Production of polyclonal antibody against M13 phage for application in nanobody technology

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ABSTRACT

The identification of specific binders requires the screening of hundreds of thousands of molecules from genetic libraries by the phage display technique, which has been routinely used for the isolation of the genetically engineered antibody fragments, including those from the heavy chain antibody of camels, also called Nanobody® (Nb), to a wide variety of antigens. M13 bacteriophage is one of the most important vectors in phage display, thus specific polyclonal antibody was produced in a rabbit after immunization with four injections with highly pure M13. Antibody purification, using protein A sepharose affinity chromatography column, was performed to obtain reactive and pure anti-M13 IgG. The potency and specificity of the purified IgG were examined by ELISA using M13 phages displaying two different Nbs against the green fluorescent protein (GFP) as antigen. Anti-M13 antibody has interesting potentials in Nb technology, including in the detection and the measurement of M13 phages, as well as in phage display biopanning.

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List of Abbreviations

BSA: bovine serum albumin
DMSO: dimethyl sulfoxide
E. coli: Escherichia coli
ELISA: enzyme–linked immunosorbant assay
FPLC: fast protein liquid chromatography
GFP: green fluorescent protein
HCAb: heavy chain antibody
HRP: horseradish peroxidase
IgG: immunoglobulin G
IPTG: isopropyl β-D-thiogalactoside
Nb: nanobody
Nb–phage: nanobody–displaying M13 phage
PBS: phosphate buffered saline
PEG: polyethylene glycol
Pfu: plaque forming unit
RT: room temperature
scFv: single chain variable fragment
TMB: 3', 3',5', 5'-tetramethylbenzidine

INTRODUCTION

Phage display technique enables the identification of antibody fragments against specific antigen through the screening of huge number of potential binders from a genetic polypeptide library [14]. It is achieved by fusing the polypeptide libraries to phage coat proteins, and the resulting phage particles (virions) display the polypeptides on their surfaces and they also contain the coding sequence in their genetic materials. Interesting members from the library can be isolated with simple biopanning selection by binding to an immobilized ligand, and the polypeptide can be identified from its encapsulated DNA [6]. Phage display libraries have become a
standard tool to identify peptides and proteins that bind antibodies, cellular receptors and other biological molecules. Using this technology, extremely rare binding clones were captured out of a huge library of recombinant fusion phage [5,27,23]. One of the most successful applications of phage display has been the isolation of the variable domains of the conventional antibodies (single-chain variable fragment; scFv) against different targets.

The 25 kDa scFv antibody fragment is created by cloning the antibody genes coding for the variable domain of the heavy and the light chains which fold in the space to form the antigen binding cavity [13]. In the camel family (Camelidae) about half of the antibodies circulating in the blood lack a light chain and these "incomplete" antibodies, referred to as heavy–chain antibodies, are able to bind their targets just as firmly as conventional antibodies [16]. The antigen–binding fragment of these antibodies is reduced into one single domain of only 15 kDa, known as a "nanobody (Nb)" [21]. The potential of Nbs as an attractive alternative to scFv in biotechnology, diagnostic and therapeutic applications has been demonstrated [13]. Nb drugs are used against cancer, rheumatoid arthritis, inflammatory bowel disease and Alzheimer's disease [11]. Nbs are not chemical hydrophobes, resistant to heat and pH, stable at room temperature (RT) and have a long shelf life without refrigeration [29].

Although other display systems have been described, the most popular vehicle for display remains the filamentous bacteriophage. The nonlytic filamentous phage, like M13, infects strains of E. coli by attaching to the tip of the pilus, and the phage genome is translocated into the cytoplasm [6]. The particle of M13, ~1 μm in length but less than 10 nm in diameter, consists of a single-stranded DNA core surrounded by a proteinaceous coat [32]. The coat contains five different proteins, but the vast majority consists of several thousand copies (~2700) of the gene–8 major coat protein (P8) which covers the length of the particle. The four minor coat proteins are present at about 5 copies per particle; (P7 and P9) cap one end of the particle while (P3 and P6) cap the other end [26]. Bacterial infection is initiated by the attachment of the phage P3 to the F pilus of a male E. coli (e.g., E. coli TG1), M13 induces a state in which the infected bacteria produce and secrete phage particles without undergoing lysis [4]. Most recently, highly engineered M13 bacteriophage has been studied as a template for the assembly of nanoribbons, including nanoparticle arrays and nanowires, which have many applications in nanotechnology [20,15].

