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Arginine-engrafted biodegradable polymer for the systemic delivery of therapeutic siRNA

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ABSTRACT

Small interfering RNA (siRNA) represent an interesting class of developmental nucleic acid-based therapeutics. Cationic carriers for deoxyribonucleic acids (DNA) are potential vehicles for siRNA delivery. However, in contrast to supercoiled plasmid DNA, the physical properties of siRNA molecules induces the formation of larger, loosely–packed complexes with most polycationic carriers, and consequently, poor target silencing. Here, we investigate a gene delivery agent, arginine-grafted bioreducible poly (disulfide amine) polymer (ABP) for siRNA delivery as it contains arginine residues with siRNA binding properties. ABP combines the attributes of polycations and poly disulfide-amines namely- excellent cell-penetrability and rapid release after disulphide bond reduction in the intracellular compartment. ABP bound siRNA, assembled into stable 150 nm sized nanoparticles and efficiently released complexed siRNA upon cellular entry. We investigated the utility of ABP in a combinatorial RNAi strategy for solid cancer therapy. Systemic administration of ABP-siRNA resulted in a preferential and enhanced accumulation of carrier-siRNA complexes in the tumor tissue. Two administrations of the formulation with a siRNA cocktail targeting Bcl-2, VEGF and Myc at 0.3 mg total siRNA/kg body weight could effectively regress advanced stage tumors. Our results establish the promise of ABP as a common systemic delivery platform for both siRNA and DNA therapeutics.

1. Introduction

RNA interference (RNAi), a biological process of sequence-specific gene silencing, has developed rapidly into a technology for investigating physiological and pathophysiological gene function as well as for the potential treatment of human diseases [1]. However, it is a widely accepted fact that *in vivo* use of small interfering RNA (siRNA), the mediators of RNAi, remains a critical barrier for therapeutic success. The inadequate penetration, suboptimal pharmacokinetics and toxicities associated with the current methods for siRNA delivery have made most of these impractical for clinical application. About 21 clinical trials are currently underway evaluating the suitability of RNAi-therapy for human diseases [2]. Most of these involve direct administration of siRNA to local target sites such as the eye, the skin and the lung (by inhalation), thereby avoiding the complexity of systemic delivery. However, to treat most 'undruggable' and 'incurable' diseases including viral infections, blood-disorders and cancers, either disseminated or localized to tissues that are not easily accessible, direct injection is very difficult or impossible. Therefore, the development of effective and safe systemic delivery approaches is of prime importance.

Non-viral carriers for plasmid DNA delivery have been researched for years. The molecular topography of supercoiled plasmid DNAs allows their condensation into small, nanometric particles when complexed with a cationic agent. This enables complete encapsulation, consequent protection against enzymatic degradation and efficient endocytic uptake into cells [3]. On the other hand, the more rigid 19–21 bp siRNA molecules [4] are difficult to condense on account of irregular interactions with cationic agents resulting in larger, loose complexes with incomplete encapsulation [5]. Further, nanoparticles in the size ranges of 100–300 nm can deliver siRNA into cells through multiple cellular mechanisms like endocytosis and macropinocytosis, however lower sizes are preferred for effective systemic delivery and

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biodistribution [6]. However, larger nanoparticles have found application in tumor treatment where the larger pore size of tumor microvessels (100–780 nm) in comparison to the tight junctions observed in microvessels in most normal tissues (2 nm) (with the exception of kidney and liver) enables enhanced fluid dynamics and accumulation of macromolecules in the tumor tissue (the enhanced permeability and retention effect-EPR effect) [7,8].

Many of the strategies adopted for non-viral DNA delivery have been applied to siRNA delivery, often with few or no changes required. Nanoparticles and liposomes (cationic, neutral) [9,10], PLGA based [11], lipid and cholesterol formulations (PEC, PEI) [12,13], synthetic and natural polymers (dendrimers, chitosan, cyclodextrin, collagen [14–17] and positively charged peptides (tat, poly-arginine) [14,18,19] have all shown promise in imparting drug-like properties to therapeutic siRNA like serum stability, cellular delivery and tissue bio-availability. However, modifications of the system, for instance- increasing the molecular weight or polycation ratio, to accommodate the smaller siRNA, can increase toxicity of the approach [20,21]. The ability of a single carrier to deliver both nucleic acid subtypes with minimal associated toxicity can potentially expand the application of delivery systems to encompass long-term gene therapeutic as well as short term treatment applications.

Our recent work has resulted in the development of an arginine grafted bioreducible poly (disulfide amine) polymer (ABP) that mediated efficient intracellular delivery of plasmid DNA in a non-toxic manner [22]. This carrier contains a bioreducible polymer cystaminebisacrylamide-diaminohexane [poly(CBA-DAH)] whose primary amine groups are all linked to arginine residues. The low toxicity of this highly polycationic polymer makes it an attractive option for siRNA delivery. Further, the unique combination of reducible polyamines for rapid release upon reduction of the disulfide bonds within the reducing environment of live cells and cell-penetration due to the positively charged arginine residues can allow delivery even in cases where tissue penetrability is the major issue.

