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RPPR Final Report
as of 04-Mar-2022

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Proposal Number: 65833LS **Agreement Number: W911NF-14-2-0091**
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Report Date: 09-Nov-2016 Date Received: 04-Mar-2022
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Report Term: 0-Other

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Distribution Statement: 1-Approved for public release; distribution is unlimited.

STEM Degrees: **STEM Participants:**

Major Goals: Aim 1. To validate and improve the application of selected known brain-targeting molecules to nanoparticle CNS delivery, using the Q? virus-like particle as the proof-of-concept platform.
Aim 2. To assess the immunogenicity, toxicity, and effects on BBB integrity of selected optimized nanoparticles from Aim 1 (and later Aim 3). Only those particles and formulations that result in no damage to the BBB (or transient disruption of the BBB that do not allow access of harmful agents) will be considered for further study and optimization. Of those, immunogenicity is the next most important potential complication, so we will determine if the immune response compromises effectiveness upon repeat dosing, and develop methods to reduce harmful immunogenicity while retaining targeting capabilities.
Aim 3. To discover nanoparticle motifs with generalized brain-targeting function by a biopanning strategy involving multiple phage display platforms in vivo.

Accomplishments: Please see uploaded file "Final_report.pdf".

Summary of activities and findings

Protein nanoparticles derived from non-enveloped viruses have several advantageous properties for the installation of new function. We used virus-like particles (VLPs) based on bacteriophage Q-beta, which means that the capsid structure has no natural and specific mammalian cell receptors. We therefore started with a structure that can interact with mammalian tissues only through nonspecific properties such as surface charge or hydrophobicity. While it can be difficult to eliminate such interactions, it is usually possible to do so, and specificity in binding and internalization can be separately introduced by the attachment of interacting groups on the particle surface.

Thus, this program was based on the idea that specific molecular entities can be added reliably and in varying density to the external surface of VLPs without damaging the stability or structural integrity of the particles. Such polyvalent display can greatly enhance binding avidity. The first part of our work therefore established both chemical and genetic methods for such particle elaboration, targeting the transferrin receptor (TfR) as the most promising method for blood-brain barrier translocation, illustrated by recent publications in the literature. Both chemical and genetic methods were implemented using tools developed in our laboratory in the preceding years. We were able to display from the protein nanoparticle both the full-length holo-transferrin protein and a 7-mer peptide previously identified and validated as a TfR ligand.

A significant amount of time and effort went in to the details of these capabilities. The most important findings were as follows. (1) Display of peptides from the N-terminus of the Q✓ capsid protein was not well tolerated, in spite of a

variety of attempts involving different expression conditions and plasmid designs. Even the co-expression of wild-type and N-extended proteins did not result in a significant number of extensions incorporated per particle, even though overall particle yields were high. We do not know the reason(s), although the amount of extended protein produced in the E. coli expression system was normal, thus eliminating concerns for aberrant translation initiation. (2) Dislay of peptides from the C-terminus was more reliable, providing excellent yields of hybrid particles. We can increase the density of peptide display even further by chemical conjugation. (3) Attachment of the large (80 kDa) transferrin molecule to the VLP was accomplished successfully, avoiding large-scale aggregation of th particle-transferring conjugates.

Peptide-labeled particles successfully bound to cultured cells in transferrin receptor-dependent fashion (the scrambled peptide analogue not showing such interaction), but particles labeled with full-length transferrin did not. We suspect misfolding of the attached protein such that the required iron cation is lost; alternatively, not enough of the attached transferrin was oriented properly to engage the target receptor. This highlights both the virtues of a more controlled approach in terms of ligation position and of simpler particles bearing multiple copies of smaller (peptide) ligands.

Administration of candidate particles to mice resulted in biodistribution profiles closely resembling unfunctionalized VLPs: in other words, the display of TfR-binding ligands did not make a significant difference. While personnel funded by this project did not assess the immunogenicity of these particles (and so the results are not described in the quarterly reports), we later determined that the VLP-Tf725 particle gave rise to a robust immune response against the capsid protein but no signs whatsoever of adverse health effects (behavioral abnormalities, weight loss), inflammation, or elevated cytokine levels. Interestingly, little antibody response was raised against the Tf7 peptide in this study.

