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Effect of Nanoparticle Conjugation on Gene Silencing by RNA Interference

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RNA interference (RNAi) is a cellular process whereby the silencing of a particular gene is mediated by short RNAs. One type is mediated by short interfering RNAs (siRNA) in which the antisense strand of a double-stranded RNA duplex guides recognition and catalytic degradation of a target mRNA by the RNA-induced silencing complex (RISC). Another is mediated by endogenous ~20−25nt short RNAs known as microRNAs (miRNAs) that either repress translation and/or enhance degradation of target mRNAs. There has been tremendous interest in advancing the fundamental understanding of both pathways and harnessing them for therapeutic applications by delivering short RNAs into cells to control gene expression; however this delivery has been challenging.[1] To achieve successful gene silencing using siRNA, several key delivery requirements must be met: the siRNA must survive degradation in the extracellular milieu, be transported to the cell surface, cross the cell membrane, and ultimately enter RISC where unwinding and pairing of the antisense strand with native mRNA occur.

In vivo, free siRNA is too rapidly cleared through the kidney to be effective; thus a variety of carriers have been explored that extend its circulation time and aid in trafficking to the site of disease. Over the past decade, systematic investigations by several groups to address this siRNA delivery challenge have started bearing fruit.[2−9] A plethora of cationic polymers (including lipids) and nanoparticles (e.g., magnetic, quantum dots, gold and carbohydrate nanoparticles) have been shown to deliver siRNAs resulting in silencing of specific genes both *in vitro* and *in vivo*.[7,10−12] As a result, methods for complexation/conjugation of siRNA with various delivery agents and a better understanding of the delivery process have emerged.[10,13] Both charge−charge complexation of cationic polymers with anionic siRNAs and covalent coupling of siRNAs with polymers or nanoparticles have been shown to be effective in delivering siRNAs.[10]

Nonetheless, reports describing the effects of nanoparticle conjugation on RISC incorporation and subsequent gene silencing have been mixed.[14] Moreover, it is unclear how the length of linker between nanoparticle and siRNA, direction of conjugation (3′ vs 5′), and strand used for conjugation (sense vs antisense) affect the relative extent of gene silencing for a given nanoparticle system. For example, a high level of gene silencing was observed *in vivo* using a nanoparticle-siRNA conjugate when the antisense strand was conjugated to the nanoparticle *via* a thioether nonlabile bond[15] while other reports suggest that a labile cross-linker forming a disulfide bond leads to greater silencing in comparison with a nonlabile amide bond forming cross-linker.[16] In another report, Dai et al. showed that a labile disulfide bond based carbon nanotube-siRNA conjugate leads to greater gene silencing in comparison with a nonlabile nanotube-siRNA conjugate.[17] Elsewhere, it has been reported that chemical modification of the 5′- terminus of the antisense strand can limit RNAi activity.[18,19] Still, nanoparticles conjugated with the 5′ antisense end of siRNA have been shown to cause effective gene silencing.[7,15] To reconcile these seemingly disparate findings, we embarked on a systematic evaluation of siRNA coupling strategies using a single nanoparticle system, cell type, and target gene.

