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Review

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Identification of Soft Matter Binding Peptide Ligands Using Phage Display

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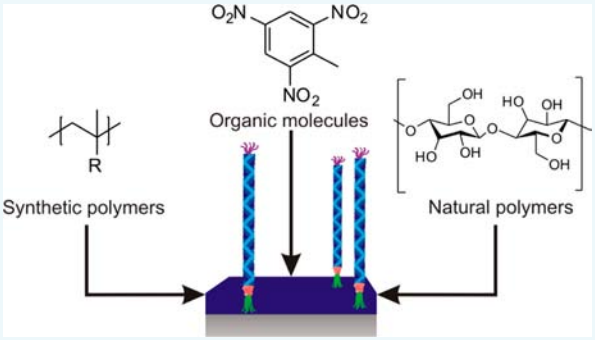
ABSTRACT: Phage display is a powerful tool for the selection of highly affine, short peptide ligands. While originally primarily used for the identification of ligands to proteins, the scope of this technique has significantly expanded over the past two decades. Phage display nowadays is also increasingly applied to identify ligands that selectively bind with high affinity to a broad range of other substrates including natural and biological polymers as well as a variety of low-molecular-weight organic molecules. Such peptides are of interest for various reasons. The ability to selectively and with high affinity bind to the substrate of interest allows the conjugation or immobilization of, e.g., nanoparticles or biomolecules, or generally, facilitates interactions at materials interfaces. On the other hand, presentation of peptide ligands that selectively bind to low-molecular-weight organic materials is of interest for the development of sensor surfaces. The aim of this article is to highlight the opportunities provided by phage display for the identification of peptide ligands that bind to synthetic or natural polymer substrates or to small organic molecules. The article will first provide an overview of the different peptide ligands that have been identified by phage display that bind to these "soft matter" targets. The second part of the article will discuss the different characterization techniques that allow the determination of the affinity of the identified ligands to the respective substrates.



INTRODUCTION

Inspired by the highly specific ligand−substrate interactions present in almost any of the biochemical processes in nature, phage display has been utilized for more than two decades for the identification of short peptide sequences that bind with high affinity and selectivity to the substrate of interest.[1−6,20] Following its first successful implementation by George P. Smith in 1985,[7] phage display has evolved into a powerful method for the identification of natural or novel ligands to a myriad of biological substrates including enzymes,[8−10] toxins,[11,12] receptors,[13,14] and structural proteins.[15,16] Phage display has also proven to be a powerful method to identify binding sites of proteins via epitope mapping.[17−19] Nowadays, phage display is used for a variety of applications including drug discovery,[20−23] vaccine development,[24] and analysis of the energetics at the binding interfaces of proteins[25] or to map the molecular diversity of receptors in the human vasculature.[26]

A typical phage display cycle consists of four steps (Figure 1). In the incubation step, a combinatorial phage library containing randomized inserts in one of the coat proteins is incubated with the substrate. Following the incubation, weakly bound phages are removed by washing the substrate several times with a buffer solution containing small amounts of a surfactant. In the next step, strongly bound phages are recovered by washing the substrate with an elution buffer and finally amplified using the *E. coli* host strain to yield a phage mixture that is enriched toward the substrate. Repetition of this cycle eventually yields a phage mixture that is predominantly composed of substrate selective phage clones. Sequencing of the genome of the individual clones allows the identification of substrate-selective, short peptides.

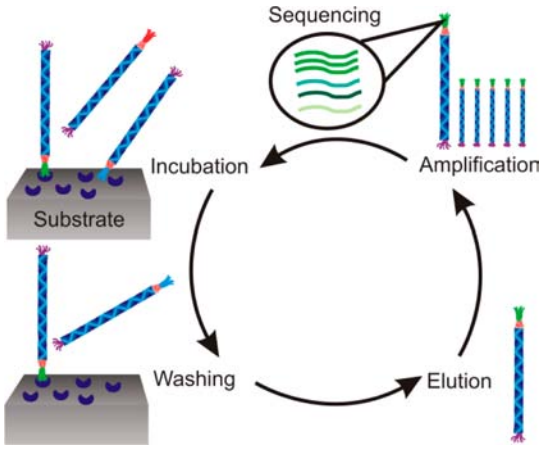


Figure 1. Schematic representation of the different steps involved in the phage display process.

While initially developed and used for biological substrates, over the past decade the scope of the phage display technique has been significantly expanded and this method is now also

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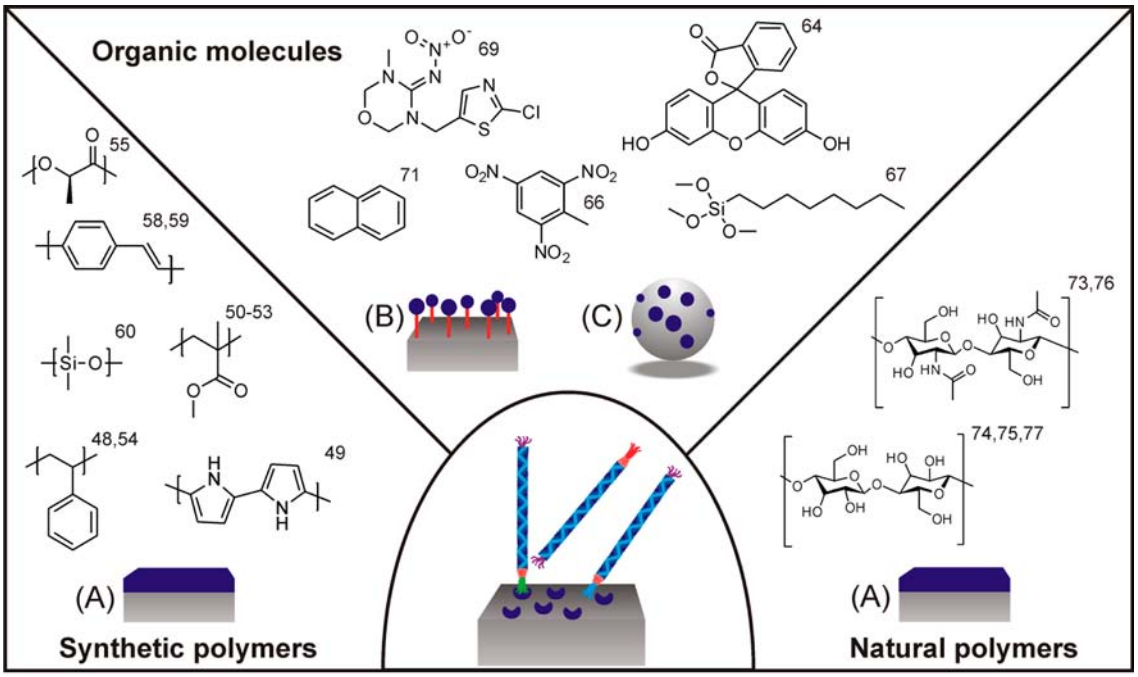

Figure 2. Phage display is a powerful tool allowing the identification of peptide ligands that are able to bind selectively to a myriad of substrates, including synthetic polymers, small organic molecules, and natural polymers. While thin, spin-coated films are most frequently used to carry out phage display on synthetic and natural polymer substrates (A), organic molecules are covalently immobilized either on flat surfaces (B) or on spherical beads (C). The numbers next to the chemical structures in this figure refer to the references that have reported peptide binders to these substrates.

increasingly used for the identification of small peptide sequences that bind to solid substrates,[27,28] such as metals,[29,30] ceramics,[31−34] carbon-based materials,[35,36] synthetic or natural polymers,[37] as well as small organic molecules. These small peptides are attractive building blocks to functionalize or to modulate the properties of surfaces and interfaces. These peptides can be conjugated with biomolecules, synthetic polymers, or nanoparticles to allow selective functionalization of the substrate of interest. This is attractive, for example, for the development of sensors,[38−40] templates for mineralization,[41−44] as well as electronic devices.[34,45−47]

The aim of this article is to present the state-of-the-art and to highlight the potential of phage display to identify peptide sequences that are able to bind with high affinity and selectivity to "soft matter" surfaces. While the use of the phage display technique to identify peptide binders to "hard" inorganic substrates has been reviewed in several other articles,[27,28,41] this contribution specifically focuses on soft matter substrates. This article consists of two main parts. The first part will summarize for three main classes of soft material substrates, viz., synthetic polymers, small organic molecules, and natural polymers, the different peptide ligands identified by phage display that have been reported in the literature (Figure 2). The second part will discuss the various techniques that have been used to characterize the affinity of these peptide ligands.