Lastly, we initiated work on the design of a system to express libraries of VLPs displaying different C-terminal extensions. This required modification of our published method to encode such variants, 1 combining this with our technique for packaging co-expressed proteins using an RNA adapter.2-4 In this case, we were able to complete the design of a system that allows for RNA to both code for the desired protein and to be packaged in the particle as a record of the identity of the protein produced.

Progress was slowed initially by delayed approval of an animal protocol at Georgia Tech, which set back initiation of the funding period, resulting in a no-cost extension. The program was further hindered by a lack of communication between our laboratory at Georgia Tech and ARL colleagues; during this period, we somehow lost touch with Dr. Fermen-Coker, our Army collaborator. Of the $150,000 budgeted grant amount, $95,446 was invoiced (covering 6 months effort by Dr. Michael Baksh and 5.5 months' effort combined by graduate students Robert Hincapie and Stephen Crooke), leaving $54,554 unclaimed.

Overall, while this work did not result in a complete set of publishable results, techniques were developed which set the stage for a larger assault on the problem of blood-brain barrier translocation by the creation of multifunctional protein nanoparticles.

Training Opportunities: This program provided significant training opportunities to three young scientists.
Dr. Michael Baksh lent his microscopy and imaging expertise to this project, but also learned new aspects of virus-like particle design. Indeed, these were his first efforts to clone something as complex as a bicistronic expression vector, thereby significantly enhancing his knowledge base.
Similarly, Mr. Stephen Crooke pioneered whole-animal imaging in our group with this project, while helping Dr. Baksh with cloning and expression efforts.
Mr. Robert Hincapie honed his skills in bioconjugation with the challenging effort to connect full-length transferrin with the nanoparticle platform.

Results Dissemination: Nothing to Report

Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: Nothing to Report

RPPR Final Report
as of 04-Mar-2022

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Participant Type: PD/PI
Participant: M.G. Finn
Person Months Worked: 1.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)
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Person Months Worked: 6.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Graduate Student (research assistant)
Participant: Stephen Crooke
Person Months Worked: 4.00 **Funding Support:**
Project Contribution:
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Participant: Robert Hincapie
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Project Contribution:
National Academy Member: N

Partners

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RPPR Final Report

as of 04-Mar-2022

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Signature: M.G. Finn
Signature Date: 3/4/22 12:02PM

Introduction

The objective of this program is to develop non-toxic and non-inflammatory protein nanoparticles that can cross the blood-brain barrier (BBB), carrying a wide variety of molecular cargos. The technology will be applicable to a large number of diagnostic and therapeutic problems involving neurological diseases and injury. Our approach uses the large amount of published work in this area as a base, leveraging these results with attention to practical issues of production and side effects, with innovative combined applications of state-of-the-art bioconjugation chemistry, polymer chemistry, and directed evolution. The major goals were as follows.

Aim 1. To validate and improve the application of selected known brain-targeting molecules to nanoparticle CNS delivery, using the Qβ virus-like particle as the proof-of-concept platform.

Aim 2. To assess the immunogenicity, toxicity, and effects on BBB integrity of selected optimized nanoparticles from Aim 1 (and later Aim 3). Only those particles and formulations that result in no damage to the BBB (or transient disruption of the BBB that do not allow access of harmful agents) will be considered for further study and optimization. Of those, immunogenicity is the next most important potential complication, so we will determine if the immune response compromises effectiveness upon repeat dosing, and develop methods to reduce harmful immunogenicity while retaining targeting capabilities.

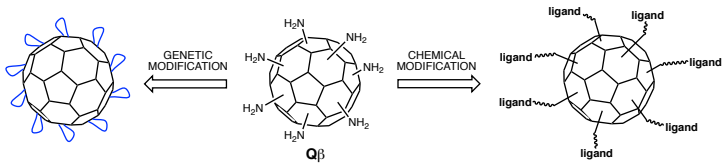
Aim 3. To discover nanoparticle motifs with generalized brain-targeting function by a biopanning strategy involving multiple phage display platforms *in vivo.*

