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Review

Combining Electrochemical Sensors with Miniaturized Sample Preparation for Rapid Detection in Clinical Samples

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Abstract: Clinical analyses benefit world-wide from rapid and reliable diagnostics tests. New tests are sought with greatest demand not only for new analytes, but also to reduce costs, complexity and lengthy analysis times of current techniques. Among the myriad of possibilities available today to develop new test systems, amperometric biosensors are prominent players—best represented by the ubiquitous amperometric-based glucose sensors. Electrochemical approaches in general require little and often enough only simple hardware components, are rugged and yet provide low limits of detection. They thus offer many of the desirable attributes for point-of-care/point-of-need tests. This review focuses on investigating the important integration of sample preparation with (primarily electrochemical) biosensors. Sample clean up requirements, miniaturized sample preparation strategies, and their potential integration with sensors will be discussed, focusing on clinical sample analyses.

Keywords: electrochemical sensor; microfluidic-based sample preparation; clinical sample analysis

1. Introduction to Biosensor

Since the first biosensors were proposed and demonstrated by Clark and Lyons in 1962 [1], the idea behind biosensors has been explored in a wealth of variations and has been defined with specific criteria by international union of pure and applied chemistry (IUPAC) in 1999 [2]. The exquisite specificity and sensitivity of biological recognition elements including antibodies [3], oligonucleotides [4], enzymes [5], and cell receptors [6] transduced through physical and chemical strategies that are not limited to electrochemical, optical or mass-based means has led to amazing analytical systems. The electrochemical glucose biosensor based on Clark's original concept is the best known, likely best studied, and surely commercially most successful biosensor to date [7,8]. As much as new sensing systems are being developed today, effort is also put toward the important aspect of integration of the detection system with an efficient and appropriate sample preparation strategy to deal with actual real-world samples. Here, great expectations are put toward miniaturized "total analysis systems" (microTAS) that hold the promise of integrating sample preparation and biosensing in one small chip, creating a portable device.

Electrochemical biosensors lend themselves well to clinical analysis as demonstrated exemplary by successful glucose sensors, the iStat, and other chemical sensors for blood gas and ion analysis [9,10]. The low-tech hardware requirements and high sensitivity are two major advantages that lead to the abundance of electrochemical biosensors. Transduction principles seen in clinical analysis include primarily amperometry, cyclic voltammetry, and differential pulse voltammetry. In addition to these electrochemical sensors, clearly no shortage of detection principles and assay formats exists ranging from optical, to mass-based, and piezoelectric formats [11], each providing unique aspects that are advantageous for specific settings, relating to limits of detection, ease-of-use, costs, assay time and alike.

The range of analytes relevant in clinical diagnostics that have been addressed by biosensors and bioanalytical systems (not limited to electrochemical transduction) is staggering [12], including cancer, genome analysis, autoimmune diseases, infectious diseases, and cardiac biomarkers. In the case of infectious disease applications, monitoring and diagnostics of pathogenic microorganisms has been described for a long list of analytes (Table 1) also including those analytes that are relevant to the food industry, water, and environmental applications [13]. Maybe not surprisingly, the typical common challenge of biosensors that are designed for application to real-world samples is the matrix of the specimens, which may likely interfere with the results or negatively affect the detection principle of the assay. In the case of clinical specimens, such as blood (whole blood, serum, or plasma), urine, saliva, stool, sputum, and tissue, this challenge of sample preparation for diagnostics has been described by J. Liao and his group recently [14]. How miniaturized biosensors solve these challenges will be addressed further along in this article.

Table 1. Summary of pathogenic organisms relevant to clinical diagnostics for which biosensors have been developed.

