Nicotinamide-Loaded Peptoid Nanotubes for Energy Regeneration in Neonatal Brain Injury

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Motivation and Background

- The brain accounts for over 20% of total oxygen metabolism.[1] Adenosine triphosphate (ATP) levels are positively associated with brain nicotinamide adenine dinucleotide (NAD^+) levels and the NAD^+/NADH ratio.[2] Acute injuries to the brain such as perinatal asphyxia, stroke, and traumatic brain injury lead to rapid consumption of NAD^+ and decreased ATP production (Figure 1).
- To prevent DNA damage and cell death, ATP levels need to be maintained, which can be achieved by replenishing NAD^+ levels immediately after the acute injury. One way to achieve energy regeneration is to deliver NAD^+ or NAD^+ precursors loaded in nanoparticles, which can transport through the blood-brain barrier, penetrate the brain, and accumulate in target cells.[3] In the brain, NAD+ is more likely to be made by nicotinamide (NAM)-derived nicotinamide mononucleotide (NMN) available locally to brain cells.
- However, since mammalian cells cannot import NAD^+ and the cell-specific targeting of NAD+ and NAM is limited,[4] cellular delivery strategies are needed to capture the potential of an NAD^+-restorative therapeutic approach.

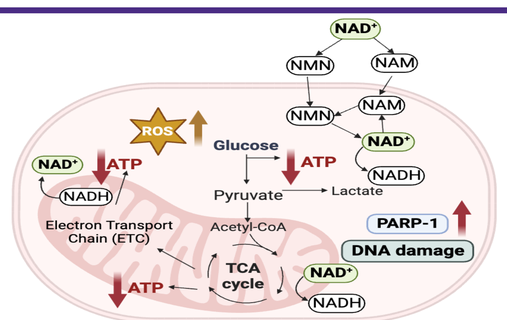

Figure 1. Schematic diagram showing metabolic pathway and NAD+ metabolism in injured cells.

Objective

In this study, we utilize a nano-based delivery strategy to conjugate NAM and NAD+ to peptoid nanotubes and achieve cell specific uptake in the neonatal brain. Peptoids are a synthetic analog of peptides synthesized by the Chen lab and can be precisely engineered with functional groups or ligands for cargo incorporation and targeted delivery. The objective of this study is to (a) demonstrate the biocompatibility and therapeutic efficacy of peptoid nanotubes as a drug delivery vehicle through in vitro, ex vivo, and in vivo models and (b) show the potential of NAD+ regenerating therapeutic approach in neonatal brain injury.

Methods

- Peptoids are highly thermally and chemically stable. The enhanced stability prevents premature drug release caused by enzymatic degradation.[5,6]
- Peptoid nanosheets are functionalized with thymine (Nthy) to conjugate NAM directly to the peptoid backbone and are then self-assembled into tubular form to create peptoid nanotubes (PNT) (Figure 2). PNT synthesis and characterization is performed by Dr. H. Trinh and Dr. C. Chen at PNNL.

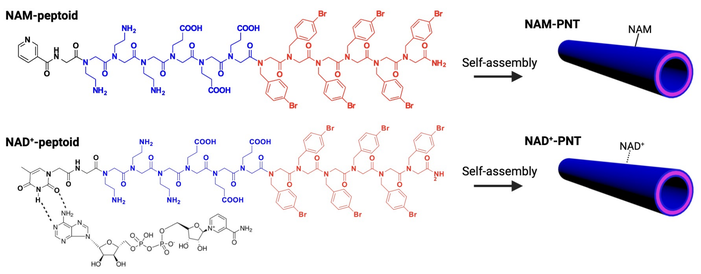

Figure 2. NAM/thymine-conjugated peptoid structure.

- **PNT length modulation**: We sonicated PNTs from 0 minutes to 360 minutes to produce 1μm long PNTs (PNT0) to 100nm long PNTs (PNT360). PNT length and tubular structure after sonicated were confirmed using Atomic Force Microscopy (AFM) (Figure 3).

