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Review

Microfluidic immunoaffinity separations for bioanalysis☆

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Abstract

Microfluidic devices often rely on antibody–antigen interactions as a means of separating analytes of interest from sample matrices. Immunoassays and immunoaffinity separations performed in miniaturized formats offer selective target isolation with minimal reagent consumption and reduced analysis times. The introduction of biological fluids and other complicated matrices often requires sample pretreatment or system modifications for compatibility with small-scale devices. Miniaturization of external equipment facilitates the potential for portable use such as in patient point-of-care settings. Microfluidic immunoaffinity systems including capillary and chip platforms have been assembled from basic instrument components for fluid control, sample introduction, and detection. The current review focuses on the use of immunoaffinity separations in microfluidic devices with an emphasis on pump-based flow and biological sample analysis.

Keywords: Immunoaffinity; Microfluidic; Immunoassay; Antibodies; Capillary:microchip

Contents

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1. Introduction

The measurement of analytes from complex and limited matrices is frequently required for analysis of biological samples. There is an increasing need for analytical instrumentation capable of minimal sample consumption and reduced analysis times, while providing selective separation of target compounds from the sample matrix. The reduction of sample requirements is advantageous for the analysis of scarce or valuable biological test materials, including research, clinical, and archived samples [1]. Miniaturized analytical devices address this requirement for reduced reagent consumption and fast time of analysis, while offering the potential for portability and patient point-of-care testing. Target analytes in biological samples are often present in low concentrations relative to the surrounding matrix components, necessitating effective separation techniques. Immunoaffinity-based separation techniques have been used to separate analytes of interest from complex biological samples based on the selective binding of antibodies to their respective antigens [1]. Immunoaffinity microfluidic devices employing antibodies as immobilized ligands may be applied to capture and concentrate target analytes from small volumes (micoliters and below) of biological matrices. The combination of miniaturized instrumentation with immunoaffinity separation science allows for potentially rapid and selective isolation of low concentration analytes from biological samples.

Immunoaffinity separations have been reviewed with respect to their use in sample preparation/extraction [2,3], post-column immunodetection [4], capillary electrophoresis [5,6], microfluidic immunosensors [7,8], and conventional scale affinity/immunoaffinity chromatography [9–11]. In the current review, recent literature will be discussed as it pertains to immunoaffinity separations, with small-scale biological sample analysis using miniaturized instrumental components and involving pumped-based systems with capillaries or microchips. The term "microfluidics" will be used to describe the movement of liquids through microchip channels or capillaries having internal dimensions less than 1 mm and the term "immunoaffinity" will encompass flow-based immunoassays as well as immunoaffinity chromatography.

2. Immunoaffinity background

2.1. Antibodies

Antibodies are large glycoproteins produced in response to a material identified as foreign by the body and immunoglobulin G (IgG) is the most commonly used antibody in immunoaffinity techniques. IgG is approximately 150 kDa, consisting of four polypeptides with two 25 kDa identical light chains and two 50 kDa identical glycosylated heavy chains [5]. Fig. 1 shows a schematic of an antibody with common functional groups diagrammed. Disulfide bonds join light and heavy chains together, as well as linking the two heavy chains to form the hinge area. The fragment antigen binding (Fab) portion is located at the N-terminus and contains a region of variable amino acid content

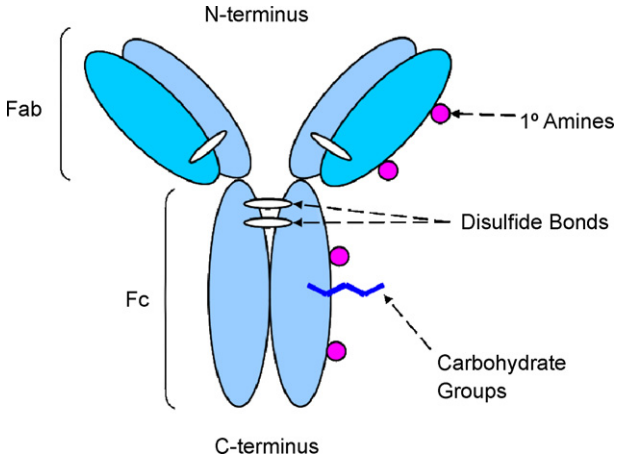

Fig. 1. Antibody structure and functional groups for labeling or solid phase attachment. Light and heavy chains are indicated by the letters L and H, respectively.

responsible for antigen specificity. The fragment crystallizable (Fc) portion spans the C-terminus to the hinge region and maintains a constant amino acid sequence within the same class of antibody. Primary amines of lysine residues are located throughout the entire antibody and carbohydrate groups are mainly located in the Fc region [12]. The carbohydrate residues and primary amines are targeted attachment sites for linking antibodies to solid supports or attaching labels for detection. Two types of antibodies employed for immunoaffinity separations are polyclonal and monoclonal. Polyclonal antibodies are natural antibodies from multiple cell lines that may bind different recognition sites or epitopes with varying strengths, whereas monoclonal antibodies are produced from a single cell line and bind to a single epitope with the same strength [10].

2.2. Antibody immobilization

Immunoaffinity-based separations typically employ immobilized antibodies as a stationary phase to capture antigens of interest from sample matrices. The unbound material is washed to waste before an elution buffer is applied to dissociate the antigen for detection [12]. The heart of an immunoaffinity analysis system is the separation column or channel, which consists of an immunosorbent of immobilized antibodies. The orientation of the stationary antibodies is critical to the binding activity [10]. Fig. 2 shows the possible orientations of an immobilized IgG on a solid support material. In (a), the antibody is attached through the Fab section and both antigen-binding sites are unavailable. In (b) the binding sites are partially available and in (c) the antibody is attached through the Fc region with both binding sites fully available. Immobilization strategies that anchor the antibody with the Fab portions facing away from the stationary support result in a higher degree of active antibody per unit of stationary support.

