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Peptide Nucleic Acid Delivered by Nanoparticles as a Gene Editing Tool: Methods of Design, Preparation and Evaluation.

Noha H. Eltaweel^{1*}, Khalda Amr¹, Ghada ElKamah², Nermeen El-moataz bellah Ahmed³, Iman Hassan Ibrahim⁴.

¹ Department of Medical Molecular Genetics, National Research Centre, Egypt

² Department of Clinical Genetics, National Research Centre, Egypt

³ Department of Oro-Dental Genetics, National Research Centre, Egypt

⁴ Department of Biochemistry and Molecular Biology, Faculty of Pharmacy (Girls), Al-Azhar University

** Corresponding Author: nohaeltaweel10@gmail.com

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Abstract: Synthetic nucleic acids could be designed in sequence-specific structure to trigger endogenous repair systems. Depending on this concept, peptide nucleic acids (or PNAs) could achieve non-enzymatic gene editing i.e. without the use of nucleases unlike CRISPR and TALENs. PNAs could bind to the DNA to form highly specific hetero-triplex structures, so PNAs have been used severally in the last decades to induce correction of different human disease-causing mutations with low off-targets. Systemic in-vivo and in-utero application of PNAs had been enabled thanks to the advances in their chemical structure, design and delivery resulting in considerable preclinical editing in mouse models. Treated animal models engineered with a human beta globin gene (HBB gene carrying a β -thalassemia mutation, showed clinically considerable protein expression, suggesting that PNAs could have a curative potential for genetic disorders. The complete methods of design, preparation and evaluation of the PNA/DNA nanoparticles formulation will be discussed in details in addition to its use in cells treatment aiming for paving the road for interested scientists in gene editing techniques for treating different single gene disorders.

Keywords: peptide nucleic acids; PNA; gene editing; triplex; nanoparticles; PLGA

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1. INTRODUCTION

Most genetic diseases are caused by mutations affecting certain gene, causing disturbance in its function e.g. thalassemia, duchenne muscle dystrophy and cystic fibrosis. Present management of these diseases depends mostly on symptomatic or replacement treatments but it is not effectively alleviating the patients' symptoms. The interest of many scientists in the last decade has been concentrated on curing genetic diseases by correcting the disease causative mutation. Peptide nucleic acids (or shortened as PNAs) are novel synthetic polymers of DNA that can bind with its alternative DNA duplex specifically to form a triplex structure which can induce DNA repair systems and produce certain intended genome modifications. PNAs have been used to correct successfully different monogenic human disease-causing mutations either in cultured cells (ex-vivo) or in mice. These gene corrections

have resulted in a parallel functional protein restoration and disease improvement 1,2 .

PNAs are synthetic DNA polymers in which the phosphodiester backbone has been replaced with N-(2-aminoethyl)-glycine units linked by peptide bonds. This change in the original phosphodiester backbone makes it highly stable and resistant to cleavage by proteases and nucleases. Being neutrally-charged, the PNA/DNA complexes have increased stability compared to DNA/DNA complexes ^{3–5}. PNA has a variety of promising medical applications especially in molecular genetics or cytogenetics ^{6–8}. The kinetics of strand invasion by different types of PNA into ds-DNA was previously described ^{9–11}.

This technique doesn't encounter any nuclease activity, so PNAs are greatly safe especially when compared to nuclease-based editing tools as

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TALENs and CRISPR/Cas9 12 . Additionally, it doesn't induce double strand DNA breaks and the frequency of off-targets is extremely low or even undetected 2 .

PNAs' intracellular delivery require special methods because it couldn't cross the cellular membrane. Techniques for PNA delivery have been successfully developed but have serious disadvantages and are inapplicable for use in-vivo². Nanotechnology-based carriers provides safe, simple and rapid method for PNA application. In 2010, McNeer et al. used poly (lactic-co-glycolic acid) (PLGA), a FDA-approved biocompatible and biodegradable polymer, in nanoparticles (NPs) formulation for a non-toxic and efficient cellular delivery of PNA and donor DNA 13.

Studies have involved different trials to improve the PNA properties and penetrability for better results. The gamma PNA or γ PNA is a novel PNA substituted at the γ -position of the PNA backbone with diethylene glycol or "miniPEG" which increases the PNA solubility, less self-aggregation and enhances its binding affinity with the DNA target ⁵.