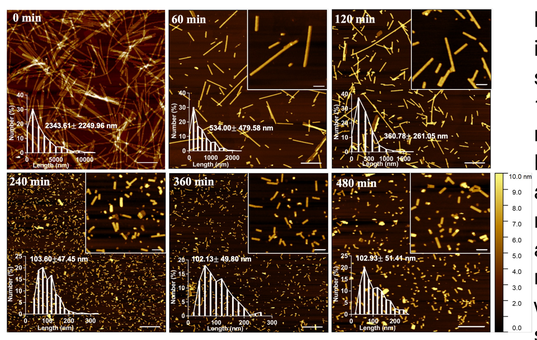

Figure 3. AFM images of PNT sonicated for 0, 60, 120, 240, and 360 min (PNT0, PNT60, PNT120, PNT240, and PNT 360, respectively) show a decrease in nanotube length with an increase in sonication time.

- **Model systems**: we use two model systems: (1) BV-2 cells were seeded at 10k and proliferated for two days; (2) Organotypic whole hemisphere (OWH) brain slices (300 μm thick) from postnatal day 10 (P10) male rats were cultured for 4 days in vitro (DIV). In both models, we mimicked hypoxia ischemic (HI) injury via oxygen-glucose deprivation (OGD) for 10 min (cells) or 30 min (slices).[7] In vivo efficacy was tested on P10 rats with unilateral left carotid artery ligation and 3 h of hypoxia exposure.
- **PNT compatibility and NAM-PNT efficacy:** We applied PNT treatments at different doses immediately after the injury to cell culture media or topically to the OWH brain slice, or intraperitoneal (i.p.) injection to P10 rats. Controls include non-OGD exposed healthy cells or slices, saline, free NAM or free NAD+, and blank PNTs. At 24h -72h after treatment, we measured cell death, ATP, NAD/NADH, cellular proliferation, RNA expression, and PNT cellular uptake.

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Patents: (1) Nance et al. Nanoparticle-mediated DNA repair through cellular delivery of NAD+ precursors. (2) Nance et al. NAD repair through nanotherapeutic delivery of NAD+ precursors.

Results

NAM-PNTs show high biocompatibility and improve cellular energy and metabolic state in the energy-depleted, acutely injured brain.

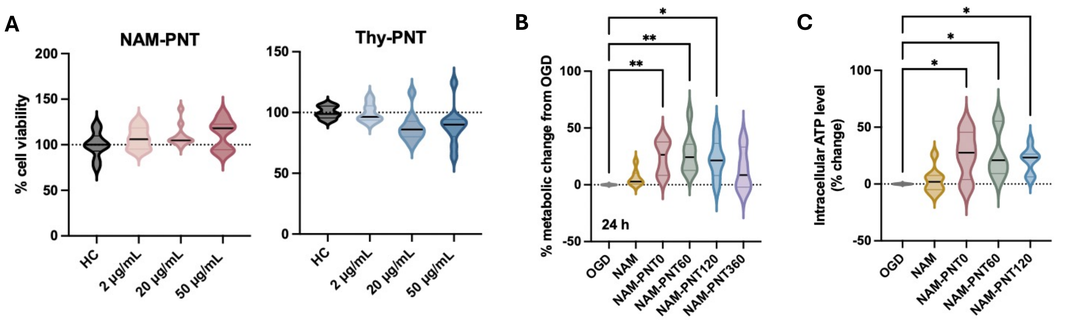
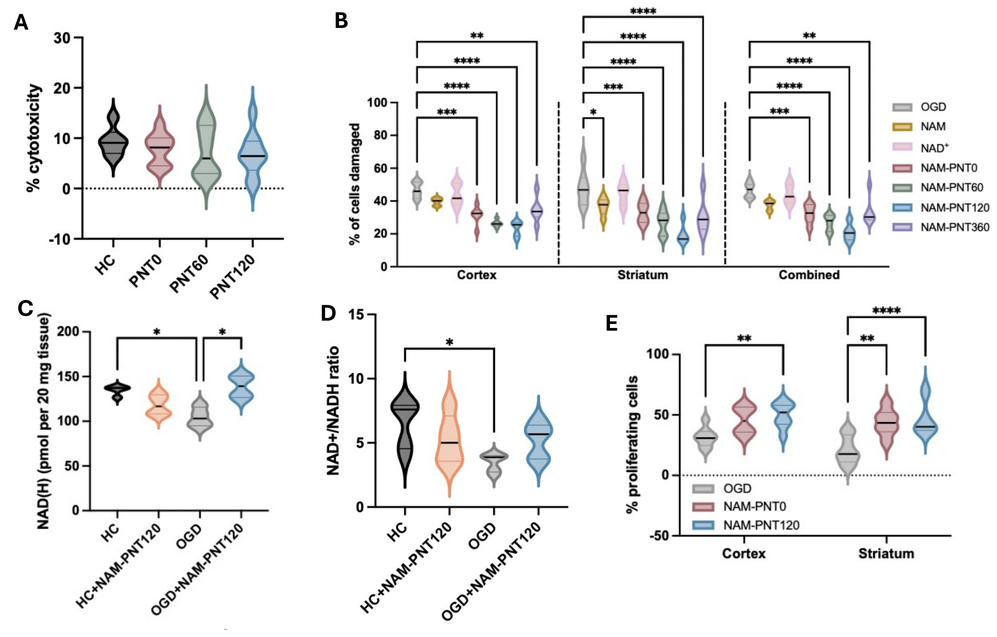