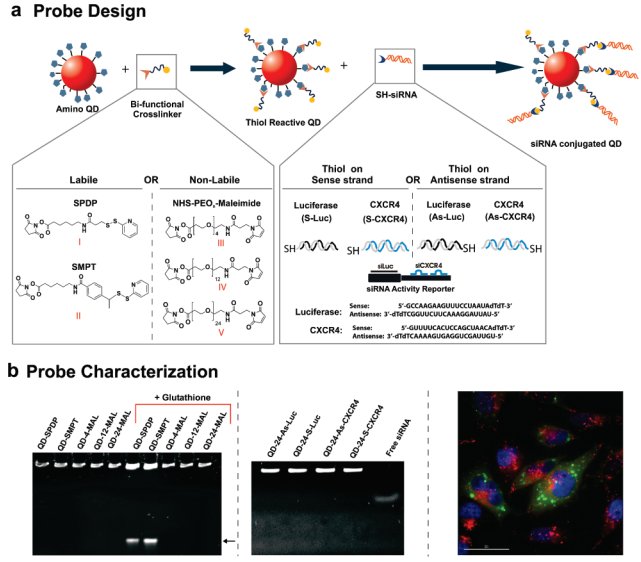

Figure 1. Probing the effect of conjugation strategy on gene silencing by QD-siRNA conjugates. (a) Scheme for probe synthesis. (b) Characterization of the probes. (Left) Gel electrophoresis of QD-siRNA conjugates. Conjugation with labile cross-linkers (SPDP and SMPT) releases the conjugated siRNA upon treatment with glutathione. Arrow indicates free siRNA. (Middle) Gel electrophoresis of QD-siRNA with nonlabile maleimide cross-linker indicating the absence of unbound siRNA. (Right) Intracellular delivery of QD-siRNA conjugates by electroporation in modified HeLa (GFP-Ago2/Luc-CXCR4) cells. QD-siRNA conjugates are in red, green is Ago2-GFP, and the nuclei are stained with DAPI (blue). Scale bar is 30 μm.

Here, we present a systematic study utilizing a single nanoparticle system to investigate the effect of siRNA-nanoparticle conjugation on gene silencing (Figure 1a). We studied gene knockdown (KD) by siRNAs that are covalently coupled to the surface of a nanoparticle *via* their sense or antisense strand using a labile (Figure 1a, I and II) or nonlabile (Figure 1a, III−V) cross-linker of varying lengths. We chose quantum dots as a model nanoparticle system

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due to their excellent photoluminiscent properties providing the ability to be monitored *via* optical imaging.[20] The sense strand (S-siRNA) or the antisense strand (As-siRNA) of thiol-modified siRNAs was coupled with the amines on QD655-PEG-NH_2 *via* labile disulfide forming sulfosuccinimidyl 6-(3′-[2-pyridyldithio]-propionamido) hexanoate (SPDP) and sulfosuccinimidyl 6-[α-methyl-α-(2-pyridyldithio)toluamido] hexanoate (SMPT) or *via* nonlabile thioether forming succinimidyl-[(*N*-maleimidopropionamido)-$_n$ethylene glycol] ester (NHS-PEO_n-Maleimide). After conjugation the QD-siRNA conjugates were purified and analyzed for siRNA release, purity, and cytosolic distribution (Figure 1b). To ensure the release of siRNAs from the nanoparticles conjugated *via* labile cross-linkers, the conjugates were incubated in a glutathione concentration (10 mM) similar to intracellular levels and analyzed by gel electrophoresis. Glutathione was able to release siRNA from nanoparticles that had labile SPDP and SMPT as cross-linkers (Figure 1b, left). On the other hand, the nanoparticles with nonlabile maleimide cross-linkers (QD-4-Mal, QD-12-Mal, and QD-24-Mal) did not release the siRNA (Figure 1b, left) irrespective of the conjugation site (Figure 1b, middle). The amount of siRNA on the nanoparticles was quantified for all the samples by SYBR gold staining. The conjugation resulted in ∼3 siRNA per QD nanoparticle. The purity of the samples (free of unbound siRNA) was confirmed by electrophoretic, UV, and gene KD experiments (Figure 1b and Supporting Information).

The nanoparticle conjugates were delivered to the cytosol of modified HeLa cells (stably transfected with GFP-Ago2/Luc-CXCR4) by electroporation to avoid membrane interactions. Electroporation resulted in an association with most cells and a cytosolic distribution as observed by epifluorescent microscopy (Figure 1b, right). It has been shown earlier by our group that electroporation can be an efficient delivery scheme for QD conjugates into the cytosol without the loss of surface ligands.[21] The modified HeLa cell line stably expressing GFP-Ago2 and *Renilla* luciferase allowed testing the siRNA activity *via* two RNAi pathways: miRNA-mediated repression and siRNA-mediated catalytic cleavage of the targeted transcript. To examine the miRNA pathway, the 3′ untranslated region of the luciferase was modified by insertion of two partially complementary binding sites for a specific siRNA, siCXCR4 (Figure 1a, siRNA activity reporter). Functioning like an endogenous miRNA, the siCXCR4 binds in a bulged configuration, resulting in translational suppression and mRNA degradation of the targeted transcript.[22,23] On the other hand, a siRNA targeting the coding region of the luciferase, siLuc, was used to examine the efficiency in siRNA-mediated cleavage of the targeted transcripts in the same cell line. The degree of KD was assessed through luciferase activity after 48 h.