Studies on mouse models had paid attention on the possibility that PNA-NP could cause clinically reliable gene editing and thus could provide the basis for a new genetic therapy for single gene disorders, such as thalassemia, sickle cell anemia (SCD) and cystic fibrosis.

The main goal of this manuscript is to focus on the design, methodology and evaluation of this technique to induce mutation-specific gene editing in any single gene disorder aiming for its therapy.

2. MATERIALS

2.1. Instruments and General Laboratory Equipment

The following are the chemicals and preparations involved in the nanoparticles formulation.

- Branson Ultrasonic Water bath.
- Centrifuge
- Digital Stirring hot plate.
- Electronic Balance for Laboratories (0.1 mg scale)
- Gel electrophoresis horizontal equipment and *gel documentation* system
- Heat block
- Magnetic stirrer.
- Nanodrop 2000C (Thermo Scientific, Waltham, MA)
- Scanning electron microscope SEM.

- Shaking water bath
- Standard calibrated pipettes (P1000, P100, and P10).
- Transmission electron microscope TEM.
- ZetaSizer Nano ZS for zeta-potential analysis.

2.2. Chemicals

- Poly (DL-lactide-co-glycolide) or PLGA 50:50 Ester Terminated (Sigma Aldrich, St. Louis, MO; cat. no.: 802182).
- Nuclease free water (Sigma Aldrich, St. Louis, MO; cat. no.: 3098).
- Phosphate Buffer Saline or PBS (Gibco, Grand Island, NY; cat. no.: 10010023)
- Poly Vinyl Alcohol or PVA (Sigma Aldrich, St. Louis, MO; cat. no.: P8136).
- Dichloromethane or DCM (Sigma Aldrich, St. Louis, MO; CAS no.: 75-09-2).
- Trehalose (Sigma Aldrich, St. Louis, MO; cat. no.: 1673715).
 - Agarose and ethidium bromide for gel electrophoresis; (Sigma Aldrich, St. Louis, MO; cat. no.: A4718 & 1.11608)

2.3. Chemical Preparations

Table 1: The composition of the chemical preparations needed for the technique

	Chemical composition	Role
PVA 5% solution	250 mg PVA in 5 ml diH ₂ O	Emulsion stabilizer
TE buffer	10 mmol/l Tris– HCl/1 mmol/l EDTA; pH 7.4	Nucleic acid extraction
TBE buffer	Tris, boric acid and EDTA (90, 90 and 2 mM, respectively) adjust pH at 8.0	Gel electrophoresis

2.4. Nucleic Acids Design

2.4.1. Donor DNA Design

Donor DNA is about 50-60 nucleotides in length homologous to the normal/desired gene edit.

- a. The sequence of the gene of interest (HBB gene) was retrieved from the Ensemble database available at <u>https://www.ensembl.org/index.html</u>., (RRID:SCR_002344).
- b. The exact site of the target mutation to be edited was selected.

c. The 60 nucleotides donor DNA's sequence was selected such that the mutation to be corrected is at

its center i.e. its position on the donor DNA might enable reliable correction of this mutation.

- d. Donor DNA were synthesized as custom-synthesized oligos by IDT Company (USA).
- e. In order to be protected against nuclease degradation, three phosphorothioate internucleosides linkages were added at the 5' and 3' ends of the donor DNA during its synthesis and then purified by reversed phase high performance liquid chromatography (HPLC).

2.4.2. PNA

Gamma-PNA is synthesized by Boc chemistry (where the tert-butyloxycarbonyl (Boc) group is used as a protecting group for amines in organic synthesis) using standard solid phase techniques or solid-phase synthesis (SPS) ¹⁴ and purified as previously described ¹⁵.

The optimum PNA total length is 12-21 bases, longer PNA oligomers are usually not preferred for hybridization. PNA oligomers can form duplexes with DNA in both orientations (figure 1). This bis-PNA consists of antiparallel Watson-Crick (hydrogen bonds) strand and parallel Hoogsteen strand each of them about seven nucleobases in length. Flexible linkers between the 2 strands of the bis-PNA is essential to enable invading the DNA in antiparallel orientation such as O linker (2 or 3 units of 8-amino-3,6-dioxaoctanoic acid), it also enhances its solubility⁷.



Figure 1. Schematic representation of PNA/DNA/PNA triplex shows the antiparallel orientation of the PNA/DNA binding.

