A helical flow, circular microreactor for separating and enriching "smart" polymer–antibody capture reagents

John M. Hoffman, Mitsuhiro Ebara, James J. Lai, Allan S. Hoffman, Albert Folch and Patrick S. Stayton*

Received 13th April 2010, Accepted 20th August 2010
DOI: 10.1039/c004978f

We report a mechanistic study of how flow and recirculation in a microreactor can be used to optimize the capture and release of stimuli-responsive polymer–protein reagents on stimuli-responsive polymer-grafted channel surfaces. Poly(*N*-isopropylacrylamide) (PNIPAAm) was grafted to polydimethylsiloxane (PDMS) channel walls, creating switchable surfaces where PNIPAAm–protein conjugates would adhere at temperatures above the lower critical solution temperature (LCST) and released below the LCST. A PNIPAAm–streptavidin conjugate that can capture biotinylated antibody–antigen targets was first characterized. The conjugate's immobilization and release were limited by mass transport to and from the functionalized PNIPAAm surface. Transport and adsorption efficiencies were dependent on the aggregate size of the PNIPAAm–streptavidin conjugate above the LCST and also were dependent on whether the conjugates were heated in the presence of the stimuli-responsive surface or pre-aggregated and then flowed across the surface. As conjugate size increased, through the addition of non-conjugated PNIPAAm, recirculation and mixing were shown to markedly improve conjugate immobilization compared to diffusion alone. Under optimized conditions of flow and reagent concentrations, approximately 60% of the streptavidin conjugate bolus could be captured at the surface and subsequently successfully released. The kinetic release profile sharpness was also strongly improved with recirculation and helical mixing. Finally, the concentration of protein–polymer conjugates could be achieved by continuous conjugate flow into the heated recirculator, allowing nearly linear enrichment of the conjugate reagent from larger volumes. This capability was shown with anti-p24 HIV monoclonal antibody reagents that were enriched over 5-fold using this protocol. These studies provide insight into the mechanism of smart polymer–protein conjugate capture and release in grafted channels and show the potential of this purification and enrichment module for processing diagnostic samples.

Introduction

Microfluidic platforms have shown promise for conducting diagnostic measurements in both clinical[1] and point of care settings.[2] While great strides have been made, the potential of this technology has not yet been fully realized and several challenges remain. One important outstanding need is the handling of dilute antigens and biomarkers and particularly their purification and enrichment from complex biological fluids.[3]

Several chromatographic strategies have been demonstrated to address analyte purification and enrichment issues in microfluidic systems. Affinity moieties have been conjugated directly to microchannel walls,[4–9] attached to particles and packed into microchannels[10–15] and immobilized within porous monolithic slabs.[16,17] These techniques suffer from several shortcomings, including complex packing and modification steps, high flow resistance and limitations in scale-up and manufacturability. These strategies also usually allow the separation of a single analyte only and cannot be modified once introduced.

Our group has been investigating the potential of using stimuli-responsive polymer–protein conjugates together with stimuli-responsive polymer-grafted channel surfaces to develop solutions to these needs. Surfaces grafted with stimuli-responsive polymers have been shown to reversibly immobilize and release biomolecules.[18–21] Stimuli-responsive conjugates have been correspondingly used to capture analytes in solution, immobilize the complex to solid substrates for purification, and subsequently to reversibly return the complex back to solution.[22–24] These two applications have been combined for specific capture of stimuli-responsive conjugates on stimuli-responsive surfaces,[25] but little attention has been paid to capture/release mechanism and to the role of flow dynamics.

Maximizing both capture/release efficiency and enrichment is crucial when working with finite volumes of dilute analytes in the short time periods required for point of care applications. Recently, Liu *et al.*[26] have shown that recirculation and helical mixing of cDNA in a microfluidic DNA microarray system increase immobilization kinetics and surface homogeneity. We hypothesized that these techniques will apply to stimuli-responsive conjugate capture, enrichment and release.

In this work, we have investigated the capture/release efficiency and enrichment of PNIPAAm–streptavidin conjugates in PNIPAAm grafted PDMS microchannels. Previously, we have shown that PNIPAAm–streptavidin conjugates undergo controlled aggregation under a temperature stimulus,[27,28] thereby

Department of Bioengineering, University of Washington, Seattle, WA, 98195, USA. E-mail: stayton@u.washington.edu; Tel: +1 (206)685-8148

limiting mass transport to the surface for immobilization. Therefore, we have incorporated an isolatable recirculator[29] within our device that is equipped with transverse flow features capable of generating helical flow.[30,31]

By isolating the recirculator from the sample stream during a capture sequence, we were able to measure capture/release efficiency and release kinetics of a finite conjugate volume under two different mass transport conditions. Using the results of these mechanistic experiments, we were able to enrich conjugates by continuous flow of conjugate through the preheated recirculator.

Experimental

Materials

Streptavidin, *N*-isopropylacrylamide (NIPAAm, recrystallized from hexanes prior to use), poly(ethylene glycol) acrylate (PEGA, M_n: 575), benzophenone, $NaIO_4$, HABA kit, and benzyl alcohol were all obtained from Aldrich. Anti-p24 monoclonal antibodies (IgG_1) were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH:HIV-1 p24 Monoclonal Antibody (183-H12-5C) from Dr Bruce Chesebro and Kathy Wehrly.[32–34] Azobis(isobutyronitrile) (AIBN) was purchased from Fluka and recrystallized from methanol and dried under vacuum prior to use. 2-Dodecylsulfanylthiocarbonylsulfanyl-2-methyl propionic acid (DMP) was obtained as a gift from Prof. Charles L. McCormick of the University of Southern Mississippi and Dr John Lai of Noveon Company. *N*,*N*′-Dicyclohexylcarbodiimide (DCC), *N*-hydroxysulfosuccinimide (NHS) and (+)-biotin-3,6-dioxaoctanediamine (EZ-Link® Biotin-PEO-Amine) were purchased from Pierce. Sylgard 184 was purchased from Dow Corning (Midland, MI). Negative photoresist SU8-50 was purchased from Microchem (Newton, MA). 3 inch silicon wafers were purchased from Silicon Sense (Nashua, NH). Tridecafluoro-(1,1,2,2-tetrahydrooctyl)-1-trichlorosilane came from United Chemical Technologies (Bristol, PA). Photomasks were from University of Washington Publication Services (3600 DPI). ITO-coated glass slides were from Delta Technologies (Stillwater, MN). AZ 1512 and 9260 were obtained from Microchemicals GmbH (Ulm, Germany). TFA and TFD etchants were from Transene Corporation (Danvers, MA). Silver Conductive Expoxy obtained from MG Chemicals (Surrey, BC, Canada). Poly-ether-ether-ketone (PEEK) was obtained from Upchurch Scientific (Oak Harbor, WA). Alexa 488–streptavidin and Alexa 488 labeling kits were obtained from Invitrogen. Syringe pumps and syringes were obtained from Kloehn (Las Vegas, NV). Solenoid valves and manifolds were from Lee Company (Westbrook, CT). LabView software was obtained from National Instruments (Austin, TX). Matlab software was obtained from The Mathworks Inc. (Natick, Massachusetts). Temperature controller and thermocouples were obtained from Omega (Stamford, CT).

