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REVIEW

Enhancing gene knockdown efficiencies by comparing siRNA-Loaded cationic nanogel particles of different sizes

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In order to silence the expression levels of pathogenic genes, small interfering RNA (siRNA) requires a nano-sized carrier for its safe and stable delivery into cells. In this research highlight, we focus on well-defined cationic nanohydrogel particles developed in our group for such purposes. To investigate the nanogels' mechanism for enhanced knockdown efficiencies, we recently synthesized two sets of particles with similar material composition and siRNA-loading characteristics, but – according to the manufacturing process – of different sizes. Within this study, 100-nm-sized nanogel particles loaded with siRNA accumulated inside the lysosomes already after 4 h and could not induce any gene knockdown, while 40-nm-sized particles were able to avoid lysosomal accumulation, and instead, generated moderate gene knockdown levels lasting for about three days. We believe that in analogy to other reports this size-dependent intracellular distribution behavior might be an essential key parameter for tuning the knockdown efficiency of the nanogel carriers. Moreover, these results might further contribute to the development of advanced siRNA carrier systems in order to enhance RNAi's translation into the clinics.

Keywords: siRNA delivery; cationic nanogel; size-dependent gene knockdown

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Already very early after its discovery RNA intereference (RNAi) [1, 2] was considered as promising therapeutic alternative for the treatment of various pathogenic genes [3, 4]. However, due to its physicochemical properties small interfering RNA (siRNA) cannot be applied as a drug systemically: Besides rapid enzymatic degradation or renal clearance the negatively charged phosphodiester backbone prevents the double-stranded oligonucleotides from cellular uptake and release into the cytosol, where an enzymatic protein machinery can be recruited for sequence specific mRNA degradation [5].

Influenced by the thriving research on nanomedicines,[6] several delivery approaches have been developed throughout the last decade progressing RNAi to be translated into the clinics [7]. Among various concepts proceeding in market or final development, nanohydrogel particles have gained more attraction recently [8]: They are pre-defined in size and shape but independent from their siRNA payload and can, thus, act as improved siRNA transporter with enhanced stability properties under physiological relevant conditions.

For such purposes, our group developed a novel synthetic approach towards cationic nanohydrogel particles for siRNA delivery by combination of controlled radical polymerization of reactive precursor block copolymers, their spontaneous self-assembly into nano-sized micellar aggregates and subsequent core cross-linking to install cationic charges for

siRNA complexation [9]. The obtained nanogels do not change their morphology after loading with siRNA but facilitate their uptake into cells over time properly. Moreover, the nanoparticles' stability under biologically relevant conditions could carefully be investigated by dynamic light scattering in human blood serum as well as by intravital video microscopy in the bloodstream of mice [10].

More recently, we paid detailed attention to the nanohydrogel's ability of mediating gene knockdown in an *in vitro* assay by its siRNA payload [11]: For that purpose two sets of well-defined cationic nanohydrogel particles were generated that – according to the manufacturing protocol – provided nanogels of similar material composition, yet, a difference in size was obtained by introducing precursor block copolymers of same block ratio but different molecular weight. Consequently, particles with diameters of about 40 nm and 100 nm were generated, which provided similar siRNA loading as well as release capabilites. Interestingly, no difference in cell viability was monitored when they were applied to luciferase-expressing HeLa cells at different nanogel concentrations (loaded either with or without siRNA targeted against luciferase as model gene). However, different results were found for the luciferase expression levels by measuring the cells' relative luminescence. Only the 40-nm-sized nanohydrogel particles seemed to induce a siRNA-mediated moderate gene knockdown (Figure 1), while for the 100-nm sized nanohydrogel particles no difference in luciferase activity was found (Figure 2). This knockdown potential could be verified by real time luminescence monitoring for the 40-nm-sized particles, too. A significant gene knockdown of could be detected compared to control nanogel particles loaded with scramble siRNA [11]. Although the knockdown efficiencies were moderate, they seemed to be quite stable and continuing over time only for the small-sized particles.

