Published in final edited form as:

Biotechnol J. 2012 April ; 7(4): 558–565. doi:10.1002/biot.201000397.

Porcine parvovirus removal using trimer and biased hexamer peptides

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Abstract

Assuring the microbiological safety of biological therapeutics remains an important concern. Our group has recently reported small trimeric peptides that have the ability to bind and remove a model non-enveloped virus, porcine parvovirus (PPV), from complex solutions containing human blood plasma. In an effort to improve the removal efficiency of these small peptides, we created a biased library of hexamer peptides that contain two previously reported trimeric peptides designated WRW and KYY. This library was screened and several hexamer peptides were discovered that also removed PPV from solution, but there was no marked improvement in removal efficiency when compared to the trimeric peptides. Based on simulated docking experiments, it appeared that hexamer peptide binding is dictated more by secondary structure, whereas the binding of trimeric peptides is dominated by charge and hydrophobicity. This study demonstrates that trimeric and hexameric peptides may have different, matrix-specific roles to play in virus removal applications. In general, the hexamer ligand may perform better for binding of specific viruses, whereas the trimer ligand may have more broadly reactive virus-binding properties.

Keywords

affinity adsorption; ligand; virus removal; bioseparations

1. Introduction

Biotherapeutic production is a growing industry with an increasing number of products coming to market each year. The industry is highly regulated with an excellent safety record, in large part because of the application of effective methods to remove contaminants (chemical and biological) from process streams. For example, nanofiltration has become the

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The authors have declared no conflict of interest.

industry standard for removal of viruses from biotherapeutics. However, this method is far from perfect. For example, it is prone to surface fouling due to small pore-sizes [1-3]. In addition, despite the fact that important enveloped viruses such as human immunodeficiency virus and hepatitis B can be removed by nanofiltration, nonenveloped viruses [e.g., hepatitis A virus (HAV) and human parvovirus B19 (HPV B19)] are small enough to pass through the filters [4]. Similarly, difficulties in inactivating these small nonenveloped viruses are experienced in monoclonal antibody production, for which the low pH treatment used to inactivate many enveloped viruses is ineffective [5].

While small nonenveloped viruses like HPV B19 and HAV tend to have less severe clinical consequences, they can be particularly troubling for elderly or immunocompromised patients, who are an important subpopulation receiving biological therapies. In fact, the transmission of HPV B19 through contaminated plasma-derived clotting factor has been demonstrated [4]. Although this situation did not result in any detrimental human health effects, it is illustrative of the capacity of small non-enveloped viruses to escape our current virus filtration and inactivation protocols. As many of these viruses are poorly characterized and under-recognized, there remains a great deal of interest in novel methods to remove them from biotherapeutic process streams.

In previous work, we reported the identification of small trimeric peptide ligands (amino acid sequence WRW and KYY) that have the ability to remove a model nonenveloped virus, porcine parvovirus (PPV) [6]. The ligands effectively removed all of the PPV in spiked saline solution, which corresponded to 4.5-5.5 log₁₀ clearance, depending on starting PPV concentration. When the peptide columns were challenged with PPV spiked into 7.5% human blood plasma, the best ligand, WRW, was able to remove all of the PPV present in the first three column volumes [6], for a total log₁₀ clearance of 4.7 from 1.5 ml of solution processed with a 0.5 ml volume of peptide-immobilized resin. However, after processing 5 ml of solution with the same amount of resin, the instantaneous clearance was reduced to 1.8 log₁₀. A 16% improvement in the selectivity of this ligand was achieved by reducing the peptide density on the chromatographic resin [7], resulting in an instantaneous clearance of 2.4 log₁₀ from 5 ml of spiked solution. This previous study, the first of its kind, provided proof-of-concept that small peptide ligands can be applied to the removal of viruses from biological solutions.