Generally several rounds of selection on an immobilized antigen are required to enrich and isolate the binding phages. The need to determine phage titers before and after each round of selection as well as to detect the increase in specific enrichment during the panning process still represents a major time and material consuming factor within this procedure. Usually, the phages are titered by infecting E. coli with serial dilutions of phage, plating the bacteria on agar plates and counting the colony (cfu) or plaque forming units (pfu) after overnight incubation [17]. However, determining the relative number of phage particles could be easily achieved by immunotitration with specific anti-M13 antibodies using "p hage titration ELISA", which offers a fast, sensitive and reproducible alternative for titration of M13 bacteriophage preparations [25]. Antibodies are widely used in diagnostic and therapeutic applications [8,28], as well as in biochemical and biological research such as ligands for the preparation of immunoaffinity columns [24]. They are also used as coating or labeling reagents for the qualitative and quantitative determination of molecules in a variety of assays, such as ELISA, double diffusion and western blot [10,7,9]. Hence was the aim of this study is to produce polyclonal antibodies specific for M13 phage. Antibody to M13 phage is instrumental in the titration of M13 phages during phage display panning and in the detection of antigen-specific phages by means of their displayed-antibody fragments, e.g. Nbs displaying phages (Nb-phage) specific to the green fluorescent protein (GFP) as antigen. Anti-M13 specific polyclonal antibody has many applications in phage display–related immunoassays and represents an important tool in Nb technology.

**MATERIALS AND METHODS**

*Preparing of M13 phages:*

A single colony of E. coli TG1 cell from fresh M9 medium (20 mM NH₄Cl, 20 mM KH₂PO₄, 10 mM NaCl, 20 mM Na₂HPO₄, 2 mM MgSO₄, 0.1 mM CaCl₂, 0.2 % glucose and 2 % agar) petri dish was cultured overnight in 10 ml LB medium (Bio Basic INC) at 37 °C. The next day 1 ml of the culture was inoculated into 250 ml 2×TY medium (1.6 % tryptone, 1 % yeast extracts and 0.5 % NaCl) in 1 L flask and incubated at 37 °C, 200 rpm, till an optical density of OD₆₀₀ = 0.5 is reached. M13K07 helper phages (GE Life Science) – 10¹⁷ pfu Øthen were added to the culture which was incubated for 30 minutes without shaking at RT, and then for further 30 minutes with gentle shaking (200 rpm) at 37 °C, in order to allow phage infection. After that Kanamycin (70 μg/ml) was added and the culture was grown overnight at 37 °C with shaking (250 rpm). The next day, the cells were pelleted by centrifugation, and M13 phages were recovered from the supernatant by precipitation in 5:1 v:v volume of polyethylene glycol (PEG)/NaCl (20 % PEG 6000 and 2.5 M NaCl). Tubes were mixed gently and incubated for at least 1 h on ice to allow phages precipitation. Finally phages were recovered by centrifugation,
removing supernatant and resuspending the pellet in 0.5 ml PBS containing 7% dimethyl sulfoxide (DMSO). Phage concentration was measured by spectrophotometer at OD_{260} (1 OD = 10^{11} pfu/ml).

Purification of M13 phage by gel filtration:

Superdex 200 10/300 GL column (GE Life Science) was used for the purification of M13 phage, which was obtained in the previous step. The column was attached to the device AKTA prime and equilibrated with 60 to 90 ml of sodium phosphate buffer at a flow rate of 1 ml/min. M13 phages sample (10^{12} pfu/ml) was filtered through a 0.45 μm filter just before it was applied into the chromatography column (0.5 ml). Eluted sample in the first peak, containing pure M13, was concentrated to 10^{11} pfu/ml (~1 mg/ml) on Vivaspin concentrators with a molecular mass cutoff of 100 kDa (Vivascience).