Antibodies may be attached directly to the walls of a capillary column or microchip channel. Antibodies can also be linked to solid phase supports and packed into columns or channels. Solid supports may consist of particles or beads made from plastic,

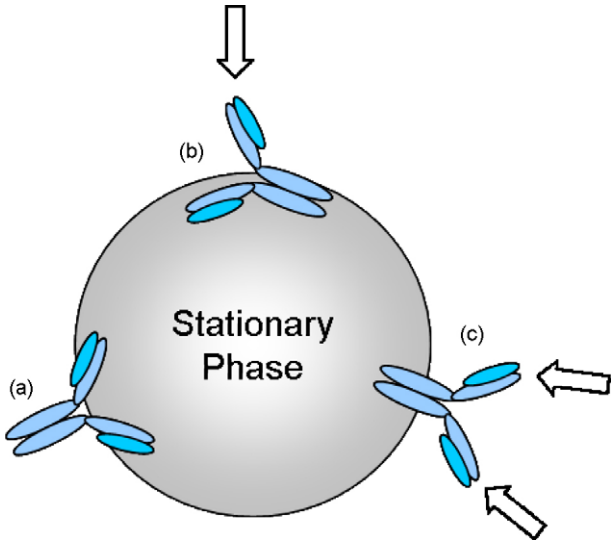

Fig. 2. Effect of immobilized IgG on antigen recognition. Arrows indicate available antigen binding sites.

silica or glass, and magnetic materials [10,13,14]. The stationary phase should possess the following characteristics [12,13,15]:

(a) Chemical stability,
(b) Low nonspecific binding,
(c) Mechanical stability for favorable flow rates,
(d) Sufficient surface area for antibody–antigen binding.

The choice of solid phase material and method of antibody attachment are important factors for developing an immunoaffinity separation. A simple approach for antibody immobilization is through direct attachment to a solid support. Antibodies may be physically adsorbed to plastic chips or beads through noncovalent bonding [16,17] or free amine groups may be reacted through activated surfaces. Immobilization of the antibodies by direct adsorption or through primary amines results in a random orientation and potentially lowered binding activity. This likely results from attachment of the antibody through the Fab arms, which can occur when linking antibodies to supports using free amino groups. Carbohydrate groups of the Fc region can be oxidized to aldehyde functional groups with periodate and reacted with hydrazide supports to give the proper orientation for antigen binding. Polyclonal antibodies are reported to have better success binding antigen versus monoclonal antibodies after periodate oxidation [18]. This may be due to differences in the degree of glycosylation between the two types of antibodies. Additionally, Fab fragments produced from enzymatic cleavage of IgG can be coupled through free sulfhydryl groups to chemically modified supports. This method results in monovalent antibody fragments with antigen receptors oriented toward the solution and away from the stationary support.

Attachment of antibodies to secondary molecules has been used for optimizing antibody orientation and also providing a protective protein coat to the solid phase material. Bacterial cell wall proteins A and G are secondary molecules that selectively bind many antibodies at the Fc region. These proteins maintain

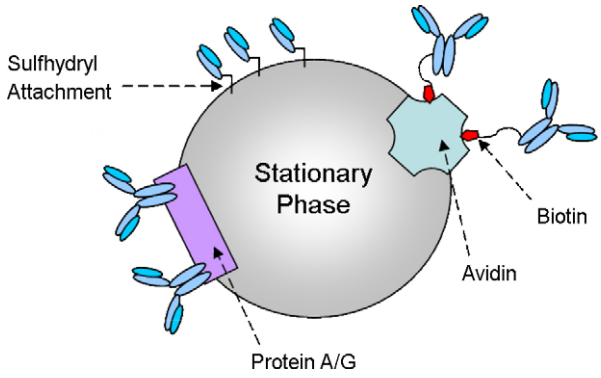

Fig. 3. Antibody immobilization strategies. Antibodies may be linked through secondary compounds such as Protein A/G and biotin/avidin or covalently attached through sulfrhydryl groups of Fab fragments.

antibody binding at physiological pH, but will release antibodies in weakly acidic conditions [9,10]. The straightforward coupling of antibodies to protein A and G is an advantage, but the antibodies must be reapplied to the column. Multiple injections on this type of column could consume large quantities of valuable antibodies; however, additional reactions may be performed to cross-link protein A or G to the antibody for a less reversible attachment [10,17].

Avidin is a protein from egg white that has a high affinity for the vitamin biotin ($K_a = 10^{15}$/M), forming strong noncovalent bonds [19,20]. Streptavidin is a bacterial form of avidin with similar binding sites for biotin, but is also a form that has less nonspecific interaction with proteins. Unlike the antibody binding of protein A or G, the streptavidin–biotin bond is nearly irreversible. Antibodies must be linked to biotin in order to react with streptavidin, but their activity is unlikely to be affected due to the small size of biotin (244 Da) [20]. Numerous biotin derivatives for linkage to antibodies are available. Biotinylation of antibodies may be achieved through primary amine groups using *N*-hydroxysuccinimide groups or through aldehydes using a hydrazide linker. The biotin hydrazide reagents will react with oxidized carbohydrates in the Fc region to form a hydrazone bond. The use of hydrazine has the same advantage as using protein A or G, which is that antibody attachment does not interfere with antigen binding sites [17]. Biotinylation of antibodies through oxidized carbohydrates or sulfhydryl groups of Fab fragments have been shown to yield stationary supports with higher degrees of antibody activity when compared to supports from amino-biotinylated antibodies [16,18]. Alternatively, many antibodies may be purchased biotinylated, although this is most often via primary amine attachment. It should be noted that biotinylated IgG has been reported to display instability in human plasma after prolonged exposure (4 h incubation) [21]. Fig. 3 shows a solid phase bead with antibodies attached by Fab sulfhydryl groups and secondary compounds. Protein A/G, avidin/streptavidin, and secondary antibody-coated beads are commercially available in polystyrene and magnetic polystyrene from numerous vendors. Sources of activated supports suitable for high performance immunoaffinity applications (i.e. silica or glass) are shown in Table 1.

Table 1
High-performance supports suitable for immobilizing antibodies through secondary compounds

Material	Coating	Vendor
Silica microspheres	Avidin	Kisker[a]
Silica microspheres	Streptavidin	Bangs Labs[b] Kisker[a] Polysciences[c]
Silica microspheres	Protein A or G	Kisker[a]
Glass particles	Streptavidin	Xenopore[d]

[a] www.kisker-biotec.com.
[b] www.bangslabs.com.
[c] www.polysciences.com.
[d] www.xenopore.com.

