THE *IN VITRO*, *EX VIVO*, AND *IN VIVO* EFFECT OF POLYMER HYDROPHOBICITY ON CHARGE-REVERSIBLE VECTORS FOR SELF-AMPLIFYING RNA

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Background: RNA vaccines have surfaced over the last decade as a cost-effective, readily manufacturable vaccine platform able to rapidly respond to any emerging disease threat. They elicit immunity by utilising synthetic RNAs encoding for a specific antigen, hijacking the host cells protein synthesis machinery to trigger vaccine production *in vivo*. However, due to its susceptibility to endogenous nucleases and negative charge, RNA must be packaged and delivered to ensure maximum antigen expression upon administration. Cationic polymers represent a commercially scalable and chemically versatile vector platform, but conventional structure-property relationships found in other gene therapy platforms may not be fully translatable to the unique vaccine setting (administration route, immunogenicity requirements etc.). In this work, we systematically evaluated the effect of polymer hydrophobicity on the expression of a model self-amplifying RNA vaccine in appropriate *in vitro*, *ex vivo* and *in vivo* models. The polymer platform chosen exhibits charge-reversal through self-activated hydrolysis to induce RNA release during delivery.

Methods: A library of six copolymers (P1-6) DP50 containing 50% cationic monomer and varied the content of hydrophobic and hydrophilic monomers to tune polymer lipophilicity were synthesised via RAFT polymerisation. Polyplex formulation was optimised with both saRNA and model DNA and the size and zeta potential measured. Transfection studies were performed *in vitro* on HEK293T cells, in an *ex vivo* skin model and *in vivo* after intramuscular injection using saRNA encoding for luciferase or saRNA encoding for GFP.

Results: All six prepared polymers showed excellent molecular weight control and narrow molar mass distributions ($Đ$ < 1.3; Table 1). When formulated with DNA, polyplexes with above than 20% HEA content hydrolysed significantly faster than more hydrophobic analogues evidenced by the negative zetapotential (-10 mV) and low particle size over the 7 d experiment in pH 7 buffer (Figure 1). Transfection studies revealed that more hydrophobic delivery vectors (P1 & 2) exhibited 30 fold higher activity that hydrophilic vectors and PEI *in vitro* albeit with relatively high cytoxicity. When applied *in vivo* however, the hydrophilic vectors (P6) performed best. Surprisingly, we observed that charge reversal plays a small role, and transfection efficiency is largely driven by membrane interaction.

Conclusions: Overall our results indicate that *in vitro* and *ex vivo* transfection follows the expected hydrophobicity trend compared to the literature, however *in vivo* we observed the opposite trend likely due to the increased toxicity of more hydrophobic vectors. We anticipate these findings will help direct future design of polymeric materials designed to efficiently delivery self-amplifying RNA vaccines.