Alexa 488–streptavidin–PNIPAAm conjugates

A telechelic PNIPAAm with carboxyl and dodecyl terminal functional groups was synthesized by reversible addition–fragmentation chain transfer (RAFT) polymerization using a chain transfer agent, DMP.[35] Briefly, NIPAAm monomer, DMP, and AIBN as an initiator were dissolved in methanol and degassed by purging with nitrogen for 20 minutes. Polymerization was carried out at 60 °C for 17 h. GPC results indicated the resulting polymer had M_w = 30 000 Da and polydispersity = 1.15. Carboxy terminated PNIPAAm was biotinylated (β-PNIPAAm) using a primary amine-terminated biotin (Biotin-PEO-Amine). Briefly, PNIPAAm and Biotin-PEO-Amine were dissolved in dioxane at a concentration of 15 mM and 30 mM, respectively. Ninety millimolar of DCC and NHS were dissolved in dioxane. The PNIPAAm solution was added to the DCC/NHS solution and the reaction proceeded overnight at room temperature. Biotinylation efficiency was determined by HABA assay to be 69%. Alexa 488–streptavidin and β-PNIPAAm were mixed in a 1 : 1.5 mole ratio (accounting for biotinylation efficiency). This mixture was allowed to incubate overnight at 4 °C with gentle mixing. HABA assay was used to confirm the conjugate protein : polymer ratio. UV spectrophotometry (Hewlett Packard 8453) was used to determine the conjugate phase transition temperature. In all experiments, the conjugate was diluted to 120 nM in various concentrations of carboxyl terminated PNIPAAm (free polymer, FPoly) in PBS.

Dynamic light scattering

Dynamic light scattering experiments were conducted as in a previously published protocol.[27,28] Briefly, sizing measurements were performed using a Brookhaven BI90Plus instrument equipped with a 535 channel correlator and a 656 nm source laser. Measurements were performed at a 90° angle. All samples (75 μl) were diluted with PBS (pH 7.4) and filtered through 0.2 μm filters at room temperature.

Control and fluid layer master fabrication

For the control layer, a silicon wafer was spin-coated with SU8-50 and baked at 95 °C for 1 h. The photoresist was exposed to UV light (Kaspar-Quintel model 2001 aligner) for 150 s through transparency masks. After exposure, the masters were baked at 95 °C for 10 min and developed with SU8 developer (Microchem) for 15 min.[36] The masters used in this study were 0.1 mm tall with various widths. Dimensions for the transverse flow component were taken from Stroock *et al.*,[30] with consideration from Hsu and Folch,[37] Schonfeld and Hardt[38] and Yang *et al.*[39] All transverse components were at 45° to a vector tangential to a circle comprising the center of the recirculator (radius = 1.4 mm) and spaced 0.2 mm center to center (Fig. 1(a)).

For the fluid layer, a silicon wafer was first treated with HMDS, then spin-coated with AZ 9260 at 350 rpm for 300 seconds. After baking at 100 °C for 6 minutes, the wafer was coated again using the same protocol, followed by 15 minutes baking at 100 °C. The photoresist was exposed to UV light (Kaspar-Quintel model 2001 aligner) for 150 s through transparency masks. To achieve a channel with parabolic cross-section, the photoresist reflow method was employed.[40,41] Following photolithographic patterning and developing, the patterned wafer was heated at 140 °C for 3 minutes then allowed to cool to room temperature resulting in channels 0.07 mm tall at apex and 0.2 mm wide at base. Patterned masters and bare silicon

