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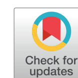
Negatively charged polypeptide-peptide nanoparticles showing efficient drug delivery to the mitochondria



Ifat Cohen-Erez [a,b], Hanna Rapaport [a,b,*]

[a] Avram and Stella Goldstein-Goren Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel
[b] Ilse Katz Institute for Nanoscale Science and Technology (IKI), Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

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ABSTRACT

Polymeric nanoparticles (NPs) represent an effective platform for drug delivery systems, albeit with various limitations including low drug loading capacity, cytotoxicity and specificity. NPs composed of the negatively charged Polypeptide, poly gamma glutamic acid (γ-PGA) and a designed amphiphilic and cationic β-sheet Peptide (denoted PoP-NPs) loaded with the drug lonidamine (LND), denoted LND-PoP-NPs were previously used in our lab to successfully target the mitochondria when coated with the peptide (LND-mPoP-NPs). In this study, we improved the drug capacity of the LND-mPoP-NPs in addition to lowering non-specific toxicity associated with the drug deficient mPoP-NPs. LND concentrations in LND-mPoP-NPs were increased (h-LND-mPoP-NPs) and the peptide coating concentration was decreased. The new h-LND-mPoP-NPs formulation shows the ability to carry the drug to the proximity of the mitochondria despite the NP's negative zeta potential.

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1. Introduction

Nanoparticles (NPs) consisting of biodegradable and biocompatible polymeric biomaterials, sized 10–100 nm have the ability to deliver a drug through the smallest blood capillary vessels, tissue gaps and penetrate cells by endocytosis [1–5]. NPs generated by such polymers may shield toxic drugs from the body, enable delivery of poorly soluble or unstable drugs thereby lowering treatment side effect and improving efficacy [6–9]. NPs exhibit limitations associated with low drug loading capacity, usually below 10% of the weight of the carrier. This limitation can be circumvented by linking the drug covalently to the polymeric carrier [3]. Nonetheless, the chemical modification associated with the covalent bonding may strongly affect the drug efficacy and pharmacokinetic properties [10]. In NPs that are prepared based on self-assembly principles, drug loading can be optimized by varying the composition and formulation routes of the NP components. Specifically, peptides can both play a role in stabilizing NPs prepared by self-assembly [11,12] as well as confer NPs with targeting to specific tissues [13,14] and even intracellular organelles.

Peptides containing both positively charged and hydrophobic amino acids, denoted cell penetrating peptides (CPPs) can facilitate intracellular internalization [15–18]. Specific peptide sequences known as mitochondria-localization sequences (MLS) [19–21] permit cytoplasmic proteins internalization through mitochondrial translocators into mitochondria [22,23]. Proteins cross the nuclear membrane while being led by nuclear localization sequences (NLS) [24]. Specific peptides were identified to target the lysosome, Golgi and endoplasmic reticulum (ER) compartments [16,25].

Many human disorders [10,26,27], such as metabolic syndrome, atherosclerosis, hypertension, type 2 diabetes and cancer can potentially be treated by targeting various drugs to the mitochondria [28–31]. Various drugs have failed in clinical trials probably due to low efficacy believed to be hampered by inadequate targeting to the mitochondria [32]. A few known mitochondria-targeted delivery moieties share the features of lipophilicity and positive charges, such as dequalinium (DQA) [33–35] and triphenylphosphonium (TPP) [36–38]. Similarly to mitochondria penetrating peptides (MPPs), these are attracted to the negatively-charged microenvironment of the mitochondrial matrix [39,40]. Synthetic MPP oligomers containing cationic (Lys and Arg) and hydrophobic (Phe and cyclohexane) residues developed by Horton et al. [40,41] were also utilized to target covalently linked DOX (mtDOX) to the mitochondria rather than to the nucleus [42,43]. Other nanocarrier systems utilized DQA decorated liposomes to target the mitochon-

* Corresponding author at: Avram and Stella Goldstein-Goren Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel.
E-mail address: hannarap@bgu.ac.il (H. Rapaport).

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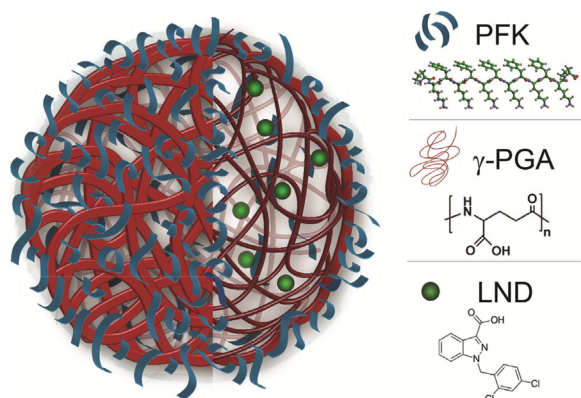

Fig. 1. Illustrations to assist in visualizing the principal components and structure of LND-mPoP-NPs. The NP core (PoP-NP) is composed of γ-PGA and PFK fibrils loaded with LND (represented in the scheme by a red thread, blue ribbons and green spheres, respectively). γ-PGA creates an anionic spherical matrix that is stabilized with fibril assemblies, formed by the cationic and amphiphilic peptide PFK. Each fibril is composed of PFK bilayers exposing the cationic side chains. This fibril arrangement is achieved by peptides in β-pleated conformation with hydrophobic and hydrophilic side chains segregated on opposite faces of the strand. PoP-NP is further on coated with the same PFK peptide fibrils, to generate the NP's shell (emphasized in the illustration on the left side of the sphere). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dria of cancer cells [44]. A polymeric drug delivery system with a covalently bond galectin-3-binding peptide that induced dual targeting to cancer cell surface and to the mitochondria was developed by Sun et al. [45].