IDENTIFICATION OF SOFT MATTER BINDING PEPTIDE LIGANDS

This section will successively present and discuss peptide ligands that have been identified via phage display and which bind selectively to synthetic polymers, small organic molecules, and natural polymer substrates. First, peptide ligands that bind to synthetic polymer substrates will be discussed, followed by small organic molecule binding peptides and peptides that bind to natural polymer substrates. The order in which these substrates are presented also reflects the historical evolution of the phage display technique. The first examples of the use of the phage display technique for synthetic polymer substrates go back to 1995.[48] Shortly after that, the first reports of the application of phage display to small organic molecule substrates were published, and over the past decade this technique has also found application for the identification of small peptides that bind to natural polymer substrates.

Peptide Binders to Synthetic Polymers. From the three classes of substrates considered in this section, most of the work that has been reported has involved the use of phage display to identify peptide ligands that bind to synthetic polymer substrates. Table 1 presents a comprehensive list of the different substrates that have been investigated. For each substrate, Table 1 includes the identified sequences, the selection conditions, as well as the techniques that were used to assess the selectivity and affinity of the peptide ligands.

The first reports, which pointed out the potential of phage display to identify peptide sequences that can selectively bind to synthetic polymers, date back to 1995 when Adey and co-workers reported sequences that bind to polystyrene (PS) and poly(vinyl chloride) (PVC) surfaces.[48] In the same year, Caparon and co-workers, while searching to identify streptavidin binding ligands, reported a group of "nuisance peptides" of the consensus sequence WHWWXW, which were speculated to originate from phages that may have bound to the plastic substrates.[62] Since then, a number of other peptide sequences rich in tryptophan, such as WXW or WXXW, as well as many others, have been reported,[63] the selection of which was usually attributed to background binding to the plastic materials used in selection experiments.

Sanghvi et al. have used phage display to identify peptide binders to a conducting chlorine-doped polypyrrole (PPyCl)

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Table 1. Overview of Synthetic Polymers That Have Been Used as Substrates in Phage Display Affinity Selection[a]

target(s)	identified peptide with the strongest affinity	selection conditions	characterization technique(s) / K_a (M^{-1}) (if provided)	comments
PS and PVC	SSRLAYDHYFPSWRSYIF WMQSWYYHWGGGETFPIR	**Incubation:** 10^{11} phages in PBST 0.1% + 0.1% BSA (pH 7.4) to blocked PS or PVC microtiter plates **Washing:** 5 x with PBS **Elution:** 50 mM Glycine.HCl (pH 2.0) **Number of rounds:** 1	Phage ELISA	Enrichment of a particular sequence was not observed. Instead, a significant increase in the abundance of Tyr and Trp in the selected sequences was observed.[48]
PPyCl	THRTSTLDYFVI	**Incubation:** Phages in TBST 0.1% (pH 7.5) to thin films of PPyCl **Washing:** 3 x with TBST 0.1% **Elution:** Glycine.HCl (pH 2.2) **Number of rounds:** 5	Phage ELISA Peptide ELISA AFM (peptide) Fluorescamine assay (peptide)	Identified sequence conjugated with GRGDS was demonstrated to promote cell adhesion to PPyCl films.[49]
it-PMMA	ELWRPTR QLQKYPS ARPHLSF TLHLSPA	**Incubation:** 4 x 10^{11} phages/mL in TBST 0.1% spin-coated it-PMMA films **Washing:** 5 x with TBST 0.1% **Elution:** 500 mM Glycine.HCl (pH 2.2) **Number of rounds:** 5	Phage ELISA ((5.4 – 10) x 10^{10}) QCM (peptide) SPR (peptide) ((4.2 – 28.0) x 10^5)	A quantitative phage ELISA protocol allowing the determination of the apparent binding strengths of the phages to polymeric thin films was established.[50] The influence of the tacticity of the polymer substrate towards the binding of selected clones was demonstrated.[50-52]
st-PMMA	HKPDANR HPVHPHR LPPWQRQ HPRWHTP	**Incubation:** 4 x 10^{11} phages/mL in TBST 0.1% to spin-coated st-PMMA films **Washing:** 5 x with TBST 0.1% **Elution:** 500 mM Glycine.HCl (pH 2.2) **Number of rounds:** 5	Phage ELISA ((2.5 – 4.2) x 10^{10})	The influence of the tacticity of the polymer substrate towards the binding of selected clones was demonstrated.[52,53]
st-PS	YLTMPTP FSWEAFA GETRAPL GETQCAA	**Incubation:** 3.3 x 10^{11} phages/mL in TBST 0.1% to st-PS films **Washing:** 5 x with TBST 0.1% **Elution:** 500 mM Glycine.HCl (pH 2.2) **Number of rounds:** 4	Phage ELISA ((1.4 – 2.0) x 10^{11})	The influence of the tacticity of the polymer substrate towards the binding of selected clones was demonstrated.[54]
α-PLLA	QLMHDYR LSQSLTR RACSKDA ANTLRSP	**Incubation:** 4.0 x 10^{11} phages/mL in TBST 0.1% to α-PLLA films **Washing:** 3 x with TBS **Elution:** 100 mM Glycine.HCl (pH 2.2) + 1 mg/mL BSA **Number of rounds:** 4	Phage ELISA ((4.8 – 6.7) x 10^9) SPR (peptide) (6.1 x 10^4)	Identified peptides were demonstrated to be able to distinguish different crystal polymorphs of PLLA.[55]
Azobenzene-containing poly(MA)'s	WPTPPNP SPSWLIQ WHTLPNA MHQGSNT	**Incubation:** 3.3 x 10^{11} phages/mL in TBST 0.1% to copolymer films **Washing:** 5 x with TBST 0.1% (pH 7.5) **Elution:** 500 mM Glycine.HCl (pH 2.2) **Number of rounds:** 4	Phage ELISA ((2.0 – 3.5) x 10^{10}) SPR (peptide) (1.4 x 10^6) QCM (peptide)	Identified peptides were able to more selectively bind to films having greater abundance of azobenzene groups having a *cis* conformation compared to the *trans* conformation.[56,57]
mpsPPV	HNAYWHWPPSMT HWDPFSLSAYFP	**Incubation:** 3.3 x 10^{11} phages/mL in TBST 0.1% to deposited polymer films **Washing:** 1 x with TBST 0.1% and 4 times with PBS **Elution:** 100 mM Glycine.HCl (pH 2.2) **Number of rounds:** 5	SPR (peptide) (1.3 x 10^5)	The immobilization of the selected peptide enhanced the fluorescence intensity of the substrate.[58]
PPV	HTDWRLGTWHHS (hyperbranched PPV binder) ELWSIDTSAHRK (linear PPV binder)	**Incubation:** 3.3 x 10^{11} phages/mL in TBST 0.1% to deposited polymer films **Washing:** 1 x with TBST 0.1% and 4 times with PBS **Elution:** 100 mM Glycine.HCl (pH 2.2) + 1 mg/mL BSA **Number of rounds:** 5	SPR (peptide) ((7.7 – 77.0) x 10^4)	The utilization of hyperbranched or linear PPV led to the identification of entirely different PPV-binding peptides, indicating that the influence of the architecture of the polymer substrate towards the binding of the selected clones were demonstrated.[59]
PDMS	LSNNNLR LQPRANF	**Incubation:** Phages in TBST 0.1 to 0.5% with PDMS slab **Washing:** Several times with TBST **Elution:** Glycine.HCl (pH 2.2) **Number of rounds:** 3	Fluorescence intentsity (phage – peptide)	The selected peptides selectively bounded to non-oxidized substrate, compared to plasma oxidized PDMS or PDMS containing microfluidic channels.[60]
Epoxy resin	TLHPAAD NERALTL SHSGYFS	**Incubation:** Phages in TBST 0.1 to 0.5% with epoxy slab **Washing:** Several times with TBST **Elution:** Glycine.HCl (pH 2.2) **Number of rounds:** 3	Fluorescence intentsity (phage-peptide)	[61]

[a]For each substrate, the identified sequences as well as the selection conditions and characterization techniques that were used are listed. (α-PLLA) α-poly(L-lactide); (AFM) atomic force microscopy; (BSA) bovine serum albumin; (CHAPS) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; (ELISA) enzyme-linked immunosorbent assay; (it) isotactic; (MA) methacrylate; (mpsPPV) poly(2-methoxy-5-propyloxysulfonate-1,4-phenylenevinylene); (PBS) phosphate-buffered saline; (PC) poly(carbonate); (PDMS) poly(dimethylsiloxane); (PMMA) poly(methyl methacrylate); (PPV) poly(phenylenevinylene); (PpyCl) chlorine-doped poly(pyrrole); (PS) polystyrene; (PVC) poly(vinyl chloride); (QCM) quartz crystal microbalance; (SPR) surface plasmon resonance; (st) syndiotactic; (TBS) Tris-buffered saline.

surface.[49] Fusion constructs of these affinity tags with the cell-adhesive GRGDS sequence were used to promote cell adhesion onto the PPyCl substrates (Figure 3).