Virus	**Bacteria**	**Fungi**
VariolaV [15,16]	*Rickettsia prowazecki* [15,16]	*Coccidioidesimmitis* [15,16]
ChikungunyaV [15,16]	*Rickettsia rickettsi* [15,16,19]	*Histoplasmacapsulatum* [15,16]
Eastern encephalitis V [15,16]	*Rickettsia tsutsugamushi* [15,16]	*Nocardiaasteroides* [15,16]
Venezuelan encephalitis V [15,16]	*Bacillus anthracis* [15,16,19]	
Western encephalitis V [15,16]	*Francisella (Pasteurella)tularensis*	
Dengue V [15,16]	[15,16,19]	
Yellow fever V [15,16]	*Pasteurellapestis* [15,16]	
Japanese encephalitis V [15,16]	*Brucellamelitensis,*	
Russian spring-summer encephalitis V	*B. suis* [15,16,19]	
[15,16]	*Coxiellaburnetti* [15,16,19]	
Argentine hemorrhagic fever V [15,16]	*Salmonella typhi* [15–17,19]	
Lassa fever V [15,16]	*Salmonella paratyphi* [15,16,19]	
Lymphocyte choriomeningitis V [15,16]	*Salmonella enteric* [17]	
Bolivian hemorrhagic fever V [15,16]	*Shigelladysenteriae* [19].	
Crimean-Congo hemorrhagic fever V	*Vibrio cholerae* [15–17,19]	
[15,16]	*Corynebacterium diphtheria*	
Haantan (Korean hemorrhagic fever) V	[15,16,19]	
[15,16]	*Actinobacillus mallei* [15,16]	
Rift Valley fever V [15,16]	*Pseudomonas aeruginosa* [17]	
Marburg V [15,16]	*Pseudomonas pseudomallei* [15,16]	
Ebola V [15,16]	*Mycobacterium tuberculosis*	
Hepatitis (A, E) V [15–17]	[15,16,19]	
Norwalk V [18]	*Burkholderiapseudomallei* [17]	
	Campylobacter jejuni [17,19]	
	Clostridium botulinum [19]	
	Escherichia coli-pathogenic [17,19]	
	E. coli O157: H7 [18]	
	Legionella spp. [17]	
	Yersinia enterocolitica [17]	
	Yersinia pestis [19]	
	Treponemapallidum [19]	
	Streptococcus pneumonia [19]	
	Staphylococcus aureus [19]	
	Listeria monocytogenes [18]	

2. Pairing (Electrochemical) Biosensors with Sample Preparation for Analyte Detection in Clinical Samples

Significant effort has to be invested in the design of a biosensor so that it can be applied to actual real-world samples. It is well known and often described how matrix effects, non-specific binding and interferences will negatively affect a biosensor signal to the point that no qualitative or quantitative analysis is possible. Sensor surfaces are therefore typically protected via membranes, films or simple blocking layers of adsorbed molecules in order to prevent any of these interferences. Examples are the

polyethylene glycol modified membrane of glucose sensors that prevent components such as ascorbic acid and uric acid to reach the electrode surface and hence render the electrochemical transduction specific [20,21]. Also, in heterogeneous immunoassays, surfaces are blocked with polymers or proteins, such as polyvinylpyrrolidone [22,23], gelatin [22,24] casein [25,26], or bovine serum albumin [27,28], respectively. Hydrogels or similar films are often applied to not only immobilize the biorecognition element but also function as diffusion barrier for interferences from the matrix [29,30].

Table 2. Important criteria for sample preparation processes considerations for the development of electrochemical (micro) sensors.

Criteria Specific to Electrochemical Sensors	Examples	Additional Important Criteria and Those Specific to Microfluidic Electrochemical Sensors	Examples
Removal of electrochemically active compounds	*In serum/plasma* [31]: - Uric acid - Ascorbic acid - Dopamine - L-cysteine - Acetaminophen - Salicylic acid *In urine* [32]: - Urea - Tartaric acid - Citric acid - Glucose - Leucine - Proline - Tyrosine *In saliva* [33]: - Uric acid - Ascorbic acid	Removal of particulate to avoid clogging of microchannels and microvalves [34]	Blood cells may form aggregates clogging the microchannels during separation of plasma from blood [35]
Adjustment of ionic strength and temperature [36,37]	- Variable ionic strength influence potentiometric, conductimetric and also voltammetric measurements. In addition, ionic strength and nature affects biological reactions [36] - Temperature affects the slope of the electrode response according to the Nernst equation [37]	Reducing non specific absorption of hydrophobic material such as PDMS [38]	Adsorption of fluorescence markers can cause a drift in the background fluorescence intensity [38]
Removal of surface fouling compounds [39]	Fouling cause by plasma proteins, lipids, and other biochemical components of the biological fluids [39]	Removal of compounds interfering with the biorecognition or signal amplification mechanisms [40]	PCR inhibitors in blood sample such as heme, hemoglobin, lactoferrin and immunoglobulin G [40]
Adjustment of pH [41]	A pH buffer can be used to reduce hydroxyl ion (OH^-) effects that interfere ISE electrodes [41]	Adjustment of pH [42]	Surface charge (Zeta potential) of the microchannels' walls is generally a function of the pH thus, the electroosmotic pumping process can be enhanced or degraded by changes in pH [42]

