Engineering of bacteriophages displaying affinity tags on its head for biosensor applications

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ABSTRACT

A method was developed for the introduction of affinity tags onto bacteriophage heads using a phage display technique. It was shown that T4 bacteriophage carrying a biotin-binding polypeptide (Biotin Carboxyl Carrier Protein, BCCP, from acetyl-CoA carboxylase of *E. coli*) or a Cellulose Binding Module (CBM, of xylanase from *Thermotoga maritime*) can be effectively immobilized onto various streptavidin- or cellulose-based materials, respectively. Interaction of the bacteriophage with the functionalized surface is strong and almost irreversible. The immobilized phages, termed BCCP-T4 and CBM-T4, retain a high level of infectivity and are capable of capturing 70-100% of *E. coli* cells from suspensions containing 10 to 10^6 cfu/ml in 30 min. The subsequent lysis of captured cells resulted in the release of multiple progeny phages. Enumeration of progeny phages by real-time PCR allowed detection of 1 cfu of *E. coli* per 5 µl sample within 2 hours.

Keywords: bacteriophage, immobilization, biotin binding domain, cellulose binding module, biosensor

1 INTRODUCTION

Bacteriophages are a valid alternative to antibodies as recognition elements in biosensors [1]. The main advantages of using bacteriophage are that they react rapidly, they are specific and exhibit a strong multivalent interaction with the host cell. In addition, there is an almost endless supply of phages in the environment that can be mined, and they can be produced cheaply. However, only a few examples were reported when bacteriophage was successfully immobilized on the surface of a biosensor while retaining its capturing and lytic activity. To date, the most popular method for phage immobilization is physical sorption [2]. Adsorption of *Salmonella* phage on a polystyrene membrane has been used to specifically separate *Salmonella* from food; however, the efficiency of cell capture was poor. Only a minor proportion of the target cells present in the sample was retained on the membrane, probably because the correct orientation of immobilized phage on the surface was sporadic. As a result, the majority of immobilized phage particles was inactive. Chemical biotinylation of phage-protein and construction of biosorbents by coating magnetic beads with phage via the biotin-streptavidin interaction significantly increased capture efficiency [3]. It was shown that up to 20% of target *Salmonella* cells could be concentrated on the surface of streptavidin-coated magnetic beads covered with biotinylated bacteriophage within 30 minutes. However, the capture efficiency was still lower than reported for immunosorbents. It was concluded that, due to the proteineous nature of both bacteriophage head and tails, there was an equal chance that the biotin moiety was introduced into sites both on the head and tail/binding site of the phage. This could result in both direct and indirect phage inactivation. Thus, introduction of affinity tags specifically on the bacteriophage head would significantly increase the number of correctly oriented phage particles immobilized on the surface; resulting in a fully active immobilized bacteriophage capable of capturing and infecting target bacteria.

Here we report a method for the introduction of affinity tags onto bacteriophage heads based on a phage display technique. As a model system, the well characterized T4 bacteriophage and its host *Escherichia coli* B were used. As affinity tags, biotin-binding polypeptide (Biotin Carboxyl Carrier Protein, BCCP, from acetyl-CoA carboxylase of *E. coli*) and a Cellulose Binding Module (CBM, of xylanase from *Thermotoga maritime*) were displayed on the bacteriophage head as fusions with the Small Outer Capsid (SOC) protein. Both recombinant bacteriophages were effectively immobilized onto streptavidin modified surfaces or cellulose-based surfaces retaining the ability to capture target cells and.high infectivity.

2 MATERIALS AND METHODS

Bacterial strains, bacteriophages and reagents. The *Escherichia coli* strain BL21(DE3), Nova blue competent cells and the expression vector pET22(b) were obtained from Novagen (Madison, WI). *E. coli B* and bacteriophage T4 (D) were obtained from the culture collection at the Canadian Research Institute for Food Safety (CRIFS). The integration plasmid pRH and bacteriophage T4-Z were kindly provided by Dr. L. Black, University of Maryland and pET28-CBM9-GFP was kindly provided by Dr. C. Haynes, University of British Columbia. Bacteria were cultured at 37°C for 14-16 h in LB broth/agar (LB-Broth/Agar, Difco) or LB supplemented with 100 µg/ml of

ampicillin as indicated. Enumeration of bacteria was performed by plate count, and enumeration of phage particles was performed by the top layer agar method.