Serizawa and co-workers have utilized phage display to identify peptide ligands for a broad variety of polymer substrates. Over the course of the past decade, they have identified peptide binders to poly(methyl methacrylate) (PMMA),[50−53] PS,[54] α-poly(L-lactide) (α-PLLA),[55] azobenzene-containing polymethacrylates,[56,57] poly(2-methoxy-5-propyloxysulfonate-1,4-phenylenevinylene) (mpsPPV),[58] and poly(phenylenevinylene) (PPV).[59] These experiments demonstrate that the tacticity, architecture, or crystallinity of the polymer or even the conformation of polymer side chain functional groups can lead to the enrichment of different peptides and as a consequence the identification of selective peptide ligands. For instance, Serizawa and co-workers have shown that phage display carried out using atactic or syndiotactic PMMA led to the isolation of entirely different peptide sequences.[50−53] The apparent binding constants of the highest affinity peptides toward one of these surfaces were reported to be 6−10 times higher as compared to

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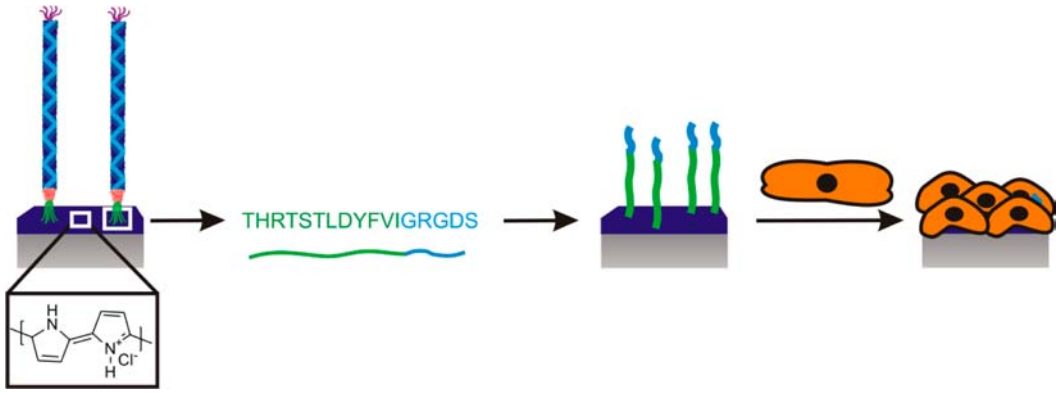

Figure 3. Incorporation of the RGD motif to the PPyCl binding peptide identified by phage display enhanced cell adhesion on PPyCl substrates as demonstrated by Sanghvi and co-workers.[49]

Table 2. Overview of Organic Molecules That Have Been Used as Substrates in Phage Display Affinity Selection[a]

target(s)	identified peptide with the strongest affinity	selection conditions	characterization technique(s) / K_a (M^{-1}) (if provided)	comments
Texas Red, Oregon Green 514 and Fluorescein	KHVQYWTQMFYS KPVQYWTQMFYT (Texas red binders) HGWDYYWDWTAW HEWEYYWDWTAW (Oregon Green 514 Binders) YPNDFEWWEYYF YPNEFDWWDYYY (Fluorescein Binders)	**Incubation:** 6.0 X 10^{10} phages/mL in TBST 0.1% (pH 7.4) to blocked fluorophore dye carrier beads **Washing:** with TBST 0.1% **Elution:** 200 mM Glycine.HCl (pH 2.2) + 1 mg/mL BSA **Number of rounds:** 3 or 4 before the random mutagenesis. Another round was carried after the mutagenesis of the binding sequences was performed.	Scatchard Plot based on phage titration (1.1 x 10^{10}) Scatchard Plot based on fluorescence intensity (peptide) (6.0 x 10^5)	Random mutagenesis of the originally identified clones led to the isolation of newer clones having 2.7 to 6.5-fold greater affinity. The K_d values of the isolated clones were in low nanomolar range.[64]
2, 4, 6-Trinitrobenzene	WHRTPSTLWGVI NHRIWESFWPSA	**Incubation:** 4.0 x 10^{10} phages in artificial seawater to TNB coated wells **Washing:** 10 x with artificial seawater containing 0.1% Tween 20 **Elution:** 100 mM Glycine.HCl (pH 2.2) + 1 mg/mL BSA **Number of rounds:** 3	Phage ELISA Fluorescence intensity assay via a flow sensor	The identified clones showed significant selectivity towards the detection of TNB in seawater compared to PBS buffer.[65]
Trinitrotoluene and 2, 4-Dinitrotoluene	WHWQRPLMPVSI (TNT binder) HPNFSKYILHQR (DNT binder)	**Incubation:** 3.9 x 10^9 independent phages in TBST 0.1% to TNT and DNT crystals **Washing:** 10 x with TBST 0.1% **Elution:** 200 mM Glycine.HCl (pH 2.2) + 1 mg/mL BSA **Number of rounds:** 3 or 4	Fluorescence intensity (peptide) ITC (1.4 x 10^7)	Identified peptides immobilized onto a gold substrate via an oligoethylene glycol linker were demonstrated to act as a selective biosensor for the detection of volatile organic compounds.[66]
Octyltrimethoxysilane	SILPYPY HAIYPRH TTYSRFP QILAFNS	**Incubation:** 7.0 x 10^{11} phages in TBST with increasing amounts of Tween 20 with respect to number of rounds to OTMS-functionalized silicon substrate **Elution:** 200 mM Glycine.HCl (pH 2.2) **Number of rounds:** 6	Fluorescence intensity	Patterned OTMS surfaces prepared via microcontact printing showed a patterned fluorescence signal upon selective immobilization of the binding clone.[67]
Polychlorinated biphenyls	DSNKLSPE LSFAADRT	**Incubation:** 1.7 x 10^{10} phages/mL in PBS + 5% milk powder to PCB-coated magnetic –COOH conjugated beads **Washing:** 6 x with PBST 0.05% **Elution:** 200 mM Glycine.HCl (pH 2.2) + 1 mg/mL BSA and 0.1 mg/mL phenol red **Number of rounds:** 4	SPR ((1.4-1.7) x 10^4)	The identified phages selectivity recognized different PCB's.[68]
Thiamethoxam	ASTLPKA HTPPVTS ALTPTPP QPQVPDA	**Incubation:** 1 x 10^{10} phages/mL in TBST to blocked TMX crystals **Washing:** Several times with TBST 0.1% **Elution:** 200 mM Glycine.HCl (pH 2.2) + 1 M BSA **Number of rounds:** 3	AFM	[69]
2, 4-dinitrotoluene	DYAMSAISYDGGSKR-DAVTPTSTTTSYYNAM-DVTGSSSNIGSNSVT-DDNQAAWDASLSG	**Incubation:** 1.5 x 10^{13} scFv-containing phages/mL in TBS or TBST 0.2% to DNT-immobilized magnetic beads **Washing:** 5 x with TBS or TBST 0.2% **Elution:** 0.1 mL triethylamine **Number of rounds:** 4	QCM Protein ELISA	[70]
Naphthalene	HFTFPQQQPPRP TLTDPAYRPHRY KLHISKDHIYPT KNVDHMATFARG	**Incubation:** 8.0 x 10^{11} phages/mL in TBS to naphthalene immobilized SAM's **Washing:** 5 x with TBST 0.1% **Elution:** 200 mM Glycine.HCl (pH 2.2) **Number of rounds:** 5	SPR (peptide) (1.4 x 10^5)	The peptide HFTFPQQQPPRP showed significant selectivity towards the recognition of naphthalene compared to benzene, anthracene and pyrene. This peptide was also demonstrated to show strong affinity towards free naphthalene in solution.[71]

[a]For each substrate, the identified sequences as well as the selection conditions and characterization techniques that were used are listed. (AFM) atomic force microscopy; (BSA) bovine serum albumin; (ELISA) enzyme-linked immunosorbent assay; (ITC) isothermal titration calorimetry; (PBS) phosphate-buffered saline; (QCM) quartz crystal microbalance; (scFv) single-chain variable fragment; (SPR) surface plasmon resonance; (TBS) tris-buffered saline.

