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Physical elution in phage display selection of inorganic-binding peptides

Senem Donatan [a,b], Hilal Yazici [b], Hakan Bermek [b], Mehmet Sarikaya [c,d], Candan Tamerler [b,c,*], Mustafa Urgen [a,*]

[a] Department of Metallurgical and Materials Engineering, Istanbul Technical University Maslak 34469, Istanbul, Turkey
[b] Department of Molecular Biology and Genetics, Istanbul Technical University, Maslak 34469, Istanbul, Turkey
[c] Department of Materials Science and Engineering, University of Washington, Seattle, WA 98195, USA
[d] Department of Chemistry and Chemical Engineering, University of Washington, Seattle, WA 98195, USA

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ABSTRACT

Phage display is a commonly utilized in vivo approach in selecting peptides specific to solid inorganic materials. In this process, traditionally, high affinity peptides are recovered by a chemical elution method, which involves contacting the phage library with the desired inorganic, washing the weak binders, and eluting the tight binders under harsh buffer conditions. This process may result in incomplete removal of all strong binders, separation of the phage from the display protein, or may modify the material surface. To overcome these potential limitations, we developed a physical elution technique based on ultrasonication. Here, we report two optimized ultrasonication protocols by which we selected peptides specific to natural mineral mica. We first performed a 30-s physical elution after the chemical elution step and increased the efficiency of screening strong binders by about 100%. Encouraged by the results, we applied physical elution-only protocol where we obtained 45% of the selected sequences as strong binders. The approach has a far shorter total elution time, i.e., seconds compared to hours in traditional chemical elution. The novel physical elution approach using ultrasonication reported herein can be a highly efficient alternate step in the screening of solid material specific peptides.

1. Introduction and background

During the last decade there has been a paradigm shift in materials science and chemistry in using selected or designed peptides as molecular tools rather than synthetic molecular units. In particular there has been a surge in the utilization of inorganic-binding peptides that are genetically selected using single-celled organisms, as molecular recognition elements in materials synthesis, assembly, and fabrication [1,2]. Using phage display and cell surface display peptide libraries, short amino acid sequences (typically 7 to 15) have been selected and isolated that are specific to various practical engineering materials including noble metals (Au, Pd, Pt, Ag) [1–4], oxides (Al_2O_3, SiO_2, ZnO, Cu_2O, TiO_2) [2,3,5–7], semiconductors (GaN, ZnS, CdS, GaAs) [8–10], minerals (mica, calcite, graphite, sapphire, hydroxyapatite) [2,3,11,12], polymers [13], zeolites [14] and carbon nanotubes [15]. These genetically engineered peptides for inorganics (GEPI) [2] have been demonstrated in a wide range of practical applications such as nanoparticle immobilization on inorganic surfaces [16–20], inorganic synthesis [4,11,21–23], biomineralization [12,24] and surface functionalization [13,15,25].

With the accumulating knowledge on the utility of GEPI in nanotechnology and bionanotechnology, there is a need for improvement in the selection of peptides with better affinity and material selectivity. For the genetic selection of these peptides, typical methods such as phage display [2–5,7–14], cell surface display [2,3,6], monoclonal antibodies [26] and yeast surface display [27] are employed. Among them, phage display has become a major approach because of the potential of screening large number of clones in a single step and obtaining enhanced library diversity. In this approach, combinatorial phage-peptide library is exposed to an inorganic solid material surface (usually powders of various sizes), weak binders or non-binders are washed out and tight binders are recovered by using chemical elution methods. The eluted phages are amplified and pooled for sequencing of the individual clones by PCR [28]. This method, known as biopanning, should be optimized for each target material [29,30]. During the biopanning experiments, the use of low pH elution buffers may result in surface modifications or deteriorations on chemically-reactive materials and alter the original interaction between peptide and the inorganic surface; this consequently may result in an incomplete removal of the binders. A considerable number of bound phage, therefore, could be left attached on the surface [30].