Nonetheless, even after this study, the need to improve the binding strength and selectivity of the trimeric peptides was apparent. The working hypothesis upon which this study was based is that longer hexamer peptides can provide improved performance by creating more chemical interactions between the peptide ligand(s) and the target virus. To test this hypothesis, we created a biased library of hexamer peptides that contained trimers WRW or KYY, and screened this library for identification of hexamer peptides with selectivity for PPV. The results of this work are presented here.

2. Materials and Methods

2.1. Materials

Phosphate buffered saline (PBS) containing 0.01 M phosphate, 0.138 M NaCl and 0.0027 M KCl, pH 7.4 was purchased from Sigma (St. Louis, MO) and human blood plasma was donated by the American Red Cross (Rockville, MD). Eagle's Minimum Essential Media (EMEM) was obtained from Quality Biologicals (Gaithersburg, MD). The sterile PBS, trypsin, penicillin/streptomycin and glutamine used for cell culture were purchased from Invitrogen (Carlsbad, CA).

2.2. Methods

Virus Propagation, Purification, and Titration—The porcine parvovirus (PPV) NADL-2 strain was titrated and propagated on porcine kidney (PK-13) cells; both the cells and the virus were gifts from the American Red Cross, as previously described [8]. Radioactive PPV was prepared by metabolically incorporating a radiolabel during virus propagation by addition of ^{35}S methionine and cysteine (Perkin-Elmer, Waltham, MA) to the cell culture media, as described by Heldt et al. [6]. Further purification of crude, radiolabelled PPV stocks was done by gel filtration using a Sephacryl 300 HR HiPrep 16/60 column (GE Healthcare, Piscataway, NJ) and the flow through fraction was collected for the equilibrium isotherm experiments [7]. All infectivity measurements were made using the MTT assay, which uses the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to detect proliferating cells. This assay has been previously correlated to the TCID₅₀ (50% tissue culture infectious dose), a common method for the titration of infectious viruses [8].

Peptide Library Construction and Screening—Solid phase peptide libraries were synthesized on Toyopearl Amino 650EC resin (Tosoh Biosciences, Montgomeryville, PA) by Peptides International (Louisville, KY). Library construction is detailed in Figure 1; note that libraries containing the constant sequence WRW were constructed with variable regions before and after the constant region, while those with KYY were synthesized only by attaching the variable region at the end of the constant region. Only one KYY library was synthesized because of the costly nature of screening. The WRW libraries served as a control to determine if library construction had a significant impact on hexamer performance. Library screening was done using ^{35}S labeled PPV, as described previously [6]. Beads that produced a high signal were excised and sequenced by the Protein Facility at Iowa State University.

Chromatography Using Peptide Resins—Selected hexamer peptide leads were synthesized on Toyopearl Amino 650M resin (Tosoh Biosciences) by Peptides International at a density of 0.10 mmol/g of dry resin and having a four unit ethylene oxide spacer arm [7]. This was calculated to be an approximate distance of 0.7 nm between peptides [7]. The peptide resins were packed into disposable PIKSI columns (ProMetic Biosciences Ltd, Cambridge, England) with a total of 0.5 ml of settled resin in PBS per column, and the columns were run at a flow rate of 0.1 ml/min. The hexamer packed resins, along with controls, were tested for their ability to remove PPV in a solution containing 7.5% human

blood plasma by comparing the titer of the virus solutions before and after passing through the investigated columns as assessed using the MTT assay [8].

Equilibrium Isotherms—The purified, radiolabelled PPV stock was prepared as described above. Various concentrations of radiolabelled PPV were added to 50 µl of settled resin and the radioactivity before and after contact was measured with a Packard 1500 Tri-Carb Liquid Scintillation Analyzer (Wellesley, MA). The bound virus was determined as the difference in the scintillation counts before and after resin contact using a total material balance.

