53

Development of Peptide-Modified Gemini Nanoplexes for Non-Viral Gene Delivery to the Retina

Lokesh Narsineni[1,2*], Ding-Wen (Roger) Chen[1,3] and Marianna Foldvari[1,4]

[1]School of Pharmacy, University of Waterloo, Waterloo, Canada, [2]Department of Biochemistry and Molecular Biology, The University of British Columbia, Vancouver, Canada, lokeshn@mail.ubc.ca; [3]Children's Hospital of Philadelphia, Philadelphia, PA, USA, [4]Waterloo Institute of Nanotechnology, University of Waterloo, Canada

Drug Delivery and Pharmaceutical Nanotechnology Laboratory
School of Pharmacy, University of Waterloo

INTRODUCTION

Current glaucoma management relies on reducing and maintaining a low intraocular pressure (IOP), an important risk factor for the progression of visual field loss, by pharmacological and invasive surgical treatments. Alternatively, gene therapy with neurotrophic factors (NF) has the potential to provide neuroprotective and neuroregenerative functions for retinal ganglion cells (RGCs) to prevent or restore visual deterioration. The design and development of these systems especially become more complicated when considering the *in vivo* structural and physicochemical barriers faced by the gene medicine intended to reach the posterior segment of eye, that is the location of neurodegenerative processes. Many lipid, surfactant and polymer based non-viral vectors were tried to deliver gene cargo into the retinal layers of the eye but crossing all the physicochemical barriers and achieving therapeutic levels at the target site has been one of the biggest challenges for these kinds of systems.

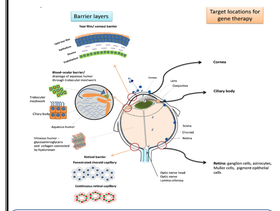


Fig 1. Barriers, penetration pathways and target locations for gene therapy in the eye (Foldvari et al, 2016)

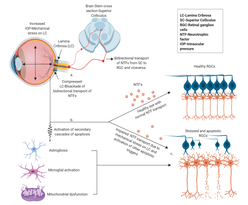
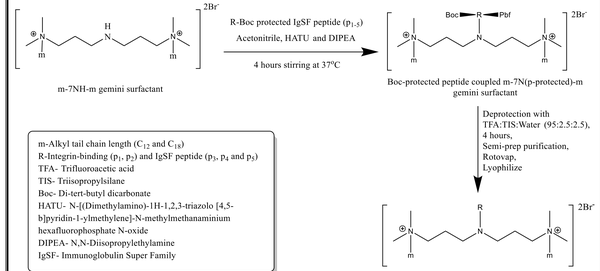


Fig 2. Mediators of RGC apoptosis and NTF deprivation. Figure generated by Lokesh Narsineni using BioRender.com

The main objective was the development of a new generation of nanoplex (NPX) system based on cell adhesion peptide (CAP)- from modified dicationic gemini surfactants (m-s(p)-m) for targeted delivery of neurotrophic factor genes to the retina.

METHODS

1. Synthesis of m-7N(p)-m (m=12 and 18, p=$p_{1\text{-}5}$) gemini surfactants



m-Alkyl tail chain length (C_{12} and C_{18})
R-Integrin-binding (p_1, p_2) and IgSF peptide (p_3, p_4 and p_5)
TFA- Trifluoroacetic acid
TIS- Triisopropylsilane
Boc- Di-tert-butyl dicarbonate
HATU- N-[(Dimethylamino)-1H-1,2,3-triazolo [4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide
DIPEA- N,N-Diisopropylethylamine
IgSF- Immunoglobulin Super Family

Scheme 1 General scheme for synthesis of integrin-binding and IgSF peptide functionalized m-7N($p_{1\text{-}5}$)-m gemini surfactant

2. Formulation of m-7NH-m, m-7N($p_{1\text{-}5}$)-m NPXs/pNPXs and lipoplexes/peptide-lipoplexes (LPXs/pLPXs)

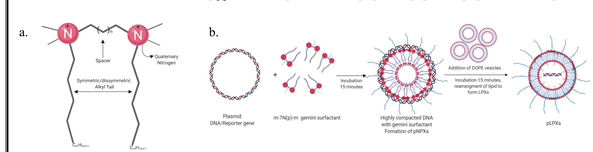


Fig 3. (a) General schematic structure of gemini surfactant, (b)General formulation steps and schematic models of compacted NPXs/pNPXs and LPXs/pLPXs. Figure generated by Lokesh Narsineni using BioRender.com.