A few solutions were proposed in the literature to overcome the limitations of phage display technique. One such approach is a bound phage recovery method [3], where the phage remained attached on

* Corresponding authors. Urgen is to be contacted at Metallurgical and Materials Engineering, Istanbul Technical University, Maslak 34469, Istanbul, Turkey. Tel.: +90 212 285 6999; fax: +90 212 285 3427. Tamerler, Molecular Biology and Genetics, Istanbul Technical University, Maslak, 34496, Istanbul, Turkey. Tel.: +90 212 286 2251; fax: +90 212 286 2253.
E-mail addresses: tamerler@itu.edu.tr, candan@u.washington.edu (C. Tamerler), urgen@itu.edu.tr (M. Urgen).

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the surface may be amplified in the presence of the material and its sequence is analyzed by PCR following the DNA extraction. Another similar approach is PCR derived phage display [30] where the bound phage amplification was eliminated and instead, phage DNA was obtained using lysis buffer and then PCR was performed to analyze the sequence of the binders. However, the question on material stability under harsh chemical conditions still remains unresolved as well as the laboriousness of the biopanning process.

We proposed that ultrasonic energy could be used during biopanning process as a rapid and reliable method for phage removal from solid surfaces. This approach was inspired from surface finishing industry where ultrasonication is one of the well-known and commonly used procedures in cleaning and degreasing solid surfaces [31,32]. Ph.D.-C7C phage library was used to select constraint 7-amino acid peptides from single crystal muscovite mica to quantitatively assess the validity of the proposed new physical elution approach. Muscovite mica was chosen because of the ease of preparation of atomically flat surfaces and its potential use in arrays or clusters for novel electronic and optical devices at the nanometer scale [33,34].

Developed ultrasonication-based physical elution approach eliminates the risk of material stability problem under strong chemical environment, increases removal efficiency and reduces the process time for the selection of mica-specific peptides, hence, providing a more convenient and alternate method for the selection of GEPI for various practical solids.

2. Experimental approach

2.1. Materials

The Ph.D.-C7C library was purchased from New England Biolabs. This library consisted of randomized 7-mer peptides fused to a minor coat protein (pIII) of M13 phage and contained 1.2×10^9 independent clones. Detailed information can be obtained from New England Biolabs instructions [35]. Single crystal (001) muscovite mica ($KAl_2(AlSi_3O_{10})(OH)_2$) sheets were obtained from Ted Pella, Inc. Substrate structure and orientation were confirmed by X-ray diffraction.

2.2. Method optimization

Ultrasonication was performed using LABSONIC U (B. Braun Biotech International) with a standard probe 5T 853810/7 at a constant frequency of 20 kHz.

The 50 μl of diluted phage library was exposed to freshly cleaved mica sheets in potassium phosphate–sodium carbonate (PC) buffer containing 55 mM KH_2PO_4 (Merck), 45 mM Na_2CO_3 (Merck) and 200 mM NaCl (Riedel-de-Haen). A 1.5 ml microfuge tube containing the phage and the mica sheet was rotated on a bench top rotator for 30 min and then subjected to ultrasonication. The tube was placed in cold water to avoid excessive heating produced during ultrasonication. Ultrasonication was applied to the water inducing an indirect effect to the tube. Duty cycle was kept constant at 0.5/s. The power and duration were varied between 120–190 W, and 30–180 s, respectively. Tittering method, which was based on the blue/white screening method, was used to compare the number of plaques formed by sonicated and non-sonicated phage samples. *E. coli* (ER2738) host cells were infected both with the sonicated and non-sonicated phage samples and plated on Luria Broth (LB) plates containing 5-bromo-4-chloro-3-indoyl-β-D-galactosidase (X-Gal, Fermentas), isopropyl-β-D-thiogalactosidase (IPTG, Sigma) and 20 mg/ml tetracycline (Sigma). Optimized ultrasonication parameters were used throughout the whole physical elution experiments.