3. Immunoaffinity separations

3.1. Antibody–antigen complexes and immunoaffinity chromatography

Antibody–antigen complexes form as the result of several intermolecular forces, the main four being:

1. Hydrogen bonding;
2. Coulombic, i.e. NH_3^+ and COO^-;
3. Van der Waals;
4. Hydrophobic interactions.

Collectively, these forces result in strong binding at physiological conditions [12,22]. In immunoaffinity chromatography, it is desirable to elute the analyte quickly by dissociating the antibody–antigen complex without damaging the immobilized antibody. The sample containing the antigen is introduced to the antibody in an application buffer with conditions to allow for maximum binding. Application buffers are typically near physiological pH and salt concentrations and phosphate or Tris-based buffers are often used. During this antigen capture step, all nonreactive sample components are washed from the separation column. Recovery of the isolated antigen for detection is achieved by the application of an appropriate elution buffer. Alternatively, detection of the antibody-bound antigen may occur directly on-chip or on-capillary; however, the antigen must still be dissociated if the system is intended to be reusable.

The selection of an effective elution buffer can vary based on the antigen under investigation and empirical tests are frequently required [12,23]. The most commonly used scheme involves lowering the pH of the running buffer down to 2 or below. Many solid supports such as silica can degrade when exposed to sharp changes from neutral to acidic conditions. Protein coatings such as protein A or streptavidin offer some protection in this regard [22]. Acidic elution buffers may lead to poor antigen recovery in cases where the antibody–antigen complex is held together predominately with hydrophobic interactions. Chaotropic agents, such as thiocyanates, are also used as elution buffers with less damaging effects to the stationary phase compared to low pH acidic buffers [24]. Chaotropes disturb the structure of water molecules and reduce hydrophobic interactions between proteins [25,26]. Other dissociation reagents have been utilized, including ionic strength modifications, denaturants, and polarity-reducing organic modifiers [22,23].

With any elution scheme, a short step-wise or linear gradient of the dissociating buffer may be introduced into the running buffer. Step gradients have the potential of providing faster antigen dissociation and shorter analysis times versus linear gradients [10]. The type of gradient may also have an effect on the shape of the eluted antigen peak; it has been reported that linear gradient elution produces sharper, more resolved peaks versus stepwise gradients [24]. For pH and chaotropic elution buffers, linear gradients are also reported to be gentler on the immobilized antibody and support material [22,24].

Different modes of immunoaffinity separations using immobilized antibody columns and microchips may be performed, including:

1. Competitive,
2. Noncompetitive,
3. Direct capture.

A competition assay may be employed, where sample antigen and a labeled antigen of known concentration compete for limited antibody binding sites. The amount of labeled antigen bound to the antibody will decrease with increasing amounts of sample antigen. A competitive assay is commonly used for smaller univalent antigens [27]. A noncompetitive sandwich immunoassay requires two antibodies with affinity for the antigen. The first antibody is immobilized to capture the antigen and a second, labeled antibody is added before or after injection of the sample. The dual antibody system offers a higher selectivity, but requires that the antigen be large enough to bind two antibodies through distinct epitopes. The sandwich assay format may also take more time to perform due to two antibody-binding steps [27]. The most straightforward approach is a direct capture assay. In this technique, the sample is injected onto the immunoaffinity column and the antigen binds to the antibody in neutral buffer. The antigen is often prelabeled (i.e. with a fluorescent conjugate) to improve detectability of low concentration samples [10]. The unbound material is pumped to waste and then the elution buffer is applied to dissociate the antigen for measurement. This results in a large initial chromatographic peak corresponding to the unbound material and excess label. The antigen peak will be a sharper, purified second peak. An inherent advantage is there is no need to purify the labeled analyte prior to analysis. An example of a typical direct capture format immunoaffinity chromatogram is shown in Fig. 4. A 250 nL injection of human parathyroid hormone (111 nM in phosphate buffer containing 0.1% human serum albumin) labeled with a laser dye was separated on a 0.175 mm × 19 mm capillary column and detected with a 650 nm diode laser LIF detector. Elution was achieved with a step gradient from a pH 7.0, 100 mM phosphate application buffer to a 2.5 M NaSCN elution buffer. The instrumentation used has been described elsewhere [28].

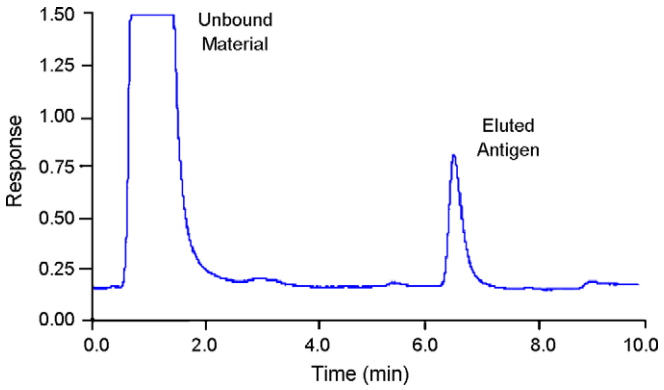

Fig. 4. Typical immunoaffinity chromatogram. The first off-scale peak corresponds to nonreactive material washing off the column during the antigen capture step. After application of a dissociation buffer, the antigen is eluted as the second peak. Conditions are described in the text.

3.2. Working lifetime of immunoaffinity devices

The working lifetime of immunoaffinity systems is important where repeat analysis is desired. Reducing the amount of material required to repeatedly prepare separation devices is an advantage considering the associated high costs of immunoassay reagents. Column and microchip lifetimes are often reported in terms of cycles, representing sample injection, elution, and/or regeneration. The useful lifetime is related to degradation of assay performance or loss of binding activity and dependent upon running/storage conditions of the system. The sample matrix, flow rate, elution conditions, and operating and storage temperature all affect the system's life expectancy. Nonspecific matrix components from repeat sample injections and incomplete elution can accumulate in the system [23] and cause increased pressure [29]. The immobilized antibody may be damaged or removed from the stationary support in the presence of high flow and pressure [24]. Flow rate also affects antigen recovery, as slower flows allow more time for antibody–antigen reactions. Harsh elution buffers may damage antibodies since the targeted forces holding antibody–antigen complexes together are the same as those responsible for maintaining the tertiary structure and binding ability of the antibody [22]. Yang et al. [30] reported that 150 cycles were possible for microfluidic chips using 10 mM glycine–HCl at pH 2.0 for elution. Additionally, the temperature of the immunoaffinity device can affect the performance and refrigerated (4 °C) conditions are reported to extend active life. Immunoaffinity column lifetimes of up to 200 cycles have been reported for columns both stored [13], and stored/operated [1] at refrigerated temperatures; although 200–250 cycles have also been reported for columns operated at room temperature [29].