METHODS

***In vitro* corneal penetration studies using the 3D EpiCorneal™ model**

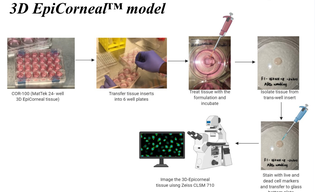


Figure 4 Illustration of nanoparticle penetration studies in the 3D-EpiCorneal™ tissue model. Figure generated by Lokesh Narsineni using BioRender.com.

Transfection efficiency and viability studies by flow cytometry

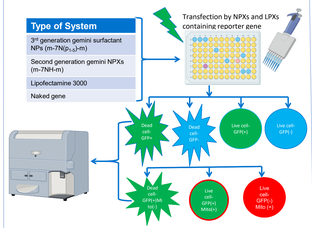


Figure 5 Schematic of *in vitro* evaluation of GP and pGP NPXs/pNPXs and LPXs/pLPXs on A7 cell line

***In vitro* transfection studies in retinal stem cell-derived neurospheres**

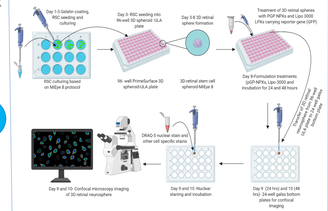


Figure 6 Schematic for evaluation of the TE and toxicity of pNPXs and pLPXs in 3D retinal stem cell derived neurospheres. Figure generated by Lokesh Narsineni using BioRender.com.

***In vivo* retinal gene transfer studies in a CD1 mice**

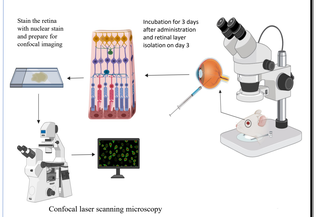


Figure 7 Schematic method for *in vivo* studies to determine the ability of pGP-NPXs in transfecting retinal cells in eye . Figure generated by Lokesh Narsineni using BioRender.com.

RESULTS

1. Synthesis and characterization of m-7N(p)-m (m=18, p=$p_{1\text{-}5}$) gemini surfactants

Peptide-modified gemini surfactants (m-7N(p_n)-m, m=12 and 18 p_n=p_1, p_2, p_3, p_4 and p_5) were synthesized using an amide bond coupling technique with HATU/DIPEA as coupling agent. Synthesized m-7N(p)-m gemini surfactants were confirmed by ESI-MS. 18-7N(p_1)-18, 18-7N(p_2)-18, 18-7N(p_3)-18, 18-7N(p_4)-18, 18-7N(p_5)-18 and 12-7N(p_5)-12 were synthesized and molecular weight (Da) was found to be 1022.65, 1092.782, 1355.09, 920.638, 1493.12 and 1324.80 respectively.

2. Formulation and characterization of m-7NH-m, m-7N($p_{1\text{-}5}$)-m NPXs/pNPXs and lipoplexes/peptide-lipoplexes (LPXs/pLPXs)

NPXs/pNPXs, LPXs and pLPXs were formulated using synthesized gemini surfactants as per the protocol listed under methods and characterizations such as particle size and zeta potential were carried out for the prepared formulations.

A. Particle size and Zeta potential

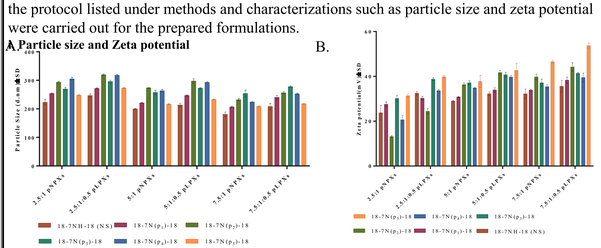
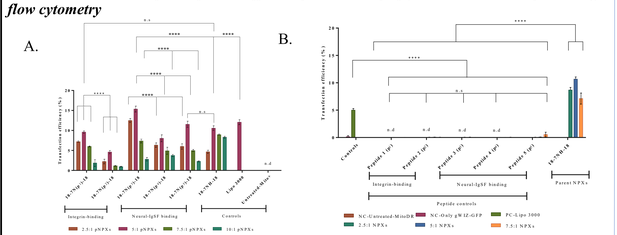


Figure 8 Physicochemical properties of 18-7NH-18 NPXs and LPXs and 18-7N($p_{1\text{-}5}$)-18 pNPXs and pLPXs. A) Z-average particle size of the five 18-7N($p_{1\text{-}5}$)-18 pNPXs and pLPXs, and the parent 18-7NH-18 NPXs and LPXs at 2.5:1, 5:1 and 7.5:1 G:P ratio and 2.5:1:0.5, 5:1:0.5 and 7.5:1:0:5 G:P:L ratios; B) Zeta potential for 18-7NH-18 NPXs and LPXs and 18-7N($p_{1\text{-}5}$)-18 pNPXs and pLPXs. All values expressed as mean ± S.D., n=3.

