RESEARCH PAPER

Bacteriophage 2:2, 61–69; April/May/June 2012; © 2012 Landes Bioscience

Detection of TNT-derivatives with recombinant phages

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Keywords: TNT, phage, scFv, recombinant, immunoassay

Abbreviations: TNT, trinitrotoluene; TNP, trinitrophenol; scFv, single chain fragment variable; BSA, bovine serum albumin; HRP, horseradish peroxidase; AP, alkaline phosphatase; TNBS, 2,4,6 trinitrobenzenesulfonic acid; TNP-Tris, trinitrophenol-Tris

New immunoreagents for detection of TNT-derivatives TNP and TNP-Tris were developed using phage display technique. The monovalent and pentavalent recombinant phages carrying scFv specific for TNT were constructed and compared with each other to define the impact of valency and molecule dimension on antibody binding in immunoassay. Also, the bifunctional phages were generated, which carried TNT-specific scFvs as well as enzyme β-lactamase as a model marker on its surface. The most sensitive recombinant phages were selected and used for detection of TNP-Tris in a competitive ELISA based on immobilized antigen. Preincubation and partial phages saturation with a sample containing antigen allowed competition with immobilized hapten and displacement of free antigen. The phages exposing enzyme were used as immunoreagents for single step detection. The other phages were detected with specific marked antibodies. To date, the results presented in this paper are the first ever published regarding the recombinant phages for the detection of TNT.

Introduction

The advantage of scFv-fragments is their ability to be exposed on the filamentous bacteriophages. One of the most used phages is bacteriophage M13, which infects *E.coli*. The phage genome consists of a single chain DNA, which is enclosed by the protein capsid.

The presentation of antibody fragments is primarily done through the direct modification of the phage genome on which the resistance and cloning sites were previously inserted. Thus, the phage vectors were generated.[1] Alternatively, so-called phagemid-vectors are used, which carry one copy of the capsid protein gene and a packing signal.[2,3] The phagemid vector contains replication origin of both plasmid and phage DNA (e.g., M13KO7) and can autonomously replicate in *E. coli*. In order to replicate itself as phage DNA, this vector needs the additional presence of helper phages and genetic information from them. In that case, both genes from phagemid and phage wild-type DNA are expressed and both recombinant and wild-type proteins can be exposed on capsid. Due to one mutation in wild-type DNA, the recombinant phages would encapsulate preferentially phagemid vector as its genetic information. By carrying cloned genes for the fusion proteins, these phages would expose them on its surface. The protein that is mostly used for the fusion with target proteins is g3p capsid protein, which could be found in 2,700 copies. Depending on the choice of the fusion genes (g3 or g8) in the phage vector, monovalent or pentavalent display can be achieved. The construction of pentavalent scFv-phages results in the enhancement of avidity, which significantly changes their binding properties and dissociation of the antigen.

During the cloning of scFv-library, the recombinant phages that carry scFvs on their surface are obtained; they constitute the fusion proteins together with the capsid proteins. Every phage can carry exactly one scFv and is coded by this phage. This technique allows selection of the single antigen-binding clones from one big library. During the selection, a target antigen is initially immobilized on the solid surface. After the selectant was added, the bound phages could be detected in ELISA. The elution is performed with a significant change of pH, enzymatic cleavage with proteases or competition with free target molecule.[4-6]

In this study, new recombinant phage immunoreagents for detection of TNT-derivatives TNP and TNP-Tris[7] were developed. From the one point of view, the monovalent phages were to be compared with the pentavalent ones to define the impact of valency and molecule dimension on the antibody binding in immunoassay. Alternatively, the bifunctional phages were planed, that would carry TNT-specific scFv-fragments as well as the multiple marker enzymes on its surface. Enzyme β-lactamase was used as a model, because it offers the optimal preconditions for expression as a fusion protein due to its low molecular weight and the structure that contains only one protein chain. The most sensitive recombinant phages were selected and

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Submitted: 01/11/12; Revised: 03/12/12; Accepted: 04/16/12
http://dx.doi.org/10.4161/bact.20408

used in immunoassay for detection of TNP-Tris depending on their binding properties and affinity. As a commonly used assay, a competitive ELISA based on immobilized antigen was performed. Small antigens, that generate an immune response only if immobilized on a large carrier such as protein, are called haptens. For the detection of TNP-Tris, the hapten-protein conjugate TNP-BSA was available. The preincubation and partial saturation of the phages with the sample containing antigen allowed competition with immobilized hapten and displacement of the free antigen. The phages exposing enzyme had to be used as direct detection reagents while the other phages should be detected with the specific marked antibodies.

To this date, the results presented in this paper are the first ever published regarding the recombinant phages for the detection of TNT.

Results

Monovalent 11B3-scFv phages. Monovalent phages were generated using the synthetic vector (phagemid), which can autonomously be replicated in *E. coli* as a plasmid. Vector pCANTAB5E was available as a phagemid. This vector possessed the gene for the ampicillin resistance and g3 for the fusion with a target molecule. The strategy was to clone the 11B3-scFv gene in front of g3p in order to create the phagemid with the fusion gene scFv-g3, which would appear as fusion protein in the protein cover. First, the 11B3-scFv gene was amplified with the appropriate oligonucleotides and inserted in the vector between the restriction sites *Sfi*I and *Not*I in front of g3p. The clones were checked for the presence of 11B3-scFv with PCR (**Fig. 1**). One of the positive clones was infected with the helper phages with MOI = 10 and recombinant phages were isolated with the titer of 1.0×10^{12}.

The fragment 11B3-scFv inserted in the vector pCANTAB5E was amplified with the oligonucleotides 11B3 Nco for and 11B3 Nco back. Five microliters of marker Eco130I (M) and 5 µl of amplificate (1) were separated on the agarose gel.