QDs with siRNA conjugated *via* labile cross-linkers SPDP (Figure 2a) and SMPT (Figure 2b) were able to KD the luciferase gene with high efficiency (>90%). This is probably due to release of siRNA from the nanoparticle driven by cleavage of the labile bond in the reducing intracellular environment[24] making siRNA available for incorporation into the RISC complex, which results in efficient gene KD consistent with the observation of Dai and co-workers with hydrophobic carbon nanotubes.[17] On the other hand, use of a short nonlabile cross-linker resulted in poor gene KD (Figure 2c) similar to what was observed by our group previously.[16] This is likely due to poor availability of siRNA for the RISC complex, driven by the steric hindrance of a large nanoparticle. We hypothesized that a longer tether may alleviate the steric hindrance and improve accessibility of siRNA on a nanoparticle. In a comparison of maleimide cross-linkers with a spacer arm length of 24.6 Å (PEO-4-Mal), 53.4 Å (PEO-12-Mal),

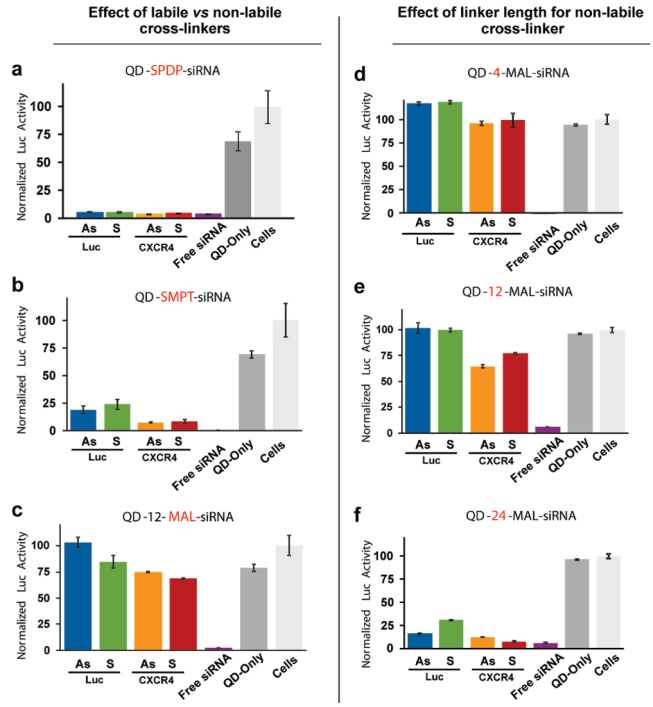

Figure 2. KD of luciferase by QD-siRNA conjugates with antisense (As) or sense (S) strand of siLuc and siCXCR4 conjugated ($n = 3$). KD by QD-siRNA conjugates with labile (a) SPDP, (b) SMPT, and short nonlabile (c) PEO-12-Mal. KD by nonlabile cross-linker of varying length (d) PEO-4-Mal, (e) PEO-12-Mal, (f) PEO-24-Mal.

and 95.2 Å (PEO-24-Mal), increasing the chain length improved the gene silencing efficiency from undetectable levels of KD for QD-4-Mal-siRNA (Figure 2d) to ∼30% for QD-12-Mal-siRNA (Figure 2e) and finally to >90% for QD-24-Mal-siRNA (Figure 2f). Thus, nonlabile cross-linkers can provide comparable efficiencies to labile cross-linkers under certain circumstances.

