

Decomplexing biofluids using microchip based acoustophoresis

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Highly efficient washing and extraction of microbeads to decomplex analytes ranging from small peptides to large viruses was realised in a microscaled continuous flow format. The bead washing principle reported herein is based on acoustophoresis, *i.e.* the primary acoustic radiation force in an ultrasonic standing wave and laminar flow properties are utilised to translate bioanalytes trapped on functionalised microbeads from one carrier fluid to another. The carry-over of non-specific material ranges from 1 to 50 ppm relative to input levels depending on application, making acoustophoresis suitable for extraction of rare species from complex environments. Selective extraction of a phosphopeptide relative to its unphosphorylated counterpart is demonstrated using metal oxide affinity capture (MOAC) beads and MALDI-TOF MS readout. Acoustophoresis of microbeads activated with specific binders could be used to capture phage viral particles. The efficiency of the acoustophoretic washing principle was demonstrated by an unspecific phage cross contamination level of only 10^{-6} of that in the input bead/phage mixture. The continuous flow format makes acoustophoretic washing flexible regarding sample volume and also allows for easy integration into a sequence of particle handling and analytical unit operations.

1 Introduction

Sample preparation prior to analytical readout is a standard procedure in all bioanalytical laboratories. When working with crude biological samples decomplexing of the original biofluid is a key step to obtain an analysis situation that optimises the final readout. In this perspective centrifugation is a major workhorse, commonly used to clarify samples containing particulate matter, microorganisms, cells or cellular fragments. When high concentrations of interfering substances prevail, dialysis is a frequently used method to reduce complexity. A well-known example is the use of on-line microdialysis to recover low molecular weight compounds from the extra-cellular matrix in tissue, enabling on-line coupling to liquid chromatography¹ analysis or direct biosensor readout.² The dialysis step removes high molecular weight compounds that would compromise a subsequent chromatography step. Likewise, removal of large molecular species, *e.g.* proteins in the case of on-line coupling of microdialysis to an electrochemical biosensor, minimizes contamination of the electrode surface and thus long-term sensor drift.³ Similar decomplexing methods encompasses centrifugal sieving through defined low molecular weight cut-off filters.⁴

More advanced sample preparation techniques are based on the use of affinity microbeads. Many times these are provided as packed columns in pipette tips, *e.g.* when desalting and concentrating proteomic samples by using solid phase extraction tips with a hydrophobic bead matrix.⁵ For more specific affinity interaction metal ion affinity capture beads, IMAC, have been

widely used to extract phosphopeptides from complex proteomic samples.^{6,7}

A most important development in sample decomplexing within the proteomics community has been the introduction of multiple immunoaffinity depletion columns, which remove high abundant proteins in blood serum. Björhall *et al.* performed an extensive investigation comparing different brands of commercially available depletion columns.⁸ More recently, superparamagnetic beads with affinity functionalities have been introduced, enabling direct mixing of the beads with the sample solution. This step improves the solid phase/sample interaction and thus extraction efficiency. To enable removal of an undesired supernatant, the superparamagnetic beads are forced to the side wall of the vial with a magnet, while excess fluid is aspirated and fresh buffer added. This procedure is subsequently repeated to perform a wash sequence of the affinity coupled bead suspension prior to displacement of the captured analyte for the following analysis. Nice examples of proteomic sample decomplexing using this strategy have been outlined by Villanueva *et al.*^{9,10} Superparamagnetic bead based sample decomplexing in a microfluidic format has also been addressed, either by targeting DNA hybridisation on single strand activated superparamagnetic microbeads¹¹ or by binding of DNA to chaotropic salts present on the silica surface of superparamagnetic microbeads.¹²

While yet capitalizing on known strategies and progress in solid phase sample preparation, this paper proposes a concept for analyte extraction from complex environments where the batch mode operation as commonly used in bead based sample decomplexing, *e.g.* superparamagnetic bead extraction, is avoided. We rather propose a continuous mode of operation, realised in a microfluidic system where affinity microbeads are washed on-line and extracted from the original complex sample solution into a pure buffer system using acoustic forces in a laminar flow format, *i.e.* acoustophoretic particle washing. The presented

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acoustophoresis chip was devised in a serial two-step particle washing configuration.

Acoustic particle manipulation in microchannels has shown great potential for concentration,^{13–15} trapping,¹⁶ washing,^{17,18} sorting¹⁹ and mixing²⁰ of cells and particles. A major advantage compared to centrifuge-based protocols is that the technique allows for continuous operation putting little restriction on sample volume. Microchip integration of the sample handling process in a continuous flow format enables serial integration of multiple particle handling building blocks in one device.

The fundamental feature to realise the proposed acoustic force particle handling relies on the primary acoustic radiation force that is the major and governing force on particles ($d > 1 \mu\text{m}$) in an acoustic standing wave field. In the case of a plane standing wave equation, (1) and (2) describe the force F_r on a particle.^{21–23} In equations (1) and (2) P_0 is the pressure amplitude of the acoustic standing wave, V_p is the particle volume, β and ρ are compressibility and density, respectively, λ is the wavelength of the standing wave and x denotes the distance to any pressure node. Indexes p and m denotes particle and suspending medium properties, respectively. As can be seen in equation (2) the sign and magnitude of the force is dependent on the difference in acoustic properties of the particle *vis-à-vis* the suspending medium. More dense particles tend to move towards the pressure nodes while lighter particles move towards the pressure antinodes. This phenomenon has previously been reported for having successfully separated blood cells from lipid particles.²⁴

$$F_r = -\left(\frac{\pi P_0^2 V_p \beta_m}{2\lambda}\right) \phi(\beta_{p,m}, \rho_{p,m}) \sin\left(\frac{4\pi x}{\lambda}\right) \quad (1)$$

$$\phi(\beta_{p,m}, \rho_{p,m}) = \frac{5\rho_p - 2\rho_m}{2\rho_p + \rho_m} - \frac{\beta_p}{\beta_m} \quad (2)$$

This paper demonstrates the use of the primary acoustic radiation force in an acoustophoresis configuration to be an attractive approach to decomplex a variety of bead suspensions containing target analytes ranging from low molecular weight compounds like peptides to viruses. More specifically, selective phosphopeptide extraction from a mixture containing both the phosphorylated and nonphosphorylated peptide was performed and validated using MALDI-TOF MS, showing no cross contamination between runs. Likewise, peptide signatures in a high BSA background were purified in the acoustophoresis microchip showing equal decomplexing performance as obtained by manual processing of the superparamagnetic beads. Antibody based affinity bead extraction was also used to demonstrate the ability to selectively extract larger biological entities like viruses from samples, a technology that can have an impact in microbiological laboratories as well as in the field of genetic engineering.

