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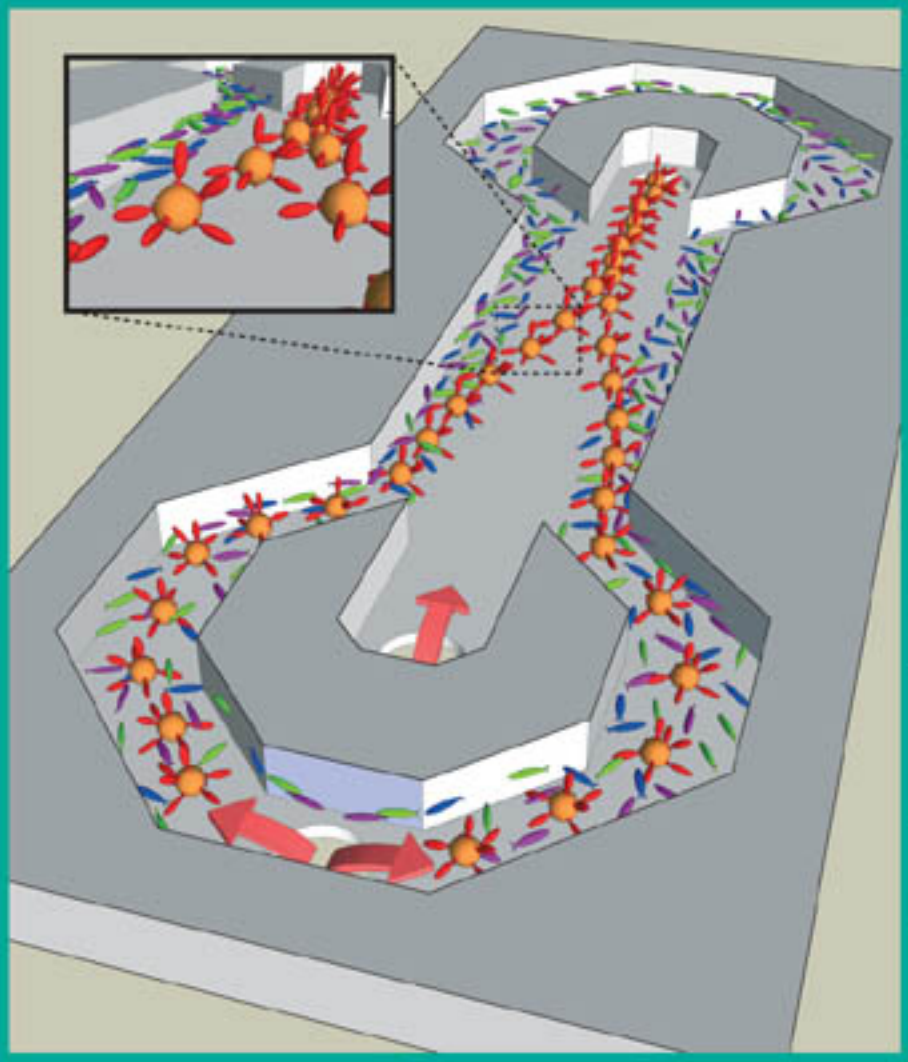

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Acoustic microfluidic chip technology to facilitate automation of phage display selection

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Keywords
acoustic standing wave forces; antigen-specific binding; microfluidic chip; phage display; selection

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Modern tools in proteomics require access to large arrays of specific binders for use in multiplex array formats, such as microarrays, to decipher complex biological processes. Combinatorial protein libraries offer a solution to the generation of collections of specific binders, but unit operations in the process to isolate binders from such libraries must be automatable to ensure an efficient procedure. In the present study, we show how a microfluidic concept that utilizes particle separation in an acoustic force field can be used to efficiently separate antigen-bound from unbound members of such libraries in a continuous flow format. Such a technology has the hallmarks for incorporation in a fully automated selection system for the isolation of specific binders.