In the parallel (Hoogsteen) strand, cytosine (denoted by C) was replaced by pseudoisocytosine (denoted by J) for pH-independent DNA binding. There are many possible gamma functional groups e.g lysine and alanine. MiniPEG gamma substitutions are the best for improved solubility and specific binding, in addition to being efficient for dsDNA invasion. As in ordinary primer design, any self-complementary sequences were avoided such as inverse repeats, hairpin forming, and palindromic sequences because PNA/PNA interaction is stronger than PNA/DNA interaction. Moreover, purine-rich sequences were also avoided because of their low solubility in aqueous solution so they tend to form aggregates. The purine content <60% were kept and purine stretch over 6 residues were avoided, especially consecutive >3 G residues.

Table 2. Example of designed PNA sequences.

	PNA sequence	Туре	Gene	Refer
				ence
1	NH2-CTTCCCTTT-OOO-	Bis-	HBB	(16)
	TTTJJJTTJT-COOH	PNA		
2	NH2-TTTTCTTCTCCC-	Bis-	HBB	(16)
	000-JJJTJTTJTTT-	PNA		
	СООН			
3	NH ₂ -	γ-	HBB	(17)
	C*TT*TC*TT*TC*TC*T-	PNA		
	000-TJTJTTTJTTTJ-			
	СООН			

Every base pair of PNA/DNA duplex roughly increase the Tm about **1°C** than the corresponding DNA/DNA duplex ¹⁸. PNA design tool was used to evaluate the PNA sequence, provided by PNA Bio and available at <u>https://www.pnabio.com/support/PNA Tool.htm</u> (figure 2).

3. METHODS

3.1. Nanoparticle Formulation

NPs were synthesized by the PLGA polymer encapsulating PNA and DNA oligomers using a modified double-emulsion solvent evaporation technique ⁽¹²⁾. The double emulsion is a two-step process of emulsification (internal w/o emulsion in external aqueous layer, in the presence of a stabilizer) ⁽¹⁹⁾. In external aqueous phase, the use of double emulsion is generally preferred than single emulsion for better loading efficiency of water-soluble encapsulated substance/drugs (such as nucleic acids) which are poorly entrapped within a single o/w emulsion resulting in rapid diffusion of the drug from the internal oil phase into the external aqueous phase ⁽²⁰⁾. To ensure quality through pharmaceutical products' life cycle, regulatory agencies had set up a science-based approach called Quality by design (QbD) approach to ensure the repeatability and replicability of PLGA nanoparticle and to allow the pharmaceutical researchers to lessen the number of experimental trials, cost and time. Panigrahi et al had discussed in details the formulation of PLGA nanoparticles (components of a double emulsion, characteristics of drugs, polymers, and used stabilizers) and the related QbD elements including the quality attributes of the product, such as particle size distribution, encapsulation efficiency, etc. ^{19.}

Different patches of NPs were prepared: combined PNA/DNA NPs, donor DNA only NPs and blank PLGA NPs. Figure 3 represents the methods of formulating PNA/donor DNA NPs either combined or alone. The beaker was placed on a stir plate. 5% PVA was prepared first then diluted to 0.3% (according to the equation CV=C'V'). Note: Dissolving PVA requires vigorous stirring.

O A https://www.pnabio.com/	80% ☆ Q Search						
CRISPR/CAS9 PRODUCTS	PNA Tool						
> Cas9 Protein with NLS							
 Custom sgRNA (RGEN) 	Enter PNA sequence (e.g., CAGTCCAGTT):						
> KO/KI Cells	CAGTCCAGTT						
Reporter for Nucleases	Enter amino acid sequence and O linker (e.g., KFEKEEXBO, O for AEEA or eg1 linker):						
	KFFKFFX80						
PNA PRODUCTS	NA PRODUCTS Submit Query						
> Custom PNA Oligos	Complete Sequence	cag too agt t - O - BXFFKFFK					
> PNA FISH Probes	Tm at 4 uM ²	55.7 °C					
> PCR Blockers	Base Count	10					
> Gamma/ Bis PNA	Base Composition	A = 2 (20.0%) T = 3 (30.0%) G = 2 (20.0%) C = 3 (30.0%)					
> Fmoc Monomers	Purines (%)	40.0%					
> MutyperR	GC Content	50.0%					
	Purine Stretch	2 (AG)					
RESOURCES	PNA Chemical Formula	C107H136N56O31					
> PNA Tool	Molecular Weight (including linker and peptide)	3876.09					
> gRNA Tool	Extinction Coefficient	97 ml/(µmole*cm)					
> About l'eptide Nucleic Acids	1 000	10.3 nmole					
> CRISPR_Cos9	PNA Sense Sequence (5' to 3')	CAGTCCAGTT					
	Reverse Complementary Sequence (5' to 3')	AACTGGACTG					
> Manuals and Brachures	Comment	Good					

Figure 2. PNA tool provided by PNA bio. It shows the properties of the proposed PNA sequence including Tm, molecular weight, purines %, GC content... etc.



