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Discovery of Stable and Selective Antibody Mimetics from Combinatorial Libraries of Polyvalent, Loop-Functionalized Peptoid Nanosheets

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ABSTRACT

The ability of antibodies to bind a wide variety of analytes with high specificity and high affinity make them ideal candidates for therapeutic and diagnostic applications. However, the poor stability and high production cost of antibodies has prompted exploration of a variety of synthetic materials capable of specific molecular recognition. Unfortunately, it remains a fundamental challenge to create a chemically-diverse population of protein-like, folded synthetic nanostructures with defined molecular conformations in water. Here we report the synthesis and screening of

combinatorial libraries of sequence-defined peptoid polymers engineered to fold into ordered, supramolecular nanosheets displaying a high spatial density of diverse, conformationally-constrained loops on their surface. These polyvalent, loop-functionalized nanosheets were screened using a homogeneous Förster resonance energy transfer (FRET) assay for binding to a variety of protein targets. Peptoid sequences were identified that bound to the heptameric protein, anthrax protective antigen, with high avidity and selectivity. These nanosheets were shown to be resistant to proteolytic degradation, and the binding to be dependent on the loop display density. This work demonstrates that key aspects of antibody structure and function – the creation of multivalent, combinatorial chemical diversity within a well-defined folded structure – can be realized with completely synthetic materials. This approach enables the rapid discovery of biomimetic affinity reagents that combine the durability of synthetic materials with the specificity of biomolecular materials.

KEYWORDS

protein-mimetic materials, multivalent molecular recognition, combinatorial display, two-dimensional nanomaterials, bioinspired polymers.

Antibodies naturally act as molecular recognition elements having high specificity and affinity to their corresponding antigens. While antibodies are nature's ultimate pathogen detectors, their utility for biomedical applications is restricted by high production cost, low solubility, loss of activity due to improper folding, and difficulties with chemical modification.[1,2] Emulating antibodies to avoid these drawbacks, various synthetic bio-macromolecules have been developed

by rational design and combinatorial approaches using natural scaffolds, such as proteins, nucleic acids, and bacterial phages.[3,4] These approaches mimic, all in some way, the sophisticated three-dimensional structures of antibody binding, where multiple, diverse chemical functionalities are presented in precise spatial geometries. However, the process for discovering artificial affinity reagents is laborious due to requisite iterative hit selection and amplification; moreover, their low stability against proteases and poor binding affinities in the micromolar range limit the utility of these materials. Antibody mimetics discovered by combinatorial approaches have been spotlighted due to their robustness and binding affinities,[5,6] but are limited in chemical diversity and their ability to display complex, folded, binding structures. Thus, there is an unmet need for methods enabling the facile and rapid discovery of molecularly-defined synthetic antibodies consisting of entirely non-natural, robust materials.

Peptoids are bio-inspired and sequence-defined *N*-substituted glycine polymers. They are ideal as building blocks for constructing protein-like architectures, because their sequence-defined linear chains can reproducibly fold into specific molecular architectures due to assorted long-range inter- and intra-molecular non-covalent interactions.[7-14] They are efficiently synthesized by the automated solid-phase submonomer method, using diverse and readily available amine synthons, to precisely modulate chain length, monomer sequence, and side chain chemistry. Like proteins, this provides the opportunity to program both folding information and biological activity into the chemical sequence of the polymer chain.[15,16] The free-floating 2-dimensional nanosheet is a fascinating example of a folded peptoid-based nanomaterial with atomically-defined structure[17]. In this work, we mimic the basic structure of the antibody binding site by positioning a diversity of well-defined peptoid loops on the surface of peptoid nanosheets. These nanosheets are made from the self-assembly of peptoid 42-mer strands that contain a central and variable chemically

diverse hexameric loop domain, flanked by two fixed nanosheet-forming domains.[18,19] Atomic force microscopy (AFM) and protein binding experiments show that these loops are displayed on the nanosheet surface.[18,19] Specific binding affinity for target proteins can be encoded by the monomer sequence of the loop-display domain, highlighting its potential as a biologically-relevant binding material. Furthermore, nanosheet self-assembly is triggered by interfacial compression of the air-water interface,[20] allowing the composition of the loops to be controlled prior to sheet formation. The zwitterionic surface of peptoid nanosheets also makes them an optimal chassis for display of functional loops, since it prevents non-specific adhesion of biomolecules.[21]

An important structural feature of loop-functionalized peptoid nanosheets is the opportunity to enable multivalent interactions with substrates due to their high spatial density of loops on the surface.[20] The ability of these high-surface-area materials to support multiple binding events simultaneously boosts selectivity and sensitivity for target proteins among various bio-macromolecules.[22] Furthermore, antibody-like peptoid nanosheets resist degradation by proteases, solvents, and extreme pH and temperature conditions, because they lack peptide bonds in their backbone structure.[16,23,24] They can be engineered to employ photo-reactive monomers for crosslinking the nanosheet interior, yielding increased chemical and mechanical stability.[25] While peptoid nanosheets thus provide valuable chemical and structural properties as affinity reagents, missing from the toolkit is a process for mimicking the recombination and affinity maturation of antibodies, which is critical to the immune system's ability to obtain specific and high affinity binders to targets. If chemists could develop an *in vitro* approach for generating specific, high affinity nanosheet binders to unfamiliar targets, these synthetic materials could overcome many of the drawbacks of antibodies.

Herein, we describe a general approach for the rapid discovery of biomimetic affinity reagents by the screening of combinatorial libraries of chemically diverse, loop-functionalized peptoid nanosheets. The discovery cycle comprised four major steps: the chemical synthesis of loop-containing peptoids (loopoids), the supramolecular assembly of loopoid nanosheets, the screening of the loopoid nanosheets against various proteins for binding activity (hits), and hit validation (Figure 1). First, the loopoid strands, which are based on a block-patterned 36-mer nanosheet-forming sequence with the insertion of 6-mers in the middle to form a conformationally rigidified surface-displayed loop, were synthesized robotically using the automated solid phase submonomer method. Drawing upon a set of 20 chemically diverse peptoid monomers, we produced a library of 256 individual loopoid strands. We then employed an auto-pipetting robot to initiate the supramolecular assembly of nanosheets in a high-throughput, parallel manner from the loopoids in multi-well plates. The assembled loopoid nanosheets were then used directly in a variety of target protein binding assays based on a Förster resonance energy transfer (FRET) assay.[20] Based on this automated process for synthesis and screening, we discovered a nanosheet binder for anthrax protective antigen [$(PA63)_7$], a ring-shaped homoheptameric toxin-related protein. Based on the chemical properties of the hit loop sequences, we identify rules for understanding the specific binding of loopoid nanosheets to $(PA63)_7$. We also demonstrated the importance of multivalency in hit interactions by bio-layer interferometry, electron microscopy, and fluorescence imaging and FRET analyses.

