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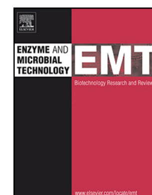

High-throughgput phage-display screening in array format

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ABSTRACT

Emerging technologies for the design and generation of human antibodies require improved approaches enabling their screening, characterization and validation. Currently, strategies based on ELISA or western blot are used to that aim. However, the ever increasing number of novel antibodies generated would benefit from the development of new high-throughput (HT) platforms facilitating rapid antibody identification and characterization. Herein, we describe a protein chip bearing recombinant phage particles and based on a large phage antibody library. In this paper we have set forth a novel implementation which provides a powerful and simple methodology enabling the identification of single-chain variable fragments (scFv). As a proof-of-principle of this method, we tested it with recombinant antigen (human recombinant interleukin 8). Additionally, we developed a novel bioinformatics tool that serves to compare this novel strategy with traditional methods. The method described here, together with associated informatics tools, is robust, relatively fast and represents a step-forward in protocols including phage library screenings.

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1. Introduction

Over the past two decades, the development of molecular display technologies allowed the design and creation of human antibodies [1,2]. Specifically, phage-display technology (PDT) was firstly described in 1985 by Smith and collaborators [3]. It constitutes a powerful tool for the generation of specific antibodies against a pre-defined antigen [4]. Based on the linkage between phenotype and genotype of phages, PDT represents a very convenient approach to study the recognition and interaction between proteins and peptides [5].

Single-chain variable fragment (scFv) is a fusion protein resulting of assembling the heavy variable region (V_H) and the light chain (L_H) of immunoglobulins (Ig_s). scFv retains the epitope specificity and affinity of the original Ig molecule against its antigen. The small size of the scFv facilitates its penetration in target cells, as well as the exploration of cryptic epitopes in the active site of enzymes and membrane proteins. Thus, scFv is the most common way employed to display antibody variable regions in phages [6].

PDT is also utilized by a broad range of applications, including drug and target discovery, protein evolution, and rational drug design [7]. Notwithstanding, the majority of current applications are related to therapy. Main advantages of PDT in comparison to conventional hybridoma-based techniques are: (i) rapid generation time, single antibody genes of Ig variable regions are displayed on bacteriophage surfaces in a short period of time constituting one of the most important advantages of phage-display strategies [8], and

Abbreviations: CCD, charge-coupled device; CDR, complementary determination regions; ELISA, enzyme-linked immunosorbent assay; hr-L8, human recombinant interleukin 8; HRP, horseradish peroxidase; HT, high-throughput; Ig, immunoglobulin; L_H, light chain; NCBI, national center for biotechnology information; NHS, *N*-hydroxysuccinimide; O/N, overnight; PCR, polymerase chain reaction; PDT, phage-display technology; PEG, polyethylene glycol; RT, room temperature; scFv, single-chain variable fragment; SD, standard deviation; TBS, tris-buffered saline; TMB, 3,3′,5,5′-tetramethylbenzidine; TSA, tyramide signal amplification; TYE, tryptone yeast extract; V_H, heavy variable region; 4BCL, 4% cross-linked agarose microbeads.

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(ii) antibody selection against an unlimited number of molecules (biological or not).

In this study, we employed the Tomlinson I+J scFv phage libraries (in phagemid/scFv format-fused to the pIII minor coat protein of M13 bateriophage). This scFv phagemid library contains synthetic V-gene (V_H–V_L) from lox library vector recloned into the pHEN2 phagemid vector. The library size is 1.47×10^8 phagemid clones in *Escherichia coli* TG1 cells, and has a high proportion of functional antibody fragments with approximately 96% of clones containing insert. Here, this library is used as a model because it is a well-known and well-characterized library in the field; also the size is quite suitable for rapid screening with the high-throughput (HT) screening platform proposed in this work.

In general, the main bottleneck putting a brake on PDT are the validation strategies employed to confirm the greatest number of scFv fragments, which requires the development of new HT techniques. Traditional and conventional strategies, as enzyme-linked immunosorbent assay (ELISA) or western blot, are high time-consuming in scFv fragments analysis. On the contrary, other strategies including arraying scFv on nitrocellulose using cellulose-binding domains fused to scFv [9] and microfluidic, microcapillary, and microengraving-based techniques [10] have been developed enable a rapid and HT system for the identification of specific antibodies. For instance, Wang et al. have applied a combination of surface-enhanced Raman scattering microspectroscopy with a microfluidic device for the detection of pathogen antigens [11].

In the present work, we have performed a method based on array technology which allows the deposition of hundreds to thousands of phages by micro-contact in a unique nitrocellulose surface in combination with a bio-informatics approach enabling rapid analysis of scFvs and its comparison with traditional screening strategies such as ELISA Array technology offers a simultaneous analysis in a HT format [12–15] providing useful advantages such as small amount of required sample compared to conventional ELISA (20 nL vs. 100 μL), better resolution, specificity and selectivity [16], which could provide a HT approach for the selection and/or validation of specific phages obtained in each selection cycle.