In comparing the effects of strand orientation, we found that luciferase activity was knocked down equivalently irrespective of whether the antisense or sense strand of siRNA was attached to the QD surface (Figure 2), suggesting that conjugation to the QD has little inhibitory effect on siRNA activity and that the site of conjugation may not be critical, which was also observed by Moore and co-workers with a different nanoparticle system.[15] Thus, having the sense strand conjugated and the antisense strand hybridized (thus easily released) does not offer a significant advantage over having the antisense strand conjugated and sense strand hybridized. Rather, the stability of the cross-linking bond and tether length were the dominant determinants of knockdown efficiency.

For *in vivo* applications, reducing agents in the bloodstream can release siRNA from the nanoparticle even before siRNA-nanoparticle conjugates are able to reach the cells. This would reduce the extent of KD due to low amounts of siRNA targeted to cells. Therefore, we investigated the stability (against disulfide bond reduction) and gene silencing efficiency of the siRNA-nanoparticle conjugates with the labile (SPDP and SMPT) and the longer nonlabile (PEO-24-Mal) cross-linker after exposure to serum. The siRNA-nanoparticle conjugates were incubated in 10% fetal bovine serum (FBS) and purified *via* filtration to remove free siRNA (released from the nanoparticle surface by serum). The purified nanoparticle conjugates were then analyzed by gel electrophoresis (Figure 3a) for the amount of siRNA that remained on the nanoparticle surface. After 8 h, all of the siRNA was released (by serum) from the QD-siRNA conjugates with labile cross-linkers SPDP and SMPT, as further treatment with glutathione did not release any

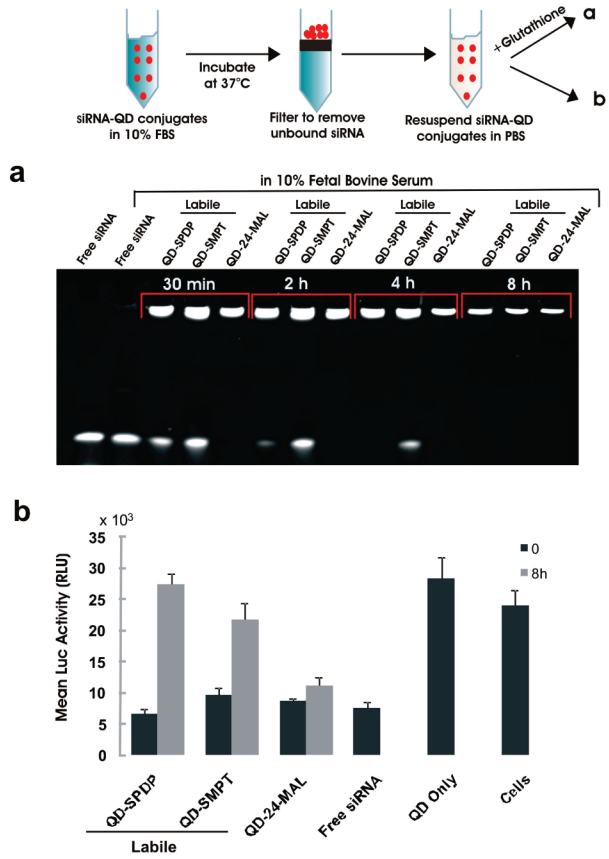

Figure 3. Performance of QD-siRNA conjugates with different conjugation chemistries after incubation in 10% FBS at 37 °C. (a) Gel electrophoresis of QD-siRNA showing the loss of siRNA after 8 h when conjugated with a labile cross-linker. (b) Comparison of the luciferase KD efficiency by QD-siRNA conjugates after incubation in 10% FBS.

siRNA. The release in serum was faster for the SPDP cross-linker (4 h) in comparison to SMPT (8 h), which is expected due to the less stable disulfide bond in SPDP. We analyzed the KD efficiency after incubating the conjugates for 8 h in 10% FBS and then removing the free siRNA from the samples. We observed that the QD-siRNA conjugates with labile cross-linkers (SPDP and SMPT) lost their KD efficiency after 8 h, whereas the conjugate with the nonlabile cross-linker (PEO-24-Mal) was able to maintain its activity and ability to efficiently KD luciferase (Figure 3b). Thus, nonlabile cross-linkers offer improved stability of the nanoparticle conjugates and do not compromise the KD efficiency as long as they are long enough to allow siRNA-RISC interaction without steric hindrance from the nanoparticle.

