Matrix metalloproteinase 2-sensitive multifunctional polymeric micelles for tumor-specific co-delivery of siRNA and hydrophobic drugs

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ABSTRACT SUMMARY

To deliver siRNAs and hydrophobic drugs, a novel matrix metalloprotease 2 (MMP2)-sensitive self-assembling copolymer, polyethylene glycol-polyethylenimine-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PEG-PEI-PE), has been developed. The micelles formed by PEG-PEI-PE possess several key features for siRNA and drug delivery, including (i) stability in physiological fluids thanks to the outer PEG "shell"; (ii) complexation of siRNA by PEI; (iii) drug-solubilization in the lipid "core"; (iv) passive tumor targeting via the EPR effect; (v) tumor targeting triggered by the MMP2-sensitivity; and (vi) tumor-cell selective endocytosis after MMP2-activated exposure of the previously hidden PEI block. (**Fig. 1**) These cooperative functions will ensure the improved tumor targetability and enhanced tumor cell internalization of co-loaded siRNAs and hydrophobic drugs.

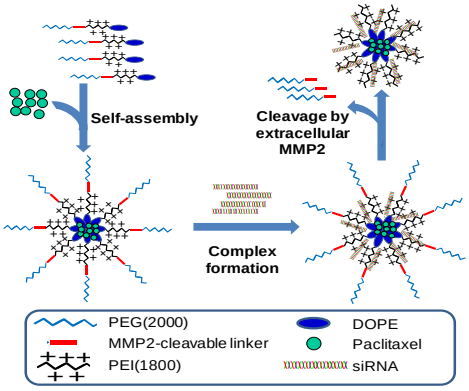


Fig.1. The MMP2-sensitive nanocarrier for siRNA and drug delivery

Key words: polymeric micelle, self-assembly, tumor targeting, matrix metalloproteinase 2, co-delivery, siRNA.

INTRODUCTION

The therapeutic effects of siRNAs are significantly compromised by their poor stability, short circulation time, non-specific tissue distribution, and insufficient cellular uptake. PEI, a widely used gene delivery carrier, is able to condense siRNAs and interact with cell membranes via electrostatic interaction. In addition, its buffering capacity facilitates the endosomal escape of siRNAs and the resultant RNA interference. However, the non-specific interaction between PEI and cell membranes may lead to impaired tumor targeting and decrease siRNA's therapeutic effect.

Paclitaxel (PTX), on the other hand, is one of the most commonly used antineoplastic agents. However, its applications are complicated by its low solubility, off-target toxicity and acquired drug resistance. Although various drug conjugates and polymeric micelles have been developed to solubilize and deliver PTX, co-delivery of hydrophobic drugs and siRNAs remains a challenge. Indeed, the combined use of siRNAs and chemotherapeutics is hindered by the current drug delivery technologies. Usually, because of their distinct physicochemical properties, siRNAs and drugs are loaded into separate carriers for simultaneous administration. However, since these molecules may not be delivered to the same cell, low synergistic effects are observed.

MMP2, is known to be involved in cancer cells' invasion, progression, and metastasis. The up-regulated MMP2 is considered as a biomarker for diagnostics and prognostics in many cancers, and also provides a strategy for tumor-targeted drug delivery via an enzyme-triggered mechanism [3].

Based on the evidence of up-regulated Bcl2 (an anti-apoptotic protein) in cancer cells, correlated with resistance to paclitaxel treatment[1] and sensitization to paclitaxel by anti Bcl2 siRNA (siBcl2)[2], we proposed co-delivery of PTX and siBcl2 by a novel MMP2-sensitive self-assembling copolymer (PEG-PEI-PE). This novel polymer is based on the lipid-polymer PEI-DOPE (PEI-PE), recently designed by our group, which possessed the advantages of both PEI and DOPE and showed the improved efficiency of siRNA delivery[4]. PEG-PEI-PE self-assembles into a "core-shell" structure in an aqueous environment (**Fig. 1**), able to load hydrophobic drugs. The presence of the MMP2-cleavable linker between PEI-PE and PEG allows targeting of aggressive tumor regions where MMP2 is up-regulated[3].

