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Protocols

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

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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.

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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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[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

positive spots on the immunoblots were detected by using the developing DAB substrate (Sigma Aldrich, life science).

2.5. ELISA test

Wells of a 96-well ELISA dish (NuncMultisorp) were coated overnight at 4 °C with 100 μl suspensions of ~10^{12} virions/ml in TBS, washed 3 times with PBS/0.05% Tween 20 on an automatic plate washer, blocked with 5% non-fat dry milk in PBS/0.05% Tween for 2 h at 37 °C and washed again; reacted with *S. aureus* ATCC 29213 cells (OD_{660} 2.0) 100 μl in PBS/1% non-fat dry milk +0,1% Tween20 for 2 h at RT with shaking; washed again (5 times); reacted with 100 μl of 1:3000 dilution of mouse anti-Lipotheicoic acid antibody (QED Biosence) for 1 h in PBS/1% non-fat dry milk +0,1% Tween20, washed again; incubated with a 1:50,000 dilution of goat anti-mouse-HRP (AbCAM ab97023) for 1 h with shaking and washed again; reacted with100 μl of TMB (3,3,5,5-tetramethylbenzidine) liquid substrate system for ELISA for 45 min at RT and stopped with 100 μl of 1 M H_2SO_4. Wells were then read on a kinetic plate reader at 405 nm (Multiskan Reader, LabSystem). Phage particles bearing no recombinant insert, from superinfection of phagemid vector pC89-containing cells, served as a negative control for evaluation of background from non-specific binding.

A similar procedure was used to test the binding capacity of selected phage against nine clinical isolated *S.aureus* and a panel of other Gram positive, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 7644and Gram negative bacteria *Pseudomonas aeruginosa* ATCC 27853,*Shigellaflexneri* ATCC 12022, *Escherichia coli* ATCC 11303, in order to assess its selectivity and specificity of binding.

2.6. DNA sequencing and peptide analysis

Phage DNAs for PCR and sequencing derived from colonies of infected bacteria. The sequencing primers M13–40 reverse (5′- GTTTTCCCAGTCACGAC −3′) and E24 forward (5′ – GCTAC-CCTCGTTCCGATGCTGTC −3′) were obtained from Proligo, Sigma (Milan, Italy). 1 μl of the suspended colony was added to the PCR reaction tube, containing 49 μl of the following PCR mixture: 10 × Mg free reaction buffer (EuroClone, Milan, Italy) (5 vol); 50 mM $MgCl_2$ (EuroClone) (5 vol); Euro-Taq DNA polymerase (5 units μl^{-1}Euro Clone) (0.5 vol); 2.5 mM dNTPs (Roche) (5 vol); primer M13–40 (10 pmol μl^{-1}) (5 vol); primer E24 (10 pmol μl^{-1}) (5 vol); doubly distilled filter-sterilized water (23.5 vol). The PCR was performed by GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA) under the following cycling conditions: one cycle at 94 °C for 5 min; 25 cycles at 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s; and one cycle at 72 °C for 7 min. The PCR products (3 μl) were analysed by agarose gel electrophoresis (1% wt/volagarose, Sigma, Milan, Italy) in 1 × TAE buffer. Gel was stained with ethidium bromide, illuminated on a Dark Reader, while DNA bands were visualized by a Kodak imaging system. PCR products were purified by NucleoSpin PCR Clean-up purification Kit (Macherey-Nagel) and sequenced using the M13–40 reverse primer.

2.7. Peptide sequence analysis

The DNA sequences were translated into amino acids by the 'translate' programon the proteomics server of the Swiss Institute of Bioinformatics Expert Protein AnalysisSystem (ExPASy [http://www.expasy.ch/]). The isoelectric points (pI) of the predicted peptide sequences were calculated by 'compute MW/pI,' also present on the ExPASy proteomics server. The amino acid sequences were aligned by the CLUSTALX sequence alignment program (available at [http://www.ebi.ac.uk/clustalw/]).GeneDoc software (http://www.psc.edu/biomed/genedoc/) was used for visualize, edit and analyze multiple sequence alignments of the peptides. Statistical analysis of the insert composition was performed.

A list of the primary amino acid sequences of the variable inserts is then compiled in the FASTA format and submitted to a motif-elucidation bioinformatics algorithm called MEME(multiple expectation-maximization for motif elicitation http://meme.sdsc.edu/meme/intro.html).

2.8. Bacterial protein extraction

The extraction procedure was performed according to the protocol of George c. Paoli et al. [23] with minor modification. Two aliquots of 5 ml in PBS of *S. aureus* (OD_{660} 1) were spun down at 3500 × *g* for 10 min, washed two times with PBS and unified. The pellet was suspended in 100 μl of extraction buffer (125 mMTris-HCl, pH 7.0 containing 2% SDS, protease inhibitor cocktail at 10 μl/mg, 20% glycerol and bromophenol blue) vortexed for 5 min. After incubation for 45 min at 37 °C in agitation 8 rpm, the cell suspension was centrifuged again, and the supernatant recuperated in a new tube.

