SELECTION OF PHAGE DISPLAYED PEPTIDES ON LIVE ADHERENT CELLS IN MICROFLUIDIC CHANNELS

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ABSTRACT

Phage display technique on whole cells has proven to be particularly effective to isolate peptide-based affinity reagents that specifically bind to transmembrane receptors because the receptors are kept in their native conformation during the selection. Here we report the first use of microfluidics technology to isolate phage displayed peptides with high affinities toward specific receptors expressed on a small number of live cells ($2 \times 10^5$). We show that the microfluidic phage selection chip (MiPS) yields superior selection results compared to conventional panning methods.

KEYWORDS: Microfluidics, Phage Display, Directed Evolution, Biomarkers

INTRODUCTION

Transmembrane receptors are an important class of diagnostic and therapeutic targets, and thus it is imperative to isolate effective affinity reagents that specifically bind to them. Toward this end, the use of phage display technique on whole cells has proven to be particularly effective because the receptors are kept in their native orientation and conformation during the selection [1]. However, conventional biopanning methods for cell surface selection suffer from several disadvantages; First, it requires a large number of cells, making the technique impractical for small cell populations harvested from organs and tissues. Second, the fixed-volume, manual washes are inefficient and laborious, often yielding irreproducible results and ineffective removal of non-specifically or weakly bound phage. Thus, there is a need for an alternative selection system capable of handling small number of cells without loss, which can also reproducibly apply highly stringent selection conditions. To address this need, we report the first use of microfluidics technology to isolate phage displayed peptides with high affinities toward specific receptors expressed on a small number of live cells ($2 \times 10^5$). We show that the microfluidic phage selection chip (MiPS) yields superior selection results compared to conventional panning methods.

EXPERIMENTAL

The microfluidic phage selection is performed with the MiPS chip, and the device fabrication scheme is briefly described in Figure 1A. First, a NUNC Lab-Tek culture slide chamber was coated with 10 µg/ml of fibronectin for 12 hrs at 4 °C to promote cell adhesion. The chamber was then seeded with ~10^4 PPC-1 human prostatic carcinoma cells in 2 mL of culture medium. Here, PPC-1 cells were utilized as a model because they express high levels of neuropilin-1 (NRP-1), which is a well known receptor that binds and internalizes peptides with C-terminal arginine residues usually in a consensus context of R/KXXR/K (the C-end Rule or CendR motif) [2]. Upon reaching 90 % confluency, the PPC-1 cells were washed twice with phosphate-buffered saline. To coat the exposed surface areas, we treated the slide with UV-treated, non-infectious G7 phage that no longer replicate but retain their phage particle-associated polysaccharide depolymerase activity ($2 \times 10^9$ pfu total in 2 mL PBS at 4 °C for 1 hr). The G7 phage displays hepta-glycine (G7) and exhibit negligible binding to PPC-1 cells. After aspirating the un-bound blocking phage, the walls of the culture slide chamber were peeled off to accommodate thin PDMS and glass layers containing the inlet and outlet for fluidic connections. Finally, the three layers were assembled by clipping the edges of the device (Figure 1A). All major components of the selection process—incubation, washing, cell lysing, and lysate collection—were performed on-chip (Figure 1B).

Figure 1: MiPS device for the selection of phage-displayed peptides targeting cell surface markers. (A) The device is fabricated from three layers consisting of glass (top, 1mm), PDMS (middle, 250 µm), and culture glass (1mm). The volume
of the chamber containing the target cells is ~50 µl. (B) Experimental schematic for phage selection on adherent cells using the assembled MiPS device. (C) Experimental scheme for selecting phage displaying strong-binding peptides to adherent PPC-1 cells in the MiPS system.

We performed de novo selection of a T7 phage library expressing random, linear 7-residue (X7) peptides (diversity approximately 5×10⁸) against PPC-1 cells using both conventional biopanning and the MiPS platform (Figure 1C). In both cases, we performed three rounds of selection under highly stringent mass-action selection conditions using 2×10⁴ cells, followed by high stringency continuous washing. A small aliquot (100 µl) of the selected phage pool was collected after each round and used to determine the enrichment fold between rounds. After the selection, the recovered phage were amplified in E. coli BLT5403 cells at 37 °C for 2 hrs, followed by phage precipitation with a polyethylene glycol (PEG)/NaCl solution and purification by CsCl gradient ultracentrifugation. The amplified phage pool was further quantified and used as starting library pool for the next round of phage selection.

RESULTS AND DISCUSSION

We found that the enrichment of high affinity phage is significantly more efficient with the MiPS system compared to conventional biopanning; after three rounds of selection in the MiPS chip, the resulting phage from the round 3 pool (R3) demonstrated ~700-fold higher binding to PPC-1 cell suspensions in comparison to the initial random library, significantly better than that obtained after three rounds of conventional cell suspension-based biopanning (100-fold) (Figure 2A).

