M13 Bacteriophages as Bioreceptors in Biosensor Device

Laura M. De Plano, Domenico Franco, Maria Giovanna Rizzo, Sara Crea, Grazia M. L. Messina, Giovanni Marletta and Salvatore P. P. Guglielmino

Abstract New recognition probes sensible, specific and robust is one of the major problems of biosensor assay. Detection biosensors has utilized antibodies or enzymes as bioreceptors; however, these have numerous disadvantages of limited binding sites and physico-chemical instabilities, can negatively affect capture and detection of target in diagnostic device. In this contest, Phage-Display provides a valuable technique for obtaining large amounts of specific and robustness bio-probes in a relatively short time. This technique relies on the ability of M13 bacteriophages (or phages) to display specific and selective target-binding peptides on major coat protein pVIII of their surface. In this work, we used P9b phage clone, displaying a foreign peptide QRKLAAKLT to selectively recognize *Pseudomonas aeruginosa* like bioreceptor. We describe different methods of functionalization to realize a selective bacteria biosensor surfaces. Several surfaces, such as latex and magnetic beads and polymeric surfaces such as mica, APTES and PEI, were functionalized by covalent bonds or physisorption with P9b. The efficiency of the surface functionalization procedures was evaluated by ELISA and AFM, while capture efficiency of the anchored phages has been assessed by plate count and Fluorescence microscopy. The results of this work pave the way to the use of phages as bioreceptor.

Keywords Phage display · Selective probes · Functionalization · Biosensor Pathogen detection

L. M. De Plano (✉) · M. G. Rizzo · S. Crea · S. P. P. Guglielmino (✉)
Department of Chemical Sciences, Biological, Pharmaceutical and Environmental, University of Messina, Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy
e-mail: ldeplano@unime.it

S. P. P. Guglielmino
e-mail: sguglielm@unime.it

D. Franco
Department of Mathematical and Computational Sciences, Physical Science and Earth Science, University of Messina, Messina, Italy

G. M. L. Messina · G. Marletta
LAMSUN (Laboratory for Molecular Surfaces and Nanotechnology), Department of Chemical Sciences, University of Catania and CSGI, Viale A. Doria 6, 95125 Catania, Italy

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1 Introduction

Advanced bio-selective sensors may meet the requests for isolation, concentration of the agents and their immediate real-time detection. Several biosensors have been described utilized, antibodies, as well as some antibiotics, proteins, DNA/RNA and aptamers, as bioreceptors [1, 2]. However many of these are numerous disadvantages including high cost of production, low availability, great susceptibility to environmental conditions and the need for laborious immobilization methods to sensor substrates. In this contest, Phage-Display provides a valuable technique for obtaining large amounts of specific bio-probes in a relatively short time [3, 4]. Phage-display allows to incorporate random peptide sequences into the major coat protein pVIII of filamentous bacteriophages. The phage able to bind a cell target have been successfully used as molecular recognition agents [5, 6]. The M13 phage is approximately 6.6 nm wide and 880 nm long with circular single-stranded DNA encapsulated in 2,700 copies of the major coat protein pVIII and capped with 5 copies of different minor coat proteins [7].The phages are structurally robust, and have a strong resistance to pH and denaturing agents [8] and its polyelectrolyte nature permit to change the spatial distributions of charges on its surfaces using different pH medium. However, the phages can be used as bioreceptors in sensor devices, as far as it necessarily exposes the binding-peptides on their surfaces. This implies that a crucial problem involves the capability to find the optimal conditions of immobilization on the different surfaces, as it rules their sensing efficiency [9].

In this work, we describe different methods of functionalization to realize a selective bacteria biosensor device, based on immobilization of affinity-selected phage. We used P9b phage clone, displaying a foreign peptide QRKLAAKLT to selectively recognize *Pseudomonas aeruginosa* [10], to tie it on biosensor surfaces. We have studied the use of functional groups present of the phage capsid surface to make the covalent bonds on latex and magnetic beads. Furthermore the analyse of isoelectric point (pI) has permit to optimize the physisorption on the polymeric surfaces such as mica, APTES and PEI using different buffer and pH values. The efficiency of the surface functionalization procedures was evaluated by means of AFM for the morphology of the anchored phages and by means of ELISA assay for the immobilized amount, while capture efficiency of the anchored phages has been assessed by Fluorescence microscopy and plate count. The results of this work pave the way to the use of immobilized phages as bioreceptor.