2.3. Application of ultrasound as a complementary elution step in biopanning

Here, four biopanning rounds were performed. In each round, phage binding, washing, chemical-based elution and then a single ultrasonication step were performed respectively (Fig. 1(a)). At the end of each round, blue/white screening was used to select phage plaques with the random peptide insert. Five plaques from chemically eluted and another five from physically eluted phages were picked randomly for sequence analysis at the end of each round.

2.3.1. Phage binding and washing

The 10 μl of the phage library was incubated with freshly cleaved muscovite mica sheet in PC buffer containing 0.1% detergent (combination of 20% (w/v) tween20 and 20% (w/v) tween80) for 30 min at room temperature. The mica sheet used was then washed several times with PC buffer with increasing detergent concentrations from 0.1% to 0.5% (v/v), at pH: 7.2.

2.3.2. Chemical-based elution

The phage was stepwise eluted from the mica sheet with increasing stringency conditions in the buffers. First, mica sample was first treated for 15 min with elution buffer 1 (EB1) consisting of 0.2 M Glycine-HCl pH 2.2 (Merck). Next, the solid was incubated with elution buffer 2 (EB2) containing Glycine–HCI pH 2.2 with 2 mg/ml BSA (Sigma) and 0.02% SDS (Merck), 1 M NaCl, 100 mM DTT (Sigma), 7 mM TCEP (Sigma), 100 mM 2-mercaptoethanol (Merck) for 15 min, and this step was repeated after replacing the used buffer with fresh buffer. The eluted phages were then transferred to a fresh tube and neutralized with Tris–HCl, pH 9.1 (Merck). By using the tittering method, the amounts of chemically eluted phage samples were determined and an enriched phage pool was generated for the following biopanning round.

2.3.3. Physical-based elution

Chemically eluted mica was placed into a fresh tube containing PC buffer. While tube was soaked in a water bath, ultrasonication was applied into the solution. The sampling was carried out in the microfuge tube during ultrasonication.

2.4. Application of physical-based elution as an alternative to the chemical-based elution

In this case, chemical-based elution was completely eliminated and the ultrasonication was utilized as the sole source for phage elution. The biopanning round was consisted of phage binding to the substrate, followed by washing the substrate and four ultrasonication steps (Fig. 1(b)). After each ultrasonication step, the sonicated phage-buffer solution was removed from the tube containing mica and replaced by fresh buffer. The physically eluted samples were, consequently, tittered and five plaques from each ultrasonication step were collected for peptide sequence analysis.

2.5. Sequence analysis

The single-stranded DNA of selected phage plaques were isolated by QIAprep® Spin M13 Kit and amplified by PCR in the presence of dye-labeled terminators (ABI, Applied Biosystems). PCR product was purified by ethanol precipitation. A 96 gIII primer or 5′-^{OH}CCC TCA TAG TTA GCG TAA CG-3′ primer was used for the amplification of ssDNA. The selected sequences of the DNA samples were analyzed by Applied Biosystems 3100 Avant genetic analyzer.

2.6. Immunofluorescence microscopy analysis of the mica-selected phage mutants

Powdered mica was used to carry out qualitative binding experiments. Particle size of mica powder was approximately 400–500 nM, determined by a Particle Size Analyzer (Malvern Instruments). The sample powder was first cleaned via several washing and ultrasonication steps with PC buffer containing 0.5% and 0.1%

