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Use of antibody gene library for the isolation of specific single chain antibodies by ampicillin–antigen conjugates

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ABSTRACT

Isolation of recombinant antibodies from antibody libraries is commonly performed by different molecular display formats including phage display and ribosome display or different cell-surface display formats. We describe a new method which allows the selection of *Escherichia coli* cells producing the required single chain antibody by cultivation in presence of ampicillin conjugated to the antigen of interest. The method utilizes the neutralization of the conjugate by the produced single chain antibody which is secreted to the periplasm. Therefore, a new expression system based on the pET26b vector was designed and a library was constructed. The method was successfully established first for the selection of *E. coli* BL21 Star (DE3) cells expressing a model single chain antibody (anti-fluorescein) by a simple selection assay on LB-agar plates. Using this selection assay, we could identify a new single chain antibody binding biotin by growing *E. coli* BL21 Star (DE3) containing the library in presence of a biotin–ampicillin conjugate. In contrast to methods as molecular or cell surface display our selection system applies the soluble single chain antibody molecule and thereby avoids undesired effects, e.g. by the phage particle or the yeast fusion protein. By selecting directly in an expression strain, production and characterization of the selected single chain antibody is possible without any further cloning or transformation steps.

1. Introduction

Poly- and monoclonal antibodies are widely used reagents in basic research, diagnostics and therapy. Applications are associated with several disadvantages, most of all the batch-to-batch-variability of polyclonal antibodies and the elevated costs and required time for the production of monoclonal antibodies. Furthermore, applications of full-length antibodies are limited in both, diagnostics and therapy by an inefficient penetration into solid tumors and the blood–brain barrier. To overcome these limitations single chain antibodies fragments (scFv) were established showing several advantages: they are easier to isolate, cheaper to produce and they can be manipulated by standard molecular biology techniques. In contrast, they may have larger conformational flexibility and a higher cross-reactivity as compared to full-length antibodies [1]. Engineering and clinical testing established scFv and scFv-based fragments as therapeutic and diagnostic alternatives to full-length antibodies, e.g. in the treatment of cancer as well as inflammatory, autoimmune and chronic viral diseases [2,3].

Antibody libraries are commonly screened by different molecular display formats including phage display and ribosome display or different cell-surface display formats. Phage display is the oldest and most commonly used molecular display technique [4]. Here, scFv are mostly displayed externally after fusion to the phage minor coat protein pIII [5]. The procedure contains several panning rounds where the antigen is immobilized on microtiter plates [6], immunotubes [7] or via biotin to streptavidin [8]. After washing steps the bound phage can be removed by pH shift [9,10], proteolytic cleavage [11] or competitive by free antigen [12]. Non-specific binding of the phage especially to the solid support remains a major challenge [13]. In contrast, ribosome display offers a completely in vitro transcription and translation system either for prokaryotic or eukaryotic systems. According to the literature, it can display much larger libraries (up to 10^{15}) and avoids problems caused by cellular expression such as different expression levels. Systematic studies revealed that different populations were isolated by phage and ribosome technique and scFv isolated by ribosome display showed a higher average affinity [14].

Cell surface display methods present thousands of copies of scFv on the cell surface, e.g. of yeast, *Escherichia coli* [15] or *Bacillus thuringiensis* [16]. In yeast, the scFv are fused to the α-agglutinin adhesion receptor, which locates the scFv to the cell wall of yeast

Abbreviations: scFv, single chain antibody fragment; PBS/NCS, phosphate-buffered saline/neonatal calf serum; POD, horseradish peroxidase; TMB, tetramethylbenzidine; FITC, fluorescein isothiocyanate; NHS, N-hydroxysuccinimide; IPTG, isopropyl-β-D-thiogalactopyranoside.

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[17]. The use of fluorescence activated cell sorting allows rapid and quantitative screening and enrichment of presenting cells binding to fluorescently labeled ligands. Thereby, yeast display overcomes problems of eluting tightly bound clones and unspecific binding to the matrix used for immobilization. In a systematic study yeast display selected twice as many new antibodies compared to phage display and was less labor intensive [18].

In order to extend the spectrum of methods available to select scFv, we describe a new selection assay method that allows the selection of *E. coli* cells producing the required antibody by cultivation in presence of a microbiostatic agent, in this case ampicillin. Ampicillin is a β-lactam antibiotic acting as a competitive inhibitor of the cell wall synthesis enzyme transpeptidase and requires no uptake into the cell. The method utilizes the neutralization of the microbiostasis of the ampicillin–antigen conjugate by the produced antibody which is secreted to the periplasm. In previous work [19] potassium clavulanate was required to inhibit the activity of β-lactamase encoded by the single chain expression plasmid. Thereby, the antibiotic activity for plasmid maintenance was also lost. To avoid this problem, a new expression system was constructed based on the pET26b vector. The method was successfully established for the selection of *E. coli* cells expressing a recombinant anti-fluorescein scFv out of a majority of *E. coli* cells producing unspecific scFv. Using this system, we could select a new scFv binding biotin out of the library in presence of a biotin–ampicillin conjugate. With this simple selection assay system, positive clones are selected by their ability to grow on the selection medium.