To explain the exclusive knockdown potential for cationic nanohydrogel particles of similar composition but different size, cell uptake and lysosomal colocalization studies were performed in HeLa cells over time, too. Thereby, we observed that both particles provided similar uptake

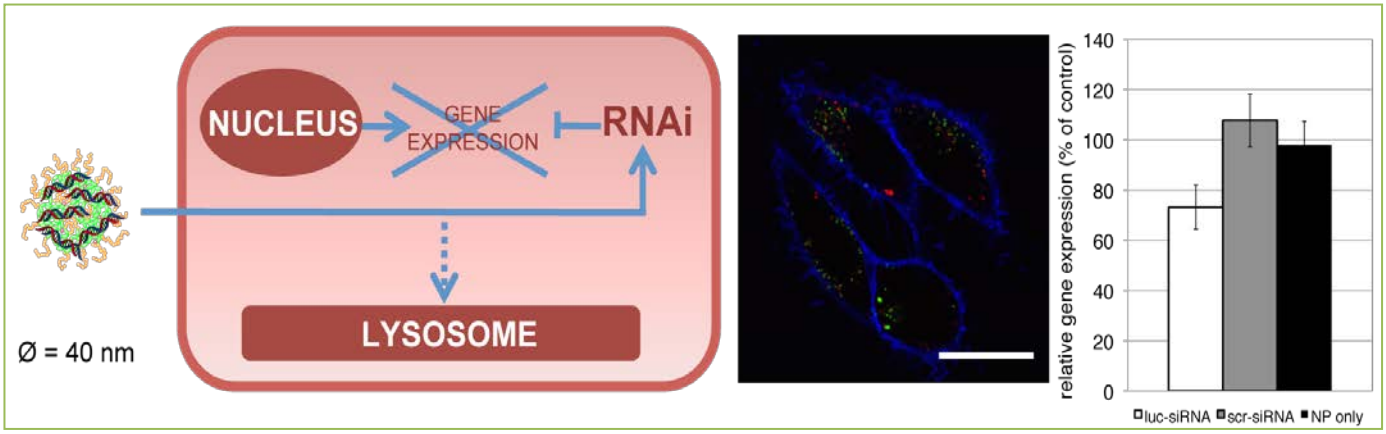

Figure 1. SiRNA-loaded cationic nanohydrogel particles with diameters of about 40 nm can avoid accumulation inside lysosomes but induce siRNA-mediated gene knockdown. Left: Schematic mechanism. Middle: Confocal image of HeLa cells (cell membranes in blue and lysosomes in red) incubated with cationic nanohydrogel particles (green) – scale bar = 25 µm. Right: Endogenous luciferase gene silencing in HeLa-Luc cells after incubation for 48 h with 400 nM siRNA. Adapted with permission from reference [11]. Copyright 2014, American Chemical Society.

