Refined multivalent display of bacterial spore-binding peptides†

Sabrina Lusvarghi,[a] Jenny Morana Kim,[a] Yehuda Creeger[b] and Bruce Alan Armitage*[a,b]

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A multiple antigen peptide display scaffold was used to create multivalent versions of a heptapeptide selected previously by phage display to bind to *Bacillus subtilis* spores. A simple flow cytometric assay was developed in which a biotinylated form of the peptide was first bound to fluorescent streptavidin, then the fluorescent streptavidin-peptide complex was bound to spores before introduction into the cytometer. This assay clearly demonstrated that the tetravalent scaffold enhanced the affinity for *B. subtilis* spores by greater than 1 and 2 orders of magnitude when compared to divalent and monovalent analogues, respectively. However, variations in the number and flexibility of spacer residues within the scaffold did not significantly affect the binding affinity of the tetravalent peptides. Similar to prior reports, these multivalent scaffolds are effective most likely because they mimic the multivalent display of the original peptide library on the phage coat. Moreover, the tetravalent peptides can be readily integrated into a variety of heterogeneous and homogeneous spore-detection assay formats.

Introduction

Bacteria able to produce spores, such as *Bacillus anthracis*, can survive for prolonged periods in extreme environments, until the right conditions allow them to germinate. Sensitive and selective detection of bacterial spores is strongly desired for proper assessment and response to possible biological contamination of air, water and food supplies. This has motivated considerable effort in the biosensor community to develop new recognition elements such as antibodies,[1–3] nucleic acid aptamers[4] and peptides[5,6] that bind with high affinity to bacterial spores. Once appropriate recognition elements have been obtained, they can be incorporated into biosensors by coupling to various signaling components. Different strategies have been developed for the detection of *Bacillus* type of spores; for example, ELISA,[7,8] PCR-based methods,[9–11] microcantilever[12,13] and quartz crystal microbalance[14] assays have all been reported. If the affinity and selectivity of the biomolecular recognition elements for their target spores are high enough, presumably they could be adapted to any detection format.

Heptapeptides able to bind *B. subtilis*[5] or *B. anthracis*[6] spores have been previously selected from a randomized library fused to the pIII coat protein of the bacteriophage M13. Affinity-based selections using this *phage display* method yielded several promising peptide ligands for bacterial spores. The relatively low molecular weight and ease of synthesis for these peptides (compared with antibodies) has led to their use in a variety of formats, the success of which depends critically on how the peptide is presented to the spore. Initial experiments indicated that, once freed from the phage surface, monovalent peptides bound to their spore targets with low affinity and/or nonspecifically, whereas multivalent display of the peptide led to high affinity and specific binding.[5] Multivalent display of these peptides has included both high-density grafting to two-dimensional surfaces (*e.g.* glass or gold) as well as immobilization on soluble nano- or microscale particles, such as semiconducting quantum dots[15] or fluorescent proteins.[5,9,16] While successful sensors were obtained, none of these strategies allows full control over the number or spatial arrangement through which the peptides are presented to the spores. Therefore, we sought to design a water-soluble scaffold from which peptides could be displayed in a multivalent fashion, effectively mimicking the phage surface environment from which the peptides were originally displayed.

Multivalency has been proved as a valid strategy to increase the binding affinity of a ligand toward a wide variety of targets, including toxins[17,18] and receptors.[19,20] Multivalent antigen peptides (MAPs) are artificial peptides consisting of multiple copies of a peptide that are displayed from a branched core or scaffold unit. MAPs were originally reported in 1988 by Tam[21] in the context of synthetic peptide vaccines, but since then they have been used for several other applications, including immunoassays, serodiagnosis, inhibitors, mimetics, epitope mapping and ligand binding.[22,23]

MAPs can be derived from relatively rigid core elements such as cyclic peptides,[19] dendrimers,[24] cholic acid,[18] carbohydrates[25] or multidomain proteins.[26,27] If designed appropriately, these MAPs will be preorganized for binding to the target, leading to maximum affinity. However, in most cases detailed structural information concerning peptide–target recognition is lacking, so preorganization cannot be designed rationally into the MAP structure. In these cases, more flexible scaffolds that allow the binding interactions of the multiple peptide copies to be optimized are desirable. Branched oligolysine scaffolds are conveniently prepared and used to display multiple peptide copies as in Tam's original MAP design[21] and later reports,[28,29] while synthetic polymers can also be effective multivalent display materials.[30,31]