2 Methods

2.1 Manufacturing of microfluidic extraction device

The acoustophoresis channel structure was etched in a $350 \mu\text{m}$ thick, 3 in. <100> silicon wafer using standard UV-lithography and KOH-etching protocols.¹⁵ The depth of the channel was $160 \mu\text{m}$ and the width of the wash channel was $375 \mu\text{m}$ corresponding

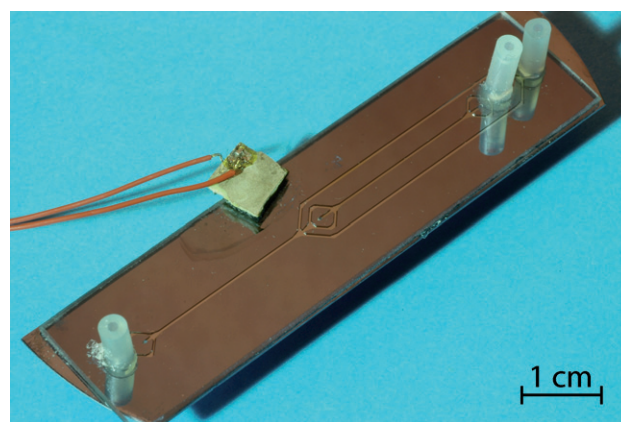


Fig. 1 Photograph of the acoustophoresis-based particle washing microchip. The square shaped piezoceramic actuator is glued onto the bonded glass lid. Tubing connectors for sample inlet and the two waste outlets are seen from lower left to top right.

to a half wavelength standing sound wave of $\sim 2 \text{ MHz}$ in water. Other segments of the channel were slightly wider in order to prevent ultrasonic resonance and thereby avoiding undesired acoustic interference. Holes were etched from the back side of the wafer to constitute three of the six fluid connections to the structure and the wafer was diced into 18 by 70 mm pieces. Borosilicate glass (1.1 mm, 18 mm, 70 mm) (Glasteknik i Emmaboda AB, Emmaboda, Sweden) with drilled holes for the three remaining fluid connections was anodically bonded to the silicon surface in order to seal the structure and to allow for visual inspection. Silicone tubings (i.d. 1 mm, o.d. 3 mm, length 5 mm) were glued (Elastosil A07, Drawin Vertriebs-GmbH, Riemerling, Germany) onto the inlets and outlets for easy attachment of external tubing (Fig. 1).

2.2 Flow system arrangement

The acoustophoretic wash chip had one sample inlet and one sample outlet. There were two inlets for wash fluid and two outlets for waste fluid. Samples were injected into the device at a volume flow rate of $50 \mu\text{L min}^{-1}$ using a syringe pump (WPI sp210iwz, World Precision Instruments Inc., Sarasota, FL, USA). Sample (typically $500 \mu\text{L}$) was aspirated into a syringe mounted in the syringe pump arranged vertically and above the channel structure. A second syringe pump with two syringes was operated in a push pull configuration to drive the wash fluids into and out from the system (WPI sp260p, World Precision Instruments Inc., Sarasota, FL, USA). The wash fluid volume flow rates were $100 \mu\text{L min}^{-1}$ and $200 \mu\text{L min}^{-1}$ in the primary and secondary wash unit, respectively.

2.3 Actuation

A 2 MHz piezoceramic plate (Pz26, Ferroperm Piezoceramics AS, Kvistgard, Denmark) was used for the generation of the ultrasound. The piezoceramic was operated at $\sim 2 \text{ MHz}$, $\sim 25 \text{ Vpp}$, $\sim 1 \text{ W}$ (net power) sine wave using a function generator (HP 3325B, Hewlett-Packard Inc., Palo Alto, CA, USA) and it was glued (Loctite 3430, Henkel Loctite Corporation, Helsinki, Finland) onto the glass lid of the device along the side of the

channel structure. A favourable positioning of the transducer was found by moving the piezoceramic along the device while performing particle focusing in the channel before the glue was completely dry.

2.4 Sample preparation and analysis

2.4.1 Dissolved species. Samples were prepared by dilution of a 20 mg mL⁻¹ stock solution of Evans blue (EB)(Merck, Darmstadt, Germany) to a final concentration of 3 mg mL⁻¹ in PBS (9.5 mg mL⁻¹, pH 7.4) (Sigma-Aldrich Chemie, Steinheim, Germany) and Tween 20 (0.01%). Polystyrene particles (~5 µm diameter, ~6 × 10⁶ mL⁻¹ when measured in final solution) (Fluka Chemie GmbH, Buchs, Germany) were thereafter added to the solution. A washing buffer was prepared by adding Tween 20 (0.01%) to PBS. Microbead number concentration was analysed using a Coulter counter (Multisizer 3, Beckman Coulter Inc., Fullerton, CA, USA) from which the particle size distribution was retrieved. By adding the size bins over the measured interval (typically from 2 to 10 µm diameter) the total amount of particles in a sample was measured. For the analysis of Evans Blue a microplate spectrophotometer (Multiskan Multisoft, Labsystems, Helsinki, Finland) was used for readouts at 595 nm.