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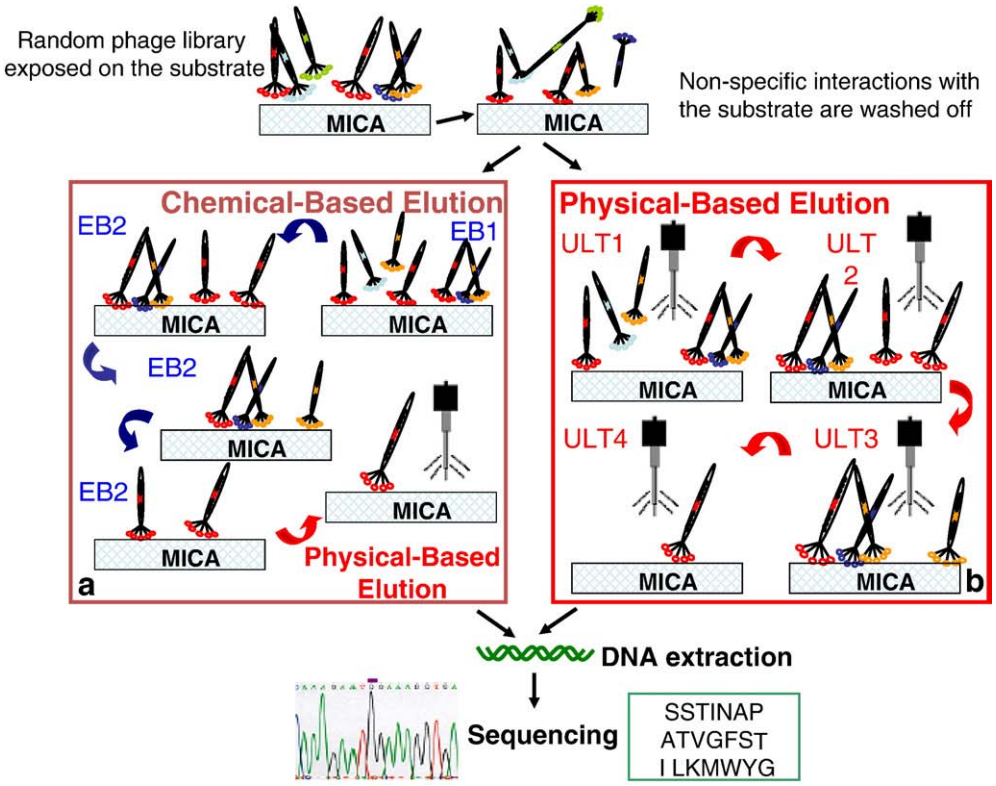

Fig. 1. (a) Schematic illustration of the two-step elution process, where ultrasonication-based physical elution step was carried out following the chemical elution step; and (b) application of the single-step physical elution process for selecting solid-binding peptides.

detergent. Then, the phage solution (10^{10} pfu/ml) was exposed to 80 mg/ml clean mica powder in PC buffer containing 0.1% detergent and rotated overnight. The solution was then discarded and powder-phage mixture was incubated with casein blocking agent containing 5% casein, 10 mM Tris–HCl, 150 mM NaCl and 1% tween20 for 10 min to inhibit non-specific interactions. The attached phage was labeled with an anti-M13 pIII monoclonal antibody (Amersham Biosciences) and Alexa-Fluor conjugated secondary antibody fragments (Zenon Alexa, Molecular Probes Inc.) for 10 min. Following this procedure, the phage was then visualized by fluorescence as well as optical microscopy (BX60, Olympus Corporation) at 20× magnification under WIB filter. The surface coverage of phage on mica powder was calculated as percentage coverage, using Image Pro Plus 5.1 (Media Cybernetics). The calculations were repeated twice, with the error range of +/−5%.

2.7. Atomic force microscopy analysis

An atomic force microscope (AFM; SPM-9500 from Shimadzu Corporation) was used in evaluating the mica substrate. The mica samples were rinsed with deionized water and dried with nitrogen before conducting any AFM measurements. Height images were recorded in air using tapping mode. The probes used were etched silicon cantilevers with spring constants of 40–60 N/m. The resonant frequencies of the tips used were between 300 and 400 kHz with a scan rate of 1 mm/s. The images were normalized using a first order plane fit to remove sample tilt, and the noisy lines were subtracted.

