Journal of Biotechnology 212 (2015) 174–180

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

A versatile puromycin-linker using cnvK for high-throughput *in vitro* selection by cDNA display

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ARTICLE INFO

Article history:
Received 11 July 2015
Received in revised form 20 August 2015
Accepted 24 August 2015
Available online 28 August 2015

Keywords:
Puromycin
cDNA display
3-Cyanovinylcarbazole nucleoside
In vitro selection
Directed evolution
High-throughput system

ABSTRACT

cDNA display is a powerful *in vitro* display technology used to explore functional peptides and proteins from a huge library by *in vitro* selection. In addition to expediting the *in vitro* selection cycle by using cDNA display, easy and rapid functional analysis of selected candidate clones is crucial for high-throughput screening of functional peptides and proteins. In this report, a versatile puromycin-linker employing an ultrafast photo-cross-linker, 3-cyanovinylcarbazole nucleoside, is introduced. Its utility for both *in vitro* selection using cDNA display and protein–protein interaction analysis using a surface plasmon resonance (SPR) system is described. Using this versatile puromycin-linker, we demonstrated the model *in vitro* selection of the FLAG epitope and a SPR-based assay to measure the dissociation constant between the B domain of protein A and immunoglobulin G. Improvement of the puromycin-linker as described herein should make the cDNA display method easier to utilize for design of protein or peptide based affinity reagents.

1. Introduction

In vitro display technology is a powerful tool to explore affinity reagents such as peptides which have a binding ability (peptide aptamers) (Gray and Brown, 2014; Nevola and Giralt, 2015), antibody fragments (Holliger and Hudson, 2005), and non-immunoglobulin based protein scaffolds (Škrlec et al., 2015). In particular, *in vitro* display technologies using a cell-free translation system [ribosome display (Hanes and Plückthun, 1997), and mRNA display (Nemoto et al., 1997; Roberts and Szostak, 1997)] are highly attractive approaches because they can handle a large size DNA library.

mRNA display is a linking technique of peptide or protein with its coding mRNA via a puromycin-linker (Pu-linker). Puromycin is an aminonucleoside antibiotic that resembles the 3′-terminus of an aminoacylated tRNA. Thus it can be fused to a nascent polypetide chain by peptidyl transferase activity in the ribosomal A site. In mRNA display, a puromycin molecule is covalently bonded with the 3′-terminus of mRNA via a DNA spacer. When a ribosome on the mRNA template could stall at the connecting point between mRNA and Pu-linker during translation reaction, the puromycin attached to the 3′-terminus of Pu-linker can enter the ribosomal A site and be fused to the nascent polypeptide chain. The mRNA can be covalently bonded with the corresponding nascent polypeptide chains via the puromycin (Nemoto et al., 1997).

Recently we developed a novel *in vitro* display technology termed cDNA display in which complementary DNA is fused to its coding peptide or protein via a Pu-linker by covalent linking (Yamaguchi et al., 2009). cDNA display is a highly stable display technology because the cDNA is directly covalently fused to the C-terminus of the peptide or protein via the Pu-linker, making it different from mRNA display. This advantage allows *in vitro* selection of protein or peptide based affinity reagents against cell surface proteins, such as G protein-coupled receptors (GPCRs), using whole cells on which the GPCR is highly expressed (Ueno et al., 2012a). Furthermore, the cDNA display method can be suitable for *in vitro* selection of disulfide-rich peptides, because post-translational reactions, including oxidative folding, are easily incorporated into the displayed peptides and proteins (Yamaguchi et al., 2009; Naimuddin et al., 2011; Mochizuki et al., 2014; Cai et al.,

Abbreviations: Pu-linker, puromycin-linker; SPR, surface plasmonresonance; cnvK, 3-cyanovinylcarbazole nucleoside; SA, streptavidin; BDA, B domain of protein A; EMSC, *N*-(6-maleimidocaproyloxy)succinimide; SBS-Pu-linker, short biotin-segment Pu-linker.

