論 文 の 内 容 の 要 旨

論文題目

Preparation, Characterization and Application of Inorganic/organic Hybrid Nanoparticles of Calcium Phosphate/Charge-Conversional Polymer for Enhanced siRNA Delivery

（リン酸カルシウム/電荷反転型ポリマーから成る
ハイブリッド型 siRNA キャリアの調製と機能評価）

氏　　名　　シルバ　ピテラ　フレデリコ

RNA interference (RNAi) is a fundamental gene silencing pathway in eukaryotic cells. Once inside cell cytoplasm, long double stranded RNAs are cleaved by Dicer enzyme into smaller fragments known as siRNA (small interfering RNA – 21-23 nucleotides long). These fragments (siRNAs) are going to be loaded into a protein complex called RNA-induced silencing complex (RISC), in which siRNA is going to be dehybridized. The siRNA-loading "activated" RISC complex will selectively seek out for mRNAs complementary to the siRNA sequence, resulting in the cleavage of the target mRNA. Direct application of naked siRNA to control gene expression is however highly limited by unstable nature of siRNA to be digested in the bloodstream and extracellular spaces. Hence, siRNA strongly needs carrier systems to protect siRNA from enzymatic degradation and overcome biological barriers, such as the cellular membranes and endosomal entrapment.

Calcium phosphate (CaP) precipitates were first used as a transfection agent in early 1970's, as they possess inherent biocompatibility similar to natural inorganic materials such as teeth and bones. Notably, CaP precipitates can bind and encapsulate polyanions including nucleic acids to protect the payload from enzymatic degradation and to deliver into cells. One of their major limitations, however, is the uncontrollable rapid growth of calcium phosphate crystal, resulting in the formation of large agglomerates that appreciably reduce the transfection efficiency. On the other hand, hydrophilic and non-ionic poly(ethylene glycol) (PEG) is widely known to provide a nanoparticle with excellent colloidal stability as well as reduced protein adsorption and immunogenicity. In this regard, several previous studies have addressed PEG coating of CaP precipitates utilizing PEG-polyanion block copolymers. In fact, the integration of PEG-block polyanions, such as poly(aspartic acid) (PAsp), poly(methacryl acid), and siRNA, into CaP precipitates led to the formation of size-controllable hybrid nanoparticles with PEG

palisade, which appreciably enhanced colloidal stability of the nanoparticles.

Herein, it was considered that the next challenge in the CaP carriers was the endosomal escape, since they are usually internalized by cells through endocytosis pathway to be delivered into acidic endosomes/lysosome, resulting in enzymatic degradation of the payload nucleic acids. In this work, a block copolymer of PEG and an endosomal escaping polyanion (PEG-PAsp(DET-Aco)) was synthesized and integrated into the CaP nanoparticles incorporating siRNA for both enhanced colloidal stability and endosomal escape of hybrid nanoparticles. PEG-PAsp(DET-Aco) possesses the flanking *cis*-aconitylamide, which is relatively stable at neutral and basic pHs but becomes cleavable at acidic pH, to produce cationic PAsp(DET) from anionic PAsp(DET-Aco) in late endosomal/lysosomal compartments, termed the charge-conversional polymer (CCP). Note that the cationic polyaspartamide PAsp(DET) has a 1,2-diaminoethane moiety in the side chain, that exerts strong membrane destabilization selectively at acidic pH, but not at neutral pH based on the change in the protonated form between neutral and acidic pHs, thereby allowing efficient endosomal escape with low cytotoxicity. The hybrid nanoparticles containing CCP and siRNA were physicochemically and biologically characterized by the comparison with non-charge-conversional control polyanions (non-CCP).