The potential for recombinant antibodies in various analytical and therapeutic applications has developed substantially over recent years. With new therapeutic targets emerging continuously for various diseases and with the completion of the human genome sequencing project [1,2], extensive efforts are now directed towards understanding how complex sets of gene products are responsible for the many different functions of living cells with respect to both health and disease. The multiplex analysis approach, employing large arrays of antibodies, is being used to expand our knowledge of how proteins participate in such processes [3]. To carry out such studies, there is a vast need for specific detection reagents. Indeed, several efforts are underway to develop binders against large sets of proteins, such as those produced by the human genome. The Human Proteome Resource Center project is designed to raise specific binders, mainly specific rabbit polyclonal antibodies, targeting sequences with a unique potential for essentially any human protein [4]. The Proteome-Binders consortium (http://www.proteomebinders.org) has been set up to establish an infrastructure to isolate and use binding molecules (not necessarily antibodies) targeting essentially every member of the human proteome [5]. Similarly, the Antibody Factory (http://www.antibody-factory.de) [6], the Sanger Institute's ATLAS of protein expression [7] and the US National Cancer Institute proteome reagent program (http://proteomics.cancer.gov) [8] have been organized to deliver reagent resources to explore proteomes. Together, these efforts are designed to raise specific binders, with an origin in the antibody scaffold or other scaffolds well suited for their intended applications.

Specific binders are raised in a relatively high-throughput format by a number of approaches, such as the development of rabbit polyclonal antibodies [9] or murine monoclonal antibodies [10]. Subsequent to its introduction as a tool for the isolation of specific binders against essentially any target [11], phage display technology has evolved into a very efficient tool

Abbreviations
CMV, cytomegalovirus; gB, glycoprotein B; scFv, single chain antibody fragment; XG, xyloglucan.

with a high utility for the very same purpose as the development of polyclonal or monoclonal antibodies. Many features of phage display and other display technologies make them amendable to automation, allowing for the efficient development of the vast arrays of specific binders that are required in proteome research efforts [6,7].

Any process designed to develop specific binders comprises a number of unit operations, each of which has the goal to produce a product that is important for a subsequent step in the process. To ensure high throughput, a maximum level of automation is required. For the development of specific binders using phage display technology, several of these unit operations can be identified (Fig. 1A). Currently, large collections of antigens are available that may serve as sources of targets from a variety of species, including *Homo sapiens*, *Mus musculus* and *Saccharomyces cerevisiae* [4,9,12–15]. Efforts to use bioinformatics antigenic epitope analysis approaches and to produce new antigens that are suitable for the development of specific binders at a high rate are on-going [4,15,16]. Large collections of binders in the form of molecular libraries intended for different selection procedures, including phage display, are available [17–21]. Similarly, automated screening systems are available that can assess binding and specificity properties of large number of selected clones [22–24]. Systems to produce and purify specific binders at a high rate [9,25] and to confirm their specificity properties [9,26] are being established. The actual selection process and, specifically, the separation of unbound phage particles displaying nonspecific antibodies is, however, still in need of an automatable process. The selection quality generally depends on a number of washing and centrifugation steps to ensure the enrichment of rare phages displaying binders specific for a given target from the large bulk of other phages. Attempts to automate the separation process by catching antigen-specific phages on paramagnetic beads that are subsequently trapped and washed on magnets have met with some success [27,28]. Systems based on antigen-immobilized on microtiter plates have also been utilized [23], but further developments would facilitate this process and increase throughput and yield. In an approach adapted to selection from bacterial display libraries, Hu *et al.* [29] developed a microfluidic system, based on dielectrophoretic forces, that could isolate rare species from such entities. We now describe a highly flexible, fast and continuous flow process, also based on microfluidics and ultrasound-based focusing of particles (Fig. 1B–E), for the efficient enrichment of phages displaying specific