For Western immunoblot analyses, protein lysate from *S. aureus* were boiled for 5 min at 95 °C and separated at 25 mA on Bio-Rad SDS-10% polyacrylamide gel electrophoresis (PAGE). Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (0.45 μm Bio-Rad) by using the Mini-Trans-Blot transfer cell (Bio-Rad) for 16–20 h at 25 mA.

The membranes were blocked at RT for 2 h in blocking buffer (5% non-fat dry milk +0.05% Tween20 in PBS), washed for 5 min with PBS/0.05% Tween 20 and then incubated with 10^{12} virions of phage-displayed peptides for 2 h at 37 °C in a gently agitation. After washing in PBS/0.05% Tween 20 for 15 min, the membranes were incubated with a 1:5000 dilution of anti-M13 peroxidase conjugate antibody in PBS/1% non-fat dry milk +0.1% Tween20 for 1 h. After the membranes were washed as described above, the bands on the immunoblots were detected by DAB (Sigma, Milan, Italy) for 15 min, dried and preserved in plastic envelop. SDS-PAGE broad-range prestained markers (Bio-Rad) were used for size estimation of the bands binding the recombinant phage.

2.9. Binding of phage to mica

As proof-of-concept, we tested the possibility of immobilizing the affinity-selected phage by simple physisorption onto mica as model surface.

In particular, 1 cm^2 of mica thin sheets were incubated for 90 min at RT with 90 μl of phage suspension at p.I. of the bacteriophage (~10^{12} virions ml^{-1}) in Tris-buffered saline (TBS) [pH 5.4. Tris hydrochloride (7.88 g/L) and sodium chloride 140 mM (8.77 g/L) were mixed and solubilized in ultrapure water. The pH was adjusted with hydrogen chloride 5 N in order at final pH values of 5.4.]. After incubation, unbound phage were removed from samples by washing three times in PBS.

The organization of the physisorbed phage layer on the mica surface was analyzed by AFM analysis.

AFM measurements were carried out in tapping mode by using a Nanoscope IIIA-MultiMode AFM (Digital Instruments, Santa Barbara, CA, USA). The images were captured in tapping mode in air, in view of the need of highlighting the deposited phage structures at the best resolution. This may imply the modification of the structure of the assembling phages during the drying step. However, in view of the high reticulation of the deposited phage networks, we suggest that its essential features are captured in the AFM measurements in air. Accordingly, the force was maintained at the lowest possible value by continuously adjusting the set point during imaging. Images were recorded using 0.5–2 Ω cm^{-1} phosphorous-doped (n) silicon tips mounted on cantilevers with a nominal force con-

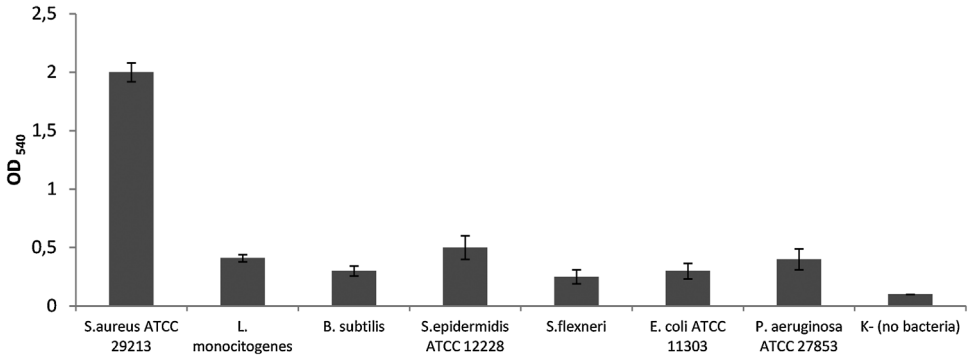

Fig. 1. Selectivity of St.au9IVS5 phage as determined by ELISA. Phage clone were coated on ELISA plate wells and incubated with bacteria cell. Mean OD_{450} is the average of three separate experiments with multiple cultures. Error bars indicate standard deviations. Paired *t*-test indicates an extremely significant difference ($P < 0.0001$) for St.au9IVS5 binding to *S. aureus* with reference to all the other challenge bacteria.

stant of 40 N m^{-1} and a resonant frequency of 300 kHz. A negative control, consisting of mica functionalized with pC89 vector was also analyzed.

2.10. Binding of S. aureus to mica-immobilized phage

An overnight culture of *S. aureus* was washed by centrifugation with PBS and diluted to prepare a final 6 ml suspension in PBS of $\approx 4 \times 10^6$ CFU/ml.

The phage layer on the mica surface samples were rehydrated and blocked at RT for 2 h in a blocking solution (4% BSA + 0.05% tween 20 in PBS), and then incubated for different time (15 min/30 min/1 h/2 h) at RT with a 500 μl suspension of *S. aureus* ATCC 29213 (with 2×10^6) pre-labeled with DAPI fluorochrome for 3 min; washed for three times and observed by fluorescence microscopy (Leica DMRE). Sequential digital images of cell binding were acquired using a CCD camera (Leica DC300F).