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http://dx.doi.org/10.1016/j.jbiotec.2015.08.020

2014). We have previously improved the preparation efficiency of the cDNA display molecule by developing additional Pu-linkers (Mochizuki et al., 2011; Ueno et al., 2012b) and modifying the preparation protocol (Mochizuki et al., 2013b), which makes cDNA display a more practical method for *in vitro* selection.

It is important to have methods to assay the binding of the obtained candidate peptides and proteins to explore functional peptides and proteins effectively. As many types of candidate sequences are obtained from the libraries, to prepare all the possible candidates via chemical synthesis or protein expression using *Escherichia coli* is very time consuming, in particular, when the candidate sequences contains several disulfide bonds. Therefore, we established a pull-down method using a biotin-attached peptide/protein prepared with a Pu-linker and a cell-free translation system (Mochizuki et al., 2013a; Tanemura et al., 2015). Our group also reported a Pu-linker containing polyA which can be used for easy and rapid purification of the synthesized biotin-attached peptide/protein from the cell-free translation system using oligo(dT)-immobilized supports (Nemoto et al., 2014). The purified biotin-attached peptide or protein is then applied to a sensor chip for detection by surface plasmon resonance (SPR), thus allowing easy measurement of the dissociation constant of the candidate peptides or proteins without chemical synthesis or protein expression.

As mentioned above, *in vitro* selection using cDNA display method is a sophisticated high-throughput system for affinity reagent design. However, several kinds of specialized Pu-linkers are required for each step (*i.e.*, *in vitro* selection and functional assay) which may be a potential barrier for researchers who want to utilize cDNA display for design of affinity reagents in a high-throughput manner. Herein, we introduce a novel Pu-linker containing 3-cyanovinylcarbazole nucleoside (cnvK) named the cnvK-Pu-linker. cnvK is an ultrafast photo-cross-linker which connects hybridized oligonucleotides using 366 nm of UV irradiation (Yoshimura and Fujimoto, 2008; Yoshimura et al., 2009). This method enables ultrafast cross-linking of the developed Pu-linker to the 3′-terminal region of an mRNA without the use of an enzymatic ligation reaction. The novel Pu-linker can also be used for exploring functional clone sequences by *in vitro* selection using cDNA display and for functional assay of the selected candidate sequences using a SPR system, making affinity reagent design via cDNA display more convenient.

2. Materials and methods

2.1. Clone and library DNA constructs

The B domain of protein A (BDA)-coding DNA was the same as described in a previous report (Mochizuki et al., 2011). The FLAG epitope (which was fused to helix IV of the Pou-specific DNA-binding domain of Oct1)-coding DNA was described previously (Nemoto et al., 2014). The library coding random peptide consisting of eight amino acids (X8 peptide) was generated by replacing the FLAG epitope-coding region with the X8 peptide-coding region.