Rabbit immunization:

For the first immunization, purified M13 phage (5×10^{10} pfu/injection) in 1 ml PBS was mixed with an equal volume of Freund's complete adjuvant (Bio Basic Inc.) to form a stable emulsion. One adult female white rabbit aged 2 months (weighing ~2 kg) was injected subcutaneously at 2 to 4 different sites. Three booster injections were given mixed with incomplete Freund's adjuvant at 15 days intervals. Serum samples were collected from the rabbit before immunization (day 0) and this pre-immunized serum was used as a negative control for the whole study. To evaluate the immune responses of the injected antigen, blood samples (0.5–1 ml) from the rabbit were collected at regular intervals before each injection and final bleeding (30 ml) was done 15 days after the last boost at day 60.

Purification of M13 polyclonal antibodies:

Polyclonal anti-M13 antibody (IgG) was purified from 5 ml rabbit serum by affinity chromatography on a 5 ml HiTrap Protein A column (GE Life Science) according to the manufacturer's instructions. Binding was performed in 0.02 M sodium phosphate, pH 7 and rabbit IgG was eluted with 0.1 M citric acid, pH 3. Eluted IgG was collected and immediately neutralized to physiological pH with 1 M Tris-base buffer, pH 9 and then concentrated to 1 mg/ml on Vivaspin concentrators with a molecular mass cutoff of 50 kDa (Vivascience).

Preparation of Nb-phages:

TG1 cells were transformed with pMES4 phagemid containing the coding sequence of two anti-GFP specific Nbs, Nb07 and 08, which were previously retrieved by phage display from a DNA library prepared from camel immunized with GFP. Single colonies were inoculated in 1 ml 2×TY containing carpenicillin (100 μg/ml) and glucose (2%) in polystyrene 24-well flat bottom tissue culture plate (Falcon) for 2–3 hours with shaking (250 rpm) at 37 °C till OD_{600} = 0.5 is reached. M13K07 helper phage ~ 10^{10} pfu then was added to each well and the plate was incubated for 30 minutes without shaking at RT, and then for 30 minutes at 37 °C with shaking at 150 rpm. The plate was centrifuged at 3500 rpm for 15 minutes and the supernatant was discarded. After drying the plate, the cells in each well were resuspended in 1 ml 2×TY containing carpenicillin (100 μg/ml) and kanamycine (70 μg/ml) and incubated overnight at 37 °C with shaking (150 rpm). Next day the plate was centrifuged at 3500 rpm for 15 minutes at 4 °C. Nb-phages were precipitated from the supernatant by PEG/NaCl as previously described, and phage concentrations were determined by phage titration ELISA and adjusted to 10^{10} pfu/ml.

ELISA:

GFPexpressing plasmid pRSET-sGFP was used to transform E. coli BL21(DE3) cells in order to produce pure GFP, which was used as ELISA immobilized antigen, as previously described (Al-Homsy, et al., 2012). An indirect ELISA format was employed for the assay of immobilized rabbit sera and the detection of Nb-phages, while sandwich ELISA format was used for the detection and measuring of M13 phages. Maxisor96-well plates (Nunc) were coated overnight at 4 °C with M13 phage (10^{9}pfu/well), GFP (0.25 μg/well) or rabbit anti-M13 diluted (1/2000) in carbonate buffer. After coating, ELISA plates were washed 3 times with the washing buffer TBS–T (20 mMTris–base, 150 mMNaCl, 0.05% Tween–20, pH 7.5). Residual protein binding sites in the wells were blocked for 1 h at 37 °C with 5% blocking buffer (3% skimmed milk and 1% BSA) in TBS–T. After the removal of blocking buffer, rabbit anti–M13 sera, serial dilutions of rabbitanti–M13 IgG, Nb–phages or the serial dilutions of M13 phage were all diluted in 1× blocking buffer and added in the wells for 1 h at RT. After 3 washes, detection of bound phages was performed with rabbit anti–M13,mouse anti–M13(P3) (New England Biolabs) or antiM13(P8) antibody conjugated to horseradish peroxidase (HRP; GE Life Science). After plate washing, all rabbit antibodies were detected by 1 h incubation at RT with goat anti–rabbit conjugated to HRP (Bethyl Laboratories Inc.) at 1:3000 in 1× blocking buffer. After an additional 5 washes, bound conjugate was detected with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate.
(Sigma), the reaction was stopped after 10 minutes with the addition 1M $\text{H}_2\text{SO}_4$. The spectroscopic absorbance of the enzymatic reaction was measured in an automated plate reader at a wavelength of 450 nm.

