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Screening Nonspecific Interactions of Peptides without Background Interference

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Abstract

The need to discover new peptide sequences to perform particular tasks has lead to a variety of peptide screening methods: phage display, yeast display, bacterial display and resin display. These are effective screening methods because the role of background binding is often insignificant. In the field of nonfouling materials, however, a premium is placed on chemistries that have extremely low levels of nonspecific binding. Due to the presence of background binding, it is not possible to use traditional peptide screening methods to select for nonfouling chemistries. Here, we developed a peptide screening method, as compared to traditional methods, that can successfully evaluate the effectiveness of nonfouling peptide sequences. We have tested the effect of different peptide lengths and chemistries on the adsorption of protein. The order of residues within a single sequence was also adjusted to determine the effect of charge segregation on protein adsorption.

Keywords

Peptide screening; peptide synthesis; protein adsorption; nonfouling

1. Introduction

It has been established that surfaces modified with hydrophilic synthetic polymers, such as poly(ethylene glycol) (PEG), are able to resist types of surface fouling, such as nonspecific protein adsorption and bacterial adhesion/biofilm formation in complex media.[2, 3] More recently, synthetic zwitterionic poly(carboxybetaine) and poly(sulfobetaine))[4–6] as well as synthetic mixed positively and negatively charged polymers[7, 8] have been developed and shown to achieve ultralow levels of fouling that have not been achieved before. Despite some progress in the fundamental understanding of molecular-level nonfouling mechanisms and the development of new materials, only a handful of nonfouling materials are currently available. Mixed charge nonfouling polymers are particularly attractive for practical applications because of their simplicity, broad variations, and low-cost.[9, 10] Among these, peptides are of particular interest due to their being natural materials. Mimicking the chemistry of zwitterionic polymers, we have also been able to achieve ultralow levels of fouling with peptide based surfaces.[7] For evaluating nonfouling materials, peptide-based nonfouling polymers offer an indispensable advantage; potentially infinite variability in structure and property, with precise control over length and sequence. In addition to the

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already rich diversity of naturally occurring amino acids, incorporating synthetically derived unnatural amino acids allows for an even greater number of potential materials.[11, 12]

Peptide libraries are tools often used to search for new functional chemistry, such as identifying enzyme binding motifs,[13] protease cleavage sites,[14] mineral binding peptides,[15] and antibiotics.[16] To accomplish this, peptide libraries require a display mechanism; commonly phage display, yeast display, bacterial display, or resin display.[17–19] These methods work by evaluating the binding performance of individual peptide sequences to specific materials. The peptide of interest usually makes up only a small portion of the presenting scaffold (i.e. cell, virus, etc.). The presence of background binding from the display mechanism does not interfere because the affinity of the peptide is far greater than the affinity of the background for the material being tested.[20]

2. Experimental methods

2.1 Materials

Triisopropylsilane (TIPS), Trifluoroacetic acid (TFA), -glycidoxypyltrimethoxysilane (GPTMS), N,N-Diisopropylethylamine (DIPEA), glass beads (212 – 300 μm), Fibrinogen from Bovine, glass beads (50–70 mesh) and octadecyltriethoxysilane were purchased from Sigma-Aldrich. Tetraethyleneglycol diamine (PEO₄-Bis Amine) was purchased from Molecular Biosciences. TentaGel MB NH₂ resin (capacity: 0.51 mmol/g) was purchased from RAAP Polymere. Alexa Fluor® 488 Carboxylic Acid, 2,3,5,6-Tetrafluorophenyl Ester (Alexa Fluor® 488 5-TFP) was purchased from Fisher Scientific. Hydroxybenzotriazole (HOBt), O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and Fmoc protected amino acids were purchased from AAPPTec.

2.2 Glass bead surface modification for peptide synthesis

100 mL of 37% hydrochloric acid was added to 20 grams of glass beads and refluxed overnight. The beads were washed several times with water and methanol, then dried under reduced pressure and heating. The glass beads were next treated with a 3:1 volumetric ratio of concentrated sulfuric acid and 30% hydrogen peroxide, and allowed to sit for 30 minutes. The beads were then rinsed several times with water, then treated with a 5:1:1 volumetric ratio of water to 29% ammonium hydroxide to 30% hydrogen peroxide, and allowed to sit for 30 minutes. The beads were washed with water several times and then washed several times with methanol. The glass was then dried under reduced pressure and heating. The beads were placed in an oven at 130°C for 2 hours to remove and trace water then allowed to cool to room temperature. For silination, the beads were added to a solution of anhydrous toluene, GPTMS, and DIPEA (94/5/1). The silination occur at 80°C for 18 hours. After silination, the beads were washed with anhydrous toluene, dichloromethane (DCM), and then dried under reduced pressure and heated at 100°C for 2 hours. The silanized beads were next added to a solution of 5% PEO₄-Bis Amine in anhydrous acetonitrile and heated to 80°C for 18 hours. The beads were then washed with acetonitrile and dried. Unreacted surface epoxide groups were deactivated by incubating in 2% sulfuric acid in water overnight. Then beads were then finally washed with water and methanol, then dried under reduced pressure and heating.