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the other surfaces. In another study, Serizawa et al. identified a crystalline α-PLLA binding peptide (QLMHDYR), which was demonstrated to have a lower affinity toward β-PLLA and amorphous PLLA as compared to α-PLLA.[55] The same group has also discovered peptides that can selectively recognize poly(methacrylates) containing cis-azobenzene groups over the ones having trans-azobenzenes.[56] In a final example, it was shown that phage display can be used to identify peptide binders that are selective to either linear or hyperbranched PPV derivatives.[59]

More recently, phage display has been used not just to identify peptide ligands that solely mediate surface immobilization, but which can also be used to generate surfaces with tunable properties. For instance, Ejima et al. have identified binders to water-soluble poly(2-methoxy-5-propyloxysulfonate-1,4-phenylenevinylene) (mpsPPV).[58] Binding of these peptides to mpsPPV in aqueous solution was found to result in an increase in fluorescence intensity. The enhancement of the increase in fluorescence intensity could be modulated by tuning the amount of the peptide. Addition of thermolysin, an enzyme that degrades the peptide, was demonstrated to allow the fluorescence to "switch off" again. It was claimed that this modulation of the fluorescence intensity was due to the peptide-mediated dispersion/aggregation of the polymer in aqueous solution. In another example, Chen and co-workers demonstrated that converting the conformation of azobenzene side chain functional groups from cis to trans by UV-irradation resulted in detachment of peptides that were identified by phage display to selectively bind to PMMA substrates that present the azobenzene groups in the cis conformation.[56,57] Swaminathan and co-workers have identified epoxy resin and poly(dimethylsiloxane) (PDMS) binding peptides in two different studies, and reported the increased localization of the bound peptides in specific regions on the respective microstructured substrates.[60,61]

Peptide Binders to Organic Molecules. Phage display has also been used to identify peptides that can selectively bind to small organic molecules. These peptide ligands are of interest for a variety of purposes, including (i) the modulation of the fluorescence intensity of fluorophores and (ii) the selective detection of explosives and polycyclic hydrocarbons, as well as for (iii) peptide-based patterning of substrates. Table 2 summarizes the different small organic molecules that have been used as targets in phage display and lists the corresponding peptide ligands that have been identified. While the synthetic polymers that were discussed in the previous section are very convenient, as they can be directly used in the form of films as substrates for phage display, this can be more challenging for small organic molecules. In some cases small organic molecules can be used directly in form of solid crystals[66,69] as substrates for phage display. In many other cases, however, the molecules are immobilized on spherical beads[64,68,70] or on planar substrates.[67,71]

One of the first examples that reported the use of phage display to identify peptide-based binding ligands to small, organic molecules was the work by Rozinov and Nolan who used a variety of fluorophores such as Texas Red, Oregon Green 514, and fluorescein as substrates.[64] They demonstrated that site-directed mutagenesis of the identified phage clones followed by their further selection toward the respective targets led to the isolation of new sequences having higher affinities than the originally selected ones. The highly affine interactions between these sequences and the fluorophores, particularly with Texas Red, were shown to be of use for the modulation of excitation and emission spectra of these molecules.

Various groups have demonstrated the feasibility of peptides identified by phage display for the selective detection of molecules that are frequently used in explosives such as 1,3,5-trinitrobenzene (TNB), 2,4-dinitrotoluene (DNT), and 2,4,6-trinitrotoluene (TNT).[65,66,70] Goldman and co-workers have identified peptides that selectively recognize TNT in seawater, but not in a standard phosphate-buffered saline (PBS) buffer using phage display.[65] Furthermore, they have integrated phages that are labeled with a fluorescence dye into a continuous flow sensor, which allowed the detection of TNT. Jaworski et al. have identified sequences that selectively recognize TNT and DNT.[66] Covalent incorporation of the identified peptides to a gold chip via an oligo(ethylene glycol) linker allowed them to selectively sense these explosives in the gas phase via headspace analysis (Figure 4). In another study, Na and co-workers used a library of single-chain variable fragments of antibodies (scFv) that were presented on a M13 bacteriophage to identify DNT binders.[70]

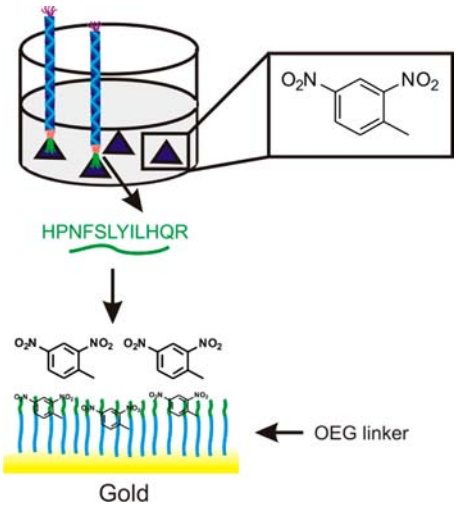

Figure 4. Detection of gaseous 2,4-dinitrotoluene (DNT) using a DNT-binding peptide coupled to a gold surface via a flexible oligoethylene glycol (OEG) linker.[66]

In addition to the detection of explosives, phage display has also been utilized for the identification of organic molecule binding peptides that can be of use for potential other sensing applications or surface patterning. Cui and co-workers have identified octyltrimethoxysilane (OTMS) binders and have prepared patterned OTMS-containing silicon surfaces via microcontact printing or photolithography.[67] Fluorescence measurements demonstrated the localization of the OTMS binding phage in the microdomains that contain surface-immobilized OTMS. Van Dorst et al. have isolated two different sets of peptides that can selectively recognize two different polychlorinated biphenyls (PCBs), which are known to be carcinogenic and can accumulate in tissue.[68,72] Cho et al. have identified binders to thiamethoxam (TMX) organic crystals and proposed that the surface manipulation of TMX crystals with the identified phages may prevent the sedimentation of their colloidal suspensions.[69] In a final example, Serizawa and co-workers have identified a naphthalene binding peptide (HFTFPQQQPPRP), which does not show any specificity toward other simple aromatic hydrocarbons, including anthracene and pyrene.[71]

Peptide Binders to Natural Polymers. The last section of the first part of this article summarizes the work that has been

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Table 3. Overview of Natural Polymers That Have Been Used as Substrates in Phage Display Affinity Selection[a]