3. In vitro assessment of transfection efficiency and toxicity of pNPXs in A7 astrocytes using flow cytometry



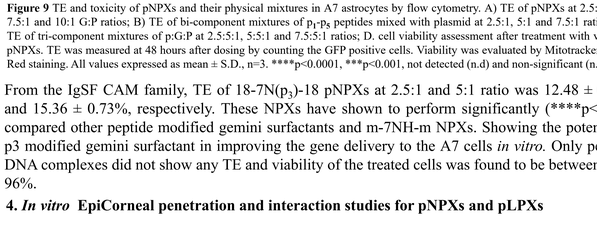


Figure 9 TE and toxicity of pNPXs and their physical mixtures in A7 astrocytes by flow cytometry. A) TE of pNPXs at 2.5:1, 5:1, 7.5:1 and 10:1 G:P ratios; B) TE of bi-component mixtures of p_1-p_6 peptides mixed with plasmid at 2.5:1, 5:1 and 7.5:1 ratios; C) TE of tri-component mixtures of p:G:P at 2.5:5:1, 5:5:1 and 7.5:5:1 ratios; D. cell viability assessment after treatment with various pNPXs. TE was measured at 48 hours after dosing by counting the GFP positive cells. Viability was evaluated by Mitotracker Deep Red staining. All values expressed as mean ± S.D., n=3. ****p<0.0001, ***p<0.001, not detected (n.d) and non-significant (n.s)

From the IgSF CAM family, TE of 18-7N(p_3)-18 pNPXs at 2.5:1 and 5:1 ratio was 12.48 ± 0.49% and 15.36 ± 0.73%, respectively. These NPXs have shown to perform significantly (****p<0.001) compared other peptide modified gemini surfactants and m-7NH-m NPXs. Showing the potential of p3 modified gemini surfactant in improving the gene delivery to the A7 cells *in vitro*. Only peptide-DNA complexes did not show any TE and viability of the treated cells was found to be between 86%-96%.

4. *In vitro* EpiCorneal penetration and interaction studies for pNPXs and pLPXs

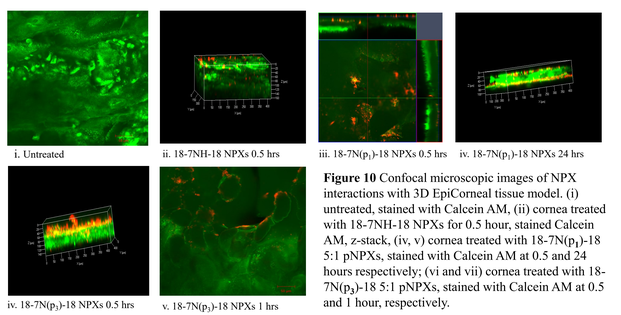


Figure 10 Confocal microscopic images of NPX interactions with 3D EpiCorneal tissue model. (i) untreated, stained with Calcein AM, (ii) cornea treated with 18-7NH-18 NPXs for 0.5 hour, stained Calcein AM, z-stack, (iv, v) cornea treated with 18-7N(p_1)-18 5:1 pNPXs, stained with Calcein AM at 0.5 and 24 hours respectively; (vi and vii) cornea treated with 18-7N(p_3)-18 5:1 pNPXs, stained with Calcein AM at 0.5 and 1 hour, respectively.

RESULTS

5. Transfection efficiency studies in 3D retinal neurosphere

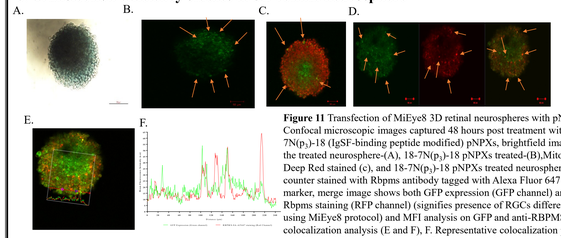


Figure 11 Transfection of MiEye8 3D retinal neurospheres with pNPXs. Confocal microscopic images captured 48 hours post treatment with 18-7N(p_3)-18 (IgSF-binding peptide modified) pNPXs, brightfield image of the treated neurosphere-(A), 18-7N(p_3)-18 pNPXs treated-(B),Mitotracker Deep Red stained (c), and 18-7N(p_3)-18 pNPXs treated neurospheres counter stained with Rbpms antibody tagged with Alexa Fluor 647 (RGC marker, merge image shows both GFP expression (GFP channel) and Rbpms staining (RFP channel) (signifies presence of RGCs differentiated using MiEye8 protocol) and MFI analysis on GFP and anti-RBPMS (red) colocalization analysis (E and F), F. Representative colocalization profile of GFP and RBPMS staining (signifies mature RGC population).