2.3 Peptide synthesis

Solid phase peptide synthesis was performed using a Titan 357 peptide synthesizer (AAPPTec inc., Louisville, KY). Coupling of Fmoc protected amino acids occurred in 80 mM amino acid, 80 mM HBTU, 80 mM HOBt, 160 mM DIPEA in dimethylformamide (DMF) for 60 minutes. Piperidine/DMF (20/80) was used to deprotect the Fmoc protected amine from the newly bound amino acid residue. A solution of DMF/DCM (50/50) was used

for washing between coupling and deprotection steps. After synthesis was complete, all terminal amine group were acetylated using acetic anhydride/pyridine/DMF (5/5/90). Deprotection of the acid cleavable side-chains occurred as the final step using a solution of TFA/phenol/water/TIPS (88/4/4/4) for 3 hours. The beads were then washed with DCM and dried under reduced pressure.

2.4 Protein adsorption and screening

Alexa Fluor 488 labeled fibrinogen was prepared by conjugation with Alexa Fluor® 488 5-TFP. Briefly, 30 mg of fibrinogen was dissolved in 3 mL of 100 mM NaHCO₃, pH 9. This solution was added to a vial containing ~1 mg of Alexa Fluor® 488 5-TFP. After 2 hours, the labeled conjugates were twice purified using 10 mL Bio-Gel P-6DG Bio-Rad disposable size exclusion columns.

Before adding labeled fibrinogen, peptide coated beads were washed several time with phosphate buffered saline (PBS). The beads were then incubated in a 0.5 mg/mL solution of Alexa Fluor 488 labeled fibrinogen for 60 minutes. After adsorption, the beads were washing several times with PBS to remove unbound protein and quickly analyzed using a Zeiss LSM 510 confocal microscope. Cross-sectional fluorescent images of the beads were taken (X-Z plane) using 488 nm argon laser for excitation, and detecting emission signal between 500–550 nm.

2.5 X-ray photonelectron spectroscopy (XPS) of glass beads

XPS experiments were performed on a Kratos Axis Ultra DLD spectrometer using a monochromatic Al K X-ray source ($h\nu = 1486.6$ eV) operated at 10 mA and 15 kV. Survey spectra were acquired with an analyzer pass energy of 80 eV. High-resolution O 1s, N 1s, C 1s and Si 2p spectra were acquired with an analyzer pass energy of 20 eV. All of the XPS data were acquired at a nominal photoelectron takeoff angle of 0°, where the takeoff angle is defined as the angle between the surface normal and the axis of the analyzer lens. Three spots on each sample were examined. The compositional data are averages of the values determined at each analysis spot.

3. Results and Discussion

In this work, we screened a large library of peptide sequences to determine nonfouling properties. Since we were looking to determine what peptide sequences have the lowest amount of nonspecific binding, traditional peptide display mechanisms would not work, due to the presence of background binding.[21] Background binding would eliminate the ability to differentiate between nonfouling sequences. For this reason, a new method for screening nonfouling peptides was developed using amino functionalized glass beads to introduce peptides to a surface through solid phase Fmoc peptide synthesis. The full surface of the glass beads were coated with synthesized peptide, thereby presenting only the peptide of interest to the protein adsorbent. We also demonstrate how this new method compared to traditional peptide screening when evaluating known nonfouling peptide sequences.

In order to grow peptides off glass beads, the glass surface was needed to be modified with accessible primary amines (Fig. 1a). To accomplish this, an epoxide functionalized silane, GPTMS, was first coated on the surface. The presence of the epoxide then allowed coupling of a tetraethylene glycol diamine linker. Tetraethylene glycol linker was used as an alternative to shorter diamines in order to improve amino acid coupling. To ensure efficient surface modification, the amino functionalized glass was evaluated by XPS. Data were collected for the unmodified bare glass beads, the silane functionalized glass beads with the epoxides deactivated, as well as the amino functionalized glass beads. As seen in Table 1,

the unmodified glass consisted or primarily of silicon and oxygen with some carbon contamination and no detectable nitrogen. Using such surface sensitive analysis techniques, contaminant carbon is often unavoidable. After silination, a significant increase in carbon was observed representing what is likely a monolayer, given the deposition conditions. After reacting the epoxide surface with the diamine linker, 2.0% nitrogen surface content was measured. This gave a 9.1/1 carbon to nitrogen ratio compared to a theoretical ratio of 7.0/1. This meant most of the deposited silane was reacted with diamine linkers. Accounting for the presence of contaminant carbon would likely bring the measured values closer to the theoretical. Overall these data show that there is a high surface density of available amine for peptide synthesis.

