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POLYMER DELIVERY SYSTEMS FOR SITE-SPECIFIC GENOME EDITING

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Abstract

Triplex-forming peptide nucleic acids (PNAs) can be used to coordinate the recombination of short 50–60 bp “donor DNA” fragments into genomic DNA, resulting in site-specific correction of genetic mutations or the introduction of advantageous genetic modifications. Site-specific gene editing in hematopoietic stem and progenitor cells (HSPCs) could result in the treatment or cure of inherited disorders of the blood such as β -thalassemia or sickle cell anemia. Gene editing in HSPCs and differentiated T cells could also help combat HIV infection by modifying the HIV co-receptor CCR5, which is necessary for R5-tropic HIV entry. However, translation of genome modification technologies to clinical practice is limited by challenges in intracellular delivery, especially in difficult-to-transfect hemolymphoid cells. Here, we review the use of engineered biodegradable polymer nanoparticles for site-specific genome editing in human hematopoietic cells, which represent a promising approach for *ex vivo* and *in vivo* gene therapy.

Keywords

Non-viral gene delivery; gene therapy; triplex forming oligonucleotides; peptide nucleic acids; nanoparticles; hematopoietic stem cells; oligonucleotide therapy; genome modification; poly(lactic-co-glycolic acid)

1. Gene therapy in hematopoietic cells

Hematopoietic stem cells (HSCs) self-renew throughout an individual’s lifetime and differentiate into progenitors that populate the diverse components of the human blood and immune system (Fig. 1). Because of these properties, genetic manipulation of HSCs—or, more broadly hematopoietic stem and progenitor cells (HSPCs)—could provide curative treatments for single-gene disorders of the blood or introduce new genomic changes to combat certain infectious diseases. For example, editing of the β -globin gene in HSCs and myeloid progenitors could provide treatments for devastating inherited hemoglobinopathies, such as β -thalassemia and sickle-cell anemia. As a further example, editing of the *CCR5* gene in HSCs (or lymphoid progenitors, or mature T cells) could provide a treatment for

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HIV infection through inhibition of HIV entry into immune cells. The chemokine receptor CCR5 is a major co-receptor used by HIV for entry into T cells, and a naturally occurring deletion mutation in *CCR5* has been shown to confer resistance to HIV [1]. A recent study has indicated that disruption of *CCR5* using zinc-finger nucleases can help reduce HIV infection in a humanized mouse model [2].

Unfortunately, gene modification remains a challenge in hematopoietic cells, especially HSPCs, due to their quiescent nature and relative resistance to both viral and non-viral methods of transfection [3, 4]. Only 5–10% of human bone marrow cells are CD34+ (a marker for HSPCs), and of these, only 10–20% express the more primitive HSC phenotypes [5], thus making cell-specific targeting important for *in vivo* gene therapy. Here, we review our recent work showing that nanoparticles—designed for gentle delivery of gene-editing oligonucleotides—can be used to modify disease relevant genes in human hematopoietic cells.

2. Gene modification with triplex-forming oligonucleotides

A major problem with the use of viral vectors for gene modification is the lack of control over the site on the host genome where the viral genome is integrated. To accomplish efficient and safe genome modification, genetic targeting—and, therefore, sequence-specificity—is critically important. Triplex forming oligonucleotides (TFOs) form unique structures by binding with high affinity and specificity in the major groove of duplex DNA. TFOs are capable of catalyzing genomic events including inhibition of transcription and DNA replication, promotion of site-specific DNA damage, and enhancement of recombination [6]. To improve them for use in *ex vivo* or *in vivo* therapies, TFOs can be produced as peptide nucleic acids (PNAs), which contain nucleobases with a polyamide backbone, conferring resistance to intracellular degradation and enhancing their binding affinity to DNA because of reduced electrostatic interactions. Triplex-forming PNAs bind via Hoogsteen and Watson-Crick bonding to a complementary DNA strand, forming a stable PNA/DNA/PNA triple helix. This abnormal structure is then recognized by cells' own DNA repair machinery, sensitizing a site for homologous recombination. Thus, intracellular delivery of a site-specific PNA can induce recombination of a short, single-stranded “donor” DNA molecule with a nearby genomic site [7] (Fig. 2). The advantages of PNA TFOs as a method for gene editing include their gene specificity [8], high binding affinity to DNA, the availability of binding sites throughout the genome, and intracellular stability.