6. Gene delivery to the retina *in vivo* in a CD1 mouse model

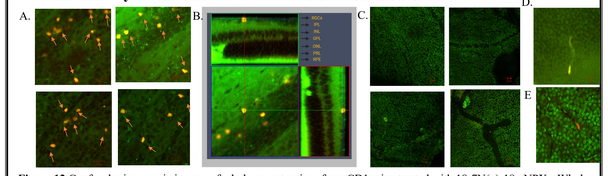


Figure 12 Confocal microscopic images of whole mount retinas from CD1 mice treated with 18-7N(p)-18 pNPXs. Whole mount images of retina treated with 18-7N(p_3)-18 5:1 pNPXs (A) and Saline controls (C). Whole mount retinas were isolated post 72 hours of administration by intravitreal injection. Retinas were stained with Syto 13 and imaged using CLSM 710 using green (Syto 13) and red channel (tdTomato), (D) Lipofectamine 3000 treated, (E) 18-7NH-18 5:1 NPXs treated and Image (B) shows 3D Z-stack orthogonal section analysis of 18-7N(p_3)-18 pNPXs treated retinas showing gene expression in the RGC layer and INL.

7. BDNF gene expression in the retina in vivo in mice treated with integrin and IgSF binding peptide modified gemini surfactant pNPXs

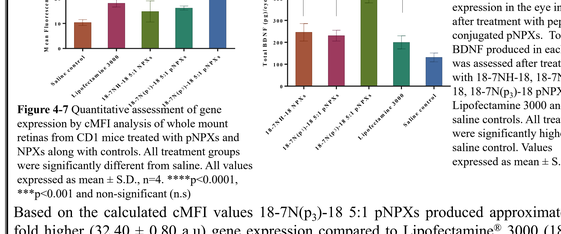


Figure 4-8 BDNF gene expression in the eye in vivo after treatment with peptide-conjugated pNPXs. Total BDNF produced in each eye was assessed after treating with 18-7NH-18, 18-7N(p_1)-18, 18-7N(p_3)-18 pNPXs, Lipofectamine 3000 and saline controls. All treatments were significantly higher than saline control. Values expressed as mean ± S.D., n=6

Figure 4-7 Quantitative assessment of gene expression by cMFI analysis of whole mount retinas from CD1 mice treated with pNPXs and NPXs along with controls. All treatment groups were significantly different from saline. All values expressed as mean ± S.D., n=4. ****p<0.0001, ***p<0.001 and non-significant (n.s)

Based on the calculated cMFI values 18-7N(p_3)-18 5:1 pNPXs produced approximately 2-fold higher (32.40 ± 0.80 a.u) gene expression compared to Lipofectamine® 3000 (18.07 ± 1.487 a.u, ****p<0.0001), 18-7N(p_1)-18 5:1 pNPXs (16.16 ± 0.80 a.u , ***p<0.001) and 18-7NH-18 5:1 NPXs (14.79 ± 4.24 a.u , ****p<0.0001). No significant difference in cMFI was observed between 18-7N(p_1)-18, Lipofectamine 3000 and 18-7NH-18 5:1 NPXs.

The highest amount of BDNF was produced after treatment with 18-7N(p_3)-18 pNPXs (422.60 ± 42.60pg/eye (average weight of an eye-17.71 mg), n=6, ****p<0.0001), followed by 18-7NH-18, 18-7N(p_1)-18 and Lipofectamine® 3000 (245.90 ± 39.72, 230.62 ± 24.47, 199.99 ± 29.90 pg/eye, respectively).

Summary

Overall, two sets of peptide-modified gemini pNPXs were formulated. Integrin-binding 18-7N($p_{1\text{-}2}$)-18 and neural IgSF CAM binding-peptide-modified 18-7N($p_{3\text{-}5}$)-18 were synthesized and pNPXs were formulated. Their efficiency in delivering reporter/therapeutic gene to the retina was evaluated *in vitro* and *in vivo*. In conclusion, the novel multifunctional IgSF peptide and integrin-binding peptide conjugated gemini NPXs provide a promising non-viral *in vivo* gene delivery approach to retinal cells.

ACKNOWLEDGEMENTS

UNIVERSITY OF WATERLOO FACULTY OF SCIENCE School of Pharmacy
UNIVERSITY OF WATERLOO
CIHR IRSC Canadian Institutes of Health Research / Instituts de recherche en santé du Canada
NSERC CRSNG
INNOVATION.CA CANADA FOUNDATION FOR INNOVATION / FONDATION CANADIENNE POUR L'INNOVATION
Ontario Research Fund
nano ontario