Molecular Docking of Peptide Ligands—To elucidate the differences between the characteristics of PPV binding with trimer and hexamer peptides, molecular docking calculations were performed using the MOE (Molecular Operating Environment) 2007.09 software (Chemical Computing Group, Montreal, Canada), and its associated DOCK function. These calculations were based on the structure of PPV viral protein 2 (VP2), the major capsid surface protein [9], which corresponds to File 1k3V.pdb from the Protein Database [10]. The protein structure was determined by free energy minimization with the Amber99 force field. The trimer and hexamer peptide structures were generated using the MOE protein builder, with specification of C-terminus amidation. This took into account the unavailability of the negative charge on the C-terminus for virus capsid interactions since this end of the peptide was bound to the spacer arm attached to the resin (Figure 1) and was not a free charge available for interaction. The peptide free energy was stochastically minimized and then the docking calculations were performed. The top 10 docking conformations, as determined by the lowest docking scores, were examined for docking location on the VP2 protein of PPV.

As a first attempt, the spacer arm was added to the peptides during docking, but the ethylene oxide repeats on the spacer arm tended to wrap around the peptide and prevent interaction with the VP2 protein. For this reason, the spacer arm was not included in the docking study. It is known that spacer arm and solid support chemistry affect binding affinity [11, 12], but the computational approach taken here was limited to examination of the interaction of the peptide with the virus capsid protein. Although docking calculations including the PEO spacer arm were apparently unreliable (since, contrary to experiments, these calculations predicted no interactions with the VP2 protein), calculations neglecting the spacer arm are still believed to be reliable. This is due to previous data demonstrating that for the WRW ligand, the inclusion of the spacer arm improved binding affinity as compared to when it was absent [7]. This was hypothesized to extend the ligand from the resin surface, and diminish any possible interaction between the resin and the peptide, as well as allow the peptide to reach into canyons on the virus surface [7, 9]. PEO was chosen for the spacer arm due to its demonstrated ability to improve ligand binding by creating an inert, yet flexible, spacer arm [7, 13, 14]. From previous evidence of PEO having the ability to extend ligands and not wrap around ligands, we concluded that the computational approach taken here that disregarded the PEO spacer arm, due to its contradictory folding around the peptide ligand, was reliable. The attachment of the PEO spacer arm to the solid support is the most likely

reason for the ability of the spacer arm to remain extended in the experimental results, but was not taken into account for the computational study.

3. Results

3.1 Experimental

Based on library screening using ^{35}S radiolabelled PPV, a total of 35 beads were excised, washed and sequenced (Table 1). The amino acids were classified by chemical composition to determine sequence homologies and to identify dominant amino acid sequences for each lead peptide. The amino acids from the XXXWRW library were predominantly aliphatic and basic; the WRWXXX library amino acids were predominantly basic and hydroxyl (hydrophilic); and the XXXKYY library produced predominantly basic and aromatic amino acids. The amino acids associated with the initial trimer library were predominantly basic and aromatic [6], and were not included in the above assessment. Because the hexamer peptides likely have different binding sites than the trimer peptides, it is not surprising that some of the dominant amino acids for the hexamer libraries differed from those obtained from the trimer library. Regardless, for both the trimer and hexamer ligands isolated from combinatorial screening, the dominant interaction appeared to be between the basic amino acids and the virus. Other research groups have studied the removal of viruses with anion exchange chromatography [15-17], demonstrating that viruses often bind to positively charged surfaces.

The sequences synthesized for continued evaluation are shown in bold in Table 1. To reduce evaluation time, we preferentially selected sequences that contained the dominant amino acids or common motifs that were repeated in different peptides. Sequences that were unique in comparison with other common motifs, (i.e. DNEKYY, which is highly acidic) were automatically excluded. We also equally sampled sequences across all three libraries. Specifically, two comparable sequences resulted from screening of the WRW libraries, and these were designated IVKWRW and WRWIVK, while two hexamers with similar sequences (VIKWRW and WRWVKV) were also found. These sequences contained lysine, valine and isoleucine, which together made up only about 0.3% of the total library, suggesting that these amino acids had a role in PPV capsid binding specificity. Hexamer WRWIFS was chosen for further evaluation in an effort to examine the effect of the hydroxyl amino acids and phenylalanine, which appear three times in the discovered sequences, while YHLWRW was chosen because of the presence of histidine, given that this amino acid contains an uncharged amine group at neutral pH. Histidine was found in one other sequence and its presence allowed us to examine the role of uncharged (H) versus charged (K, R) amine groups.