3. Results and discussion

We first carried out the conventional phage display method based on our developed procedures of chemical elution for selecting mica-specific binders [3]. Five mica-binding peptide sequences at the end of each round were selected (Table 1). We characterized the binding affinities of the peptides to the substrate using an immunofluorescent microscopy technique [3]. Surface coverage of each phage clones with selected sequence on the substrate were obtained by comparing the fluorescence and optical microscopy images. We qualitatively classified the sequences that cover 70–100% of the surface as strong binders, 20–70% as moderate binders or lower than 20% as weak binders. We next utilized AFM to elucidate removal efficiency of the phage by the chemical elution technique. The AFM studies showed that a large number of phage particles remained on the surface (Fig. 2(a)). As expected, these images demonstrate that the chemical-based elution may be insufficient for the removal of all the specific binders from the surfaces. A similar observation was also reported by Stone et al. [30].

Table 1
The sequences of mica-binders obtained via chemical elution (strong binders are shown in bold)

	Sample code	(C7C) sequence
Round 1	**R1,E1**	**QTTSLPE**
	R1,E2.1	QMAQGLI
	R1,E2.2	LQKPEPN
	R1,E2.3	SQPSTAL
	R1,E2.3.1	NTMTPTS
Round 2	**R2,E1**	**IQSGHPQ**
	R2,E2.1	KNIETSR
	R2,E2.2	**VHPMPFI**
	R2,E2.3	KGDWTRN
	R2,E2.3.1	**TNTITHS**
Round 3	**R3,E1**	**PKSASHY**
	R3,E2.1	ITTEYQD
	R3,E2.2	QLYHANA
	R3,E2.3	PLPNKYE
	R3,E2.3.1	FNSPMHQ
Round 4	R4,E1	SGMTGAY
	R4,E2.1	CHNTARN
	R4,E2.2	KHLCGGG
	R4,E2.3	QKWASPL
	R4,E2.3.1	**TANSTAL**

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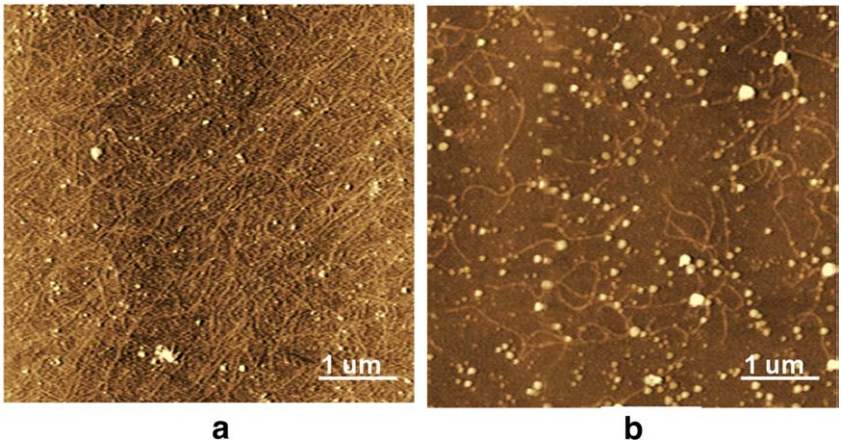

Fig. 2. (a) An AFM image of mica surface treated with chemical-based elution. (b) An AFM image of the mica surface treated using the two-step elution process.

As a high-energy process, ultrasound was expected to be capable of breaking the bonds between mica and the phage molecules during the elution process. We first conducted systematic experiments to optimize the ultrasonication parameters for physical elution. The applied power and duration of the ultrasonic energy are the critical parameters in this process. The shear force due to high frequency of vibration could potentially destroy phage and may also damage material surface. A very low shear force, however, may be insufficient for the removal of the phage that was strongly bound to the surface. We, therefore, utilized phage tittering method to optimize ultrasonication power and duration. The number of phage removed from the mica surface at various levels of ultrasound is shown in Fig. 3. Highest phage removal was found at 150 W in 30 s. At 150 W, durations varied from 30 s to 180 s did not affect the phage removal significantly. Ultrasonication parameters were, therefore, selected as 150 W and 30 s to minimize the possible damage to the phage or the mica surface.