A quantitative evaluation of cell binding were performed as percentage of input bacterial cells (2×10^6 CFU) and cells number estimated by Scion Image Software (Windows version of NIH Image Software), in terms of integrated density (I.D. = $N \times (M - B)$, where N is the number of pixels in the selection, M is the average grey value of the pixels, and B is the most common pixel value) [24].

To assess the stability of the phage adsorbed on the surface, the mica-immobilized phages were dried and preserved at RT for a week. Indeed, the same procedure were used to test the functionalized.

3. Results and discussion

In this study, we used a phage-displayed random peptide library, to isolate phage clones displaying peptides capable of specific binding to whole *S. aureus* cell surface.

Phage clones that bound to *S. aureus* cells were affinity selected from the landscape M13 9mer-phage library by means of a series of four rounds of a selection procedure against the whole cell suspension [14,15]. Following the fourth round, individual phage clones were randomly selected from the eluted phage population and independently propagated for further analysis.

3.1. DNA sequencing and peptide analysis

Thirty phage clones were amplified and their DNA were sequenced to determine the peptide sequence displayed in their major coat protein. Different sequences were present more than once, their frequencies being shown in Table 1.

In particular, they have four amino acid (RVRS), present with a high frequency, immediately adjacent to the N-terminus of the peptide insert, indicating that the invariant amino acid is likely identical to the native sequence. The amino acids more represented are the hydrophilic amino acid serine (S) in the 4 and 7 positions, the basic amino acid arginine (R) in 1 and 3 positions, the hydrophobic amino acid valine (V) in the 2 position, and alanine (A) in the 5 position. Also the presence of the serine in the fourth and seventh position, on the sequence peptide, could have an important role in bacteria detection since three sequences, isolated more than once, have in common this amino acid.

Table 1
peptide sequence, displaying on the major coat protein of the engineered bacteriophage isolated of the phage display selection against *S. aureus* ATCC 29213.

Phage clones selected		
Clone ID	Motif sequence	frequency
St.au9IVS1	RVRSSPVVQ	1
St.au9IVS2	RVRSIPHVA	1
St.au9IVS3	RVRSYSPHL	6
St.au9IVS4	RVRSNAASM	3
St.au9IVS5	RVRSAPSSS	17
Consensus by MEME server	RVRS-P-S-	

The sequences showed the most significant similarity with consensus (MEME output) when were re-aligned with this. These data suggest that the group is essential for the recognition of target bacteria. The relative target binding of the phage clones was estimated by ELISA test, using as negative control the insert-less vector pC89 (data not shown), and the phage clone displaying the deduced sequence RVRSAPSSS, named St.au9IVS5, was chosen as the most representative.

3.2. Specificity and selectivity of phage binding to S. aureus

To determine the recognition specificity, we investigated the ability of clone St.au9IVS5 to preferentially interact with the selection target (*S. aureus*) in comparison to other potential targets (Fig. 1), including Gram-positives bacteria as *S. epidermidis*, *L. monocytogenes*, *B. subtilis,* and Gram-negatives bacteria as *Escherichia coli* ATCC 11303, *P. aeruginosa*, *S. flexneri*. The selected St.au9IVS5 clone is found to interact more efficiently (roughly a factor 4) with the selection target than with each of the others tested gram positive or gram negative bacteria.

In addition, St.au9IVS5 binding capacity was assessed against nine clinical *S. aureus* isolates strains included a MRSA strain (Fig. 2). ELISA assay confirmed that the St.au9IVS5 is the most specific and selective phage clone for different *S. aureus* strains, included the MRSA strain.

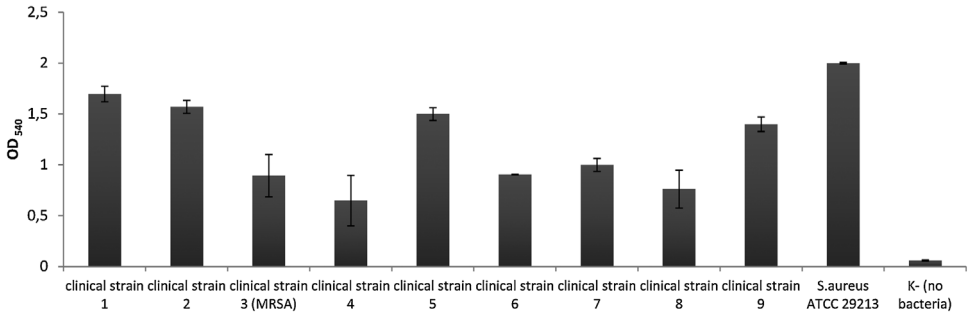

Fig. 2. St.au9IVS5 phage capacity to detect clinical *S. aureus* isolates determined by ELISA. Mean OD_{450} is the average of three separate experiments with multiple cultures. Error bars indicate standard deviations.

