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Engineered Virus-like Nanoparticle Heparin Antagonists*

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Abstract— **Virus nanoparticles provide a self-assembling, reproducible multivalent platform that can be chemically and genetically manipulated for the presentation of a wide array of epitopes. Presented herein are engineered bacteriophage Qβ nanoparticles that function as potent heparin antagonists. Three successful approaches have been used: 1) chemically appending poly-Arg peptides; 2) point mutations to Arg on the virus capsid; 3) incorporation of heparin-binding peptides displayed externally on the virus surface. Each approach generates particles with good heparin antagonist activity with none of the toxic side effects of protamine, the only drug currently FDA-approved for clinical use as a heparin antagonist.**

I. Introduction

Heparin is a naturally occurring glycosaminoglycan with a wide array of physiological functions that include angiogenesis, cell-cell communication, multiple aspects of developmental biology, etc [1]. It is comprised of repeating D-glucosamine/L-iduronic acid dissacharide units that can be up to 100 units in length. With an average of 2.7 sulfate groups per unit, heparin is among the most negatively charged biomolecules in nature and can range anywhere from -40 (12 kDa heparin with disaccharide repeat of -2 charge) to -90 per polymer (18 kDa heparin with disaccharide repeat of -3 charge).

Heparin is perhaps best known for its ubiquitous clinical use as the gold standard anticoagulant, dating back to the 1940s [2]. Treatment of patients with heparin is common for several medical procedures to prevent thrombosis (e.g., most commonly during cardiac procedures), while it is also applied on machine surfaces used to pump blood to prevent clots. A long standing and serious danger with the use of heparin is the potential for uncontrolled bleeding [3]. To counteract the effects of heparin, the drug protamine is administered. Protamine is a cocktail of short peptides (~ 4 kDa) that contain approximately 70% arginine by composition [4]. It is the only FDA-approved agent that can be used clinically to reverse the anticoagulant activity of heparin, presumably by charge complementation with the positive arginine residues interacting electrostatically with the negative sulfate groups on heparin.

Despite its widespread use, there are significant safety issues regarding the use of protamine that include allergic reaction, hypotension, and paradoxically heparin-like anticoagulant activity; deaths due to protamine are annually reported [5]. These adverse effects are known to occur with even a slight excess of protamine circulating in the body. Consequently, constant monitoring of protamine via titration following heparin treatment is necessary. Given the ubiquity of heparin treatment and the toxicity of protamine, there is intensive research aimed at finding alternatives to protamine.

The multiple sulfate groups on heparin suggest that an effective antidote would have a polyvalent structure that displays several positive charges, while being otherwise biologically benign. Based on these criteria, the use of bacteriophage Qβ as multivalent platform has been explored in an effort to engineer heparin antagonists. Qβ is an RNA virus with an icosahedral capsid that is approximately 28 nm in diameter; Fig. 1 is a transmission electron micrograph (TEM) showing wild-type particles. The capsid is composed of 180 copies of a 14.1 kDa coat protein [6]. The virus can be readily expressed from *E. coli* in gram-scale quantities as a non-infectious agent, and it has been repeatedly validated as a scaffold that can be used to display desired epitopes polyvalently [7, 8]. Described herein are efforts aimed at exploiting this phage for heparin antagonism.

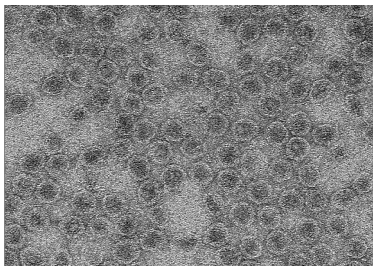

Figure 1. TEM showing intact wild-type Qβ particles (28 nm diameter).