2.4.2 Phosphopeptide extraction. Samples of the phosphorylated peptide ALGADDS(pY)YTAR and the corresponding unphosphorylated peptide ALGADDSYYTAR was prepared daily by dilution of a 10 µM stock solution in 0.1% trifluoroacetic acid (TFA), and diluted to a working solution of 50 nM with 0.1% TFA. This working solution was further diluted 1 : 10 with binding buffer (supplied within Phos-Trap™ phosphopeptide enrichment kit PRT301001KT, Perkin Elmer Inc., Waltham, MA, USA), to provide a 5 nM sample solution. Different volumes of the sample solution were used to provide the specified amounts of peptides.

For the complex sample a solution of 10 mg mL⁻¹ bovine serum albumin (BSA) (Sigma-Aldrich Chemie, Steinheim, Germany) in binding buffer was spiked with the phosphopeptide ALGADDS(pY)YTAR to a concentration of 10 nM.

Acoustic chip purification of phosphopeptides was performed using 4–7 µm superparamagnetic Metal Oxide Affinity Capture (MOAC) beads and buffers from Phos-Trap™ phosphopeptide enrichment kit (PRT301001KT, Perkin Elmer Inc., Waltham, MA, US). Briefly, bead suspension 10 µL in binding buffer was added to the sample solution and allowed to incubate at RT for 1 h. The sample solution containing the MOAC beads was then diluted to a total volume of 100 µL if necessary and injected into the acoustic washing chip. In the acoustic chip the sample beads were washed with binding buffer in the first step and washing buffer in the second step to remove unspecific binders (*i.e.* non-phosphorylated peptides). This wash protocol was chosen in order to emulate the standard Phos-Trap™ sample preparation protocol (see below). After in chip wash, 100 µL of beads in washing buffer were collected into an Eppendorf tube directly from the outlet. The beads were subsequently pelleted by placing the tube on a magnet, the supernatant removed and 5 µL of elution solution was added followed by a final pelleting of the beads. Finally, 2 µL of the elution solution was spotted directly on a standard stainless steel target followed by 1 µL of matrix, 5

mg mL⁻¹ α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile, 0.1% TFA.

Reference samples were prepared according to the standard Phos-Trap™ phosphopeptide enrichment kit protocol. Briefly, incubation and capture was performed in 0.6 mL Eppendorf tubes and a strong magnet was placed at the bottom/side of the Eppendorf tube to retain the superparamagnetic beads during 4 × 200 µL washes with binding buffer, followed by a final wash with 200 µL washing buffer. Care was taken during pipetting to avoid bead loss. For comparative reasons the same amount of beads and sample was used in the chip and reference sample preparation protocol. Elution was performed with a volume of 5 µL, elution buffer and this volume was used to make MALDI spots of 2 µL eluate and 1 µL matrix.

MALDI-TOF MS analysis was performed on a M@ldi LR (Waters/Micromass, Milford, MA, USA) instrument in reflector mode. Prior to data acquisition the MALDI settings were optimised to provide the best possible resolution and a three point calibration was made on each plate. From each sample spot a spectrum of 100 summed laser shots was acquired.

2.4.3 Affinity extraction of bacteriophages. Filamentous bacteriophage was investigated as a model for separation of larger bioparticles using on-chip acoustic manipulation. Phage particle preparations were prepared by infecting *Escherichia coli* Top10F' (Invitrogen, Carlsbad, CA, USA) harbouring a phagemid vector encoding a fusion between the AE11F antibody fragment²⁵ and the C-terminal domain of filamentous phage protein 3 with VCS-M13 helper phage (Stratagene, La Jolla, CA, USA).

Beads coated with antibodies specific for mouse immunoglobulin (Dynabeads Pan Mouse IgG, Invitrogen Dynal AS, Oslo, Norway) were coated with mouse anti-M13 filamentous phage antibodies (GE Healthcare, Uppsala, Sweden) that bind protein 8, a highly abundant bacteriophage protein (>2000 copies per phage particle).²⁶ The mixture was incubated for 2 h. Excess antibody was removed by centrifugation and washing of the beads in PBS containing 3% BSA, 0.05% Tween 20. The beads were resuspended in the same buffer containing phage particles displaying the AE11F antibody fragment. For reference, beads not carrying the anti-protein 8 antibody but otherwise treated in the same way were also incubated with the phage-containing solution.

Streptavidin-coated microbeads (M280, Dynal) were separately coated with a biotinylated peptide corresponding to the AD-2-sequence of cytomegalovirus glycoprotein B²⁵ on rotator for 2 h. The beads were subsequently centrifuged and the supernatant containing excess antigen was removed by manually washing 5 times with 1 mL 3% BSA, 0.05% Tween-20 in PBS. The beads were resuspended in a solution of 3% BSA, 0.05% Tween-20 containing phage particles displaying antibody fragment AE11F that binds the AD-2 epitope.²⁵ As these antibody fragments fused to the C-terminal domain of phage protein 3 were produced from a phagemid vector co-existing with the VCS-M13 helper phage, they were displayed on only a fraction of the protein 3 molecules found on the phage particles.²⁷ As protein 3 is found in only 3 to 5 copies on the bacteriophage particle,²⁶ and as not all protein 3 units actually carry the antibody fragments, the display of these proteins is substantially lower than in the case of the presence of protein 8. The bead/bacteriophage mixture was

incubated on rotator for 2 h. For reference, beads not carrying the peptide but otherwise treated as described above were also incubated with the phage solution. Beads were then extracted from the solution by either manual washing or acoustic extraction procedure.

Analysis of infectious phage particle number was performed, before and after manual or acoustic extraction, by adding bead slurry of a sample to exponentially growing *E. coli* carrying F-pili (Top10F') that can be infected by these bacteriophages. The bacterial suspension was incubated for 30 min at 37 °C without shaking. The bacteria were then spread on LB agar culture plates containing antibiotics (100 µg mL⁻¹ ampicillin) so that only infected bacteria could survive on the plates and form colonies. The number of colony forming units per ml (cfu mL⁻¹) was counted after culture for 16 h at 37 °C.