Bacteriophage M13 are coated by several proteins that protect the single-stranded DNA genome until after entry into bacteria. Peptide libraries are commonly fused to the pIII coat protein, five

[a]*Department of Chemistry, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213-3890, USA. E-mail: army@cmu.edu; Fax: +1-412-268-1061; Tel: +1-412-268-4196*
[b]*Molecular Biosensor and Imaging Center, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213-3890, USA*
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copies of which are present on one end of the rod-shaped phage.[32] Prior work has shown that attaching phage-selected peptides to a multivalent, branched lysine scaffold to create a MAP[21,33] can lead to significant increases in affinity.[34] Therefore we used a MAP scaffold to mimic the display of mono-, di- and tetravalent versions of a heptapeptide reported to bind specifically to *B. subtilis* spores. Binding of these peptides to specific and nonspecific spores was analyzed by a homogeneous flow cytometry assay and verified the need for multivalent display in order to achieve spore recognition with high affinity and specificity. Moreover, the simple design of the MAP-displayed peptides is amenable to the other heterogeneous analysis formats previously reported.

Results and discussion

Design

Our goal was to create a scaffold that would allow display of spore-binding peptides in controlled, multivalent structures (Fig. 1 and Table 1). Since the identity of the spore-surface target recognized by the heptapeptide is unknown, a flexible scaffold was desired. The amino acid lysine, with its two primary amino groups, provided a simple branching element to achieve this goal.[21,33] We used solid-phase peptide synthesis to prepare mono- (**M**), di- (**D**) and three tetravalent (**T1**–**T3**) versions of the *B. subtilis* spore-binding heptapeptide originally identified by Turnbough and coworkers.[5] The three tetravalent constructs were distinguished by the degree of flexibility in the spacer between the spore-binding heptapeptide and the branched scaffold, with **T1** featuring no spacer, **T2** having a flexible and hydrophilic single miniPEG unit, and **T3** bearing a rigid benzene ring within the spacer. (The mono- and divalent constructs were analogous to **T1**, *i.e.* no spacer was used.) Two scrambled versions of the first two tetravalent peptides (**T1s** and **T2s**) were also prepared to assess nonspecific binding. Our design also included an anchoring motif, biotin, which allowed us to attach our peptides to fluorescein-labeled streptavidin (F-STA), providing a fluorescent label to the various peptide constructs that was useful for flow cytometric assays. The biotin also allows our MAPs to be incorporated into other assay formats that require surface immobilization, since streptavidin is readily deposited onto surfaces. Three flexible "miniPEG" units were used to separate the biotin from the heptapeptides in each case. All peptides were satisfactorily purified by reverse-phase HPLC and characterized by MALDI-MS.

Table 1 Sequences of the synthesized peptides. (mp = 3,6-dioxa-8-aminooctanoic acid, "miniPEG", z = aminomethyl phenyl acetic acid)

Peptide	Sequence	Calculated *m/z*	Observed *m/z*
M	K(biotin) mp mp mp G G G V K P L F H N	1814.1	1815.2
D	(biotin) mp mp mp K (G G G V K P L F H N)$_2$	2949.4	2950.0
T1	(biotin) mp mp mp K [K (G G G V K P L F H N) $_2$]$_2$	5220.1	5224.4
T2	(biotin) mp mp mp K [mp K (mp G G G V K P L F H N)$_2$]$_2$	6092.0	6096.8
T3	(biotin) mp mp mp K [z K (z G G G V K P L F H N)$_2$]$_2$	6103.1	6103.2
T1s	(biotin) mp mp mp K [K (G G G L F N K H V P)$_2$]$_2$	5220.1	5225.6
T2s	(biotin) mp mp mp K [mp K (mp G G G L F N K H V P)$_2$]$_2$	6092.0	6095.3

Spore-binding properties

The relative affinity of the different constructs for binding to spores was determined using a flow cytometry assay. As illustrated in Fig. 2, the biotinylated peptides were first incubated with fluorescein-labeled streptavidin (F-STA) and then with *B. subtilis* spores. Unbound peptide was removed by centrifugation and the amount of fluorescence of each spore was measured by flow cytometry.