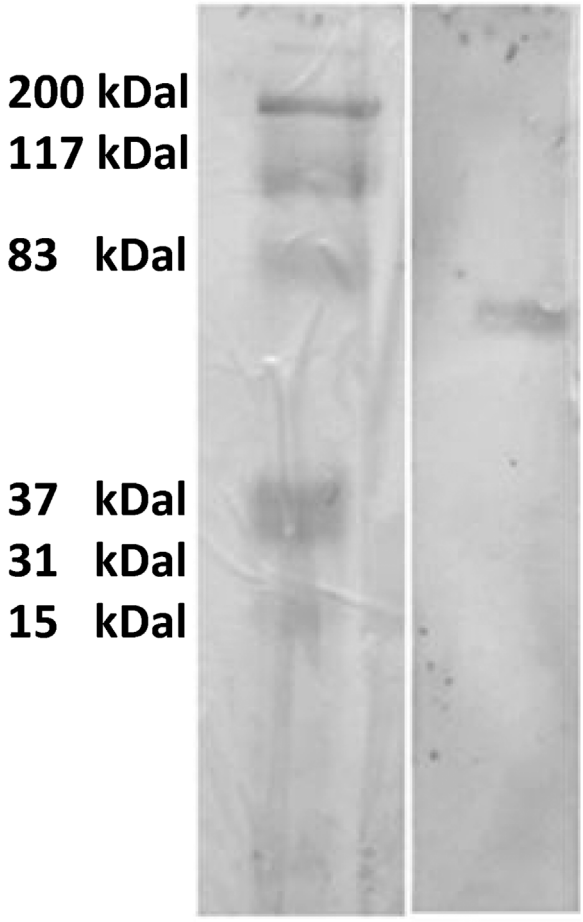

Fig. 3. Western blot analysis with the selected phage-displayed clone. Proteins isolated from *S. aureus* ATCC 29213 strain were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The membrane were probed with phage clone St.au9IVS5. SDS-PAGE broad-range pre-stained markers were used (Bio Rad, MW indicated in kDa).

3.3. Characterization of the binding specificity of the phage-displayed peptide

The Western blot analysis revealed that St.au9IVS5 phage clone binds with high avidity to a band with a mobility of about 78 kDa, confirming its selective and specific binding to *S. aureus* cell surface (Fig. 3). We propose that, probably, this band is associated at a membrane protein, namely a cell-division protein [25].

This protein is specific to *S. aureus* as demonstrated by no reactivity both with other gram-positive and gram negative bacteria, and is present in many *S. aureus* strains. Although only a few clinical strains were tested, this phage clone was shown to be able to detect all clinical *S. aureus* isolates, also the *S. aureus* MRSA strains.

Rao et al. [1] identified a phage display peptide VPHNPGLISLQG that binds specifically S. aureus, recognizing probably a surface receptor corresponding to an epitope of around 60 kDa and also with some affinity towards a 50 and a 40 kDa protein bands, which correspond to a fibrinogen-binding protein and adhesive matrix respectively. Subsequently the peptide can be evaluated its diagnostic potential just at reseach level by fluorometry analysis. However, for more end-user friendly diagnostic assays, detection platforms with lesser requirement of expertise are needed. Pei Liu et al. [17] reported a *S. aureus*-specific recognition element, GQTTLTTS, isolated by phage-display, and proposed an ingenious and simple *S. aureus* colorimetric detection system where gold nanoparticles were complexed with protein p8 extracted from f8/8 virion, expressing a peptide capable to binding *S. aureus* cells. However, the techniques of protein extraction and purification must take care of the tendency of the p8 proteins to aggregate into complexes with reduced functionality and are still time expensive. Interestingly, all of the sequences reported, including ours RVRSAPSSS, are able to bind with high specificity and affinity different components of the cell surface of *Staphylococcus aureus* and this may be useful when, for example, you want to use multiple peptides capable of binding different targets of the same cell to improve bacterial capture efficiency or in multiple detection systems.

3.4. Phages deposition onto mica

10^{12} virions/ml St.au9IVS5 phage in TBS at pH 5.4, i.e., the pI of the phage clone calculated by using "compute MW/p.I" on server (ExPASy), were physically adsorbed onto mica.

Atomic Force Microscopy (AFM) analysis has been performed after a careful washing in PBS to remove from the surfaces unbound phages. Fig. 4 shows the formation of a highly reticulated phage network densely covering the substrates. The network shows a two-levels organization, roughly corresponding to two strikingly different organization mode of the phage particles. The first level organization occurs at the very substrate surface, where the phages self-assemble in large bundles, respectively 5.68 ± 0.68 nm thick and 34.42 ± 3.29 nm wide, forming a network of relatively short segments (in average less than 200 nm) of much longer and multi-connected fibers. The dimensions of the linking bundles is, in average very homogeneous. The second-level organization mode occurs above the layer of phage bundles and basically involves the formation of thin circular platelets, respectively 3.13 ± 0.24 nm thick and with an average diameter of 40.04 ± 2.56 nm, closely adhering to the underlying fibers.