However, coatings and blocking strategies cannot circumvent all negative sample matrix effects, including fouling of surfaces, interference with biorecognition reactions, clogging of fluid channels, *etc*., and sample preparation is hence of imminent importance. Different criteria apply for different transduction principle in order to avoid matrix-effects. For example, turbidity is a common problem for optical sensors, auto-fluorescence for any fluorescence-based system, non-specific adherence of any particle is a challenge for mass-based systems, and the avoidance of electrochemically active compounds is mandatory for electrochemical sensors. In Table 2 specific criteria for sample preparation processes are listed as they relate to applications of clinical sample analyses with electrochemical sensors and those when used in microfluidic systems.

The most often applied sample preparation steps are summarized in Figure 1. Whenever possible, the sample is being diluted in order to shift the effect of interferences below a tolerable threshold, *i.e*., when blocking and protecting functionalities of the biosensor design can be effective against undesired matrix components. This has been demonstrated, for example with glucose analyzers, such as those developed by Yellow Springs Instrument Company (Yellow Springs, OH, USA). Glucose oxidase is immobilized between two membrane layers. The outer polycarbonate membrane retains the enzyme, allows glucose to pass, but prevents larger molecules from entering, thus reducing interferences. The inner membrane is gas selective and necessary for the selectivity of the sensor [43]. Another example is the multilayered membranes developed by Matsumoto *et al*., which are able to measure glucose concentrations in a high enough range so that no sample dilution is required. Furthermore, the sensor provides a rapid response, a wide measuring range, and practical immunity to interference species (ascorbic acid, uric acid, and p-acetaminophen) [44,45]. However dilution or thick protective layers are obviously only applicable, if the analyte is present at high enough concentrations. Instead, other, frequently used simple sample preparation procedures include centrifugation, filtration, precipitation and deproteinization.

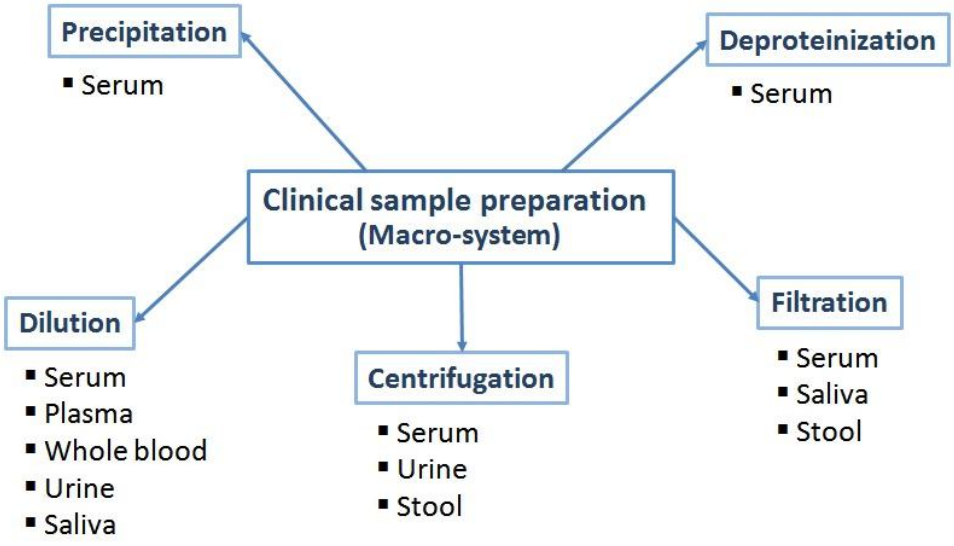

Figure 1. Summary of the most often applied macro-system sample preparation procedures for clinical samples.