Binding specificity. Fig. 3 illustrates the results from the flow cytometry assay for three different concentrations of the tetravalent peptide **T1** and its scrambled control peptide **T1s**. At the highest concentration (500 nM) both peptides label the spores, as indicated by the stronger intensity relative to a control sample containing spores and fluorescent streptavidin, but no peptide (blue line). The fact that there is a bimodal distribution for **T1s** in Fig. 4 indicates that the spores are aggregating in the presence of this construct, whereas binding of the specific peptide **T1** shows a simple unimodal distribution.

As the peptide concentration decreases to 50 nM and then 5 nM the fluorescence intensity of the spores mixed with the scrambled control peptide decreases to a level that is indistinguishable from

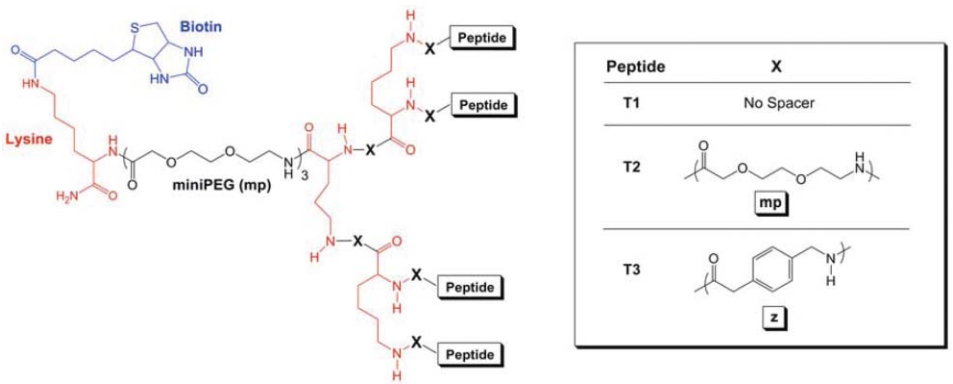

Fig. 1 Chemical structure of the tetravalent *Bacillus subtilis* spore-binding peptides. Peptides were linked to the tetravalent core by three different types of spacer: none (T1), flexible (T2) and semi-rigid (T3). Peptide sequence: Gly-Gly-Gly-Val-Lys-Pro-Leu-Phe-His-Asn (C→N terminus).

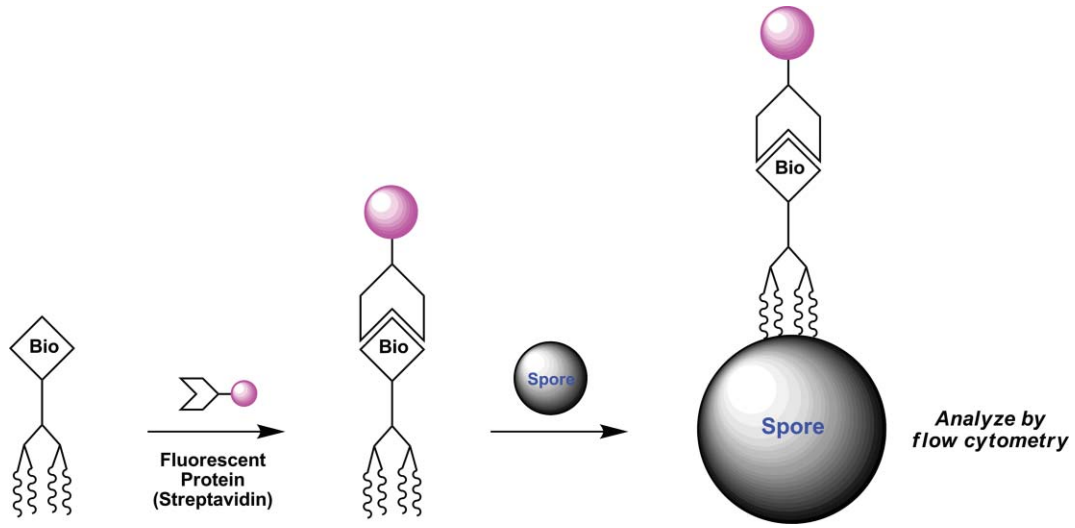

Fig. 2 Fluorescent labeling scheme. Biotinylated peptide is incubated with fluorescent streptavidin followed by spores. After removal of unbound peptide and streptavidin, spores are analyzed by flow cytometry.