DNA extraction was performed using Ultra Clean Microbial DNA Isolation Kit (Biolab, Inc.), and QIA Quick Spin kit, (Qiagen) for bacteria and bacteriophage, respectively.

To determine ATP concentration, bioluminescent ATP reagent from BioTrace (UK) was used according to the manufacturer's instructions. The bioluminescence intensity was measured using a luminometer (GEM Biomedical, Hamden, CT).

Construction of *soc-bccp* and *soc-cbm* expression vectors. The *bccp, soc and cbd* DNA fragments were generated by PCR using respective DNA templates and primers and sub-cloned into pET22 plasmid. Expression of fusion proteins was confirmed by SDS-PAGE and RT-PCR.

Construction of T4-soc integration vectors was performed using pRH plasmid with cloned *soc-bccp* and *soc-cbm* fusions as described previously [4].

Homologous recombination was performed according to Ren et. al. [5]. Display of BCCP, biotin and CBM on the bacteriophage head was confirmed by SDS-PAGE and Western Blotting.

Immobilization of recombinant bacteriophages was performed by overnight incubation of the phages with streptavidin-labeled magnetic beads (Dynal Biotech), microcrystalline cellulose beads (CP-102, Asthi Kasei Chem. Corp. Japan) or cellulose-based filter paper (Whatman No.4) at room temperature and with gentle shaking. Unbound phage was removed by washing 3 times with PBS-Tween 20 solution.

The ability of immobilized bacteriophages to capture the target bacterial cells was tested by incubating bacterial cell suspension with immobilized bacteriophage for one hour at room temperature and assessing the extent of capture by the decline of bacterial cell numbers in the supernatant.

The ability of immobilized bacteriophages to lyze the target bacterial cells was assessed by measuring the release of intracellular ATP during the incubation of bacterial cells with immobilized phage. The zone of lysis produced by immobilized bacteriophage on a lawn of *E. coli* cells was also assessed visually.

3 RESULTS AND DISCUSSION

3.1 Immobilization of bacteriophages

Recombinant T4 bacteriophages displaying biotin (BCCP-T4) and cellulose binding module (T4-CBM) were constructed as described in Materials and Methods section and immobilized on streptavidin-coated magnetic beads and cellulose-based materials, respectively. The specificity of binding was investigated by comparing the extent of removal of both recombinant and wild type phage from suspension in the presence of the respective media.

For biotin labeled bacteriophage (BCCP-T4), the number of bacteriophage particles (plaque forming units, pfu) was determined in a 1 ml sample both before adding 20 µl of streptavidin-labeled magnetic beads containing ~ 2×10^7 particles and following removal of the beads. The amount of bound bacteriophage was estimated by subtraction, and expressed as a percentage of the original phage population. The results are presented in Table 1.

Table 1. Capturing of the bacteriophage by Streptavidin-labeled Dynabeads™.

BCCP-T4, log pfu/ml			T4, log pfu/ml		
Before	After	%	Before	After	%
7.4	5.6	99.4	7.5	7.3	33
5.7	4.3	96	6	5.9	20
3.1	0	100	4.3	4	50
2.7	0	100	2.7	2.4	50
1.3	0	100	1.3	0	100

Table 2. Capturing of the bacteriophage by microcrystalline cellulose.

Beads mg	CBM-T4, log pfu/ml			T4, log pfu/ml		
	Before	After	%	Before	After	%
10	4.48	4.11	56	5.70	5.66	8
	4.47	4.11	57	4.43	4.21	40
	4.18	3.68	68	4.13	3.89	43
	3.88	3.44	64	3.83	3.43	60
	3.57	3.16	62	3.53	3.33	37
100	4.48	3.78	82	5.47	4.63	86

For BCCP-T4 bacteriophage at a concentration below 10^3 pfu/ml, no phage particles were detected in the supernatant after removal of the magnetic beads coated with immobilized phage. At phage concentrations between 10^5 - 10^7 pfu/ml, a greater than one log cycle reduction in the number of phage particles was observed; indicating up to 90-100% binding of bacteriophage by the beads. In contrast, the extent of binding was much less (20-50%) for wild type bacteriophage treated with streptavidin-coated magnetic beads, thus confirming the specificity of binding through biotin-streptavidin interaction in the case of BCCP-T4.