Blood as clinical sample has the advantage that it is the most rich with respect to variety of relevant analytes, yet also has the disadvantage to be the most rich with respect to matrix complexity and viscosity [14]. It can be divided into three types of specimen for each of which many amperometric biosensors have been presented, *i.e.*, whole blood [46,47], serum [48–58], and plasma [59–61]. For whole blood and plasma, dilution is the most frequently used sample preparation step and was, for example used for the analysis of Zn^{2+} [46], neuropathy target esterase [47], glucose [20], pyrazinamide [59], prostate specific antigen [60], and nitrite/nitrate [61].

Similarly, also for serum samples, dilution is the most often utilized technique and is combined with additional processing steps, such as centrifugation for dopamine [48], uric acid [48], glucose [49], and immunoglobulin A [62] analysis; precipitation for dopamine [53] and biphenyl [58] analysis; deproteinization with acid and filtration for glucose [55] analysis. It is important to keep in mind, though, that in some instances, especially in single-use devices, biosensors are described that can deal with the complex blood matrix without sample pretreatment step such as shown for glucose where Nafion membranes are known to cut down the most prevalent interferences such as ascorbic and uric acid [63] and nucleic acids (miRNAs) [64].

In the case of urine samples, the wide range of pH values found in samples can be challenging [14]. In addition to pH adjustment, centrifugation and dilution are two of the most often used sample preparation techniques as described for analytes, such as pirazinamide [58], anti-malarial drug (Artesunate) [65], testosterone [66], homocysteine [67], nuclear matrix protein 22 [68], dopamine [69], and uric acid [70–72].

Similar to blood, saliva samples suffer from an immense component complexity and variation of compositions. Here, filtration and dilution methods are for example utilized for lactate [73] and nitrite/nitrate [62] analysis, respectively.

Challenges associated with stool samples are most prominently similar to those of other solid materials such as soil, and solid food samples, but also the presence of high concentrations of bile. Centrifugation or filtration is typically a must in order to remove particulates, especially when considering microfluidic sensor developments [74].

3. Recent Strategies of Miniaturized Sample Preparation and Their Comparison to Bench-Top Standards

When miniaturizing biosensors for clinical analysis, requirements for and necessity of analyte isolation from the sample matrix remain of utmost importance, in fact, additional challenges are added (Table 2). Microfluidic-based sample preparation can be classified into two groups (Figure 2). Most simply put, microtechniques are developed that copy one-to-one those techniques found in the macro-system, alternatively micro-phenomena are exploited to produce the same sample preparation result. The comparison of microtechniques with corresponding bench-top strategies (Table 3) can be done either by directly comparing performance characteristics or by comparing final limits of detection reported for the respective target or model analytes. In some cases, this comparison is straight forward based on published data, in other cases this is more challenging due to limited data available. This section provides a few case studies for these important comparative evaluations.