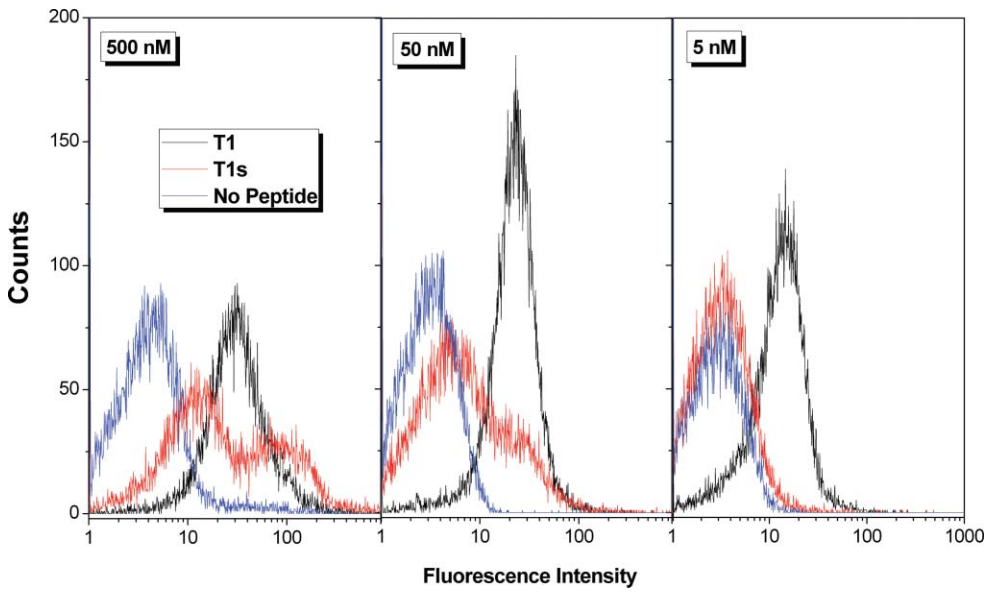

Fig. 3 Flow cytometry results for binding of tetravalent peptides T1 and T1s to *B. subtilis* spores at different peptide concentrations.

the control sample. In contrast, the intensity of the sample labeled with specific peptide **T1** is relatively unaffected by the 100-fold decrease in peptide concentration. This result indicates that binding of the specific peptide occurs with sufficiently high affinity to allow the assay to be performed at low nanomolar peptide concentrations, where nonspecific binding by the control peptide is eliminated.

The aggregating effect of the scrambled peptide is a likely consequence of the nonspecific nature of the peptide-spore interaction. In the case of the specific peptide, binding likely relies on multi-point recognition, where entropy favors binding of a single tetravalent peptide to the spore, rather than four peptides from separate ligands. In contrast, nonspecific binding by the scrambled peptide requires high concentrations and presumably does not require multi-point recognition. Thus, a single tetravalent scrambled ligand can bind to multiple spores by using each peptide branch to bind to a different spore, leading to the aggregation.

The other aspect of specificity that needs to be considered is how well the recognition peptides distinguish between spores from different bacterial species. The heptapeptide selected by Turnbough and coworkers exhibited selectivity for *B. subtilis* over *B. licheniformis* spores[5] and this is preserved in our new tetravalent constructs, as shown in Fig. 4 for **T1**. In contrast, the scrambled peptides **T1s** and **T2s** do bind to *B. licheniformis* spores (Fig. S1†), further indicating that the low-affinity binding by these control peptides to *B. subtilis* shown in Fig. 3 likely does not involve recognition of the same target as for specific **T1** binding.

Multivalency improves binding affinity. We next analyzed spore-binding as a function of peptide valency. As expected, the tetravalent peptide **T1** showed much stronger binding toward the

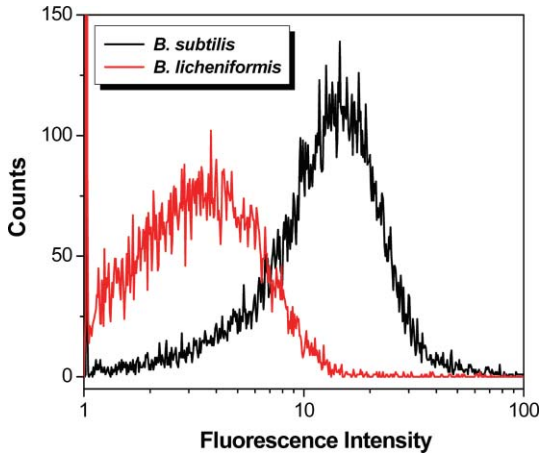

Fig. 4 Flow cytometry results for binding of 5 nM T1 to *B. subtilis* or *B. licheniformis* spores.

spores than either the divalent (**D**) or monovalent (**M**) peptides (Fig. 5). Fifty percent binding (EC_{50}) was observed for **T1** at 1.2 nM whereas EC_{50} = 17 nM for **D** and $EC_{50} \geq 720$ nM for **M**. (Since the binding curve for the monovalent peptide has not reached its maximum value at the highest peptide concentration, the stated EC_{50} value is a lower limit.) Thus, each increase in valency leads to an improvement in the binding affinity of at least one order of magnitude.