Keeping these points in mind, in the present study, we used the ABP polymer to deliver therapeutic anti-cancer siRNA. A combinatorial drug regimen is the typical treatment paradigm for cancer therapy. The overexpression of Bcl-2, c-Myc and VEGF in a wide variety of tumors makes them a popular target for anti-cancer therapeutics. VEGF, a tyrosine kinase, is a key mediator of angiogenesis, characteristically upregulated in many tumors and is, therefore a strategic target for anti-cancer therapeutics [23,24]. The Bcl-2 protein is postulated to block cytochrome C activity preventing the propagation of death signals and promoting cell survival. Targeting the anti-apoptotic Bcl-2 family of proteins induces apoptosis making Bcl-2 another prime target of cancer therapeutics [25–27]. Myc is a transcription factor that recruits histone deacetylases (HDAC1, 2 and SIRT1) to induce expression of growth factors such as EGFR and anti-apoptotic factors like Bcl-2, XIAP, cIAP1, cIAP2 and again, an appealing target for cancer therapy [28,29]. Knockdown of each of these gene products is known to have potent anti-tumor effects [30–32].

We used a combinatorial RNAi strategy targeting the three genes Bcl-2, VEGF and c-Myc, involved in distinct pathways of cancer progression. We investigate the efficacy of ABP/siRNA complexes in regressing pre-established tumors in a murine melanoma model.

2. Materials and methods

2.1. ABP

ABP was synthesized according to procedures published previous by our group [22].

2.2. siRNA

Duplexed siRNA were purchased from Genolution (Seoul, Korea). The gene target sequences are previously published and depicted below.

c-Myc: 5′-GAACAUCAUCAUCCAGGAC-3′ [30],
VEGF: 5′-CGAUGAAGCCCUGGAGUGC-3′ [30]
Bcl-2: 5′-GCAUGCGACCUCUGUUUGA-3′ [33]
CyPB: 5′-GGAAAGACUGUUCCAAA-3′ [34]
Control Luciferase- 5′-GGACAUUACUAGUGACUCA-3′ [18]

siRNAs were used at 2.4 μg and 0.3 mg siRNA/kg body weight of the animal for *in vitro* and *in vivo* experiments respectively after preliminary optimization experiments. For combinatorial treatment, the total amount of siRNA corresponded to 2.4 μg and 0.3 mg/kg.

2.3. Cell culture

B16-F10 murine melanoma cells were obtained from ATCC (Rockville, MD) and cultured in DMEM containing 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml).

2.4. Size and surface charge measurements

2.4 μg (200 pmoles) of siRNA was suspended in 100 μl of DPBS (pH 7.4) with varying amounts of ABP (ranging from 1 to 60 weight ratio). Polyplexes were prepared by adding the siRNA into ABP-containing tubes, simple mixing followed by incubation at room temperature for 30 min. The samples were then diluted 6 times with RNAse-free deionized water. The mean hydrodynamic diameter and ζ-potential of the nanoparticles formed were measured using dynamic light scattering (Zetasizer-nano analyzer ZS; Malvern instruments, Worcestershire, UK).

2.5. Atomic force microscopy

Polyplexes were prepared as detailed above. 10 μl of the polyplex formed was placed on cleaved mica. Morphological characteristics of ABP/siRNA complexes were visualized in a close contact mode in air using a Nano-R atomic force microscope AFM (Pacific Nanotechnology Santa Clara, CA).

2.6. Electrophoretic mobility shift assay (EMSA)

siRNA (2.4 μg or 200 pmoles) was complexed with different weight ratios (ranging from 1 to 40) of ABP in DPBS (pH 7.4) and incubated at room temperature for 30 min. Samples were then loaded onto 1.5% agarose gels containing ethidium bromide and subjected to electrophoresis.

2.7. Ethidium bromide (EtBr) exclusion assay

Dose -dependent condensation of siRNA by ABP was examined by the quenching of EtBr fluorescence in a EtBr exclusion assay. A fixed amount of siRNA (5 μg or 400 pmoles of siRNA in 100 μl of RNAse-free DPBS) was mixed with increasing amounts of ABP contained in 100 μl of DPBS corresponding to 0–20 weight ratios of ABP:siRNA and incubated at room temperature for 30 min. This was followed by addition of 1.5 μg EtBr in 100 μl of DPBS (pH 7.4) and the samples were further incubated for 15 min. The fluorescence was measured using UV/fluorescence reader (spectraMax M2; Molecular devices, Sunnyvale, CA) at an excitation wavelength of 544 nm and emission wavelength of 590 nm. The relative fluorescence intensity was calculated relative to the uncomplexed siRNA-EtBr sample and expressed as a percentage.

2.8. In vitro cellular uptake of siRNA

ABP/siRNA complexes at 25: 1 weight ratio of ABP:siRNA {60 μg of ABP and 2.4 μg or 200 pmoles of FITC-labeled siLuc (siFITC)} were prepared in 100 μl DPBS (pH 7.4) by incubating at room temperature for 30 min. The complexes were then added on to B16-F10 cells (1×10^5/well) seeded in 12 well tissue culture plates in a volume of 400 μl DMEM. Cells were analyzed for siRNA uptake using flow cytometry 4 h or 16 h post-transfection.

2.9. Cell viability and proliferation assays

Cell viability and proliferation was evaluated using a tetrazolium dye-based metabolic assay measuring the mitochondrial reduction of WST-8 reagent to water soluble formazan [Cell Counting Kit-8 or CCK-8; Dojindo] according to manufacturer's protocol. B16-F10 cells (1×10^5/well) in 12 well plates were transfected after 24 h with ABP/siRNA or lipofectamine 2000/siRNA as positive control in triplicate as described above. After 24 h (for cell viability assays) and multiple days (for cell proliferation assays), cells were incubated with WST-8 assay reagent for 1 h and the amount formazan dye formed was determined by measuring absorbance at 450 nm.