It is to stress that, as mentioned above, the working pH corresponds to the isoelectric point of the phage clone, i.e., to the formation of neutral zwitterionic systems, which in turn either interact directly with negatively charged mica surfaces or each

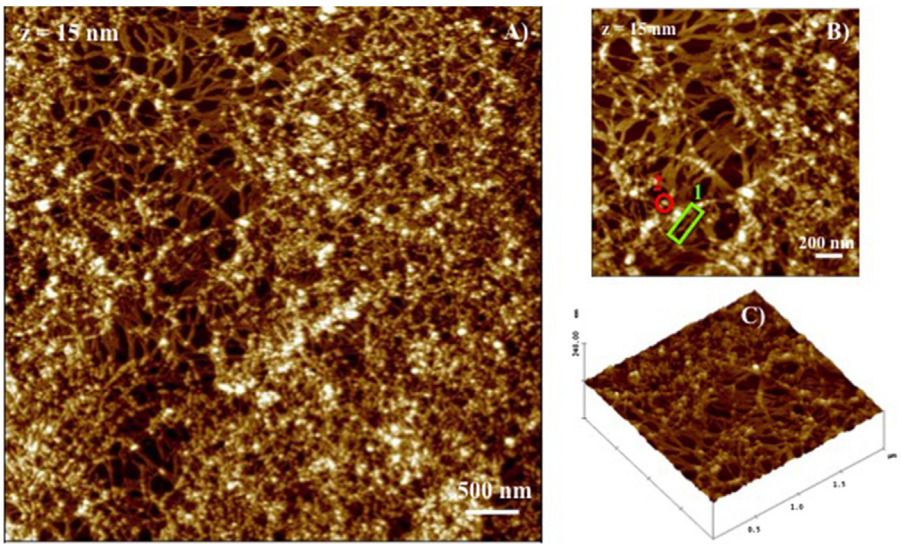

Fig. 4. AFM images of St.au9IVS5 deposited onto the surface of mica. The phage clones appear to form a highly reticulated, two-level network, entirely covering the substrate. A characteristic filamentary bundle of phage strains, assembled at the very surfaces, is shown in the (A) box, while circular platelets, overlaying the bundle fibers, are shown in the (B) box.

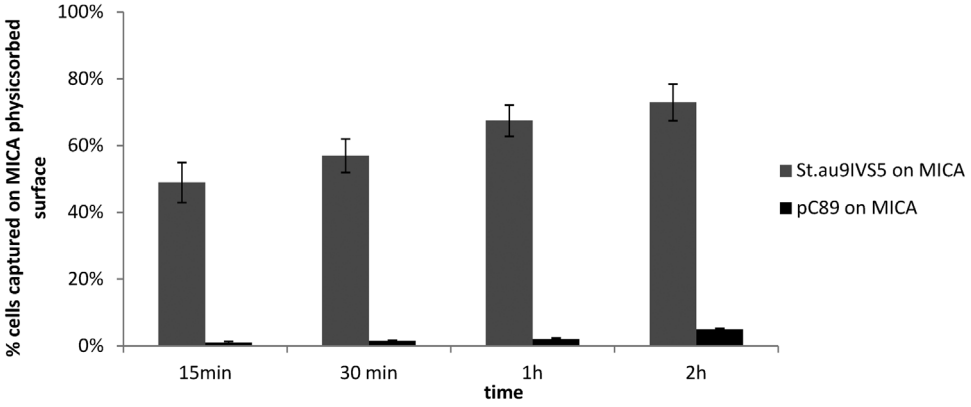

Fig. 5. Kinetic phage-capture test on mica-physisorbed phages results. Mean OD_{450} is the average of three separate experiments with multiple cultures. Each test was conducted at four different times15 min, 30 min, 1 h and 2 h at RT with shaking St.au9IVS5 (gray) and control pC89 vector (black).

other. In the first case, the globally neutrals phages can be oriented by the negative charges on mica surface, prompting their assembly in fibrillar bundles. In the second case, circular platelets would be formed, due to the average isotropic non-specific forces dominating the phage-phage interactions as far as their direct interaction with mica surface is hindered by the fibrillary bundles.

At the present stage, the eventual dependence of the relative abundance of the two different levels of organization upon the phage concentration has not been investigated, in view of the specific interest of the present study in setting the relationship the biological response of a well-defined concentration with the corresponding phage organization. However, we suggest that the relative abundance of the two phage structures should have a low dependence on the solution concentration, due to the close link between bundles of phages and circular platelets, formed on the bundles."

3.5. *Binding of* S. aureus *to the mica-physisorbed phages*

The phage layer on the mica surface samples were rehydrated and used to evaluated the binding efficiency of *S. aureus* cells. In particular, the recognition capability of *S. aureus* by phage layers deposited onto mica was studied as a function of time, by measuring the percentage of the bacteria cell capture by fluorescence microscopy (Fig. 5).

The mica-physisorbed phages were incubated with *S. aureus* cells, labeled with a fluorochrome DAPI, and the bound cells were counted at different incubation times (15 min/30 min/1 h/2 h). A quantitative evaluation of cell binding was performed as percentage of input bacterial cells (2×10^6 CFU) and cells number estimated by Scion Image Software (Windows version of NIH Image Software), in terms of integrated density (I.D. = $N \times (M - B)$). To verify the affinity of the clone, the same protocol was used for pC89 vector.

