Purified Anelloviral Capsid Proteins can Bind and Encapsidate Different Nucleic Acid Payloads into AnelloVectors® for Gene Delivery

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Background

Human anelloviruses are an abundant and highly divergent class of commensal viruses with no known disease association and low immunogenicity. In addition, their diversity and abundance in all tissues presents potential for tissue tropism. These features position them as ideal candidates for potentially safe and redosable gene delivery of a diverse range of therapeutic nucleic acid payloads for various disease indications.

These characteristics of anelloviruses inspired us to develop AnelloBricks®, a cell-free, in-vitro assembled genetic medicines platform which is composed principally of two components: recombinant anellovirus capsid protein and a nucleic acid payload (e.g., DNA, RNA), offering payload diversity while also simplifying production to conventional and readily scalable systems at dramatically lower cost.

Using electrophoretic mobility shift assays followed by in-house electron microscopy (EM), we evaluated the binding and encapsidation of different nucleic acids with purified capsid proteins. The tested payloads included single and double stranded DNA and RNA with different structures and sizes. Successful in-vitro formation of a virus-like particle, the anelloviral vector (AnelloVector®/ANV), was achieved with DNA and RNA payloads as confirmed by electron microscopy. In-vitro transduction in cell culture is currently being utilized to inform the development and enhancement of these ANVs towards becoming a truly versatile gene delivery platform.

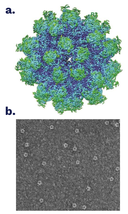


Figure 1:
a. Cryo-EM map of an ANV produced by Ring Therapeutics[1].
b. EM micrograph of capsid protein without nucleic acid payload.

EMSA-Guided VLP Assembly

Electrophoretic mobility shift assays (EMSAs) are commonly used in research to assess capsid protein interactions with nucleic acids and demonstrate in-vitro VLP formation[2,3]. This technique involves mixing a nucleic acid substrate with increasing amounts of capsid protein and running the samples in a non-denaturing agarose gel. Interaction between the nucleic acid and capsid protein causes the nucleic acid signal to "shift" in the gel due to the increased mass of the complex.

Assembly often results in a distinct band in the gel, corresponding to VLP, when the correct stoichiometric ratio of protein to nucleic acid is achieved. However, due to the overall positive charge of anelloviral capsid protein, ANV formation in our EMSA gels is indicated by a shift of nucleic acid signal entirely into the well of the gel.

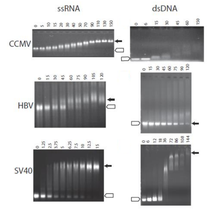


Figure 2: ***Examples of EMSA-guided assembly of different VLPs encapsidating ssRNA and dsDNA visualized with nucleic acid stain. Empty arrows denote unbound nucleic acid; closed arrows denote known migration of capsid[2].***

ANV Production Workflow

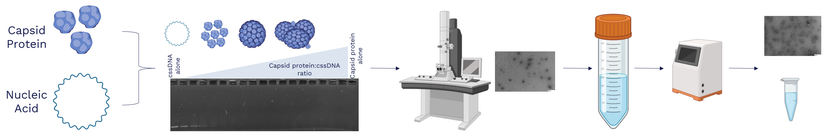
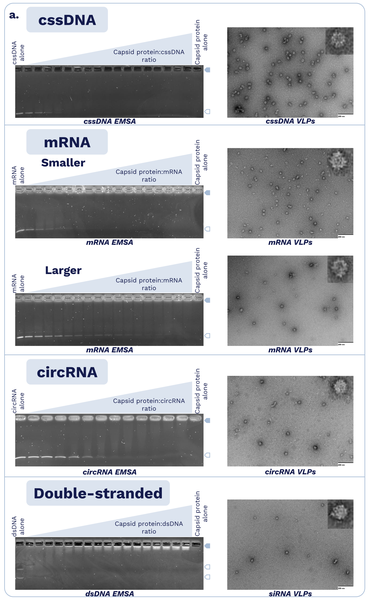


