Microbead display by in vitro compartmentalisation: selection for binding using flow cytometry

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Abstract In vitro compartmentalisation in an emulsion was used to physically link proteins to the DNA that encodes them via microbeads. These microbeads can be selected for catalysis, or, as demonstrated here, for binding. Genes encoding a peptide containing an epitope (haemagglutinin) were enriched to near purity from a 10^6-fold excess of genes encoding a different peptide by two rounds of selection using flow cytometry, indicating ~1000-fold enrichment per round. Single beads can be isolated using flow sorting and the single gene on the bead amplified by polymerase chain reaction. Hence, the entire process can be performed completely in vitro.

Key words: In vitro compartmentalization; Microbead; Selection; Binding; Flow cytometry; Directed evolution

1. Introduction

Both natural evolution and directed evolution require a link between genotype and phenotype [1]. This can be achieved in the laboratory by creating a physical genotype–phenotype linkage – either the gene is the active molecule (in the case of nucleic acids [2]) or the gene and the protein it encodes are physically connected.

The first, and most widely adopted technique for selecting protein ligands is phage display, where the protein is displayed on the surface of a filamentous bacteriophage [3]. However, proteins can also be displayed on the surface of other bacteriophage, eukaryotic viruses, bacteria, yeast, and even directly bound to the encoding plasmid [4–7].

Two completely in vitro display technologies have also been developed, which use cell-free translation systems: polysome display [8] and RNA–peptide fusion [9]. They promise much larger repertoire sizes [10,11] and the possibility of selecting proteins with hitherto unattainable properties [12].

We have recently described an entirely different strategy, based on in vitro compartmentalisation (IVC) of reactions in the aqueous droplets of a water-in-oil emulsion [1]. We have previously shown how IVC can be used to select for *catalysis* by selecting DNA-methyltransferases [13] and IVC has also been used for the directed evolution of *Taq* DNA polymerase [14].

Here, we describe a novel IVC strategy based on creating repertoires of microbeads, each displaying a gene and the protein it encodes (Fig. 1). These beads can be selected by the catalytic activity of the displayed protein [29], or, as described here, selected for binding.

2. Materials and methods

2.1. Synthesis of genes

The vector pETFLAG was created by annealing the oligonucleotides FLAG-NB and FLAG-BN (Table 1) and cloning into *Nco*I–*Bam*HI-cut pET23d. The FLAG-haemagglutinin (HA) gene (Fig. 2) was created by polymerase chain reaction (PCR) amplifying pETFLAG with the primers Biotin-pETRev and FLAG-HA. The FLAG-*fol*A gene (Fig. 2A), encoding dihydrofolate reductase (DHFR) with an N-terminal FLAG tag was created by amplifying the *fol*A gene [16] from *Escherichia coli* using primers folA-FW and folA-BW, cloning it into *Hin*dIII–*Xho*I-cut pETFLAG (creating pETFLAG-*fol*A) and amplifying this construct using primers Biotin-pETRev and pETFor.

2.2. Microbead display of peptides

10^9 1.0-μm diameter streptavidin-coated polystyrene beads (Bangs Laboratories) were centrifuged at 2300×*g* for 4 min (as for all washing steps) and then resuspended in 50 μl TNTB (0.1 M Tris 7.5, 0.15 M NaCl, 0.05% Tween-20, 0.5% bovine serum albumin). 8 μg of biotinylated M5 anti-FLAG antibody (Sigma) was added (60 000 antibodies/bead) and incubated for 5 h at 5°C. The beads were washed three times in TNTB before addition of biotinylated FLAG-*fol*A or FLAG-HA DNA at a ratio of two genes/bead, and incubated overnight at 4°C. As ~50% of genes bind (assayed using ^{32}P-labelled DNA), about one gene is bound per bead. The beads were washed twice in TNTB then resuspended in 100 μl of ice-cooled *E. coli* S30 in vitro translation system and emulsified as in [13]. The emulsions were incubated at 30°C for 90 min, spun at 2300×*g* for 10 min and the oil phase removed. 200 μl of TNTB was added and the emulsion broken by extracting three times with 1 ml of hexane. The recovered beads were washed twice in TNTB and resuspended in 0.2 ml of TNTB.

2.3. Labelling of microbeads displaying peptides with an HA epitope

0.5 μl of 25 mU/μl rat anti-HA peroxidase conjugate (3F10, Roche) was added to the recovered beads and incubated for 20 min at 25°C. The beads were washed three times with TNTB then resuspended at 5×10^7 beads per ml in 50 mM Tris–HCl, pH 8.0 containing 0.004% H_2O_2 and 4 μM fluorescein tyramide [17] and sonicated for 1 min. After 5 min at room temperature, the beads were washed three times in TNTB, resuspended in phosphate-buffered saline, sonicated for 1 min and analysed on a FACScan cytometer (Becton Dickinson).