Figure 3. Schematic representation of the steps of double emulsion solvent evaporation technique for NP formulation.

1. PVA solutions was prepared with a stir bar (2 mL of 5% PVA in a test tube and 25 mL of 0.3% PVA in a 150 mL flat-bottom beaker).

- 2. Eighty mg of PLGA was dissolved in $800 \ \mu$ l of dichloromethane DCM with a magnetic stirrer to facilitate the dissolution of PLGA in a well-covered bottle because the DCM is highly volatile at room temperature.
- 3. The PNA and the donor DNA were dissolved separately in nuclease free water to reach 1 mM stock of each.
- 4. Eighty nmole of PNA (80 μl of 1 mM stock) and 40 nmole of donor DNA (40μl of 1 mM stock) were heated separately at 65°C for 10 minutes using a heat block. Such that, the final ratio of the starting material will be 2 nmole PNA: 1 nmole DNA: 1 mg PLGA.

Analysis Results #1: GATGAAGTTGGTGGTGAGG							
Rating	:	100.0		3' end stability	:	-8.2	kcal/mol
Molecular Wt	:	6003.96		ΔH	:	-134.9	kcal/mol
Tm	:	52.48	°C	ΔS	:	-0.35	kcal/°K/mol
GC%	:	52.63		5' end ∆G	:	-6.58	kcal/mol
GC Clamp	:	2		Self Dimer (ΔG)	:		kcal/mol
nmol/A ₂₆₀	:	5.09		Hairpin (ΔG)	:		kcal/mol
ug/A ₂₆₀	:	30.59		Repeats (# of pairs)	:		kcal/mol
ΔG	:	-29.83	kcal/mol	Run (# of bases)	:		kcal/mol

Analysis Results #2: AACCTGTCTTGTAACCTTGATA							
Rating	:	100.0		3' end stability	:	-5.96	kcal/mol
Molecular Wt	:	6684.46		ΔН	:	-157.0	kcal/mol
Tm	:	51.38	°C	ΔS	:	-0.42	kcal/°K/mol
GC%	:	36.36		5' end ∆G	:	-7.95	kcal/mol
GC Clamp	:	1		Self Dimer (ΔG)	:		kcal/mol
nmol/A ₂₆₀	:	4.74		Hairpin (ΔG)	:		kcal/mol
ug/A ₂₆₀	:	31.69		Repeats (# of pairs)	:		kcal/mol
ΔG	:	-31.99	kcal/mol	Run (# of bases)	;		kcal/mol

Figure 4. NetPrimer parameters for primer design of a proposed pair of primers showing Tm, GC%, self dimering.... etc.

- 5. The PNA with the donor DNA solutions were added and mixed together just before the formation of the 1st w/o emulsion.
- 6. The PLGA solution was mixed well using a vortex, then the nucleic acids mixture was added slowly and dropwise to the polymer solution. Blank NPs were loaded with nuclease free water instead of the nucleic acids solution, and formulated using the same method.
- 7. To formulate the first water-in-oil emulsion (w/o), the mixture was sonicated using an ultrasonic processor set at amplitude of 38% for 3 separate sonication steps, 10 seconds each. After each sonication step, if the mixture was hot, the device was stopped and the mixture was allowed to cool on ice for approximately 5 seconds.
- 8. The first emulsion was added slowly to 1.6 ml 5% PVA solution dropwise then the resulting w/o/w emulsion was sonicated (3×10 sec.) as described before to form the second emulsion.
- 9. The resulting w/o/w emulsion was directly poured into 20 ml of 0.3% aqueous PVA solution then sonicated for 3 hrs at room

temperature or until the DCM completely evaporates.