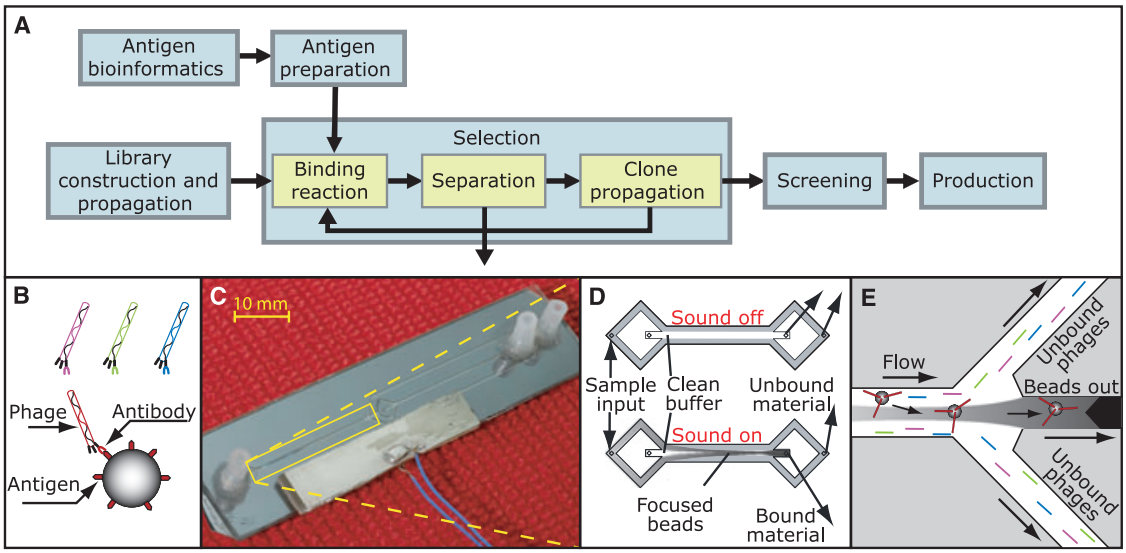

Fig. 1. Acoustic microfluidic chip technology in phage display. (A) Unit operations in a procedure to isolate antigen-specific binders by phage display. The selection unit is further divided into tasks to define the placement of the herein-designed separation unit in the process. (B) Specific antibody fragment-displaying phage particles bind to an antigen-coated bead as opposed to other phage particles. (C) Photograph of the microfluidic separation device. (D) Schematic of the separation device (only one unit illustrated). A mixture of beads and phage particles (light gray) is flow-laminated along both sides of phage-free buffer in the channel center (upper). Beads are focused towards the center of the flow under the influence of an ultrasonic standing wave field, whereas unbound phage particles, not being affected by the ultrasound, remain in their flow-laminated position near the side walls (lower). (E) Illustration of trifurcation outlet collecting the bead-containing center fraction (dark gray) of the flow while unbound phage-particles are effectively removed.

binders from commonly used phage display libraries. Beads carrying the target of interest are continuously translated from a complex buffer solution (phage particle-containing mixture) into a clean carrier buffer laminated in the center of a flow channel using acoustic standing wave forces. This procedure has the hallmarks of a process that lends itself to full automation. We envisage that this technology will be used in high-throughput operations for the development of a unit operation involving the selection and separation of specific binders from large combinatorial libraries.

Results

System design

Using an artificial mixture of two different affinity molecules [i.e. the carbohydrate-binding module XG-34 that binds xyloglucan (XG) and the single chain antibody fragment (scFv) GgB1 that binds cytomegalovirus glycoprotein B (CMV gB)] displayed on the surface of phage particles, optimal conditions were sought for enriching either of these two clones from a 1000-fold excess of the other clone using antigen immobilized on microbeads. The separation of bound and unbound phages was achieved using two serially linked acoustic separation channels because the use of a single channel device had proven insufficient. Generally, a 1000-fold enrichment factor of the phage displaying the protein binding the immobilized target was observed in a single round of selection (Fig. 2A).

Complex library selections

To validate the efficiency of the microchip-based separation system and to compare it with the classic manual separation method, parallel selections were performed using a conventional antibody fragment-displayed library by selecting binders for one specific target, the grass pollen allergen Phl p 5. Titration of input phagestocks and phagestocks made after selection and reinfection in *Escherichia coli* demonstrated that the microchip-based separation system was at least as efficient as conventional, manual separation in producing a population enriched for specific phages (Fig. 2B). After a single round of selection, 16 of 30 and nine of 30 randomly picked clones obtained after microchip-based or manual separation, respectively,