In conclusion, using a single nanoparticle system with varying conjugation schemes and model cell line, we have shown that the accessibility of the siRNA linked to the nanoparticle may be critical for efficient gene KD mediated by both siRNA and miRNA pathways. In this model system, the efficiency of KD is governed by the conjugation strategy (labile vs nonlabile) used for attaching the siRNA to the nanoparticle and the tether length in the nonlabile case. These findings were independent of the strand orientation. While our findings may be specific to a surface chemistry, target mRNA, and cell line, we believe these data provide a useful framework that can be used to guide siRNA conjugation strategies across different model systems to achieve efficient gene silencing.

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Supporting Information Available: Experimental details and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Effect of nanoparticle conjugation on siRNA gene silencing

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Materials. Quantum dots with emission maxima of 655 nm and modified with PEG and amino groups were obtained from invitrogen. QD concentrations were measured by optical absorbance at 490 nm, using extinction coefficients provided by the supplier. Cross-linkers used were sulfosuccinimidyl 6-(3-[2-pyridyldithio]- propionamido)hexanoate (SPDP), sulfosuccinimidyl 6-[α-methyl-α-(2-pyridyldithio)toluamido] hexanoate (SMPT) and non-labile thioether forming Succinimidyl-[(N-maleimidopropionamido)-$_n$ethylene glycol] ester (NHS-PEO$_n$-Maleimide) with n=4,12 and 24. All the crosslinkers were purchased from Pierce. Synthetic RNA duplexes directed against Luciferase and CXCR4 gene were synthesized (Alnylam), with the sense strand or the antisense strand modified to contain a 3' thiol group. The sequence for Luc siRNA used is 5'-GCCAAGAAGUUUCCUAAUAdTdT (sense strand) and 5'-UAUUAGGAAACUUCUUGGCdTdT (antisense strand). The sequence for CXCR4 siRNA used is 5'-GUUUUCACUCCAGCUAACAdTdT-3' (sense strand) and 5'-UGUUAGCUGGAGUGAAAACdTdT-3' (antisense strand).

Conjugation of siRNAs to QDs:

Amino-modified QDs were conjugated to thiol-containing siRNA using SPDP, SMPT and NHS-PEO$_n$-Maleimide (n= 4, 12 and 24) cross-linkers. QDs were resuspended in RNAse free 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.2, using Saphadex G-25 gravity column (NAP-5, GE Healthcare). Cross-linker (1000-fold excess) was added to QDs and allowed to react for 2 h. Samples were filtered on a NAP-5 column (to remove excess cross-linker) into similar buffer supplemented with 10 mM EDTA. siRNA was treated with 0.5 M DTT for 1 h and filtered on a NAP-5 column into EDTA-containing buffer. A 10-fold molar excess of siRNA was

added to the filtered QDs and allowed to react overnight at 4 °C. Using Amicon filters, product was filtered twice with Dulbecco's phosphate buffered saline (PBS), twice with a high salt buffer (1.0 M sodium chloride, 100 mM sodium citrate, pH 7.2) required to remove electrostatically bound siRNA. The samples were filtered several times (~12) with PBS until the supernatant showed no free siRNA by UV-Vis spectroscopy (Figure S1a). The conjugation of siRNA to the QD was confirmed and quantified using gel electrophoresis and Fluorescence spectroscopy. The samples were incubated with 10 mM Glutathione for 2h at 37 °C and run on a precast 15% TBE gel (BioRad Laboratories) at 80 mV for 1 h. The siRNAs in the gel were stained with SYBR Gold (Invitrogen Inc., Carlsbad, CA). To quantify the number of siRNA on QD, the samples were run along with free siRNA standards on the gel. The number of siRNA was found to be similar for both the labile (SPDP and SMPT) cross-linkers (Figure S1b). Since, the siRNA on the QDs with non-labile cross-linkers cannot be reduced by glutathione, to confirm and quantify the amount of siRNA on the QD with NHS-PEOn-Mal cross-linkers were stained with SYBR Gold and measured with a fluorimeter (SpectraMax Gemini XS, Molecular Devices). Similar loading of siRNA was observed for both non-labile (Mal-12 and Mal-24) cross-linkers (Figure S1c). To assess the purity of the QD-siRNA conjugates with non-labile cross-linkers knockdown experiment (Figure S2) was performed with the filtrate obtained after the tenth centrifugation cycle of the non-labile samples.