3 Results and discussion

The acoustophoresis based particle washing chip reported herein was developed with the intention of achieving a washing efficiency, well matching the performance of commonplace manual washing procedures. The design of the washing device was based on a previous design¹⁸ that was shown to wash away 95% of dissolved species (Evans Blue colour compound). Particles suspended in contaminated medium were transferred by means of acoustophoresis from their initial flow path near the side walls of a microchannel, towards the centre into a clean buffer zone of the channel by actuating the chip with an ultrasonic frequency matching the channel width by a half wavelength. The contaminated medium was not affected by the obtained standing wave and consequently continued along the side walls of the channel. Particles and contaminated medium could thereby be separated and collected through different outlets at the end of the channel (Fig 2). The new particle washing chip performed two sequential cycles of acoustic particle transfer (buffer exchange).

3.1 Buffer exchange principle

Particles suspended in contaminated carrier fluid were injected along the sidewalls of a separation channel while clean carrier

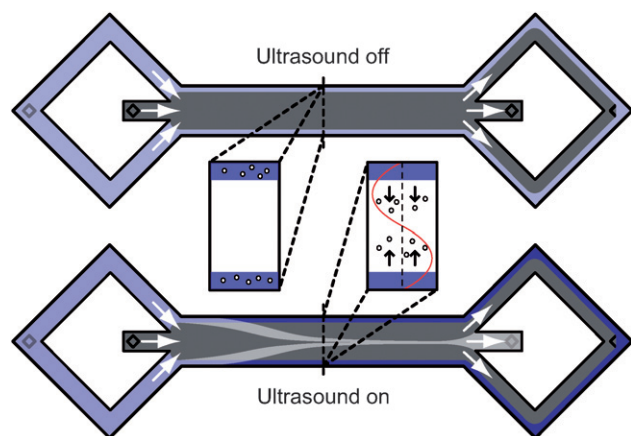


Fig. 2 Principle of an acoustic microfluidic particle washing device. The force field experienced by the microparticles is indicated with a sinusoidal wave.

fluid was injected in the centre of the same channel. The acoustophoretic transport of particles into clean wash fluid was performed twice in the device in order to raise washing efficiency substantially compared to previous work. The detailed two step microfluidic solution is given in Fig. 3. Sample (beads in a contaminated buffer) enters the wash channel structure through inlet 1 and is split into two streams that are laminated along both sides of the central wash buffer stream, injected at inlet 2. The fluids do not mix due to the highly laminar flow conditions in the channel. The microparticles will stay in their flow laminated position, consequently ending up in outlet 6, as long as the ultrasound is turned off. When the ultrasound is activated, particles will migrate under influence of the employed acoustic force towards the centre of channel segment I, leaving most molecules or small fragments in its original side wall laminated flow path. The focused particles are then again divided and are transferred to the next wash step (B) where fresh wash fluid is injected through inlet 3 and the process of focusing particles is repeated in segment II. Particles focused in the clean carrier fluid (unit II) exit the channel through outlet 4. Material

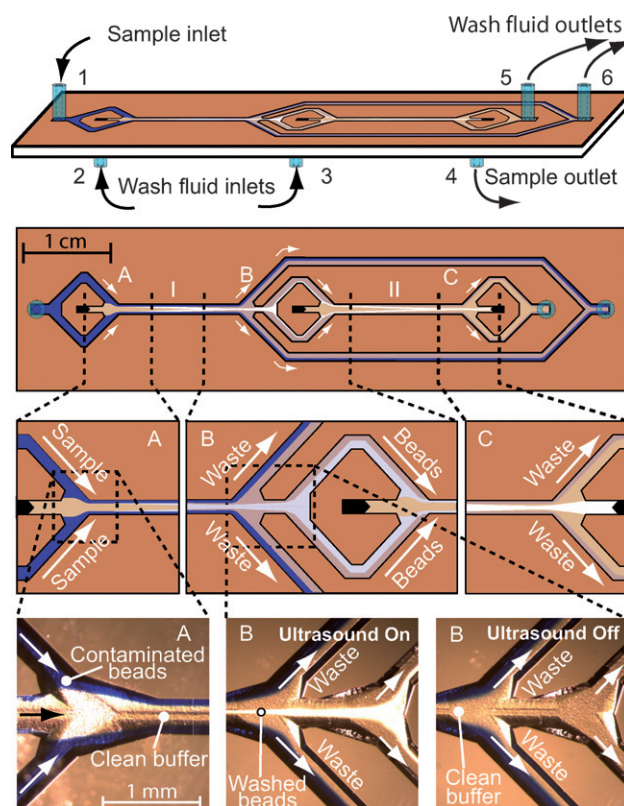


Fig. 3 Schematic of the acoustically controlled particle washing principle. (A) Beads in contaminated carrier fluid (sample) are laminated on both sides of the clean buffer flow (A). When ultrasound is turned off, beads in contaminated carrier fluid exit the channel through a pair of waste fluid side outlets of wash unit I (B). When ultrasound is turned on, beads are focused before the trifurcation outlet of extraction unit I (B). Contaminated carrier fluid exit through the waste outlets while beads exit through the centre outlet and are transferred to extraction unit II. The particle stream is subsequently split and proceeds into the entrance of the 2nd wash unit (II), where (C) yet one acoustic washing/extraction takes place. Photo inserts (bottom) show the corresponding microfluidic states.

that is not affected by the ultrasound will exit through outlet 5 and 6.

3.2 Particle extraction, dissolved species

Samples containing Evans blue and microparticles based on polystyrene (5 μm diameter) were processed in the device as a model system to evaluate the washing performance of the acoustophoresis chip. The aim was set to produce a clean suspension of particles in PBS containing the lowest possible amount of Evans blue. The frequency was tuned to 1.989 MHz and the voltage, ~ 25 Vpp, over the transducer was set to focus particles immediately before the trifurcation outlet of wash unit I. The sample throughput in the device was set to a volume flow rate of 50 $\mu\text{L min}^{-1}$. It was noted that in the second wash channel, particles reached the centre of the channel at an earlier stage than in the first channel under equal flow conditions. This is explained by an uneven ultrasonic energy distribution in the microchip. In order to accommodate for the stronger particle focusing in wash unit II the centre inlet buffer flow was increased to 200 $\mu\text{L min}^{-1}$, giving equal focusing conditions as in unit I. A high ultrasound intensity to flow ratio was also found to cause asymmetric bead paths and trapping (Fig. 4).