Successful synthesis of PEG-CCP and its controls was confirmed by GPC and ^{1}H NMR measurements. Size controlled nanoparticles were then obtained after two step mixing of a solution of siRNA coding vascular endothelial growth factor (VEGF) in HEPES buffer (pH 7.5) with another solution of HEPES containing PO_4^{-3} (pH 7.5), and then with the other containing the polymer (pH 7.5) and $CaCl_2$ in Tris buffer (pH 7.5~10). The cumulant size and polydispersity index (PdI) of prepared nanoparticles were determined by dynamic light scattering, and consequently the particles size was in the range between 50-100 nm, while the PdIs were from 0.06-0.14, indicating the narrow size distribution. Transmission electron and atomic force microscopic images further reveled the spherical morphology of the nanoparticles. Effective encapsulation of siRNA in the nanoparticles (around 80%) was confirmed in preparation with PEG-PAsp(DET-Aco) at the initial concentration between 600 and 2000 μg/mL, while a slight decrease was observed at the concentration of 3000 μg/mL (around 70%).

The hybrid nanoparticles were confirmed to have negligible cytotoxicity to a pancreatic cancer cell (PanC-1) even at the highest concentration tested (50 μg/mL PEG-polyanion, 1.5 μM siRNA). Confocal laser scanning microscopy showed that all the tested nanoparticles were efficiently uptaken by PanC-1 cells, but only nanoparticles containing CCP presented excellent endosomal escape after 3 hours of application.

Precisely, a large fraction of Cy5-labeled siRNAs was not colocalized with late endosome/lysosome stained by LysoTracker, indicating early escape from endosomes. On the other hand, at 24 hours colocalization of siRNAs with LysoTracker was decreased in all the tested nanoparticles, suggesting that calcium phosphate core disassembly in a low ionic condition may inherently facilitate endosomal escape of siRNA, but not as early as the case of CCP. The real-time PCR analysis revealed that VEGF siRNA delivered to PanC-1 cells by CCP-containing hybrid nanoparticles promoted the highest gene knockdown *in vitro* up to 80% among the tested samples including the control with the non-CCP, consistent with the finding in confocal microscopic observation showing the efficient endosomal escape of the hybrid nanoparticles with PEG-PAsp(DET-Aco).

After the optimization in nanoparticle preparation for purification, hybrid nanoparticles were applied for *in vivo* experiments. Treatment of mice bearing BxPC3 (tumorigenic pancreatic cancer cell line) subcutaneous tumors with nanoparticles containing CCP and VEGF siRNA presented a slower tumor growth compared to that treated by scramble siRNA and HEPES buffer as a negative control. After 3 injections of 25μg VEGF siRNA per mouse (n=6), the relative tumor volume was significantly smaller at days 9, 11 and 13, compared to the negative control. Although from the day 15 all the tumors reached the similar growth rate, VEGF siRNA-treated tumors were smaller than those treated by the control at the day 26. Indeed, VEGF siRNA delivered by CCP-containing nanoparticles was confirmed to promote VEGF mRNA knockdown in the tumor tissue by real-time PCR, indicating that the slow growth rate was correlated to the inhibition of the pro-angiogenic factor. Using a different payload (luciferase siRNA), hybrid nanoparticles promoted the luminescence decrease in an extra *in vivo* model, after intravenous injection. The siRNA sequence was previously validated *in vitro* in luciferase expressing cells. The systemically injected nanoparticle is believed to accumulate in the tumor tissue by Enhanced Permeation and Retention (EPR) effect.

Finally, freeze-thawing cycles and freeze-drying of the nanoparticle solution were investigated for possible improvements in stability under *in vivo* conditions and long-term storage. The successful procedures allow the translation of the system into pharmaceutics.

In conclusion, this work is presenting a novel carrier system to explore RNAi therapeutics with the improved colloidal stability and endosomal escape. The *in vitro* results suggest that the siRNA is safely and efficiently delivered to cytoplasm to promote VEGF gene knockdown. Further, intravenously injected hybrid nanoparticles demonstrated the *in vivo* utility in cancer treatment as a promising candidate to realize therapeutic applications of siRNA**.**