The initial objectives relied on three fundamental capabilities and targets: (1) the well-known role of the transferrin receptor in BBB translocation,[1-4] (2) biocompatible virus-like particles that can be chemically modified,[5,6] and (3) our ability to display genetically-encoded peptides on the exterior surface of VLPs.[7] Key to the approach was the realization that a moderate affinity for the transferrin receptor (TfR) is likely to be needed, derived from published studies of TfR-binding antibodies[8,9] and peptides.[10,11]

Summary of activities and findings

Protein nanoparticles derived from non-enveloped viruses have several advantageous properties for the installation of new function. We used virus-like particles (VLPs) based on bacteriophage Qβ, which means that the capsid structure has no natural and specific mammalian cell receptors. We therefore started with a structure that can interact with mammalian tissues only through nonspecific properties such as surface charge or hydrophobicity. While it can be difficult to eliminate such interactions, it is usually possible to do so, and specificity in binding and internalization can be separately introduced by the attachment of interacting groups on the particle surface.

Thus, this program was based on the idea that specific molecular entities can be added reliably and in varying density to the external surface of VLPs without damaging the stability or structural integrity of the particles. Such polyvalent display can greatly enhance binding avidity. The first part of our work therefore established both chemical and genetic methods for such particle elaboration, targeting the transferrin receptor (TfR) as the most promising method for blood-brain barrier translocation, illustrated by recent publications in the literature. Both chemical and genetic methods were implemented using tools developed in our laboratory in the



preceding years. We were able to display from the protein nanoparticle both the full-length holo-transferrin protein and a 7-mer peptide previously identified and validated as a TfR ligand.

A significant amount of time and effort went in to the details of these capabilities. The most important findings were as follows. (1) Display of peptides from the N-terminus of the Qβ capsid protein was not well tolerated, in spite of a variety of attempts involving different expression conditions and plasmid designs. Even the co-expression of wild-type and N-extended proteins did not result in a significant number of extensions incorporated per particle, even though overall particle yields were high. We do not know the reason(s), although the amount of extended protein produced in the *E. coli* expression system was normal, thus eliminating concerns for aberrant translation initiation. (2) Dislay of peptides from the C-terminus was more reliable, providing excellent yields of hybrid particles, as summarized in Table 1 and illustrated in Figure 1. We can increase the density of peptide display even further by chemical conjugation. (3) Attachment of the large (80 kDa) transferrin molecule to the VLP was accomplished successfully, avoiding large-scale aggregation of th particle-transferring conjugates (Figure 2).