According to the AFM results above summarized, we suggest that the *S. aureus* recognition is mainly achieved by the circular platelet moieties of St.au9IVS5, which in fact are in direct contact with the bacteria-containing suspensions and may expose a rich variety of epitopes to the randomly interacting bacteria, with respect to the relatively "closed" assembly of the bundle-forming structures. Indeed, at the present stage we only may speculate that phages organized in circular platelets make more accessible the phage domains relevant to the bacteria recognition. Further experiments, focusing the bacteria-phage binding at the molecular level are planned to unravel this point. In particular, after 2 h, while only a sporadic adhesion of *S. aureus* is observed to surface bearing the control phage pC89, a significant adhesion was found

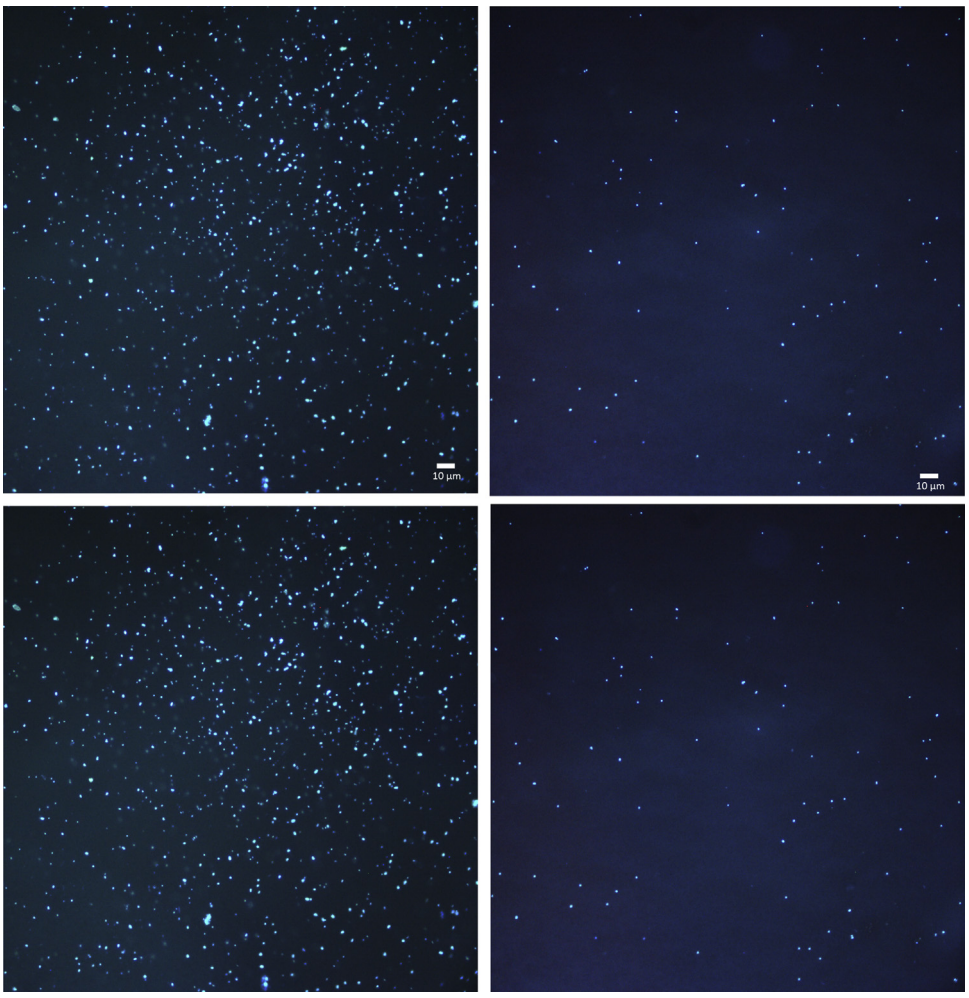

Fig. 6. Fluorescence microscopy images of *S.aureus* attached to the surface of mica with the affinity-selected St.au9IVS5 (left) and to mica previously adsorbed with pC89 vector as negative control (right) phage 50 × magnification.

for the mica surfaces bearing the St.au9IVS5 adsorbed layers above described. Statistical analysis in terms of I.D. shows a significant difference between the two types of samples, the I.D. mean values measured at 15 min, for *S. aureus* on the St.au9IVS5-coated surface being significantly higher ($P < 0.005$) than the ones measured for pC89 vector-physisorbed mica substrates (Fig. 6); this different of value increase during the time get to $P < 0.0001$ at 2 h. The selectivity of mica surface functionalized with St.au9IVS5 phage clone was tested also to detect other potential targets (in particular *L. monocitogenes* and *P. aeruginosa* strains). We were observed only a sporadic adhesion of tested bacteria, which was assigned to non-specific bond of the bacteria to the mica surface.