The KYY library lead sequences were dominated by aromatic, aliphatic and basic amino acids. In this case, hexamer sequence KKYKYY was chosen due to its similarity to the KYY parent trimer, suggesting that only the terminal three amino acids are responsible for binding. For comparative purposes, sequence YKLKYY was also chosen in an effort to determine if only the three amino acids closest to the resin were responsible for PPV binding. If this were the case, one would expect that the binding of the sequence YHLWRW would be very similar to that of YKLKYY.

The ability of the four highest performing hexamers to remove PPV from 7.5% human blood plasma, as compared to the acetylated control, is shown in Figure 2A. The acetylated resin was specifically designed to have low protein binding affinity so as to minimize non-specific binding of the virus to the resin backbone. Other resins or support matrices, including more flexible membranes, may allow for improved virus binding to the peptides, but these were not explored in this study. The interaction of the peptide with the resin was not specifically characterized here, but this could be done either computationally or experimentally by comparing the binding capabilities of the peptides with multiple, alternative resins [12].

None of the hexamers identified from the WRW ligand libraries were able to capture as much PPV as the original WRW trimer ligand. The only ligand that showed any substantial ability to remove PPV was YKLKYY, and its virus clearance approximated that of the original trimeric peptide WRW. Overall, the performance of the WRW ligand was significantly better than that of the KYY ligand [6, 7]. Nonetheless, none of the hexamers performed better than the trimers. The other hexamers not shown in Figure 2B performed in a manner similar to YKLKYY, such that the first column volume had a detectable clearance of above 10%, but clearance quickly dropped to about 0.01% by the ninth column volume.

The resins were also examined for their ability to bind PPV in buffer using a static binding isotherm, as described elsewhere [7]. This was calculated using the linear form of the Langmuir isotherm,

$$q = \frac{q_{\max} C}{K_d} \quad (1)$$

where q is the amount of the virus bound to the resin, $q_{\max}$ is the maximum capacity of the resin, C is the unbound concentration of virus in solution, and K_d is the affinity dissociation constant. The slope of the linear isotherm is represented by $q_{\max}$ divided by K_d . The maximum capacity of the resin is not known, but it can be assumed to be consistent with respect to the peptides investigated since it is greatly dependent on the stacking of the virus around the resin. Since the virus is icosahedral, the stacking around the resin should not be dependent upon the presence of different binding sites, and hence should be the same for all resins. Therefore, the slopes of the linear isotherm can be compared directly and assumed to be inversely proportional to K_d [7]. Using the assumptions mentioned above, it was determined that all of the resins shown were able to bind PPV at a similar binding affinity as WRW (Figure 2B). All other resins that were tested (shown as the highlighted sequences in Table 1) were able to bind less than one-third of the PPV captured by WRW (data not shown).

The experimental data demonstrated that library design is critical. For example, none of the sequences derived from the WRWXXX library showed promise. It is likely that the variable region needs to be physically separated from the resin surface to promote accessibility for virus binding. For this reason, we did not further pursue the KYYXXX library.

3.2 Molecular Docking

To better understand the lack of improvement in virus binding efficiency that was observed when the original trimeric ligands were extended to hexamers, qualitative molecular docking modeling was performed. This provided a better understanding of the specific binding sites that the trimer and hexamer ligands were likely to occupy. Based on these models, it was suggested that trimer WRW was able to dock at multiple locations on the capsid VP2. This might be anticipated as a small trimeric ligand does not have the secondary structure that would be necessary for selective steric interaction with “pockets” on the protein surface. The PPV VP2 protein is highly hydrophobic and it is likely that trimer WRW binds more as a multi-mode hydrophobic and positively charged ligand rather than as a specific affinity ligand. Multi-mode ligands [18], containing both hydrophobic and charge interactions, are becoming more common in industrial applications, such as in antibody purification [19]. The multi-modal nature of trimer peptide binding may well find many applications in future industrial processes.