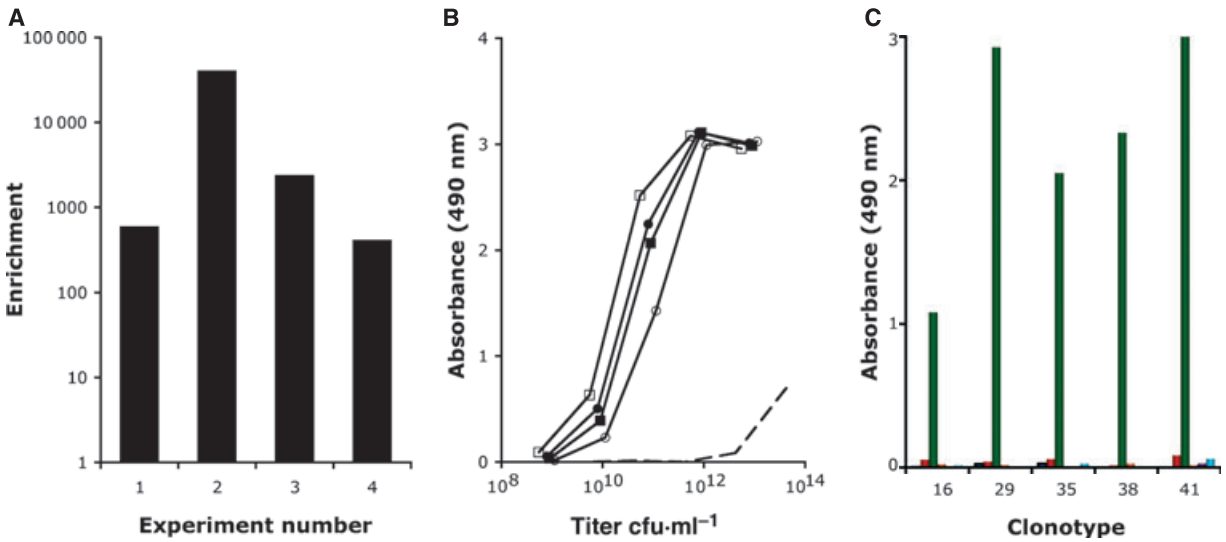

Fig. 2. Performance of acoustic microfluidic chip separation in phage display. (A) Enrichment factor of antigen-specific phages using the microchip-based washing principle. The results show the enrichment factor of CMV gB-specific antibody fragment GgB1 (experiments 1 and 2; duplicate experiments) and carbohydrate-binding module XG-34 (experiments 3 and 4; duplicate experiments) in the presence of a 1000-fold excess of phages displaying the other protein. (B) Titration by antigen (Phl p 5)-specific ELISA of polyclonal phage stocks to illustrate the enhanced recognition of allergen after enrichment of the antibody fragment library displayed on phage. Samples include a phage stock of the original antibody fragment library population before selection (dashed line) and phage stocks made after one round of selection for Phl p 5-specificity employing either a manual (closed symbols) or a microchip-based (open symbols) separation approach. (C) Antigen-specificity of selected binders. Representative clones of the five clonotypes (Fig. 3) identified after the use of microchip-based (clonotypes 16, 29, 35 and 38) and manual (clonotypes 29, 35 and 41) separation systems were assessed for specificity. Their binding to recombinant allergen Phl p 5 (green) (the antigen used in selection) but not recombinant Phl p 2 (dark blue), Phl p 6 (orange), Phl p 7 (magenta), natural Phl p 4 (red) or streptavidin (light blue) demonstrated that selected clones were specific for the intended target.

were specific for the target antigen, as determined by ELISA. To assess the diversity of this selected population, we performed sequencing of randomly picked clones that produced antibody fragments specific for the allergen. This procedure identified a diverse set of sequences in both selected populations (Fig. 3) [30,31]. Because genes encoding the heavy chain variable domain sequences of the library had been amplified from the transcriptome encoding IgE, a population restricted in the number of clonotypes that are contained within it [30,32], several of the clones were similar, as expected. The obtained clones could be divided into five groups based on their genetic resemblance. Clones from four of the five groups were extracted when using the microchip-based separation system, whereas three of the five groups were identified among the sequences found after the manual separation method. The presence of different mutations and light