Using the proposed polymeric micelle, both siBcl2 and PTX can be simultaneously and specifically delivered into tumor cells. The tumor cells will be efficiently killed by the synergism of therapeutics, resulting in improved anticancer efficacy and minimized toxicity. Here, we describe the synthesis and characterization of PEG-PEI-PE, siRNA polyplex formation, MMP2-dependent cellular uptake and *in vitro* co-delivery of siRNA and paclitaxel.

EXPERIMENTAL METHODS

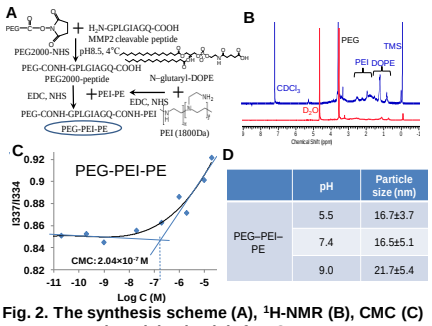
The synthesis scheme is shown in **Fig. 2A**. First, the MMP2-cleavable peptide (GPLGIAGQ)[3] and PEG2000-NHS (1.2:1, molar ratio) were mixed in carbonate buffer (pH 8.5) at 4°C overnight, followed by dialysis (MWCO 2,000 Da) against water. Then, the branched PEI (1.8kDa) was reacted with N-glutaryl-DOPE at a 1:1 molar ratio to form PEI-PE [4]. Finally, PEG2000-peptide reacted with PEI-PE (1:1) in the presence of an excessive amount of NHS/EDC at RT overnight. The product was purified by dialysis (MWCO 8,000Da) and characterized by ^{1}H-NMR. The critical micelle concentration (CMC) was determined using pyrene. The particle size and size distribution were determined by dynamic light scattering. To determine the MMP2-sensitivity, 1 mg/mL of PEG-PEI-PE was incubated with 5ng/μ?mL human MMP2 in HEPES-buffered saline (HBS) containing 10mM $CaCl_2$ at 37°C overnight, followed by

analysis using thin layer chromatography (TLC), size exclusion HPLC, and zeta potential.

The siRNA polyplexes were formed by incubation of siRNA with PEG-PEI-PE in HBS for 20 min at RT at various Nitrogen to Phosphate (N/P) ratios. The complexes were examined by transmission electronic microscopy (TEM), light scattering, and gel retardation assay.

Cellular uptake and gene silencing of siRNA or siRNA/PTX containing nanopreparations were evaluated by fluorescence-activated cell sorting (FACS) and confocal microscopy after incubation in media containing 10% fetal bovine serum. All tests were performed with human non-small cell lung cancer (A549) cells.

RESULTS AND DISCUSSION



	pH	Particle size (nm)
PEG–PEI–PE	5.5	16.7±3.7
	7.4	16.5±5.1
	9.0	21.7±5.4

Fig. 2. The synthesis scheme (A), ^{1}H-NMR (B), CMC (C) and particle size (D)of PEG-PEI-PE

In the ^{1}H-NMR (**Fig. 2B**), the peaks of hydrophobic DOPE were observed in chloroform but not in water suggesting the formation of a "core-shell" structure, which was further confirmed by their low CMC value (**Fig. 2C**). The formed PEG-PEI-PE micelles had a small size in a broad range of pH indicating their stability against pH change during the preparation and administration.

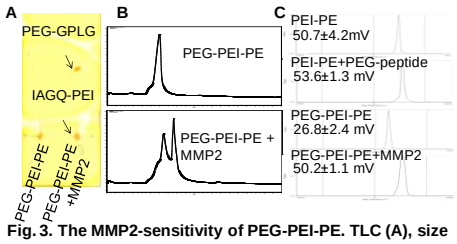


Fig. 3. The MMP2-sensitivity of PEG-PEI-PE. TLC (A), size exclusion-HPLC (B) and zeta potential (C).