Pentavalent phages. For the pentavalent display of 11B3-scFv, a phage fd-Tet vector was used. Fd-Tet vector contained all the genes for phage replication and encapsulation as well as the tetracycline resistance and could independently replicate within *E. coli*.

The pentavalent monofunctional phages had to be constructed with all of the capsid proteins in the wild-type form excluding the g3p, which had to be fused with 11B3-scFv. The insertion of 11B3-scFv was performed between the restriction sites *Sfi*I and *Not*I. The clones were characterized in PCR with the fragment-specific oligonucleotides (**Fig. 2**).

The fragment 11B3-scFv inserted in the vector fd-Tet was amplified with the oligonucleotides 11B3 Nco for and 11B3 Nco back. Five microliters of the marker Eco130I (M) and 5 µl (1) of the amplificate were separated on 1% agarose gel.

Being that fd-Tet was the phage vector, it was necessary to bring this vector into the TG1-cells. The phages replicated autonomously and were secreted from the bacterial cells into the medium. After incubation of the 500 ml culture overnight in the medium containing tetracycline, the phages were isolated with the titer 8.0×10^{9}.

Pentavalent bifunctional 11B3-scFv-phages with β-lactamase. For the phage production two vectors were used: phage vector fd-Tet with 11B3-scFv-gene and plasmid pCANTAB5E with the gene for β-lactamase. The vector pCANTAB5E already contained the gene g8p as a capsid gene and the gene for β-lactamase had to be cloned in front of the gene g8p. The ligation of amplified β-lactamase fragment occurred between the restriction sites *Sfi*I and *Not*I. The clones were checked for the presence of fragment using the restriction enzymes *Sfi*I and *Not*I. Additionally, the clones were analyzed in PCR with the fragment-specific oligonucleotides (**Fig. 3**). The presence of β-lactamase gene was verified with both methods.

The gene for β-lactamase in the vector pCANTAB5E was amplified with the oligonucleotides betaLac Sfi for and betaLac Not back (pET/pHEN2). Five µl of the marker Eco130I (M) and 5 µl of the amplificate (1) were separated on 1% agarose gel.

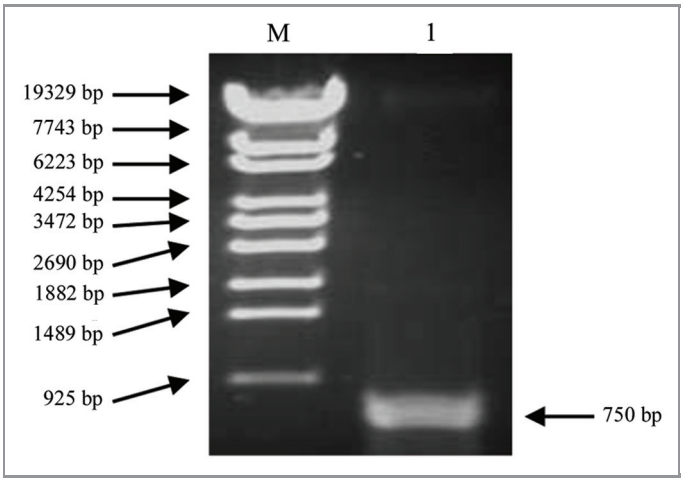

Figure 1. Amplificate of 11B3-scFv with 750 bp.

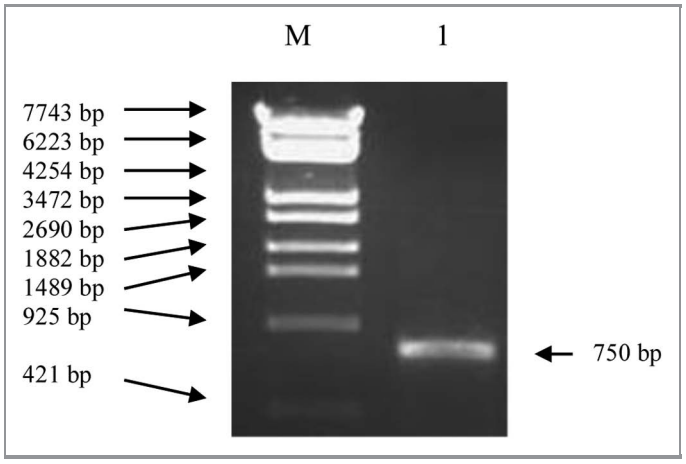

Figure 2. Amplificate of 11B3-scFv with 750 bp.

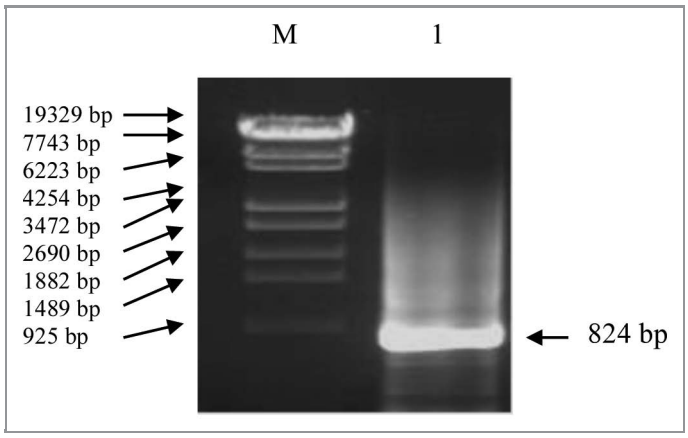

Figure 3. Amplificate of β-lactamase with 824 bp.

To generate pentavalent bifunctional phages, TG1 cells containing the vector pCANTAB5E-g8-β-lactamase were infected with the pentavalent monofunctional phages at the density of OD = 0.5. The infection was done at MOI = 1 followed by the 30 min incubation. The culture was centrifuged and the cells were placed into a 500 ml 2YT-medium containing tetracycline and ampicillin. After the overnight growth the phages were isolated, but the titer could not be determined (see the Discussion).