2 Materials and Methods

2.1 Bacteriophages

P9b phage clone, and St.au 9IVS5 were derived from M13-pVIII-9aa phage peptide library, constructed in the vector pC89, and displayed the foreign peptide

QRKLAAKLT and RVRSAPSSS, which represents a specific and selective probes for *P. aeruginosa* [10] and *S. aureus* [11], respectively. The isoelectric points (pI) of the predicted peptide sequences were calculated by 'compute MW/pI,' also present on the ExPASy proteomics server.

2.2 Functionalization of Magnetic and Latex Beads

ScreenMAG-Amine superparamagnetic beads (1 μm diameter, Chemicell GmbH (Berlin, Germany) and Carboxyl-polystyrene latex beads (0.8 μm diameter, SERVA Electrophoresis GmbH (Heidelberg, Germany) were functionalized using protocol described previous [12, 13]. Phage suspensions in ultrapure water were functionalized with the ratio about 360 phage clones/magnetic beads or with the ratio w/v of 320 μl of phage (title of $1.3 \cdot 10^{12}$ PFU/ml) and 1 ml of beads (10% w/v in ultrapure water). In order to verify phage coating of the beads, we performed an ELISA test with M13-pVIII antibody.

In order to determine maximum capture efficiency, 10^3 *Pseudomonas aeruginosa* ATCC 27853 cells/ml were incubated for 30 min against scalar concentration of phage-coated beads (for latex beads: 5, 7.5, 10 and 12.5% w/v; for magnetic beads: 10^2, 10^4, 10^6 and 10^8 beads). Colony Forming units per millilitre (CFU/ml) counts were determined before and after beads incubation with bacteria and the capture efficiency percentage was calculated. The same capture test was performed with unfunctionalized beads (Blank). Tests were performed in triplicate and results were reported as percentage average of capture.

2.3 Binding of Phage to Polymeric Surface and Capture of Bacteria Target

Surfaces such as quartz and polymeric surface mica, APTES and PEI were functionalized using physisorption protocol described previous work [10, 11]. Phage suspensions in ultrapure water at pH7 and Tris-buffered saline (TBS) (Tris hydrochloride (7.88 g/L) and sodium chloride 140 mM (8.77 g/L) at pI of the bacteriophage, 4.2, 6.3 and 5.4 for pC89 vector, P9b and St.au9IVS5 respectively. The organization of the physisorbed phage layer on the 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). A negative control, consisting of surface functionalized with pC89 vector was also analyzed. An culture of *P. aeruginosa* or *S. aureus* in PBS at $\approx 4 \times 10^6$ CFU/ml were pre-labeled with DAPI fluorochrome, then was incubated for 15 min at RT with the functionalized surface; 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) and cells number estimated by Scion Image Software (Windows version of NIH Image Software), in terms of integrated density (I.D.).

3 Results and Discussion

In this work, we describe different methods of functionalization to realize a selective bacteria biosensor device, based on immobilization of affinity-selected phage. The surface anchoring has been based on the following two approaches, covalent bonds and physisorption tuned by optimizing pH, ionic force and species. It's possible obtained an optimal functionalization of phage clone using both amino or carboxyl groups present on the its surface. The activation agent EDC in MES reacts with the carboxyl groups, forming highly reactive O-acylisourea derivatives that react readily with the amino groups. In particular to performed phage-coated latex beads the activated carboxyl-groups, exposed on bead surface, covalently bond specific NH_2 phage-chemical groups. On the contrary to obtain phage-coated magnetic beads the P9b phage clone bond covalently the amino groups exposed on magnetic beads, due to intermedia of reaction formed on the COO^- phage-chemical groups. In both cases the data show an efficient coupling of phage onto beads compared to the negative control (Fig. 1a).