2.2. Synthesis of the cnvK-Pu-linker

The modified oligonucleotides Puro-F-S and the cnvK-I-polyA-B fragment were obtained from Tsukuba Oligo Service (Japan). The Puro-F-S fragment represents 5′-(S)-TC-(F)- (Spacer18)-(Spacer18)-CC-(Puro)-3′, where S is the 5′-thiol-Modifier C6, F is fluorescein-dT, Puro is puromycin CPG and Spacer18 is the spacer phosphoramidite 18. The Biotin-polyA-Inosine-cnvK fragment represents 5′-(B)- AAAAAAAAAAAAAAAAAAAA-(I)-TTCCA-(K)-GCCGCCCCCCG-(T)-CT-3′, where B is 5′-biotinTEG, I is inosine, K is cnvK, and T is the amino-modifier C6 dT. The cross-linking reaction of the Puro-F-S fragment and the Biotin-polyA-Inosine-cnvK fragment via N-(6-maleimidocaproyloxy) succinimide (EMSC, Dojindo Laboratories, Kumamoto, Japan) was performed according to a previous report (Yamaguchi et al., 2009; Mochizuki et al., 2011). A total of 30 nmol of the Puro-F-S fragment was reduced by 50 mM dithiothreitol in 1 M disodium hydrogen phosphate for 1 h at room temperature and then desalted on a NAP-5 column (GE Healthcare, Waukesha, WI, USA) just before use. A total of 10 nmol of the Biotin-polyA-Inosine-cnvK fragment and 2 mmol of EMCS were mixed in 100 ml of 0.2 M sodium phosphate buffer (pH 7.2). The mixture was subsequently incubated for 30 min at 37 °C and excess EMCS was removed by ethanol precipitation with a coprecipitating agent (Quick-Precip Plus Solution, Edge BioSystems, Gaithersburg, MD, USA). The reduced Puro-F-S fragment was immediately added to the precipitate and incubated at 4 °C overnight. Dithiothreitol was added (final conc. of 50 mM) to the sample and incubated for 30 min at 37 °C to stop the reaction. The non-reacted Puro-F-S fragment was removed by ethanol precipitation with the coprecipitating agent. The precipitate was dissolved with nuclease-free water and purified with a C18HPLC column under the following conditions: column: AR-300, 4.6 × 250 mm (Nacalai Tesque, Kyoto, Japan); solvent A: 0.1 M Triethyl Ammonium Acetate (TEAA, Glen Research, Sterling, VA, USA); solvent B: acetonitrile/water (80:20, v/v); 15–35% B over 45 min; flow rate: 1 ml/min; detection by ultraviolet (UV) absorbance at 260 nm and fluorescence excitation/emission at 488/520 nm. The fraction from the last peak at an absorbance of 260 nm (corresponding to a single peak at an emission at 520 nm) was collected. After drying, the cnvK-Pu-linker was resuspended in nuclease-free water.

2.3. Photo-induced cross-linking and enzymatic ligation

The cnvK-Pu-linker was hybridized to the 3'-terminal region of mRNA in 25 mM Tris-HCl (pH 7.5) with 100 mM NaCl under the following annealing conditions: heating at 90 °C for 1 min followed by lowering the temperature to 70 °C at a rate of 0.4 °C/s, incubating for 1 min, then cooling to 25 °C at a rate of 0.08 °C/s. The sample was irradiated with UV light at 365 nm using CL-1000 UV Crosslinker (UVP, Upland, CA, USA) for 30 s. The cross-linked products were analyzed by 8 M urea containing 7% denaturing polyacrylamide gel electrophoresis (PAGE). The cross-linked mRNAs were visualized by FITC fluorescence using a fluoroimager (Pharos Fx, BioRad, Hercules, CA, USA), then staining with SYBR Gold Nucleic Acid Gel Stain (Life Technologies, Gaithersburg, MD, USA). The short biotin-segment Pu-linker (SBS-Pu-linker) was ligated to the 3'-terminal region of each length of mRNA as reported previously (Mochizuki et al., 2011) with a minor change as follows: the ligation reaction was performed by incubating at 25 °C for 30 min.

2.4. Preparation of the mRNA display molecule and cDNA display molecule

Three pmol of linker cross-linked mRNA was added to 25 μL of reaction mixture containing nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI, USA). After the reaction mixture was incubated at 30 °C for 10 min (BDA) or 15 min (X8 peptide-coding library DNA containing FLAG epitope-coding DNA), 3 M KCl and 1 M $MgCl_2$ were added (final conc. was 900 mM and 75 mM, respectively) then incubated at 37 °C for 1 h. mRNA display molecule formation was analyzed by 8 M urea containing 4% stacking-6% separating SDS-PAGE. mRNA display molecules were immobilized on streptavidin (SA) magnetic beads (Dynabeads MyOne Streptavidin C1, Thermo Fisher Scientific, Somerset, NJ, USA) in the same manner as in the previous report (Mochizuki et al., 2013b) and reverse transcribed to synthesize cDNA display molecules on the beads. After