Assay with the monovalent 11B3-scFv phages. The monovalent phages were produced with the titer of 1.0×10^{12}. The functionality of these phages had to be verified in ELISA for the detection of TNP.

Detection with anti-M13-IgG-HRP conjugate. ELISA was performed with the phage dilutions of 1:2 and 1:5 in 2% milk powder. The detection was performed only with the primary antibody anti-M13-IgG-HRP in dilutions of 1:500, 1:1,000, 1:2,500, 1:5,000 and 1:10,000. Because of the high background only the antibody dilution 1:10,000 was considered and showed on **Figure 4**.

After covering with TNP-BSA in PBS overnight and BSA as a negative control (overall dark lines), the phage dilutions 1:2 (1) and 1:5 (2) in 2% MPBS were incubated for 60 min. After the 60 min incubation period of the primary antibody anti-M13-IgG-HRP (1:10,000) ended the substrate ABTS was added and the signal was measured at 405 nm.

Detection with anti-M13-IgG and anti-mouse-IgG-AP conjugate. A direct assay using these detection reagents was performed with the phage dilution of 1:5. The working dilutions were 1:100 for the primary antibody anti-M13-IgG and 1:5,000 for the secondary antibody anti-mouse-IgG-AP (both recommended by the manufacturer). The result is shown on the **Figure 5**.

After covering with TNP-BSA in PBS (1) over night and BSA as a negative control (2), the phage dilution 1:5 in 2% MPBS was incubated for 60 min. After the 60 min incubation of the primary antibody anti-M13-IgG (1:100) and secondary antibody anti-mouse-IgG-AP (1:5,000), the signal was detected at 405 nm using the substrate pNPP.

Pentavalent monofunctional 11B3-scFv phages. Pentavalent phages were produced with the titer of 8.0×10^{9}. The functionality of these phages also had to be verified in ELISA for the detection of TNP.

Detection with anti-M13-IgG-HRP conjugate. The direct assay was performed similarly to the one with the monovalent phages. The used phage dilutions were in the range of 1:2 to 1:3,000. The most favorable dilution for the subsequent performing of the

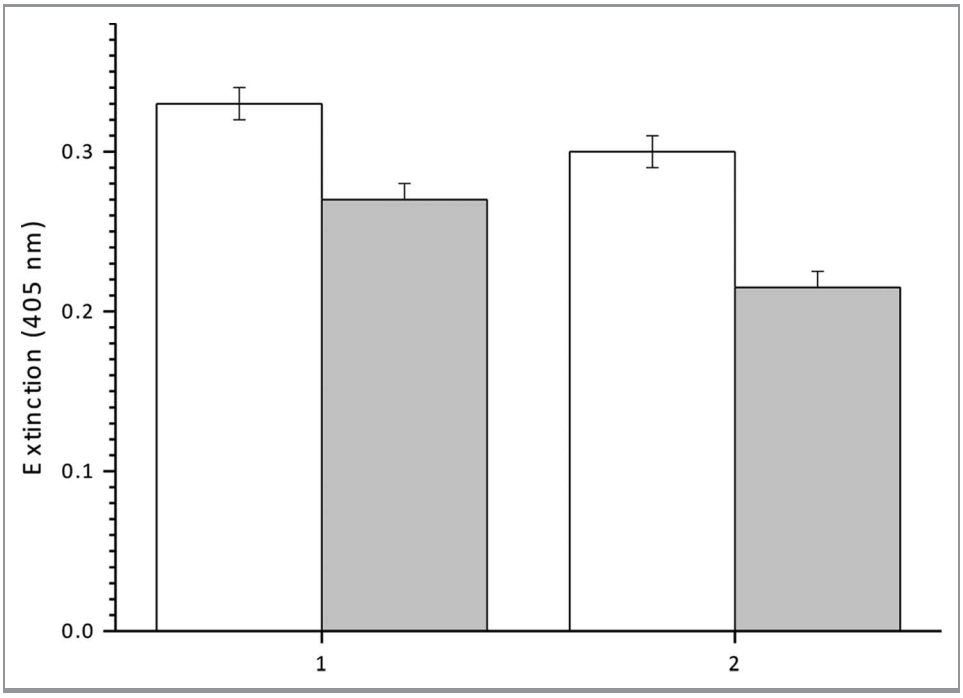

Figure 4. Detection of monovalent phages in ELISA with the primary antibody.

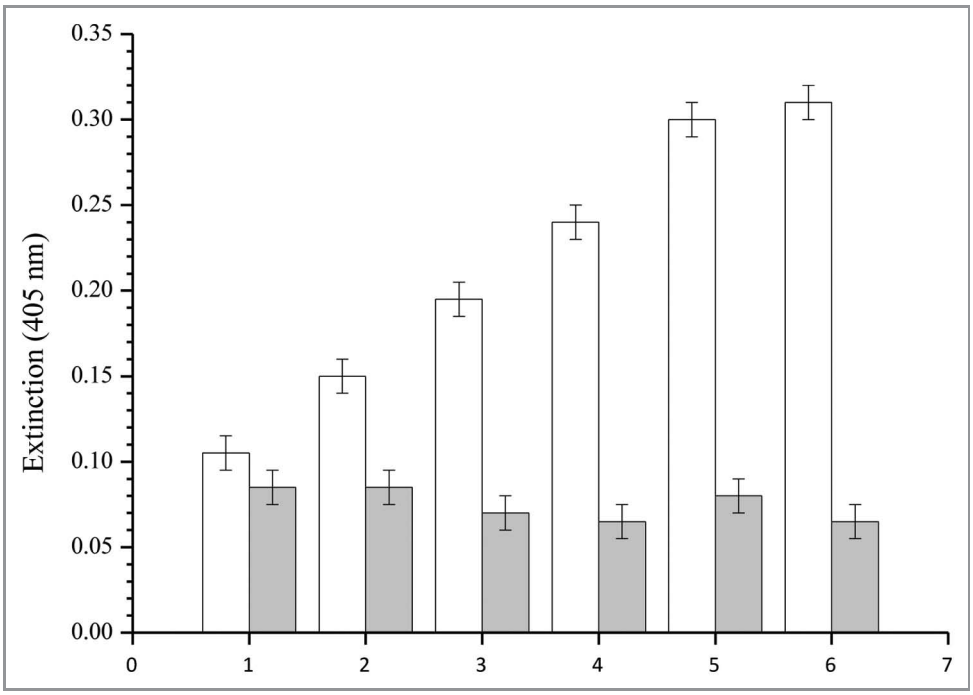

Figure 5. Detection of monovalent phages in ELISA with the primary and secondary antibodies.

competitive assay proved to be 1:3,000 with signal of 0.335 compared with the control signal of 0.126 (**Fig. 6**).