The results of capture efficiency were reported as percentage average of captured bacteria (Fig. 1b, c). The phages, coated on the beads using both NH_2 or the COOH present on their surface, maintained the recognition capacity on both type of functionalization despite the covalent bind. In particular, the percentage of cells captured by the phage-coated latex beads increased from 13 to 39.63% as phage-coated beads increased from 5 to 10% w/v. On the contrary, the capture efficiency decreases from 39.63 to 30% when phage-coated beads further increase, suggesting that the ratio between phage-coated beads and *P. aeruginosa* cells was a major factor influencing

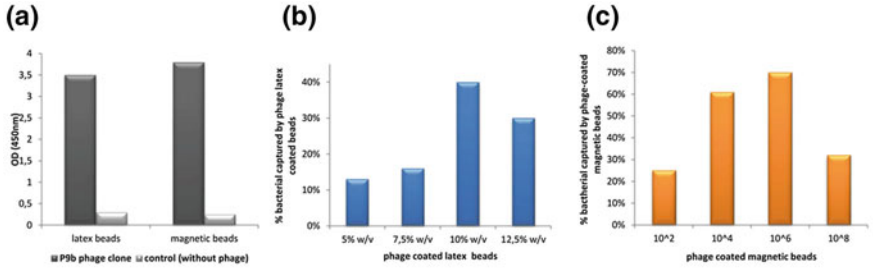

Fig. 1 **a** ELISA assay on phage-coated beads. Beads functionalized with P9b phage clone and respective negative controls (not coated beads); **b** Dependence of capture efficiency on the amount of used phage-coated latex beads; **c** Dependence of capture efficiency on the amount of used phage-coated magnetic beads. All tests were performed in triplicate and results were reported as percentage average of capture

the capture efficiency. Similarly, phage-coated magnetic beads, at same bacteria concentration, the capture efficiency were clearly increasing until 67% only until at one optimal concentration of 10^6 phage-coated magnetic beads. This is possible because the probability meeting between two elements is low when was presented both small and high quantity of phage-coated beads in the solution with same bacteria concentration. The data show that 10% w/v of P9b-coated latex beads and 106 P9b-coated magnetic beads permit the better capture efficiency of bacteria target.

The wild-type phage coat protein pVIII of pC89 vector (a phage control without the exogenous peptides), influences the isoelectric point (pI) calculated and experimentally confirmed as pH 4.2 [14]. However, the phage clones, present the exogenous amino-acid sequence "in frame" the wild-type shifts the pI at a different value. To evaluate the effect of physisorption for the different pI due to peptides exposed, we used pC89 vector, P9b and St.au9IVS5 specific and selective for *S. aureus* in process. The pI of the peptide sequences, exposed on the clones surfaces, were value at 6.3 and 5.4 for P9b and St.au9IVS5 by protein calculator, respectively. The protein surface charge is positive at pH below pI, and negative at pH above pI, so in the case of pH 7 resulted −3, −1 and −0.1 charge negative for pC89, St.au9IVS5 and P9b, respectively. Our preliminary data showed as the peptide exposed on the pVIII protein of the phages influenced the functionalization according to the phage used, the pH, the buffer solution and the characteristics of surface (Fig. 2).

Only sporadic adhesion for pC89 vector on the mica surface in H_2O pH 7, unlike of St.au9IVS5 which showed a bubbles disposition only when the functionalization was conducted a pH 9. Instead, P9b phage clone showed an adhesion in insula disposition at pH 7, condition in which his charge is near at neutrality. The repulsion between phages clones and mica surface is attributable to electrostatic effects, mica has a surface with negative potential and all the phage clones, in the pH medium used (pH 7), have negative charges exposed on their surfaces. When the phages clone were in TBS pI buffer the ions present influenced the pC89 vector which covers whole the surfaces; likewise St.au9IVS5 phage clone was a similar behavior, however, an better arrangement was observed along the surface. Probably the peptide plays an important role in the stabilization of salt–bridge, which are highly dependent upon factors such as the cost of desolvating the charged groups and the relative flexibility of the side chains involved in the ion pair.