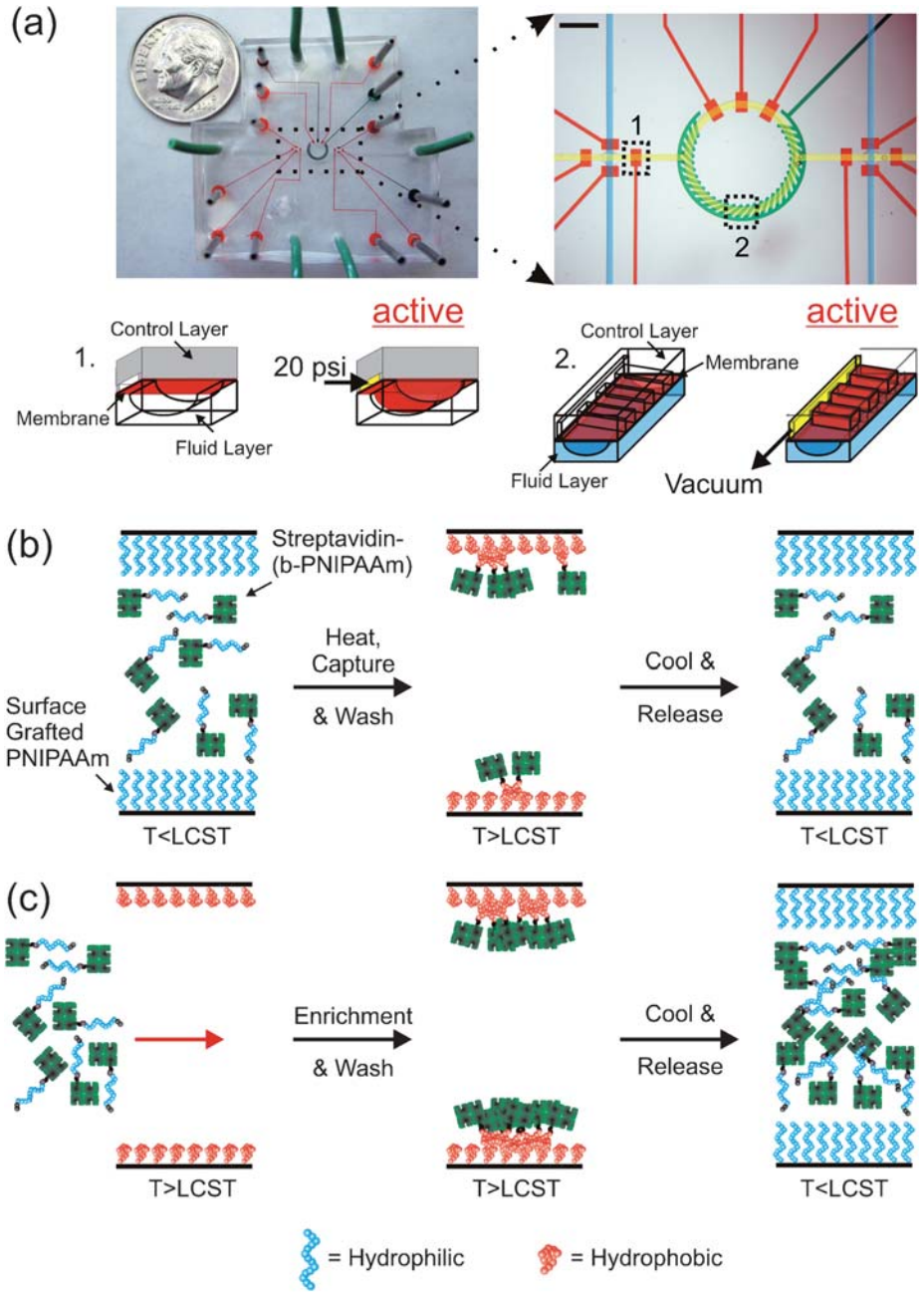

Fig. 1 Microfluidic device and experimental schematics are shown. (a) Assembled device where red (valves and recirculator) and green (transverse flow feature) channels represent features in the control layer. Recirculator region where false colour depicts polymer grafting regions. Yellow regions were grafted with PNIPAAm and blue regions were grafted with PEGA (scale = 1 mm). (1) Schematic view of valve activation. A thin PDMS membrane separates the fluid layer from the control layer. Valve is closed (ACTIVE) when positive pressure is applied to the control layer, deflecting the membrane into the fluid layer. (2) Schematic view of transverse flow feature activation. Similar to (1), a thin PDMS membrane separates the fluid layer from the control layer. Features are ACTIVE when vacuum is applied, and membrane is deflected into the control layer. (b) Conjugate capture schematic (cold start). Conjugate is loaded into the recirculator at room temperature and isolated by the valves. When the temperature rises above the phase transition temperature, conjugates begin to aggregate through hydrophobic interactions. Next, peristaltic flow within the recirculator is activated, and transverse flow moves the aggregates into contact with the PNIPAAm-modified surface. Again, hydrophobic interactions between the conjugates and the surface PNIPAAm immobilize aggregates on the surface. The recirculator is then washed with warm buffer to remove unbound conjugates. The recirculator is then cooled to 25 °C, and peristaltic flow re-initiated. Surface bound conjugates are released from the re-hydrated PNIPAAm surface grafts, diluted and homogenized. (c) Conjugate enrichment schematic (Hot Start). The recirculator is heated above the phase transition temperature and room temperature conjugate is introduced to the recirculator under continuous flow to sequentially capture the conjugates from larger total volumes as they aggregate and are concentrated at the PNIPAAm surface. After a buffer wash, the recirculator is cooled to 25 °C and peristaltic flow is initiated. Surface bound conjugates are released from the surface and homogenized within the recirculator, resulting in a concentration higher than that in the original sample stream (enrichment).

wafers were passivated by 10 minute exposure to fluorosilane under vacuum.[36]

PDMS device fabrication

PDMS prepolymer was prepared by mixing PDMS base with a curing agent in a 10 : 1 ratio by weight and degassing the mixture under vacuum. To fabricate fluid and control layers, the mixture was cast against the patterned silicon master, degassed and cured at 65 °C for 2 hours. Access holes in the control layer were formed by punching holes at predefined locations with a blunt syringe. Control layers were bonded to 0.017 mm thick membranes using O_2 plasma treatment following Hoffman *et al.*[36] Following removal of the membrane from fluid layer access holes, the fluid layer and membrane sealed control layer were treated with O_2 plasma. Quickly, the fluid layer was covered with a fine layer of methanol, placed on a 50 °C hot plate and aligned to the control layer under a microscope.[37,42] Once aligned and bonded, the devices were transferred to an oven and baked overnight at 65 °C. The completed device is shown in Fig. 1(a).

PEEK tubing was placed into the access holes for fluid access. Valve and recirculator control channels are filled with deionized H_2O. The transverse component control channel was not filled with liquid and was left open to atmosphere.

ITO heater fabrication

ITO heaters were patterned according to a previously published protocol.[43] AZ 1512 was spun onto ITO slides and exposed to UV through transparency photomasks. ITO etching was performed by immersing photoresist patterned slides in HNO_3 : HCl : H_2O (1 : 4 : 15 by volume) warmed to 55 °C for 7.5 minutes. To fabricate electrical conductors, patterned ITO slides were then coated by 20 nm of chromium followed by 150 nm of gold by electron beam evaporation. Conductors were patterned photolithographically as described above. Gold and chromium were etched by 5 minutes exposure to TFA gold etchant followed by 5 minutes exposure to TFD chromium etchant. Copper wires were attached to the heater conductors using silver conductive epoxy.

UV-induced graft polymerization

UV-mediated grafting was directed according to a previously published protocol.[43,44] Briefly, benzophenone was dissolved in acetone (20%) and flowed into the horizontal recirculator channel for approximately one minute. The channel was then washed extensively with water. A monomer solution containing NIPAAm (10% in water), $NaIO_4$ (0.5 mM), and benzyl alcohol (0.5 wt%) was loaded into the channel, which was then irradiated with UV light (100 W, 365 nm) for 15 minutes. The channel was washed extensively with water resulting in a PNIPAAm grafted channel (Fig. 1(a2)). Poly(ethylene glycol) (PEG)-grafted surfaces were prepared using the same protocol, with PEG acrylate (PEGA) substituted for NIPAAM in the monomer solution. The two vertical channels were irradiated for 7 minutes resulting in PEGA grafted surfaces (Fig. 1(a)). One device was used for all capture and release experiments and another was used for all enrichment experiments.

Optical microscopy, fluid and temperature control

Images were acquired using a monochrome CCD camera (Retiga-1300, Q Imaging, Burnaby, British Columbia) mounted to an inverted microscope (Eclipse TE2000U, Nikon USA). Image analysis was performed using Matlab.

Positive displacement syringe pumps controlled by LabView software facilitated flow control. Hydraulic on-chip valves and transverse flow components were actuated off-chip *via* LabView-NIDaq controlled solenoid valves connected to a compressed nitrogen tank (regulated to 20 psi) *via* an 8-port manifold. In this case, off chip actuation resulted in the PDMS membrane deflecting downwards, sealing the fluid channel (Fig. 1(a)). Peristaltic flow in the recirculator was accomplished by actuating three valves with a 120° phase delayed pulse train at 5 Hz.[40,41] The transverse flow control structure was actuated using the same hardware and software, with a single valve inserted in a manifold connected to house vacuum (−19 mmHg). In this case, off chip actuation resulted in the PDMS membrane deflecting upwards, exposing features in the control layer to the fluid channel (Fig. 1(a)).[37]

Temperature control within the device is accomplished through an I-series temperature controller and J-type thermocouple embedded within the control layer aligned above the ITO heater. The thermocouple tips were separated from the fluid layer by the PDMS membrane.

Conjugate capture and release protocols

Fig. 1(b) gives the general strategy for conjugate capture. Initially, conjugates are brought into a PNIPAAm grafted PDMS channel and heated above the phase transition temperature of the conjugate. As the temperature increases, and the polymers begin to phase separate, the polymer conjugate can be either incorporated into growing aggregates or interact and adsorb onto the grafted channel surface. The aggregates themselves can also be transported by diffusion or mixing to the channel surface where they can be immobilized.