3.3. Biological samples and complex matrices

Complex sample matrices require extensive sample preparation to become compatible with microfluidic systems and small-scale instrument components [31]. Biological samples for clinical and research purposes are problematic for microfluidic devices due to nonspecific binding of biomolecules, particularly proteins. Nonspecific adsorption to surfaces of capillary tubing and microchip materials, including stationary supports, can lead to increased system pressures and obstruction of small dimension flow paths. Immunoaffinity separations are adversely affected by nonspecific binding of biomolecules, which can cause higher background signals [32]. Blood proteins have been reported to quench fluorescently labeled antigens upon binding. Hatch et al. [33] have reported spiking iophenoxate into human whole-blood samples to preferentially bind serum albumin and reduce this quenching effect. Syringe filtration of human plasma has also been reported for sample pretreatment prior to immunoaffinity chromatography [29]. Filtration removes particulate matter that can clog pores of retaining frits or connecting tubing. Molecular weight cutoff filters (MWCO) have been employed for removing proteins and macromolecules from biological samples that could interfere with immunoaffinity separations [34]. These filters are available in centrifugal devices capable of processing small volumes of fluid. Filter selection is based upon the molecular weight of the target antigen; compounds of a larger molecular weight are retained on the filter and the desired analyte passes through to the filtrate. Biological fluids have been diluted in buffers prior to introduction into microfluidic devices to minimize matrix effects. The total protein content of samples has been adjusted to a constant amount to better control fluorescent labeling reactions [34,35]. This type of normalization maintains consistent reaction conditions for conjugation reactions that are dependent on the amount of excess reagents. For example, a fluorescent dye that reacts with primary amines would potentially label all protein material present in a biological sample, requiring large concentrations of excess dye.

Capillary columns and connecting tubing for microfluidic devices are often made of fused-silica and polyetheretherketone (PEEK). Salim et al. [36] have studied the effects of nonspecific protein binding on glass capillaries, fused-silica tubing, and PEEK tubing, noting the latter two as common microfluidic transfer lines. Adsorption of the human blood plasma protein fibrinogen was monitored by an enzyme-linked immunosorbent assay (ELISA) using an anti-fibrinogen antibody conjugated to horseradish peroxidase (HRP). The ELISA reagents were introduced into capillaries of each material after incubation with fibrinogen and flushed into microplate wells for measurement. It was determined that in addition to glass capillaries, PEEK tubing and fused-silica capillaries exhibited significant amounts of fibrinogen adsorption. The use of these materials in microfluidic immunoaffinity devices may contribute to nonspecific binding. Moreover, targeted antigens that are protein in nature may also bind to transfer tubing prior to separation, resulting in a loss of detectable analyte.

Microfluidic chips are commonly made from polymeric materials that are mechanically and chemically stable, as well as inexpensive to manufacture. Materials such as polystyrene and poly(dimethylsiloxane) (PDMS) are popular polymers for microfluidics, but have an inherent disadvantage of nonspecific protein binding [37,38]. The surfaces of these devices are often modified to reduce their hydrophobic nature, which

is believed to be involved in the nonspecific interaction of biomolecules. Sibrani et al. [37] have reported the use of phospholipid polymers to modify PDMS microchip channels for reduced nonspecific protein adsorption. Human serum albumin conjugated with fluorescein isothiocyanate (FITC) was incubated in the PDMS channels, washed, and the channel-bound albumin conjugate measured with fluorescence microscopy. The polymer modification contained hydrophilic phosphorylcholine groups, which reduced nonspecific adsorption of the albumin and also facilitated sample loading versus unmodified PDMS. A similar study involved coating poly(methyl methacrylate) (PMMA) microchannels with phospholipid polymers to reduce nonspecific interactions [39]. In this study, FITC-bovine serum albumin (BSA) was shown to be bound to unmodified PMMA microchannels, but this binding was significantly reduced when incubated with the phospholipid-coated surface. Additionally, human serum and rabbit plasma were incubated on the modified PMMA substrates and scanning electron microscopy images showed minimized nonspecific adsorption. The authors note that high water content of phospholipid polymers helps prevent hydrophobic interactions between biomolecules and the substrate. A microfluidic immunoassay for glucagon has been demonstrated using antibodies anchored to mobile polymer chains of poly(ethylene glycol) (PEG) acrylate [40]. The polymer chain was reported to reduce nonspecific binding of proteins to the substrate materials and also increase accessibility by extending the antibody into the sample solution. A detection limit of 1×10^{-13} M was reported for glucagon in phosphate buffered saline and no blocking steps were required. However, the sensitivity was decreased 53% and 66% for assays in 20% plasma and 20% whole blood, respectively.

Protein solutions and surfactants have been employed in immunoassays as blocking reagents to prevent nonspecific binding of proteins and matrix components [41,42]. Proteins such as BSA or casein are typically added as incubation steps in static assays and allowed to react with the microfluidic surface before being removed with wash steps [32,41]. In flow-based assays such as immunoaffinity chromatography, surfactants may be added to the application and elution mobile phase buffers to reduce nonspecific binding. Nonionic surfactants commonly used include Triton, Tween, and Brij, which are all effective in reducing hydrophobic protein aggregation and adsorption to solid support materials [41,43].

4. Microfluidic immunoaffinity instrumentation

The ideal lab-on-a-chip system is a microfluidic device that allows complete analysis, from sample introduction to detection, all on-chip [44]. As discussed previously, biological samples may require extensive pretreatment before introduction into a microfluidic system. Additionally, fluid manipulation and detection are often performed by off-chip external equipment [31]. Miniaturized instrument components facilitate microfluidic analyses and may fit onto mobile laboratory carts for potential point-of-care applications. Instrumentation used in the construction and operation of pump-based immunoaffinity microfluidic devices typically involves fluid control, sample introduction, separation columns or channels, and detectors.

