Hemocompatible pH-responsive polymeric nanoparticle for intravenous siRNA Delivery

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Statement of Purpose: Small interfering RNA (siRNA) is emerging as a powerful therapeutic approach for potent, gene-specific silencing [1] but clinical use of this promising class of drugs hinges on the development of safe and effective delivery technologies. Key siRNA carrier design criteria include serum stability, extended blood circulation times that allow for targeting (i.e. tumor EPR), and mechanisms for endo-lysosomal escape. Traditional cationic polymers, such as PEI, are effective for gene delivery *in vitro* because they form polyplexes with positive surface charge that increase interactions with anionic cell membranes and trigger endocytosis [2]. However, polycationic carriers delivered IV suffer from non-specific interactions and aggregation with serum proteins and red blood cells, preferential biodistribution to the capillary bed of the lungs, and acute toxicity. In this work, we have used RAFT polymerization to synthesize a small library of copolymers designed to identify an optimal polymer that: condenses siRNA into serum stable nanoparticles (NPs), forms a PEG corona that minimizes interaction with blood cells and serum proteins, and displays pH-dependent membrane disruptive activity fine-tuned to mediate escape from endo-lysosomal vesicles.

Methods: A series of 12 poly(EG_{5kDa}-*b*-DMAEMA-*co*-BMA) polymers was synthesized by RAFT polymerization. In this design, the DMAEMA was intended to initiate electrostatic siRNA condensation and formation of polyplex NPs with a PEG corona. The BMA was intended to increase hydrophobicity in the polyplex core in order to enhance NP stability and to enable fine-tuning of pH-dependent membrane disruptive behavior for endosomal escape. All polymers were characterized for molecular weight, polydispersity, and composition by GPC and NMR, respectively. Optimal formulation conditions for siRNA packaging were determined by gel electrophoresis. DLS, TEM, and ζ-potential were used to confirm the size, morphology, and surface charge of the particles. NPs were characterized for pH-dependent membrane disruption using a hemolysis assay where RBCs model the endosomal membrane. NP uptake, gene knockdown of the model gene luciferase, and cytotoxicity were assessed *in vitro*. To investigate endosome escape *in vitro,* confocal microscopy was performed to assess the degree of colocalization of fluorescently labeled siRNA with the endosome and lysosome staining Lysotracker® (Invitrogen). Finally, a new Förster Resonance Energy Transfer (FRET)-based assay was developed where NP stability and nonspecific interaction with RBCs were assessed in human whole blood using NPs dual-loaded with two siRNAs containing FRET-paired fluorophores.

Results: A library of 12 polymers was screened, and the 50% BMA polymer (18.7 kDa, PDI=1.04) performed optimally. This polymer was stable at physiologic pH (7.4) but destabilized and became membrane disruptive at acidic endosomal pH, as demonstrated by a hemolysis assay (**Fig 1A**). Cell viability was preserved at all doses measured (<300nM siRNA). Cell uptake of the novel, PEG-shielded NPs was lower than commercial standards, but pronounced gene silencing was observed (**Fig 1B**), suggesting efficient endosomal escape of endocytosed NPs. To this end, confocal microscopy indicated efficient endo-lysosomal escape of the NPs based on lack of colocalization with Lysotracker in human breast cancer cells (**Fig. 1C**). When incubated in human whole blood, a significantly higher ($p<0.01$) percentage of the NPs remain in the serum relative to commercial reagents that nonspecifically partitioned more into the RBC fraction. (**Fig. 1D**). In addition, the NPs had significantly better serum stability based on retaining 77% of the FRET signal compared to 35% ($p<0.01$) in commercial reagents. We anticipate that these results are predictive of hemo-compatibility/stability and long-circulation time *in vivo*.

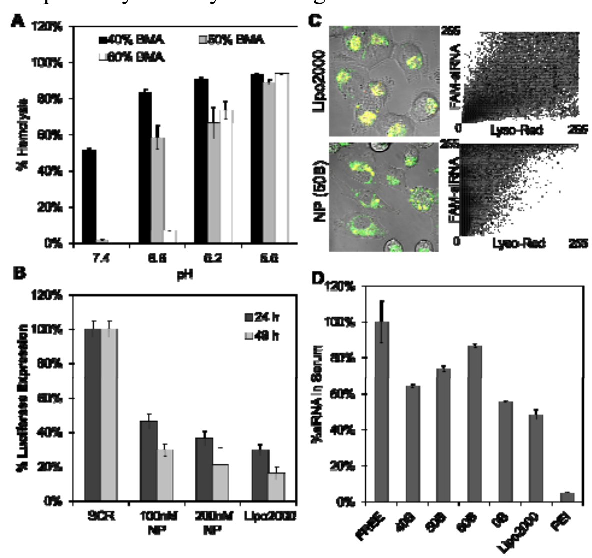

Fig. 1 – (A) NPs disrupt RBCs at acidic but not physiologic pH, (B) mediate protein-level silencing of a model gene, (C) avoid colocalization with endosomes relative to Lipo2000, and (D) improve serum retention of siRNA indicating stability and inertness in whole blood.

Conclusions: We have synthesized and screened a library of pH-responsive polymers for formulation of *in vivo*-ready siRNA nanocarriers. The library was designed in order to optimize pH-dependent membrane disruptive activity for endosomal escape and stable siRNA packaging through balancing cationic and hydrophobic content of a core-forming block. PEG was used in the corona to impart hemocompatibilty and long circulation times. The most optimal polymer formulation is highly efficient at siRNA cytoplasmic delivery once internalized. Future work will investigate EPR-driven delivery to tumors *in vivo* and incorporation of cell-specific targeting ligands.

References: [1] Fire A et al. Nature. 1998;391:806-11
[2] Oh YK et al. Adv Drug Deliv Rev. 2009;61:850-62