To evaluate the performance of this new method of peptide screening, five amino acids were chosen to rationally design peptide sequences of interest. Glutamic acid (E) and lysine (K) were chosen due to their negative and positive charges, respectively. Glycine (G) was included because it constitutes the bare amide backbone found in all peptides, since it has no side-chain. Leucine (L) was included due to its hydrophobic characteristics, and glutamine (Q) for its uncharged, hydrophilic amide side-chain. The structures of these amino acids, with their chemical characteristics, can be seen in Fig. 1b. The beads were synthesized using standard Fmoc peptide synthesis. Since the peptide surfaces were synthesized as large groups of beads, samples were able to be taken during the syntheses to provide surfaces that had shorter length peptides, thereby increasing the number of peptide surfaces capable of being analyzed.

Fouling was evaluated by performing protein adsorption experiments using 0.5 mg/mL fluorescently labeled (Alexa Fluor 488) bovine fibrinogen in phosphate buffered saline (PBS). Fibrinogen is a large protein found in the blood that is highly susceptible to surface adsorption and plays a vital role initiating the foreign body response in the body. Fibrinogen is therefore useful to determine the nonfouling characteristics of surfaces. Beads were incubated in the protein solution, and then washed with PBS to remove unbound protein. The beads were analyzed using confocal microscopy in order to accurately measure the fluorescent intensity of bound protein at a bead's surface.

Fig. 2 demonstrates the effectiveness of the new screening method as compared to a traditional screening method that uses TentaGel resin. The fluorescent images are cross sections of the beads or resin taken using a confocal microscope. Like most resins, TentaGel resin consists of a polymer scaffold that has peptides synthesized throughout the scaffold. As seen in Fig. 2, both the "non peptide" and poly(glycine, G) samples had moderate to low levels of fibrinogen adsorption, however, the mixed charge peptide (EKEKEKEK) had extremely high levels of adsorption for the TentaGel resin and very low levels of adsorption for the glass beads. As published previously, this mixed charge sequence was shown to have ultralow levels of adsorption.[7, 12] The unintended result observed from the TentaGel resin was likely due to the protein entering the resin and salting out within the surface of the polymer scaffold. The salting out was likely caused by the presence of the ionic species of the peptide (E, negative; K, positive). These complex adsorption events can be avoided if the peptides are presented on a rigid surface, removing background effects. The lower row in Fig. 2 shows the new method with expected adsorption levels, in contrast to what was observed with TentaGel.

Fig. 3 shows fluorescent cross sections in the XY plane of beads with different peptide surfaces after exposure to fluorescent protein. A glass bead was also silinated with an alkyl silane (octadecyltriethoxysilane) as a positive control for protein binding due to its hydrophobicity, labeled as "alkyl". It is apparent that there are significant differences based on peptide sequence. It was observed that hydrophobic and positively charged surfaces

showed increased levels of protein adsorption while the negative and hydrophilic beads had low levels of protein adsorption. Unfortunately, quantifying protein adsorption using XY cross sectional images was inaccurate, varying greatly with the selected Z plane. This made it difficult to quantify the difference between sequences. In order to achieve reproducible fluorescent surface intensities, XZ fluorescent cross sections were measured. Data achieved in this manner gave consistent measurements of protein adsorption. All collected fibrinogen fouling data for synthesized peptide surfaces can be seen in Fig. 4a. Values were calculated as percentages relative to the positive control: octadecyltriethoxysilane modified beads. An example of a XZ fluorescent cross sectional image can be seen in Fig. 4b. Integrating across the surface of the bead was used to quantify the amount of protein fouling (Fig. 4c).

The collected fouling data was organized to elucidate trends and patterns based on a peptide's sequence and chemistry. Fig. 5 shows the trend in fouling of homologous sequences for the five amino acids used. Since fibrinogen is a negatively charged protein, there is a distinct effect of surface charge on fouling. Glutamic acid surfaces showed low levels of fouling, with lower fouling correlated to the number of amino acids in length. The opposite was apparent with lysine a positively charged residue, with longer chains having corresponding to higher levels of fouling. Hydrophobic surfaces were also susceptible to higher levels of protein fouling, due to hydrophobic interactions with the protein core, resulting in irreversible unfolding and adsorption. Glycine with no side-chain and glutamine with a non-charged amide side chain display low fouling properties. The lower fouling levels seen for glutamine compared to glycine show that its amide side-chain plays a significant role for reducing protein fouling. At longer peptide lengths, glutamine was seen to be one of the lowest fouling peptides.