4.1. Pumping systems and mixers

Immunoaffinity separations for capillary electrophoresis and capillary electrochromatography involve the use of electrokinetic flow and have been reviewed elsewhere [6,45,46]. Flow-based immunoassays and immunoaffinity chromatography in microfluidic platforms use pumps to push or pull liquids through the device. Syringe pumps [47,48] and peristaltic pumps [49,50] are frequently used to generate flow and introduce reagents. These pumps are available in micro-formats capable of μL/min to nL/min flow rates. Simple or disposable systems may require only one pump to deliver antigens into a capture/detection zone with an application buffer. When antigen elution or regeneration of the microfluidic device is desired, a second buffer or elution solvent is introduced. A single syringe pump, for example, would require the syringe to be changed mid-run for antigen elution. Simple step gradients can be generated using a switching valve connected to two buffer reservoirs and a single pump; however, flexible linear gradients require two pumping sources and a mixing device [24]. Mixing of buffers occurs in tees or microfluidic chips with branching channels. Gradient mixers are placed in-line between the pumps and the separation column or channel. Dual syringe pumps [28] and microdialysis pumps [1] have been successfully used to produce immunoaffinity gradients with microfluidic mixing chips. Capillary action has also been applied for immunoassay fluid movement in microchannels [51]. Devices employing capillary action forces do not require external pumping equipment or power to generate flow and are therefore well suited for portable applications. Capillary flow is manipulated by the shape of the flow path and the hydrophobic/hydrophilic character of the surface [52]. Wolf et al. [51] have described a microfluidic system using capillary action force to perform a C-reactive protein sandwich immunoassay on polymer substrates. A capillary pump was created by setting a piece of clean room paper over the exit channels. A flow rate of approximately 0.6 μL/min was achieved using the tissue for flow promotion. Examples of representative pumping mechanisms and separation devices are given in Table 2.

Table 2
Types of pump-based flow for immunoaffinity devices

Flow control	Separation device	Ref.
Syringe pumps	Packed fused-silica capillary	[1]
	Packed PEEK/fused-silica capillary	[28]
	PDMS/glass channel	[73]
Peristaltic pumps	PEEK capillary/stainless steel	[50]
	Fused-silica capillary chip	[49]
Capillary action	PDMS/PMMA channel	[52]
	PDMS/silicon channel	[51]
	Glass capillary	[67]

4.2. Sample introduction

The analysis of limited biological samples often involves processing a small volume of material and sample introduction into microfluidic devices requires instrumentation capable of sub-microliter injections. Manual injection valves suitable for micro- and nanoliter volumes of fluid may be fitted with laboratory-constructed sample loops on a capillary scale. PEEK and fused-silica capillary tubing can be cut to desired lengths corresponding to loop volumes based on the calculation for the volume of a cylinder. The chief limitation with manual injectors is limited automation and throughput; however, many clinical and point-of-care tests only require testing small numbers of samples. Microsyringes may be used to transfer samples to wells or ports of microchips and syringe pumps have been applied as well. Sato et al. have described a syringe pump connected to a microchip outlet hole with a capillary to pull samples from the inlet into the reaction region [47]. As commercial autosamplers for liquid chromatography become miniaturized (i.e. nano-LC), microfluidic immunoaffinity separations will benefit from a higher degree of automation and increased throughput.

4.3. Separation column or channel

Capillary columns or microchip channels serve as the reaction area for immunoaffinity microfluidic separations. Resolution of the unbound peak and antigen peaks in immunoaffinity chromatography is controlled by the elution step, not necessarily the column dimensions [53]. Immunoaffinity columns are therefore amenable to miniaturization as long as the separation zone is large enough to provide adequate antigen binding capacity. For clinical samples, a 50:1 ratio of capture antibody to potential antigen amount has been suggested for efficient binding [34].

Separation capillaries and channels may be open with antibodies attached to the inner surfaces or packed with an immunosorbent such as beads. The use of reduced-dimension open channels or packed bead beds has inherent advantages over conventional scale immunoassay platforms. Compared to microtiter wells commonly used in immunoassays, the reactive surface area to solution volume ratio is larger and diffusion distances are reduced in capillaries and microchannels [54]. These effects are more pronounced when beds of packed microbeads are used for antigen capture in microfluidics [44,55]. Increasing the reactive binding surface and reducing the internal volume required for antibody–antigen recognition enables the concentration of dilute antigens in small sample volumes [55,56]. In flow-through systems, sample volumes greater than the volume of the packed bed may be injected and eluted in a concentrated, smaller volume. The increased reactive surface area and reduced diffusion distances in immunoaffinity microfluidics result in a faster time of analysis versus conventional immunoassay techniques [44]. For example, the analysis time of human immunoglobulin A on a polystyrene bead-packed microchip was reduced to 1/90 of the time needed for an immunoassay in a microplate [57].

Capillary columns used in microfluidic immunoaffinity separations have been constructed from fused-silica [49,58,59], PEEK [36], and PEEK-coated fused-silica (PEEKsil) [28]. These materials are commercially available in a variety of internal and outer diameters and may be cut to desired lengths. Retaining frits are required for bead-based separations and have been made by heat annealing silica particles to form porous glass frits [1,59]. Alternatively, capillary-scale stainless steel frits may be purchased and attached to micro-column end fittings [28]. Capillary columns made from monolithic stationary supports do not require the use of retaining frits [58] and offer reduced backpressures with fast mass transfer properties [60]. Fused-silica capillaries have been used for the direct attachment of antibodies to the inner walls of the column [48,49]. Open capillary tubes would have less back pressure in pump-based systems versus packed capillaries and could be operated at faster flow rates.

Increasing the flow rate in immunoaffinity separations may result in incomplete antibody–antigen reactions; however, it is not always possible or necessary to reach equilibrium of antibody–antigen binding in microfluidic systems. Wolf et al. [51] have noted that the binding step can be optimized by picking the time that produces a sufficiently measured response. In flow-through devices, this means choosing the fastest flow rate that still allows enough antigen binding to provide a desired signal.

Microfluidic immunoaffinity separations have been performed on chips made from silicon, glass, and polymeric materials, such as PDMS, PMMA, and polystyrene [37,44]. PDMS has been most commonly used [7,37], but as discussed previously requires surface treatments or blocking steps to inhibit high nonspecific protein interactions. Microchips may contain antibodies immobilized to the surface of the substrate or to beads packed in channels. Antibody Fab fragments have been directly attached to ports of glass chips for immunoaffinity-CE of neuropeptides [34] and cytokines [61] in clinical samples. Beads are retained in microchips by creating weirs or physical barriers to trap beads that are larger than the outlet channel [55,56]. Magnetic beads do not require alterations of channel dimensions and are instead held in place by use of an external magnet [44,55]. In the bead-based chips, the stationary support is typically back flushed from the weirs [62] or the magnetic field is removed [14] to release the beads and reuse the device. Although this eliminates the need for regeneration of the immunoaffinity solid phase, the beads must be reapplied with each sample, which may consume valuable materials.

Capillaries and chips that use beads for antibody–antigen reactions must be packed to form separation zones. Positive pressure is often applied via syringe pumps to pack slurries of beads into microfluidic devices [62], although high pressures may lead to damage of the immobilized antibody [22]. Negative pressure may also be used for packing bead solutions using vacuum or syringe pumps to pull the slurry from the end of the column or channel [28,47,63]. Slurries of beads may also be introduced into columns by a series of injections under positive or negative pressure [64,65]. Packed beds using magnetic beads are made by pumping or pulling the beads past a magnet located at the desired location of the capillary or chip [14].