2. Materials and methods

2.1. Strains and growth conditions

Strains and plasmids used in this study are listed in Table 1. *E. coli* NEB 5-alpha chemically competent cells were used for cloning and maintenance of the single chain library. *E. coli* BL21 Star (DE3) cells were used for screening and expression of scFv. Strains were routinely grown aerobically at 37 °C in LB medium at 50 μg/ml kanamycin during cloning procedures and maintenance and in liquid pre- and expression cultures.

2.2. Construction of a vector encoding the anti-fluorescein single chain antibody and a naïve single chain antibody library

A schematic view of the DNA construct is shown in Fig. 1 using the indicated restriction sites. DNA fragments encoding the variable regions of the light and the heavy chain of the monoclonal anti-fluorescein antibody B13-DE1 [20] were amplified introducing *NcoI* and *BamHI* sites for the heavy chain and *HindIII* and *NotI* sites for the light chain from plasmid pCantab6-B13DE1 [21] and cloned into the library vector resulting in plasmid pNG36.

Fig. 1. Schematic view of the vector set-up used for library construction. Shown is the insert construction of pET26b(+), coding sequences for OmpA leader peptide (dotted), for the variable region of the heavy chain (V_H, diagonally down), for the glycine–serine linker (GlySer-linker, white), for the variable region of the light chain (V_L, diagonally up), and for the c-myc- and His_6-tag (gray). Indicated are the restriction enzyme sites used for cloning.

mRNA of naïve Balb/c and C57/bl6 mice spleen cells was isolated and converted into cDNA using Oligo$(dT)_{18}$ and random hexamer primers (Fermentas, St. Leon-Rot, Germany). Variable regions of the light and the heavy chain were generated using adequate degenerated primers (Table 2). The PCR products were purified by gel electrophoresis and digested with either *NcoI-BamHI* (heavy chain) or *HindIII-NotI* (light chain). The obtained fragments were ligated into the vector construct shown in Fig. 1. Several primary transformants were randomly chosen and sequenced. The library was transformed into *E. coli* BL21 star (DE3).

2.3. Verifying expression under selection assay conditions

To verify expression of scFv under selection assay conditions, *E. coli* BL21 Star (DE3) was grown under induced and non-induced preculture conditions and the LB-agar plates were incubated overnight at either 30 °C or 37 °C. Expression of scFv was tested by resuspending single colonies from LB-agar plates containing 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) in 10 μl water and transferring them to a microtiter plate. 50 μl of 50% ethanol was added to lyse the cells and to allow adsorption of the scFv to the microtiter plate. Microtiter plates were incubated overnight at 37 °C. After washing the microtiter plates were blocked with PBS containing 5% newborn calf serum (PBS/NCS) for 60 min. After an additional washing step the microtiter plates were incubated with the mouse anti-c-myc antibody E910 (1:350) and with a horseradish peroxidase-labeled goat anti-mouse immunoglobulin antibody (POD-goat-anti-mouse Ig, Dianova, 1:5000 diluted). The volume was 50 μl per well. Between incubation steps the microtiter plates were washed at least five times with tap water. Bound peroxidase was determined by the substrate 0.04% H_2O_2 and 0.12 mg/ml

Table 1
E. coli plasmids and strains.

Plasmid or strain	Genotype or relevant characteristics	Source or reference
Plasmids		
pET26b(+)	Km^R	Novagen
pCantab6-B13DE1	Anti-fluorescein scFv	[21]
pNG36	pET26b derivative for the production of OmpA-V_H (anti-FITC)-GlySer-V_L (anti-FITC)-MycHis	This work
pMN195	pET26b derivative for the production of anti-biotin scFv (scBiotin)	This work
Strains		
NEB 5-alpha	*fhuA2* Δ(*argF-lacZ)U169 phoA glnV44* Φ*80*Δ (*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
BL21 Star (DE3)	F^- *ompT* $hsdS_B$ ($r_B^-m_B^-$) *gal dcm rne131* (DE3)	Invitrogen

Table 2
Primer sequences for PCR amplification of V_H and V_L (κ- and λ-chain), introduced restriction sites are underlined.

V_H	
Forward primer	GCG AAC CAT GGC ASA RGT SMA RVT GCA G
	GCG AAC CAT GGC ASA RCT KCT CGA GTC
	CGA ATA GTC CAT GGC ACA GGT SCA GCT GCA G
	CGA ATA GTC CAT GGC ACA GGT SMA RCT GCA G
	CGA ATA GTC CAT GGC ACA GGT GCA GCT GCA G
Reverse primer	GCG AAG GAT CCT GMR GAG ACD GTG ASH
	GAG ATA GGC GGA TCC TGC AGA GAC AGT GAC CAG
V_L (λ-chain)	
Forward primer	CGA AGT AAG CTT ATG ACT TGG GCT CCA CTA
	CGA AGT AAG CTT ATG GCC TGG AYT YCW CTY
Reverse primer	CGA AGT GCG GCC GCT AGG ACA GTS ASR TTG GT
	CGA AGT GCG GCC GCC AGT GAC CTT GGT TCC AC
V_L (κ-chain)	
Forward primer	CGA ACC AAG CTT GAT GTT YTR ATG ACC CAR ACT CCA
	CGA ACC AAG CTT GAK MTC DTG MTG ACC CAR WCT CCA
	CGA ACC AAG CTT GAT ATC GTG ATR ACM CAR GAT GAA
Reverse primer	CGA ACC GCG GCC GCC CGT TTB AKY TCC AGC TT