Herein, we describe a technology for the affinity screening of antibody-displaying phages based on protein arrays containing scFv fragments as capture agents immobilized onto a hydrophobic nitrocellulose surface. In addition, a user-friendly informatics tool was developed to compare the results obtained from traditional and innovative screening platforms in a fast way.

2. Materials and methods

2.1. Materials

Ampicillin, kanamycin, sulfuric acid, Tween® 20, 3,3′,5,5′-Tetramethylbenzidine (TMB), trypsin, *N*-hydroxysuccinimide (NHS)-biotin, agarose microbeads covered with streptavidin or neutravidin, polyethylene-glycol (PEG) solution (20% (v/v) PEG, 2.5 M NaCl), bovine serum albumin (BSA), and Corning® Hybridization Chambers were supplied by Sigma–Aldrich (St. Louis/MO, USA); anti-cMyc antibody, anti-M13 horseradish peroxidase (HRP)-linked antibody, anti-mouse IgG HRP, Streptavidin-Cy™3, and neutravidin or streptavidin empty disposable PD-10 columns were obtained by GE Healthcare (Little Chalfont, Buckinghamshire, UK); SuperSignal West Pico Chemiluminescent Substrate, tris-buffered saline (TBS), SuperBlock® blocking buffer in TBS, recombinant protein L, and neutravidin agarose from Thermo Scientific (Rockford/IL, USA), and mSeries LifterSlip™ cover slips 25 × 60 from Thermo Scientific (Portsmouth/NH, USA); glucose from VWR (Radnor/PA, USA); covered plates High BindStreptawell from Roche (Basel, Switzerland); human recombinant interleukin 8 (hr-IL8) from Immunostep, SL (Salamanca, Spain); Tyramide signal amplification (TSA) reagent from PerkinElmer (Waltham/MA, USA); PVDF membrane from Immobilon-P^{SQ}-Millipore (Billerica/MA, USA); nitrocellulose-coated FAST slides from Schleicher & Schuell Whatman (Sanford/ME, USA); petri dish by Corning Life Science (Tewksbury MA, USA); ELISA reader from Sunrise™-TECAN (Männedorf, Switzerland); PBS (100 mM NaH_2PO_4, 150 mM NaCl); 1% fat powdered milk without *Lactobacillus*; Tryptone Yeast Extract (TYE) medium (18.75 g/L agar, 10 g/L NaCl, 12.5 g/L BD Bacto™-Tryptone, 6.25 g/L yeast extract in deionized water); 2 × TY medium (16 g/L BD Bacto™-Tryptone, 10 g/L of yeast extract, 5 g/L NaCl in deionized water).

Tomlinson I+J libraries (MRC HGMP Resource Centre, University of Cambridge, UK) were built in the pIT2 vector (derived from pHEN1). pIT2 vector contains a pelB promoter located upstream of the V_H-$(G_4S)_3$-V_L insert followed by His and Myc tags, a stop codon and the *G3P* anchor gene. These libraries are based on a germinal fragment from a V_H3–23 which is paired with a L_H designed by mutagenesis in three complementary determination regions (CDR1-3).

2.2. Methods

2.2.1. Phage selection

In order to screen the specific phages for the test recombinant antigen (hr-IL8), the antigen was immobilized by conjugating the human recombinant IL8 protein (hr-IL8) with biotin. Subsequently, it was incubated with 4% cross-linked agarose microbeads (4BCL) coated with streptavidin (Fig. 1). Next, the phage repertoire was added to the bead solution and incubated for 8 h at 4 °C with mild stirring using an orbital shaker. Afterwards, an empty column was packed with the mixture of antigen and phage-coated microbeads. After two washing steps, antigen-specific phages were eluted by trypsinization. Two more selection cycles were performed.

2.2.2. Library construction and phage production

All incubations were carried out at 37 °C, and all media were supplemented with 1% (w/v) glucose and 0.1% (w/v) ampicillin. Phage repertoire (~100 million copies) was transformed in TG1 bacteria. Initially, bacteria were grown in plates with TYE medium. After O/N incubation, between 100 and 200 colonies were observed on each plate. These colonies were scraped and grown in 2 × TY. The flask was incubated using a shaker at 300 rpm. When the bacteria culture reached suitable concentration, 2×10^{12} M13 co-operator phages were added. The culture was incubated for 16–20 h and then centrifuged and precipitated with PEG solution. Phage titer was calculated according to Eq. (1).

$$\frac{\text{phages}}{\text{mL}} = \text{number of plaques} \times \text{phage suspension dilution} \times \text{volume plated} \quad (1)$$

In order to validate the infectivity of phages, they were incubated with trypsin protease solution for 30 min at room temperature (RT). Dilutions of these treated phages were used to infect TG1 bacteria, which were cultured on standard Petri dish, resulting in the correct range (the number of colonies obtained from trypsin-treated phage was 10^6-fold lower than for non-treated phage, and should be 10^5–10^8 lower than for non-trypsin treated phage).

2.2.3. scFvs Phage-display microarrays

2.2.3.1. Array preparation. The full process for array construction is briefly described in Fig. 2. Two different 384-well plates (one plate per library, I or J) coming from six 96-well microtiter plates containing the clones (96 clones per plate) were centrifuged

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