4.3.1. Open systems

Antibodies may be attached to internal walls of capillaries or channels by direct adsorption or through functional group modifications. For example, an "immunostack" of reagents was formed in the channels of plastic chips by first directly adsorbing C-reactive protein from human serum [66]. The blocking buffer, primary antibody, and secondary detection antibody were then added sequentially. This procedure was simple and took 25 min to complete versus several hours for an ELISA of the same antigen. Open glass capillaries were used to perform sandwich immunoassays of myocardial proteins in diluted human plasma [67]. The capture antibodies were immobilized to silane or silane/glutaraldehyde-modified capillaries and the effects of sonication were studied. The silane surface treatment of the internal walls was enhanced by sonication, although the antibody–antigen-binding step was negatively affected.

Fused-silica capillary columns (250 μm internal diameter (i.d.)) have been modified with glutaraldehyde for the immobilization of anti-*E. coli* O157:H7 antibodies [48]. A sandwich immunoassay was demonstrated using a syringe pump to deliver reagents and off-line absorbance detection of an enzymatic reaction product was employed. A separate sandwich immunoassay for *E. coli* O157:H7 was performed in 530 μm i.d. fused-silica capillaries using fluorescence detection [68]. The capture antibody was immobilized to the internal capillary wall through a protein A modification and covalently anchored with dimethyl pimelidate. The detection antibodies were conjugated to 250 nm liposomes that encapsulated a fluorescent dye. Once the sandwich complex was formed, a detergent was added to lyse the bound liposome and release the dye for detection.

4.3.2. Packed systems

Microfluidic chips and capillaries are frequently packed with solid particles that have been modified with immobilized antibodies or antigens. Polystyrene beads coated with anti-C-reactive protein have been used in a PDMS chip for a competitive chromatographic immunoassay [63]. The beads were held in a chamber by a frit structure and a 20-fold amplification of the fluorescence signal was achieved compared to the assay without beads. Polystyrene beads trapped in weirs of a PDMS chip were demonstrated for a sandwich immunoassay of tacrolimus with on-chip fluorescence detection [69]. The assay was performed using a single channel and took 15 min to complete versus 2–4 h for commercial systems. It should be noted that microchip time was for one sample and the commercial systems were capable of processing 24–48 samples. Malmstadt et al. [70] have reported a temperature-responsive stationary phase in poly(ethylene terephthalate) (PET) channels for a competitive immunoassay of digoxin. Biotinylated antibodies were immobilized to streptavidin anchored to the beads through a biotin-PEG linkage. The beads were also coated with a temperature sensitive polymer, poly (*N*-isopropylacrylamide) (PNIPAAm). A hydrophilic-to-hydrophobic phase transition of the polymer occurs at temperatures above the lower critical solution temperature (about 28 °C). The temperature of the microfluidic device was maintained at 33–37 °C, which caused aggregation of the beads and adhesion to the channel walls. When the temperature was adjusted below 28 °C the beads were no longer in a hydrophobic phase and were released for fluorescence measurement of the bound antigen. The advantages of the reversible immobilized beads were that one device platform could be reused multiple times and different antigens could be assayed by applying different immobilized antibody beads.

A direct capture microfluidic immunoaffinity chromatography system was demonstrated for the analysis of substance P in human plasma, serum, urine, cerebral spinal fluid, tissue lymph, and cell lysates [1]. Biotinylated antibodies were attached to glass beads and packed into 12 mm long fused-silica capillaries with a 50 μm internal diameter. A dual syringe microdialysis pump was used to deliver buffers and only 50 nL injection volumes were required. HPLC microcolumns were reported for fast separations of fluorescein and BSA using a packed bed of immunoaffinity beads sandwiched between layers of diol-bonded silica [64]. Although the size of the columns was 2.1 mm i.d. × 1.0 cm, the length of active stationary support used in the study varied from 60 μm to 1.1 mm. A competitive chromatographic immunoassay of BSA required only 5–25 s for signal measurement. In a similar study by the same research group, affinity microcolumns were demonstrated using monolithic disks as the stationary support [71]. Anti-FITC antibodies immobilized on a 4.5 mm i.d. × 0.95 mm monolithic support were used to measure fluorescein in phosphate buffer. A 95% extraction of fluorescein was achieved when the residence time in the column was 100 ms using a flow rate of 3 mL/min. The authors note that nonspecific binding could be reduced in small-scale columns that employ relatively high flow rates. The monolithic columns also exhibited lower backpressures and greater stability to higher flow rates versus the silica bead-packed sandwich microcolumns.

Monolithic supports in 250 μm i.d. × 10 cm fused-silica capillaries were reported for immunoaffinity separation of fluorescein with laser-induced fluorescence detection [58]. The antigen was concentrated prior to detection by applying 150 column bed volumes of an aqueous solution and eluted with 20% methanol in a neutral buffer. Affinity microcolumns in PDMS channels have been demonstrated for simultaneous on-column measurement of multiple analytes [65]. Affinity receptors included immobilized fluorescein-labeled anti-carninoembryonic antigen, FLAG peptide, and biotin. The fluorescence resonance energy transfer of the receptor beads to bound analytes was measured. Glass receptor beads were packed into 600 μm segments separated by uncoated beads to form approximately 5.4 mm columns. The columns were mounted vertically and scanned up and down with a laser so that each analyte segment provided a distinct signal.

4.3.3. Magnetic systems

Magnetic particles may be modified with immunoaffinity ligands and used as solid supports that are manipulated by placing a magnet near channels or capillaries. Paramagnetic beads coated with streptavidin were used to demonstrate a flow immunoassay for inerleukin-5 and parathyroid hormone [14]. The beads were conjugated with biotinylated primary antibodies and pulled by vacuum into 50 μm i.d. fused-silica capillaries. A rare earth mag-

net placed near the capillary allowed the beads to form a partially packed bed. The packed bed did not cross the entire capillary, which resulted in lower pressure and potentially higher flow rates versus a densely packed bed. Magnetic beads have also been employed for mixing in microfluidic devices. Herrmann et al. [72] have reported the use of streptavidin magnetic beads in a stop-flow ELISA for anti-streptavidin antibodies on PDMS chips. The beads served not only as a reactive stationary support, but also as an internal mixing element. A rare earth magnet was used to trap the beads in the reaction chamber to form a loose bed. During the stop-flow incubation period, the magnet was moved back and forth along the channel to mix reagents and improve the reaction.