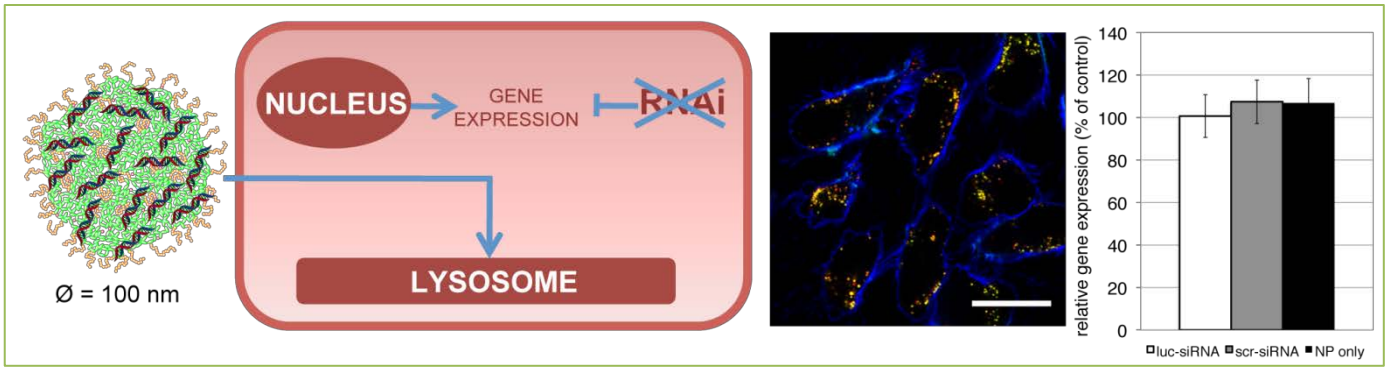

Figure 2. SiRNA-loaded cationic nanohydrogel particles with diameters of about 100 nm accumulate inside lysosomes and, thus, cannot induce siRNA-mediated gene knockdown. Left: Schematic mechanism. Middle: Confocal image of HeLa cells (cell membranes in blue and lysosomes in red) incubated with cationic nanohydrogel particles (green) – scale bar = 25 µm. Right: Endogenious luciferase gene silencing in HeLa-Luc cells after incubation for 48 h with 400 nM siRNA. Adapted with permission from reference [11]. Copyright 2014, American Chemical Society.

efficiencies, yet, their intracellular distribution differed again significantly. While the 100-nm-sized particles accumulated inside lysosomes already after 4h (Figure 2), most of the 40-nm-sized particles seemed to avoid these compartments even after incubation for 24h (Figure 1) [11]. In conclusion, the nanohydrogel particles' knockdown efficiency can directly be correlated to less lysosomal colocalization. In that context, the nanogels' size already seems to be an essential key parameter for affecting their intracellular trafficking and distribution.

Avoiding cellular uptake pathways that are subsequently directed to lysosomal compartments, where oligonucleotides can be degraded rapidly, are generally considered to be one of the major bottlenecks in siRNA delivery. Moreover, by methods to retain siRNA inside endosomal compartments Sahay et al. could recently enhance the gene silencing potential effectively [12, 13]. Consequently, increased siRNA residence time in non-lysosomal compartments might contribute to a slow, controlled diffusion of the oligonucleotide from such reservoirs into the cytosol, followed by robust gene knockdown. We assume that the small-sized cationic nanohydrogel particles presented in our last study [11] might have found access to an cellular uptake pathway that avoids lysosomal accumulation. Among several endocytotic uptake mechanisms some are well-known to avoid vesicle fusion with lysosomes (e.g. caveolin-mediated endocytosis [14]). As a result, the internalized particles can be stored in non-acidic, non-degrading cellular compartments, where the carriers act as reservoirs for slow, long-lasting release of their payload. So far, the nanohydrogel particles do not provide any membrane-disruptive elements, which might increase their penetration into the cytosol for better access to the RNAi-machinery. This, however, might on the other hand also induce decreased cell viability, which could not be found so far for the nanogel carriers at the current stage. The moderate silencing efficiencies observed for the 40-nm-sized nanogel carriers are probably due to their high siRNA-complex stabilities under physiological relevant conditions [10]. To that respect, we are currently focusing on advanced cationic nanohydrogel systems with stimuli-responsive groups that allow side-specific carrier degradation as well as efficient siRNA release. As an example, we recently introduced novel reductive-degradable cationic nanohydrogel particles that showed both redox-triggered particle degradation into its polymer fragments as well as release of complexed siRNA payload under reductive conditions of the cytosol [15].

In analogy to previously reported studies, we assume that the size-dependent knockdown efficiency, which we found in our study for the 40-nm-sized cationic nanohydrogel particles compared to the 100-nm-sized carriers exclusively [11], might open possibilities to improve the knockdown potential of other siRNA delivery systems, too, by simply addressing subcellular uptake pathways that avoid lysosomal accumulation. This might perhaps already be accessible for carrier systems of smaller sizes (e.g. around 40 nm). To that respect, we believe that our observations could also contribute to the development of further advanced siRNA carrier systems, which may enhance RNAi's translation into the clinics.

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