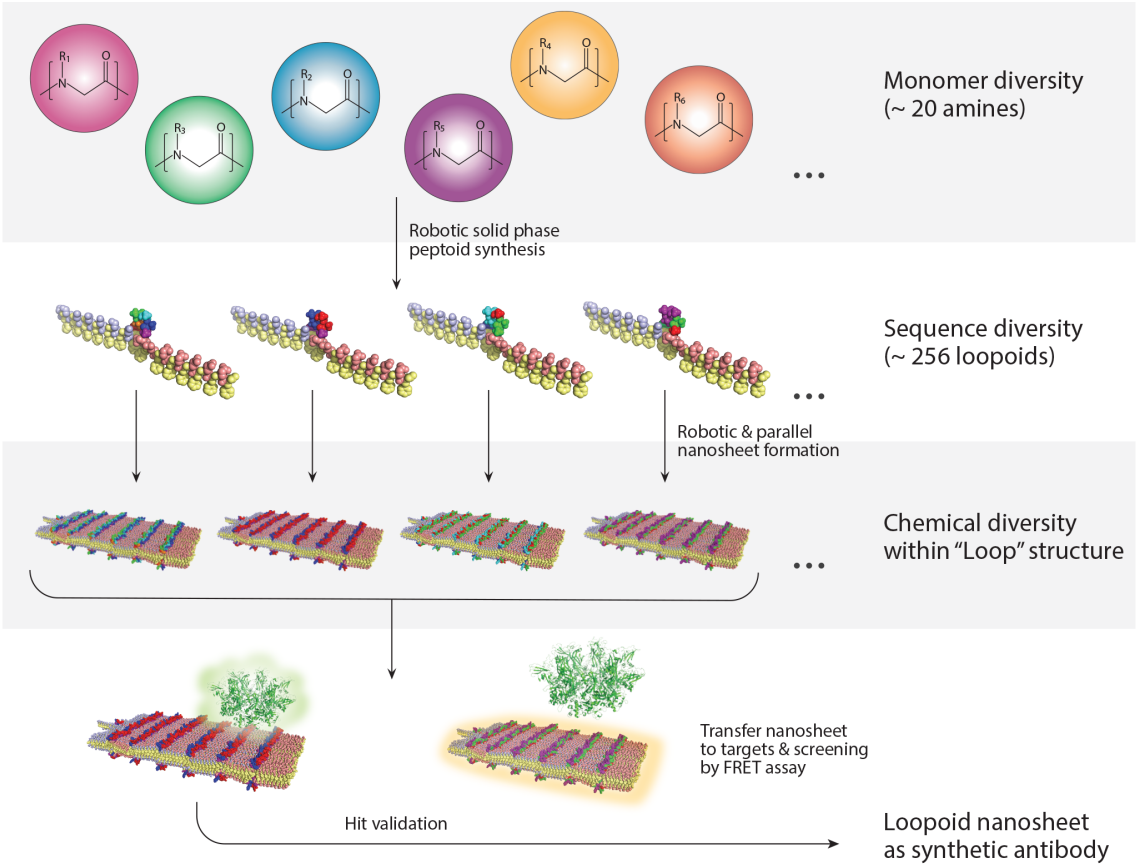

Figure 1. Combinatorial discovery of loop-functionalized peptoid nanosheets as chemically diverse, biomimetic affinity reagents. The hierarchical assembly creates polyvalent chemical diversity within a conformationally-constrained, folded context.

RESULTS AND DISCUSSION

Design of Loopoid Libraries. The diverse antigenic specificity of antibodies is achieved by the chemical diversity (*i.e.*, combination of 20 amino acids) within the structural context of complementarity-determining regions (CDR). To replicate the binding specificity of antibodies to their corresponding antigens, the loop-functionalized nanosheet is an ideal scaffold because of the opportunity to display chemically diverse moieties within a conformationally constrained loop domain projected from the nanosheet polar surface. For these displayed loops, we identified a set of 7 hydrophilic (**P**) and 13 hydrophobic (**H**) peptoid monomers possessing distinct chemical

properties similar to natural amino acids (Figure 2a). Combinatorial loop libraries were designed to contain specific combinations of **H** and **P** monomers to avoid extremes of hydrophobicity or charge within the loop domains. These combinations were selected using design rules based on monomer chemical properties, the feasibility of polypeptoid synthesis, and its capacity to fold into nanosheets (Figure 2b). We fixed the length of the loop domain to be six monomers in order to protrude far enough from the surface of nanosheets for efficient protein binding.[20] A peptoid loop six monomer length is expected to have conformational rigidity, as has been shown in similarly-sized peptoid macrocycles.[26] There are 64 possible amphiphilic **HP** patterns of loop sequence in a 6-mer by considering only the **H** and **P** property of monomers. Our previous studies determined that the nanosheet formation mechanism involves a key intermediate where the peptoids must adsorb to the air-water interface to form an ordered monolayer.[18] Hence, the overall water solubility and surface activity must be balanced to promote self-assembly and the correct folding of loops on the surface of nanosheets. For this reason, we eliminated sequences with alternating **H** and **P** monomers, sequences with ≥ 3 **H** monomers, and sequences with ≥ 2 large **H** monomers per sequence. Employing these rules, we randomly selected 12 hexameric **HP** patterns. Chemical diversity was introduced into the loop sequence by classifying each monomer according to their primary and secondary chemical characteristics: aromatic, small aliphatic, large aliphatic, large hydrophobic, cationic, anionic, hydrogen bond donor/acceptor, and heterocycle. Considering the chemical characteristics of these monomers, the 12 initial **HP** patterns were enumerated to a set of 256 specific sequences with a bias toward minimizing the number of different monomers within the same sequence. In this way the chemical distinctness of each loop could be maximized, making it easier to later identify key chemical properties essential for molecular recognition (Supplementary Table S1).