We have recently developed a NP platform composed of the negatively charged Polypeptide, poly gamma glutamic acid (γ-PGA) and a designed amphiphilic and cationic β-sheet Peptide, Pro-Lys-(Phe-Lys)$_5$-Pro, PFK (denoted PoP-NPs) [11]. This peptide has an intrinsic tendency to form bilayer-fibril assemblies that interact electrostatically with the anionic polypeptide while creating hydrophobic niches within the NPs. The γ-PGA and the PFK peptide were shown to co-assemble simultaneously, with γ-PGA acting as the NP scaffold matrix while being stabilized with fibril assemblies formed by the cationic and amphiphilic peptide PFK (Fig. 1). When coated with the same peptide, this unique PoP-NP system (denoted mPoP-NPs) enabled intracellular delivery to the proximity of the mitochondria [11]. The negatively charged chemotherapeutic molecule lonidamine (LND) [46,47] which inhibits the mitochondrial outer membrane enzyme hexokinase [47,48] was loaded on the mPoP-NPs to form LND-mPoP-NPs. LND was used in the past for the treatment of benign prostate hypertrophy, breast, ovarian and lung cancer [49–52] however, the Food and Drug Administration (FDA) suspended its use, due to severe liver toxicity attributed to poor distribution, pharmacokinetic properties and low solubility [44]. LND-mPoP-NPs were found to reduce IC50 by two orders of magnitude compared to the free drug in osteosarcoma (Saos2) cell cultures. Yet, this formulation, deprived of the LND drug (mPoP-NPs), also exhibited certain levels of cytotoxicity suspected to be attributed to the peptide coating [11]. In this study, we aimed to improve the formulation of mPoP-NPs so to exhibit better effective drug delivery and reduced cytotoxicity. For this purpose, LND concentrations in LND-mPoP-NPs were increased and the peptide coating concentration was decreased (Fig. 1). We examined the effect of these formulation changes on NP size, shape, surface charge and efficacy versus cytotoxicity in osteosarcoma, Saos2 cell culture.

2. Materials and methods

2.1. Materials

Peptides were synthesized and then purified by high performance liquid chromatography to 95% (GenScript, Piscataway, NJ). Pro-Lys-(Phe-Lys)$_5$-Pro (Mw of 1717.16), denoted PFK was co-assembled with γ-PGA, 200–500 KDa (Wako Chemicals, Tokyo, Japan) to generate the PoP-NPs. A FITC N-terminal-labeled PFK peptide was incorporated into NPs to enable fluorescence measurements. Lonidamine (LND) was purchased from Abcam (Cambridge, UK). Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (Rehovot, Israel) and were of the highest available purity. All solutions were prepared with deionized water (DIW), 18.2 MΩcm (Thermo Scientific, Waltham, Massachusetts, USA).

2.2. LND loaded PoP-NPs formulations

LND solution was prepared by dissolving the drug in 1 mM NaOH at room temperature that was next heated to 60 °C until a clear solution was achieved. PFK was dissolved in DIW. The LND and PFK solutions were mixed using a magnetic stirrer overnight. PoP-NPs loaded with LND, were prepared by first mixing 1 ml of 0.5 mg/ml PFK with the same volume of 0.15 mg/ml or 0.5 mg/ml LND solution, denoted LND-PoP-NPs and h-LND-PoP-NPs, respectively, overnight. In LND-PoP-NPs and h-LND-PoP-NPs there is a ratio of 1.2:0.3 and 1.2:1 between the formal positive charges of PFK and the negative charges of LND, respectively. One ml of 0.5 mg/ml γ-PGA dissolved in 0.1 M NaOH was added to each of the PFK-LND solutions, followed by adjusting the pH to 7.4 by aliquots of 0.1 M HCl. The three-component solutions (0.167 mg/ml PFK and γ-PGA and 0.05 or 0.167 mg/ml LND) were stirred overnight then centrifuged at 3000*g* for 20 min, and finally the supernatant was passed through a syringe-driven 0.22 μm filter. These final NPs filtered solutions (as well as other formulations described below) were examined on the same day of their preparation.

Control PoP-NPs were prepared as a drug-free version of the LND-PoP-NPs. One ml of the 0.5 mg/ml PFK was mixed with the same volume of 1 mM NaOH (representing the LND solution). This PFK in NaOH solution was then stirred using a magnetic stirrer overnight and supplemented with one ml of 0.5 mg/ml γ-PGA. This PoP-NPs solution was next stirred, centrifuged and filtered as mentioned above for the drug loaded version.

2.3. PFK coating to generate mitochondria targeted NPs

PFK solutions at different concentrations were subsequently applied to the NPs described above to generate the NP coating layer. Since PFK tends to induce PoP-NP aggregation, a range of peptide concentrations was tested to identify that which after applied to h-LND-PoP-NPs would still yield a clear solution. h-LND-PoP-NP solutions, 0.9 ml, prepared as above, were subjected to coating with 0.1 ml of PFK solutions, 0.5–10 mg/ml (adjusted to pH 7.4 with aliquots of NaOH), such that the final peptide coating concentration ranged from 0.05 to 1 mg/ml. The selected concentration, 0.05 mg/ml (see text, Section 3.2) which appeared clear to the naked eye, was passed through a syringe-driven 0.22 μm filter, to yield the final h-LND-mPoP-NP solution.

2.4. NP size and zeta potential characterization

The NP hydrodynamic radius was measured by dynamic light scattering, DLS (CGS-3 LSE-5003, ALV, Langen, Germany) applying the detector at a 90° angle. Zeta potential was measured by Zetasizer (Zetasizer Nano ZS, Malvern, Worcestershire, UK) and data

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