The performance of wash unit I & II was evaluated individually, measuring the concentration of Evans blue in the purified bead suspension, collected at the central outlets. The two serially linked wash units (dual extraction unit) were also evaluated using the Evans blue contamination model, (Fig. 5). In order to relate the chip based extraction efficiency to a manual procedure, samples were washed manually 3 times using a micro-centrifuge and a vortex mixer. The dual unit chip based wash could clearly compete with the manual washing protocol regarding particle recovery and extraction efficiency. Analysis of particle numbers before and after extraction showed that $\sim 75\%$ of the particles

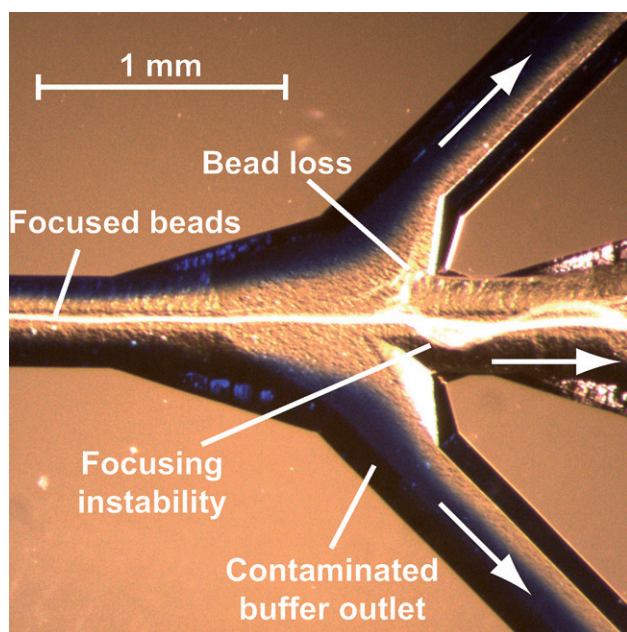


Fig. 4 Bead focusing instability at low flow rate relative to the acoustic power input. An increased flow rate suppresses this effect.

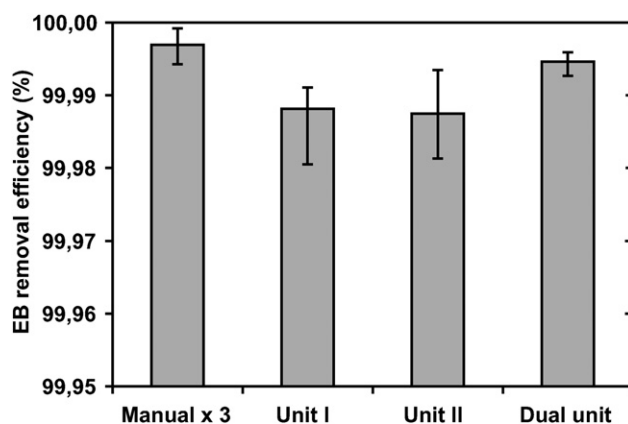


Fig. 5 Washing efficiency presented as percentage of removed Evans blue (EB) contamination relative to initial contamination level in the supernatant of the washed samples. The bars represent manual washing repeated 3 times, the wash performance of the individual wash units I and II and the wash performance of wash units I and II linked together in a dual unit, respectively. $N = 5$ for all bars and the error bars indicate the minimum and maximum values of the retrieved data.

were retained both for chip based extraction and for manual washing. The Evans blue removal efficiency, defined as $(\text{abs}_{\text{in}} - \text{abs}_{\text{out}})/\text{abs}_{\text{in}}$, was found to be 99.995% whereas the manual wash protocol yielded a 99.997% removal efficiency. Notably the efficiency of a single unit automated extraction was also very high (99.988% Evans blue removal), consequently leaving very little contamination to be removed in the second unit. (Fig. 5)

The high contaminant removal efficiency is significantly higher than what has previously been reported¹⁸ (95–98% removal efficiency). The deviation is proposed to be attributed to the relatively low particle content ($\sim 0.05\%$) of the processed samples, reducing any bead associated contaminant transfer. To support this idea, samples containing particle concentrations ranging from 0.01%w to 2%w were processed in the device, demonstrating a strong relationship between particle content and washing efficiency, (Fig. 6). We conclude that the acoustophoresis wash chip has the efficiency required for decomplexing of samples *e.g.* for bioanalysis where high washing efficiencies are required in order to suppress the high levels of unbound species.

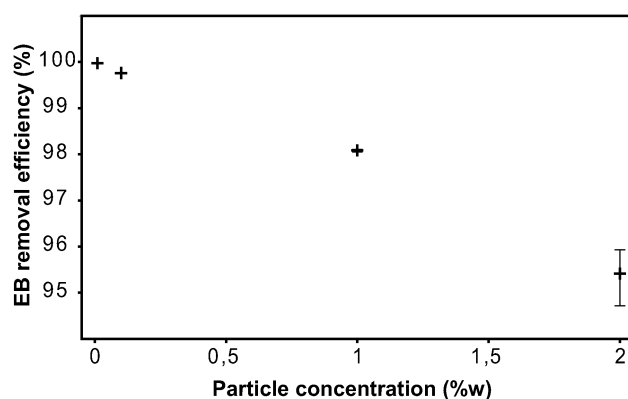


Fig. 6 Evans blue (EB) washing efficiency *versus* particle concentration. Single wash unit (I) operation. $N = 4$ for all bars and the error bars indicate the minimum and maximum values of the retrieved data.

3.3 Phosphopeptide affinity extraction

The recent introduction of metal oxide affinity capture media (MOAC) for selective enrichment of phosphopeptides has greatly improved the analysis of this biologically important class of peptides.^{28,29} Superparamagnetic MOAC beads were used to enable direct comparison of the analytical performance of the acoustophoretic buffer exchange sample preparation and a standard magnetic bead sample preparation protocol. Two experiments were carried out in order to evaluate the potential use of acoustophoretic washing for extraction of molecular species from complex environments.