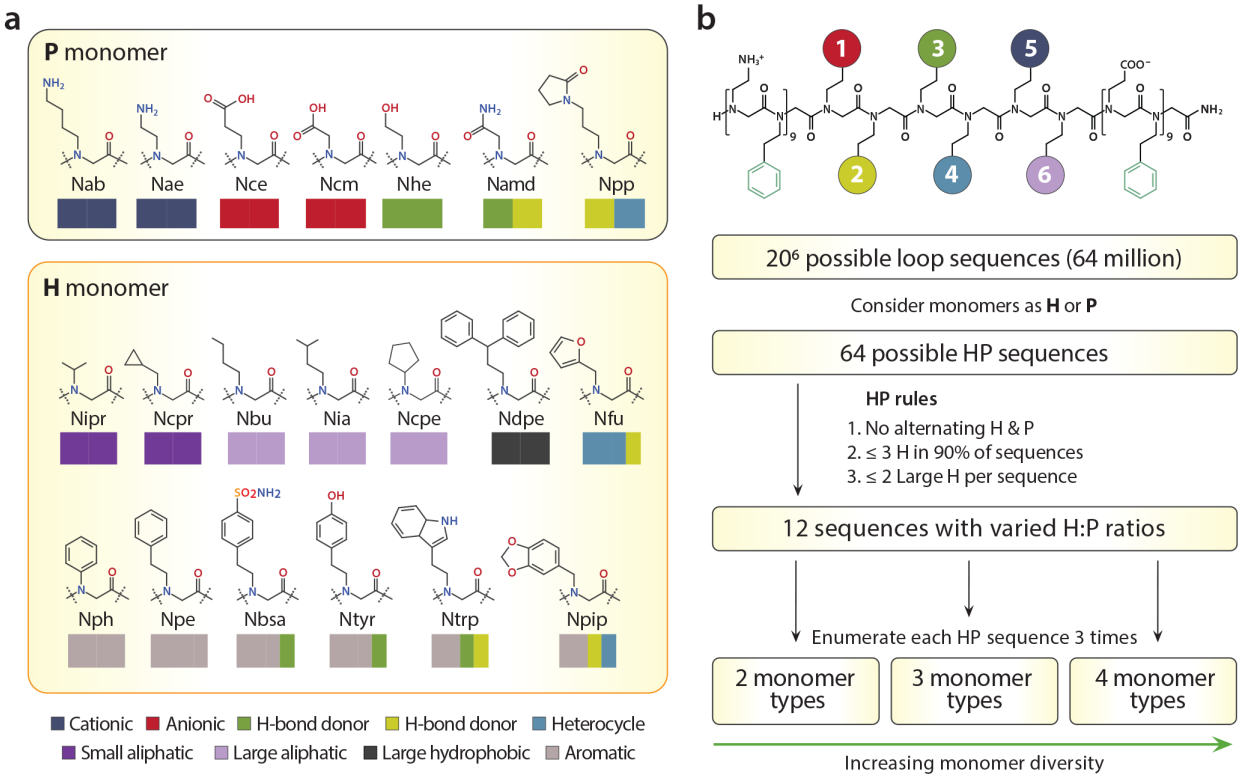

Figure 2. Design of the chemically diverse binding loops, built from a basis of 20 efficiently incorporated monomers. (a) **P** (hydrophilic) and **H** (hydrophobic) amine monomers exhibiting various properties. Colors in rectangular represent primary and secondary properties of monomers. All abbreviations of monomers are included in supplementary information. (b) Design rules of loop sequences regarding its folding into nanosheet and the creation of chemical diversity.

Screening of Loopoid Library and Hit Validation. The first hurdle to discovering a nanosheet affinity reagent is to effectively convert the library of individual peptoid chains into loop-functionalized nanosheet libraries. Conventionally, this supramolecular assembly process has been accomplished by the lateral compression of peptoid monolayers formed at the air/water or oil/water interface.[27,28] A device to rotate a glass vial containing milliliter volumes of peptoid-dissolved solutions from vertical to horizontal was designed to induce a 3-fold change of interfacial area for obtaining high-quality peptoid nanosheets. However, the vial-rocking method is too difficult to parallelize for high-throughput library generation and screening and operates at the milliliter scale,

which results in unnecessary waste of material and makes materials cost prohibitively high as the library size increases. To address this challenge, we established a pipetting method for effective preparation of nanosheets that can be performed in multi-well plates with an automated pipetting robot. Withdrawing an aqueous solution by pipetting creates an air/water interface inside the pipet tip. Due to the conical geometry of the tip, the interfacial area changes depending on the volume of liquid aspirated (Supplementary Figure S1). By repeating aspiration and dispense cycles, surface compression comparable to the vial rocking method could be achieved at much smaller volume scales (~ 10 μL) (Figure 3a). The quality (size, thickness, and uniformity) of nanosheets synthesized by the pipetting method were in line with that prepared by vial rocking (Figure 3b and Supplementary Figure S2). Attractive aspects of the pipetting method are that it is readily automated, can be performed in a multi-well plate formats, and produces nanosheets at concentrations and in buffers suitable for direct use in subsequent protein-binding screens.

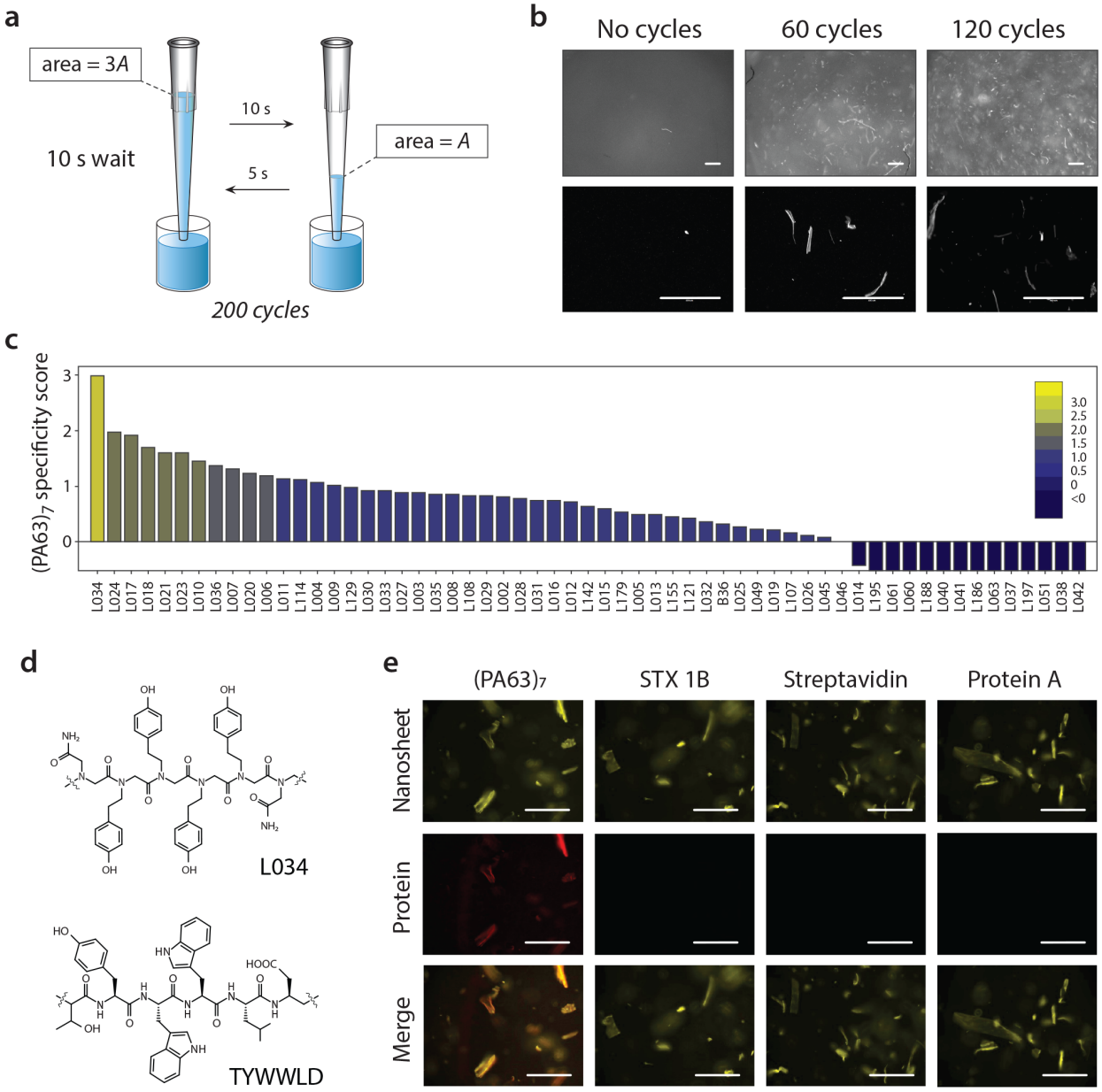

Figure 3. Automated production and screening of a loop-functionalized peptoid nanosheet library for specific binding to $(PA63)_7$. (a) Schematic illustration of peptoid nanosheet formation by interfacial compression through robotic pipetting. (b) Fluorescence microscopic images of pipetting-induced peptoid nanosheets. All scale bars represent 100 μm. (c) $(PA63)_7$ specificity score of various loopoid nanosheets from screening process. The detail of the calculation of specificity score described in supplementary information. (d) Chemical structure of loop domain of L034 and TYWWLD nanosheet. (e) Validation of $(PA63)_7$ binding specificity of L034 nanosheet by fluorescent microscopy. All scale bars represent 100 μm.