After covering with TNP-BSA in PBS overnight and BSA as a negative control (overall dark lines), the phage dilutions 1:2 (1), 1:20 (2), 1:200 (3), 1:1,000 (4) and 1:3,000 (5) in 2% MPBS were incubated for 60 min. After the 60 min incubation of primary antibody anti-M13-IgG-HRP (1:5,000) and adding the substrate ABTS, the signal was measured at 405 nm.

The competitive assay was performed with the established dilutions 1:3,000 for the phages (titer 2.67 × 10^6) and 1:5,000

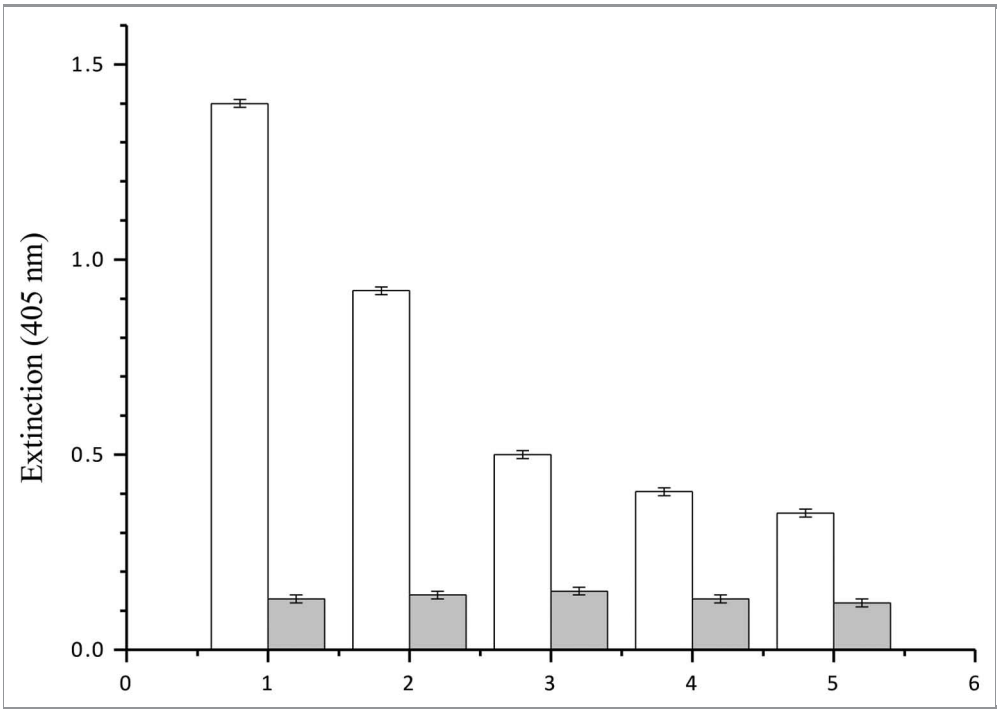

Figure 6. Detection of pentavalent monofunctional phages in ELISA with the primary antibody.

for anti-M13-IgG-HRP conjugate. Five minute-incubation period of the phage dilution with a concentration range of the competitor TNP-Tris (2.5×10^{-5} to 2.5×10^{-8}) gave no signal inhibition of the bound phages compared with a positive control without the inhibitor.

Detection with the anti-M13-IgG and anti-mouse-IgG-AP conjugate. A direct assay with these detection reagents was performed with the phage dilution of 1:3,000. The working dilutions were 1:100 for the primary antibody anti-M13-IgG and 1:5,000 for the secondary antibody anti-mouse-IgG-AP (both recommended by the manufacturer). The phages were detected with the strong signal and the low background (**Fig. 7**).

After covering with TNP-BSA in PBS overnight and BSA as a negative control (overall dark lines), the phage dilutions 1:2 (1), 1:20 (2), 1:200 (3), 1:1,000 (4) and 1:3,000 (5) in 2% MPBS were incubated for 60 min. After the 60 min incubation of the primary antibody anti-M13-IgG (1:100) and the secondary antibody anti-mouse-IgG-AP (1:5,000), the signal was detected at 405 nm using substrate pNPP.

The application of pentavalent phages for detection of TNP-Tris. In order to use these phages for the detection of TNP-Tris, the attempt was made to increase significantly the concentration of the competitor that would allow to see if they could be inhibited at all. The concentration of TNP-Tris was made in the range 2.5 M to 2.5×10^{-4} M and it was incubated with the phage dilution of 1:3,000 for different time periods (5, 10, 15, 20, 30 and 60 min). Afterwards, an assay was performed as described above with the anti-M13-HRP conjugate (1:5,000) as a detecting reagent. During the optimization of preincubation time, it was concluded, that no signal difference occurred using different time periods. As the optimal preincubation time for an assay, the period of 5 min was chosen.