In the first experiment MOAC beads were incubated in a sample solution containing equal amounts of unphosphorylated and phosphorylated peptide. The beads were thereafter subjected to the acoustophoretic washing procedure in order to remove the unphosphorylated variant of the peptide, while retaining the phosphorylated peptide. The analytes bound on the washed beads were subsequently eluted and analyzed with MALDI-TOF MS. Between each processed sample, the acoustophoresis chip was flushed briefly with buffer solution and a sample containing blank capture beads (*i.e.* no bound peptides) was run in order to reveal any carry over effects. As reference, identical samples were washed manually according to a standard manual protocol. The resulting mass spectra after the manual and the acoustic wash are shown in Fig. 7 and 8, respectively. When analyzing the starting sample prior to MOAC purification both the unphosphorylated and the phosphorylated peptides were observed. Due to the negative charge of the phospho-group the unphosphorylated peptide will always provide the most intense signal (Fig. 7a). After metal oxide affinity capture and manual wash, only the phosphorylated peptide was observed in

the mass spectra (Fig. 7b). The acoustophoretic washing performed in the microchip was equally successful in purifying the phosphorylated peptide without any cross-contamination between runs (Fig. 8a–c).

In the second experiment, a more complex sample was purified. A phosphorylated peptide was spiked into a sample solution containing 10 mg mL⁻¹ BSA (Fig. 9a). Using this sample no data could be obtained without purification, but after either manual or acoustophoretic washing a signal for the spiked phosphorylated peptide could be observed. Although other peptides were also captured from the sample solution, their presence was not dependent on the used extraction protocol. Rather they were observed both when using the manual or the acoustic procedures (Fig. 9b–c). The main reason for the capture of these peptides is probably due to an overall acidic character. It is well known that acidic peptides bind to MOAC beads and a number of different strategies can be employed to improve analysis.^{30,31} Thus, the data suggest that on-chip acoustic affinity purification processes are highly compatible with this type of biomolecular assays.

3.4 Affinity extraction of bacteriophages

The other extreme of analytes that could be addressed by on-chip acoustic separation systems are microorganisms and viruses. An experiment was set up to evaluate the possibility of using the acoustophoretic wash chip for the extraction of bioparticles, more specifically bacteriophages. Filamentous bacteriophage, like M13, are long slender rods (approximately 1 μ m long, for review see Rasched *et al.*²⁶) that infect F-pili-carrying *E. coli*. Beads coated with an M13-specific antibody binding a highly abundant phage protein, were incubated in a suspension containing phage particles ($\sim 10^9$ mL⁻¹) prior to washing in the chip.

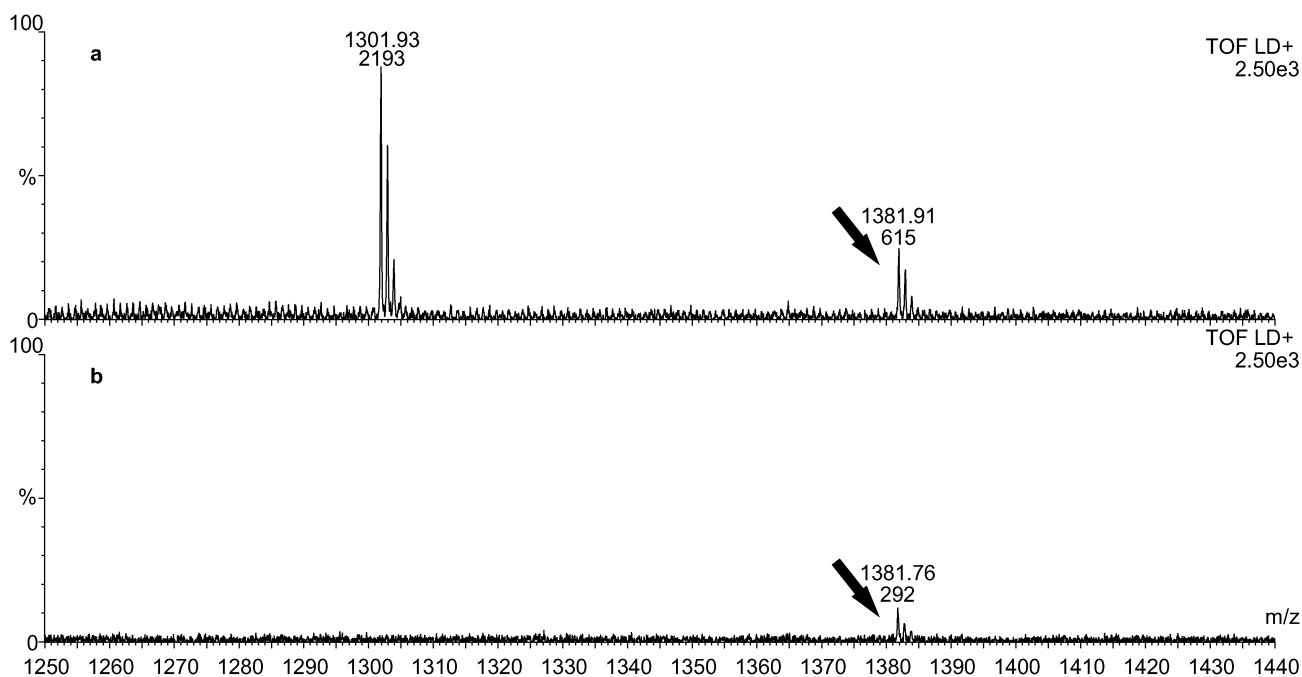


Fig. 7 (a) A mass spectrum showing a sample of an equimolar amount (50 nM) of an unphosphorylated peptide (1301 Da) and the same peptide with a phosphorylation (1381 Da) prior to dilution and washing. (b) A mass spectrum that was obtained after standard manual magnetic metal affinity oxide purification of the phosphorylated peptide. The arrows indicate the phosphopeptide.