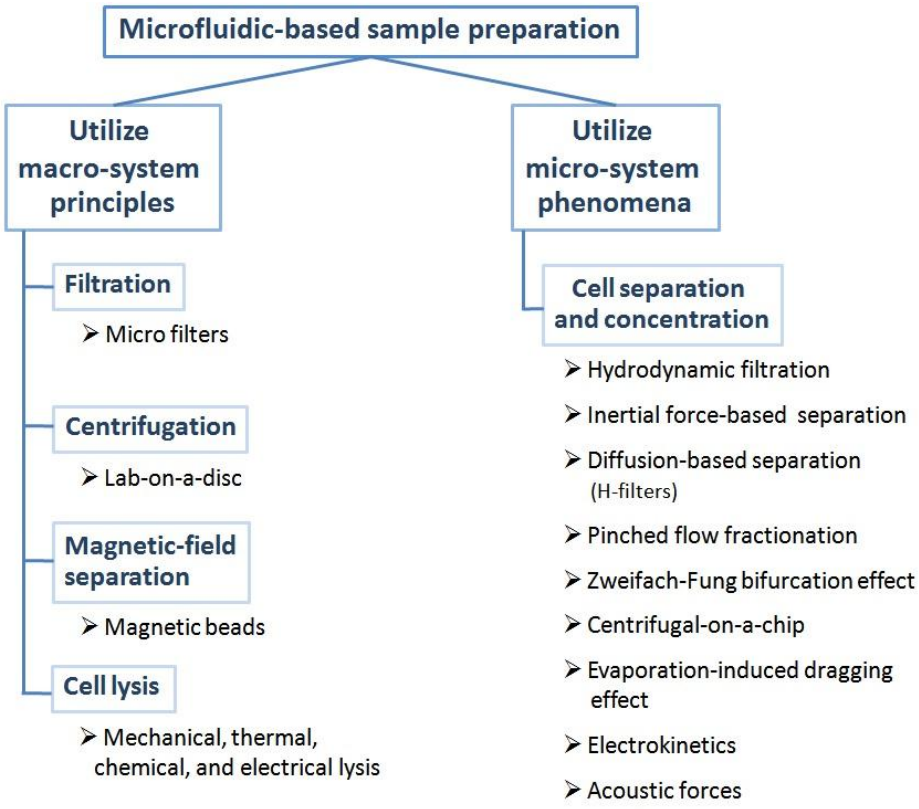

Figure 2. Summary of microfluidic-based sample preparation techniques that are classified into two groups: (1) those obtained by scaling down a macro-system and (2) utilization of micro-system phenomena.

Table 3. Comparison between micro techniques to corresponding bench-top methods for sample preparation based on published data.

On-Chip Sample Preparation Techniques	Bench-Top Methods	Comparison Result of On-Chip To the Bench-Top Method	References
Microfilter membrane (Paper-based)	Centrifugation	Comparable	[75]
Microfilter membrane (Parylene)	Immunomagnetic separation	Better	[76]
Magnetic bead-based separation	ELISA	Comparable	[77]
Lab-on-a-disc	ELISA	Comparable	[78]
Miniaturized bead-beating	In-tube bead-beating	Comparable	[40]
Inertial force-based	Flow cytometry	Comparable	[79,80]
Dielectrophoresis	Centrifugation	Comparable (for purity)	[81]
Zweifach-Fung bifurcation	Centrifugation	Worse	[82]
Pinched- flow fractionation	Centrifugation	Worse	[83]
Acoustic force-based	Centrifugation	Better	[84]
Diffusion-based (H-filter)	Centrifugation	Comparable	[85]

With respect to microfluidic techniques that utilizing macro-principles, filtration is an excellent example, such as the filtration of red blood cell agglutination complexes via paper-based microfluidics in order to detect the target analyte present in the plasma [75]. Microfilters [86–88] have also been developed as the straightforward method for cell separation in micro-system. Alternatively, centrifugation has been realized using lab-on-a-disc for the separation of target cells [78]. Similarly, magnetic field separation is realized in micro-systems by bead-based analyte capture integrated with microfluidic systems [89,90]. Cell lysis techniques used in the macro-system have also been realized in micro devices, such as mechanical [40,91], thermal [92–94], chemical [95], and electrical lysis [96]. All of these techniques can reduce the volume of sample/reagent, which is the main advantage of the scaling down devices while keeping the scientific principle of the sample preparation step the same.

Comparing their efficiency to standard bench-top methods has been described by some researchers. An excellent example is the use of microfilter membranes for cell separation or concentration in microdevices. Yang *et al*. [75] developed a paper-based microfilter membrane for the separation of plasma from whole blood with the purpose of plasma glucose determination using a glucose oxidase-based colorimetric assay. The researchers compared this sample preparation technique with the conventional centrifugation method (800 rcf, 15 min) and found a good correlation of the results for both techniques. Similarly, parylene microfilter membranes, which were developed by Lin *et al*. [76], were applied to the identification of circulating tumor cells (CTCs) in whole blood. This system was shown to achieve more than 90% recovery and in fact showed better CTC identification when compared with CellSearch, a bench-top immunomagnetic separation technique.

Also for magnetic bead and centrifugal force principles, the scaling down resulted in comparable results. For example, a magnetic bead-based proximity ligation assay was developed in which magnetic field-enhanced separation of the target analyte from human plasma was performed [77]. The detection range of this micro-system was found to be at 5–100 pg/mL. This compared well with respect to the limit of detection of a bench-top ELISA (2.2–50,000 pg/mL) for TNF-quantification, but fell short with respect to the dynamic range achievable. Lee *et al*. [78] developed a disc-based assay for anti-HBs and HBsAg from whole blood utilizing centrifugal forces for fluid movements. Their "Lab-on-a-disc" technique demonstrated comparable limits of detection to a bench-top ELISA for both analytes.