To evaluate the protein-binding capability of each loop-displayed nanosheet, we developed a screening method based on our previously developed homogenous FRET binding assay.[20] Nanosheet-forming solutions were mixed with octadecyl rhodamine (OR), which anchors its aliphatic tail in the sheet hydrophobic core, and serves as the FRET donor. These OR-labeled sheets yield a FRET signal upon binding of Alexa Fluor 647 (AF647)-conjugated target proteins on the nanosheet surface (Supplementary Figure S3). By analyzing fluorescence spectra of control (loopless), biotinylated, and globotriosylated nanosheets in the absence and presence of protein, a positive FRET signal was generated by a binding event of nanosheets to their corresponding targets (Supplementary Figure S4). Spectral scanning of the surface of globotriosylated nanosheet by confocal microscopy verified the FRET signal originating from protein binding onto the nanosheet surface (Supplementary Figure S5). To estimate the degree of binding for the selection of hits, the FRET ratio was calculated based on curve fitting to prevent potential errors caused by noise in the fluorescence spectra. We found that this method could satisfactorily identify high-FRET hits similar to the conventional FRET ratio measurements (~ $I_{Acceptor}/I_{Donor}$) (Supplementary Figure S6). Analysis of the fitting-corrected ratio for 10 samples of each loopless, biotinylated, and globotriosylated nanosheets in the presence of Shiga toxin 1 subunit B (STX 1B) (Supplementary Figure S7), revealed a Z-factor of approximately 0.73, indicating that this assay is sufficient for high-throughput screening.[29]

We next applied our automated screening platform to discover loop-functionalized nanosheets capable of selective binding to anthrax protective antigen $(PA63)_7$ which is homo-heptameric toxin-related protein. To gauge binding specificity, FRET values of each loopoid nanosheets were obtained against fluorescently labeled $(PA63)_7$ and other multimeric control

proteins, STX 1B and streptavidin, and the ratiometric score for binding specificity was calculated. From the parallel screening of a nanosheet library of 60 variants, sample L034 nanosheet was found to have the highest specificity score for $(PA63)_7$ (Figure 3c).

Upon successful identification of a hit for $(PA63)_7$, we scaled up the preparation of several nanosheet candidates to the milliliter scale using the vial-rocking method to allow for more stringent validation and characterization of binding affinity.[28] This seamless transition from microscale screening to macroscale production highlights the utility of this platform for rapid affinity reagent development. After scale-up, we examined the consistency of $(PA63)_7$ binding specificity to the L034 nanosheet by the FRET assay and by fluorescence microscopy. As a positive control, we created a nanosheet that displays a loop containing a known $(PA63)_7$ binding motif composed of a peptide hexamer, TYWWLD, previously discovered by phage-display methods[30,31] (Fig. 3d). Fluorescence microscope images and the FRET assay indicate that only $(PA63)_7$ was selectively captured by the L034 nanosheet as well as the specific binding by TYWWLD nanosheet (Figure 3e, Supplementary Figure S8 and S9). This result demonstrates the ability to rapidly discover antibody mimetics from microscale screening of combinatorial libraries, and immediate scale up of hits to the milligram scale for further testing and use.

Binding Specificity: Chemical Properties and Multivalency of Loop-functionalized Nanosheets. The $(PA63)_7$ binding hit, L034, has a loop insert sequence of Namd-Ntyr-Ntyr-Ntyr-Ntyr-Namd, which consists of two small polar carboxamidomethyl (Namd) side chains, and four aromatic hydroxyphenethyl (Ntyr) side chains (Figure 3d). Interestingly, this falls into the same **HP** family as the positive control loop (TYWWLD), where there are four central hydrophobic groups flanked by two polar groups (*i.e.* **PHHHHP**). Additionally, both contain three aromatic

residues in the hydrophobic loop region, allowing us to consider the impact of specific chemical features of the loop on $(PA63)_7$ recognition (Supplementary Table S2). Given the predominance of hydrophobic side chains in both our hit and the positive control sequence, we first examined the binding of other hydrophobic library members. There were five other loop-functionalized nanosheets with the identical **PHHHHP** pattern that did not recognize $(PA63)_7$ (Supplementary Figure S10) indicating that aromatic richness is the major factor for $(PA63)_7$ recognition. We also explored the importance of multiple aromatic derivatives within the loop domain by designing an all-peptoid analog (rationally designed peptoid, Rtoid) inspired from the known TYWWLD peptide sequence, which has three aromatic groups (Supplementary Figure S11). The Rtoid nanosheets exhibited strong binding, indicating that the aromatic side chains are playing a dominant role in binding. Motivated by these observations, we plotted the $(PA63)_7$ specific binding ability against the number of aromatic monomers within the loop region (Figure 4a), which revealed that ≥ 3 aromatic monomers were necessary but insufficient for binding. A secondary chemical property, a hydrogen bonding donor, is also required for binding. Additional secondary properties like a hydrogen bonding acceptor or chirality were not required.