Hahn et al. [73] have demonstrated a novel magnetophoretic immunoassay for human IgE in a PDMS-based microfluidic chip. The antigen, dust mite allergen, was conjugated to 5.6 μm microbeads and used to capture allergen-specific human IgE. A sandwich complex was formed by adding 9 nm superparamagnetic particles conjugated with anti-human IgG. The superparamagnetic particle-microbead sandwich was pumped through a microchannel parallel to a nickel microstructure under external magnetic field. The deflection velocity of the microbeads to the nickel was measured with a charge coupled device (CCD) camera on an inverted microscope. The microbead velocity (μm/s) was proportional to the amount of superparamagnetic particles and thus the IgE. For two dust mites, *Dermatophagoides farinae* and *D. pteronyssinus*, the detection limits of IgE in human serum were 565 and 268 fM, respectively.

4.3.4. Devices for multiple assays

Some biomedical applications require the measurement of many analytes from a single small volume of sample. The analysis of multiple analytes or multiple samples simultaneously by microfluidic immunoaffinity devices requires more complex system designs. These typically involve the use of multiple reaction channels or capillary columns. For example, 30 fused-silica columns were connected in series to analyze multiple analytes from a single 10 μL human serum sample [59]. Each capillary was packed with antibody-coated glass beads corresponding to a specific antigen for a direct capture assay. The antigens were fluorescently labeled and detected on-column by laser-induced fluorescence (LIF). In another study, four samples were simultaneously assayed for interferon using glass chips containing branching channels packed with antibody-coated polystyrene beads [47]. A similar eight-channel system has been developed in PDMS chips for parallel ELISA using a magnetic bead separation [72]. The branching channels and columns connected in series have the advantage of using a single pumping system to deliver reagents and samples. A unique system for the analysis of biological warfare agents has been developed by combing antibody-coated capillary columns with chips [49]. Fused-silica capillary columns were glued into grooves of a polymer chip to create ten separate channels. Ten simultaneous sandwich immunoassays for one antigen each and multiplexed sandwich immunoassays for three antigens were demonstrated in aqueous solutions. Microperistaltic pumps and a multianode-photomultiplier array were used for fluid control and detection in each of the channels. Immunosensors have also been reported for monitoring multiple antigens using three [74] or four [75] distinct antibody bands in a single plastic capillary.

4.4. Detection

Detectors for microfluidic systems must be capable of providing sensitive measurements in low volumes and small physical dimensions are a significant advantage. Common detection methods for microfluidic immunoaffinity separations include fluorescence and LIF, electrochemical detection, and chemiluminescence [7]. Labeling of the antigen or a secondary antibody is often performed to enhance the detectable signal [7,10]. Enzyme labels have been used in microfluidic immunoaffinity systems with absorbance [48], fluorescence [69,72], and chemiluminescence [66,67] detection. Table 3 lists some representative detection methods for microfluidic immunoaffinity separations.

Immunoaffinity devices involve directly measuring the antibody-bound antigen on the solid support or in a moving liquid after elution occurs. On-chip measurements typically require focusing detectors onto a small, transparent detection zone. Fused-silica capillaries can be made into flow cells by burning off the polyimide coating and connecting to the outlet of chips or capillary columns [28,58]. Square capillaries are available in 50, 75, and 100 μm internal diameters (Polymicro Technologies, Phoenix, AZ) and may be used to construct low-volume flow cells. On-column detection zones for fused-silica capillaries have been created by removing the outer coating [14,59]. When the detection zone is made of packed beads, background scatter may be problematic in optical measurements. Alternatively, offline sample fractions have been collected post-system and measured using absorbance [48] or fluorescence [70] spectrophotometers.

Fluorescence and LIF are the most commonly used methods of detection and can be measured by simple instrumentation including an excitation source, microscope, and photomultiplier tube (PMT) or CCD [7,76,77]. Laser-induced fluorescence is a highly sensitive technique, well suited for analyzing analytes in small volumes of fluid in capillary or chip formats [78]. Diode lasers are compact, stable, and inexpensive [79], and are more compatible with portable microfluidic devices than larger lasers, such as gas lasers [76]. The analysis of biological matrices by visible diode LIF is advantageous because of the minimal biological sample background exhibited in the far-red (>620 nm) spectrum [80]. Since most sample antigens do not fluoresce in the far-red region of the spectrum, derivatization of functional groups with fluorochrome labels is often required [81]. Amine-reactive dyes excitable by lasers may be used to readily label protein antigens, antibodies, or peptides for immunoaffinity separations.

Chemiluminescence detection is increasingly being applied to microfluidic immunoassays and offers the potential for portable field use or point-of-care devices. Light is generated from the chemiluminescent reaction itself and therefore no light sources are required as in fluorescent techniques. In addition to the low background signal, power requirements and external

Table 3
Detection methods used in microfluidic immunoaffinity separations

Detection	Antigen	Matrix	Sensitivity[a]	Ref.
Absorbance	*E. coli* O157:H7	Buffer	5.0×10^2 cfu/mL (LLOQ)	[48]
Fluorescence	C-reactive protein	Human plasma	30 ng/mL	[51]
	C-reactive protein	Buffer	1.4 nM	[63]
	Anti-streptavidin Antibodies	Buffer	0.1 pg/mL (LLOQ)	[72]
Laser-induced fluorescence	Substance P	Unclear	<500 fg/mL	[1]
	Fluorescein	Aqueous	0.1 nM	[58]
	Cholera toxin	Unclear	~210 pM	[38]
	Naproxen	Human plasma or buffer	5 ng/mL	[35]
Chemiluminescence	Myoglobin	Human plasma diluted 1:8 in buffer	1.2 ng/mL	[67]
	Creatine kinase mb	containing 12.5% plasma	0.6 ng/mL	
	Troponin I		5.6 ng/mL	
	Fatty acid-binding protein		4 ng/mL	
	Staphylococcal enferotoxins	Aqueous	0.1 ng/mL	[49]
	C-reactive protein	Human serum	100 ng/mL	[66]
Surface plasmon resonance	Interleukin-8	Buffer	2.5 pM	[30]
	B-type natriuretic peptide	Human saliva	184 pM	
	Phenytoin	Buffer	5 pg/mL (LLOQ)	[83]
		Buffer	75 nM (LLOQ)	[84]
Thermal lens microscope	Carcinoembryonic antigen	Human serum	~0.03 ng/mL	[62]
	Interferon-γ	Buffer	~0.01 ng/mL	[47]
Electrochemical	Human α-fetoprotein	Buffer	0.1 ng	[87]