Figure 4. Compatibility of PNTs in healthy BV-2 cells the efficacy of NAM-PNT in OGD-exposed BV-2 cells. (A) MTT data showing both NAM-PNT and Nthy-PNT gave negligible effect on cell viability compared to healthy control at all three dosages. (n = 10). (B) PNT length impacts the extent of viability improvement for 20μg/mL of NAM delivered via PNTs. n = 4-6 independent experiments for all studies. (C) Different lengths of NAM-PNTs dosed at 20ug/mL improved intracellular ATP levels in OGD-exposed BV-2 cells compared to healthy controls. (n = 7-9). Data are presented as violin plots that show the full range with lines at the median with interquartile range. *P<0.05, **P<0.01.

NAM-PNT treatment reduces the % of damaged cells in a PNT length dependent manner in OGD exposed brain slices and drives cellular proliferation.



NAM-PNT treatment reduces global injury, area loss, pro-inflammatory cytokine expression levels and in HI-treated brain.

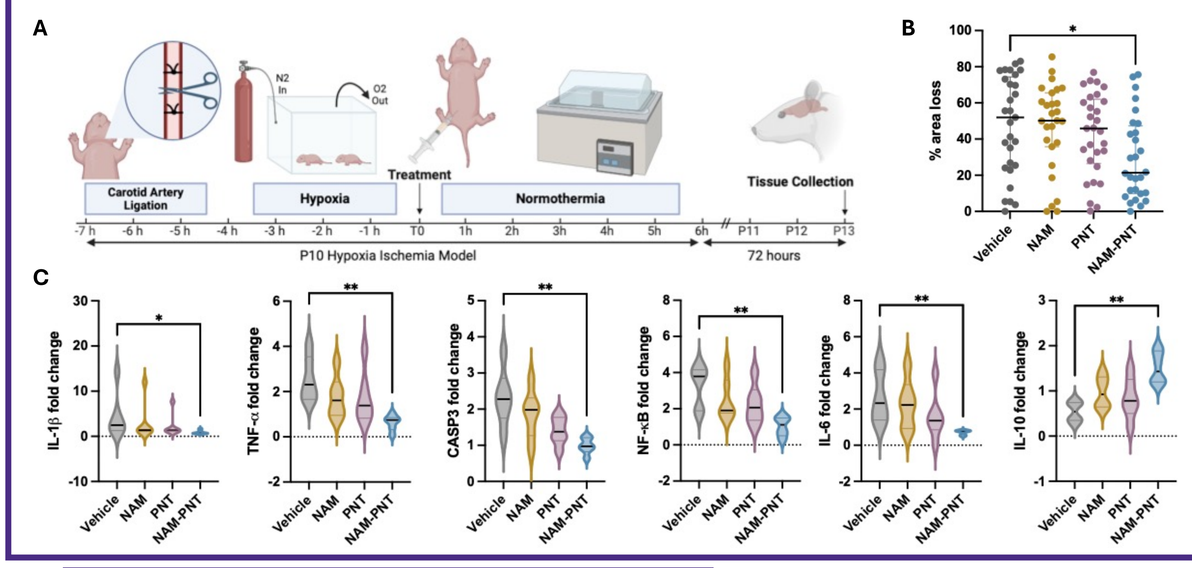


Figure 7. (A) HI pups were treated 30 min after HI injury with saline (vehicle. 10 mL/kg), free NAM (25 mg/kg), blank PNT (500 mg/kg), or NAM-PNT (500 mg/kg). The pups were euthanized on P13, 72 h after HI injury. All analyses were performed in a blinded manner. (B) Total area of loss was calculated by assessing the percent area of tissue lost in the ipsilateral hemisphere, normalized to the contralateral hemisphere: vehicle, n = 29; free NAM, n = 27; PNT, n = 29; NAM-PNT, n = 28. (C) Fold-changes of mRNA markers compared to the contralateral hemisphere showing NAM-PNT helps regulating RNA expression in P10 rats after HI injury with PNT treatments. (n = 6). Data are presented as violin plots that show the full range with lines at the median with interquartile range. *p<0.05, **p<0.01.