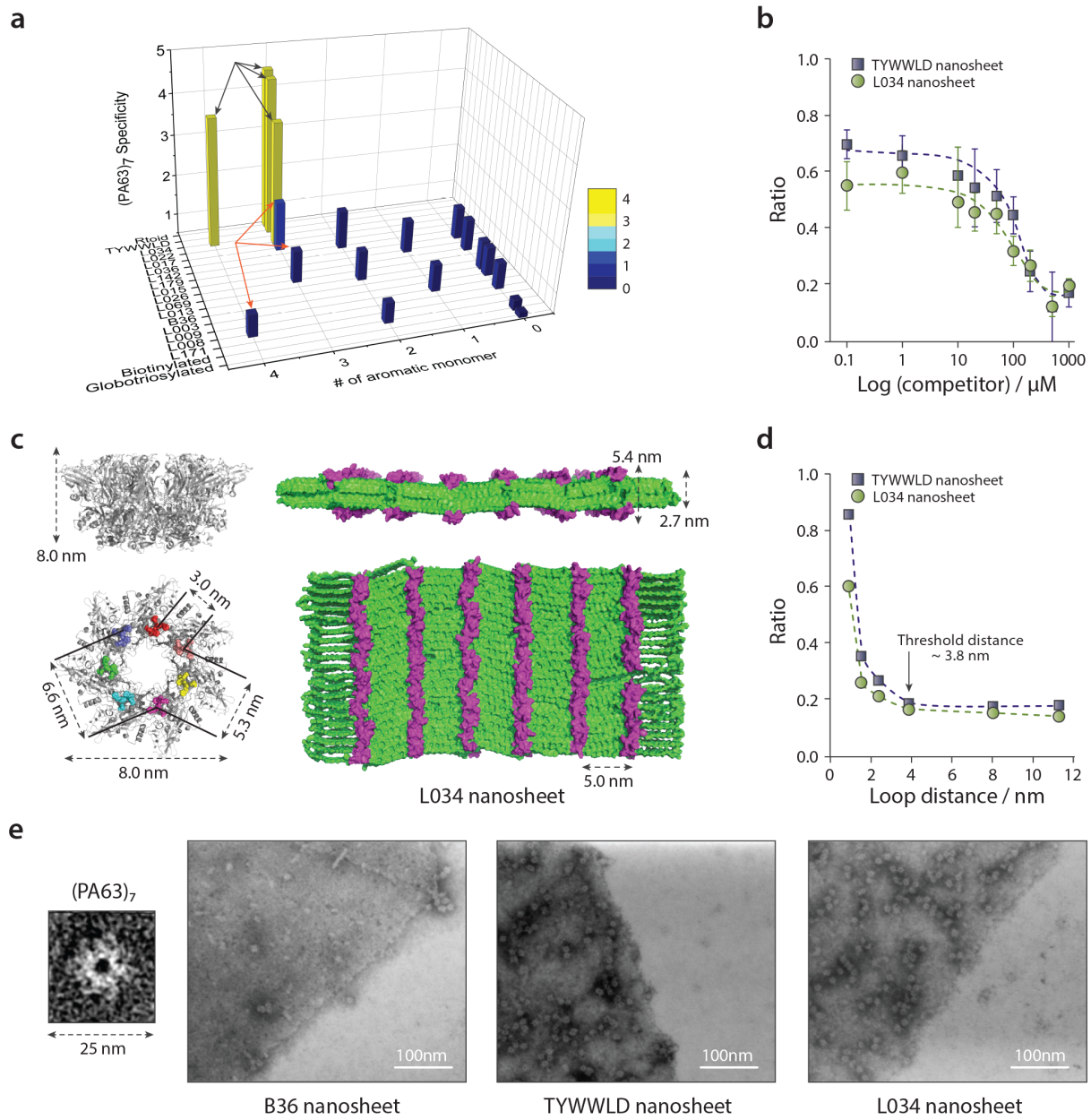

Figure 4. Characterization of selective $(PA63)_7$ binding behavior of L034 nanosheet by monomeric chemical property and multivalent configuration (a) Relationship of the number of aromatic monomers within loop domain to $(PA63)_7$ specificity score. Arrows indicate loop sequences with aromatic monomers possessing the secondary property as hydrogen bonding donor (black arrows) or others (orange arrows). (b) Competitive binding assay with TYWWLD hexamer as a competitor. (c) Molecular structure of $(PA63)_7$ (PDB 1TZO) and L034 nanosheet. Colored regions in $(PA63)_7$ represent binding site for TYWWLD ligand. L034 nanosheet structure shows the spatial configuration and inter-spacing of loops (purple) on the backbone chains (green) (d) Degree of $(PA63)_7$ binding as a function of the shortest average loop separation on the nanosheet. (e) Electron microscopic images of B36 (*i.e.*, loopless), TYWWLD and L034 nanosheet with bound $(PA63)_7$.

The specific binding affinity of loops containing aromatic and hydrogen bonding donor properties hints that they interact with a specific site of $(PA63)_7$. By performing competitive binding experiments of L034 or TYWWLD nanosheet in the presence of the free TYWWLD hexamer as a competitor (Figure 4b), the half maximal inhibitory concentration (IC_{50}) value of L034 nanosheet of 78 μM is similar to that of the TYWWLD nanosheet (98 μM), indicating they interact with the same binding site of $(PA63)_7$. A computational docking simulation of the TYWWLD peptide in a loop conformation binding to $(PA63)_7$ provided insight into the molecular interactions involved. The central YWW residues are in close contact (≤ 4.5Å) to P184, F202, F203, S204, and P205 which are known to form a hydrophobic binding pocket in $(PA63)_7$.[32] Phenylalanine facilitates an interaction with aromatic peptide residues *via* π-π interactions. In addition, proline is known to preferentially interact with electron-rich aromatics, such as tyrosine and tryptophan, by CH-π interactions in contrast to electron-poor aromatics and non-aromatics.[33] Taken together, we envisioned that a loop domain comprised of many electron-rich aromatic functionalities can recognize the hydrophobic pocket of $(PA63)_7$ *via* π-π and CH-π interactions.

From a multivalent binding point of view, the spatial arrangement of binding loops on the surface of the peptoid nanosheet plays a key role in its specificity. The distance between loops in the direction of the chain is approximately 5.1 nm, which is close to the second shortest distance, 5.4 nm, between $(PA63)_7$ binding sites, as shown in the simulated structure of the L034 nanosheet (Figure 4c). According to the model, the hydrophobic binding pocket of $(PA63)_7$ covers several loops on the sheet surface, indicating that simultaneous multiple binding events, *i.e.*, multivalent binding, may occur. Typically, multivalency leads to non-linear binding responses such as super-selectivity and high binding affinity.[22] We explored the effect of loop display density on $(PA63)_7$ binding by diluting the TYWWLD and L034 loop strands with varying amounts of a loopless

nanosheet-forming peptoid. This results in a series of co-assembled nanosheets with different inter-loop spacings. The degree of binding exhibited a non-linear response *versus* loop spacing (Figure 4d). The threshold distance of both nanosheets is ~3.8 nm, which is close to the shortest distance between binding sites of $(PA63)_7$. This suggests that the close loop spacing is required for cooperative and higher affinity binding. The multivalent interaction between loops and $(PA63)_7$ was also supported by direct observation *via* transmission electron microscopy which revealed that the central pore of $(PA63)_7$ was vertically aligned on the surface of both the TYWWLD and L034 nanosheets (Figure 4e).

Binding Performance of Loop-functionalized Nanosheets: Binding Affinity and Proteolytic Stability. To evaluate the feasibility of our identified affinity reagents for practical use, we evaluated their binding performance using bio-layer interferometry (BLI) according to our previously developed method.[34] We transferred a loop-functionalized peptoid monolayer onto the hydrophobic surface of the optical fiber by Langmuir-Schaefer deposition, exposing loop domains that can capture target proteins. BLI allows real-time, label-free monitoring of bound protein based on interference patterns formed by reflection of white light from a functional surface, allowing measurement of binding affinity. Both L034 and TYWWLD monolayers immediately captured and tightly immobilized $(PA63)_7$ on their surface in accordance with association and dissociation stages. In contrast there was no binding response with the loopless peptoid monolayer (Figure 5a). Analysis of the binding curves of L034 and TYWWLD revealed that these loop-functionalized monolayers had single-digit nanomolar binding affinity, 2.0 nM and 1.6 nM respectively, (Supplementary Table S3), which is comparable to the K_D of recombinant antibodies and natural complementary components (*i.e.*, edema factor and lethal factor) for $(PA63)_7$.[30,35,36] The binding

affinity of these materials is strikingly high in comparison with the known micromolar binding of the monovalent TYWWLD ligand. Taken together, these results indicate a significant enhancement of binding affinity due to the high avidity of designed and engineered multimeric structures.[37]