Triplex-forming PNAs facilitate genomic modification in human hematopoietic cells. For example, triplex-forming PNAs can bind the human β -globin gene and stimulate modification at a β -thalassemia associated site in human CD34+ HSPCs without loss of pluripotency [9]. Chin *et al* designed PNAs that mediate recombination at the first position of intron 2 (IVS2-1) of the β -globin gene (Fig. 3A), achieving recombination frequencies of 0.1–0.5% in a CHO cell GFP β -globin fusion model (Fig. 3B) [9]. Gene correction was verified at the genomic (by sequencing), mRNA (by qRT-PCR), and protein levels. Primary human CD34+ HSPCs transfected with PNA and DNA were found to contain the desired mutation (as determined by allele-specific PCR, or AS-PCR), even after differentiation along erythroid and neutrophil lineages (Fig. 3C) [9]. In addition, the Glazer lab has developed a PNA and DNA combination for modification of the human *CCR5* gene, which confers HIV resistance in THP-1 cells (manuscript in revision)[10]. Transfection in these studies was accomplished via electroporation/nucleofection [11], which although useful for proof-of-principal studies, is relatively toxic to hematopoietic cells and cannot be used *in vivo*. Cationic lipid methods cannot be used for PNA delivery due to their neutral or net positive charge, and other methods of PNA transfection have drawbacks including dependence on complementary or conjugated DNA carriers, direct conjugation to peptides or lipids, and the use of non-biodegradable materials [12–18]. Because of the difficulty of

transfection of HSPCs, many prior studies have been performed in cell line reporter systems, which makes it difficult to predict the value of these approaches in a clinical setting.

3. PLGA nanoparticles for gene modification

Here, we review our novel solution to the problem of intracellular delivery of PNA and DNA molecules for genome editing. Poly(lactic-co-glycolic acid) (PLGA) is an FDA-approved biocompatible polymer used clinically for delivery of drugs for numerous indications including treatment of prostate cancer (Lupron® and Trelstar®). In prior work, we have shown that PLGA nanoparticles can provide reliable intracellular delivery of nucleic acid polymers and oligomers, including plasmid DNA [19, 20] and siRNAs for sustained gene silencing [21]. These PLGA nanoparticles are taken up readily by numerous cell types through a likely endocytic mechanism, followed by escape from endosomes and association with exocytic organelles[22]. However, the exact mechanism of endosomal escape and subsequent delivery of nucleic acid cargo to cytoplasm and nucleus is not well understood, and requires continuing study. We have developed methods to formulate nanoparticles containing PNA and DNA (PNA-DNA) for enhanced delivery to HSPCs (Fig. 4). Our studies indicate that PLGA nanoparticles can be used to deliver PNA and DNA for gene modification in hematopoietic progenitors[23].

To design nanoparticles for intracellular delivery of PNA, we first found compositions for PLGA nanoparticles that allowed for their internalization in HSPCs. As in prior work, we used coumarin 6 (C6) as a fluorescent probe, which can be loaded into PLGA nanoparticles. Nanoparticles loaded with C6 can be used to track particle association, internalization, and distribution, because the hydrophobic C6 is not released readily from PLGA particles [24, 25]. C6 PLGA nanoparticles were formulated using a single-emulsion solvent evaporation technique we have previously described [21]: the particles are small and spherical (Fig. 4 inset), with C6 loadings of ~4 nmoles C6/mg PLGA. When added to cells in culture, C6 PLGA nanoparticles associated with and were taken up by CD34+ HSPCs (Fig. 5A), although a large percentage of particles still remained externally associated without internalization.

These PLGA nanoparticles become associated with HSPCs in the bone marrow after intravenous injection. In a small pilot experiment, unmodified C6 nanoparticles injected intravenously or intraperitoneally into mice localized in small percentages to the bone marrow after 6 hours, with clearance by 24 hours (Fig. 5B). Intravenous injection led to a higher percentage of nanoparticles in the bone marrow compared to intraperitoneal injection (Fig. 5B), and in this small sample, nanoparticle localization to CD4+ cells (rare in the bone marrow) was also seen. These results are in contrast with what is seen with free oligonucleotides, which are cleared more rapidly after intravenous injection than after intraperitoneal administration [26]. These findings are consistent with recent reports that nanoparticles in the 200 nm diameter range can localize to hematopoietic cells in mice following systemic delivery[27]. We hypothesize that after intravenous delivery, there is widespread distribution of nanoparticles throughout the mice, and some of these particles in the smaller size range may be able to access the bone marrow. It is possible that bone marrow accumulation after intraperitoneal delivery is reduced because of increased liver clearance following administration by this route[28].

