A Layer-by-layer Gene Therapy Approach for Promoting Exogenous and Inhibiting Endogenous Protein Expression

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Statement of Purpose: Multi-functional nanoparticles which enable imaging and diagnostics, in addition to delivering therapeutics are needed. Gold nanoparticles (AuNP) are capable of being imaged both *in vitro*, *ex vivo* and *in vivo*, are easily chemically modifiable, and have interesting optical properties for colorimetric diagnostics, as well as photothermal therapy. As the root cause of cancer and diseases are genetic, the potential pay-off for successfully and safely modifying gene expression is tremendous. However, delivering macromolecules capable of positively and negatively regulating gene expression such as DNA and short interfering RNA (siRNA), respectively, remains a challenge due to their size and charge. We sought to co-deliver both DNA and siRNA using a modifiable charge-alternating layer-by-layer (LbL)[1-3] technique constituting AuNPs and two polymers which degrade mechanistically differently via hydrolysis (ester bonds via water) and intracellular reduction upon cytosolic entry (disulfide bonds via glutathione). Co-delivery could promote apoptosis and inhibit cell migration as an anti-cancer therapeutic or rectify a disease by replacing an aberrant protein.

Methods: The disulfide-containing poly(amido amine) (SS37)[4] and the poly(beta-amino ester) (447)[5] polymers were synthesized as previously reported and characterized via gel permeation chromatography. The AuNPs were synthesized via the Frens method (CAu) and were thiolated with carboxyl groups (MAu; confirmed by surface plasmon resonance (SPR) shift). Polyelectrolyte solutions were added in successive order to the AuNPs: polyethyleneimine, dsRed plasmid DNA, SS37, anti-eGFP siRNA, and 447. The diameter and the zeta potential were assessed using nanoparticle tracking analysis (NTA) software (NanoSight NS500) and dynamic light scattering (Malvern Zetasizer), respectively. Aggregation was assessed using transmission electron microscopy (TEM) at each subsequent layer. DNA and siRNA content was quantified using nucleic acid-specific intercalating dyes, namely Picogreen® and Ribogreen®, respectively, which was subsequently used to calculate the polymer mass to nucleic acid mass (weight/weight or w/w) values. Knockdown was assessed in human glioblastoma multiforme cells *in vitro* derived from brain tumor cells (79 year old patient) stably expressing GFP on days 1-7 using a fluorescence plate reader (Synergy2). Fluorescence microscopy (FM; Zeiss) and flow cytometry (Accuri) was used to assess dsRed expression on day 2 and GFP knockdown on day 7. Cytotoxicity was accomplished using an MTS cell titer (CellTiter 96®) assay. Expression and knockdown was compared to Lipofectamine® 2000. TEM was used to assess cellular uptake at 2 hours using fixed, dehydrated and Epon-infiltrated cells.

Results: The 17 ± 2 nm AuNPs when carboxylated had a 6 nm SPR redshift. The SS37 and 447 had a number-average molecular weight of 2.5 and 10.2 kDa. According to NTA and TEM the largest aggregation occurred at the DNA layer and resulted in approximately 200 nm particles (**Fig. 1**; top row). The zeta potential after multiple washings at each layer was reversed (**Fig. 1**; bottom left). Two dosages of DNA and siRNA per well were delivered and are referred to as the high and low doses (HD and LD). The HD and LD formulations contained 300 ± 40 and 200 ± 20 ng of DNA and 240 ± 10 and 160 ± 9 ng of siRNA, respectively. The DNA and siRNA w/w values ranged from 14-83 and 17-104. According to the plate reader, the LD and HD formulations peak knockdown days were 5 and 6, respectively. The knockdown ranged from 0% to 44 ± 5% and near 0% to 34 ± 3% according to the plate reader and flow cytometry. The higher w/w values were associated with stronger knockdown for each of the dosages assessed in all cases. The FM images of the HD 240 ng siRNA formulation at 69 w/w on day 7 is shown in **Fig. 1** (bottom right).

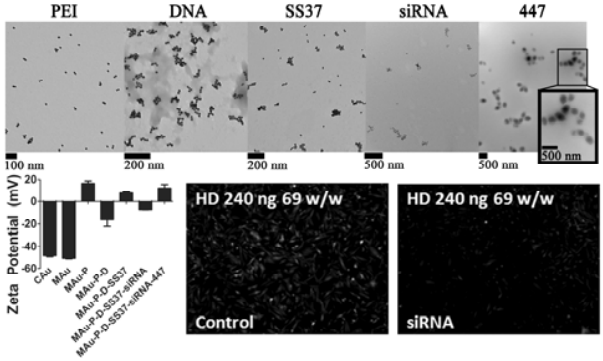

Figure 1. Top row: TEM at each LbL layer; bottom left: ZP at each layer (P=PEI; D = DNA); bottom right: FM image of HD 240 ng siRNA at 69 w/w

Lipofectamine at comparable dosages were optimized and found to peak at 20 ± 2% and 25 ± 7% according to the flow cytometry and plate reader, respectively. Expression peaked on day 2 and increased with increasing dosages and w/w, ranging from near 0 to 10.8 ± 0.5% which was inferior to Lipofectamine (14 ± 2% at a 100 ng dosage). The relative metabolic activity according to the MTS assay ranged from 0.73 ± 0.04 to 0.91 ± 0.06.

Conclusions: We have successfully shown that siRNA and DNA can be co-delivered using AuNPs and two polymers which degrade mechanistically differently to human glioblastoma multiforme using an easily modifiable LbL technique. This proof of concept is a theranostic enabling platform technology.

References: [1] Elbakry A. Small 2012;8:3847. [2] Lee SK. Small 2011;7:364. [3] Lee JS. Nano Lett. 2009;9:2402. [4] Lin C. J Control. Release 2008;126:166. [5] Bishop CJ. J. Am. Chem. Soc. 2013;135:6951. [6] Frens G. Nature-Physical Sciences 1973;241:20.

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