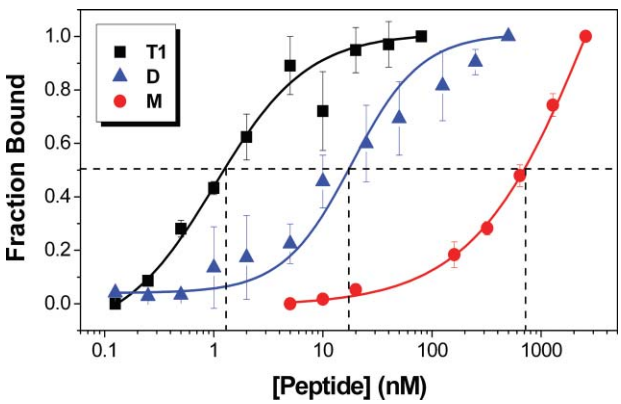

Fig. 5 Multivalency improves the binding ability of the peptides towards *B. subtilis*. Data points and error bars correspond to mean and standard deviations of three separate trials. Lines through data points correspond to sigmoidal fit and are shown only to guide the eye.

Presence of spacer in the core does not affect the affinity. In addition to varying the number of peptides attached to the branched lysine scaffold, we also tested different tetravalent constructs in which the nature of the spacer between the peptides and the core was varied (**T1–T3**, see Fig. 1 for structures). As shown in Fig. 6, similar binding plots were obtained for each of the tetravalent constructs, indicating that the spacer length and rigidity can be varied without compromising affinity. The lower affinity binding for the scrambled control peptides previously shown for **T1s** in the histograms (Fig. 3) is reproduced here, where >100-fold more peptide is required to bind to the spores.

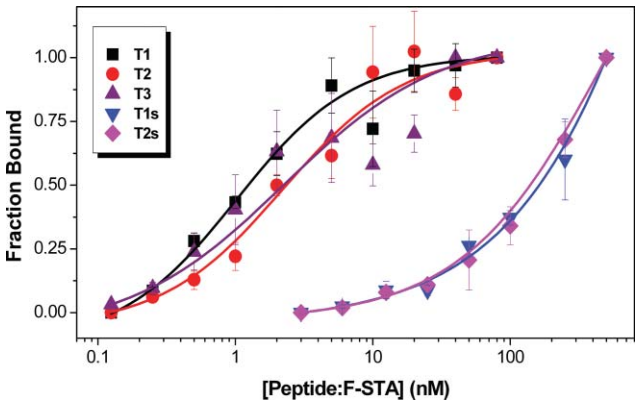

Fig. 6 Concentration dependence of binding of the tetravalent specific (**T1–T3**) and scrambled (**T1s** and **T2s**) peptides to *B. subtilis* spores.

Quantification of peptide-binding sites

While the data shown in Fig. 5 clearly demonstrate the advantage of multivalent display scaffolds for the spore-binding peptide, the histograms shown in Figs. 3 and 4 indicate that there is relatively low fluorescence intensity even when the peptide is >50% bound. This suggests that there are relatively few binding sites for the peptide on the spore surface. To estimate the actual number of peptide-binding sites on a spore, we used as a reference polystyrene beads coated with a known number of biotin groups. The fluorescein-labeled streptavidin (F-STA) was then added to the beads in increasing amounts and the fluorescence intensity of the beads was measured in the flow cytometer. The resulting calibration curve was then used to determine the corresponding number of F-STA molecules bound to spores in the presence of the tetravalent peptide **T1**. The fluorescence intensity under saturating concentrations of the peptide corresponded to $(5.6 \pm 1.0) \times 10^3$ peptide-binding sites per spore. For a spore of 500 nm radius, this corresponds to one binding site per 560 nm^2 of surface area, although it is possible that the epitope recognized by the multivalent peptide is clustered heterogeneously on the spore surface. It is also possible that each tetravalent peptide binds to a single molecular target on the spore, rather than to a cluster of targets.