We have recently developed a method to convert these particles into PNA and DNA delivery systems[23]. We formulated PLGA nanoparticles by a double-emulsion solvent-evaporation technique [29], which allowed us to encapsulate PNA, DNA (in which the DNA was neutralized using spermidine as a counterion), or both PNA and DNA (in which the lysinated PNA served as the counterion for the DNA). These particles exhibited spherical morphology and size in the 150 nm diameter range, loaded densely with PNA and DNA, and

exhibited burst release of contents, which occurred predominately within the first 24 hours of incubation in PBS at 37°C [23].

To test for the ability of these particles to enhance genome modification, we loaded nanoparticles with PNA that targets a site in the human β -globin gene near a β -thalassemia associated mutation (as in Fig. 3). We treated human CD34+ HSPCs with nanoparticles containing PNA, DNA, PNA-DNA, or PBS (blank) in dosages of 0.25–2 mg PLGA/mL media, and corresponding dosages with nucleofection, followed by quantification of cell survival. Cells treated with nanoparticles exhibited greater cell recovery and viability after treatment with nanoparticles than with nucleofection (Fig. 6A), with similar if not increased retention of CD34 expression over time [23]. Particle treatment led to much higher rates of recombination as determined by two independent methods (Fig. 6B), corresponding to an at least 60-fold increase in modified and viable cells. In addition, genomic modification persisted after differentiation of HSPCs into erythroid and neutrophil lineages and expansion (Fig. 6C). Our modification was also present after gel purification of genomic DNA to exclude possible persisting oligonucleotides, and spiking of the genomic DNA with high doses of donor DNA did not lead to false detection by AS-PCR [23]. In addition, we demonstrated modification in the human *CCR5* gene using a *CCR5* targeted PNA with a *CCR5* donor DNA [23].

4. Potential impact of this new technology

Our work provides the first demonstration that nanoparticles can be used for site-specific genome editing in human HSPCs. Although early in development, there are several reasons to be optimistic about the potential impact of this technology on human health. First, this approach is translatable into targeted gene therapy in human hematopoietic cells, bridging the gap between the use of triplex-forming oligonucleotides for gene modification as a research tool and as a clinical treatment modality. It is suggested that gene modification frequencies of 10–15% are required in hematopoietic stem and progenitor cells for treatment of clinical hemoglobinopathies [4], and 17% disruption of the human *CCR5* gene was sufficient to confer HIV resistance in a humanized mouse model [2]. While our current results show efficiencies that are still lower than these targets, we are confident that further optimization will lead to levels of gene modification that are clinically relevant, particularly for *ex vivo* HSPC treatment, in which HSPCs are harvested from donors, modified by treatment with particles outside the body, and then re-infused back into the same donor for re-engraftment.

Second, this approach provides a framework for development of a translatable system for direct *in vivo* genomic modification of human hematopoietic cells. While *ex vivo* therapies can provide the most immediate clinical application of this technology, direct *in vivo* genome editing eliminates the need to harvest and manipulate hematopoietic cells *ex vivo*. Since PLGA nanoparticles have low toxicity and are easily administered, multiple treatments could be used to increase the cumulative gene modification frequencies. In addition, because genome editing using triplex-forming oligonucleotides is gene-specific, nucleic acid delivery to non-targeted cells (such as to liver parenchyma) would not be detrimental, for instance in the editing of human β -globin. However, genome editing in non-targeted cells would be of concern if edited genes have additional systemic functions. Alternatively, direct *in vivo* gene editing may provide a mechanism for gene modification in systemic disease where multiple cell types and organs are affected.

Third, this work suggests a versatile method for targeted drug delivery to human hematopoietic populations. Methods have been described for surface modification of PLGA nanoparticles for specific targeting of hematology cell types, providing a framework for cell-specific delivery of payloads [29, 30] Development of efficient nucleic acid delivery

tools for HSCs and T cells could be expanded to the delivery of payloads other than PNA and donor DNA, such as plasmid DNA, siRNA, microRNA, and other oligonucleotides. And finally, this work suggests a new method for gentle and versatile PNA delivery, which can be used in other applications for PNAs, such as delivery of PNA antisense. The use of peptide nucleic acids in a therapeutic context is currently limited by barriers to delivery in relevant human cells and organs such as the hematopoietic system.