We further investigated the compositional differences in the peptide motifs enriched by the two selection methods. To do so, we randomly picked 20 individual phage clones from each R3 pool and obtained their amino acid sequences. Previously, it has been shown that that peptides with a C-terminal R/KXXR/K sequence exhibit significantly higher affinity to NRP-1-expressing cells than peptides with a C-terminal arginine alone (XXXR) [2, 3]. We found that MiPS chip enables more efficient enrichment of phage displaying higher affinity motifs (Figure 2B): 90 % of the phage selected using MiPS displayed the high affinity (R/KXXR/K) motifs and 5 % phage displaying lower affinity motifs (XXXR). In contrast, conventional cell suspension-based panning yielded at best 50 % phage displaying R/KXXR/K motifs and 30 % phage displaying XXXR motifs.

Next, we investigated the specificity of individual phage clones obtained with MiPS by picking 6 phage clones, and measuring their relative binding to PPC-1 and M21 melanoma cells (Figure 3A). Of note, PPC-1 cells express high levels of NRP-1, while M21 cells express only minimal amounts of this protein. As shown in Figure 3A, the selected clones expressing R/KXXR motifs exhibited significantly better specificity towards PPC-1 compared to the XXXR motifs. The best motif obtained from conventional biopanning (RPARPAR, green color) [2] was also measured at the same time for comparison. Two out of three of the measured R/KXXR motifs selected from MiPS chip showed higher specificity compared to the RPARPAR motif (Figure 3B).

We synthesized the peptide sequences of the selected clones and measured their equilibrium dissociation Constants (Kd) to NRP-1 receptor via ELISA (Figure 3A). Briefly, microtiter wells were coated with 50 µL of 5 µg/mL of the purified NRP-1 protein overnight at 4 °C. After washing with PBS added with 0.01% Tween 20, the wells were incubated for 1 h at room temperature with 50 µL of various concentrations of biotinylated peptide in PBS. After washing with the same washing buffer (PBS added with 0.01% Tween 20), streptavidin-conjugated horseradish peroxidase was added to the wells and incubated for 30 min at room temperature. Horseradish peroxidase binding was quantified with 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) as a substrate. The dissociation constants (Kd) of the peptide-NRP-1 interaction are

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**Figure 2:** Results from de novo selection with a random linear X7 peptide library. (A) After three rounds of selection using the MiPS chip, the resulting phage pool (R3) demonstrated ~700-fold higher binding to PPC-1 cells in comparison to the initial random library. In contrast, the conventional cell suspension-based biopanning yielded ~100-fold enrichment. (B) Sequences of selected peptides resulting from three different selection conditions, with clones containing the strong-binding motif (R/KXXR) shown in black, sequences containing the weak-binding motif (XXXR) displayed in blue and non-CendR sequences shown in red.

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**Figure 3:** Next, we investigated the specificity of individual phage clones obtained with MiPS by picking 6 phage clones, and measuring their relative binding to PPC-1 and M21 melanoma cells (Figure 3A). Of note, PPC-1 cells express high levels of NRP-1, while M21 cells express only minimal amounts of this protein. As shown in Figure 3A, the selected clones expressing R/KXXR motifs exhibited significantly better specificity towards PPC-1 compared to the XXXR motifs. The best motif obtained from conventional biopanning (RPARPAR, green color) [2] was also measured at the same time for comparison. Two out of three of the measured R/KXXR motifs selected from MiPS chip showed higher specificity compared to the RPARPAR motif (Figure 3B).

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obtained by fitting the dependence of OD 405 value of specific binding on the concentration of peptides to the one site binding saturation equation $Y = \frac{B_{\max} X}{K_d + X}$. A number of sequences showed comparable, or superior binding affinities to that of the RPARPAR sequence, including NGARKPR ($K_d = 28.8 \pm 3.5 \mu M$), GKRPAR ($K_d = 22.2 \pm 0.3 \mu M$), and RGRPLR ($K_d = 18.8 \pm 3.2 \mu M$). Interestingly, the sequences with high affinities showed more specific binding to PPC-1 cells versus M-21 cells (Figure 3B).

![Figure 3: The affinity and specificity of individual peptide motif. (A) Affinity measurement using ELISA and one site binding saturation equation $Y = \frac{B_{\max} X}{K_d + X}$ fitting. (B) The sequences of the amplified phage clones, their ratio of binding to PPC-1/M21, and the dissociation constant of the synthesized peptide. The clones with CendR motif (R/KXXR) are displayed in black. Clones with weaker-bind XXXR motif are shown in blue and non-CendR sequences are in red. Best clone got from former conventional biopanning selection is shown in green.]

**CONCLUSION**

We show that the MiPS system offers two significant performance advantages. First, it is able to impose highly stringent mass-action selection pressure, yielding strong-binding peptides. Second, it applies continuous washing, promoting rapid enrichment of strong-binding peptides and efficient elimination of nonspecific, weak binders with minimal cell loss. Based on these results, we believe that the MiPS chip will be especially useful for processing limited numbers of primary cells derived from biopsies and other primary tissues.

**ACKNOWLEDGEMENTS**

We are grateful for the financial support of the ARO Institute for Collaborative Biotechnologies, Office of Naval Research, and National Institutes of Health. We thank Andrew Csordas, Seung Soo Oh and Scott Ferguson for their help and discussions, Joshua A. Olson for technical help and Dr. David Cheresh for the M21 cell line.

**REFERENCES**


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