In a typical experiment, the sample solution (containing conjugate and FPoly) was introduced into the device through a PEGA-grafted input and the recirculator was filled with wash buffer. The fluorescence intensity from the buffer filled recirculator was taken as I_{BG}. The transverse flow component was activated throughout the entire experiment (Fig. 2(a)). Sample solution was then pumped into the PNIPAAm-grafted recirculator at 5 μL min^{-1} for 30 s (I_{Flood}, Fig. 2(b)). Once the solution was isolated in the recirculator, the temperature was raised to 36 °C and the solution was captured for either 30 seconds or two minutes (termed "Cold Start" protocol). Once the recirculator reached 36 °C, one of two different protocols was completed. The solution was either held static (diffusion dominated transport) or mixed at 5 Hz (Fig. 2(c)). Following the capture step, the recirculator was hot washed with 25 μL PBS (pH 7.4) at 1.2 μL min^{-1} (Fig. 2(d)). Once washed, the recirculator was isolated and the solution was allowed to cool to 25 °C. The contents of the recirculator were then mixed at 5 Hz (cool mix) allowing all conjugates and free polymers to release from the surface and return to solution (Fig. 2(e)). Finally, the recirculator was cold washed with buffer to remove the released conjugate and free

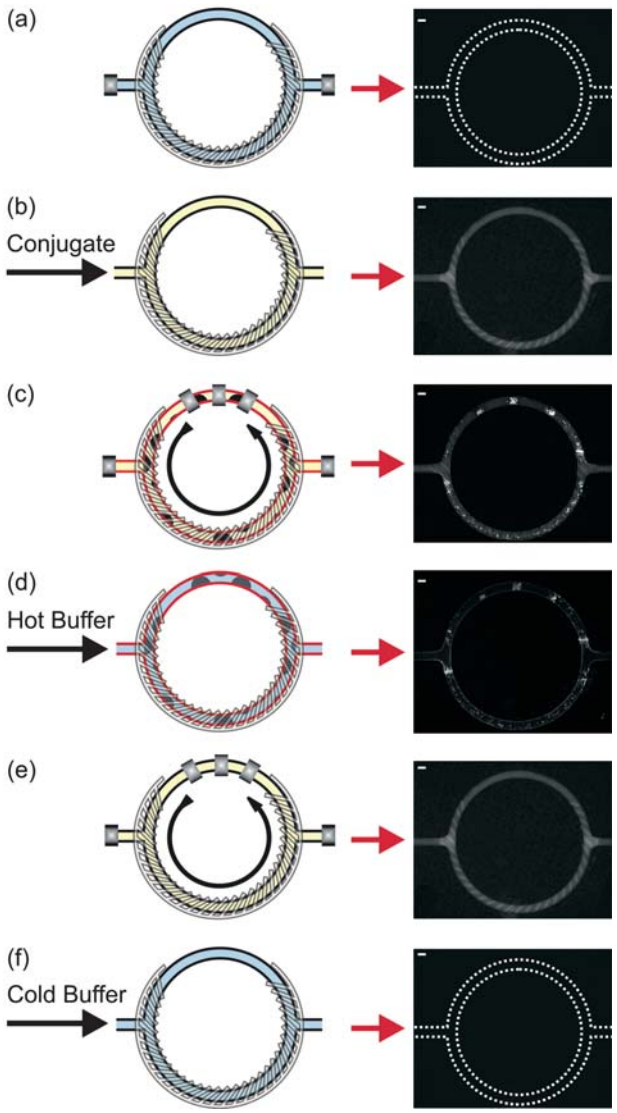

Fig. 2 Schematic of conjugate capture and release protocols (shaded valves indicate CLOSED) and representative experimental images for the corresponding steps. Black solid arrows indicate flow direction. The left column gives a top down schematic view with the temperature condition (black walls = room temperature and red walls = 36 °C). The right column shows the images acquired at 4× magnification (scale = 0.2 mm) with fluorescently labelled conjugate (with 83 μM free polymer) after the recirculator action displayed on the left. (a) The recirculator was filled with PBS buffer (blue). The dotted white line depicts channel boundaries. (b) At 25 °C, conjugate and free polymer solution was pumped into the recirculator. (c) The recirculator region was isolated and the temperature was raised to 36 °C. The solution was then either mixed or allowed to diffuse for 30 seconds or two minutes. (d) While at 36 °C, the recirculator was "hot washed" with PBS buffer removing loosely bound aggregates. (e) After the hot wash, the recirculator was isolated and allowed to cool to room temperature. Once cooled, the recirculator contents were mixed at 5 Hz, resuspending and homogenizing conjugate, thus allowing quantification of capture and release. (f) The recirculator was then washed with room temperature buffer to clear the resuspended material, leaving the device ready for another capture experiment.

polymer solution and ready the device for another experiment (Fig. 2(f)).

To measure conjugate captured on the surface and subsequently released after cooling, the fluorescence intensity within the channel was measured before heating (I_{BG}, Fig. 2(a) and I_{flood}, Fig. 2(b)) and after the cold mix ($I_{released}$, Fig. 2(e)). Taking the measurement at room temperature after mixing reduced self-quenching effects. Given the initial conjugate concentration (C_I) and the reactor volume (V), the mass of conjugate captured and released could be calculated. Conjugate captured and released (fmol) was calculated as:

$$\text{captured and released (fmol)} = \left(\frac{I_{released} - I_{BG}}{I_{flood} - I_{BG}}\right) \times C_I \times V \times 10^3 \quad (1)$$

Conjugate release from PNIPAAm grafted surfaces

Following the Cold Start protocol, we used time lapse fluorescence microscopy to measure conjugate release from the PNIPAAm grafted recirculator channel. Image acquisition (10 seconds between exposures) began after the hot wash was completed, as the device cooled from 36 °C to room temperature. Temperature was recorded simultaneously. During cooling, two different protocols were run. Once the device temperature reached 25 °C, the recirculator was either (i) actuated at 5 Hz (mixing) or (ii) held static (desorption).

Conjugate enrichment protocol

Fig. 1(c) gives the general strategy for conjugate enrichment. Enrichment involved the introduction of increasing conjugate volumes to the PNIPAAm-grafted microreactor with the aim of capturing and enriching conjugate at the surface. The enrichment protocol employed was the same as the cold start, with two exceptions. First, the recirculator was heated to 36 °C prior to flowing the conjugate into the recirculator (termed "Hot Start", Fig. 3(a)). Second, the polymer conjugate solution (5 nM conjugate with 83 μM free polymer) was flowed into the recirculator for defined times at 5 μL min^{-1}. Capture, hot wash, release, and cold wash steps are the same as those in the cold start experiments. Enrichment was quantified by comparing the fluorescence intensity after release to a standard curve. Finally, this protocol was used to enrich an anti-p24 monoclonal antibody (mAB). mAB was biotinylated and labeled with Alexa 488 according to manufacturer's protocols. The enrichment protocol, as described above, was conducted with a solution of mAB (10 nM) mixed with streptavidin (100 nM) and biotinylated PNIPAAm (83 μM) in PBS.