3.1. Physical elution as a complementary step to chemical elution

Here, we introduced a two-step elution process where ultrasonication-based physical elution step was carried out following the chemical elution step (Fig. 1(a)). Five different mica-binding peptide sequences were selected by physical elution following the completion of each chemical elution round (Table 2). One additional strong binder in the first round and two additional strong binders in the second, third and fourth rounds were recovered. A single additional step of ultrasonication, which takes only 30 s, increased the number of strong mica-binders from six to thirteen, and moderate binders from nine to sixteen; but this step also increased the number of weak binders from five to eleven (Fig. 4). The recovery of high affinity binders is a crucial step in achieving the overarching goal of peptide based materials and systems which relies on engineering and controlling the peptide material interaction. We believe therefore, that the method developed will have a high utility in the selection of inorganic-binding peptides.

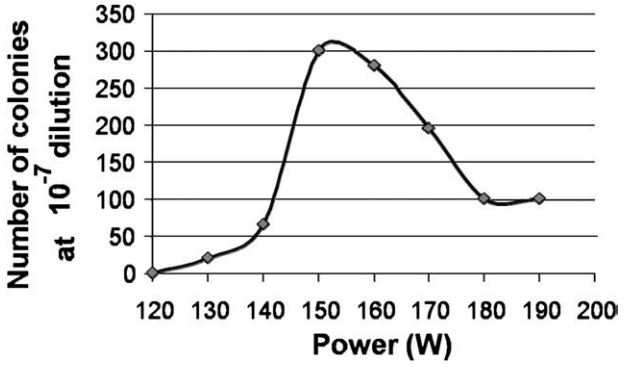

Fig. 3. Optimization of ultrasonication parameters: Ultrasonic power *versus* number of colonies produced by the eluted phage at 10^{-7} dilution.

The AFM images obtained from mica surface following the two-step elution process demonstrated the efficiency of ultrasonication on the removal of bound phage from the surface (Fig. 2(b)). We performed further investigation on the removal efficiency of the two-step elution process *via* phage tittering experiments. In Fig. 5, the number of the removed phage obtained *via* washing with the elution buffers (chemical-based elution) and ultrasonication (physical-based elution) is shown. Here, an EB1 treatment was followed by the EB2 washes three times, and a final ultrasonication step. The highest number of phage was obtained through ultrasonication, despite of the fact that it was applied as a last step. These results prove that the proposed two-step elution method, which includes an additional ultrasonication-based physical elution step, is a reliable technique that significantly enhances the ability to identify strong binding peptides to a solid material selected through phage display.

3.2. Physical elution as an alternative to chemical elution

The successful results obtained with two-step elution led us to suggest a protocol where chemical-based elution was completely

Table 2
The sequences of mica-binders obtained via ultrasonication following chemical elution (strong binders are shown in bold)

	Sample code	(C7C) sequence
Round 1	R1,ULT1.1	LRQHLNS
	R1,ULT1.2	**PLQPLPT**
	R1,ULT1.3	TPNLTQH
	R1,ULT1.4	NTLNPLM
	R1,ULT1.5	HPYYTFL
Round 2	R2,ULT1.1	NSLNGSA
	R2,ULT1.2	**SAPTLRQ**
	R2,ULT1.3	**SPSPTGT**
	R2,ULT1.4	DACRGTH
	R2,ULT1.5	RHPSADV
Round 3	R3,ULT1.1	TGNEYSS
	R3,ULT1.2	SSTLPDH
	R3,ULT1.3	**PQPNEDN**
	R3,ULT1.4	RVHEHPH
	R3,ULT1.5	**HSVQKSL**
Round 4	R4,ULT1.1	SMSPASL
	R4,ULT1.2	**QPASSRY**
	R4,ULT1.3	SKSDPRQ
	R4,ULT1.4	LPAERSQ
	R4,ULT1.5	**PFTNAFG**