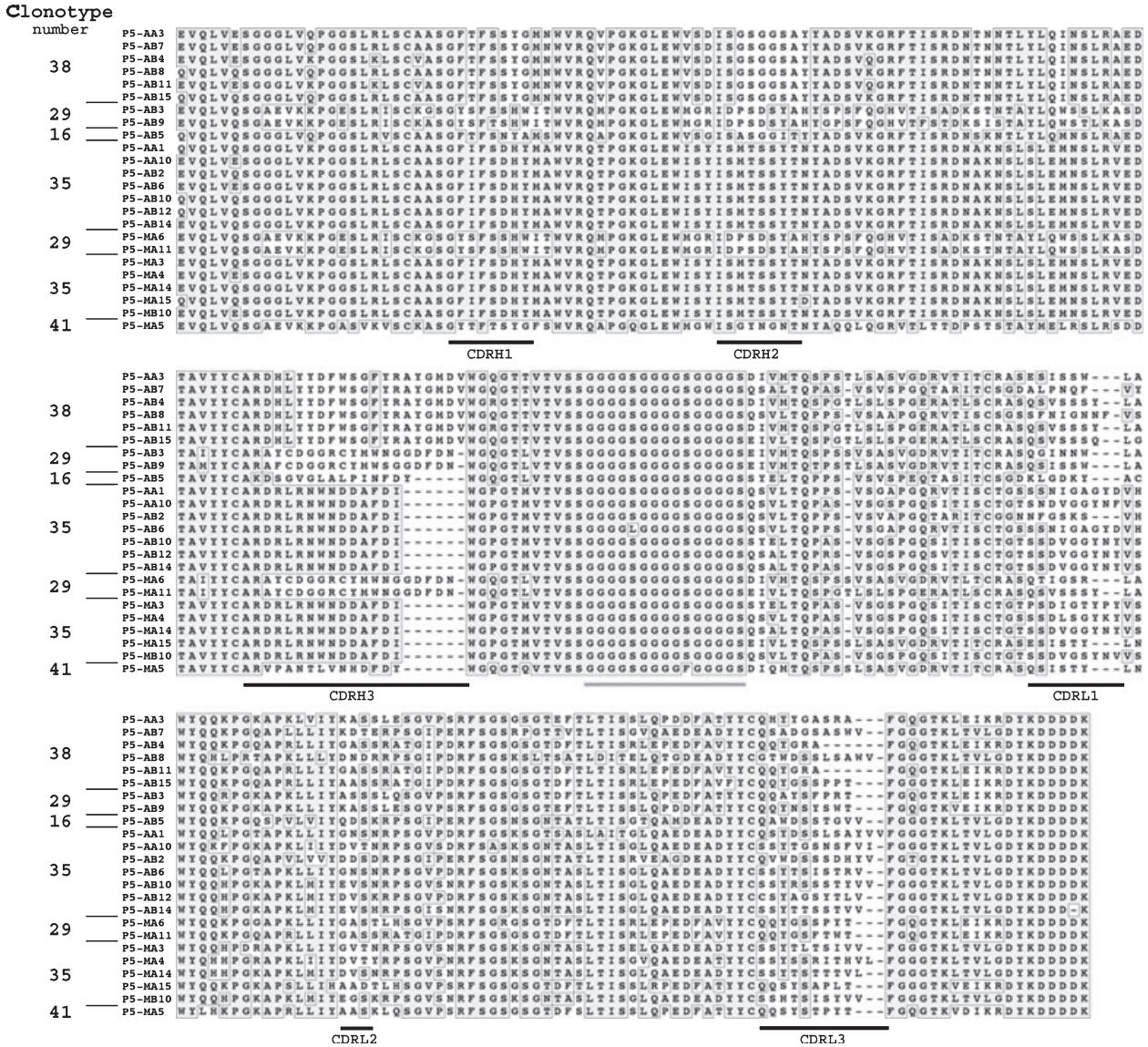

Fig. 3. Sequences of selected Phl p 5-specific scFv. Sequences of proteins selected by the microchip-based separation method (clones denoted P5-AA and P5-AB) and the conventional manual wash procedure (clones denoted P5-MA and P5-MB). Clones are arranged according to the separation method and their origin in a common clonotype as defined by Persson *et al.* [30] with the addition of clone P5-MA5 that represents a novel clonotype, number 41. All sequences, except P5-AB4 and P5-AB11, are unique. Complementarity determining regions (CDR) of the heavy (H) and light (L) chains, as defined by ImMunoGeneTics nomenclature [31], are underlined (black line). The linker region inbetween the H and L chain variable domains are underlined (gray line). Residues found in ≥ 50% of the sequences are boxed.

chain variable domains nevertheless demonstrated that many different sequences were selected in each group. The microchip-based separation method thus did not bias the selection to one or a few clones. In addition to sequences similar to those that had been selected previously [30,32], entirely new binders were selected, one each from studies employing the two different separation methods (clones P5-AB5 and P5-MA5). The specificity of representatives from the five groups for the target antigen was investigated. It was shown that binding to the target antigen was specific, demonstrating that the selection approaches were appropriate and selected for specific binders (Fig. 2C). In conclusion, the microchip-based separation method efficiently enriched phages displaying specific antibody fragments and retrieved a diverse population of specific sequence variants.