Results

Characterization of the streptavidin–PNIPAAm conjugate

HABA assays performed in triplicate demonstrated that mixing streptavidin with 30 kDa β-PNIPAAm in a 1 : 1.5 mole ratio resulted in one polymer per streptavidin tetramer stoichiometry. Phase transition measurements showed that the streptavidin conjugate phase separation was complete above 34 °C.

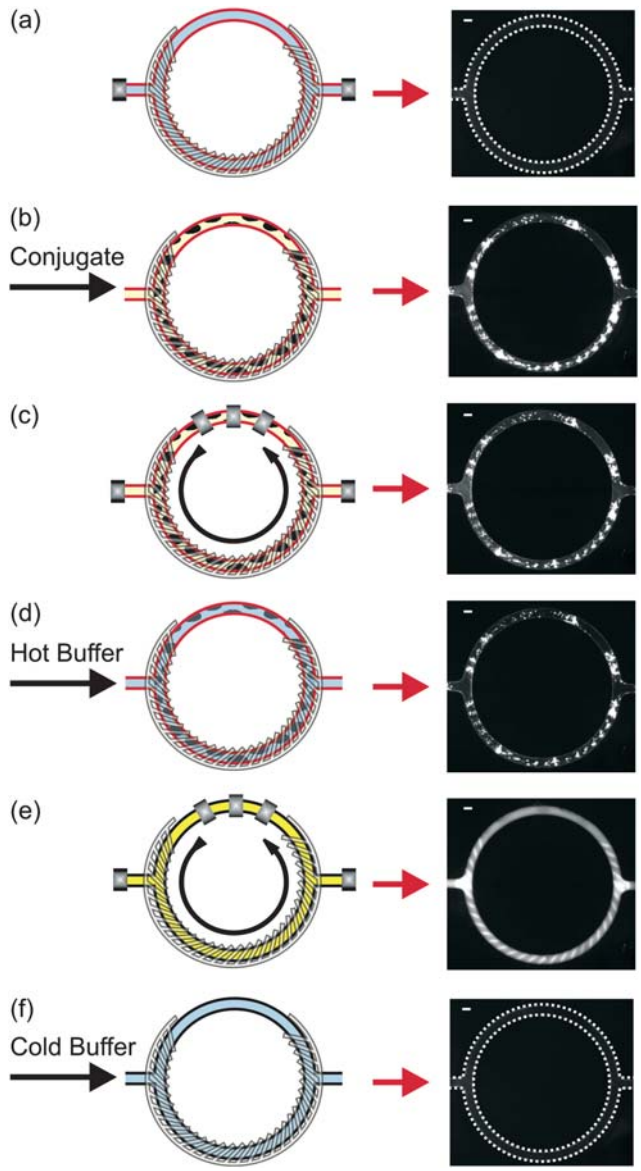

Fig. 3 Conjugate enrichment protocol (shaded valves indicate CLOSED) where black solid arrows indicate flow direction. The left column gives a top down schematic view with the temperature condition (black walls = room temperature and red walls = 36 °C). The right column shows representative images (after 30 seconds of conjugate flow) of the fluorescently labelled conjugates acquired at 4× magnification (scale = 0.2 mm) after the action displayed on the left. (a) PNIPAAm modified recirculator was filled with PBS buffer (blue) and heated to 36 °C. (b) At 36 °C, the recirculator was filled with a specific volume of conjugate solution. Aggregates formed and immobilized as soon as the solution reached the heated region (Fig. 1(c)). (c) Flow was then halted, the recirculator region was isolated and the solution was either mixed or allowed to diffuse for two minutes. (d) The recirculator was hot washed with PBS buffer removing unbound aggregates. (e) The recirculator was isolated and allowed to cool to 25 °C. Once cooled, the recirculator contents were mixed at 5 Hz, which resuspended and homogenized the conjugates and allowed the quantitation of conjugate enrichment. (f) The recirculator was then with room temperature buffer to clear the resuspended material from the recirculator, leaving the device ready for another concentration experiment.

Therefore, 36 °C was used as the working temperature above the LCST in all DLS and microfluidic experiments.

Dynamic light scattering analysis of conjugate aggregation

Aggregate formation was investigated under two free polymer concentrations and temperature conditions using a 120 nM conjugate solution. As summarized in Table 1, at 25 °C with free PNIPAAm at 0.83 μM, there was no measurable aggregate formation. When the free PNIPAAm concentration was raised to 83 μM, 32 nm diameter aggregates with narrow size distributions were formed at 25 °C. When raised above the phase transition temperature and incubated for 5 minutes, aggregate diameter increased to 106 nm for 0.83 μM PNIPAAm and 688 nm for 83 μM while maintaining a relatively narrow size distribution. We attribute the narrow aggregate size distribution to the amphiphilic nature of the streptavidin conjugate.[27]

UV-induced graft polymerization

PEGA graft polymerization was performed on the vertical device inputs (Fig. 1(a), blue channel color) to prevent sample adsorption onto PDMS surfaces. PNIPAAm graft polymerization (Fig. 1(a), yellow channel color) was performed within the horizontal recirculator channel to assist conjugate capture and release. Previous studies[43,46] have shown that PNIPAAm conjugated nanoparticles could be reversibly immobilized within a PDMS microfluidic channel (by raising the local temperature above the polymers LCST), and that UV-induced PNIPAAm graft polymerization increased the polymer conjugate release rate from the surface.

Conjugate capture and release on PNIPAAm-grafted channel surfaces in microreactor

The experiments were designed to study the effects of three main system parameters on capture and release properties: (1) mixing *vs.* diffusion transport, (2) the conjugate aggregate size and role of free PNIPAAm, and (3) time dependence of conjugate capture/release. Four different capture protocols were investigated, with active transverse flow mixing or diffusion for either 2 min or 30 s period. The streptavidin conjugate was diluted to 120 nM in PBS (pH 7.4) with final free polymer of either 83 μM or 0.83 μM. Images shown on the right side of Fig. 2(a–f) show results from a 2 minute mixing experiment with 83 μM free PNIPAAm. In Fig. 2(a), the recirculator was filled with PBS buffer at room temperature and imaged (dotted white line depicts channel boundaries). Next in Fig. 2(b), the recirculator was flooded with conjugate, the contents isolated, and imaged. The temperature was then raised to 36 °C above the LCST of the conjugates and the surface grafts. After the recirculator reached 36 °C, the recirculation valves were actuated at 5 Hz and the streptavidin conjugate was immobilized on the recirculator surface. The average fluid velocity was measured to be 0.28 mm s^{-1} with this flow condition.[47] As seen in Fig. 2(c), after heating and mixing large aggregates appear in the recirculator. Fig. 2(d) shows the results of the hot wash where many large aggregates remained attached to the channel wall. Once the solution was cooled, and mixed again at 5 Hz, Fig. 2(e) shows that aggregates had returned to solution off the PNIPAAm surface with

Table 1 Dynamic light scattering results[a]