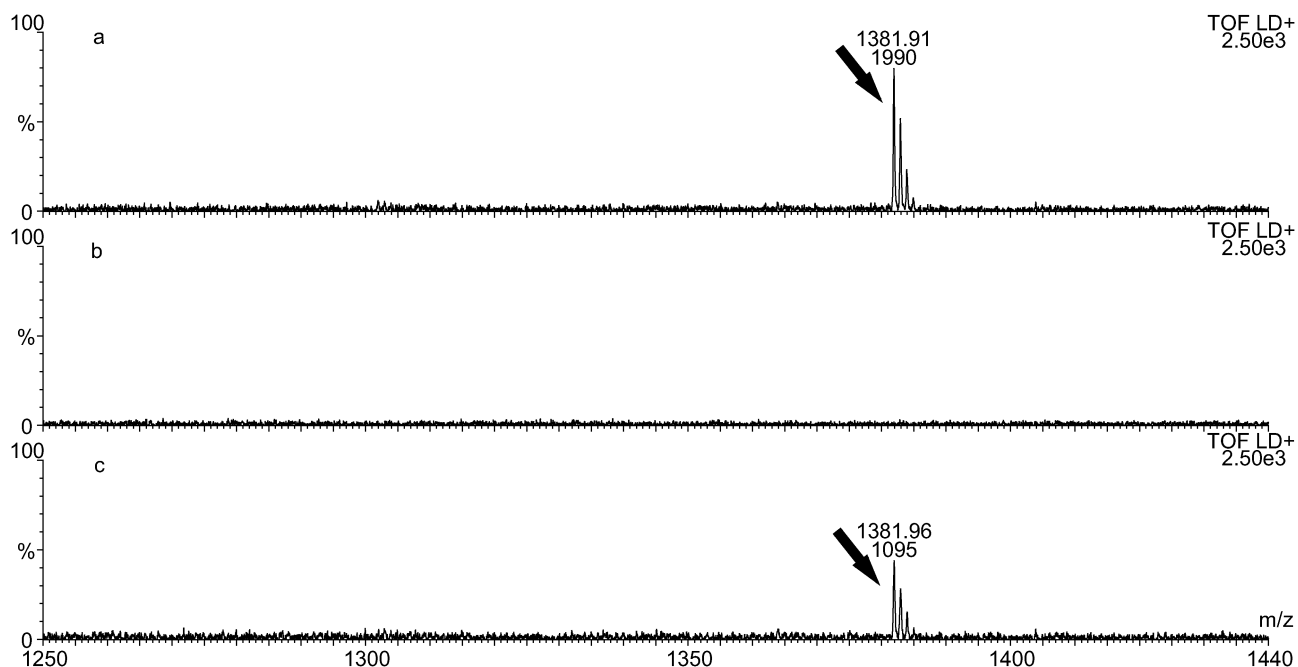


Fig. 8 Resulting mass spectra (a and c) after purification, using the acoustophoretic microchip washing, of the phosphorylated and unphosphorylated peptide. Mass spectrum (b) of a blank sample containing beads but no peptides, processed in between the two peptide-containing samples. The arrows indicate the captured phosphopeptide.

As a reference, the same bead type without antibodies were also incubated with phage suspension and washed. Beads and supernatant were separated from the washed samples and the result showed almost 700 times higher number of phages on the collected beads when coated with the antibody than was the case for non-coated beads. In a second experiment, phage particles

displaying low amounts of a recombinant antibody fragment (AE11F) were incubated with beads coated with an antigen (AD-2 peptide) recognized by the antibody fragment prior to acoustophoretic bead purification. Also in this configuration the level of phage particles were 700 times higher than for the non-coated beads (Fig. 10). Thus both structures (phage protein 8 and

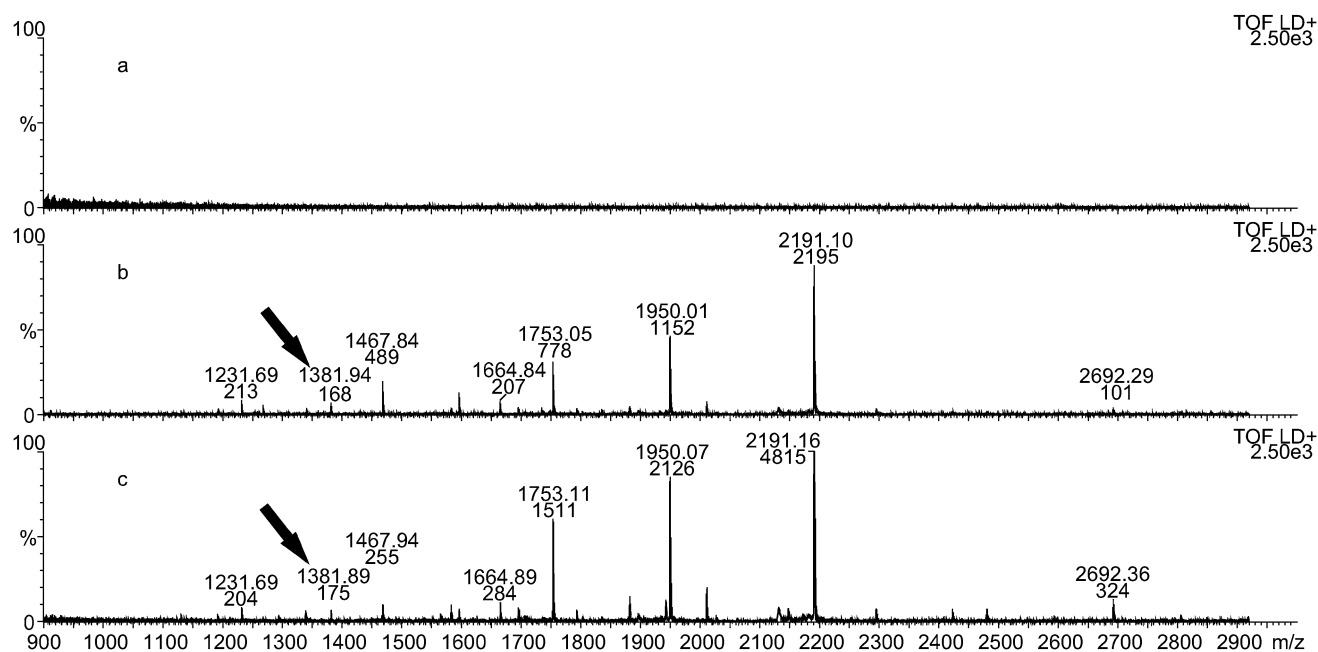


Fig. 9 (a) Mass spectrum of the phosphopeptide spiked BSA sample prior to purification, note that no peptides are observed. Two mass spectra below showing the MALDI read-out after purification using (b) manual washing and (c) dual acoustic extraction chip. The arrows indicate the captured phosphopeptide.