As final example, cell lysis [40] using a miniaturized magnetically actuated bead-beating system was compared to the standard in-tube bead beating lysis method. In both cases, centrifugation and RT-PCR followed the initial lysis step for the detection of respiratory pathogens in nasopharyngeal aspirates. No difference in lysis efficiency was found between the micro- and macro systems.

The second strategy to realize sample preparation in a miniaturized system takes advantage of phenomena unique to microfluidic systems or utilizes those that are very easy to realize in the micro-world in comparison to the macro-system. For example, cell separation and concentration can be accomplished using hydrodynamic phenomena, such as the Zweifach-Fung bifurcation effect [97,98], inertial force-based cell separation [99–102], centrifugal-on-a-chip (Figure 3) [103], evaporation-induced dragging effect [104], hydrodynamic filtration [105,106], pinched flow fractionation [107,108], and diffusion-based cell separation by using H-filters [109]. Cell separation has also been demonstrated using active separation techniques such as electrokinetic strategies [110–113] and acoustic forces [84,114].

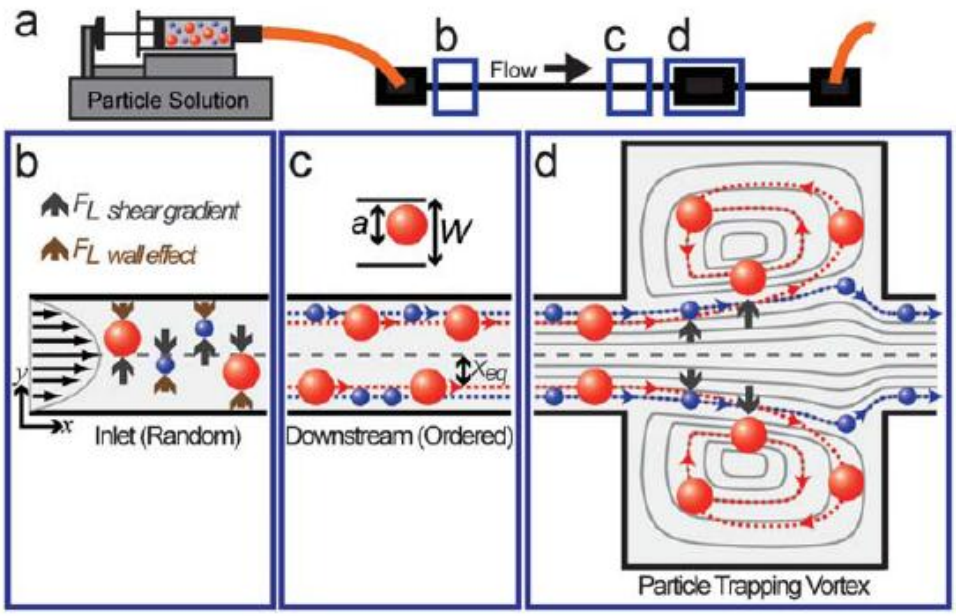

Figure 3. Particle entry mechanism in laminar microvortices. (**a**) For a polydisperse particle solution injected into a device with a straight high aspect ratio channel leading into an expansion-contraction chamber we expect size-dependent entry into the laminar vortices created; (**b**, **c**) Particles are subjected to a shear gradient lift force, which directs particles toward the channel wall, and a wall effect lift force, directed toward the channel center, which leads to entrainment of particles at dynamic equilibrium positions, X_{eq}; (**d**) As focused particles enter the vortex chamber, the lift forces are decoupled due to the absence of a nearby wall, resulting in a dominate shear gradient lift force. Larger particles (red) experience larger lift forces and are able to migrate across fluid streamlines into the vortices while smaller particles (blue) follow fluid streamlines and flow out of the system [103] with permission of The Royal Society of Chemistry.