When optimizing the inhibition concentrations of TNP-Tris, it was shown that the lowest dilution of 2.5×10^{-4} M gave no signal inhibition at all (**Fig. 8**). The lowest measurable signal inhibition occurred with a TNP-Tris concentration of 2.5×10^{-3} M. TNP-Tris-concentration that reduced signal completely was 2.5 M. Through these facts, it was found that: (A) the complete inhibition of 1.6×10^{5} phages (60 µl of 2.67×10^{-6} dilution) occurred with a TNP-Tris quantity of 2.5 µmol (1 µl of 2.5 M solution), (B) with these phages, the detection limit for TNP-Tris was 2.5 nmol (1 µl of 2.5×10^{-3} M solution).

The same assay was performed with identical parameters using anti-M13-IgG and anti-mouse-IgG-AP conjugate as the detection reagents. Detection limit of 2.5 nmol TNP-Tris (1 µl of 2.5×10^{-3} M solution) was also confirmed (data not shown).

After covering with TNP-BSA in PBS overnight and BSA as a negative control (overall dark lines), the phage dilution 1:3,000 in 2% MPBS was incubated for 5 min with different quantities of TNP-Tris [2.5 µmol (1), 250 nmol (2), 25 nmol (3), 2.5 nmol (4) and 250 pmol (5)], respectively. Positive control was the phage dilution without the competitor (6). All probes were then placed in ELISA-wells and incubated for 1 h. After the 60 min incubation of the primary antibody anti-M13-IgG-HRP (1:5,000) was over and the substrate ABTS was added, the signal was measured at 405 nm.

Pentavalent bifunctional phages. The pentavalent bifunctional phages were produced with the unknown titer. For the verification of β-lactamase activity nitrocefin was used as a substrate.[8] The color change in the phage solution (diluted

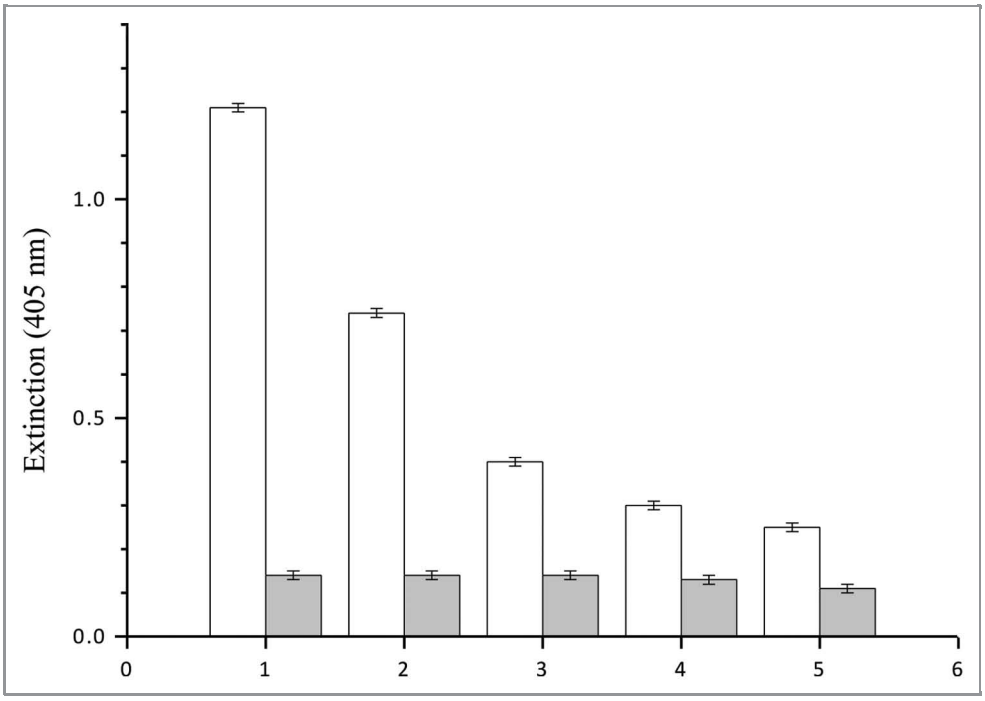

Figure 7. Detection of pentavalent monofunctional phages in ELISA with primary and secondary antibodies.

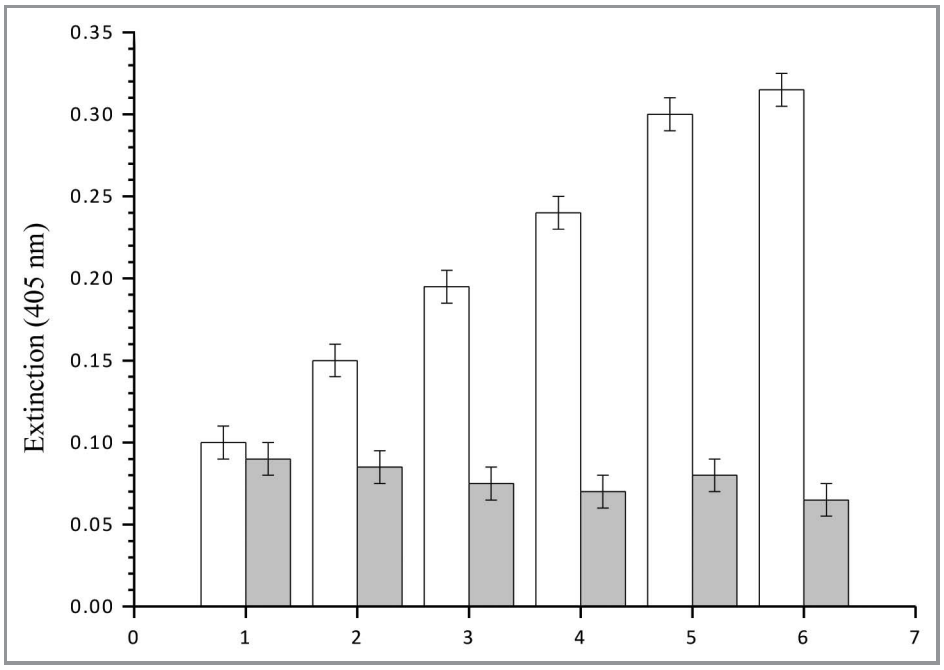

Figure 8. Determination of inhibition area for pentavalent phages in TNP-BSA-ELISA.

maximal to 1:100) confirmed the functionality of the expressed enzyme.