Conclusion

Phage display is a widely used method for selecting peptide and protein ligands from combinatorial libraries.[32] The ability to present the library members on the phage surface through genetic fusion to coat proteins facilitates straightforward selection *via* magnetic bead capture, while the ability of the phage to infect bacteria and co-opt the bacterial gene expression machinery allows the amplification step that distinguishes selection methods from screening methods. The technical simplicity of "biopanning" a phage-displayed peptide library has spread throughout the biotechnology community, resulting in rapid identification of ligands for a wide variety of targets.

Once a promising peptide ligand has been selected by phage display, consideration must be given to how it will be utilized for a particular application. More specifically, how will the peptide be presented to its target? An interesting result was reported by Turnbough and coworkers, who identified the *B. subtilis*

spore-binding peptide used in our studies.[5] When the soluble peptide was mixed with their cognate spores, only weak, nonspecific binding occurred, whereas the phage-displayed peptide bound at sub-micromolar concentrations. We report a similar result with our new scaffold (Fig. 5). This observation can be explained by an "avidity effect",[30] in which weak binders survive the selection process because each phage displays several copies of a given peptide, each of which can make a binding contact to the target molecule or surface.[32] When the peptide is freed from the phage coat, it can only bind monovalently and the loss of avidity leads to substantially weaker binding.

In many applications, the peptide is immobilized on a surface at sufficiently high density that the avidity returns and tight binding of the target can be achieved. This has been the case for numerous spore-binding assays developed based on the peptides selected by phage display. However, in order to retain avidity in a homogeneous assay, multiple copies of the peptide should be linked together *via* a multivalent scaffold. Turnbough and coworkers accomplished this by covalently crosslinking approximately 10 copies of a spore-binding peptide to the fluorescent protein phycoerythrin (PE),[5] while Levy and coworkers synthesized lower density peptide-PE conjugates where only 3 peptides were bound to each protein.[9] The benefit of this approach was that the PE not only presented multiple copies of the peptide to the spore, it provided a strong fluorescent signal that could be used to detect spores in a flow cytometry assay or by microscopy. However, the crosslinking sites on the PE protein are heterogeneously displayed and this likely leads to lower affinity. Moreover, it is difficult to control the number of peptides attached to the protein; the relatively high number of peptides on the PE in the original study[5] led to dimerization, where two spores were presumably bound to a single peptide–PE conjugate.

In our design, inspired by original work by Tam,[21,33] four copies of the same spore-binding peptide were displayed from a soluble, branched lysine scaffold. The results shown in Fig. 5 illustrate the value of the multivalent display scaffold, which resembles the selection environment, where 5 copies of a given peptide are displayed asymmetrically on one end of the rod-shaped phage when the library is genetically fused to the pIII coat protein. The lower number of peptides attached to our scaffold compared to the PE conjugate reported previously also appears to discourage crosslinking of two spores by a single multivalent peptide since only a single peak is observed over a 100-fold range in **T1** peptide concentration in the flow cytometry histogram (Fig. 3).

The use of a branched, lysine MAP scaffold for multivalent display of peptides was pioneered by Tam in the context of antigen presentation for vaccine development.[21,33] Our scaffold is similar to Tam's original design, with the exception of the terminal biotin group and the tri-miniPEG spacer to allow the peptides to extend away from the streptavidin surface. Our peptide display scaffold could be used in a recently reported multiparameter flow cytometry method for detecting spores,[16] but could also be used in heterogeneous formats, where immobilization would be *via* the terminal biotin to a streptavidin-coated surface. Alternatively, the biotin could be replaced by a thiol-containing group (*e.g.* cysteine) to allow immobilization on gold, or a variety of other chemistries that are compatible with the growing catalogue of functionalizable surfaces. These "phage-mimetic" scaffolds could significantly improve the performance of selected peptide ligands, as was shown previously for peptides selected for binding to the E2F protein.[34] It is even possible that MAP scaffolds would improve binding of phage-selected peptides to small molecules, where the multiple peptide chains could form a cage-like structure around the ligand.

The design of our assay relies on biotin to bridge the tetravalent peptide to a fluorescent streptavidin. The femtomolar affinity of biotin-streptavidin allows the assay to be performed without concern for dissociation of the streptavidin from the labeled spores, since the peptide-spore interaction is a nanomolar affinity interaction. Since the bacteriophage display only 5 copies of the peptide, it is unlikely that further increasing the valency of our MAP will lead to significant increases in affinity.