	25 °C		36 °C	
[FPoly]/μM	Aggregate diameter/nm	$D_{25} \times 10^{-7}$ cm^2 s^{-1}	Aggregate diameter/nm	$D_{36} \times 10^{-8}$ cm^2 s^{-1}
0.83	—	—	106 (±2.1)	6.53
83	32 (±1.0)	1.34	688 (±42.7)	0.97

[a] Size data for the aggregates formed from streptavidin–PNIPAAm conjugates (120 nM) with different free PNIPAAm concentrations. Aggregate sizes are shown after 5 minutes incubation at both 25 °C and 36 °C. Aggregate Diameter represents the average effective hydrodynamic diameter with ± standard deviation from $n = 3$ samples. D_{25} and D_{36} are the diffusion constants at 25 °C and 36 °C, respectively, and are calculated from the aggregate diameter using Stoke's law.[45] Sizing data for the 0.83 μM free polymer solution at 25 °C could not be obtained due to insufficient scattering from the aggregates.

fluorescence comparable to that shown in the initial load, indicating that the conjugate had been captured and released back into solution. Following a final buffer wash (Fig. 2(f)), the fluorescence intensity within the channel was comparable to that seen in the initial buffer load.

The quantitative results are shown in Fig. 4 where femtomols of conjugate captured and released are plotted for each experimental condition (diffusion *vs.* mixing and capture duration) for the two free polymer concentrations investigated. With free PNIPAAm at 83 μM where the PNIPAAm–streptavidin conjugate forms larger aggregated particles above the LCST, mixing enhances conjugate capture when compared to diffusion. Using the data in Table 1, with Stoke's law and the Einstein equation as guides, 688 nm diameter aggregates have a diffusion constant of 9.7×10^{-9} cm^2 s^{-1} at 36 °C and take ~10 minutes to diffuse 35 μm, the distance from the center of the fluid channel to the top sidewall. Mixing, through the introduction of transverse (helical) flow,[30,31] assists the conjugate capture by shortening the distance an aggregate must travel to come in contact with the PNIPAAm modified sidewall or another immobilized aggregate. Also, recirculation increases conjugate capture in microfluidic systems,[26,48] by providing additional opportunities for aggregates to accumulate on the channel surface. With these larger aggregated conjugates, it is clear that transverse flow is important to capture efficiencies and this condition leads to the overall greatest level of capture. However, shear forces generated by recirculation at 5 Hz act to offset capture by removing conjugates from growing surface aggregates. Assuming a simplified channel model[49] that does not include negligible shear contribution from the grooves,[50] the shear force at the sidewalls was estimated to be 1–10 N m^{-2}. Work by Ishida and Kobayashi[51] has shown that the adhesive force between a PNIPAAm grafted surface and hydrophobic microparticle to be in the 1–10 nN range. Assuming surface aggregates seen in Fig. 2(c) have a major dimension in the micron range, the adhesive force is approximately 10^3 greater than the viscous force, allowing conjugates to aggregate on the surface. However, as aggregates increase in size, the local velocity gradient increases due to the fixed flow rate. The corresponding increase in viscous force may prevent further conjugate aggregation and limit overall capture efficiency.

When free polymer was reduced to 0.83 μM and the conjugate aggregate size was much smaller above the LCST, the diffusion and mixing regimes both gave similar results, at both time conditions. Again using Stoke's law and the Einstein equation as guides, 106 nm diameter aggregates have a diffusion constant of 6.53×10^{-8} cm^2 s^{-1} at 36 °C and take ~1.6 minutes to diffuse 35 μm. This timescale is well within the experimental adsorption time conditions and the experimental results are consistent in that little difference in capture was observed between the transverse flow mixing and diffusion at either 2 minutes or 30 seconds (Fig. 4(a)).

Characterization of release with the combination of free polymer and transverse flow mixing (after the cold wash) showed that conjugate retention was minimized on the channel surface after the conjugate is resuspended and the recirculator washed (Fig. 2(f)). The retained fluorescence was less than 1% of the initial solution intensity in all cases investigated (data not shown).

Conjugate enrichment under constant flow

This recirculating microreactor with transverse mixing might be especially useful in the continuous processing of smart polymer–protein capture reagents from larger diagnostic sample volumes. In the enrichment experiments we investigated the ability of our system to concentrate conjugates on the recirculator channel walls and subsequently release the conjugate into a smaller concentrated volume. The initial conjugate concentration was set to 5 nM to represent a dilute diagnostic target.[52] Conjugate was flown into the recirculator when the temperature reached 36 °C. Fig. 3(b) shows that 30 seconds of flow lead to substantial conjugate aggregation within the recirculator and that mixing (Fig. 3(c)) and a hot wash (Fig. 3(d)) did not substantially displace conjugate aggregates. As the conjugate was flowed into a heated recirculator, transverse flow components acted to bring the polymer conjugates into close proximity to channel walls and facilitated conjugate immobilization. Recirculation after immobilization acted to redistribute conjugate within the recirculator and prevent conjugate loss to shear forces during the hot wash. Fig. 3(e) shows that recirculation at room temperature removed aggregated conjugate from the sidewalls and homogenized the solution. Similar to the results seen in Fig. 2(f), the release was nearly complete and the fluorescence intensity in the channel (Fig. 3(f)) was less than 1% greater than that measured in Fig. 3(a).

As shown in Fig. 4(b), with 83 μM free polymer and 5–30 seconds of continuous conjugate flow at 5 μL min^{-1}, conjugate enrichment increased monotonically from 1.2 times to ~9 times with only 30 seconds of conjugate flow. The utility of this protocol was further demonstrated by enriching an anti-p24 monoclonal IgG (Fig. 4(c)). Biotinylated and Alexa 488 labeled mAB (10 nM) was conjugated to biotinylated PNIPAAm