These phages were used in a direct ELISA, which was done in the same manner as for their analogs, in the serial phage dilutions from 1:2 to 1:1,000 with a single modification in the detection step. The detection was done directly by an expressed β-lactamase and its substrate nitrocefin, but no signal was registered.

Discussion

Monovalent phages. The monovalent monofunctional phages were produced in the high titer. The strategy was to clone the 11B3-scFv gene in front of g3p in order to create the phagemid with fusion gene scFv-g3, which would after the infection with the helper phages appear on a capsid as a fusion protein. Because the protein g3p is normally present on a capsid in five copies, it is possible to vary the ratio between the wild-type and the fusion protein using different MOI (multiplicity of infection). To present only one copy of scFv on a phage surface (monovalent display), the cells carrying phagemids had to be infected with the high MOI of the helper phages, so that preferentially wild-type g3 protein was incorporated into capsid.

In a direct ELISA with the monovalent phages, only the primary antibody as a single detection reagent was used at the beginning. All the phage concentrations caused very high background (0.21–0.29), whereas their positive signal was between 0.24 and 0.36. This is the consequence of the fact that the most phages tend generally to bind unspecifically at almost all surfaces. The lowest background of 0.21 was obtained with the phage dilution 1:5 and the anti-M13-IgG-HRP dilution of 1:10,000 (**Fig. 4**). Positive signal was in this case around 0.29. Being that the background was too high compared with the positive signal, no competitive assay could be performed. These phages were alternatively detected with the anti-M13-IgG and the anti-mouse-IgG-AP conjugate. The problem was, again, extremely high background (value of 0.25) compared with a positive signal 0.51 (**Fig. 5**). However, the space between the positive signal and the negative control (background) was better than in the one component detection system. This could be explained through the observation that more specificity and sensitivity were achieved by introducing the two antibody detection system. In the experiments that followed, with and without the competitor TNP-Tris, the undesirable background signal could not be eliminated, although the additional washing steps were attempted. For this reason, it was decided not to perform the competitive assay.

Pentavalent monofunctional phages. To generate the pentavalent phages, five copies of 11B3-scFv were needed on the phage surface. g3p is present in five copies in a capsid, so that a number of five fusion proteins were achieved with insertion of scFv-gene in front of g3 in the phage vector fd-Tet.

The pentavalent monofunctional phages were produced in the lower titer than the monovalent phages. Low titers can be usually explained with the fact that longer incubation than 4–5 h may result in the deletions and lower yield of the amplified phages. Also, it is critical for cultures to be well aerated and the phages should either be added to an early-log culture, $A_{600} < 0.01$, or to a 1:100 dilution of an overnight culture. Despite of the low titer, the pentavalent phages were highly detectable in a direct ELISA. The dilutions under 1:200 were not considered, because the obtained signal was too high. Compared with the monovalent phages, no high background signal occurred here, as well as in the high concentrated dilutions. The most favorable dilution for the competitive assay proved to be 1:3,000 with a signal of 0.33 compared with a negative control of 0.13 (**Fig. 6**).

Table 1. Genotypes of used bacterial strains

TG1:	supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5 (rK– mK–) [F′ traD36 proAB lacIqZΔ*M15*]
DH5:	F-, endA1, gyrA96, thi-1, hsdR17(r_K^-,m_K^+), supE44, relA1. Φ 80Δ lacZΔ M15, Δ (lacZYA-argF),U169

In the experiments done for establishing a competitive assay, a 5 min-incubation of the phage dilution with a concentration range of the competitor TNP-Tris (2.5×10^{-5} - 2.5×10^{-8} M) gave no signal inhibition of the bound phages compared with a positive control without the inhibitor. Based on this fact, one can conclude that these phages cannot be inhibited with the low TNP-Tris concentrations because of their pentavalency. Only high levels of the inhibitor (range 2.5 M to 2.5×10^{-4} M) gave measurable signal inhibition (**Fig. 8**) as the result of high antigen quantity for the saturation of five antibody units per phage particle. The same assay was performed with two-antibody detection system, which gave slightly lower signal than the one component system (**Figs. 6 and 7**). The two-component system has a disadvantage that the dissociation is increasingly favored with a growing number of the binding components. Thus, this system carries not only the advantages of the higher sensitivity and specificity, but also a risk of the favored dissociation.

Pentavalent bifunctional phages. Pentavalent bifunctional phages were thought to carry five copies of 11B3-scFv and a high number of β-lactamase in the protein cover. 11B3-scFv was fused with g3p and β-lactamase with g8p. These bifunctional agents, which carried simultaneous immune and enzyme component should possess on the one hand, high sensitivity due to the large number of detecting molecules and on the other hand very good binding properties due to the high avidity of five scFvs acting together.

To generate the pentavalent bifunctional phages, TG1 cells containing vector pCANTAB5E-g8-β-lactamase were infected with the pentavalent monofunctional phages. The infection was done at MOI = 1, which meant that recombinant g8p from pCANTAB5E was preferred. Because pCANTAB5E in this case did not contain g3, the pentavalent display of recombinant g3p-11B3-scFv from the phage fd-Tet vector was ensured at the arbitrary MOI.

For the pentavalent bifunctional phages, the titer determination was impossible because in each experiment the same number of clones persisted in all dilutions, which was the indication of a permanent contamination.

Materials and Methods

Cell lines/bacterial species. The experimental part of the study was performed with the different bacterial strains *E. coli* K12. For cloning and standard usage in molecular biology, DH5α, Top10, etc. were primarily used. When working with antibody libraries F+ supE-strain TG1 was most frequently used (**Table 1**).