Discussion

The aim of the present study was to develop an efficient and easy-to-use separation method optimized for high-throughput development of affinity binders towards a multitude of targets, in order to cope with the growing demand for such reagents in applications such as global proteome analysis. These approaches use large arrays of different specific binders such as antibodies or antibody fragments towards the various targets in a proteome. When aiming to generate large enough numbers of antibodies, enormous pressure is placed on the development and selection stages [33]. Several of the different steps in the process of obtaining new antibodies through phage display, a state-of-the-art source of specific binders, are already automatable for high-throughput strategies. The actual selection process and, specifically, the separation of unbound phage particles displaying nonspecific antibodies is, however, still in need of an automatable process. We believe that the results presented in the present study comprise a substantial step towards a solution to this bottleneck in high-throughput phage display selection. To this end, a chip-based microfluidic wash system has been designed and tested because such a system has the potential to be easily incorporated into an automated liquid handling system. Subsequent to its introduction in 2001 [34,35], chip integrated ultrasonic standing wave technology has demonstrated important advancements in the precise control of particles in microfluidic systems [36]. A major development was the discovery that the induction of an acoustic standing wave in microchannels orthogonal to the incident sound wave allowed for acoustic force manipulation of cells and particles in microfluidic networks [37]. Advanced acoustic microchip particle separation approaches have subsequently been successfully exploited in biomedicine and biotechnology [38–41]. Acoustic microfluidic chip technology has recently also enabled noncontact particle and cell trapping and manipulation for online bioassaying [42–45]. The results of the present study now extend microchip acoustic particle separation into selective targeting of biomolecular entities, facilitating functional molecular evolution by genetic engineering. The microscale environment yields a low Reynolds number, and ensures perfect laminar conditions in the flow system, facilitating its separation efficiency. We have previously demonstrated the possibility of using acoustic forces to extract particles from a contaminated environment in a continuous flow format [41]. A system for continuous flow phage library selection is now proposed based on this concept. A detailed chip design and fundamental microfluidic and acoustic performances in conventional bioanalytical procedures evaluation have recently been described (P. Augustsson, J. Persson, S. Ekström, M. Ohlin & T. Laurell, unpublished results). We now define optimum operation conditions for the phage library selection performed in the present study. The initial assessment of the system indicated that it was capable of separating bound and unbound phage particles and that it achieved an enrichment factor in the order of 1000 in a single chip comprising two serially coupled separation channels. The exact level of enrichment will be dependent not only on the separation approach itself, but also on the specific character (level of display, affinity, etc.) of the molecules displayed on the phage particles. The achieved enrichment, therefore, does not define the upper limit of enrichment but rather a realistic level. Assessment of contamination of phages in an antigen-free system indicated that the efficiency of separation can be as high as 99.9999% for a double channel chip. Efficiencies approaching an at least 1000-fold enrichment may then be achievable depending on level and nature of the displayed molecules. Importantly, the separation step requires no manual intervention and it is completed in approximately 8 min when applying a 500 μL sample, which is a volume typical of many selection procedures, suggesting that even a single unit can handle large numbers of samples in 1 day even when considering the need for automated wash cycles between different runs. Moreover, the throughput of beads was approximately 5×10^4 s^{-1}, which is considerably high in a microfluidic chip context.

The usefulness of a unit operation in phage selection depends not only on the speed, but also on its ability to maintain diversity in the population of selected

molecules. By assessing the diversity of clones obtained after selection on Phl p 5, we determined that a variety of clones could be obtained. It is evident that this system is addressing a very similar antibody repertoire, and certainly a no less diverse one, compared to the manual wash system.

In conclusion, the chip-based microfluidic wash system that separates bound and unbound phages, displaying proteins with a specific binding property, is at least as efficient as conventional separation approaches, such as those involving washing of microtiter plates or microbeads. However, it has several advantages, including an automatable fluidic system approach and the potential for high throughput. In addition, it has the capacity to use a variety of beads and cells [39,46] as antigen carriers because very different types of particles can be focused by ultrasound. The system is thus highly flexible and can be adopted to virtually any kind of antigen carrier. Altogether, we foresee that the proposed chip-based microfluidic wash system for antigen-bound phage enrichment/extraction will be used as an automated unit operation in approaches to isolate binders specific for members of entire proteomes.

Experimental procedures

Proteins, genes, vectors and libraries

Recombinant CMV gB [47] and biotinylated XG [48] was kindly provided by Sanofi-Pasteur (Marcy l'Etoile, France) and H. Brumer (the Royal Institute of Technology, Stockholm, Sweden), respectively. Recombinant timothy allergens (Phl p 2, Phl p 5, Phl p 6 and Phl p 7) were obtained from BioMay (Vienna, Austria). The natural allergen Phl p 4 was kindly provided by J. Lidholm (Phadia AB, Uppsala, Sweden). Recombinant gB and Phl p 5, biotinylated using sulfo-NHS-biotin and sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA), respectively, and extensively dialyzed against NaCl/P_i, were kindly provided by Fredrika Axelsson and Kristina Lundberg (Lund University, Lund, Sweden).