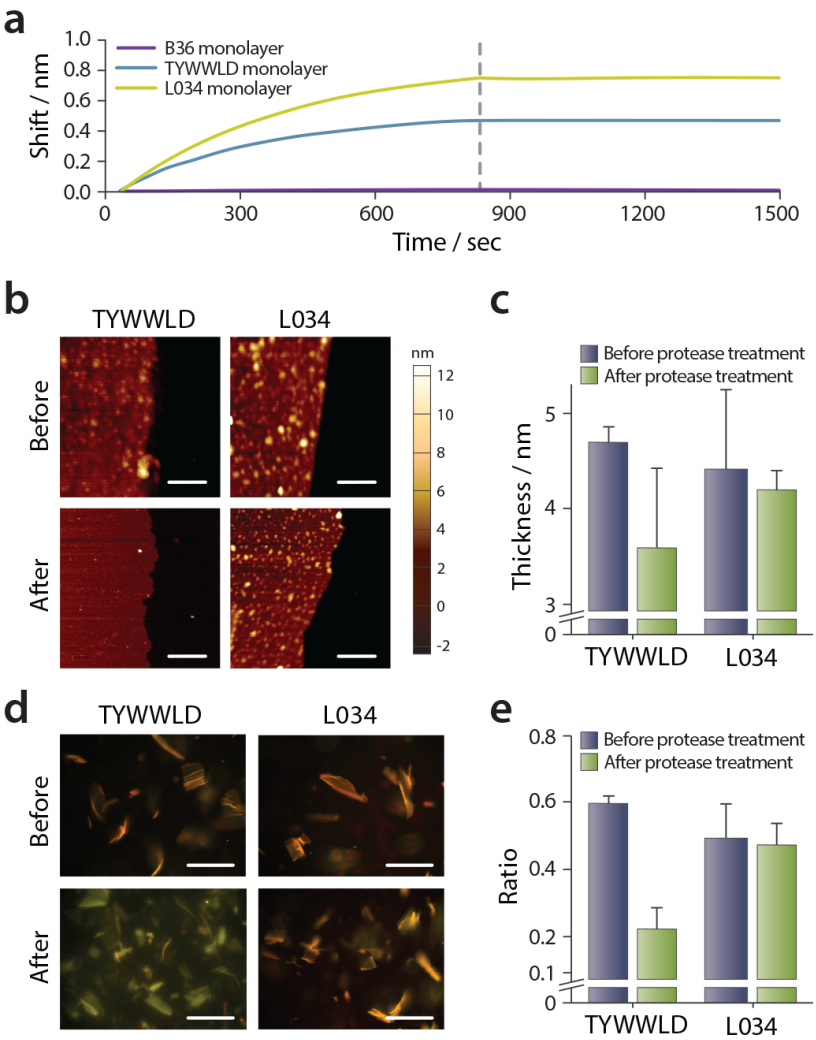

Figure 5. Binding performance and proteolytic stability of L034 nanosheet.(a) Binding response of B36 (*i.e.*, loopless), TYWWLD, and L034 monolayer-deposited optical fiber against $(PA63)_7$ by using bio-layer interferometric technique. Area before and after dotted line represents association and dissociation phase, respectively. (b) AFM images of TYWWLD and L034 nanosheets before and after protease treatment. Scale bars represent 1 μm. (c) Quantification of the thickness upon protease treatment calculated from AFM images. Each thickness was estimated by averaging three AFM measurements. (d) Fluorescence microscopic images of loopoid nanosheets before and after protease treatment. All images were obtained in the presence of $(PA63)_7$ under TR (yellow) and Cy5 (red) filter. Scale bars represent100 μm. (e) Quantification of

the proteolytic stability of TYWWLD and L034 nanosheet by FRET-based assay. L034 nanosheet retains binding affinity after protease treatment, demonstrating resistance to proteolysis.

Beyond their exceptional binding performance, these materials hold promise as a robust affinity reagent scaffold due to the chemical stability and durability of peptoids against proteases.[38] To demonstrate this, we monitored the proteolytic stability of the L034 nanosheet by atomic force microscopy. While the all-peptoid L034 nanosheet maintained loop architectures after protease treatment, the initially rough surface of the peptide-containing TYWWLD nanosheet smoothed (Figure 5b). Additionally, the thickness of the L034 nanosheet did not change, whereas the TYWWLD nanosheet thinned from 4.7 nm to 3.4 nm after protease treatment (Figure 5c). These results indicated that while the peptide loops of the TYWWLD nanosheet were eliminated through proteolytic degradation, the all-peptoid L034 nanosheet has high proteolytic resistance. We further studied the influence of proteolytic degradation on the binding activity of the L034 and TYWWLD nanosheets using multi-color fluorescence imaging, with the TR filter for nanosheets and the Cy5 filter for the $(PA63)_7$ protein as yellow and red color, respectively (Figure 5d). The orange color of the TYWWLD nanosheet incubated with $(PA63)_7$ turned to yellow after protease treatment, indicating that the removal of TYWWLD loops led to the loss of binding affinity to $(PA63)_7$. The L034 nanosheet, however, remained orange in the merged fluorescence image after the incubation of protease, indicating that they retained $(PA63)_7$ binding activity. We corroborated these results with our FRET assay, where proteolytic degradation of TYWWLD loops also demonstrated loss of $(PA63)_7$ binding in contrast to negligible change in FRET signal from L034 nanosheet (Figure 5e). The durability of these peptoid constructs against proteolysis highlights a major advantage of this synthetic system.

CONCLUSION

We have described an approach to generate 2D antibody mimetic materials with specific protein binding using a purely synthetic approach. The method uses high-throughput screening of combinatorial libraries of non-natural, sequence-defined peptoid polymers folded into protein-like architectures. The selective binding affinity of the L034 nanosheet hit to $(PA63)_7$ sensitively depends on the chemical characteristics of the surface-displayed loops. Furthermore, the spatially dense display of surface loops enables high binding avidity that significantly improves binding affinity to the target protein *via* multivalent interactions. This is a major advantage of these non-natural sheets which have a massive binding surface area relative to antibodies, and make them particularly well-suited to engage with large, multivalent targets. Moreover, their non-natural peptoid chemistry makes them robust to proteolytic attack as demonstrated by detailed analysis using AFM, fluorescence imaging, and FRET binding assay. Robustness is a valuable property of using non-natural backbone chemistry, allowing screening in less stringently-purified biofluids and discovery of affinity reagents that operate under harsher and more practical use cases. This work advances on several key aspects of antibody mimetic materials, demonstrating a rational and scalable strategy for generating and screening large chemical libraries, generation of materials with high and selective binding affinity *via* multivalency, and the ability to immediately scale production of hits by simple batch synthesis approaches that do not involve animals. Screening capacity is limited only by the fluidic approach for mixing and testing the combinatorial library members. Using established microfluidic miniaturization approaches, screening hundred-million-member libraries should be feasible, allowing multiple loopoid strands to be combined prior to nanosheet assembly to access enormous chemical diversity akin to the natural immune system. Combined, these properties should allow rapid and effective discovery of robust recognition

elements for pathogens (*e.g.*, toxin proteins, viruses, and cells) important to many biomedical applications such as sensing, diagnostics, and therapeutics.

MATERIALS AND METHODS

Instrumentation. Fluorescence data was collected with a BioTek Synergy H1 microplate reader. Fluorescence microscopy was performed on an Olympus IX81 inverted microscope and confocal FRET experiments were conducted on a Zeiss LSM710 microscope equipped with a spectral detector. Confocal image spectra were collected from images by image thresholding across all channels and background subtraction through ImageJ. Bio-layer interferometry measurements were conducted on Octet Red384 system from ForteBio.