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Figure 1. Hematopoiesis

A schematic of hematopoiesis, the process by which hematopoietic stem cells form the components of the blood and immune system. Some examples of cell surface markers are included.

Figure 2. Genome modification using PNA and DNA

The 50 to 60-mer donor DNA (black) is homologous to the gene target of choice (grey) except for a several base-pair mutation (X X X). The PNA (red) binds near the target and induces homologous recombination of the donor strand into the target. Allele-specific PCR (AS-PCR) can distinguish between modified (mutant) and unmodified (wild-type) genomic DNA.

Figure 3. Triplex-forming PNAs induce recombination at a thalassemia mutation site
(A) PNAs were designed to bind to intron 2 sequences of the human *b-globin* gene at a distance of 35 to 830 bp from the targeted thalassemia mutation at the first position of intron 2. **(B)** PNA-mediated gene correction frequencies in CHO cells using a GFP-expression assay. **(C)** Human CD34⁺ cells treated with PNAs and short donor DNAs (HBB donor) were capable of differentiation into erythroid and neutrophil lineages; both lineages showed targeted gene modification by AS-PCR up to 21 days following oligonucleotide transfection. Reproduced with permission from [9] (permission pending).

Figure 4. Schematic of nanoparticle formulation

PNA and DNA were loaded into nanoparticles using a previously described double-emulsion solvent evaporation technique [29]. In combined PNA-DNA particles, PNA acts as the counterion for DNA. In DNA only particles, spermidine is added to act as the counterion. Scanning electron micrograph shows sample batch of PNA-DNA nanoparticles.

Figure 5. PLGA nanoparticles deliver cargo into hematopoietic cells *in vitro* and *in vivo*
 (A) Human CD34⁺ cells were treated with 0.2 mg/mL of C6 PLGA particles at a cell concentration of 1 million cells/mL. Cells were harvested for FACS for analysis 24 hours post-treatment, and either co-stained with CD34 APC (an HSPC marker) or trypan blue (quenches external fluorescence). Co-staining with trypan blue indicated that 39% of fluorescence was from internalized particles. (B) 17 week old NCr nu/nu mice were injected with either 9.6 mg IP or 6 mg IV PLGA nanoparticles containing C6, in RPMI media (320 μ L or 200 μ L respectively). Mice were then sacrificed at 6 hours to assess for nanoparticle uptake in bone marrow cells by FACS. Total percentages of C6 positive cells or percentages co-staining for C6 and specific cell markers are given.

Fig. 6. PNA-DNA nanoparticles mediate genomic modification in the β -globin gene in HSPCs with low toxicity and high efficiency

Blank particles contain PBS. For nucleofection, cells were nucleofected as per the Amaxa protocol. Mock nucleofection was without nucleic acid. **(A)** Nucleic acid delivery by nanoparticles has higher live cell recovery than nucleofection. Cell counts were performed after 3 days of treatment using Trypan blue to stain dead cells. Error bars give SD. *** $p = 5 \times 10^{-12}$ for two-tailed t-test, 2-sample unequal variance. **(B)** PNA-DNA delivery by nanoparticles leads to higher gene modification frequencies than nucleofection. Cells were treated with 2 mg/mL nanoparticles or optimized Amaxa nucleofection. Modification frequencies were determined by two independent methods after three days of treatment, mean and 95% confidence intervals given. Standard curve qPCR: Quantitative AS-PCR was performed on genomic DNA from treated cells, and relative values were compared to a standard curve generated by known amounts of mutant plasmid copies in wild-type genomic DNA. Limiting dilution: after the three day treatment, cells were replated at 20 cells/well in 96-well format and expanded 4 weeks in replica plates. Genomic DNA was harvested and assessed by AS-PCR. Positive wells were validated in the replica. Limiting dilution analysis (<http://bioinf.wehi.edu.au/software/elda>) was used to determine frequencies. **(C)** Cells maintain mutation after differentiation and expansion. CD34+ cells were treated with 0.5 mg/mL particles and then switched to erythroid- or neutrophil-differentiating conditions, or in media with expansion (non-differentiating) cytokines. Reproduced with permission from [23].