The cleavage of the linker released the PEG block as evidenced by a new spot in the TLC and a new peak in size exclusion HPLC. PEG deshielding was further confirmed by the increase of the zeta potential from 26.8±2.4mV to 50.2±1.1mV after exposure of PEI block, increased charge promoting the cellular uptake of PEG-PEI-PE micelles by tumor cells (**Fig. 3**).

The siRNA/ PEG-PEI-PE complexes organized as uniform spherical nanoparticles were determined by TEM (**Fig. 4A**) The size of the siRNA polyplexes didn't change before and after MMP2 cleavage, suggesting that the structure of the siRNA polyplexes was stable (**Fig. 4B**). At a N/P of 40, siRNA was completely complexed

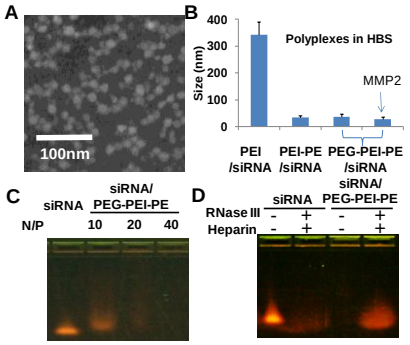


Fig. 4. The formation of siRNA complexes. The morphology (A), stability (B), siRNA condensation (C), and RNase III protection (D).

(**Fig. 4C**) and protected from RNase III degradation (**Fig. 4D**). The FACS data showed that PEG-PEI-PE was able to efficiently transfer siRNAs into A549 cells in the presence of serum while no fluorescence was detected with uncleavable complexes, suggesting that the up-regulated MMP2 in tumor cells cleaved the peptide linker resulting in PEG deshielding and the resultant cell internalization (**Fig. 5**).

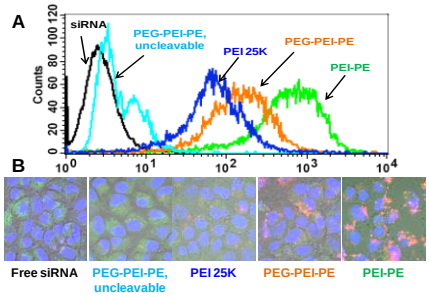


Fig. 5. Cellular uptake of siRNA (red) polyplexes in complete growth media (FACS (A) and confocal microscopy (B))

In addition to the loading of siRNAs, hydrophobic molecules such as paclitaxel can be loaded into the lipid core of PEG-PEI-PE micelles. Successful *in vitro* co-delivery of siRNA and paclitaxel was observed in almost all cells (98.2% cells positive for both siRNA and paclitaxel) with PEG-PEI-PE in serum-containing media whereas no co-delivery was observed with PEI 25kDa (**Fig. 6A**). Intracellular co-delivery of siGLO Red siRNA and Oregon Green paclitaxel was confirmed by confocal microscopy (**Fig. 6B**).

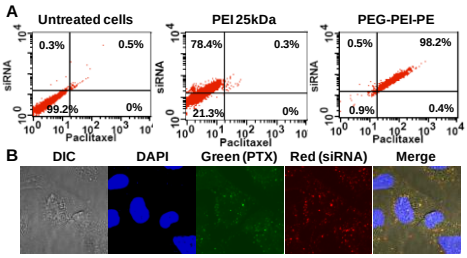


Fig. 6. Co-delivery of siRNA (red) and paclitaxel (green). FACS analysis (A) and Confocal microscopy (B).

CONCLUSION

The cationic PEG-PEI-PE self-assembles into micelles with a small size and uniform distribution, could co-load siRNA and hydrophobic paclitaxel. MMP2-cleavage could remove the PEG shell and expose the positively charged PEI, allowing cancer cell internalization.

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ACKNOWLEDGMENT

This work was supported by the NIH Grant 1R01CA121838 in black to Vladimir P Torchilin.