PNTs localize primarily in Iba1+ microglia after 24h and show slow endocytosis profile.

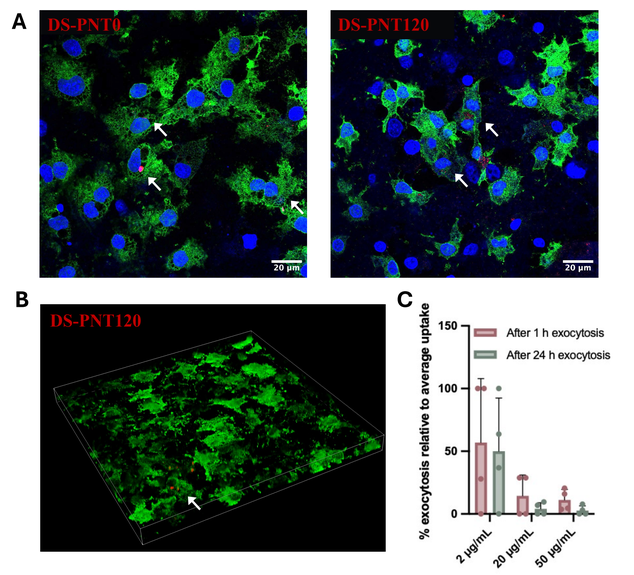


Figure 5. (A) Localization of different length Dansyl-labeled-PNTs (red) in Iba-1+ cells (green) in the cortex and striatum of healthy OWH brain slices at 24h after exposure. Scale bar: 20 μm. (B) Z-stacked image showing PNT localization is likely cellular. (C) PNT endocytosis (percentage) after 1h and 24h relative to the average uptake in the BV-2 cell model. n = 4.

Figure 6. (A) Cytotoxicity of NAM-PNT of varying lengths on healthy OWH brain slices. Lactate dehydrogenase (LDH) release (%) values are normalized to the LDH release of acute slices immediately treated with TX-100 (positive cell death control). n = 6-8 slices per group. (B) NAM-PNT treatment reduces the % of damaged cells, assessed by PI quantification, in a PNT length-dependent manner. (n = 7 slices). (C-D) Intracellular total NAD (C) and NAD+/NAD(H) ratio (D) in healthy (HC) and OGD-treated slices with or without NAM-PNT120 for 24 h. (n = 5). (E) Percentage of proliferating cells in after OGD at the cortex and the striatum. Data are presented as violin plots that show the full range with lines at the median with interquartile range (n = 6-8). *p<0.05, **p<0.01, ***p<0.001, ****p<0.001.

Conclusions and Ongoing Work

- PNTs are biocompatible without inducing an inflammatory response in the healthy brain and show localization in microglia. NAM-PNTs improved cell viability, replenished cellular energy supply and NAD(H) levels, suppressed inflammation, and reduced cell death after acute brain injury.
- Our findings highlight the strong therapeutic potential of NAM-PNTs for for cell-specific targeted delivery and energy restoration in the acutely injured neonatal brain.
- Further study will be focused on pharmacokinetics and biodistribution of PNT in healthy and HI-treated neonatal Rats. Preliminary study has shown that rhodamine-PNT accumulates in the brain (Figure 8).
- Though we have explored the therapeutic efficacy of NAM-PNT, the mechanism of PNT cellular internalization still remains unclear. By using endocytosis inhibitors, we are aiming to discover microglia nanoparticle uptake mechanism and their changes in response to the properties of PNTs.

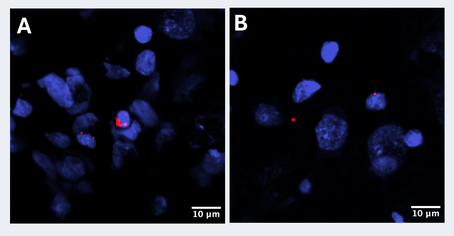


Figure 8. Rhodamine-PNT (red) colocalized with brain cells (nuclei: blue) ipsilateral (A) and contralateral (B) to the carotid artery ligation. Scale bar: 10 μm.