On the contrary the peptide exposed on pVIII proteins of the P9b, influence negatively the density of disposition of the phage on the mica surface. In this surface P9b shows sporadic adhesion in elongated form and in insula disposition in both buffer used.

On surfaces with positive charge such as PEI and APTES the functionalization was conditioned with these charged exposed on the surfaces. Pc89 vector in H_2O pH 7, is strongly attracted of the positive surfaces permit the multi-stratification of the phage on the surfaces. However, in TBS pI, the pC89 vector the present Na^+ ions cover phage surface, reduced the interaction with the positive charges exposed on surface. On the contrary St.au9IVS5 clone has a better distribution in salts presence (TBS pI) compared to H_2O pH7 where the phage clone was conditioned in the self-assembling only for its negative charges exposed. The P9b clone, which is neutral in all the

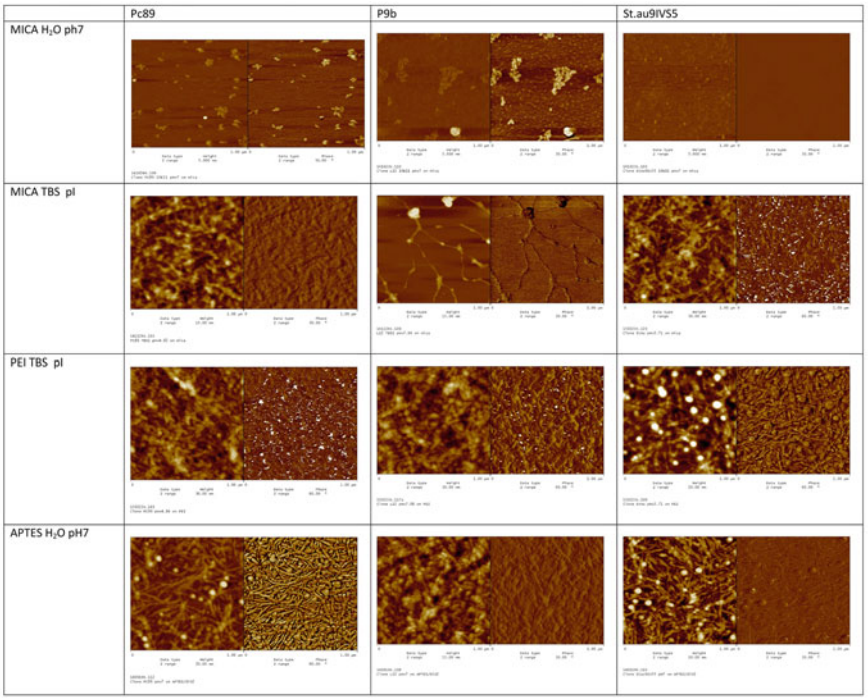

Fig. 2 AFM images of Pc89 vector, P9b and St.au9IVS5 on mica in H_2O at pH 7 (the first line); mica in Tris-buffered saline (TBS) buffer at pI (the second line), PEI in TBS buffer at pI (the third line); APTES in H_2O at pH 7 (the last line)

conditions tested, immobilization on positive surfaces produces the formation of a highly reticulated phage-networks roughly covering the whole substrates; however in TBS pI permits an organized stratification on the surface compared to H_2O pH7.

Also in this case is possible notice that the peptides have an important role in the formation of a shell on the phage structure obtaining the stabilization of salt–bridge and consequently complete stratification compared to pC89 vector. Then the optimal condition of immobilization were on PEI in TBS pI for P9b clone, on mica in TBS pI for St.au9IVS5 and on APTES in H_2O solution for pC89 vector. In these surfaces phage networks show a two-levels organization, respectively corresponding to a phage self-assembly in short fibers (less than 200 nm wide) and large bundles of 5.68 ± 0.68 nm thick and 34.42 ± 3.29 nm wide in the first-level; second-level organization mode occurring on the phage bundles with circular platelets of 3.13 ± 0.24 nm thick and with an average diameter of 40.04 ± 2.56 nm (second-level). Thus, the recognition capability of the targeted bacteria was performed on the optimal functionalized surface. Statistical analysis in terms of I.D. shows a significant coverage of the surface, in particular *P. aeruginosa* and *S. aureus* display the same pattern of the