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Abbreviations: IVC, in vitro compartmentalisation; TSA, tyramide signal amplification; DHFR, dihydrofolate reductase

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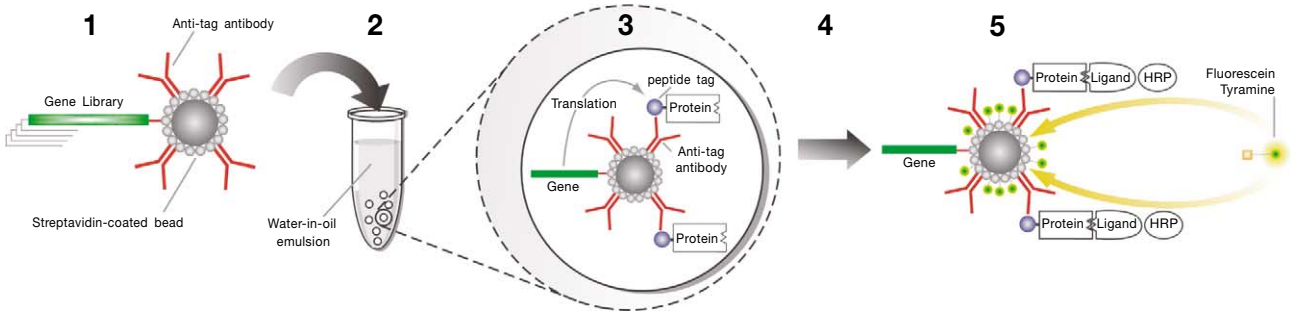

Fig. 1. Creation of microbead display libraries by IVC and selection for binding using flow cytometry. A repertoire of genes encoding protein variants, each with a common N- or C-terminal epitope tag, are linked to streptavidin-coated beads carrying antibodies that bind the epitope tag at, on average, ≤ 1 gene per bead (1). The beads are compartmentalised in a water-in-oil emulsion to give, on average, <1 bead per compartment (2), and transcribed and translated in vitro in the compartments. Consequently, in each compartment, multiple copies of the translated protein become attached to the gene that encodes it via the bead (3). The emulsion is broken (4), and the microbeads carrying the display library isolated. The beads are incubated with ligand coupled to horseradish peroxidase (HRP), washed to remove unbound ligand and incubated with hydrogen peroxide and fluorescein tyramide (5). Immobilised HRP converts the fluorescein tyramide into a short-lived, free-radical intermediate which reacts with adjacent proteins. Hence, beads displaying proteins that bind ligand become labelled with multiple fluorescein molecules. These beads can then be enriched (together with the genes attached to them) by flow cytometry.

2.4. Sorting of microbeads

Beads were sorted using a MoFlo cell sorter (Cytomation) directly into PCR buffer. DNA was amplified (using Super Taq, HT Biotechnology) with primers Biotin-pETRev and FLAGRev1. 0.5 μl of the first PCR reaction was re-amplified using primers Biotin-pETRevN (which primes inside Biotin-pETRev) and FLAGRev1. The DNA was purified from a 1% agarose gel for a second round of selection. Beads sorted from the second round were amplified using primers Biotin-pETRevN and FLAGRev1 for the first PCR then primers Biotin-pETRevN1 (which primes inside Biotin-pETRevN) and FLAGRev1 for the second. All PCRs were cycled 30 times (94°C, 0.5 min, 60°C, 0.5 min, 72°C, 2.0 min).

Single beads were sorted into individual wells of a 96-well PCR plate into PCR buffer and amplified as above with primers Biotin-pETRev and FLAGRev1 for 37 cycles.

3. Results

3.1. Microbeads displaying multiple copies of a peptide ligand can be created by IVC and identified by flow cytometry

Biotinylated FLAG-HA genes, encoding a 30-residue peptide with a FLAG epitope tag at the N-terminus and an HA epitope at the C-terminus, or FLAG-*fol*A genes, encoding DHFR with an N-terminal FLAG epitope tag (Fig. 2) were attached to 1-μm diameter streptavidin-coated beads coated with anti-HA antibodies at one gene/bead. Microbeads displaying the translated protein were then created by translation in an emulsion and the beads labelled with peroxidase-conjugated anti-HA antibody and fluorescein tyramide signal amplification (TSA) [18] as in Fig. 1.