For the purpose of the present study, we used phagemid vectors designed for display of proteins on protein 3 of filamentous phage. These included a vector based on pAK100 [49] encoding chloramphenicol resistance, which encodes a scFv, GgB1, specific for CMV gB (F. Axelsson, J. Persson, E. Moreau, M. H. Côté, A. Lamarre & M. Ohlin, unpublished data), and a vector based on a modified version of pFab5c.His [50] encoding ampicillin resistance, which codes for the carbohydrate-binding module XG-34 [48] specific for XG.

A library [32] encoding scFv cloned into the pFab5c.His vector was also used. The heavy chain variable domain-encoding-sequences of this library had been amplified from transcripts encoding IgE of an allergic donor. This library has previously been used successfully to select a range of scFv specific for a number of allergens [30,32].

Acoustic particle washing microchip

To create a chip for microbead separation, similar to that relevant in a system designed to potentially enable automated selection from combinatorial protein libraries such as those displayed on phage, we constructed a new microfluidic washing device (Fig. 1C), based on previous work (P. Augustsson, J. Persson, S. Ekström, M. Ohlin & T. Laurell, unpublished results). The manufacturing of the device was based on standard microfabrication techniques that are accessible in most clean-room facilities. The basic silicon processing scheme has been described in more detail by Nilsson *et al.* [37]. Briefly, the separation channel was etched in (100) silicon using standard KOH wet etch techniques creating channels of rectangular cross section (width = 375 μm, height = 160 μm). The channel width was selected to match a $\lambda/2$ wavelength resonance criterion in aqueous media. Borosilica glass was anodically bonded to the silicon to enclose the flow structure and to allow for optical surveillance. Particles passing along the channel while actuated at 2 MHz will experience a primary acoustic radiation force that will position them either in the center of the channel or near the side walls. The magnitude and direction of the force is dependent on the acoustic properties (density and compressibility) of the particles as well as the suspending media. Most biological and fabricated particles are slightly denser than water, which makes them move towards the center of the channel. Because the ultrasound has little or no effect on the suspending media, it is possible to utilize the force field to move particles from one media to another by flow lamination of the two media in the presence of an acoustic force field (Fig. 1D).

The separation chip was actuated using a 7×35 mm piezoceramic (PZT 27; Ferroperm Piezoceramics A/S, Kvistgard, Denmark) resonant at 2 MHz. The transducer was glued to the upper side of the glass alongside to the channel structure. A function generator (HP 3325A; Hewlett-Packard Inc, Palo Alto, CA, USA) coupled to a power amplifier (Amplifier Research Model 50A15; Amplifier Research, Souderton, PA, USA) fed the transducer with a 2 MHz sine wave. The net power (transmitted minus reflected) was monitored using a wattmeter (43 Thruline Wattmeter; Bird Electronic Corporation, Cleveland, OH, USA).

Sample containing beads and unbound molecular material entered the structure and was bifurcated to each side of the first of two wash fluid inlets. The sample and wash fluid did not mix due to the highly laminar flow condition in the microchannels. The fluids passed a 2-cm long channel segment where the beads were acoustically focused towards the center of the channel, whereas the unbound material

remained in its flow-laminated position near the side walls. By splitting the flow outlet in three, the undesired material was separated from the beads that continued via yet another bifurcation to a second identical wash step (Fig. 1B–E).

Production of phage stocks

All phage stocks were produced by standard procedures. Briefly, F-pili-carrying *E. coli* were grown in medium containing 1% glucose and relevant antibiotics. When the culture had reached exponential growth phase, the bacteria were infected with VCS-M13 helper phages (Stratagene, La Jolla, CA, USA) for 30 min at 37 °C. Phage stocks were produced by culture in glucose-free medium containing antibiotics and 0.25 mM isopropyl thio-β-D-galactoside at 30 °C overnight. In some cases, phages were precipitated by the addition of 0.25 volumes of 20% PEG6000/2.5 M NaCl and resuspended in NaCl/P_i. Phage stock of the library with an origin in IgE-encoding transcripts were used as such, whereas phage stocks displaying XG-34 and GgB1 were mixed in ratios of approximately 1 : 1000 and 1000 : 1 to prepare model mini-libraries useful for evaluation of phage purification efficiency.