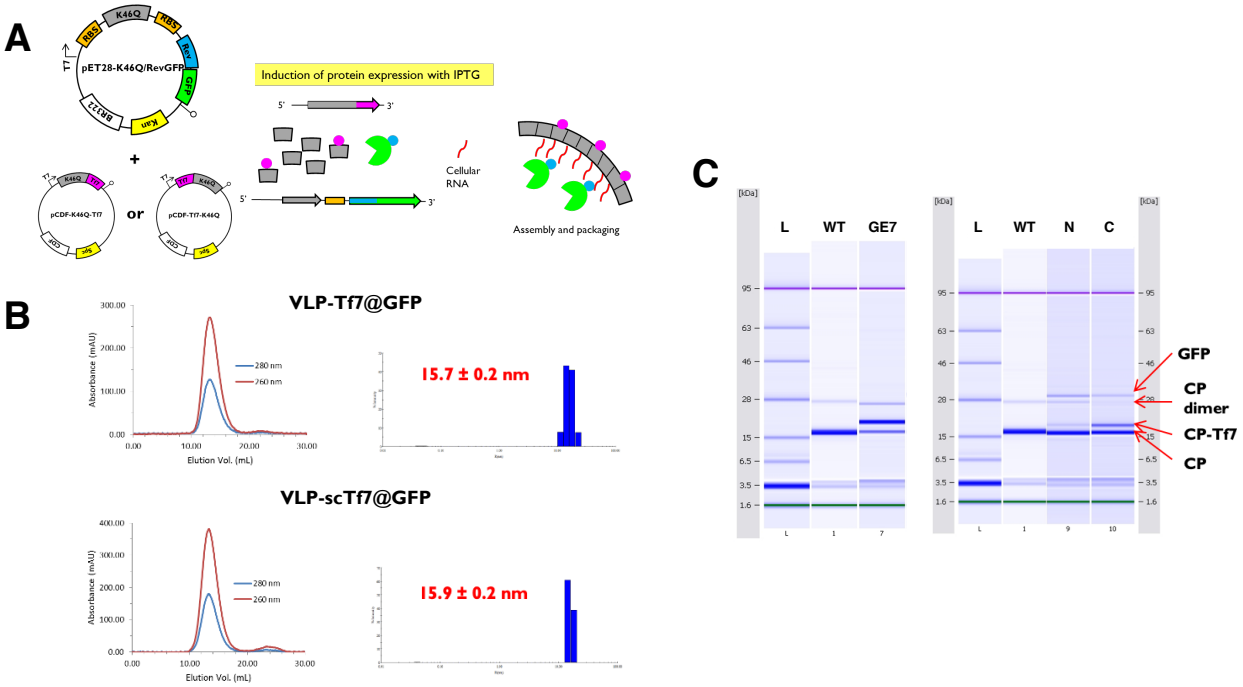


Figure 1. VLP production and representative characterization data. (A) Method for expression of hybrid particles. (B) Size-exclusion chromatography (Superose-6) and dynamic light scattering of the indicated Qβ-peptide particles. (C) Microfluidic electrophoresis (Agilent Bioanalyzer) of hybrid PP7 particles the EGFR-binding GE7 peptide (*left*) and hybrid Qβ particles bearing the TfR-binding Tf7 peptide (*right*). Integration of the band intensities allows calculation of the average composition of the capsid proteins in each particle. Lanes: L = MW ladder; WT = wild-type Qβ particle; GE7 = hybrid VLP containing CP with C-terminal GE7 peptide extension; N = TF7-VLP@GFP = hybrid VLP containing CP with N-terminal Tf7 peptide extension and packaged GFP; C = VLP-Tf7@GFP = hybrid VLP containing CP with C-terminal Tf7 peptide extension and packaged GFP. Band labels: CP = wild-type coat protein, CP-Tf7 = coat protein with Tf7 peptide extension, CP dimer = noncovalent dimer of coat protein, GFP = packaged green fluorescent protein

Table 1. Production of Qβ VLPs bearing TfR-binding peptide (or scrambled negative control).

sample	location [a]	# peptides [b]	sample	location [a]	# peptides [b]
VLP-Tf7	C	25, 6	**Tf7-VLP**	N	2
VLP-Tf7@GFP	C	35	**TF7-VLP@GFP**	N	4
VLP-Tf7sc	C	30	**scTF7-VLP@GFP**	N	0
VLP-scTf7@GFP	C	45			

a) location of peptide extension on coat protein; C = C-terminus, N = N-terminus

b) number of coat proteins (out of 180 per particle) having the peptide extension at the indicated terminus