One specific pocket on the VP2 surface appeared to be consistently associated with hexamer ligand binding (Figure 3A). This pocket was dominated by D99 and D100 which acted as hydrogen bond donors to the basic amino acids of the ligands. The D99/D100 pocket corresponded to the most favorable docking score for each hexamer examined, however hexamer YKLKYY was the only one that seemed to have exclusive specificity for this location. All of hexamer ligands, except for YKLKYY, also docked to other regions of the VP2 protein without a consistent pattern. WRW also bound to the D99/D100 pocket, but it was calculated as the eighth lowest docking score (or lowest energy binding site).

The degree of virus clearance associated with the first five column volumes was compared to the consistency of each particular ligand to dock to the D99/D100 pocket (Figure 3B). The YKLKYY ligand demonstrated both the highest virus clearance and the greatest tendency to dock to this location. Linear regression analysis of the relationship between docking percentage (defined as the number of docking conformations that were found in the D99/D100 pocket as compared to other locations on the VP2 protein) and PPV clearance (without inclusion of the data for peptide WRW) produced a slope of 0.88 and an R^2 value of 0.82. These data further support the correlation between docking tendency and virus clearance, providing quantitative evidence for the specificity of the ligands for the D99/D100 pocket.

Trimeric peptide WRW was not included in the linear regression because it did not dock in the same manner as did the hexamer peptides. Specifically, it sampled multiple regions on the VP2 surface that included both binding and non-binding pockets. Based on MOE analysis, it appeared that the hexamer ligands tended to dock at designated pockets into which the folded hexamers could occupy the entire space. On the other hand, WRW bound to both pocket and non-pocket binding sites. When a random acidic peptide, designated DEPDEP, was docked as a control, it too preferred designated pockets, but did not bind to the D99/D100 pocket in its top ten docking configurations.

The results of the simulations also imply that the secondary structure of hexamer ligands is an important factor in binding specificity. This sort of secondary structure does not exist in

trimer ligands, which probably exert their binding as a function of charge and hydrophobicity, not because of steric or specific conformational fit. Because of the differences in binding behavior, the evolution of trimers into hexamer ligands may not be as simple as originally anticipated.

The finding of YKLKYY as the lead hexamer was unexpected. This ligand comes from the KYY library, which was considered to be the weaker library because KYY has a lower dynamic binding capacity than does WRW [6]. The high prevalence of lysine, valine and isoleucine in multiple WRW based sequences did not appear to have any effect on binding affinity. From docking studies, it is likely that the base trimer ligand played a very small role in influencing the binding of the YKLKYY hexamer to the virus. It appears that, in this case, secondary structure was the more dominant factor influencing binding affinity, suggesting that the evolutionary approach may not be appropriate for identification of hexamer peptides.

4. Discussion

The question remains as to which sort of binding mechanism will result in better peptide ligand performance. Clearly, both binding mechanisms are common in protein purification chromatography and each has its own advantages and disadvantages [11]. Specific ligands that bind to defined pockets on a protein's surface allow for high binding efficiency, but their use often requires a pre-treatment to remove contaminating proteins or nucleic acids. Lower binding specificity associated with hydrophobicity and charge can be useful in initial capture steps, but often results in unacceptable product loss. We maintain that both of these mechanisms may have a role when optimizing virus removal or purification, and the choice of mechanism could easily depend on specific application and/or sample matrix. For example, one can envision use of the more specific hexamer ligands for production of highly purified virus suspensions as would be required for gene therapies or vaccines. For this application, the virus would be eluted off of the resin and proper cleaning would promote multiple uses of the affinity peptide resin. On the other hand, trimers may be better suited for removal of viruses from relatively simple sample matrices, such as for nanofiltration of water. For more complex samples such as blood or plasma, the specific hexamer peptides with purportedly higher specificity might be more appropriate as these would likely perform better in the presence of competing proteins [6].