The beads displaying FLAG-HA peptides could easily be distinguished from the beads displaying FLAG-*fol*A by flow cytometry: the mean fluorescence of beads carrying the FLAG-HA genes was 57 times that of beads with FLAG-*fol*A genes (Fig. 3). A calibration curve constructed by labelling beads coated with biotinylated peroxidase molecules indicated that each bead captured 200–300 FLAG-HA peptides.

Table 1
Oligonucleotides

FLAG-NB	5′-CATGGACTACAAAGATGACGATGATAAAATGC-ATGGCAACGAAGGTACCG-3′
FLAG-BN	5′-GATCCGGTACCTTCGTTGCCATGCATTTTATC-ATCGTCATCTTTGTAGTC-3′
Biotin-pETRev	5′-Biotin-GGTTTTCACCGTCATCACCG-3′
FLAG-HA	5′-AACTCAGCTTCCTTTCGGGCTTTGTTAGGATC-CTCCTCCCGCATAATCCGGCACATCATACGGATA-TCCTCCTCCGGATCCGGTACCTTCGTTGCC-3′
folA-FW	5′-GCGCGAAGCTTCGATCAGTCTGATTGCGGCG-3′
folA-BW	5′-GCGCCTCGAGTTCCGCCGCTCCAGAATCTC-3′
pETFor	5′-GACTCCAACGTCAAAGGGCG-3′
FLAGRev1	5′-AACTCAGCTTCCTTTCGGGC-3′
Biotin-pETRevN	5′-Biotin-AAACGCGCGAGGCAGCTGC-3′
Biotin-pETRevN1	5′-Biotin-GCGAGGCAGCTGCGGTAAAG-3′

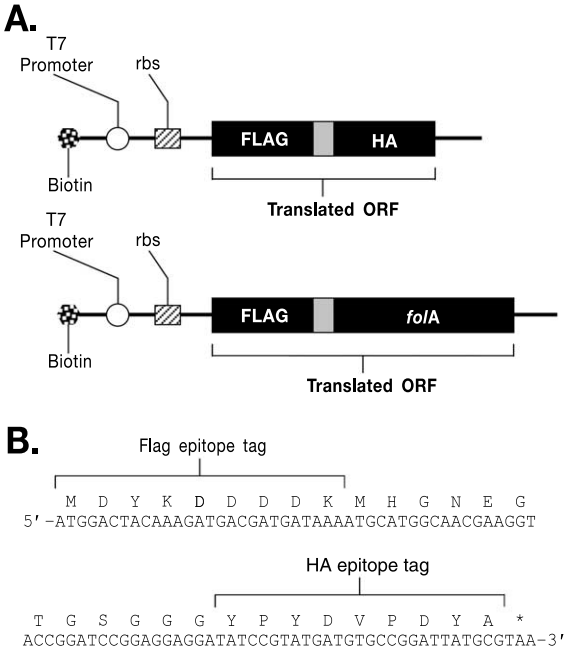

Fig. 2. The selected genes. A: Schematic representation of the gene encoding the 30-residue FLAG-HA peptide with an N-terminal FLAG [15] and a C-terminal HA [28] epitope tag, and the control gene, FLAG-*fol*A, encoding *E. coli* dihydrofolate reductase [16] with an N-terminal FLAG tag. Both genes contain a T7 promoter and ribosome binding site (rbs) and are biotinylated at one end. B: The sequence of the translated open reading frame (Translated ORF) of the FLAG-HA gene.

3.2. A gene encoding a peptide containing an epitope (HA) is enriched to near purity from a 10^6-fold excess of genes encoding a peptide without the epitope after two rounds of selection

Biotinylated FLAG-HA genes and biotinylated FLAG-*fol*A genes (Fig. 2) were mixed in a ratio of $1:10^6$ and attached to beads coated with anti-HA antibodies at one gene/bead, as above. Microbeads displaying the translated protein were then created by translation in an emulsion and the beads labelled with peroxidase-conjugated anti-HA antibody and fluorescein TSA (Fig. 1). 6.6×10^4 beads out of 3.6×10^7 (i.e. ~0.2%) of single, unaggregated beads were sorted using a flow cytometer. The genes on the sorted beads were amplified by PCR using a biotinylated primer, and subjected to a second round of selection as above, except at 0.1 gene/bead. 2.0×10^3 beads out of 2.4×10^5 were collected (i.e. ~1%). After one round of selection a faint band corresponding to the FLAG-HA gene was visible on an agarose gel and after two rounds the FLAG-HA gene had been enriched to near purity (Fig. 4). This indicates an enrichment of about 1000-fold per round. No enrichment was observed when the DNA on the beads was amplified without sorting.