From a microfluidic device development point of view, the use of "microfluidic phenomena" comparability of results is very important, as completely new parameters are applied in bench-top and microsystems. Following are a few interesting studies reported. For example, for the separation of cancer cells from whole blood, an inertial force-based method was developed [79] and compared with flow cytometry. The microdevice showed superb cancer cell recovery rates in whole blood of 99.1%, blood cell rejection ratio of 88.9%, and a throughput of 1.1×10^8 cells/min which is comparable to the commercial flow cytometry systems' achieved throughput (~2.4 million cells/min) [115]. The same inertial force-based technique was also applied for neural cell separation from cell culture medium [80]. Here, a throughput of ~1 million cells/min was found to be comparable to the commercial macroscale flow cytometer with an 80% efficiency and high relative viability (>90%).

When comparing dielectrophoresis with macro-system centrifugation for blood plasma separation [81], plasma yield of 15.6% ± 2.5% and purity efficiency of 94.2% ± 3.6% were found for dielectrophoresis and plasma yield of 95% and purity efficiency of 99% were found for the centrifugation technique. Blood plasma separation by other microfluidic-based methods was also studied. Plasma yield of 40% and purity efficiency of 53% were found for the development of blood plasma separation by using the

Zweifach-Fung effect [82] and 80% of erythrocyte separation efficiency was found for the development of a Pinched-flow fractionation [83] microdevice. In other cases, lipid particle separation from blood was investigated which are relevant for intra-operative blood wash applications [84]. Here, Petersson *et al*. utilized an acoustic force-based technique and removed more than 80% of the lipid particles from the blood while collecting ~70% of the erythrocytes (recovery). The researchers discussed the quality of the separation to be excellent and additionally avoid standard problems of macroscale wash steps based on centrifugation including hemolysis, discontinuity, and a demand for large volumes (~500 mL) of blood.

The Yager research group [85] developed an H-filter diffusion-based technique for the separation of small molecular analytes (Phenytoin, 252 Da) from saliva samples. The H-filters were comparable to centrifugal techniques [85,116], which were used to extract the analyte from the remaining large molecular weight species in the filtered saliva sample. Specifically, the H-filter processed saliva sample retained 23% of the analyte with 97% and 92% reduction in glycoproteins and proteins, respectively. Furthermore, subsequent detection processes were improved as the H-filter processed sample caused significantly less fouling of biosensor surfaces.

Gillers *et al*. [117] developed microfluidic-based DNA extraction from crude stool samples prior to PCR amplification. While no direct comparison to the bench-top DNA extraction method was provided, the authors could demonstrate that their on-chip method resulted in extract purity suitable for subsequent PCR.

4. Conclusions

Bioanalytical sensors and miniaturized sample preparation strategies have been described and successfully applied to a variety of clinical samples. We conclude that the combination of several of the miniaturized sample preparation assays are ideally suited for the integration with electrochemical detection strategies. For example, the above-described acoustic force-based technique used for the separation of lipid particles [84] can easily be combined with a simple miniaturized amperometric detection strategy [74]. Here, electrochemical sensors such as those using nanomaterials integrated with the screen-printed electrodes (SPE) surface for cardiac biomarkers [118,119] will benefit from such a sample preparation step as electrode fouling through lipid particles will be avoided. Similarly, the dielectrophoretic generation of plasma from blood samples [81] would mean that plasma tests performed for human health diagnosis and treatment can be performed by simply applying the finger tip's whole blood sample onto the microfluidic device and waiting for the results (sample-to-answer concept) [120]. In addition, saliva samples can be prepared and analyzed within microdevices for the detection of antibodies to HIV, therapeutic drugs and steroids [121] if an H-filter diffusion-based separation technique is directly integrated on chip.

Assay systems like these can overcome the greatest shortcoming of today's bioanalytical detection systems and be developed into commercially viable diagnostic tests. They will be effective, simple and rugged self-contained assays for point-of-care and point-of-need testing that on the one hand integrate innovative and novel concepts and on the other hand rely on well-established concepts that can be trusted for clinical diagnostics.

Abbreviations

CTCs	circulating tumor cells
ELISA	enzyme-linked immunosorbent assay
IUPAC	international union of pure and applied chemistry
microTAS	micro total analysis systems
RT-PCR	reverse transcription polymerase chain reaction
SPE	screen-printed electrodes
TNF	tumor necrosis factors

Conflicts of Interest

The authors declare no conflict of interest.

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