Loopoid Library Synthesis and Purification. Methods for the solid-phase synthesis and purification of peptoid polymers were previously reported by our group and others.[15] Submonomer peptoid synthesis was automated using a Protein Technologies Symphony X or AAPPTEC Apex 396 peptide synthesizers to allow for the parallel synthesis of up to 24 different loopoid sequences at a time. β-Alanine tert-butyl ester (Nce) and glycine tert-butyl ester (Ncm) were prepared from the HCl salt using 1 M aq. NaOH and extracted with DCM before preparation of 1 M solutions in DMF. Glycinamide HCl (Namd) was neutralized in DMF by adding an equimolar amount of KOH (50% w/w aq. solution) and filtered by centrifugation immediately before use. Protected ethanolamine (Nhe) was prepared according to published procedures.[39] All other amines were purchased from commercial suppliers and used without further purification. Peptoid loopoids were prepared using a Rink amide resin (200 mg) from Protein Technologies with a standard amine

loading of 0.64 mmol/g. Standard peptoid coupling proceeded in two steps, initial bromoacetylation with bromoacetic acid (BrAA, 0.8 M) and *N*,*N*-diisopropylcarbodiimide (DIC, 0.8 M) in *N*,*N*-dimethylformamide (DMF) for 2.5 minutes. Subsequent displacement with amine (1 M) in DMF lasted for 5 minutes. Anilines, and other low nucleophilicity amines, were bromoacetylated for 20 minutes and their displacement lasted for 1 hour using amine (2 M) solutions with added potassium iodide (1 M).[40]

TYWWLD loopoid strand was prepared on a low loading (200 mg, 0.2 mmol/g) Rink amide resin from Protein Technologies to counter aggregation. Peptoid coupling proceeded with the standard reagents using 1 hour displacement and bromoaceylation times. Amino acids in the loop were coupled twice with HCTU (0.4 M), *N*-methylmorpholine (0.4 M), and Fmoc-protected amino acids (0.4 M) in DMF for 1 hour and then deprotected with 4-methylpiperidine (20% v/v) for 1 hour. The TYWWLD hexamer was prepared on standard Rink amide resin using DIC (0.8 M) as coupling agent with 1 hour coupling and deprotection times.

Crude loopoids were cleaved from the resin using 95% TFA/2.5% H_2O/2.5% TIPS while mixing on a rotary shaker for 30 minutes. The cleaved peptoid was filtered and resin was washed with DCM before concentration of the combined organics on a Biotage V10 evaporator. The loopoids were purified by reverse-phase HPLC using a Waters XBridge Prep C18 (5 µm, 19 x 100 mm) column. The typical gradient was 35-65% over 30 minutes with H_2O+0.1% TFA as solvent A and ACN+0.1% TFA as solvent B. The pure fractions were combined and concentrated with a Biotage V10 evaporator before dissolving with MilliQ water and lyophilizing to produce a typical yield of 15-30 mg. Purified loopoids were evaluated by analytical reverse-phase HPLC and MALDI-TOF mass spec to meet a standard of ≥85% purity for inclusion in the library screening.

Loop-functionalized Nanosheet Assembly and Screening. A library of loopoid nanosheet was prepared with a robotic liquid handling system (Biomek FX, Beckman Coulter) as follows. Each well of 96-well plates (polypropylene, round bottom; Greiner Bio-One, cat # 655209) was filled with 225 μL of 5 μM R18 (octadecyl rhodamine B chloride; ThermoFisher, cat # O246) in 10 mM Tris buffer (pH 8.0). 2.5 μL of 2 mM loopoid stock solution in 2:1 (v/v) DMSO:water was added to each well to make a final loopoid concentration of 20 μM. The robotic pipettor with a 96-channel head (AP96) was programmed to perform 3:1 interfacial area compression inside a P250 pipet tip as follows (Fig. S1; Beckman Coulter, cat # 717252): (a) aspirate 200 μL of loopoid solution; (b) 200 cycles of dispense 160 μL at 16 μL/s flow rate, aspirate 160 μL at 32 μL/s with a delay time of 10 s after the aspiration; (c) dispense 200 μL. Then, 50 μL of nanosheet-containing solutions were transferred to the plates pre-filled with 50 μL of Alexa 647-conjugated substrate protein solutions and mixed with the 96-channel pipet head. After 30-min incubation at room temperature, the fluorescence intensities and/or spectra of both donor and acceptor fluorophores were obtained with a plate reader (Infinite M200 PRO, Tecan; excitation at 550 nm, donor emission at 590 nm, isosbestic point at 645 nm, acceptor emission at 675 nm) and analyzed to calculate FRET ratios. Fluorescence images of produced nanosheets were acquired with an automated microscope (EVOS FL Auto, Life Technologies) using the RFP light cube (Ex. 531/40 nm, Em. 593/40 nm) with a raster scan mode and the images from the same well were stitched together with the manufacturer provided software (PerlScope, revision # 31201).

To compare binding specificity quantitatively, we calculate specificity score (S) for each loopoid ($i = 1,2,\cdots,n$) and each target protein ($j = 1,2,\cdots,m$) from FRET ratio values (R_{ij}) as follows: (1) filter out loopoids with negative FRET ratio values due to background noise ($R_{ij} >$

0); (2) normalize FRET ratio values for each target protein group by dividing the values with the maximum FRET value from each group ($R'_{ij} = \frac{R_{ij}}{\max_i(R_{ij})}$); (3) calculate the relative ratio as defined:

$$S_{ik} = \frac{(m-1)R'_{ik}}{\sum_{j \neq k} R'_{ij}}$$

For example, when FRET ratio values for three target proteins (anthrax, shiga, and strep) are available from a binding affinity screen, the anthrax binding specificity score for loopoid i is calculated as:

$$S_{i,anth} = \frac{R'_{i,anth}}{(R'_{i,shiga} + R'_{i,strep})/2}$$

For hit validation, nanosheets were prepared by conventional vial-rocking method according to the literature.[28] Briefly, 1 mL Tris buffer (10 mM, pH 8.0) containing 20 μM peptoid strands were prepared in 4 dram glass vial. The vial was tilted by custom-built machine from horizontal to vertical for 200 repetitions and 10 s horizontal wait times.

$(PA63)_7$ Expression, Purification and Dye Labeling. A plasmid for expression of the 83 kDa anthrax protective antigen (PA83; K563C, D425A mutant) cloned into a pD444-ompA vector (ATUM, Newark, CA) with ampicillin resistance, IPTG inducible T5 promoter and ompA signal peptide was a gift from the Pentelute lab at MIT. Similar protocols for purification of $(PA63)_7$ have been described previously.[41,42] BL21 (DE3) *Escherichia coli* (New England Biosciences, Ipswich, MA) transformants were grown from overnight cultures in 0.5 L batches at 37°C with shaking at 250 rpm to OD 0.6 - 0.8. Over-expression was induced with 1 mM IPTG at 30°C and 250 rpm for 3 hours. Cells were harvested by centrifugation and stored as pellets at -80°C until protein

purification. Cells were exposed to osmotic shock to extract proteins from the periplasmic space by dispersion in 10 mM bis-tris propane pH 8.9 with 1 mM EDTA and 30% glucose, followed by centrifugation and redispersion into 5 mM $MgCl_2$. A round of ultra-centrifugation was used to clarify the supernatant (50,000 g, 30 minutes), then the solution was brought to 10 mM bis-tris propane pH 8.9 with 5 mM TCEP (Buffer A) with concentrated stocks. This solution was applied to a 5 mL HiTrap Q Fast Flow anion exchange column (GE Healthcare Life Sciences, Marlborough, MA) and eluted over 20 column volumes with a 0-60% gradient of Buffer A + 1 M NaCl. The fraction containing PA83 was identified by SDS-PAGE and dialyzed into Buffer A overnight at 4°C. Cleavage of PA83 to the active PA63 form was done by adding 1:1000 (g/g) trypsin:PA63 for 30 minutes at room temperature, followed by the addition of a 10 times excess of trypsin inhibitor. It was found that heptamerization of PA63 did not occur until a second round of anion exchange (this time over a 1 mL column) after cleavage to separate PA63 from the 20 kDa cleaved unit. The $(PA63)_7$-containing fraction was dialyzed into phosphate buffered saline pH 7.4 (PBS; 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) overnight at 4°C.