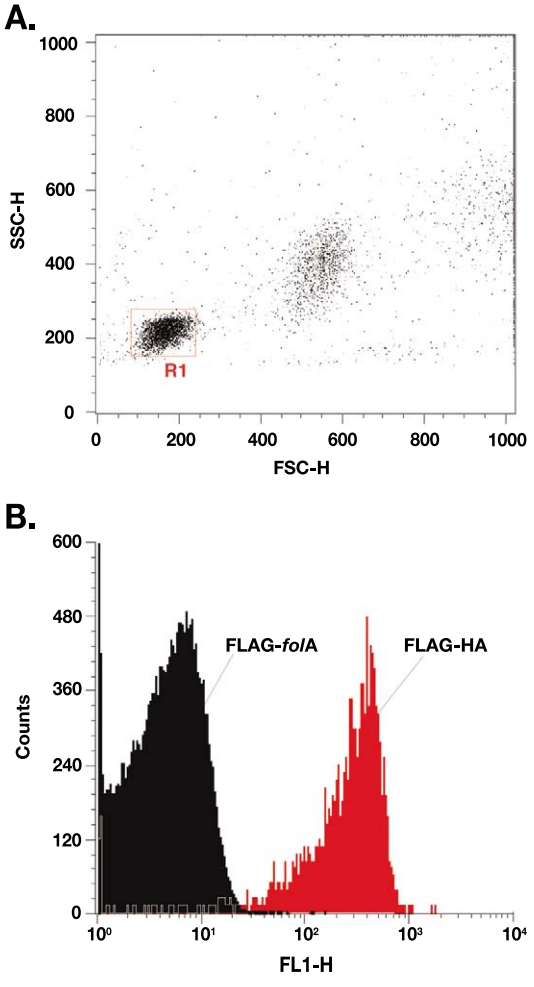

Fig. 3. Flow cytometry of microbead–gene complexes which do, or do not, contain peptides that bind to an anti-HA antibody. A: Forward scatter (FSC-H) and side scatter (SSC-H) of beads analysed by flow cytometry. Single unaggregated beads were gated through R1. B: Fluorescence of beads (gated through R1) coated with either FLAG-*fol*A genes or FLAG-HA genes following translation and capture of peptides onto the beads in an emulsion, incubation with peroxidase-conjugated anti-HA antibody and fluorescein TSA.

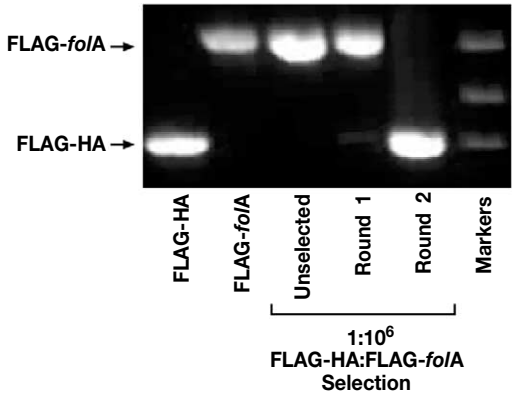

Fig. 4. Selections for genes encoding peptides that bind to an anti-HA antibody from a starting ratio of $1:10^6$ FLAG-HA:FLAG-*fol*A genes. Pure FLAG-HA genes (877 bp), pure FLAG-*fol*A genes (1371 bp), unselected DNA and DNA recovered after round 1 and round 2 of selection were amplified by PCR and analysed on a 1% agarose gel. Markers, ϕX174-*Hae*III digest.

3.3. Individual genes encoding peptides with binding activity can be isolated by single-bead sorting

Beads were coated with a $1:10^3$ mixture of FLAG-HA:FLAG-*fol*A genes at one gene/bead, and selected as above. The most highly fluorescent beads (in the top 0.1%) were sorted by flow cytometry and the genes attached to a pool of 500 sorted beads amplified by PCR (Fig. 5A). After sorting, the band corresponding to the FLAG-HA gene was of