**Results:**

**Purification of M13 phage:**

M13 helper phage (M13K07) was propagated by infecting exponentially growing *E. coli* TG1 strain to ensure $\text{F}^-\text{pilus}$ production. Phages were recovered from the culture medium by precipitation with PEG and then further purification step was performed by gel filtration in order to obtain a pure virus (Fig. 1A). In this technique, which separates proteins on the basis of differences in molecular size, separation is achieved using a porous matrix to which smaller molecules have greater access and larger molecules less and the sample is thus eluted in decreasing order of size. The protein fractions eluted are detected by an in–line UV monitor and collected for subsequent specific analysis or further preparation steps. In this result, M13 phages are large molecules and they were eluted rapidly from the column in the void volume as the first peak (Fig. 1A). In this regards, gel filtration is mainly used in the final purification stage to remove a few remaining contaminants from the target. By adding this purification step, M13 phage was purified from the contaminant bacterial extract traces which were not eliminated during the preparation of the virus. For further confirmation, ELISA test was set up in which equal antigen quantities from all gel filtration peaks were immobilized and detected by mouse anti–M13(P8) and anti–M13(P3) antibodies. The reactivity was obviously seen in the first second fraction peaks (Fig. 1B), confirming that those two fractions contain M13 phage. However, because of the low ELISA signal of anti–M13(P3) in the second peak and its low yield after gel filtration, it was neglected and the first one was considered as the source of pure M13 phage for this study.

**Fig. 1:** Purification of M13 phage by gel filtration

(A) M13 phage obtained after infection of TG1 bacterial cells with M13K07 helper phage and precipitation of virus from the supernatant by PEG. M13 sample ($10^{12}$ pfu/ml, 500 µl) was injected in Superdex 200 10/300 GL column at flow rate of 1 ml/min, and peaks of protein fractions were collected. (B) Equal quantities (0.25 µg/well) from all fraction peaks (from 1 to 9), resulted from gel filtration, were ELISA tested using monoclonal anti–M13(P3) and (P8) antibodies, and no antibody (No Ab) as negative control. These two commercial antibodies target the main coat proteins of M13 phage (inset).

**Rabbit immunization with M13 phage:**

An adult female rabbit was immunized with four doses of pure M13 phage by subcutaneous injections. Blood samples were collected at several time points from the start of the immunization and tested in ELISA against immobilized M13 phage to evaluate the raise of specific immune response (Fig. 2A). A solid phase ELISA test of the serum dilution (1:2000) prepared from these samples revealed an increase in reactivity toward M13 phage after two weeks of immunization onwards. Rabbit immune response against M13 phage increased almost exponentially up to day 30, after which it maintained the same level until the final bleeding at day 60 (Fig. 2A).

**Purification of anti–M13 IgG:**

IgG from immunized rabbit serum sample at day 60, were purified by affinity chromatography using proteinA column. An automated purification procedure was established using the AKTA prime system allowing a direct and confirmed purification of total IgG from 5 ml serum. A second purification step was done using the
flow-through from the first injection to confirm the total purification of IgG from the serum (Fig. 2B). Titration of purified anti-M13 IgG showed a high reactivity and important median effective concentration (EC50) towards its antigen, in the range of 20 ng/ml (Fig. 2C).