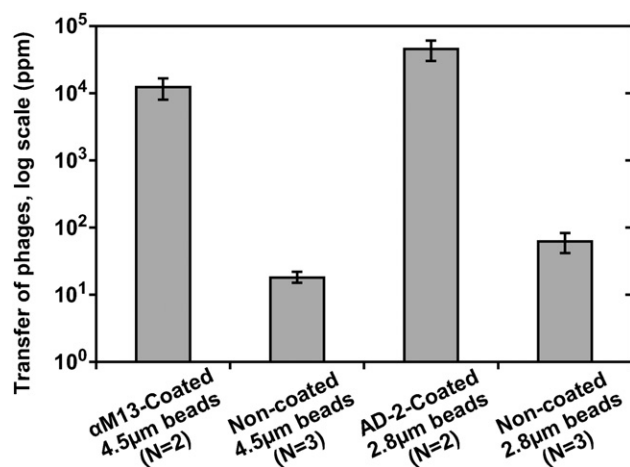


Fig. 10 Transfer of phage particles for 4.5 μm beads coated with an antibody specific for phage major coat protein 8, and for 2.8 μm beads coated with AD-2 peptide. The peptide is recognized by antibody fragments displayed on the minor phage protein 3 on these phage particles. The level of phage bound to these particles is compared to the transfer of phages to corresponding beads without specific antibody or peptide. The error bars indicate the minimum and maximum values of the retrieved data.

antibody fragment on protein 3) present at high and low abundance, respectively, on bacteriophages can be used to extract these bioparticles from one regime of the flow into the other.

Although being efficient it would be desirable to obtain phage particle extraction efficiencies that are even more competitive to manual superparamagnetic bead washing procedures, a common practice in phage display³²-based selection of specific binders, which typically display efficiencies in the order of 1000 times elimination of non-specific binders.

In order to accomplish this, the actuation of the wash chip was modified to provide a more homogenous ultrasonic excitation of the flow channels. A five times longer transducer (7 by 35 mm) was glued to the side of a wash chip. The driving voltage was tuned to, ~ 60 Vpp (~ 2 W), obtaining equal particle focusing dynamics in both separation channels. Both wash units could now be operated under a centre inlet wash buffer flow of $100 \mu\text{L min}^{-1}$.

The modified wash chip was evaluated in terms of ability to suppress phage contamination/carry-over when extracting non-coated superparamagnetic microbeads. A suppression ratio of 10^6 (corresponding wash efficiency 99.9999%) was obtained with the new chip, which should be compared to a factor of 10^4 for the earlier chip version, denoting a substantial improvement. As a comparison a manual superparamagnetic bead wash sequence of 4 consecutive wash steps was performed. The suppression ratio was monitored after wash step 2, 3 and 4 and compared to the new wash chip phage suppression ratio (Fig. 11). The acoustophoretic wash system performed better than the manual superparamagnetic bead process. These data demonstrate a performance that certainly would allow acoustophoretic washing to be used in enrichment of bacteriophages (or other viruses) from various samples, as well as in the washing of microbeads in an automated approach to high throughput selection of molecular phage-displayed libraries.

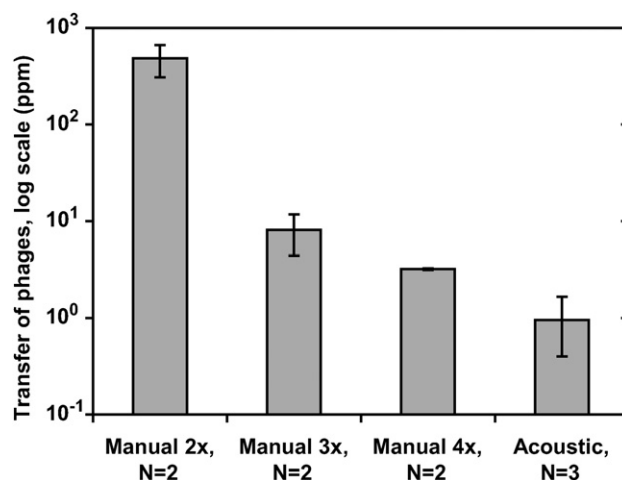


Fig. 11 Transfer ratio relative to input of phages using non-coated superparamagnetic beads in manual (2, 3 and 4 times washing) and acoustophoretic microchip based washing. The rightmost bar indicates phage particles suppression ratio using the improved version of the wash chip. The error bars indicate the minimum and maximum values of the retrieved data.

We have lately been able to show that this is indeed possible to accomplish.³³

4 Conclusions

The current work demonstrates an improved set-up for chip integrated acoustic washing of microparticles, where extraction of particulate matter from complex environments is shown in a continuous flow format. Washing efficiencies of particles from dissolved species (Evans blue) ranging up to 99.995% are reported. Moreover the throughput of particles was $\sim 5 \times 10^5 \text{ s}^{-1}$ with a particle recovery of $\sim 75\%$. An application for microbead based sample preparation in complex proteomic samples are further shown, where phosphopeptide extraction is performed. Also, affinity specific extraction of phage particles using acoustophoretic washing has proven possible, yielding suppression ratios of 1 to 100 ppm for unbound material. The generic nature of the acoustophoretic particle-washing platform is clearly demonstrated in this paper. It is anticipated that acoustic standing wave force manipulation can be further developed as a standard tool in on-chip microbead manipulation in the development of integrated bioanalytical concepts.

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