target(s))	identified peptide with the strongest affinity	selection conditions	characterization technique(s) / K_a (M^{-1}) (if provided)	comments
Chitin	CSRTTRTRC	**Incubation:** 10^{10} phages in PBS:EtOH 1:1 to a packed chitin column **Washing:** 1 x PBS:EtOH 1:1 **Elution:** PBS:EtOH 1:1 + 50 mg/mL *N*-acetylglucosamine **Number of rounds:** 5	SPR (Peptide) (9.1×10^3)	Identified clone bound to chitin only after intramolecular disulfide-bond formation via oxidation.[73]
Cellulose	HAIYPRH SHTLSAK TQMTSPR YAGPYQH	**Incubation:** 5 x 10^9 phages/mL in TBST 0.1% to dispersed cellulose microcrystals **Washing:** 5 x with TBST 0.1% or 5 x with TBST 0.1% + varying amounts of cellobiose **Elution:** 500 mM Glycine.HCl (pH 2.2) or TBST 0.1% + 100 mM cellobiose **Number of rounds:** 5	Phage ELISA ($(4.6 - 5.2) \times 10^{10}$)	The isolated binding clones can be subdivided into two different groups, which were speculated to show affinity towards crystalline and amorphous domains, respectively.[74]
Carbon Black and Cellulose	FHENWPS MPPPLMQ WHLSWSPVPLPT (Carbon black binders) THKTSTQRRLLAA KCCYVNVGSVFS (Cellulose binders)	**Incubation:** 2 x 10^{11} phages in TBST 0.1% containing 1 mg/mL BSA to solid carbon black or cellulose samples **Washing:** 6 x with TBST 0.1% **Elution:** 200 mM Glycine.HCl (pH 2.2) + 1 mg/mL BSA **Number of rounds:** 4	Phage ELISA GST activity assay Peptide ELISA	The preparation of a construct comprised of a carbon black and cellulose binder connected with a linking sequence was demonstrated to disperse the carbon black particles and subsequently attach them to paper surfaces.[75]
Chitin	EGKGVEAVGDGR GEVGEQEKARVG	**Incubation:** 10^{10} phages in PBST 0.1% to chitin-immobilized wells **Washing:** 5 x with PBST 0.1% **Elution:** 50 mM Glycine.HCl (pH 2.0) **Number of rounds:** 3	Phage ELISA Peptide ELISA	The specific interaction between the identified peptide and the clone allowed the assisted formation of a biomacromolecular network.[76]
CNWs	WHWRAWY WHWTYYW	**Incubation:** 2 x10^{11} phages/mL in TBST 0.1% to cellulose-immobilized dishes **Washing:** 10 x with TBST with increased concentration of Tween 20 w.r.t. number of rounds **Elution:** Not indicated **Number of rounds:** 3	Phage ELISA ($(1.0\text{-}10.0) \times 10^{10}$) ITC (Peptide) ($1.1 \times 10^5$) Fluorescence quenching (Peptide) ($(5.0 - 10.0) \times 10^4$) NMR (Peptide)	NMR and molecular modelling techniques allowed a more precise understanding of the interaction between the target and the substrate.[77]

[a]For each substrate, the identified sequences as well as the selection conditions and characterization techniques that were used are listed. (BSA) bovine serum albumin; (CNWs) cellulose nanowhiskers; (ELISA) enzyme-linked immunosorbent assay; (GST) gluthathione *s*-transferase; (ITC) isothermal titration calorimetry; (NMR) nuclear magnetic resonance; (PBS) phosphate-buffered saline; (TBS) tris-buffered saline; (SPR) surface plasmon resonance.

done using phage display for the identification of peptide ligands that selectively bind to natural polymers, especially to polysaccharides such as cellulose derivatives and chitin. These peptide binders are summarized in Table 3.

Fukusaki and co-workers have utilized a random phage library displaying cyclic peptides that are formed via disulfide bond formation of the flanking cysteine residues in order to identify chitin binders.[73] The identified peptide (CSRTTRTRC) was found to recognize chitin only in its cyclic conformation, whereas the linear derivative that was produced via reduction of the cyclic peptide did not show any affinity toward the target. Later, Khousab et al. identified linear chitin binders and reported the formation of porous chitin−peptide networks upon addition of the peptide to a mixture containing 0.5% colloidal chitin in water.[76] These biocompatible networks were proposed as possible scaffold materials for tissue engineering.

A variety of cellulose derivatives has also been used as target substrates for the identification of peptide binding ligands. Serizawa and co-workers have identified a variety of heptapeptides using microcrystalline cellulose as the substrate. These ligands were subdivided in two different groups. The first group was characterized by the presence of an −OH containing amino acid (S, T, Y) in the first and fifth position and a cationic amino acid (H, R, K) in the seventh position, while the second group of peptides was primarily composed of aliphatic residues.[74] The origin of this difference between the two groups was proposed to arise either from the affinity of different groups separately to crystalline and amorphous domains or from different modes of peptide−crystalline cellulose interactions. Later, Guo and co-workers identified a crystalline cellulose nanowhisker binding peptide that was predominantly composed of aromatic residues (WHWTYYW).[77] The binding of this peptide to cellulose nanowhiskers (CNW) was studied using a variety of techniques, such as phage ELISA, isothermal titration calorimetry (ITC), and fluorescence quenching as well as nuclear magnetic resonance (NMR) and molecular modeling, which allowed a better understanding of the role of the individual amino acids. These studies indicated that the peptide ligand adopts a bent conformation, which allows the tyrosine residue in the fifth position to form CH/π stacking interactions and a hydrogen bond with the cellulose glucose ring. In a final example, Qi and co-workers have identified binders to fibrous cellulose as well as to carbon black by using phage display.[75] A fusion construct that incorporated both the cellulose and carbon black binding sequence was able to disperse carbon black particles and allowed to subsequently attach these to paper surfaces, which are primarily composed of fibrous cellulose (Figure 5).

CHARACTERIZATION OF SOFT MATTER BINDING PEPTIDES IDENTIFIED VIA PHAGE DISPLAY

The second part of this Review will present and discuss the techniques that are most commonly used to characterize the affinity of peptide ligands, which have been identified via phage display, toward soft matter surfaces. This part is organized in five sections, which will successively discuss ELISA and fluorescent based techniques, surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), atomic force microscopy (AFM), and quartz crystal microbalance (QCM). These techniques are illustrated in Figure 6. Following a brief introduction to each technique, examples describing the use of these techniques for the determination of the binding strengths of soft matter binding peptide ligands will be presented.

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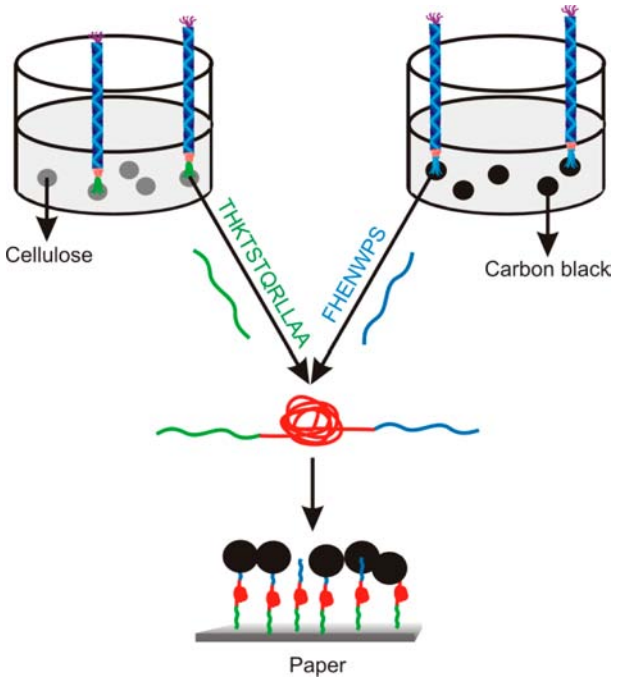

Figure 5. Triblock fusion construct composed of a central, rigid, and hydrophilic interdomain linker (PTPTPTPTPTPTPTPTPTPTPTPT) flanked by cellulose and carbon black binding peptide ligands allows the deposition of carbon black particles onto paper surfaces.[75]

ELISA and Fluorescence-Based Techniques. Phage ELISA is a robust technique that is frequently used to obtain insight into the binding affinity of phage clones (Figure 6A). A typical direct phage ELISA experiment consists of three steps: First, a phage solution is incubated with the substrate of interest and the unbound phages are washed with a buffer that usually contains small amounts of surfactant. In the next step, a primary antibody−enzyme conjugate, such as, for example, antiM13−horseradish peroxidase (HRP), which binds with the substrate bound phages, is added. Following the removal of the excess primary antibody−enzyme conjugate by repeating the washing step several times, an HRP substrate that yields a colorimetric response upon reaction with HRP is introduced and the intensity of this signal is measured. As it does not require the isolation of individual phage clones, phage ELISA can even be used as a monitoring step between successive affinity selection cycles. However, to calculate absolute binding affinities of the identified clones, a substrate concentration is required, which is ambiguous for solid substrates. As a consequence, phage ELISA is predominantly used to obtain qualitative information by comparing the ELISA readout from an experiment with a selected phage clone with that from an experiment with the starting, random phage library at a specific concentration with a predetermined amount (mass) of the substate.[49,65,75,76] This simple experiment provides the relative ratio of the deposition of different phage clones at a particular phage concentration; however, it does not yield absolute quantitative information about the affinity of the phage.