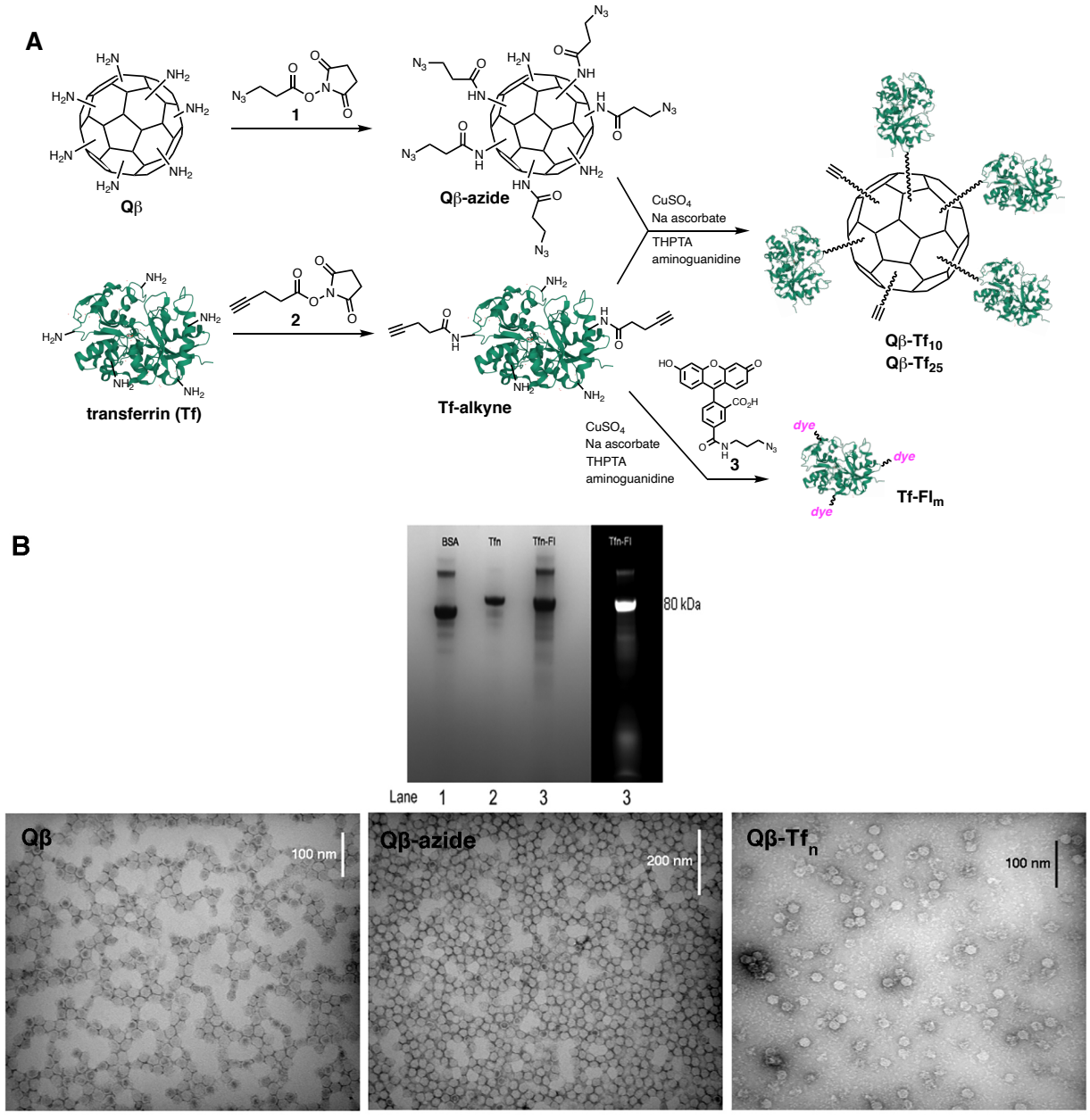


Figure 2. Preparation of transferrin conjugates. (A) Reaction schemes. Initial protein labeling reactions were performed with protein at 1 mg/mL and the use of 50 and 4 molar equivalents of **1** and **2**, respectively, vs. VLP particles and transferrin monomer. (B) (*top*) SDS-PAGE analysis of **Tf-Fl$_m$**, showing covalent dye labeling of the protein. (*bottom*) Transmission electron micrographs of the indicated particles (n = 25).

Peptide-labeled particles successfully bound to cultured cells in transferrin receptor-dependent fashion (the scrambled peptide analogue not showing such interaction; Figure 3), but particles labeled with full-length transferrin did not. We suspect misfolding of the attached protein such that the required iron cation is lost; alternatively, not enough of the attached transferrin was oriented properly to engage the target receptor. This highlights both the virtues of a more controlled approach in terms of ligation position and of simpler particles bearing multiple copies of smaller (peptide) ligands.