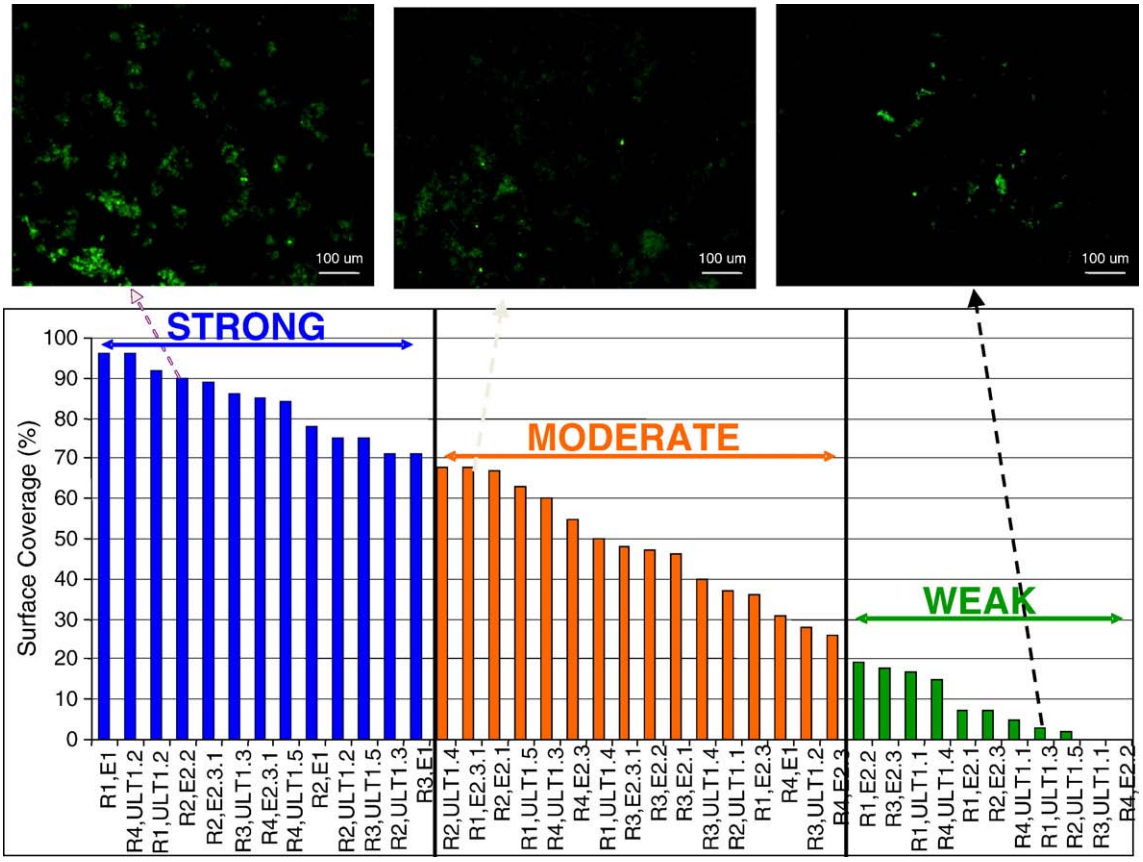

Fig. 4. Surface coverage percentages of the 40 mica-binding sequences obtained through the single-step and two-step elution processes.

replaced by a physical-based elution process (Fig. 1(b)). The applicability of this single-step physical elution approach was examined through sequence identification and binding characterization via immunofluorescent microscopy analysis. We identified 9 strong and 11 moderate mica-binders using the single-step physical-based elution. No weak binders were obtained among the 20 sequences selected. Although only a small amount of clones were identified in a single-step elution process, the percentage of the strong binders recovered in the whole set of selected peptides was 45%. The AFM images of the surfaces obtained following the single physical-based elution were also similar to those treated at the end of the two-step elution, i.e., chemical followed by physical elution (Fig. 2(b)). The possibility of obtaining a substantial amount of strong binders by solely using physical-based elution clearly demonstrated the validity of this technique as a novel approach in phage display selection (Table 3).