Charge position was also investigated to determine how the arrangements of positive and negative charges (lysine and glutamic acid) affected fouling. It is expected that surfaces with positive and negative charges, that are uniformly mixed, give the lowest levels of fouling by minimizing charge localization and dipole formation. In contrast, with enough charge localization, with separated areas of negative and positive charges, on an overall net neutral surface, there will be higher degrees of fouling. Fig. 6a shows levels of fouling on several mixed charged surfaces, which have a net surface charge of zero. Little distinction was seen between peptides that had alternating charges or charges fully segregated. These results indicate two possibilities. First is the presence of salt bridging between positive and negative regions of peptides, which reduces the degree of charge separation experienced on the surface. Second, the maximum peptide length tested was only 8 residues. It is expected that longer peptides with larger regions of segregated charges will experience higher degrees of fouling. It was apparent that sequences tested in this study were not long enough to experience this effect. Here, the primary variable affecting protein fouling was peptide length. This was due to higher degrees of surface hydration, with an overall increase in hydrated peptide chains. However, for glutamic acid and lysine mixtures that were not charge neutral, there were observable differences in fouling as expected (Fig. 6b). Electrostatics between a charged surface and charged protein can play a dominant role in attracting or repelling a protein onto a surface, depending on different charges on a protein and a surface. A charged surface which repels the adsorption of similarly charged proteins cannot be considered a nonfouling surface since proteins of the opposite charge would certainly lead to fouling.

4. Conclusions

Here, we developed a new platform of peptide screening that eliminates the effect of background binding. This was done by using a bead display that allowed for the synthesis of peptides off the full surface of the bead. This is a valuable technique for measuring the

performance of nonfouling peptide sequences since it is essential to eliminate the role of background binding. We have shown how this technique can be used to differentiate between beads consisting of different peptide coatings. Confocal microscopy was an ideal method for observing bound fluorescent protein, which was able to accurately measure the adsorbed protein film across the peptide surface. From our results, it was observed that glutamine and mixtures of charged residues of overall neutral charge had the lowest degrees of fouling. Using this new method, the problem of background binding found in traditional peptide screening methods can be avoided, allowing for the screening of nonfouling chemistries.

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Figure 2.

Steps for surface modification of glass beads. GPTMS was first used to introduce epoxide groups to the glass surface. The epoxidized surface was exposed to PEO₄-Bis Amine in order to attach reactive amine groups needed for peptide synthesis. Finally, amino acids, with diverse chemical properties, were selected to grow peptides from the glass beads using standard Fmoc peptide synthesis.

Figure 2. Comparison between the traditionally used TentaGel resin and our glass beads for evaluating nonspecific protein adsorption. It can be observed for peptides like EKEKEKEK that TentaGel resins are susceptible to swelling and protein entrapment, where the glass beads provide a more rigid surface that represents the chemistry of the surface more accurately.

Figure 3. Fluorescent images in the XY plane of peptide coated glass beads after exposure to Alexa Fluor 488 labeled fibrinogen. The first tile is a light field image without fluorescence of the alkyl surface.

Figure 4.

a) Nonspecific fibrinogen adsorption of all synthesized peptide surfaces. Values listed of percents fouling compared to an alkyl silane surface. b) An example of a protein bound bead scanned in the X-Z plane. The top of the bead is integrated c) to measure the level of adsorbed protein.

Figure 5.

The effect of peptide length on protein adsorption for homologous peptide sequences. The addition of positively charged lysine and hydrophobic leucine resulted in increased protein adsorption. Increased length on glycine, glutamic acid and glutamine showed reduced protein adsorption, with glutamic acid and glutamine showing the lowest adsorption levels (5%).

Figure 6.

a) The effect of charge position on protein adsorption for charge neutral peptide sequences. No effect was observed as a result of charge segregation except for peptides of only two residues. The primary factor in reducing protein adsorption for charge neutral ionic peptides was length. b) Net charged peptides showed differences in protein adsorption only as a result of total charge.

Table 1

Summary of XPS-Determined Elemental Composition of bare glass, deactivated epoxy and amine surfaces (atom %).

Sample	Atom %				
	O	N	C	Si	C/N [*]
Bare Glass	67.5 ± 1.0	-	4.4 ± 1.1	28.1 ± 0.6	-
Deactivated Epoxy	57.6 ± 2.3	-	21.7 ± 1.6	20.7 ± 0.7	-
Amine Surface	57.0 ± 2.1	2.0 ± 0.5	18.3 ± 0.6	22.6 ± 1.4	9.1 7.0

* Theoretical C/N ratio for 100% coupling efficiency of the diamine linker onto the surface.