In order to circumvent the uncertainty in the substrate concentration, Serizawa and co-workers have developed a method that allows the determination of the apparent binding affinities of phages to solid surfaces by phage ELISA.[50] Their method is based on the assumption that the surface deposition of the phages follows a Langmuir-type adsorption and the ratio of the ELISA signals obtained from the assay with the individual phage clones and that with the library on an identical substrate as a function of phage concentration would yield an adsorption isotherm. This isotherm, then, can be utilized to calculate the apparent affinities of individual phage clones. This method is based on the assumption that, in a given set of experiments at each phage concentration, both for the selected clone as well as for the random library, all the substrates (at an equivalent surface area), present the same surface concentration of binding sites, which may not be applicable to substrates having high surface roughness, which is a general limitation of a Langmuir-type adsorption model. Serizawa and co-workers have successfully used this approach to calculate the apparent affinities of selected phages to thin, spin-coated films of a broad range of synthetic and natural polymers, including PMMA, PS, PLLA, PPVs, and cellulose, among others.[50,52,54−56,74]

The calculated binding affinities obtained by phage ELISA are typically in the range of 10^{10}−10^{11} M^{-1}, which is ∼2−5 orders of magnitude greater than the calculated binding constants of the corresponding peptides to solid surfaces. This apparent discrepancy between the phage and peptide affinities is due to multivalency effects. Whereas each peptide can only act as a single binder, the most frequently used M13 phage displays multiple copies of each peptide, which allows for multivalent interactions. From experiments that have compared single peptides with multivalent constructs, it is known that multivalency can significantly increase the binding strength.[16,78,79] From this point, it can be speculated that the binding strengths obtained from phage ELISA experiments are an overestimate. It is also worth noting that the calculated binding affinities of identified phage clones using phage ELISA experiments depend on the extent of nonspecific interactions between the random phage library and the surfaces and, therefore, are apparent values. This is because the affinity of an individual phage clone is calculated from the ratio between the adsorption isotherm of that particular phage clone to the random phage library in phage ELISA.

The binding of phages and peptides has also been assessed using fluorescently labeled primary or secondary antibodies. These allow the direct observation and analysis of the substrate bound phages/peptides by fluorescence microscopy techniques.[60,61,65−67] Sanghvi and co-workers have used streptavidin−fluorescein isothiocyanate (FITC) either as a primary or secondary antibody, respectively, to compare the extent of binding of different phage clones or biotinylated variants of the peptide that was previously isolated as the strongest binder to PPyCl.[49] Similarly, Jaworski and co-workers have used Atto-425-streptavidin to detect the extent of binding of biotinylated DNT-binding peptide and its alanine-substituted derivatives.[66] These fluorometric assays were not performed to determine the binding strengths of the individual clones, but rather used to qualitatively assess the amount of phage/peptide that was bound to the desired substrate. Sanghvi and co-workers directly determined the amount of bound peptide to PPyCl surfaces with fluorescamine, which can rapidly react with N-terminal amine groups to yield a strong fluorescent signal.[49] Guo et al. used a method previously designed by Yoon and co-workers[80] to calculate the binding strength of the CNW-binding peptide sequence WHWTYYW to cello-oligosaccharides. This method was based on the quenching of the intrinsic fluorescence of the tryptophan and tyrosine residues upon binding of the peptide ligands to the substrates. The binding affinity of this peptide was found to be on the order of 10^5 M^{-1}, which was approximately 5

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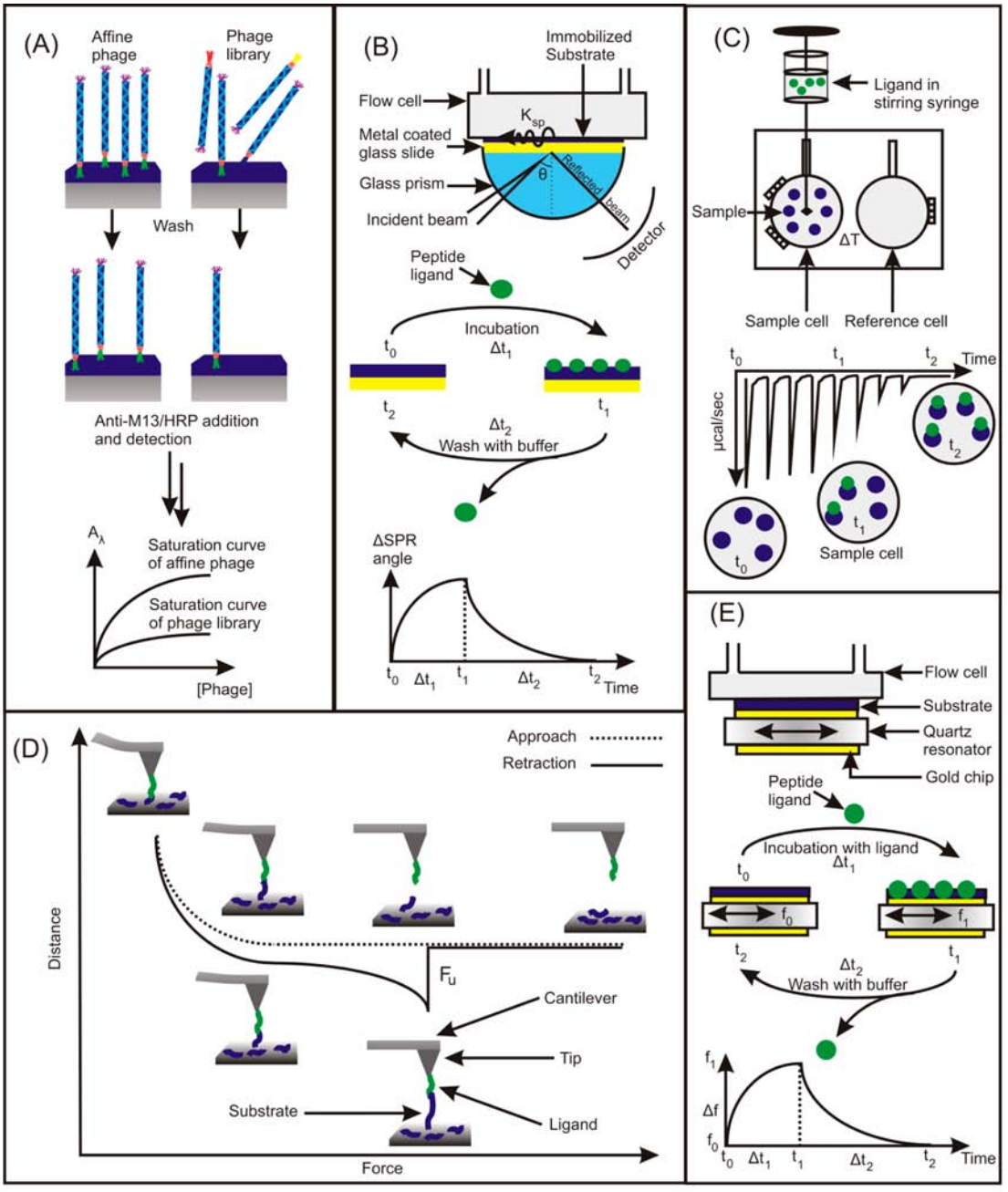

Figure 6. Schematic overview of the most frequently utilized techniques to assess the strength of phage−soft matter interactions. (A) Phage ELISA, (B) surface plasmon resonance (SPR), (C) isothermal titration calorimetry (ITC), (D) atomic force microscopy (AFM), and (E) quartz crystal microbalance (QCM). In a typical SPR experiment (B), the substrate is incubated in a solution containing the ligand at t_0 until all of the substrate binding sites are populated with the ligand (t_1). At this point, the SPR angle no longer changes as a function of time. Next, the ligand bound substrate is washed with a buffer until the SPR angle returns to its original value (t_2). In a typical ITC experiment (C), the concentration of the ligand in the sample cell is zero at t_0. Titration of the sample with the ligand leads to the release or absorption of heat with each titration; the amount of heat gradually decreases due to the decrease in the number of possible ligand sample interactions (t_1) and eventually reaches zero (t_2), which indicates the saturation of substrate binding sites. Similar to the SPR experiment, in QCM (E), the substrate immobilized on the QCM chip is incubated with the ligand at t_0 until saturation of the substrate binding sites (t_1). At this point, the resonance frequency of the QCM chip stays constant as a function of time. Next, incubation of the chip with the buffer solution leads to the detachment of the ligand from the substrate, and complete detachment occurs at the point that the resonance frequency of the QCM chip returns to its initial value (t_2).

orders of magnitude smaller than the affinity of the phage-containing this peptide.