Cell Culture:

The knockdown experiments were performed on HeLa(EGFP-Ago2) cell line generated as described previously. Growth media was Dulbecco's modified Eagle's medium (DMEM)

containing 4.5 g/L glucose and supplemented with 10% FBS, 500 ng/mL puromycin and 600 μg/ml G418 for selection. One day before transfection, cells were seeded at 5 x 10^5 cells/well in a 6-well plate such that they would be 95% confluent at the time of transfection. For transfection, QD-siRNA conjugates, free siRNA (5 μM) and non-conjugated QD were electroporated by using I-13 protocol (Amaxa, Gaithersburg, MD). As transfection efficiency is affected by the concentration of siRNA, we optimized the concentration of free siRNA that gives the most efficient transfection by this method of delivery (electroporation). The dose-dependent relationship between the Luciferase gene silencing and the siRNA concentration can be seen in Figure S3. A final QD concentration of 20 nM was used per well for each sample. The medium was changed 4 h post transfection, and cells were incubated for another 44 h before they were washed with PBS, lysed with passive lysis buffer (Promega), and assayed for luciferase expression with a Dual-Glo luciferase assay kit (Promega) on a single tube luminometer (GloMax, Promega). The cell lysates were also used for measuring the total protein concentration for each sample using the Bradford reagent (Bio-Rad, Hercules, CA, USA). Since the efficiencies of the free siRNA (5 μM) and 20 nM QD-MAL-siRNA (60-80 nM siRNA concentration) are not directly comparable six different concentrations of free CXCR4 siRNA and 60-80 nM of the QD conjugated siRNA (S-QD-4-CXCR4 and S-QD-24-CXCR4) were electroporated in the modified Hela cells. Assessment of the luciferase activity after 48 h of transfection revealed that the knockdown due to the QD-conjugated siRNA was similar to the knockdown obtained by free siRNA at the concentration of 100 nM. The results indicate that the conjugates (S-QD-24-CXCR4) have comparable or marginally higher silencing efficiency compared to the free siRNA at similar concentrations.

Stability of QD-siRNA conjugates in serum:

The QD-siRNA conjugates (20 nM) were incubated in 10% FBS for specified amount of time at 37 °C. The samples were then centrifuged using Amicon filters (MWCO 30,000) twice to remove all the free siRNA (not remaining on the NP) from the sample after serum exposure. The clean samples were then used for visualizing the siRNA remaining on the QD and to perform knockdown experiments with the conjugates after serum treatment. For visualizing the siRNA remaining on the QD (after serum treatment), the filtered samples were further incubated with 10 μM glutathione for 2h at 37 °C and analyzed by gel electrophoresis (15% TBE gel) and SYBR gold staining as described above. The knockdown experiments with filtered-serum-treated QDs were performed using a similar protocol as described above.