The significantly short application time (seconds compared to hours in chemical-based elution) and utilization of PC buffer without any detergent are the additional significant advantages of this novel technique. Using the new approach, material instability problem, which could be the major drawback of the traditional approaches due to the use of harsh chemicals, can also be avoided.

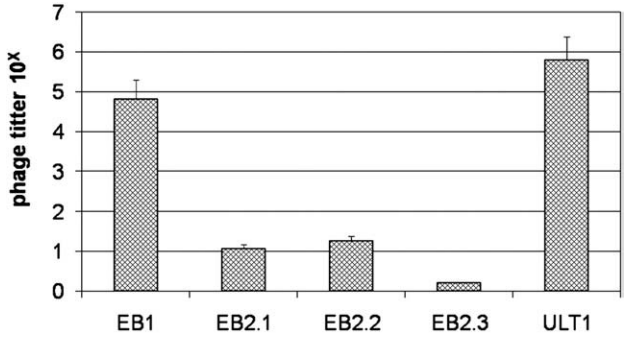

Fig. 5. Number of phage removed from mica surface through one EB1 (Elution Buffer 1) and then three EB2 (Elution Buffer 2) washes followed by an ultrasonication step.

4. Conclusions

We utilized ultrasonication as a physical-based elution method for the removal of strongly-binding phage mutants from solid surfaces during the selection of inorganic-binding peptides. The approach is

Table 3
The sequences and surface coverage results of mica-binders obtained via physical elution alone (strong binders are shown in bold, M: moderate binder, S: strong binder)

Sample code	(C7C) sequence	% binding	Affinity
ULT1.2	TNLFGHL	60	M
ULT1.3	THADHAK	64	M
ULT1.4	DETPHTT	63	M
ULT1.5	STPVNKT	42	M
ULT1.7	PYGDTDW	64	M
ULT2.1	DRAWPIG	43	M
ULT2.2	PPTSLNL	30	M
ULT2.3	**TLKMPLP**	**83**	**S**
ULT2.4	**MVSPRPS**	**74**	**S**
ULT2.5	**DLQSQSC**	**77**	**S**
ULT2.6	**HQNPLPL**	**80**	**S**
ULT2.7	**HAALTMQ**	**90**	**S**
ULT3.1	**TLTRVGW**	**75**	**S**
ULT3.2	ISNQKHT	38	M
ULT3.4	SLQRATP	66	M
ULT3.5	**PITLQPA**	**80**	**S**
ULT3.6	ETAGQNT	51	M
ULT4.1	**QPHDSSS**	**84**	**S**
ULT4.4	**PMALDPL**	**75**	**S**
ULT4.5	LPDSAPK	60	M

highly efficient and easy to use; it is a necessary step for the removal of strong binding peptides from a given solid substrate during the biocombinatorial selection process. Initially selected strong binders are a requirement in practical applications as molecular linkers and erectors. Binding characterization studies indicated that higher number of strong binders was recovered from the mica surface *via* the application of ultrasound following the chemical-based elution in a two-step process. Use of a physical-based elution alone, as a single step, in phage display selection process increased removal efficiency of solid surface-specific binders, and in the meantime, shortened the time required for biopanning. This novel protocol based on a single-step physical elution speeds up the selection of inorganic-binding peptides while retaining surface of the solid unharmed. Further work is underway to apply the new method to a number of materials with different surface characteristics (e.g., minerals and oxides) to investigate the effects of the chemical and physical elution steps to the surfaces of these materials.

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