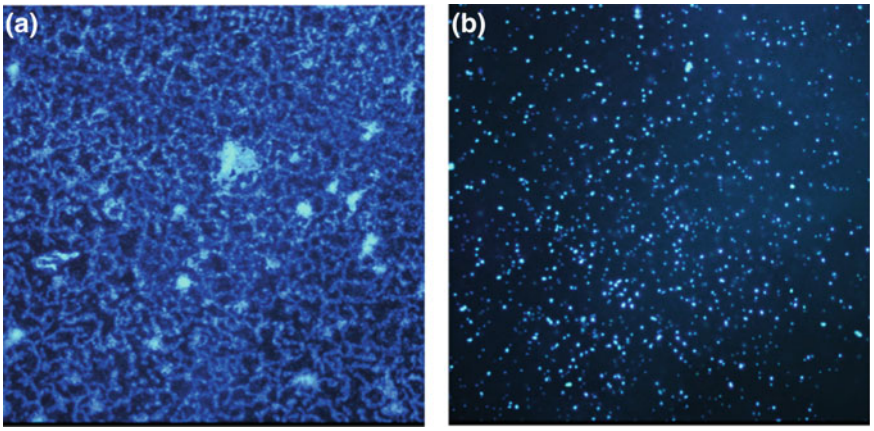

Fig. 3 *P. aeruginosa* captured on PEI physisorbed P9b at PEI; *S. aureus* captured on mica physisorbed St.au9IVS5 at TBS pI

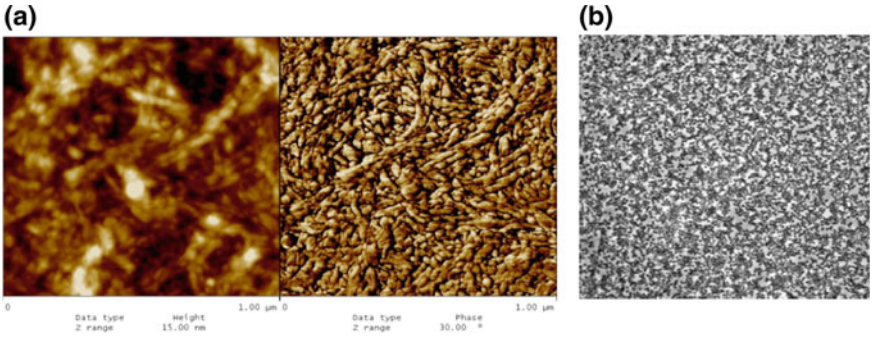

Fig. 4 **a** P9b immobilized on quartz; **b** Phase contrast microscopy images of *P. aeruginosa* captured on P9b-immobilized quartz 1000 × magnification

P9b-coated and St.au9IVS5-coated surface confirmed that the P9b and St.au9IVS5 maintained the capacity to bind the targets (Fig. 3a, b).

In the quartz, which does not present litching characteristic, the phage suspension in TBS pH7 permit an optimal functionalization to have a surface covered (Fig. 4a). The phage functionalized maintains the ability to recognize and capture the target as observed by high-power optical phase contrast microscopy (Fig. 4b). Also in this case the salts play a fundamental role in the multilayer functionalization of phages on the surface.

4 Conclusions

The results of this work pave the way to the use of immobilized phages as bioreceptor. In fact the phage probe could be used to build a micro-biosensor system in which biological sensing element is the selected phage-displayed peptide. Through the use of functional groups on the capsid surface or the analysis of the isoelectric point of the phage clone it was possible to find the optimal conditions of functionalization of different surfaces maintaining the ability to bind and capture target. Then phage-based biosensor offer many unique advantages in the context of product development and commercialization, low time and cost, sensibility and specific. Furthermore, the nature of the bioreceptor layer holds potential utilization for detection of others pathogens agents as bacteria, cancer cells, virus or toxin to which a corresponding phage was selected for. Sensors prepared with phage as probes could be an effective analytical method for detecting and monitoring quantitative changes of bacterial agents under any conditions, including clinical based diagnostics, food monitoring and industrial use.

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