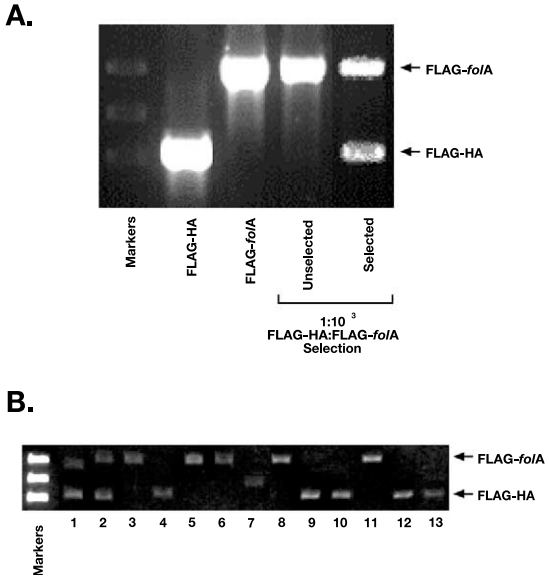

Fig. 5. Direct sorting of individual FLAG-HA genes from a $1:10^3$ mixture of FLAG-HA:FLAG-*fol*A genes by flow cytometry of beads. A: Pure FLAG-HA genes (903 bp), pure FLAG-*fol*A genes (1397 bp), unselected DNA and DNA recovered from 500 sorted beads (pooled together) after one round of selection were amplified by PCR and analysed on a 1% agarose gel. B: DNA amplified by PCR from *single* positive beads sorted into the wells of a 96-well PCR plate, 13 of which are shown analysed on a 1% agarose gel. Markers, ϕX174-*Hae*III digest.

similar intensity to the band corresponding to the FLAG-*fol*A genes indicating an enrichment of ~500-fold. No enrichment was observed when the beads were not sorted. In addition, *single*, highly fluorescent beads (in the top 0.1%) were sorted into the wells of a 96-well PCR plate. DNA was successfully amplified by PCR from 22 of the 96 wells and 13 of these are shown in Fig. 5B. Hence, ~23% of the input genes were recovered intact. Of the wells that amplified, nine gave single bands of the size expected for FLAG-HA, and 11 gave single bands of the size expected for FLAG-*fol*A. This indicates that the FLAG-HA genes were enriched ~500-fold and is consistent with the enrichments observed above (Figs. 4 and 5A).

4. Discussion

We demonstrate here how IVC provides an efficient way of physically linking proteins to the genes that encode them and selecting them for binding. IVC has also been used for the selection of gene–protein complexes created by translation of biotinylated genes encoding peptides fused to streptavidin [19]. However, the enrichment observed in a model selection was very low (10-fold; v. >500-fold observed here) and the efficiency of formation of the protein–DNA complexes was only ~1%.

Proteins can of course be physically linked to genes that encode them by a variety of techniques. These 'display technologies' have proven highly successful in the selection of binding proteins [10,12,20–25]. Nevertheless, microbead display libraries made by IVC have a number of attractive features.

First, they are formed completely in vitro; hence no transformation or cloning is required (unlike systems with an in vivo step such as phage display [20,21]).

Second, the gene is DNA, which is more stable than the RNA used in other completely in vitro systems [10,12,25]. Indeed, 23% of the input genes survived intact at the end of the selection.

Third, the efficiency of assembly is high. Nearly all the beads coated with FLAG-HA, translated in an emulsion and labelled using peroxidase-conjugated anti-HA antibody and fluorescein TSA, were highly fluorescent (Fig. 3B). Furthermore, in the selection of a $1{:}10^6$ FLAG-HA:FLAG-*fol*A gene mixture, most of the beads (at least one in eight) carrying FLAG-HA genes must have become labelled with fluorescein and sorted (as 23% of genes survived, only 8×10^6 of the 3.6×10^7 beads sorted carried a gene).

Fourth, multiple copies of each protein can be displayed (as opposed to single copies in ribosome display or mRNA–peptide fusion) providing a potential advantage in the selection of low affinity ligands. In this case 200–300 peptides were displayed per bead.

Fifth, with other display technologies the peptides to be selected are fused to proteins for display on phage or cells, attached to ribosomes or fused to mRNA and this can lead to the selection of peptides with poor solubility [12]. With microbead display the expressed peptides are only fused to a short epitope tag and peptides that are too insoluble to be captured by the beads will not to be selected.

Finally, the microbead display libraries can be selected by flow cytometry, which has a variety of practical advantages for the selection of ligand binding [26]. Ligand binding equilibria and dissociation kinetics can be determined and clones selected accordingly. Indeed, flow cytometry has been used to select an extremely high affinity anti-fluorescein single-chain Fv antibody (K_d = 48 fM) from libraries displayed on yeast [27].

Throughput is relatively high (up to 100 000 s^{-1}; http://www.cytomation.com), but flow cytometry does impose an upper limit of ~10^9 on the size of libraries that can be selected. The small size of the compartments (~5 fl) means that very large gene libraries could potentially be selected: a 100 μl reaction mix dispersed in 0.5 ml oil forms ~2×10^{10} aqueous compartments. However, to do so it is probably necessary to select the microbead display libraries by affinity purification, as with other display technologies.

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