Colloids and Surfaces B: Biointerfaces 157 (2017) 473–480

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Protocols

# Specific and selective probes for *Staphylococcus aureus* from phage-displayed random peptide libraries

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ARTICLE INFO

*Article history:*
Received 3 March 2017
Received in revised form 12 May 2017
Accepted 29 May 2017
Available online 17 June 2017

*Keywords:*
Phage display
*Staphylococcus aureus*
Biosensor selective probes
Pathogen detection
Functionalization of mica surface

ABSTRACT

*Staphylococcus aureus* is a major human pathogen causing health care-associated and community-associated infections. Early diagnosis is essential to prevent disease progression and to reduce complications that can be serious.

In this study, we selected, from a 9-mer phage peptide library, a phage clone displaying peptide capable of specific binding to *S. aureus* cell surface, namely St.au9IVS5 (sequence peptide RVRSAPSSS).The ability of the isolated phage clone to interact specifically with *S. aureus* and the efficacy of its bacteria-binding properties were established by using enzyme linked immune-sorbent assay (ELISA). We also demonstrated by Western blot analysis that the most reactive and selective phage peptide binds a 78 KDa protein on the bacterial cell surface. Furthermore, we observed selectivity of phage–bacteria-binding allowing to identify clinical isolates of *S. aureus* in comparison with a panel of other bacterial species.

In order to explore the possibility of realizing a selective bacteria biosensor device, based on immobilization of affinity-selected phage, we have studied the physisorbed phage deposition onto a mica surface. Atomic Force Microscopy (AFM) was used to determine the organization of phage on mica surface and then the binding performance of mica-physisorbed phage to bacterial target was evaluated during the time by fluorescent microscopy. The system is able to bind specifically about 50% of *S. aureus* cells after 15′ and 90% after one hour. Due to specificity and rapidness, this biosensing strategy paves the way to the further development of new cheap biosensors to be used in developing countries, as lab-on-chip (LOC) to detect bacterial agents in clinical diagnostics applications.



## 1. Introduction

*Staphylococcus aureus* is a Gram-positive bacterium often associated with a broad spectrum of human diseases ranging from mild skin infections to endocarditis, sepsis, and pneumonia [1,2], and its pathogenic potential is enhanced by several bacterial virulence factors including exotoxins and adhesins [3]. It also causes infections in farm animals [4], and the contamination of primary food products, become a risk for public health [5,6]. The widespread habitat of *S. aureus* in nature makes very difficult controlling the organism, and prevention of contamination is almost impossible. Therefore, it is important for human safety to develop accurate and rapid methods to detect *S. aureus*. Despite the recent development of different detection methods with no need for enrichment as real-time fluorescent quantitative PCR for detecting of specific nucleic acid [7], this requires skilled operators and expensive equipment. Advanced bio-selective sensors are able meet for isolation requests, concentration of the agents and their immediate real-time detection. So many researchers have recently geared their efforts towards the development of quick and reliable strategies [8].

Several biosensors have been described utilized, antibodies, as well as some antibiotics, proteins, DNA/RNA aptamers and carbohydrates, as targeting ligands to probe *S. aureus* cells [9], as bioreceptors [10,11] many of these are usually susceptible to environmental conditions and they need for laborious immobilization methods to sensor substrates [12]. Only recent studies employed phage display technology to screen new single-chain variable fragment (scFv) [26,27] or peptides from landscape phage library against *S. aureus* [1,17]. Phage display technology has been applied to numerous fields as a valid substitute for antibodies or peptides

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http://dx.doi.org/10.1016/j.colsurfb.2017.05.081
0927-7765/

[13–16]. This technique uses filamentous virus (commonly called bacteriophage or phage) that infect the bacterium *Escherichia coli*. This phage consists in a cylindrical shell, mostly made up of P8 (major coat protein) and P3 (minor coat protein), that encloses a circular single-stranded DNA molecule. Phage-display allows to incorporate random peptide sequences into the coat proteins. The phages pool, composed by several phages each of them express only a peptide sequence (phage clones), was selected against a definite target. Consequently, the phage able to bind a molecular target can be used in therapeutic target validation, drug design, vaccine development, as probes to detection of specific targets. Phage displayed peptides and phage function proteins have been successfully used as molecular recognition agents for numerous bacteria and also for *S. aureus* to achieve rapid screening. For the detection of *S. aureus*, recently Pei Liu et al. [17] immobilized gold nanoparticles with target-specific pVIII protein, obtained by isolation from protein capsid of engineeredf8/8 bacteriophages, and used them in a colorimetric biosensor. Rao et al. [1] proposed a synthetic peptide, representing the consensus sequence of the bacteria-binding phage-display peptides, as a potential diagnostic tool for *S. aureus* detection in biological samples. In previous works, we demonstrated that whole bacteriophage displaying peptides on the major capsid protein can be used for biosensor applications [14,15,18,19].

However, the whole engineered bacteriophages to be used are more robust, stable and resistant to temperature variations and hard pH conditions and, in the last, every clone presents several copies of peptide of interest, thus increasing avidity of the specific target binding [18,19]. Furthermore, the use of the whole phage structure as the recognition system of detection devices responds better to the need to develop systems based on more simple and economic methods, such as the molecular recognition mode. On the other hand, the use of the whole phage could have problems to find the optimal conditions of immobilization on the different surface, bringing at the change on the capture capacity [20].