For CBM-T4 bacteriophage, two capture media were used for immobilization – microcrystalline cellulose beads and filter paper. The extent of binding by microcrystalline cellulose beads is shown in Table 2. In this case, 1 ml of bacteriophage suspension was incubated with 10 or 100 mg of microcrystalline cellulose beads overnight at room temperature with gentle shaking. The supernatant was then removed by decanting, beads were washed with PBS-Tween and bacteriophage particles were enumerated in the supernatant before and after addition of beads, as well as in wash solutions. The amount of bound phage was calculated

as described above. At a low concentration of cellulose beads (10 mg/ml), approximately 62.7% of CBM-T4 phage and 45% of wild type T4 were bound when the initial phage concentration was 3×10^3 - 3×10^4 pfu/ml. However, at a higher concentration of bacteriophage (5×10^5 pfu/ml) only 8% of phage particles were bound to cellulose beads. At a higher cellulose concentration (100 mg/ml), the percentage of binding was similar for both CBM-T4 and T4, being 82 and 86%, respectively. The high level of binding for wild type T4 bacteriophage could be explained by the fact that, in general, bacteriophage binding receptors located on their tail fibers are specific for certain carbohydrate moieties of the LPS layer of the cell wall, such as glucose and galactose. The surface of microcrystalline cellulose on the other hand is composed of oriented glucose rings that may mimic the surface of the cell wall and thus bind bacteriophage particles through their receptors. In this case the infectivity of immobilized bacteriophage would be compromised.

In order to investigate the strength of bond between CBM-T4 bacteriophage and cellulose beads, extensive washing of the immobilized CBM-T4 and T4 was performed every 24 h for 4 days; followed by enumeration of phage particles in all washing solutions. The results are presented in Table 3. It was observed that for the recombinant CBM-T4 bacteriophage, only 5% of bound phage were removed by washing; while for wild type T4, 20% of bound phage were removed. This indicates that the specific interaction of CBM with cellulose results in stronger, almost irreversible, binding as compared with the non-specific binding of wild type T4 with cellulose-based surfaces.

Table 3. De-sorption of bacteriophage from cellulose beads by extensive washing with buffer.

	CBM-T4, pfu per sample	T4, pfu per sample
Initial phage concentration	2.7×10^4	5×10^5
Supernatant	1.2×10^4	4.6×10^5
Bound	1.5×10^4	4×10^4
1st wash	220±80	5×10^3
2nd wash	65±5	2.3×10^3
3rd wash	420±80	640
4th wash	65±5	340
Total washed off (% of bound)	5	20.7

From the data obtained, it was concluded that recombinant bacteriophages specifically bind to the respective support through the interaction of the displayed affinity tag with the active group on the surface of the solid support. A high degree of non-specific binding was observed when T4 bacteriophage was immobilized on cellulose.

3.2 Infectivity of immobilized bacteriophages

The infectivity of immobilized bacteriophages was assessed in three ways: i) by measuring their ability to capture target bacteria; ii) by estimating the extent of bacterial lysis by monitoring the release of intracellular ATP; and iii). by determining the zone of lysis produced on a bacterial lawn.

The extent of capture of *E. coli* by immobilized BCCP-T4 bacteriophage is presented in Figure 1. Wild type T4 bacteriophage immobilized non-specifically on streptavidin-coated magnetic beads was used as a control. It was observed that 65-100% of *E. coli* cells were captured by immobilized BCCP-T4 compared with 12-30% captured by non-specifically immobilized wild type T4.