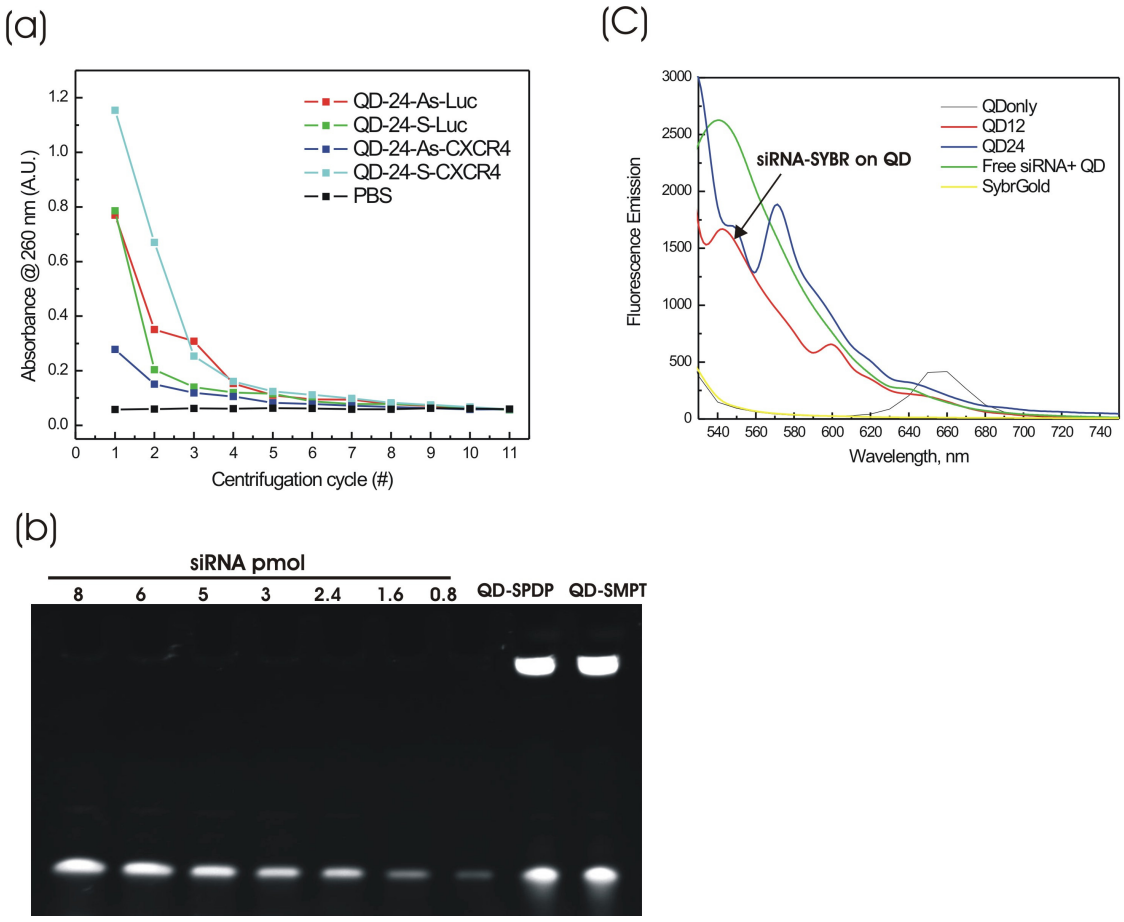

Figure S1. (a) Purification of QD-siRNA conjugate by centrifugation. The removal of free siRNA was monitored in the sample by measuring the absorbance of the filterate after each cycle at 260 nm. (b) Amount of siRNA conjugated to the QDs using labile cross-linkers. (c) Presence of siRNA on QD-siRNA conjugates with non-labile cross-linkers.