In this study, we selected M13 phage display library against *S. aureus* whole cells to isolate peptides that specifically bind to surface surface of bacterium. The ability of isolated phage clone to interact specifically with *S. aureus* and the efficacy of its bacteria-binding properties during the time were demonstrated using enzyme linked immunosorbent assay (ELISA), also Western blot analysis confirming a 78 KDa protein as the specific recognized target. We have also tested the selectivity of phage-bacteria-binding by comparison with a panel of other bacterial species and the phage ability to identify clinical isolates, including MRSA strains. Finally the organization of phage in the Mica surface was analyzed by Atomic Force Microscopy (AFM) and it was evaluated the capacity to maintain the selectivity in recognizing *S. aureus* during the time by fluorescence microscopy, also after one week from the drying process.

## 2. Materials and methods

### 2.1. Bacteria

*Staphylococcus aureus* ATCC 29213 obtained from the American Type Culture Collection (ATCC, LGC Promochem, Milan, Italy) was propagated in tryptone soya broth (TSB) broth and Mannitol Salt Agar (MSA). *Escherichia coli* TG1 was used for propagation of phage clones. *Pseudomonas aeruginosa* ATCC 27853, *Shigella flexneri* ATCC 12022, *Escherichia coli* ATCC 11303, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 7644 and nine *S. aureus* clinical isolates, were used in selectivity assessment of phage–bacteria binding. Stock organisms were maintained in LB broth (or TSB, tryptone soya broth, for *S. epidermidis*, *S. aureus*, *L. monocytogenes*, *B. subtilis* and nine clinical isolates) containing 20% (v/v) glycerol at −80 °C.

### 2.2. Landscape phage library

The landscape phage library (kindly donated by Professor F. Felici) consists of filamentous phage particles that displays random 9mer peptides, fused to phage the major coat protein (pVIII). The nonapeptide library was constructed in the vector pC89 [21], by cloning a random DNA insert between the third and fifth codon of the mature pVIII-encoding segments of gene VIII [22].

### 2.3. Phage peptide selection

Firstly, the library was used for pretreatment with the plastic materials which will be used to the selection protocolon-binding phage clones were used for four rounds of affinity selection against *S. aureus* ATCC 29213 whole cells by incubating $10^{12}$ phage with *S. aureus* cells ($OD_{660}$ 0.5) in phosphate-buffered saline (PBS, 137 mM NaCl,2.7 mM KCl, 10 mM phosphate buffer, pH 7.4; 1 ml) for 60 min at room temperature (RT) with gentle agitation. Bacteria-phage complex were precipitated by spinning for 5 min at 16,000 × *g*, and separated from unbound phage in solution by a series of 10 washing and centrifugation steps (16,000 × *g*, 5 min) with 1mlTBS/Tween buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl,0.05% (v/v) Tween 20) each time. Bound phage were pelleted with cells and finally eluted with 250 μl of 0.2 M glycine–HCl (pH 2.2) with gentle shaking at RT for 20 min, and sonicated in ice bath at 20KHzfor 10 min. So the solutions were been neutralized with 25 μl of 1MTris–HCl (pH 9.1). Eluted phage from the four round of affinity selection against *S. aureus* cells were used to infect *E. coli* TG1cells and then These were plated on LB agar plates containing ampicillin. This cultural condition was used to select bacterial colonies, each containing phage from a single library clone, were randomly selected and propagated for subsequent analyses.

### 2.4. Colony immunoscreening

An aliquot of 200 μl of eluted phage suspension was used to infect 800 μl of TG1 *E. coli* cells in exponential phase grow for 45 min at 37 °C. After infection, the bacteria were spread on large (150 × 20 mm) LB agar plates containing ampicillin–1% glucose. After overnight incubation at 37 °C, the colonies were recovered from the agar plates with a glass spreader, and 5–10 ml of LB broth containing ampicillin was added to obtain a homogeneous suspension. 2 ml of LB–ampicillin was inoculated with 10 μl of bacterial suspension ($OD_{660}$ of 0.05–0.1). After growth up to an $OD_{660}$ of 0.4–0.5 at 37 °C, 500 μl culture was infected with 0.5 μl M13KO7 helper phage (~$10^{12}$ TU $ml^{-1}$, Stratagene). The infected bacteria were recovered by centrifugation at 6000 × *g* for 10 min. Bacteria pellet was gently resuspended in LB–ampicillin–kanamycin and incubated in agitation for 35 min at 37 °C, then diluted and plated in LB plates, containing ampicillin, and incubated overnight at 37 °C, in order to obtain single colonies. Bacterial colonies, each containing phage from a single library clone, were transferred onto a nitrocellulose (NC) membrane (Millipore) for 1 h at RT. The NC membranes were blocked at RT for 2 h in a blocking solution (5% non-fat dry milk + 0,05% tween20 in PBS), and then incubated with a suspension of *S.aureus* ($OD_{660}$ 0.5) in PBS/1% non-fat dry milk +0,1% Tween20 for 2 h at RT. After washing in PBS/0.05% Tween-20 three times for 3 min, the membranes were incubated with a 1:3000 dilution of mouse anti-Lipotheicoic acid antibody(QED Bioscence) for 2 h in PBS/1% non-fat dry milk +0,1% Tween20. The membranes were washed for five times (as described above) and then incubated with a 1:50,000 dilution of goat anti-mouse (AbCAM ab97023) for 1 h. The membranes were again washed as described above, and the