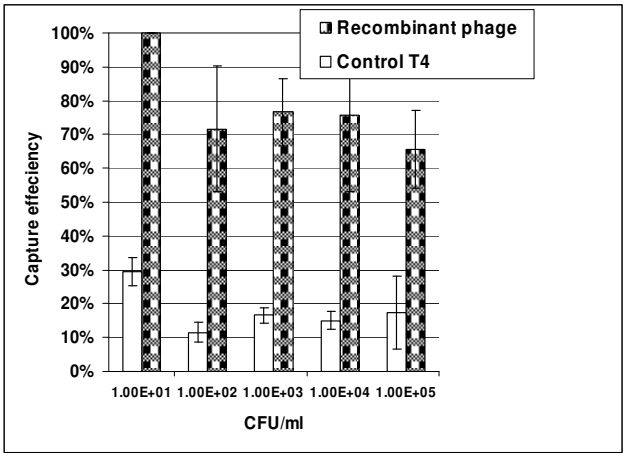

Figure 1. Bacteria capture efficiency for biosorbent based on BCCP-T4 bacteriophage immobilized on Streptavidin magnetic beads.

The captured bacteria were lyzed both by the addition of ATP-releasing agent, and 'naturally' by bacteriophage-mediated lysis. The released intracellular ATP in both cases was measured using bioluminescent ATP reagent. The results are presented in Figure 2.

It was observed that phage-mediated lysis of captured bacterial cells resulted in the release of 10-fold more intracellular ATP (point 3, Figure 2) than chemical lysis (line 2, Figure 2). This confirms that immobilized BCCP-T4 bacteriophage retain a high level of infectivity.

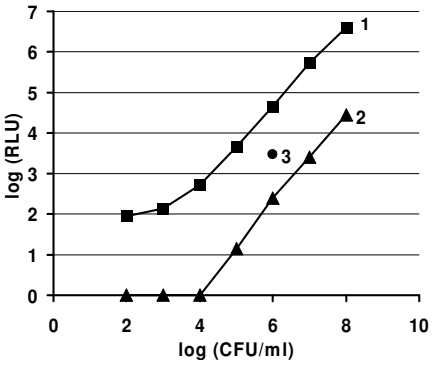

Figure 2. ATP-bioluminescence in Relative Light Units (RLU) for *E. coli* cells: 1- cell suspension in broth, ATP released by ATP-releasing agent; 2 - cells captured by the BCCP-T4 bacteriophage immobilized on Streptavidin magnetic beads and ATP released by ATP releasing agent: 3, cells captured by the beads and ATP released by phage-mediated lysis.

The extent of *E. coli* capture by CBM-T4 phage immobilized on cellulose beads is presented in Table 4.

Table 4. Bacteria capture efficiency for biosorbent based on T4 bacteriophage immobilized on cellulose beads.

Initial concentration of bacterial cells, CFU/ml	*E.coli* in supernatant, after 30 min incubation	
	CBM-T4	T4
5×10^2	$(7\pm2)\times10^2$	5.4×10^2
5×10^3	5×10^3	$(7\pm2)\times10^3$

Though the level of bacterial capture by immobilized T4-CBM phage was undetectable by the plate count technique, the immobilized phage showed a high level of infectivity compared with wild type T4 non-specifically immobilized on cellulose beads. The zone of lysis observed for T4-CBM immobilized on filter paper was significantly greater than for wild type T4 (Figure 3).

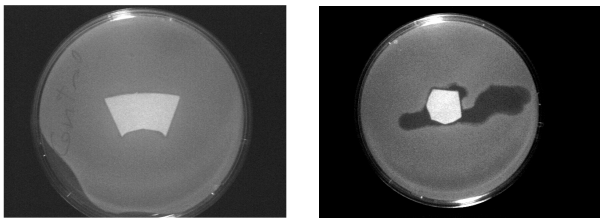

Figure 3. Zone of *E.coli* lysis produced by bacteriophage T4 (left) and T4-CBM (right) immobilized onto filter paper.

This supports our previous conclusion that wild type T4 non-specifically immobilized onto cellulose-based solid surfaces might have lower infectivity due to interaction of phage binding receptors with sugar rings of cellulose.

4 CONCLUSIONS

We have shown that T4 bacteriophage displaying biotin binding polypeptide (BCCP) or cellulose binding module (CBM) on the head could be efficiently immobilized on the respective functionalized surfaces. The immobilized bacteriophages retain their ability to capture and lyze target *E. coli* cells. The interaction between the phage head and solid support is very strong, and almost irreversible. The proposed method of phage immobilization was validated for biosensor application [6]. The proposed approach could be extended for different bacteriophages, provided that information on their genetic structure is available to identify non-essential genes encoding head proteins.

5 ACKNOWLEGEMENTS

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