The same results were obtained for test on functionalized surface dried and preserved at RT for a week (data not show).

Instead, phage layers deposited onto mica that we have proposed forthe recognition of *S.aureus* is easy to realize and useful for clinical settings. Furthermore, it has a low-cost for rapid detection of bacteria target.

4. Conclusions

In this work we showed that the use of whole engineered bacteriophages immobilized onto a mica surface leads to the formation of a biosensor selective for bacterial detection. Through the analysis of the isoelectric point of the phage clone it was possible to find the optimal conditions of immobilization on the mica surface. The phage deposition provided a at two-level network on mica surfaces that permit to maintain the recognition capability of St.au9IVS5 phage clone. The physisorbed phage is able to bind and capture about 50% of S. *aureus* cells in a few minutes, and the same results have been obtained after that biosensor surface was dried and stored at room temperature for a week.

Then phage-based reagents offer many unique advantages in the context of product development and commercialization, as a functionalization with low time and cost.

So, it was possible to achieve a, sensible, specific and rapidness biosensor which may be also used in developing countries or in resource-constrained settings, as lab-on-chip (LOC) to detect bacterial agents in clinical diagnostics applications without using any costly instruments.

Conflict of interest

The authors report no conflicts of interest in this work.

Author contributions

L.D.P. and S.C. performed phage display screening and validated the Staphylococcus-binding selected phage clone. L.D.P. performed ELISA tests to verify the specificity and selectivity of the phage clones. G.M.L.M. and G.M. performed and analyzed the phage deposition onto a mica surface. L.D.P. and S.C. performed and analyzed

the time dependence of binding of mica-physisorbed phages to bacterial target. L.D.P., S.C., G.M.L.M., M.G.R., G.M. and S.G. discussed and analyzed data, and wrote the manuscript. All authors read and approved the manuscript.

