condense siRNA to small nanoplexes (100–300 nm) which are compatible with cellular uptake (Huang, Fong, Khor, & Lim, 2005).

In order to maintain these advantageous characteristics under physiological conditions, the functional modification of chitosan

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with hydrophilic PEG polymers is a strategy widely used because of its unique physicochemical properties. Characteristic properties of PEG are its formal electrostatic neutrality and its unique hydration ability, allowing the formation of a tightly bound interfacial hydration layer and reducing the protein-surface interaction. These go together with entropically driven steric exclusion, leading to a steric stabilization effect (Lee, Lee, & Andrade, 1995; Rauscher, Perucca, & Buyle, 2010; Zheng et al., 2010). PEG has been reported to enhance chitosan solubility at higher pH values, increase stability of the nanoplexes in the biological environment, and prevent inter-nanoplexes aggregation (Casettari et al., 2012; Kolate et al., 2014; Roberts, Bentley, & Harris, 2002; Varum, Ottoy, & Smidsrod, 1994). Furthermore, it has been described to increase bioavailability, reduce cytotoxicity and decrease immunogenicity (Lee et al., 1995; Veronese & Pasut, 2005). PEG can also be used as a linker or a spacer between a targeting moiety and the surface of the nanoplexes for a cell specific siRNA delivery (Lee & Kim, 2005).

The modification of chitosan with PEG is an established process (Aktas et al., 2005; Kulkarni, Hukkeri, Sung, & Liang, 2005; Prego et al., 2006). However, contrary effects of PEG on physicochemical characteristics and bioactivity of nanoplexes are still a topic in ongoing research. In the literature, improvement of chitosan nanoplex characteristics with PEG has been shown concerning stability, cell uptake and even 10 times enhanced transfection rates for DNA delivery (Germershaus, Mao, Sitterberg, Bakowsky, & Kissel, 2008). Other groups describe negative effects on cell uptake, especially for high degrees of PEGylation (Vader, van der Aa, Engbersen, Storm, & Schiffelers, 2012). Even for phagocytotic active cells PEG was described to significantly decrease cell uptake (Xie, Xu, Kohler, Hou, & Sun, 2007).

Until now, the PEGylation phenomenon has been extensively studied for chitosan and various other nanocarriers without a clear understanding of the parameters required for it to function optimally. While it has been previously shown that PEG with shorter chains (550 Da) can interfere with the nanoplex formation and stability (Mao et al., 2006), longer PEG chains can stick on the surface of the nanoplexes, participating in the stealth shielding and facilitating the addition of a targeting moiety at the distal end of the PEG chains (Knorr, Allmendinger, Walker, Paintner, & Wagner, 2007). In particular, a PEG chain length with Mw greater than 2 kDa showed better protection against RNAse digestion, and a positive influence on the transfection efficiency (Mao et al., 2006; Zheng et al., 2010). However, reduced protein adsorption and successful transfection efficiency depend on both PEG chain lengths and surface coverage (Fang et al., 2006; Lee et al., 1995; Zheng et al., 2010; Zhou et al., 2007). According to Zheng *et al.* (Zheng et al., 2010), a PEG density of greater than 7% is necessary for higher shielding against unspecific interactions with proteins and cells. In the literature, various PEGylated chitosans with increasing degrees of substitution have been described as being successful, ranging from 0.5% up to 10% or < 28% (Cho, Kim, Kwon, & Oh, 2012). High PEG density is also known to replace a certain percentage of the primary amino groups necessary for nanoplex formation, as well as to decrease the transfection efficiency: due to reduced cellular uptake (Sung et al., 2003). Maurstad *et al.* demonstrated in the case of PEGylated chitosan/DNA nanoplexes, that an increase in transfection efficiency was proportional to an increase in the PEG grafting ratio up to 1.9%: assuming the higher degree of PEGylation to be favorable (Maurstad, Stokke, Vårum, & Strand, 2013).

We performed systematic analyses with gradually increasing degrees of PEGylation and investigated the resulting effects on nanoplex characteristics and biological performance. Based on these studies we wanted to combine the advantages of enhanced chitosan solubility, nanoplex stability and PEG shielding properties with successful siRNA transfection efficiency. The main novelty of our study is that the degree of PEGylation can be balanced to maintain the appropriate physicochemical nanoplex characteristics while satisfying cell biological performance.

2. Materials and methods

2.1. Materials

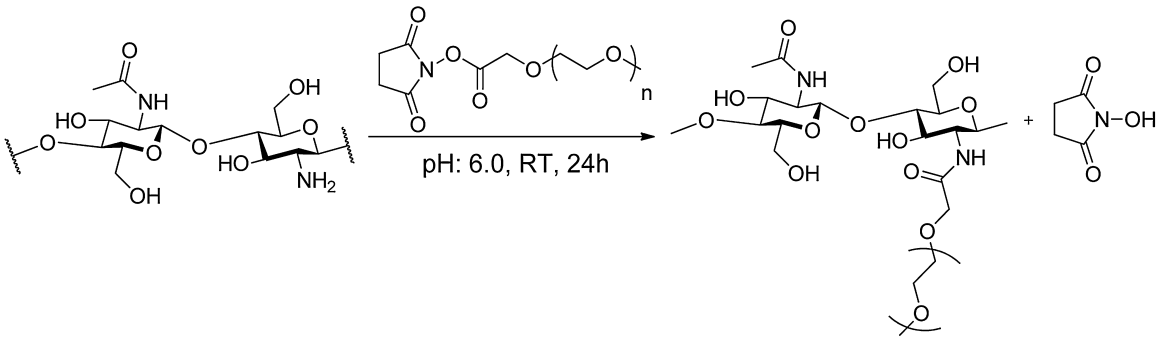
Chitosan with a degree of deacetylation (DD) of 92.6% and Mw 152 kDa was purchased from Heppe Medical Chitosan GmbH 95/50 (Halle, Germany), Methoxypolyethylene glycol 5,000 acetic acid N-succinimidyl ester (mPEG–NHS, PEG average M_n 5,000 Da) was purchased from Sigma Aldrich (Taufkirchen, Germany), TYE 563 DS was purchased from Integrated DNA Technologies (Leuven, Belgium).

2.2. Synthesis of PEG-chitosan

In this study, N-hydroxysuccinimide methoxy PEG (mPEG-NHS) with a MW of 5 kDa was used to modify chitosan at various degrees of substitution. PEG-chitosan (PEG-chi) was prepared as shown in Scheme 1. In summary, 100 mg of chitosan was dissolved in 2% CH_3COOH by mixing overnight at room temperature. The following day, the pH was adjusted to 6.0–6.3 with sodium hydroxide (1 N). mPEG–NHS was then added stepwise to the chitosan solution, and the reaction was conducted for 24 h at room temperature. The crude product was extensively dialyzed against de-ionized water (ddH_2O), using a cut off dialysis membrane of 50 kDa for 48 h. This process was followed by freeze drying.

2.3. Characterization of chitosan–g–PEG

The DD or percentage of free amine groups ($-NH_2$) on chitosan, and degree of substitution (DS) of PEG to chitosan were calculated based on ^{1}H NMR spectra of chitosan and PEG-chi, which



Scheme 1. Synthesis of PEG-chitosan. Deacetylated chitosan was modified at pH 6.0 using NHS–methoxy PEG at different degress of substitution.