Vectors. For cloning, the phage vector fd-Tet was obtained from G.Winter, Center of Protein Engineering, MRC Cambridge. pCANTAB vector was purchased from Amersham Pharmacia Biotech.

Antibody fragments and enzymes. scFv-fragment 11B3 and the gene for β-lactamase were available. 11B3 is a murine antibody specific for TNT.[9] Beta-lactamases are enzymes which inactivate β-lactam-antibiotics. In this study, the β-lactamase RTEM1 from *E. coli* was used.[10]

Helper phages. Within the diverse phage production, the helper phages M13K07 were used and they were purchased from Amersham Pharmacia Biotech.

Oligonucleotides. The used oligonucleotides were synthesized by Metabion (**Table 2**).

Standard molecular biology techniques. PCR, ligation, restriction, DNA dephosphorylation, agarose gel electrophoresis, DNA extraction from agarose gels, classic plasmid preparation, alcohol precipitation, DNA quantification, production of competent *E. coli*-cells, transformation with electroporation, etc. were done according to the standard protocols.[11]

Production of M13KO7 helper phages. Volume of 0.5 ml logarithmic growing culture of the suppressor strain *E. coli* TG1 was placed into 4 ml soft-agar-solution, which was incubated at 42°C, shortly mixed and poured out on preheated 1× YT-plate, on which the helper phages M13K07 were immediately placed. After hardening, the plate was incubated for 12 h at 37°C. The regions with good formed plaques were inoculated in 200–400 ml of 2× YT-kanamycin-medium and hold for 10–16 h at 37°C and 250 rpm on a shaker. After centrifugation, the supernatant was mixed with 1/5 volume fraction of PEG 6000 / NaCl and the phages were precipitated at 4°C for 2–12 h. After centrifugation at 4,000 U/min and 4°C for 30 min the supernatant was discarded and after repeated centrifugation, the pellet was resuspended in 5 ml PBS. After centrifugation at 10,000 ×g and 4°C for 30 min the supernatant with the phages was separated from the cell rests, mixed with glycerol to the final concentration of 15–30% and stored at –20°C. Finally, the titer of the stock solution was determined.

Table 2. The oligonucleotides used in the study

Name	Sequence 5′-3′	Target sequence	Restriction site
11B3 Nco for	CATGCCATGGCCCAGGTGAAGCTG	11B3-scFv	*Nco*I
11B3 Nco back	CATGCCATGGCCCGTTTTATTTCCA	11B3-scFv	*Nco*I
βLac Not back (pET/pHEN2)	GATCGCGGCCGCCCAATGCTTAATCAGTGA	βLac	*Not*I
11B3 Sfi for (pHEN2)	ATAATGGCCCAGCCGGCCATGGCCCAGGTGAAGCTG	11B3-scFv	*Sfi*I
βLac Sfi for	ATAATGGCCCAGCCGGCCATGCACCCAGAAACGCTGGTGAA	βLac	*Sfi*I
11B3 Not back (pHEN2)	GATCGCGGCCGCCCGTTTTATTTCCAGCTT	11B3-scFv	*Not*I

Titer determination of M13 phages. For the exact adjusting of the infection multiplicity, the titer of a certain stock solution must be known. For this purpose, 200 µl of logarithmic growing culture of suppressor strain *E. coli* TG1 was mixed with 200 µl of suitable phage dilution in 2× YT-medium and incubated for 30 min at 37°C without shaking. Afterwards, 100 µl of this solution was placed on TYE-plates with the appropriate resistance. Beside controls, the serial dilutions from 10^{-6} to 10^{-12} were made. After incubation at 37°C for 12 h, the resulting clones were countered and the titer was determined. The average titer value was in the area of 10^{12}–10^{13} cfu/ml.

Recombinant phages production (rescue). The cells of the suppressor *E. coli* strain TG1 carrying phagemid were cultured in 2× YT-AG-medium until density of OD_{600} = 0.5 was reached. This value correlates with the cell number of 4×10^8 per ml and is the basis for the infection and its infection multiplicity. In the cultures with high densities the linear dependence was assumed, whereas the cultures with the densities over OD_{600} = 0.8 were not used. The infection multiplicity varied between 1 and 20 depending on the probes. High densities assured the monovalent display (wild type was forced) and the low ones allowed the multivalent one. After the helper phages in a suitable quantity of cells were added, the incubation was performed at 37°C without shaking for 30 min. The centrifugation was done at 2,500 ×g for 10 min and the cells were resuspended in the 2× YT-AK-medium. The phage secretion occurred over night for the maximum of 16 h at 30°C and 250 rpm. For the selection, the volumes of 10 ml were usually infected and then resuspended in 50 ml volumes. After centrifugation at 4,000 ×g and 4°C for 30 min, the cell rests were separated from the culture and the phages were precipitated from the supernatant using 1/5 volume parts PEG 6,000/NaCl for at least 2 h on ice. After centrifugation at 3,000 ×g for 30 min at 4°C, the supernatant was removed and the precipitate was resuspended in PBS. After recentrifugation at 10,000 ×g and 4°C for 10 min, the supernatant with the phages was separated from the cell components and stored at 4°C. Finally, the titer of the phage suspension was determined.

ELISA. *Direct assay.* For the ELISA analysis, the 96-wells plate was used, which was covered over night at 4°C with a strong diluted TNP-BSA conjugate (50 µg/ml). The wells were covered with BSA, which was a negative control in each assay. After the washing procedure (three times with cold PBS and TPBS, respectively), the wells were blocked with BSA for 45 min at room temperature. After the identical washing process, the anti-TNT-phages were incubated for 1 h at the room temperature on a rocker platform. After the same washing procedure, the primary antibody for detection was added and incubated for 1h at room temperature on a rocker platform. If the secondary antibodies were used, this step was repeated with the specific antibody-HRP or -AP conjugates. Following the washing process, the appropriate solution for detection was added and the signal was measured in the ELISA-reader after 10 min. ABTS was used as a detection solution for HRP and 16 mM para-nitrophenyl-phosphate (pNPP) in TBS pH 9.5 served as a detection solution for AP.[12] The measuring procedure was always performed twice.