The main limitation of our system is the relatively small difference between the histograms for specifically labeled and unlabeled spores. The saturation evident in the binding plots (Figs. 5 and 6) demonstrates that the weak signal is not due to insufficient binding, but rather to the low copy number of the binding epitope on the spore surface. It should be possible to improve the fluorescence intensity using brighter labels, such as a commercially available streptavidin conjugated to a phycoerythrin protein or a quantum dot.

Finally we note that the scaffold used here for tetravalent display of the spore-binding peptide is easily prepared by conventional solid-phase peptide synthesis and thus is readily generalized to virtually any peptide selected by phage display, including the *B. anthracis*-binding heptamers selected by Turnbough and coworkers.[6]

Experimental

Reagents and bacterial strains

Boc-protected amino acids, resins and coupling reagents were obtained from Novabiochem (La Jolla, CA) or Peptides International (Louisville, KT). Fluorescein (DTAF) conjugated streptavidin (F-STA) was obtained from Jackson ImmunoResearch Laboratories (West Groove, PA) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). *B. subtilis* ATCC6051 and *B. licheniformis* ATCC14580 where purchased from ATCC (Manassas, VA).

Peptide synthesis

All peptides were synthesized by standard solid-phase synthesis. Boc-Lys-Fmoc-OH was first attached to MBHA resin and the Fmoc protecting group was then selectively removed from the side chain using 20% piperidine/DMF. The free amino group was then reacted overnight with 5 equivalents of biotin using 5 eq of HBTU and 10 eq of MDCHA. Standard t-Boc-protected amino acids were used to synthesize the rest of the peptide sequence. After cleavage from the resin using a mixture of trifluoroacetic acid and trifluoromethane sulfonic acid, peptides were purified by high performance liquid chromatography, characterized by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS; Applied Biosystems, Voyager DE sSTR), and quantified using the BCA protein assay (Pierce Biotechnology; Rockford, IL). Sequences of the synthesized peptides are presented in Table 1.

Preparation of *B. subtilis* and *B. licheniformis* spores

Bacillus spores were prepared, and quantified as previously described in literature.[35] Briefly, spores, as provided by ATCC, were suspended in 1 mL of LB broth and then diluted to 6 mL; then allowed to grow in a LB broth-agar plate at 32 °C for 24 h. Independent colonies were picked and grown in 500 mL of LB broth until $OD_{600nm} = 1.6–2.0$ at 32 °C. Solutions of bacteria were diluted four times in synthetic replacement sporulation media at 37 °C for 2 days. Spores were collected by centrifugation at 10,000*g* for 10 min and then suspended in 1–2 mL of urografin 35%. This suspension was then layered over 20 mL of 50% urografin and centrifuged for 1 h at 10,000*g*. Upper layers were disposed and pellet was resuspended in water, centrifuged at 8000*g* for 10 min and washed again with water. Small fractions were prepared, lyophilized and kept at 4 °C for future use. The spores were quantified by preparing different concentrations, growing in LB-agar plates and counting the independent colonies.

Binding of peptides to *Bacillus* spores

Biotinylated peptides were mixed with the fluorescently labeled streptavidin (F-STA) in a 1:1 ratio of biotinylated peptide to F-STA, and allowed to bind in PBS with continuous shaking for one hour. Spores (10^6 in number) and BSA (2 μM final concentration) were added to 200 μL of the solution and allowed to bind to the peptides for one additional hour. Spores were centrifuged at 10,000*g* for 5 min, the upper solution was discarded and the spores were washed once with PBS to remove unbound peptides. Spores were then analyzed by fluorescence assisted cell sorting (FACS, Coulter Epics Elite) using 15 mW Ar laser excitation at 488 as the light source and a 530/30 emission filter. 25,000 events were counted and the data were analyzed with the WinMDI 2.9 software.

Quantification of the peptide-binding sites

Biotin coated polystyrene particles (Spherotech, TFP-5058-5, Lake Forest, IL) were mixed with known concentrations of F-STA (from 5000 pM to 0.05 pM). Change in fluorescence *vs.* concentration/number of particles was used to create a calibration curve that was then used to calculate the number of peptides bound to each spore. For this calculation it was assumed that individual spores, rather than aggregates, were detected. This assumption is supported by the observation of monomodal histograms in the flow cytometry experiments.

Acknowledgements

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