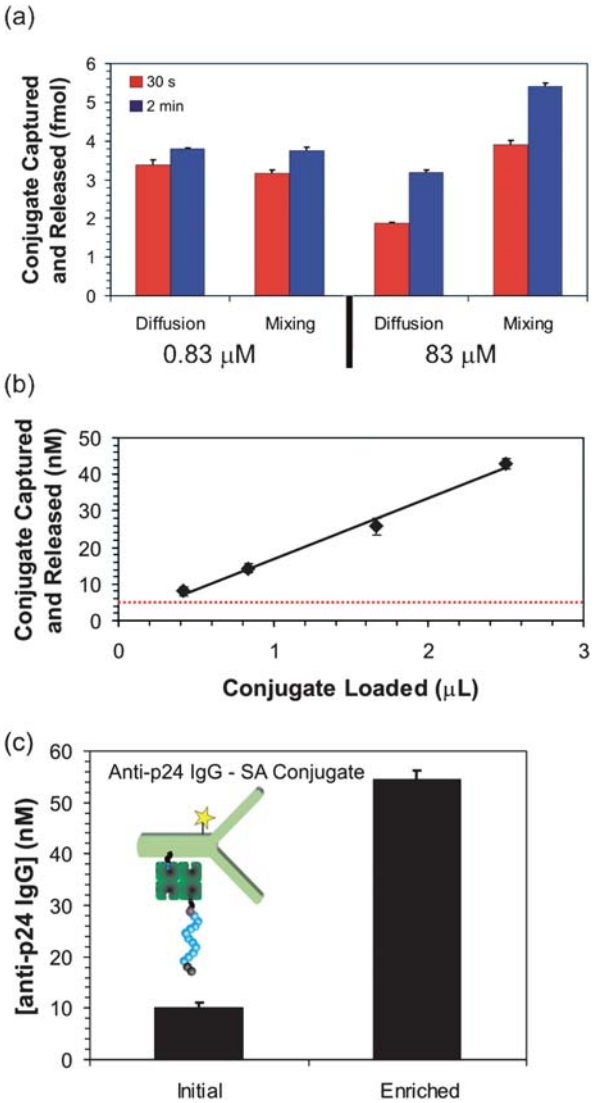

Fig. 4 Conjugate capture and release results and enrichment characterization ($n = 3$ in all experiments and error bars show standard deviation). (a) Results from conjugate capture and release experiments (images shown in Fig. 2), capture duration did not enhance conjugate capture and release at low free polymer concentrations. When free polymer concentration was increased, an increase in capture duration with mixing greatly increased conjugate capture and release. (b) Results from conjugate enrichment experiments (images shown in Fig. 3). Enrichment increased monotonically with increasing conjugate load volume. 5 nM conjugate loaded into the recirculator for 30 seconds (2.5 μL) was enriched 9 times. (c) Schematic of anti-p24 IgG–SA conjugate where Alexa 488 label on anti-p24 IgG provided measure of IgG concentration. Conjugates were enriched 6 times by loading 2.5 μL of conjugate using the protocol shown in Fig. 3.

(83 μM) by streptavidin (100 nM) and enriched using the best practices from the experiment detailed above. As shown in Fig. 4(c), mAB was enriched over 5 times by flowing conjugate at 5 μL min^{-1} for 30 seconds.

Dependence of conjugate release kinetics on mixing

Using fluorescence microscopy, the kinetics of PNIPAAm–streptavidin release were measured from the PNIPAAm grafted surface as the device was cooled to room temperature after the hot wash (Fig. 2(e)). Specifically, we measured the impact of transverse mixing on conjugate release as the temperature within the device fell below the phase transition temperature. As shown in Fig. 5, once the device temperature reached 25 °C and the recirculator was actuated at 5 Hz, the streptavidin conjugate was rapidly released from the surface. If the conjugate was allowed to desorb from the surface without perturbation, the release rate was significantly slower. Recirculation with helical flow likely transported water to the channel surface facilitating the rehydration of collapsed surface grafts. When surface grafts were below the LCST and rehydrated, there was little to no adhesive force.[51] Therefore, once conjugates were released from the surface, fluid shear acted to break up aggregates, which were quickly homogenized through a combination of Taylor dispersion and helical flow.

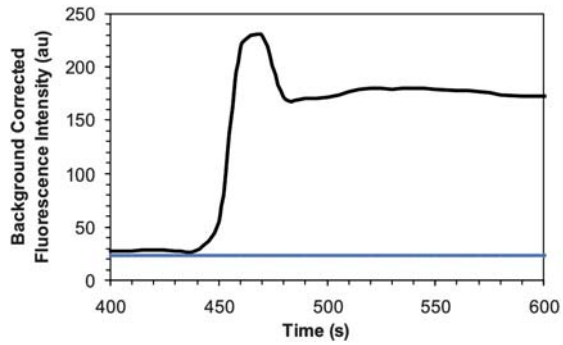

Fig. 5 Mixing during the release step sharpens the conjugate release profile. The fluorescence intensity within the reactor is measured as conjugate immobilized on the recirculator walls was cooled and released into solution. The x-axis corresponds to the time after the heater was turned off ($t = 0$ s). The y-axis corresponds to the fluorescence intensity in a small section of the reactor at a given time. In the system where the solution was allowed to cool to room temperature without perturbation, the conjugate did not desorb from the channel wall and return to solution over this timescale (blue line). In the second case, the solution was cooled to 25 °C ($t = 430$ s) then the recirculator was activated at 5 Hz (black line). As surface bound aggregates were initially released from the surface, the local concentration was high, resulting in a small spike of fluorescence that levelled out to a final concentration after homogenization.

Conclusions

We have described a microfluidic system capable of capturing, enriching and releasing "smart" polymer–protein conjugates that have been used as capture reagents for diagnostic biomarkers. We have shown that the combination of sample recirculation and helical flow can greatly increase the smart polymer–protein reagent capture above the phase transition temperature when free PNIPAAm is employed. We have also shown that mixing and recirculation substantially increase conjugate release rate and sharpness once temperature has dropped below the phase transition temperature. Lastly, we have shown this system is capable of strongly enriching conjugates and sharply releasing them into a finite volume for concentration of diagnostic reagents. The microreactor module thus clarifies designs for optimizing the purification and concentration of biomarkers

using smart polymer–protein capture reagents with smart channel surfaces.

Acknowledgements

The authors would like to express their gratitude to the NIH for funding (grant EB000252-09). The authors would like to thank the Washington Technology Center for the use of clean room facilities and equipment. The authors would like to thank the Bill and Melinda Gates Foundation for Grand Challenges in Global Health.