- 10. The formed NPs were collected in a sterile 50 mL Falcon tube, washed three times with 20 ml diH2O followed by centrifugation for 10 minutes at 12,000 rpm, 4°C. After each wash, the supernatant was discarded, and the NPs were resuspended in new diH2O using a water bath sonicator and vortex until the pellet was fully resuspended.
- 11. The final supernatant was discarded and the pellet was resuspend in 4 mL diH2O.
- 12. Optional: trehalose (as a cryoprotectant) could be added at the same weight of PLGA in the final NP yield, not the starting mass of PLGA. (empty NPs were initially prepared to determine typical final yields)
- 13. The NPs were freezed at -80°C then lyophilized for 72 hours.
- 14. Lyophilized NPs are stored at -20°C.

3.2. Nanoparticle Characterization and testing

3.2.1. Gel mobility shift assay

To assess the binding efficiency of PNAs with its complementary DNA sequence to form triplex complex.

a. Primers for amplification of the desired sequence were designed using NetPrimer online tool (Premier Biosoft, Palo Alto, CA, USA; figure 4). The difference in Tm between both the forward and reverse didn't exceed 2°C, with no excessive dimering.

- b. PCR product of gDNA was prepared containing the target sequence corresponding to the PNA.
- c. The PCR product with the PNA oligomers were incubated at different proportions at 37 °C.
- d. Electrophoresis in a 1% agarose gel stained with ethidium bromide, in TBE buffer, with the use of the PCR product alone as a blank was performed. The shift of the PCR product mobility to a higher molecular weight compound indicates positive results.

3.2.2. NPs physical characterization

Measuring the NPs diameter by DLS (Dynamic Light Scattering), the surface charge of the NPs (Zeta potential), and the particle morphology by TEM and SEM. Each is measured three times independently. When characterizing NPs by zeta potential and DLS, it is critical to report NP concentration and buffer selection, as these factors greatly influence the values obtained. It is also recommended to perform these measurements before lyophilization.

A 0.05 mg/ml solution of NPs in diH2O is prepared for DLS, TEM and Zeta potential with complete resuspension the nanoparticles through vortexing and ultrasonic water bath.

For SEM, the dried NPs are used. The cryoprotectant should be removed from the final product by washing it three times with diH2O, because the presence of cryoprotectant may introduce artifacts during SEM imaging. Images could be analyzed using ImageJ software (National Institutes of Health).

3.2.3. Nucleic acids release from NPs

a. 4-6 mg of NPs were incubated from each batch in 600 µl of PBS in a 37 °C shaking incubator.

- b. At indicated time points (0, 3, 6, 9, 12, 24 and 48 hr), the tubes were spinned down and the supernatant was removed.
- c. The nucleic acid content of the supernatant was measured as the absorbance at 260 nm using the NanoDrop (Thermo Scientific) using PBS as a blank.

3.2.4. NPs loading test

NPs loading is the amount of nucleic acid encapsulated in the NPs, it is determined using aqueous phase extraction.

- a. 4 mg of NPs from each batch were dissolved in 0.5 ml of DCM at room temperature for at least 1 hour or untill complete dissolution.
- b. 0.5 mL of TE buffer was added to the dissolved NPs.
- c. Vigorous mixing by vortex for 2 min then centrifuging at high speed to spin it down. The aqueous phase was removed.
- d. Steps b and c were repeated with another 0.5 ml TE buffer for a total extraction volume of 1 ml TE buffer.
- e. The absorbance at 260 nm was measured of the combined 1 ml fraction with a Nanodrop.

3.3. Cells treatment

a. The choice of the cells to be cultured depends on the target gene such that this gene has to be expressed in these cells to test for the technique efficacy. The Human Protein Atlas (available at <u>https://www.proteinatlas.org/</u>) could be used to know where genes are expressed.

b. The dose to be used are either low dose (0.5 mg/ml), medium dose (1 mg/ml) or high dose (2 mg/ml). Sometimes, higher dose of 3 mg/ml could be used.



Figure 5. NPs loading results of loaded and blank NPs show difference in loading between both.



Figure 6. Gel mobility shift assay shows shift of the band to a higher molecular weight which indicates binding with PNA.

c. The amount of NPs was weighed according to the dose and the amount of media (which is related to the cell count in the plate).

d. 0.5 ml of the media was added to the NPs, mixed well and sonicated on ice for 30 sec till uniformly suspended, then added to the cells and the media was completed to the final volume.

e. The cells were watched, a sample for DNA and RNA extraction was taken at indicated time points.