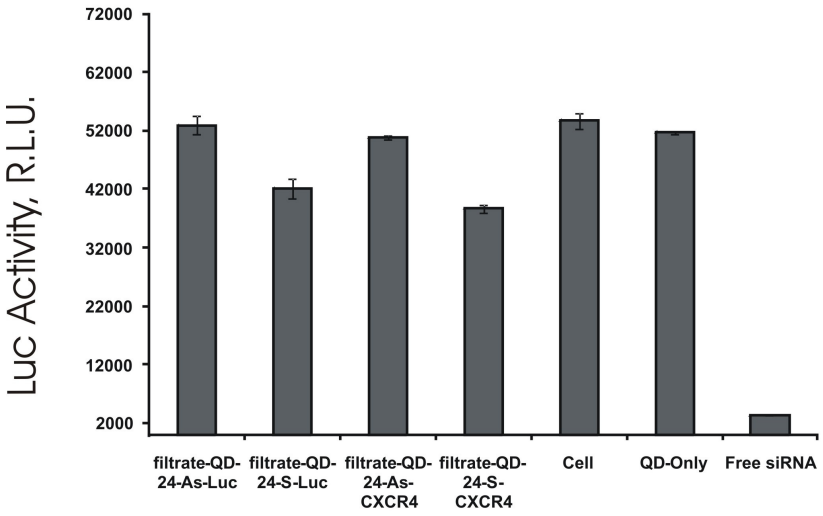

Figure S2. Luciferase knockdown by the filtrate obtained from the non-labile sample purification cycle. This demonstrates that the filtration process results in highly pure siRNA conjugated nanoparticles and there is no free siRNAs in the nanoparticle solution, hence the KD obtained by the nanoparticles is due to the conjugated siRNA.

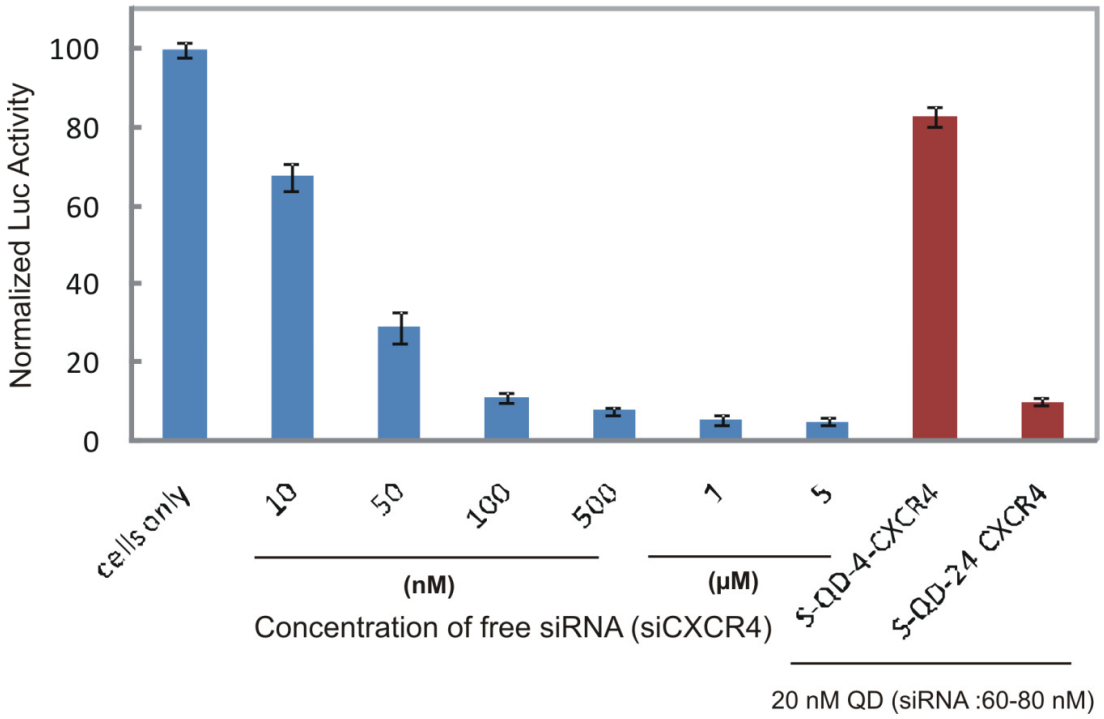

Figure S3. Luciferase knockdown by various concentrations of free siCXCR4 and 20 nM S-QD-4-CXCR4 (siRNA concentration of 60-80 nM). This indicates that the conjugates (S-QD-24-CXCR4) have comparable or marginally higher silencing efficiency compared to the free siRNA at similar concentrations.