**Fig. 2:** Purification and testing of rabbit anti-M13 IgG

(A) The reactivity of rabbit sera (diluted to 1:2000) taken at different time points (days) during immunization, were ELISA tested in the absence (No Ag) or the presence of immobilized M13 phage (10^8 pfu/well). Antibody/antigen interaction was detected by goat anti-rabbit HRP conjugated antibody. (B) Purification of IgG from rabbit serum by affinity chromatography using AKTA prime FPLC system. Five ml of rabbit serum (day 60) was injected (inject 1) onto HiTrap Protein A column and washed with phosphate buffer (void) to remove the unbound proteins (flow-through), before eluting pure IgGs (eluate). A second purification step (inject 2) was done using the flow-through from the first injection to confirm the total purification of IgG from the serum. (C) Purified IgG (1 mg/ml) was titrated by ELISA using the indicated serial concentrations (ng/ml) in the absence or the presence (No Ag) of antigen (M13 phage, 10^8 pfu/well). Estimated EC50 (ng/ml) for anti-M13 IgG was indicated.

**Phage titration ELISA:**

Rabbit anti-M13 specific IgG can not only detect M13 phage but also can be used for the titration of M13 phage in unnecessarily pure samples, basing on a sandwich ELISA technique. Rabbit IgG was coated onto microtiterplate and was used to capture M13 particles from the specimen. Captured M13 particles are detected by a peroxidase conjugated monoclonal antibody to P8. This result showed a typical titration curve when using serial dilutions of M13 phage (Fig. 3A) and allows the quantitation of samples of an unknown M13 phage according to the following formula: x = \[\text{exponent} \left(\frac{y + 7.7}{0.515}\right)\], where “y” is the absorbance of the tested sample and “x” is its calculated concentration (pfu/ml), and the accuracy of the equation was R^2 = 0.9727 (Fig. 3B).

**Nb−phage ELISA:**

Another application of anti-M13 IgG was for the detection of antigen−bound M13 phages which display specific Nbs at their tips. After the construction of Nb library, the standard phage display panning procedure started by infecting E. coli TG1 cells of the library with M13KO7 helper phage to produce virus particles with the cloned Nbs at their tips. After several rounds of panning, phage particles with specific Nbs are enriched. Clear antigen−specific enrichment among rescued phage particles from the primary library and after each round of panning could be assessed by “polyclonal phage ELISA” against immobilized antigen (Fig. 4A). The utility of anti-M13 IgG in this phage ELISA was tested using M13 phages displaying two different Nbs, Nb07 and Nb08, which are specific to GFP (Fig. 4B). Anti-GFP specific Nb−phages were prepared by infecting transformed TG1 colonies, containing Nb genes within pMES4 phagemid, with M13 helper phage. Rescued virions were precipitated from culture supernatant and dosed by the previous titration ELISA in order to use
equal quantities of both Nb-phages (10^{10} pfu/ml). Comparing to control M13 helper phage, this result showed that both Nb-phages were able to recognize their target immobilized antigen. However, detection signal using rabbit anti-M13 was much more important than using the commercial mouse anti-M13(P8) antibody (Fig. 4C).

Fig. 3: Phage immunoassay by sandwich ELISA
(A) Sandwich ELISA test for the detection of serial concentrations of pure M13 phages (pfu/ml), using rabbit anti-M13 IgG (R—a—M13, 1/2000) as capturing immobilized antibody and mouse anti-M13(P8) conjugated to HRP (1/3000) as detecting antibody (inset). Same virus concentrations were tested in the absence of immobilized antibody. (B) The linear fit curve of a the data set from the previous ELISA experiment, using the Least Square method, where x is M13 phage concentrations (pfu/ml) and y is their absorbance values (OD_{450}). Unknown phage concentrations could by calculated using the equation \[ y = a \ln(x) + b \], where a is slope (= 0.5), and b is intercept (-7.7). The calculated correlation coefficient (R^2), which is an indicator of the "goodness of fit" is shown as well.