It should be noted that the high degree of positive charge associated with the hexamer peptides, contributed by basic amino acids, may result in some unexpected difficulties. Specifically, these amino acids are likely to bind to negatively charged species that may be present, including nucleic acids and cellular membrane components, resulting in non-specific adsorption and the blocking of virus binding sites. Through sequence and solution optimization, it may be possible to fine-tune the selectivity of the peptide(s) for viruses compared to other contaminants. The combined use of trimer and hexamer peptides may also have the potential to enhance our current virus removal and purification methods.

Using PPV, a model non-enveloped virus, this study demonstrates that more complex hexamer ligands produced from a trimeric support do not necessarily exhibit improved

binding capacity. Further analysis of the differences in binding mechanisms revealed that trimer-PPV binding is likely mediated by amino acid charge and hydrophobicity, while hexamer-PPV binding may rely more heavily on ligand secondary structure which allows penetration into a specific pocket on the virus capsid surface. This secondary structure, while apparently the dominant characteristic of the hexamer ligands, is by the primary amino acid sequence. This body of work further supports the usefulness of computational design to simplify and speed the process of screening and evaluating candidate ligands resulting from random combinatorial peptide libraries.

Acknowledgments

The authors would like to thank Deborah McGuinn for assistance in preparing the PIKSI kits for resin evaluation. The authors would also like to thank Pathogen Removal and Diagnostic Technologies (PRDT), a joint venture between the American Red Cross and ProMetic BioSciences Inc. and the NIH/NCSU Molecular Biotechnology Training Program (Grant 2T32GM008776) for funding.

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Figure 1.

Orientation of the ligands to the resin particle. (A) XXXWRW library, (B) WRWXXX library, and (C) XXXKYY library.

Figure 2.

PPV clearance by hexamer peptide ligands. (A) About $5 \log_{10}$ (MTT/ml) was consistently pumped onto the columns at 0.1 ml/min and 0.5 ml fractions (equivalent to one column volume) were evaluated for infectious PPV using the MTT assay. 100% detectable clearance represents the removal of all detectable virus. Error bars represent the range of duplicate experiments. (B) Equilibrium isotherms constructed using radiolabeled PPV (CPM) [7]. The data was fit to the linear Langmuir isotherm for comparison of binding affinities. Experiments run in triplicate and all data is shown.

Figure 3. Docking and clearance of peptides. **(A)** Docking of YKLKYY into the pocket of PPV VP2. The peptide is in red with the C-terminus extending out of the pocket, which would allow this conformation to exist even when the ligand is attached to the resin. The surface of the protein was color coded to represent the different surface-exposed chemical groups, where green represents hydrophobic amino acids, pink represents hydrogen bonding amino acids and blue represents polar amino acids. **(B)** Comparison of ligand docking and PPV clearance. Black bars correspond to PPV clearance associated with the first five column volumes, which was calculated as percent clearance relative to that of the WRW peptide (arbitrarily set at 100%). White bars correspond to docking tendency, which was calculated as the percent of the top 10 ligand conformations from one docking experiment that docked or bound to the pocket shown in Figure 3A.

Table 1

Sequences identified during primary screening of biased hexamer libraries. The sequences in bold were used in additional screening studies.

Sequence			
Wash	XXXWRW	WRWXXX	XXXKYY
Acetic Acid	IVKWRW	WRWGTK	DNEKYY
	WRKWRW	WRWEFK	FITKYY
	ASRWRW	WRWLNL	ISPKYY
	VTRWRW	WRWIVK	SGLKYY
	R(V,W)RWRW ^{a)}	WRWVKV	
1M NaCl and 0.1% Tween 20	HLIWRW	WRWITS	YKLKYY
	YHLWRW	WRWFKQ	KKYKYY
	VIKWRW	WRWTIP	YAKKYY
	KYLWRW	WRWHKQ	SYKKYY
		WRWIFS	GFAKYY

^{a)} The sequencing reactions could not distinguish between V and W as the second amino acid in this sequence.