LLOQ refers to the lowest reported level of calibration.
[a] Sensitivity reported as limit of detection (LOD), unless noted otherwise.

equipment are reduced, making miniaturization more feasible [82]. Bhattacharyya and Klapperich [66] have reported chemiluminescence detection for C-reactive protein in human serum using cyclic polyolefin chips. Secondary antibodies labeled with horseradish peroxidase were exposed to luminol as a substrate in the presence of hydrogen peroxide and the chemiluminscence light was measured on either photographic film or using a commercial imaging device. The assay provided detection limits of 100 ng/mL in serum using the imaging device. Although no quantitative data was given, the use of photographic film shows potential for point-of-care applications by reducing the amount of external equipment required for analysis. The instrumentation needed to measure chemiluminescent signals has also been miniaturized. A photodiode chip was used to measure several cardiac proteins in diluted human plasma based on the reaction of HRP-conjugated secondary antibodies with luminol [67]. A multi-channel immunodetector was reported for a sandwich assay of biological agents using a portable chip system containing a multianode-photomultiplier array to measure the response of HRP-labeled secondary antibodies [49].

Electrochemical detection has also been applied in microfluidics, with amperometric methods reported as the most common [7,77]. The instrumentation used is simple to miniaturize and inexpensive; however, the electrodes may be difficult to control in reduced dimensions and the system may require shielding [7,77,83]. Cyclic voltammetry has been applied to affinity separations using gold electrodes on a polymer substrate [52]. The sensing surface was comprised of gold electrodes modified with immobilized biotin or ferritin as models. HRP-conjugated streptavidin or HRP-conjugated anti-IgG were introduced into the device to form complexes with biotin or anti-ferritin/ferritin, respectively. Adding 4-chloro-1-naphthol to the HRP conjugates resulted in an enzyme-catalyzed precipitation reaction and the insoluble products were measured. Lim and Matsunaga [50] have reported the use of ferrocene-conjugated IgG for a flow immunoassay of human chorionic gonadotropin with electrochemical detection. Antibody–antigen complexes were separated from unreacted ferrocene-labeled antibodies by an inline cation exchange column. Current from the ferrocene label was measured by pumping the samples into a three-electrode flow cell. The authors note that in addition to the simplicity of the assay, the time of analysis is reduced versus other electrochemical immunoassays because no enzyme-catalyzed reactions were necessary.

Surface plasmon resonance (SPR) detectors for immunoaffinity separations offer an alternative technology that is well-suited for detection on microfluidic platforms. SPR measurements are based upon changes in optical properties on the surface of chips coated with immobilized ligands, such as antibodies. The binding of associated antigens and formation of an immunocomplex causes changes in the refractive index and shifts the SPR angle [30]. SPR is a fast, simple technique and does not require the use of labeled reagents [83,84]. A commercial SPR system was used to perform a sandwich immunoassay of interleukin-8 (IL-8) in human saliva in microfluidic channels [30]. A capture antibody immobilized on the surface of the sensor chip was exposed to the antigen and secondary antibody, sequentially. A detection limit of 184 pM was reported for IL-8 in centrifuged saliva. To reduce nonspecific interactions on the carboxymethyl dextran surface of the sensor, carboxymethyl dextran sodium salt

was added to samples prior to analysis. Nonspecific adsorption of biomolecules to the sensor surface in SPR is problematic since the technique responds to refractive index changes produced by specific or nonspecific binding [83]. Kurita et al. [83] have reported that heating of human serum was necessary to reduce nonspecific interference with an SPR immunoassay for B-type natriuretic peptide (BNP). Antibodies labeled with acetylcholine esterase were exposed to acetylthiocholine, which produced thiocholine. The thiocholine was then pumped to a gold film sensor for measurement. Plasma diluted 1:10 contained pseudocholinesterase and caused large amounts of thiocholine production. Heat treatment of the plasma inactivated the esterase and allowed recovery of BNP from 10 pg/mL to 100 ng/mL in diluted plasma.

Other detection methods have been used for microfluidic immunoaffinity separations. Thermal lens microscopy (TLM), which is a technique based on detection of heat generated by the absorption of light [85], has been reported for chip-based immunoassays. Colloidal gold has been used as a label for TLM to detect human IgA [57], carcinoembryonic antigen [62], and interferon [47] in polystyrene bead-packed channels. The use of mass spectrometry (MS) has been reported for micro-immunoaffinity chromatography of various amino acids [86]. Stereo-selective antibodies were immobilized to silica beads packed in PEEK columns and used for isocratic chiral separations. Direct analysis of antigens in immunoaffinity chromatography commonly involves non-volatile buffers, such as phosphate, which are not compatible with MS analysis. An ammonium bicarbonate buffer was used that had the advantages of neutral buffering capacity for antibody–antigen reactions and volatility for MS detection.

5. Conclusions

Microfluidic devices are capable of performing a wide range of analytical functions with low reagent and sample requirements. Miniaturization of analytical instrumentation towards portable platforms is beneficial for point-of-care testing or other field applications. The combination of immunoaffinity separation techniques with microfluidic systems offers selective measurement of target analytes based on antibody–antigen interactions. Specific compounds of interest may be isolated from limited sample sources and immunoaffinity concentration facilitates measurement of trace levels.

Research in clinical and biomedical fields commonly requires analysis of complex biological matrices. Directly introducing biological material in to a microfluidic system may result in unwanted nonspecific binding of proteins or other compounds. Much of the published work reviewed here has been conducted in aqueous systems and may not be easily transferable to real biologic samples. Sample pretreatment, buffer additives, and modifications of device surfaces are frequently required to make biological samples compatible with small-scale instrumentation. Some published works have demonstrated application to real samples by analyte quantification in diluted real matrix. The corresponding effect on the lower limit of quantification in undiluted samples should be understood by those who attempt to reproduce this work.

Microfluidic chips and capillary systems using immunoaffinity separations range from simple to elegant in design. Many of these systems are based on pressure-driven fluid control and are assembled from several external miniaturized and conventional scale components, including components for sample introduction and detection. Compared to immunoassays performed in microplates, microfluidic immunoaffinity devices provide the potential for faster analysis times and reduced sample size.

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