Figure 3: ***Anelloviral capsid protein and nucleic acid payload are prepared separately for encapsidation. Both components are used for small-scale EMSA reactions. Select reactions are spot-checked by electron microscopy to determine optimal capsid protein:payload ratio for ANV formation. Too little capsid protein leads to unprotected, protein-bound payload. Too much leads to increased asymmetrical clusters. The identified optimal ratio is scaled up volumetrically and then concentrated and formulated to produce ANVs ready for transduction.***

ANV Packaging Versatility



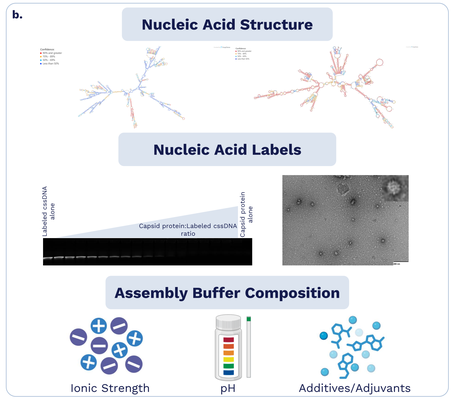
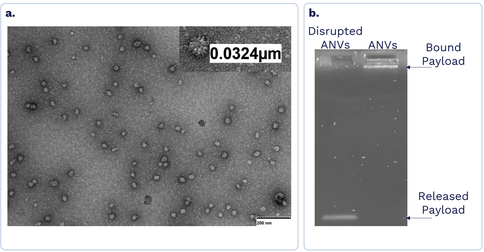


Figure 4:
a. In-vitro ANV assembly enables modularity in payload packaging. Left: EMSA assay with specified payload and anellovirus capsid protein. Empty arrows denote unbound nucleic acid; closed arrows denote capsid protein-bound nucleic acid. Right: EM micrograph of the ANVs produced with an example particle enlarged to show detail.
b. Factors amenable with in-vitro ANV assembly. Top: Different single-stranded nucleic acid structures successfully encapsidated in ANVs. Middle: Example of a labeled nucleic acid (fluorophore-labeled) encapsidated in ANVs. Bottom: In-vitro assembly conditions that can be adjusted to study particle formation.

ANV Characterization



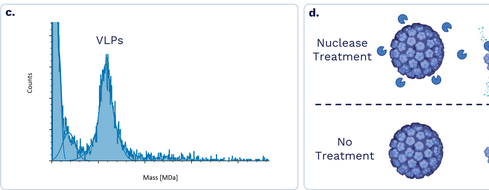


Figure 5:
a. Electron microscopy can determine ANV diameter and morphology.
b. Agarose gel electrophoresis confirms absence of unbound nucleic acid in ANV samples and recovery of intact nucleic acid payload from disrupted particles.
c. Mass photometry is employed to assess average ANV mass and distribution.
d. qPCR assay quantifies protected nucleic acid. ANVs are treated with nuclease that digests non-protected nucleic acid. Inactivation of nuclease and removal of capsid protein yields protected nucleic acid which can be quantified in qPCR.

In-vitro Protein Expression Confirms Activity of Assembled ANVs

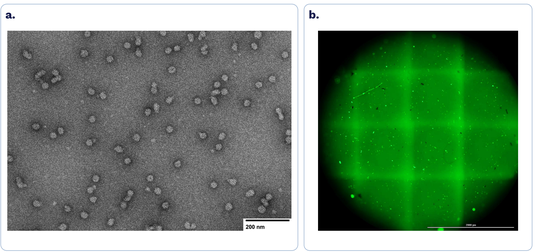


Figure 6:
a. In-vitro produced ANVs containing cssDNA payload encoding eGFP.
b. HEK-293 cells transduced with ANVs to express eGFP. eGFP expression was confirmed by various analytical methods including microscopy, shown here, confirming the activity of the in-vitro produced ANVs and validating HEK-293 transduction as a suitable method to measure activity of the various particles produced.

Acknowledgements

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In-vivo poster

Tues, May 13, 6:00-7:30 PM, Poster Hall I2, Poster 880
Infectivity and Expression in Mice of an Anelloviral Vector Produced by In Vitro Assembly.

Oral presentation

Thurs, May 15, 4:30-4:45 PM, New Orleans Theater B
AnelloBricks: Development of a Scalable, Low-Cost, In-Vitro Assembled Anellovirus-Derived Platform for Gene Therapy Applications.

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