Surface Plasmon Resonance. Surface plasmon resonance (SPR) is a powerful optical technique that is frequently utilized for the real-time determination of the affinities of ligands in solution to surface-immobilized substrates. As it allows the analysis of thin polymer coatings deposited onto silver or gold metallic films, it is a very valuable tool for the investigation of degradation,[81] adsorption,[82] or hydration[83] processes of synthetic or natural polymeric films, as well as for the characterization of ligands that selectively bind to these substrates.[84] A major advantage of SPR is that it does not

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require chemical modification of the ligand or the substrate. As the measurement does not rely on the signal of a reporting molecule (i.e., primary or secondary antibody) it represents a direct method for the determination of binding affinities.

Figure 6B schematically outlines the SPR analysis of surface binding peptides and phages. Briefly, in the Kretschmann configuration, which is based on the principle of attenuated total reflectance (ATR), the instrument is composed of a glass/metal/dielectric interface, where the thickness of the metal layer is less than the wavelength of the incident light. While gold and silver are the most suitable thin metallic films, the dielectric medium can be, e.g., a thin polymer film in an aqueous solution. When a p-polarized incident beam in the near-IR range undergoes total internal reflection (TIR) at the glass/metal interface, it generates an evanescent wave parallel to the metal/dielectric interface that can resonantly excite a surface plasmon at a specific angle of incidence of the incoming beam.[85,86] Since the excitation of the surface plasmon depends on the refractive index of the medium in close proximity to the metal/dielectric interface, any event that results in a change in the refractive index of this medium, such as ligand-binding or dissociation, leads to a change in the angle of incidence required to excite the surface plasmons.[87] Therefore, an SPR coupled with a flow cell can first allow the determination of the rate constant of association (k_{ass}) by using a constant flow of a solution containing a predetermined concentration of the ligand until the change in the SPR angle becomes zero over time, which is an indication of the surface saturation. Next, the rate constant of dissociation (k_{diss}) can be calculated by passing a blank solution over the saturated substrate until the SPR angle reaches the initial value, which indicates complete dissociation. From the determined k_{ass} and k_{diss} values, the binding constant can be calculated ($K_a = k_{ass}/k_{diss}$).[84,88]

In an early example, Fukusaki et al. have immobilized a chitin-binding peptide onto a gold chip and determined a binding affinity of 9×10^3 M^{-1} to a soluble derivative of chitin, chitotriose, which was passed through the flow cell.[73] It is important to note that while the affinity selection was carried out using chito-oligoagarose as the substrate, chitotriose was utilized in the SPR measurements, which may result in lower binding affinities measured via SPR as slight differences in the properties of polymeric substrates were reported to significantly affect the strength of the phage/peptide−substrate interactions.[50,52,55,59] Matsuno and co-workers have prepared thin films of α, β, and atactic (at) PLLA on gold substrates and compared the binding affinity of a β-PLLA-binding peptide (QLMHDYR) to these substrates.[55] A K_a of 6.1×10^4 M^{-1} was found for α-PLLA, which was approximately 10-fold higher than the affinity of this peptide toward β and at-PLLA, illustrating the selective binding of this peptide to α-PLLA. It is worth noting that a K_a value of 6.7×10^9 M^{-1} was obtained when phage ELISA was performed using the phage clone displaying this sequence and α-PLLA as the substrate. Possible reasons for this discrepancy were discussed in the previous section. Ejima et al. found K_a values of 7.7×10^5 M^{-1} and 7.7×10^4 M^{-1} for two different peptides toward hyperbranched (hyp) and linear PPVs, respectively.[59] Furthermore, SPR analysis of the hypPPV-binding peptide variants prepared via alanine scanning provided important insights into the binding mechanism of this peptide to these polymeric surfaces. The same authors also used SPR to identify the binding affinity of a peptide recognizing poly(2-methoxy-5-propyloxysulfonate-1,4-phenylenevinylene) (mpsPPV) surfaces ($K_a = 1.3 \times 10^5$ M^{-1}).[58]

SPR has also been used to determine the binding affinity of small peptide ligands toward organic molecule targets. For example, Van Dorst and co-workers have chemically immobilized polychlorinated bisphenyl (PCB)-binding phages onto an SPR chip and monitored the rate of change of SPR angle as a function of PCB concentration in the aqueous phase.[68] The resulting saturation curves allowed the calculation of K_a in the range of $(1.4–2) \times 10^4$ M^{-1}. Sawada and co-workers have immobilized 2-naphthylamine on gold substrates and determined a K_a value of 1.4×10^5 M^{-1} for a naphthalene-binding peptide identified by phage display and demonstrated its selectivity toward naphthalene over other aromatic hydrocarbons such as benzene, anthracene, and pyrene using SPR.[71]

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) is a powerful technique that allows the determination of the thermodynamic interaction parameters between two or possibly more components in solution.[89−91] An isothermal titration calorimeter consists of two cells. The reference cell only contains a buffer solution. The sample cell is loaded with the substrate of interest at a defined concentration in the same buffer solution. The method relies on the measurement of the temperature difference between these two cells upon gradual titration of the substrate with the corresponding ligand until saturation is reached. Each addition results in heat that is released or absorbed as a result of the reaction or interaction between the species that are added together. The heat is measured by the isothermal titration calorimeter and appears as spikes in the titration curves, as shown in Figure 6C. Integration of these spikes does not only provide information about the binding affinity but can also allow calculating the changes in the enthalpy and entropy associated with the ligand−substrate interactions.[90,91]

ITC is often not a very convenient technique to determine the binding affinities of ligands to solid substrates since the concentration of the binding sites that are available for the phage or the peptide, which is a required parameter for the calculation of the binding affinities, is ambiguous when solid surfaces are used. Several groups, however, have successfully utilized ITC for the determination of binding affinities of peptide ligands to small organic molecules having a known concentration. Jaworski and co-workers, for example, have calculated a binding constant of 1.4×10^7 M^{-1} of a trinitrotoluene (TNT) binding peptide to TNT in pure acetonitrile.[66] Guo and co-workers have titrated homogeneous suspensions of CNWs with the corresponding CNW-binding peptide and have calculated a binding constant of $\sim 10^5$ M^{-1}, which was in the same range obtained from fluorescence quenching measurements.[77]

Atomic Force Microscopy. Due to its ability to accurately measure forces in the piconewton range, atomic force microscopy can be used to determine the strength of noncovalent ligand−substrate interactions. The determination of the unbinding force between the ligand and the substrate is usually carried out using AFM tips and substrates that are functionalized with the respective molecules.[92−94] Briefly, a typical AFM force measurement cycle reveals a hysteresis between the force−distance curves that are recorded during approach and retraction of the AFM tip (Figure 6D). This hysteresis reflects the unbinding force that is required to separate an interacting pair immobilized to the tip and the substrate at a given loading rate. The loading rate is defined as the time derivative of the force applied to a bound pair and depends on the retraction velocity and the spring constant of the cantilever.[94] As a consequence, the unbinding force is an "apparent" value that

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allows comparison of the ligand−substrate interactions at a particular loading rate. It is also worth noting that although AFM force measurements in theory can allow the calculation of binding affinities, the determination of the rate of association may not be feasible or require laborious scans and a careful interpretation of the data for many of the ligand−substrate interactions.[95] As a consequence, binding affinities ($K_a = k_{ass}/k_{diss}$) are not frequently reported. A detailed theoretical and practical framework for the utilization of AFM for the determination of the strength of ligand−substrate interactions is provided in several excellent reviews.[96,97]

While AFM force measurements potentially are a powerful complementary technique to study the interaction between phages or peptide ligands and surfaces, this technique has been used less as compared to, for example, SPR or QCM, which is presumably due to the relative difficulties in the optimization of the experiments and the interpretation of the results. Sanghvi and co-workers have determined an unbinding force of 112 pN between a PPyCl-binding peptide selected via phage display and thin films of PPyCl.[49] Experiments with biotin−avidin (as a positive control) and GRGDS nonspecifically adsorbed on the PPyCl films as a negative control afforded forces of 309 and 43 pN. In another interesting experiment, Cho et al. covalently immobilized a thiamethoxam (TMX)-binding phage as well as a random phage library onto Si_3N_4 tips and assessed the unbinding forces between these tips and TMX immobilized to a glass substrate via an epoxy resin at a constant loading rate.[69] The experiments revealed an approximately 8-fold greater unbinding force for the phage displaying TMX-binding peptide (∼147 pN) as compared to the random phage library (∼18 pN), highlighting its specificity toward TMX surfaces.