References

1 J. M. Gonzalez-Buitrago and C. Gonzalez, *Clin. Chim. Acta*, 2006, **365**, 50–57.
2 P. Yager, T. Edwards, E. Fu, K. Helton, K. Nelson, M. R. Tam and B. H. Weigl, *Nature*, 2006, **442**, 412–418.
3 H. Wang, S. G. Clouthier, V. Galchev, D. E. Misek, U. Duffner, C. K. Min, R. Zhao, J. Tra, G. S. Omenn, J. L. M. Ferrara and S. M. Hanash, *Mol. Cell. Proteomics*, 2005, **4**, 618–625.
4 A. Schwarz, J. S. Rossier, E. Roulet, N. Mermod, M. A. Roberts and H. H. Girault, *Langmuir*, 1998, **14**, 5526–5531.
5 A. Dodge, K. Fluri, E. Verpoorte and N. F. de Rooij, *Anal. Chem.*, 2001, **73**, 3400–3409.
6 A. Papra, A. Bernard, D. Juncker, N. B. Larsen, B. Michel and E. Delamarche, *Langmuir*, 2001, **17**, 4090–4095.
7 T. L. Yang, S. Y. Jung, H. B. Mao and P. S. Cremer, *Anal. Chem.*, 2001, **73**, 165–169.
8 H. B. Mao, T. L. Yang and P. S. Cremer, *Anal. Chem.*, 2002, **74**, 379–385.
9 L. M. Shamansky, C. B. Davis, J. K. Stuart and W. G. Kuhr, *Talanta*, 2001, **55**, 909–918.
10 H. Andersson, W. van der Wijngaart, P. Enoksson and G. Stemme, *Sens. Actuators, B*, 2000, **67**, 203–208.
11 M. E. Piyasena, T. Buranda, Y. Wu, J. M. Huang, L. A. Sklar and G. P. Lopez, *Anal. Chem.*, 2004, **76**, 6266–6273.
12 S. Ekstrom, J. Malmstrom, L. Wallman, M. Lofgren, J. Nilsson, T. Laurell and G. Marko-Varga, *Proteomics*, 2002, **2**, 413–421.
13 K. Sato, M. Yamanaka, H. Takahashi, M. Tokeshi, H. Kimura and T. Kitamori, *Electrophoresis*, 2002, **23**, 734–739.
14 T. Richter, L. L. Shultz-Lockyear, R. D. Oleschuk, U. Bilitewski and D. J. Harrison, *Sens. Actuators, B*, 2002, **81**, 369–376.
15 L. Ceriotti, N. F. de Rooij and E. Verpoorte, *Anal. Chem.*, 2002, **74**, 639–647.
16 C. Ericson, J. Holm, T. Ericson and S. Hjerten, *Anal. Chem.*, 2000, **72**, 81–87.
17 D. S. Peterson, T. Rohr, F. Svec and J. M. J. Frechet, *Anal. Chem.*, 2003, **75**, 5328–5335.
18 D. L. Huber, R. P. Manginell, M. A. Samara, B. I. Kim and B. C. Bunker, *Science*, 2003, **301**, 352–354.
19 C. D. H. Alarcon, T. Farhan, V. L. Osborne, W. T. S. Huck and C. Alexander, *J. Mater. Chem.*, 2005, **15**, 2089–2094.
20 A. E. Ivanov, J. Ekeroth, L. Nilsson, B. Mattiasson, B. Bergenstahl and I. Y. Galaev, *J. Colloid Interface Sci.*, 2006, **296**, 538–544.
21 N. Idota, A. Kikuchi, J. Kobayashi, Y. Akiyama and T. Okano, *Langmuir*, 2006, **22**, 425–430.
22 K. Auditorehargreaves, R. L. Houghton, N. Monji, J. H. Priest, A. S. Hoffman and R. C. Nowinski, *Clin. Chem. (Washington, DC, U. S.)*, 1987, **33**, 1509–1516.
23 N. Monji, C. A. Cole and A. S. Hoffman, *J. Biomater. Sci., Polym. Ed.*, 1994, **5**, 407–420.
24 N. Malmstadt, A. S. Hoffman and P. S. Stayton, *Lab Chip*, 2004, **4**, 412–415.
25 J. Hyun, W. K. Lee, N. Nath, A. Chilkoti and S. Zauscher, *J. Am. Chem. Soc.*, 2004, **126**, 7330–7335.
26 J. Liu, B. A. Williams, R. M. Gwirtz, B. J. Wold and S. Quake, *Angew. Chem., Int. Ed.*, 2006, **45**, 3618–3623.
27 S. Kulkarni, C. Schilli, A. H. E. Muller, A. S. Hoffman and P. S. Stayton, *Bioconjugate Chem.*, 2004, **15**, 747–753.
28 S. Kulkarni, C. Schilli, B. Grin, A. H. E. Muller, A. S. Hoffman and P. S. Stayton, *Biomacromolecules*, 2006, **7**, 2736–2741.
29 J. W. Hong, V. Studer, G. Hang, W. F. Anderson and S. R. Quake, *Nat. Biotechnol.*, 2004, **22**, 435–439.
30 A. D. Stroock, S. K. W. Dertinger, A. Ajdari, I. Mezic, H. A. Stone and G. M. Whitesides, *Science*, 2002, **295**, 647–651.
31 A. D. Stroock, S. K. Dertinger, G. M. Whitesides and A. Ajdari, *Anal. Chem.*, 2002, **74**, 5306–5312.
32 K. Wehrly and B. Chesebro, *Methods*, 1997, **12**, 288–293.
33 K. Toohey, K. Wehrly, J. Nishio, S. Perryman and B. Chesebro, *Virology*, 1995, **213**, 70–79.
34 B. Chesebro, K. Wehrly, J. Nishio and S. Perryman, *J. Virol.*, 1992, **66**, 6547–6554.
35 X. Yin, A. S. Hoffman and P. S. Stayton, *Biomacromolecules*, 2006, **7**, 1381–1385.
36 J. M. Hoffman, J. Shao, C. H. Hsu and A. Folch, *Adv. Mater.*, 2004, **16**, 2201–2206.
37 C. H. Hsu and A. Folch, *Appl. Phys. Lett.*, 2006, **89**, 144102.
38 F. Schonfeld and S. Hardt, *AIChE J.*, 2004, **50**, 771–778.
39 J. T. Yang, K. J. Huang and Y. C. Lin, *Lab Chip*, 2005, **5**, 1140–1147.
40 M. A. Unger, H. P. Chou, T. Thorsen, A. Scherer and S. R. Quake, *Science*, 2000, **288**, 113–116.
41 V. Studer, G. Hang, A. Pandolfi, M. Ortiz, W. F. Anderson and S. R. Quake, *J. Appl. Phys.*, 2004, **95**, 393–398.
42 B. H. Jo, L. M. Van Lerberghe, K. M. Motsegood and D. J. Beebe, *J. Microelectromech. Syst.*, 2000, **9**, 76–81.
43 M. Ebara, J. M. Hoffman, A. S. Hoffman and P. S. Stayton, *Lab Chip*, 2006, **6**, 843–848.
44 S. W. Hu, X. Q. Ren, M. Bachman, C. E. Sims, G. P. Li and N. Allbritton, *Anal. Chem.*, 2002, **74**, 4117–4123.
45 A. E. Kamholz and P. Yager, *Sens. Actuators, B*, 2002, **82**, 117–121.
46 M. Ebara, J. M. Hoffman, P. S. Stayton and A. S. Hoffman, *Radiat. Phys. Chem.*, 2007, **76**, 1409–1413.
47 N. Bontoux, A. Pepin, Y. Chen, A. Ajdari and H. A. Stone, *Lab Chip*, 2006, **6**, 930–935.
48 M. F. Ali, R. Kirby, A. P. Goodey, M. D. Rodriguez, A. D. Ellington, D. P. Neikirk and J. T. McDevitt, *Anal. Chem.*, 2003, **75**, 4732–4739.
49 S. R. Bannister, C. H. Lohmann, Y. Liu, V. L. Sylvia, D. L. Cochran, D. D. Dean, B. D. Boyan and Z. Schwartz, *J. Biomed. Mater. Res.*, 2002, **60**, 167–174.
50 M. S. Williams, K. J. Longmuir and P. Yager, *Lab Chip*, 2008, **8**, 1121–1129.
51 N. Ishida and M. Kobayashi, *J. Colloid Interface Sci.*, 2006, **297**, 513–519.
52 N. L. Anderson and N. G. Anderson, *Mol. Cell. Proteomics*, 2002, **1**, 845–867.