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References

[1] S.S. Rao, K.V.K. Mohan, Y.M. Gao, C.D. Atreya, Identification and evaluation of a novel peptide binding to the cell surface of Staphylococcus aureus, Microbiol. Res. 168 (2) (2013) 106–112, http://dx.doi.org/10.1016/j.micres.2012.07.004.
[2] J. Paulsen, A. Mehl, A. Askim, E. Solligard, B.O. Asvold, J.K. Damas, Epidemiology and outcome of Staphylococcus aureus bloodstream infection and sepsis in a Norwegian county 1996–2011: an observational study, BMC Infect. Dis. 15 (2015) 116, http://dx.doi.org/10.1186/s12879-015-0849-4.
[3] F.D. Lowry, *Staphylococcus aureus* infections, N. Engl. J. Med. 339 (1998) 520–532, http://dx.doi.org/10.1056/NEJM199808203390806.
[4] A.C. Fluit, Livestock-associated *Staphylococcus aureus*, Clin. Microbiol. Infect. 18 (2012) 735–744, http://dx.doi.org/10.1111/j.1469-0691.2012.03846.x.
[5] J. Kadariya, T.C. Smith, D. Thapaliya, Staphylococcus aureus and staphylococcal food-borne disease: an ongoing challenge in public health, BioMed Res. Int. 82796 (2014) 5, http://dx.doi.org/10.1155/2014/827965.
[6] K. Nawattanapaiboon, W. Kiatpathomchai, P. Santanirand, A. Vongsakulyanon, R. Amarit, A. Somboonkaew, B. Sutapun, T. Srikhirin, SPR-DNA array for detection of methicillin-resistant Staphylococcus aureus (MRSA) in combination with loop-mediated isothermal amplification, Biosens. Bioelectron. 74 (2015) 335–340, http://dx.doi.org/10.1016/j.bios.2015.06.038.
[7] N.T. Thet, S.H. Hong, S. Marshall, M. Laabei, A.T.A. Jenkins, Visible, colorimetric dissemination between pathogenic strains of Staphylococcus aureus and Pseudomonas aeruginosa using fluorescent dye containing lipid vesicles, Biosens. Bioelectron. 41 (2013) 538–543, http://dx.doi.org/10.1016/j.bios.2012.09.01.
[8] J. Brigati, D.D. Williams, I.B. Sorokulova, V. Nanduri, I.H. Chen, C.L. Turnbough, V.A. Petrenko, Diagnostic probes for Bacillus anthracis spores selected from a landscape phage library, Clin. Chem. 50 (10) (2004) 1899–1906.
[9] R.A. Bohara, S.H. Pawar, Innovative developments in bacterial detection with magnetic nanoparticles, Appl. Biochem. Biotechnol. 176 (4) (2015) 1044–1058, http://dx.doi.org/10.1007/s12010-015-1628-9.
[10] U.B. Olsen, Human mononuclear cell chemiluminescence and interleukin-1 release induced by Staphylococcus aureus protein A, Immunopharmacology 22 (1991) 107–113, PMID: 1761398.
[11] H. Yue, Y. Zhou, P. Wang, X. Wang, Z. Wang, L. Wang, Z. Fu, A facile label-free electrochemiluminescent biosensor for specific detection of Staphylococcus aureus utilizing the binding between immunoglobulin G and protein A, Talanta 153 (2016) 401–406, http://dx.doi.org/10.1016/j.talanta.2016.03.043.
[12] V.A. Petrenko, V.J. Vodyanoy, Phage display for detection of biological threat agents, J. Microbiol. Methods 53 (2003) 253–262, PMID: 12654496.
[13] V.A. Petrenko, G.P. Smith, Phages from landscape libraries as substitute antibodies, Protein Eng. 13 (8) (2000) 589–592, PMID: 10964989.
[14] S. Carnazza, G. Gioffrè, F. Felici, S. Guglielmino, Recombinant phage probes for Listeria monocytogenes, J. Phys.: Condens. Matter 19 (2007), http://dx.doi.org/10.1088/0953-8984/19/39/395011, 395011 (13pp).
[15] S. Carnazza, C. Foti, G. Gioffrè, F. Felici, S. Guglielmino, Specific and selective probes for Pseudomonas aeruginosa from phage-displayed random peptide libraries, Biosens. Bioelectron. 23 (2008) 1137–1144, http://dx.doi.org/10.1016/j.bios.2007.11.001.
[16] H. Qi, H. Lu, H.J. Qiu, V.A. Petrenko, A. Liu, Phagemid vectors for phage display: properties, characteristics and construction, J. Mol. Biol. 417 (3) (2012) 129–143, http://dx.doi.org/10.1016/j.jmb.2012.01.038.
[17] P. Liu, L. Han, F. Wanga, V.A. Petrenko, A. Liu, Gold nanoprobe functionalized with specific fusion protein selection from phage display and its application in rapid, selective and sensitive colorimetric biosensing of Staphylococcus aureus, Biosens. Bioelectron. 82 (2016) 195–203, http://dx.doi.org/10.1016/j.bios.2016.03.075.
[18] G. Lentini, E. Fazio, F. Calabrese, L.M. De Plano, M. Puliafico, D. Franco, M.S. Nicolò, S. Carnazza, S. Trusso, A. Allegra, F. Neri, C. Musolino, S.P.P. Guglielmino, Phage-AgNPs complex as SERS probe for U937 cell identification, Biosens. Bioelectron. 74 (2015) 398–405, http://dx.doi.org/10.1016/j.bios.2015.05.073.
[19] F. Calabrese, S. Carnazza, L.M. De Plano, G. Lentini, D. Franco, S.P.P. Guglielmino, XVIII AISEM Annual Conference (2015).
[20] S. Huang, H. Yang, R.S. Lakshmanan, M.L. Johnson, I. Chen, J. Wan, H.C. Wikle, V.A. Petrenko, J.M. Barbaree, Z.Y. Cheng, B.A. Chin, The effect of salt and phage concentrations on the binding sensitivity of magnetoelastic biosensors for Bacillus anthracis detection, Biotechnol. Bioeng. 101 (2008) 1014–1021, http://dx.doi.org/10.1002/bit.21995.
[21] F. Felici, L. Castagnoli, A. Musacchio, R. Jappelli, G. Cesareni, Selection of antibodies ligands from a large library of oligopeptides expressed on a multivalent exposition vector, J. Mol. Biol. 222 (1991) 301–310, http://dx.doi.org/10.1016/0022-2836(91)90213-p.
[22] A. Luzzago, F. Felici, Construction of disulfide-constrained random peptide libraries displayed on phage coat protein VIII, Methods Mol. Biol. 87 (1998) 155–164.
[23] G.C. Paoli, J.d. Brewster, A listeria monocytogenes-specific phage-displayed antibody fragment recognizes a cell surface protein whose expression is regulated by physiological conditions, J. Rapid Methods Autom. Microbiol. 15 (2007) 77–91, http://dx.doi.org/10.1111/j.1745-4581.2007.00079.x.
[24] S. Carnazza, S. Satriano, S. Guglielmino, Self-organization of yeast cells on modified polymer surfaces after dewetting: new perspectives in cellular patterning, J. Phys.: Condens. Matter 18 (2006) S2221–S2230.
[25] R. Nandakumar, M.P. Nandakumar, R. Marten Mark, Julia M. Ross, Proteome analysis of membrane and cell wall associated proteins from Staphylococcus aureus, J. Proteome Res. 4 (2005) 250–257, http://dx.doi.org/10.1021/pr049866k.
[26] S. Nian, T. Wu, Y. Ye, X. Wang, W. Xu, Q. Yuan, Development and identification of fully human scFv-Fcs against Staphylococcus aureus, BMC Immunol. 17 (8) (2016), http://dx.doi.org/10.1186/s12865-016-0146-z.
[27] J. Li, Y. Xu, X. Wang, Y. Li, L. Wang, X. Li, Construction and characterization of a highly reactive chicken-derived single-chain variable fragment (scFv) antibody against Staphylococcus aureus developed with the T7 phage display system, Int. Immunopharmacol. 35 (2016) 149–154, http://dx.doi.org/10.1016/j.intimp.2016.02.024.