4. RESULTS and DISCUSSION

pharmaceutical In synthesis, the reproducibility is one of the important challenges. Variables during PLGA NPs synthesis, isolation (washing and filtration), and characterization could affect the particle size and loading efficiency. Synthesis include parameters the chosen emulsification technique, the type of PLGA polymer and used stabilizer (21). Overall, all the variables must be chosen carefully and are listed in details.

The choice of the composition of the PLGA (i.e. the L:G or the lactate:glycolate ratio) is also crucial. For example, high lactate PLGA polymer produce NPs with increased crystallinity, leading to a slower rate of NPs degradation and a slower drug release ^{(22).}

There are several methods of PLGA NPs preparation techniques including emulsion solvent evaporation (ESE), spray-drying and nanoprecipitation. The PLGA NPs formulation technique is chosen according to its application, in addition to the physico–chemical properties of the encapsulated substance ⁽²¹⁾.

The ideal size and shape of NPs differ according to its intended application. For example,

PLGA NPs (size range: 120-150 nm) showed enhanced tumor accumulation when administrated intravenously in cancers, however, submicron NPs (500 nm) showed optimal sustained drug release (23,24). The diameter of PLGA PNA/DNA NPs are typically between 250-290 nm as measured by DLS (25) while in other patches the mean diameter was 150 nm ¹³. The results of the NPs characterization of the 2 patches; the loaded NPs and the blank NPs, should be compared concerning their size, shape and surface morphology. Differences between both indicates successful nucleic acids loading. Complete details regarding buffers used, concentrations ... etc. must be written to ensure reproducibility of the results because the above tests might vary among different conditions.

Loading of donor DNA and PNA is measured as mentioned above using absorbance at 260 nm, which are normalized to the amount of NPs used for extraction (**OD/mg/ml**); figure 5. Typical OD/mg/ml values range between 0.4 and 0.6. So far, *Schleifman et al.* have found that NPs with nucleic acids loading below 0.4 OD/mg/ml don't successfully edit genes in vitro ⁽²⁶⁾.

In the gel mobility shift assay, increasing the amount of the added PNA vs the amount of the PCR shift the band migration upwards as in figure 6. No noticed migration of the PCR band indicates strong binding between the DNA and the designed sequence. This happens because binding of the cationic peptides of the PNA to the charged DNA neutralize its charge in addition to the formed large complex is hard to move.

Successful gene editing could be determined by observing the new introduced sequence in the DNA either by direct sequencing or by allele specific PCR (AS-PCR). The efficacy of this edit could be determined by measuring the expression of the edited gene and comparing it to negative control. Higher doses and using multiple doses shall give better results.

Ricciardi and co-workers demonstrate safe intravenous and intra-amniotic administration of polymeric NP (containing PNAs and donor DNAs) to fetal mouse tissues at selected gestational ages with no effect on survival or postnatal growth. *Inutero* introduction of these NP corrects a diseasecausing mutation in the β -globin gene in a mouse model of human β -thalassemia, yielding sustained postnatal elevation of blood hemoglobin levels, reduced reticulocyte counts, and improved survival, with no detected off-target mutations in partially homologous loci ¹⁷.

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These recent successes in mouse models 2. raise the possibility that PNA-NP mediated gene editing may eventually provide the basis for a new genetic therapy for single-gene hereditary disorders, such as sickle cell anemia, thalassemia, and cystic fibrosis. It also may provide the basis for a safe method of fetal gene editing for human monogenic disorders.

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Conflicts of Interest: None to declare.

Ethical Statement: the study was approved by the ethical committee of Faculty of Pharmacy (Girls), Al-Azhar University (code: 199; session 21 at 25/3/2019).

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List of Abbreviations: AS-qPCR: Allele Specific quantitative PCR, Boc: Butyloxycarbonyl group, CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9, DCM: Dichloromethane, DLS: Dynamic Light Scattering, G6PD: Glucose-6-phosphate Dehydrogenase, HBB: Hemoglobin Beta (Beta Globin) Gene, IVS: intervening sequence, NPs: Nanoparticles, **OD**: Optical Denisty, PBS: Phosphate Buffer Saline, PLGA: Polylactic coglycolic Acid, PNA: Peptide Nucleic Acids, PVA: Polyvinyl Alcohol, QbD: Quality by design, SCD: Sickle cell Disease, SEM: Scanning Electron Microscope, TALENs: Transcription Activator-Like Effector Nucleases, TBE: Tris-Borate EDTA buffer, TEM: Transmission Electron Microscope.

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