II. Results and Discussion

A. Chemically appended poly-Arg motifs

Inspired by protamine, it was first attempted to append synthesized poly-Arg motifs to the capsid exterior [9]. The attachment chemistry is summarized in Fig. 2. The peptide $N_3Arg_8Gly_2$ (**1**) was synthesized using standard solid phase techniques with Fmoc chemistry, where N_3 represents an azide functional group at the end of the sequence. For the virus scaffold, wild-type Qβ was used. Surface amine residues (four per coat protein, 720 total) were modified with N-hydroxysuccinimide linkers that terminated in an alkyne (**4**), generating the intermediate alkyne-derivatized particle. Finally, exploiting the copper-catalyzed azide-alkyne cycloaddition reaction [10], **1** was conjugated to the intermediate yielding **9**. The number of appended peptides for construct **9** was determined by MALDI-MS (data not shown); relative to the wild-type particle, the theoretical positive charge (assuming all residues ionized) is +40. Particle integrity after chemical derivatization was confirmed

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using transmission electron microscopy, which revealed particles indistinguishable from wild-type (not shown).

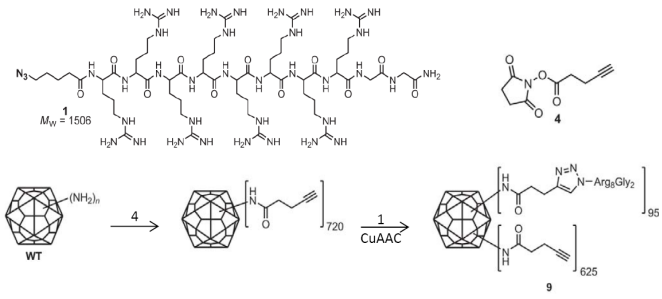

Figure 2. Synthetic scheme for generating chemically modified viruses.

B. Point mutations to generate cationic particles

Chemical manipulation of the virus scaffold, while possible, is cumbersome. An alternative strategy takes advantage of the repeating nature of the capsid: a single mutation on the coat protein is immediately replicated 180 times over the capsid surface, presenting a facile method for generating polycationic particles if the mutation is tolerated. Analysis of the Qβ crystal structure revealed that the coat protein loop comprising residues 10-18 is the most solvent exposed (Fig. 3). Focused on this region, particles D14R and T18R were generated [9]. T18R was readily isolated in large quantities (50-100 mg per liter of culture) and showed good stability. By contrast, D14R appeared to be a disruptive mutation as particles were isolated in poor yields (a few mg per liter of culture) and appeared to disassemble with time following purification. Subsequent studies focused only on T18R; relative to the wild-type particle, the theoretical positive charge is +180. Particle integrity was confirmed by TEM (not shown).

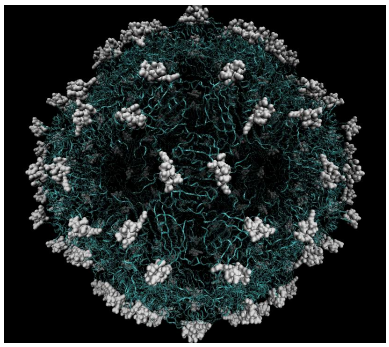

Figure 3. Qβ crystal structure. The solvent exposed loop on each coat protein containing amino acids 10-18 are hilighted in silver.

C. Co-expression to generate mixed particles

A final method to generate Qβ-based heparin antagonists combines the efficacy of heparin-binding peptides (e.g., poly-Arg) with the ease of genetic manipulation. Prior work has established a two-plasmid system wherein one plasmid codes for the wild-type coat protein while the second has a C-terminal extension appended to the coat protein [11]. Co-expression and subsequent particle self-assembly leads to the generation of mixed particles, with the ratio of wild-type to modified coat protein dictated by what nature samples as the most stable configuration. This allows one to incorporate peptides that would otherwise be too disruptive if the addition were forced to be present in each of the 180 copies of the coat protein on the capsid.