Quartz Crystal Microbalance. The quartz crystal microbalance (QCM) technique allows determination of the mass of material that is adsorbed or deposited on a surface from the change in resonance frequency of an oscillating quartz crystal (Figure 6E).[98−101] The QCM technique can also be used in liquid solutions, which makes this approach an attractive tool to study (bio)interfacial phenomena. The QCM technique has been utilized to assess the interaction between antibody presenting phages and the complementary antigens.[33,102] In principle, the QCM technique could represent an alternative method to obtain (semi)quantitative insight into the binding affinities of soft matter binding peptides. QCM can be used in two ways to (semi)quantitatively characterize binding affinities. The first approach involves measuring in real time the adsorption and desorption of molecules by monitoring the changes in the frequency of the QCM sensor upon successively exposing the chip to a solution containing the ligand and a washing buffer similar to an SPR measurement highlighted above.[103] The second method is based on the measurement of the resonance frequency at different concentrations of the ligand subjected to a fixed amount of substrate until the frequency no longer changes as a function of ligand concentration. The resulting saturation curves can be treated as a Langmuir adsorption isotherm, which can eventually provide K_a values.[104]

In spite of the potential opportunities to also provide (semi)quantitative information, so far the QCM technique has been mainly used to qualitatively monitor the interactions between soft matter binding ligands and their target substrates. In one example, Serizawa and co-workers have exposed QCM chips, which were coated either with isotactic (it) or syndiotactic (sn)-PMMA films, to a solution containing the peptide selected via phage display.[50] The QCM experiment confirmed the ability of the peptide to selectively recognize the it-PMMA surface. In another example, Chen et al. prepared spin-coated films of poly(methacrylates) containing cis or trans-azobenzene side chain functional groups on a QCM chip, which were then subjected to a solution containing a biotinylated derivative of the peptide recognizing cis-azobenzene groups.[57] Next, the QCM chip was exposed to a solution containing streptavidin and the QCM frequency changes were used to assess the extent of deposition of streptavidin on the surfaces and provided further evidence for the selectivity of this sequence toward cis-azobenzene over its trans conformer.

CHALLENGES AND OPPORTUNITIES

The examples presented in this Review demonstrate the feasibility of phage display to identify soft matter binding peptide ligands for a variety of substrates. These peptide ligands can be used to promote cell adhesion to polymer surfaces,[49] to modulate the fluorescence of organic molecules or polymers,[58,64] to direct scaffold formation,[76] to selectively detect explosives,[65,66] and in printing applications.[75] However, the applicability of the phage display technique and the utilization of the identified soft matter binding peptide ligands may present some limitations. In what follows below, some of these limitations will be discussed and potential solutions or alternatives that may allow us to overcome these challenges will be presented.

First of all, unlike biological substrates such as enzymes and proteins, synthetic polymer films or organic crystals generally do not have well-defined binding pockets that can be occupied with the corresponding ligands with very high affinity. As a consequence, the binding constants of soft matter binding peptides are typically relatively low, in the range of 10^4−10^6 M^{-1}. For practical applications, it may be desirable to enhance the binding strengths of these ligands. One possibility to enhance the affinities of the selected peptides is the use of mutagenesis.[64] Another approach is to explore multivalency, since multivalent ligands have been shown to have few orders of magnitude higher binding constants as compared to monovalent ligands.[105,106] This can be achieved, for example, by using scaffolds (polymers, particles, etc.), which present multiple copies of the peptide ligand of interest.[16,78,79] These multivalent constructs may also allow a reduction in the discrepancy between the reported phage and peptide binding constants as the most frequently used M13 phage contains five copies of each peptide. Another way to overcome the discrepancy between phage and peptide affinities would be to use phage platforms that only display a single copy of the peptides in affinity selection experiments.[107]

Most of the examples presented in this article have used commercially available, linear 7- or 12-mer M13 phage libraries. There are a number of other phage display formats, however, which can also be used to screen and identify binding ligands to soft matter substrates. In addition, the use of phage libraries displaying conformationally constrained cyclic sequences may allow the isolation of cyclic peptide binders with higher affinities as compared to flexible linear sequences.[108−111] When comparing phage display formats that generate peptide ligands of different lengths, it is worth mentioning that the diversity of phage libraries is typically in the range of 10^8−10^9 clones, indicating that any insert having more than 7 amino acids ($20^7 = 1.28 \times 10^9$) would cover only a small fraction of all possible sequences.

In addition to peptide phage display, there are several other combinational techniques that can be used to identify soft matter binding ligands. Antibody phage display[112−114] and bacterial cell

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surface display,[115,116] for example, have been used to identify either short peptide sequences or large antibodies that bind to soft matter substrates. Particularly, single-chain variable fragment (scFv) antibody phage display may yield soft matter binding proteins having higher binding strengths as compared to short, flexible peptides. For instance, calculated binding constants of strongest DNT-binding antibodies were an order of magnitude (10^8 M^{-1}) higher than those of previously identified DNT-binding peptides (10^7 M^{-1}), which were identified via peptide phage display.[66,113] However, the use of antibodies in hybrid conjugates may present some challenges due to their lower stability and higher costs compared to short peptides.[23] Another technique that is of potential interest for the identification of small ligands that bind to soft matter substrates is systematic evolution of ligands by exponential enrichment (SELEX).[117−119] SELEX generates nucleic acid ligands, so-called aptamers, and is particularly advantageous to identify small molecule binding ligands owing to the small size of nucleotides compared to phages. An interesting aspect of SELEX is the diversity of the initial random library. SELEX typically starts from a chemically synthesized random oligonucleotide library composed of ~10^{15} molecules,[119] whereas phage display typically involves 10^{10} different clones.[3] Aptamer selection has been reported to be facilitated by the presence of positively charged groups as well as groups that can act as hydrogen bond donors and acceptors, and be more difficult on substrates that are hydrophobic or present negatively charged groups.[119] Nevertheless, this technique has been very successfully used to identify highly affine binders to a broad range of substrates including, e.g., sugars, drugs, as well as polysaccharides such as chitin.[119−121]

A final challenge is the elucidation of the binding mechanisms of peptides identified via phage display. Few recent studies have used NMR techniques combined with computational modeling to understand the molecular structures of peptides/proteins bound onto well-defined solid surfaces.[122−124] However, the applicability of these techniques to more complex targets, such as structurally heterogeneous polymer films, is yet to be demonstrated. Furthermore, it is important to highlight that most of the phage display experiments cited in this Review did not yield consensus sequences or motifs, but rather compositionally diverse peptides that do not necessarily share similar properties, i.e., hydrophobicity or charge. As a consequence, the role of each amino acid in each of the identified sequences toward the binding onto these substrates should be analyzed separately, for example, using alanine scan variants of each sequence,[59] which is a time-consuming task. The lack of consensus may also require the sequencing of a greater number of clones following the affinity selection to identify otherwise undetected binders, which can be carried using high-throughput, next-generation sequencing.[125−127] Furthermore, polymeric substrates often are structurally heterogeneous, such that they, for example, simultaneously present crystalline and amorphous domains, have different tacticities, architectures, and side chain functional groups. Each of these features can lead to the selection of entirely different peptide binders, as shown by Serizawa and co-workers,[37] which can make establishing unambiguous sequence−property relationships daunting task.

CONCLUSIONS

With its rapidly expanding scope, it is evident that the phage display technique is a powerful tool that allows the identification of specific peptide binders to a myriad of artificial or natural substrates including synthetic polymers, small organic molecules, as well as natural macromolecules. These peptide ligands open new avenues to functionalize and interface both man-made and biological materials. While a number of experimental techniques can be used to (semi-)quantitatively assess binding of phages and peptides to soft matter substrates, an accurate, molecular-level characterization remains a challenge. Possibly the use of advanced molecular modeling and simulations combined with experimental techniques such as NMR and next-generation sequencing may pave the way for a better understanding of the interactions between phages/peptides and their substrates.

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Notes

The authors declare no competing financial interest.

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