Selection system

Biotinylated ligands, XG (20 μg), gB (5 μg) or Phl p 5 (20 μg), were added to 50 μL of streptavidin-coated M280 Dynabeads (Invitrogen, Carlsbad, CA, USA) and incubated for 2 h on a rotator at room temperature. These beads were washed three times with 3% BSA and 0.05% Tween-20 in NaCl/P_i (NaCl/P_i-Tween) to remove excess ligand prior to use. Phage populations, either artificial mixtures of those displaying XG-34 and GgB1 or those displaying scFv with an origin in the IgE-encoding population, were added in NaCl/P_i-Tween to beads ($\sim 5 \times 10^7$ beads·mL^{-1} in final suspension) coated with the ligand. The mix was incubated on a rotator for 1–2 h at room temperature.

Microchip-based wash procedure

Samples containing phages and antigen coated-microbeads were aspirated into a 1 mL disposable syringe that was inserted into a syringe pump (WPI SP210IWC; World Precision Instruments Inc., Sarasota, FL, USA) vertically and above the microfluidic washing device. Wash fluid was loaded into a pair of 10 mL glass syringes (1010 TLL; Hamilton Bonaduz AG, Bonaduz, Switzerland) positioned in a dual push–pull syringe pump (WPI SP2 60P; World Precision Instruments Inc.) with an additional pair of syringes mounted reversely in the same pump for waste fluid aspiration. TFE Teflon™ Tubing (inner diameter 0.3 mm) (Supelco, Bellefonte, PA, USA) was used for guiding fluids in and out from the device. The sample outlet was open to atmospheric pressure through a short piece of tubing emanating in a sample collection test tube. The system was primed with wash fluid (NaCl/P_i-Tween) by compressing the wash syringes until all air bubbles were completely removed from the channel structure and all external tubing. Prior to connecting the sample injection syringe, the wash syringe pump was run for approximately 1 min to stabilize flow in the system. The wash fluid flows were set to 120 μL·min^{-1} into each washing chamber and the sample injection and throughput flow was set to 60 μL·min^{-1}. The ultrasound was subsequently turned on at a frequency of 2 MHz delivering a net power of 1.1 W to the transducer. Washed bead suspensions were collected from the device in a continuously running process in fractions of 0.2 mL.

Manual wash procedure

Mixtures of antigen-coated beads and phage stocks were washed five times with NaCl/P_i-Tween and three times with NaCl/P_i using a magnet to retrieve the microbeads.

Bacterial infection procedure

A slurry of beads obtained after the manual wash procedure or as the output from the outlet of the microchip washing device was added to exponentially growing *E. coli* carrying F-pili (Top10F′) for 30 min at 37 °C (without shaking). Dilutions of bacteria infected with artificial mixtures of phages displaying GgB1 and XG-34 were spread on culture plates (LB agar) containing chloramphenicol (25 μg·mL^{-1}) or ampicillin (100 μg·mL^{-1}). The relations between the two clones in the libraries after the phage binding and subsequent wash procedure were determined from the numbers of colonies on plates with the different antibiotics. The output after selection on Phl p 5 was grown on plates containing ampicillin and 1% glucose. After culture for 16 h at 37 °C, the number of colonies was counted.

Immunological analysis

To assess the quality of the output of selection of scFv on the recombinant allergen, fifteen clones were picked from each of four selections performed on Phl p 5 using the conventional, manual washing approach (clones named with prefixes P5-MA and MB) or the microchip-based washing approach (clones named with prefixes P5-AA and AB). Phage stocks for each of the 60 clones were analyzed in ELISA to determine their ability to bind the antigen Phl p 5 in addition to several other antigens from grass pollen. Bound phages were detected with horseradish peroxidase-conjugated M13-specific monoclonal antibody (GE Healthcare Biosciences Corp., NJ, USA) using *o*-phenylenediamine as chromogen. Phage stock for the

entire polyclonal outputs after selections (MA and MB, AA and AB), in addition to the parental IgE-based library in serial dilutions, was also analyzed by antigen-specific ELISA to determine the accumulated specificity relative to the parent library.

Sequencing and sequence analysis

Plasmids from the clones producing Phl p 5-specific scFv were purified from bacterial cell pellets using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and subsequently sequenced (MWG Biotech, Martinsried, Germany). Sequences (GenBank Accession Numbers EF601881–EF601896 and EU090053–EU090060) were compared with previously selected clones from the library specific for Phl p 5 [30,32]. Clones were named using the following nomenclature: P5 (defining timothy group 5 allergen specificity), a letter combination denoting an origin in selections employing either manual (MA and MB) or microchip-based (AA and AB) washing approaches, and a clone number.

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