Alexa Fluor 647 succinimidyl ester (ThermoFisher Scientific, Waltham, MA) was conjugated to solvent-exposed lysine residues of $(PA63)_7$. The protein in PBS pH 7.4 solution was made basic with 1 M sodium bicarbonate pH 9.0 and dye was dissolved in dry DMSO. 10 molar equivalents of the dye (relative to heptamer concentration obtained by UV-Vis absorbance at 280 nm using a measured extinction coefficient of 4.39×10^5 $M^{-1}cm^{-1}$) was added to protein solution while stirring vigorously and the reaction was allowed to proceed for 1 hour. The reaction was quenched with 0.2 M tris(hydroxymethyl)aminomethane hydrochloride pH 7.5 and the protein was purified as a heptamer *via* gel filtration with a Superdex 200 Increase 10/300 GL column (GE

Healthcare Life Sciences, Marlborough, MA). Final protein concentrations were measured by UV-Vis and samples were stored in PBS pH 7.4 at 4°C.

Scanning Transmission Electron Microscope Sample Preparation. Nanosheet samples were combined 1:1 with 1 mM $(PA63)_7$ in PBS pH 7.4. The solution was drop-cast onto copper TEM grids (ultrathin carbon with lacey carbon film, 400 mesh, Ted Pella, Redding, CA) and allowed to incubate for 10 minutes. After wicking away the sample solution and washing twice with water, an organotungsten negative stain (Nano-W®, Nanoprobes, Yaphank, NY) was applied for 5 minutes. The stain was wicked away and the samples were allowed to air-dry overnight before imaging at 30 kV in STEM mode on a Zeiss Gemini Ultra-55 analytical FE-SEM.

Transmission Electron Microscope Sample Preparation. Negative staining EM specimen preparation: PA63 Heptamer sample was prepared by optimized negative staining method as descripted.[43-45] In brief, the sample was diluted to 0.01 mg/mL with Dulbecco's phosphate buffered saline (DPBS). An aliquot (~4 μL) of sample was placed on a glow-discharged thin-carbon-coated 200 mesh copper grid (CF200-Cu-UL, Electron Microscopy Sciences, Hatfield, PA 19440, USA). After ~1 min incubation, excess solution was blotted with filter paper. Then, the grid was stained by 1% (w/v) uranyl formate (UF) on Parafilm. The grid was dried with nitrogen.

EM data acquisition and image processing: The Negative staining EM specimen was examined on a Zeiss Libra 120 Plus TEM (Carl Zeiss NTS, Oberkochen, Germany) operating at 120 kV with 20 eV in-column energy filtering at room temperature. The micrographs were

acquired by a Gatan UltraScan 4Kx4K CCD at 80,000x magnification (each pixel of the micrographs corresponded to 1.48 Å) under near Scherzer focus (0.1 μm) and defocus of 0.4 μm.

Protease Treatment of Peptoid Nanosheets. For the observation of proteolytic stability of loop-functionalized nanosheet, a fresh stock solution of 20 mg/mL proteinase K, 4 mM $CaCl_2$ was prepared in DI H_2O. 500 mL nanosheet solution was incubated with proteinase K at 60 ^oC, and agitated on a rotary shaker at 300 rpm overnight. Final concentration is 0.25 mg/mL of Proteinase K, 4 mM $CaCl_2$, and 50 mM Tris pH 7.5 buffer. After incubation with Proteinase K, nanosheets were dialyzed overnight, using a 1 mL Spectra-Por Float-A-Lyzer device with 100 kD MWCO to remove Proteinase K. The dialyzed sample were transferred onto the surface of freshly cleaved mica to observe morphology by AFM.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: (insert link here). Design and synthesis of the peptoid library, microscale formation of nanosheet libraries, nanosheet characterization, FRET assay validation and binding data, analysis of binding specificity.

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Author Contributions

R.N.Z., A.R.A., K.K. and C.L.C. conceived the project and directed the work. M.A.K., B.W.T., D.C.D., Y.C., V.U., T.J. and M.D.C. performed the synthesis and purification of the peptoid polymers. J.H.K., S.C.K., J.C. and M.K. produced the nanosheet libraries for screening. J.H.K., S.C.K. and E.M.G. developed the screening method and performed the screening assays. D.J.M. developed the sensor-coating method. L.Y. and J.C. characterized the nanosheets produced for screening. J.L. and G.R. performed electron microscopy of the nanosheets binding to target proteins. J.H.K., S.C.K., A.R.A. and R.N.Z wrote the manuscript with input from others.

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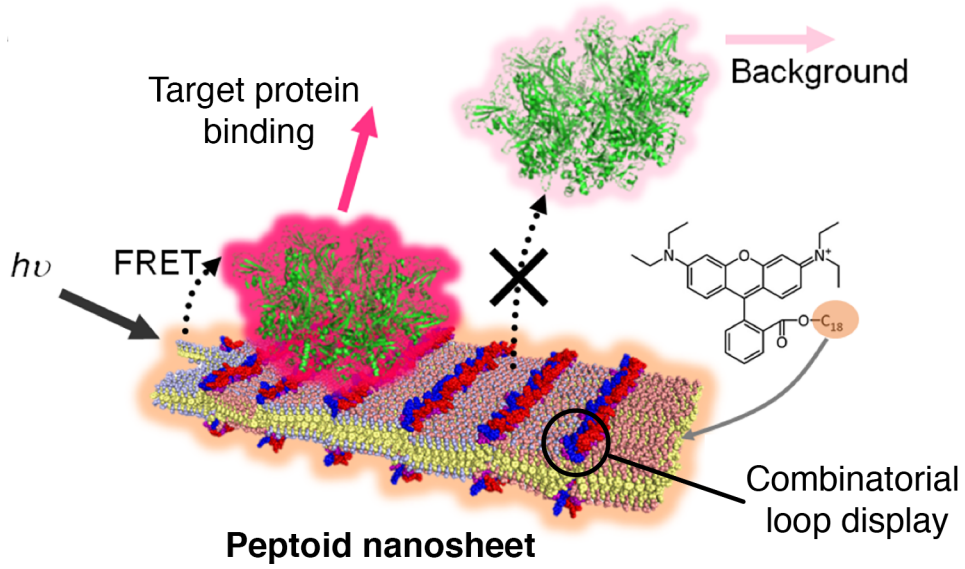
Target protein
binding
Background
hυ
FRET
Combinatorial
loop display
Peptoid nanosheet