Cardin and Weintraub previously identified the amino acid sequence XBBBXBX as a potent heparin-binding motif, where B and X are any basic and non-basic amino acids, respectfully [12]. Guided by this, the sequence *SGSG*ARKKAAKA was incorporated at the C-terminus of the wild-type coat protein, where the underlined portion corresponds to a Cardin-Weintraub sequence and the italicized portion is an amino acid spacer. Co-expression and purification resulted in assembled particles (confirmed with TEM, not shown) that included both wild-type and modified coat proteins, as shown by SDS-PAGE (Fig. 4). Analysis of the peaks from MALDI-MS of the particles (not shown) indicated that of the 180 coat proteins per particle, on average 28 contained the C-terminal tail. This mixed particle containing the C-terminal Cardin-Weintraub sequence is designated as CCW. Relative to the WT particle, the theoretical surface charge is +112; notably, this particle has the added advantage of containing a specific sequence that is known to interact selectively with heparin.

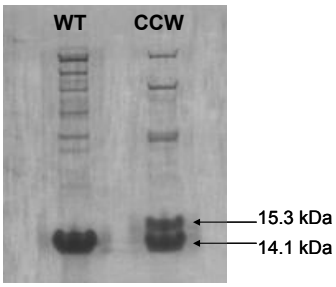

Figure 4. SDS-PAGE of purified wild-type and CCW viruses. The lane for CCW shows both WT (14.1 kDa) and modified coat proteins (15.3 kDa).

D. Heparin antagonist activity

The three methods detailed above have therefore produced a battery of particles – **9**, T18R, CCW – that, in their own way, display polyvalently positive charges that may be able to interact with heparin, thereby neutralizing its anticoagulant activity. To test for this, the particles were assessed using the activated partial thrombin time (APTT) assay. The assay measures the time it takes for a blood clot to form, using as reagents human plasma, a platelet surrogate, and an aqueous calcium solution to initiate clotting (see [9] for details).

The results in Fig. 5A clearly show that all of the particles generated are effective at reversing heparin anticoagulant activity. This was expected given the highly cationic nature of the particles and the presumption of an electrostatic interaction with the anionic heparin. Fig. 5A also shows the efficacy of protamine, which is both highly effective yet acutely toxic at even slight overdose. The toxicity is explicitly observed in Fig. 5B; this figure also shows the

acute toxicity of the naked peptide **1**. By contrast, none of the virus particles show this acutely toxic effect.

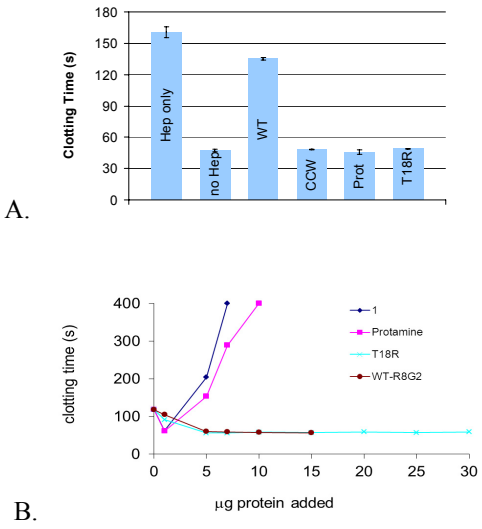

Figure 5. APTT clotting assays with 0.6 µg/µL of heparin in 150 uL reaction volume. A. Virus concentrations were maintained at 15 µg/µL. Normal clotting takes approximately 50 seconds. Abbreviations are Hep (heparin) and Prot (protamine). The wild-type particle (WT) serves as a negative control. B. Clotting assays with increasing concentration of protein. Only the naked peptides protamine and **1** show aberrant behaviour as protein concentration increases.

III. Conclusion

By exploiting polyvalent virus particle scaffolds, the results presented herein validate the use of engineered viruses as safe heparin antagonists. Significantly, none of the particles showed the toxicity exhibited by protamine. The varied methods by which the particles were created and the differing physical properties of each presents several malleable scaffolds from which a potential therapeutic can be generated. Future work will further explore the efficacy of the particles *in vivo*.

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