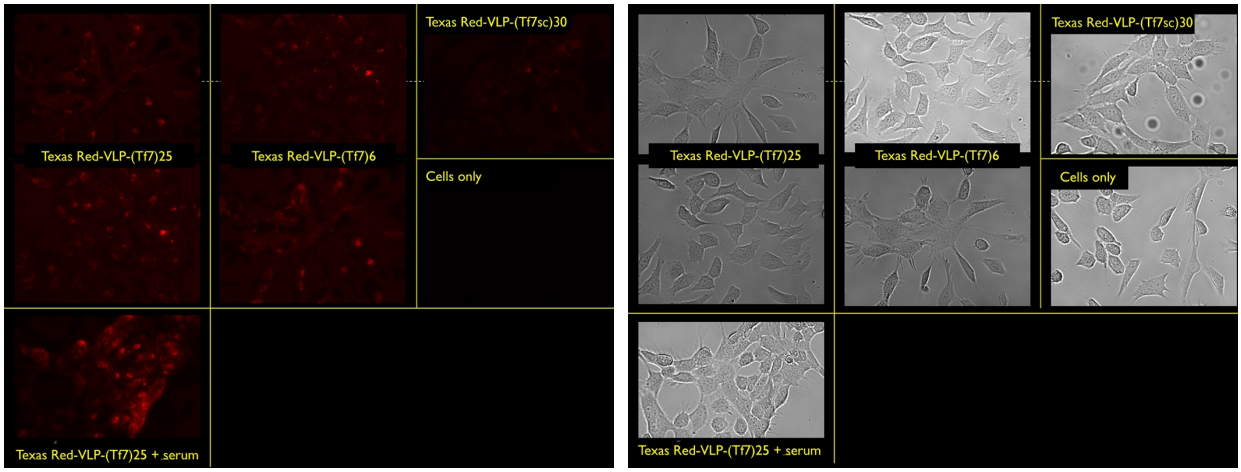


Figure 3. Representative fluorescent (*left*) and brightfield (*right*) images of HT-29 cells incubated with 10 nM dye-labeled VLP-$Tf7_{25}$ and VLP-$Tf7_6$ cells (1 hour, 37°C).

Administration of candidate particles to mice resulted in biodistribution profiles closely resembling unfunctionalized VLPs (Figure 4 shows representative data): in other words, the display of TfR-binding ligands did not make a significant difference. While personnel funded by this project did not assess the immunogenicity of these particles (and so the results are not described in the quarterly reports), we later determined that the VLP-$Tf7_{25}$ particle gave rise to a robust immune response against the capsid protein but no signs whatsoever of adverse health effects (behavioral abnormalities, weight loss), inflammation, or elevated cytokine levels. Interestingly, little antibody response was raised against the Tf7 peptide in this study.

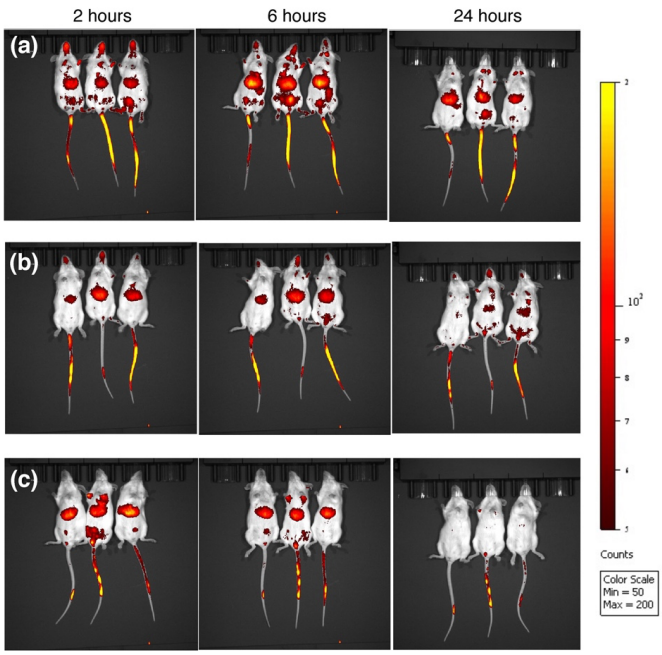


Figure 4. Whole-animal imaging of CD1 mice after administration of 50 μg of the following particles, all bearing an average of 15 copies of near-IR dye: (a) VLP-Tf7$_6$, (b) VLP-Tf7$_{25}$, (c) Qβ VLPs with no TfR ligand. Measurements were made on an IVIS Spectrum imager.

Lastly, we initiated work on the design of a system to express libraries of VLPs displaying different C-terminal extensions. This required modification of our published method to encode such variants,[7] combining this with our technique for packaging co-expressed proteins using an RNA adapter.[12-14] In this case, we were able to complete the design of a system that allows for RNA to both code for the desired protein and to be packaged in the particle as a record of the identity of the protein produced.

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