Fig. 4: Immunodetection of Nb-phages by anti-M13IgG
(A) Detection of two anti-GFP specific Nb-phages (Nb07 and Nb08) was done using phage ELISA in the absence (No Ag) or the presence of immobilized GFP (0.25 µg/well). Detection of bound Nb-phages (10^{10} pfu/ml) was achieved by either rabbit anti-M13 IgG followed by goat anti-rabbit (G—a—R—HRP) HRP conjugated antibody (B), or with commercial mouse anti-M13(P8)HRP conjugated antibody (C). M13 helper phage (10^{10} pfu/ml) was used as negative control.

Discussion:
The phage display technology is a powerful and well-established tool for the investigation of protein–protein interactions and for the generation of human antibodies for in vitro diagnostics and in vivo therapy(Nuttall and Walsh, 2008). One of the most important applications of phage display is the selection and screening of recombinant antibody, scFV for Nb, libraries [12,19,14,1]. This work describes the production of anti-M13 phage polyclonal antibody, which provides a useful tool for phage display technology. Grace of the high reactivity of this antibody, as demonstrated by its elevated EC_{50}~20 ng/ml, it is considered as an important tool for the detection of M13 phages which are displaying specific peptides or recombinant antibodies at their tips. When recombinant antibodies are displayed on M13 phage surface and bind to their antigen, the M13 bound phage can then be detected by commercial mouse monoclonal anti-M13(P8) HRP labeled antibody.
that recognizes the P8 coat protein [4]. If peptides were fused to P3, bound phage can be detected by rat monoclonal antibody that recognizes an epitope localized in the N-terminal portion of P3 [18]. Although monoclonal M13 antibodies are very specific, they are very hard to produce, expensive and target one epitope which can be covered in case of M13 fusion with Nb or other recombinant proteins. However, polyclonal anti–M13 antibodies can help in amplifying the signal from target phage even with low expression level, as M13 phage will be bound by several antibody molecules. Nevertheless, time scale of producing M13 monoclonal antibodies is long considering hybridomas preparation [33].

In the present study, gel filtration chromatography was carried out in order to obtain highly purified M13 phages from the supernatant of the inoculated E. coli cells. Gel filtration has also been used for the purification of bacteriophages DNA [3]. After immunization, rabbit antisera have demonstrated a specific activity towards M13 bacteriophages. Since a strong immune response was mounted a few days after rabbit immunization start, M13 phages were presumed to be highly immunogenic. Specific polyclonal anti-M13 IgGs were then obtained by affinity chromatography using protein A sepharose affinity column. Protein A and Gaffinity chromatography is the fastest method for purifying antibodies. Protein A purification is recommended for rabbit antibodies and is the best candidate to isolate monoclonal and polyclonal IgG from ascites, serum, tissue culture and bioreactor supernatants [30].

The sandwich ELISA method, which was designed in this study, using anti–M13 polyclonal antibody was successfully led to create a simple formulaseful for the determination of M13 phages concentration. This method might replace the traditional way for measuring M13 titration based on viral infectivity, which are generally time consuming. Later conjugating of anti–M13 polyclonal antibody with HRP or alkaline phosphatase enzymes could omit the need of conjugated secondary antibody in immunoassays [31]. Furthermore, sandwich ELISA test will be structured with homemade anti–M13 and anti–M13 conjugated antibodies without more need for commercial ones. In addition, this pure and specific anti–M13 rabbit polyclonal antibody serves as a valuable tool to detect M13 phage in fusion with specific Nbs displayed on P3 coat protein. It effectively amplifies the detection signal more than commercial monoclonal anti–M13 antibody, since several thousand copies of P8 exist on the phage surface. Also, the detection of M13 phages alone or in fusion could be carried out even when phages are in the crude supernatant and before purification steps are performed.

**Conclusion:**

The highly reactive polyclonal anti–M13 IgG, which was produced in rabbit after immunization with gel filtrated M13 phage, has multiple applications in many biological and molecular applications, especially in the immunotitration and the detection of the interesting phages issued from the phage display panning, which is an indispensable step in Nb technology.

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**Disclosure:**

The authors report no potential conflicts of interest in this work.

**REFERENCES**