Competitive assay. Competitive ELISA was performed similarly to the direct assay. The only change was the incubation of phages with a certain concentration of the antigen (competitor, inhibitor) TNBS for 5–60 min before placing into ELISA-wells, in order to cause the competition and subsequent signal inhibition. The amount of antigen could be calculated based on the signal reduction.

Future Perspectives

In this study, the TNT-specific scFv-phages were constructed and for the first time used for the detection of TNT-derivatives. With the detection limit of 2.5 nmol TNP-Tris (1 µl of 2.5×10^{-3} M solution), they proved to be 10^5 less sensitive than the bivalent anti-TNT-antibodies.[7] Additionally to standard detection protocols (1° and 2° antibodies), a new approach that included the bifunctionality was designed for the direct detection with the exposed enzymes. The detection enzyme β-lactamase proved to be insensitive both in the experiments with the phages and with some scFv-enzyme fusion proteins (in press). The future works would concern the enzyme sensitivity enhancement. An alternative approach concerning higher sensitivity of β-lactamase could be achieved with the fluorescent substrate CCF2.[13] Enzymes such as alkali phosphatase with a large turnover number could potentially serve as the high sensitive one-component detection systems. Alternatively, on the solid phase covered with the streptavidin, the phages exposing both scFv and biotin can be applied. Using fluorescently marked TNP-Tris molecules, a competitive assay can also be established.

With the change from optical to electrochemical detection, the recombinant fusion protein would be cloned and expressed with a suitable enzyme (alkaline phosphatase, β-galactosidase), for which the substrates already exist with the changeable redox potentials.[14,15] With no enhancement of assay sensitivity, electrochemical detection would allow the rapid identification of the TNT-derivatives in the turbid solutions as well.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

One part of this work was supported by the Ministry of Education and Science of Republic of Serbia, grant 31093. Special thanks to Professor Dr R. Bredehorst and Dr E. Spillner from the University of Hamburg, Germany, for their support during the experimental part of this work.

References

1. McCafferty J, Fitzgerald KJ, Earnshaw J, Chiswell DJ, Link J, Smith R, et al. Selection and rapid purification of murine antibody fragments that bind a transition-state analog by phage display. Appl Biochem Biotechnol 1994; 47:157-71, discussion 171-3; PMID:7944335; http://dx.doi.org/10.1007/BF02787932
2. Short JM, Fernandez JM, Sorge JA, Huse WD. Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. Nucleic Acids Res 1988; 16:7583-600; PMID:2970625; http://dx.doi.org/10.1093/nar/16.15.7583
3. Barbas CF, 3rd, Kang AS, Lerner RA, Benkovic SJ. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc Natl Acad Sci U S A 1991; 88:7978-82; PMID:1896445; http://dx.doi.org/10.1073/pnas.88.18.7978
4. Griffiths AD, Williams SC, Hartley O, Tomlinson IM, Waterhouse P, Crosby WL, et al. Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J 1994; 13:3245-60; PMID:8045255
5. Ward RL, Clark MA, Lees J, Hawkins NJ. Retrieval of human antibodies from phage-display libraries using enzymatic cleavage. J Immunol Methods 1996; 189:73-82; PMID:8576582; http://dx.doi.org/10.1016/0022-1759(95)00231-6
6. Meulemans EV, Slobbe R, Wasterval P, Ramaekers FC, van Eys GJ. Selection of phage-displayed antibodies specific for a cytoskeletal antigen by competitive elution with a monoclonal antibody. J Mol Biol 1994; 244:353-60; PMID:7527464; http://dx.doi.org/10.1006/jmbi.1994.1735
7. Simonovic M, Milosevic-Zlatanovic S, Milosavic N, Vrvic M, Simonovic B. Characterization of recombinant antibodies for detection of TNT and its derivatives. Chemical Papers 2009; 63:391-8; http://dx.doi.org/10.2478/s11696-009-0043-5
8. Guay R, et al. IRCS Med Sci 1980; 8:209.
9. Whelan JP, Kusterbeck AW, Wemhoff GA, Bredehorst R, Ligler FS. Continuous flow Immunosensor for detection of explosives. Anal Chem 1993; 65:3561-5; http://dx.doi.org/10.1021/ac00072a005
10. Rodrigues ML, Presta LG, Kotts CE, Wirth C, Mordenti J, Osaka G, et al. Development of a humanized disulfide-stabilized anti-p185HER2 Fv-β-lactamase fusion protein for activation of a cephalosporin doxorubicin prodrug. Cancer Res 1995; 55:63-70; PMID:7805042
11. Ausubel FM. Current Protocols in Molecular Biology. New York: Wiley Interscience, 1996.
12. Bretaudiere J, Spillmann T. Methods Enzym Anal 1984; 4:75-82.
13. Zlokarnik G, Negulescu PA, Knapp TE, Mere L, Burres N, Feng L, et al. Quantitation of transcription and clonal selection of single living cells with β-lactamase as reporter. Science 1998; 279:84-8; PMID:9417030; http://dx.doi.org/10.1126/science.279.5347.84
14. Thomas JH, Kim SK, Hesketh PJ, Halsall HB, Heineman WR. Bead-based electrochemical immunoassay for bacteriophage MS2. Anal Chem 2004; 76:2700-7; PMID:15144178; http://dx.doi.org/10.1021/ac035503c
15. Yu Z, Xu Y, Ip MP. An ultra-sensitive electrochemical enzyme immunoassay for thyroid stimulating hormone in human serum. J Pharm Biomed Anal 1994; 12:787-93; PMID:7918781; http://dx.doi.org/10.1016/0731-7085(94)E0005-L