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REVIEW

Chemiluminescence microarrays in analytical chemistry: a critical review

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Abstract Multi-analyte immunoassays on microarrays and on multiplex DNA microarrays have been described for quantitative analysis of small organic molecules (e.g., antibiotics, drugs of abuse, small molecule toxins), proteins (e.g., antibodies or protein toxins), and microorganisms, viruses, and eukaryotic cells. In analytical chemistry, multi-analyte detection by use of analytical microarrays has become an innovative research topic because of the possibility of generating several sets of quantitative data for different analyte classes in a short time. Chemiluminescence (CL) microarrays are powerful tools for rapid multiplex analysis of complex matrices. A wide range of applications for CL microarrays is described in the literature dealing with analytical microarrays. The motivation for this review is to summarize the current state of CL-based analytical microarrays. Combining analysis of different compound classes on CL microarrays reduces analysis time, cost of reagents, and use of laboratory space. Applications are discussed, with examples from food safety, water safety, environmental monitoring, diagnostics, forensics, toxicology, and biosecurity. The potential and limitations of research on multiplex analysis by use of CL microarrays are discussed in this review.

Keywords Chemiluminescence · DNA microarrays · Antibody microarrays · Multiplexed bioassays · Rapid methods · Food and water safety

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Introduction

Microarray technology is an emerging field of analytical chemistry and a powerful analytical tool for simultaneous detection of several analytes in a single sample. A microarray consists of a matrix of reactive spots on a supporting material. (Bio)chemically selective receptors (e.g., antibodies, DNA, aptamers, lectins, membrane receptors, enzymes, cells, or molecularly imprinted polymers) can be deposited by microdispensing and can be immobilized on a surface by physical or chemical interactions. The spots generated have diameters in the micrometer range. Hundreds or more (bio)analytical reactions can be performed on an area of one square centimeter. The reaction on each spot is traceable by knowing the position of each selective receptor. Multi-analysis by use of analytical microarrays has become an innovative research topic in analytical chemistry, because of the possibility of generating several sets of quantitative data for different classes of analyte in a short time. Applications described in the literature deal with pharmaceuticals, drugs of abuse, toxins, allergens, proteins, and (pathogenic) microorganisms and viruses. Multi-analyte quantitative methods are important if a group of analytes must be quantified. For many different analytes, critical levels in food and water safety are defined. Forensics, diagnostics, and biosecurity are other fields in analytical chemistry in which multiplex analysis is valuable. Small organic molecules, proteins, microorganisms, and viruses can be quantified by use of microarray immunoassays (MIAs). Nucleic acids of microorganisms, viruses, or eukaryotic cells can be analyzed by use of nucleic acid amplification tests (NATs). For multiplex measurement, hybridization assays on DNA microarrays can be performed. The principle of the test, i.e. combined nucleic acid amplification and DNA microarray analysis can be abbreviated to NAMA (nucleic acid amplification and microarray analysis).

Analytical microarrays have been refined in different ways in the last 20 years. Static-incubated segmented microarrays and flow-injection-based microarrays have been investigated. Techniques are available for automated processing of analytical microarrays [1]. Analytical microarrays can be differentiated by the readout system used, and fluorescence [2], electrochemical [3, 4], chemiluminescence (CL) [5–8], or label-free microarray readout systems [9–11] have been used. This review focuses on CL microarrays. CL is based on generation of photons by a chemical reaction. Photons produced on each spot of the microarray are detected with appropriate spatial resolution by use of optoelectronic imaging sensors.

As depicted in Fig. 1, microarray technology covers the entire process used for quantitative analysis by use of CL microarrays, including microarray manufacturing, measurement techniques for microarray readout, establishment of multi-analyte assays, and processing of several sets of quantitative data.

Establishment of CL-based analytical microarrays has involved research in engineering, analytical chemistry, and biology. Current research on multi-analyte applications using CL microarrays is reviewed. Applications in a variety of end-user industry is envisaged, for example, use by veterinarians, physicians, food chemists, pharmacologists, toxicologists, quality-control inspectors, in the pharmaceutical, food, and water industries, and in diagnostic applications.

Detection principle for CL microarrays

The generation of photons by chemical reaction is called chemiluminescence (CL). A metastable intermediate is produced by the reaction and CL molecules are chemically excited. On return to the ground state, some of the chemical energy is converted to electromagnetic energy. The reaction can be initiated by enzymes or purely chemical [12]. The intensity of CL (I_{CL}) emission depends on the rate of the chemical reaction and on the overall efficiency of the CL reaction (Φ_{CL}) [13]. The direct proportionality of I_{CL} to the concentration of a limiting reactant is used for analytical purposes. CL microarrays use a CL reaction that is localized on each spot of the microarray [14]. A limiting reagent (the "label") on each spot of the CL microarray produces a CL signal that correlates quantitatively with the amount of label. Analytes can be quantified after calibration with multiplex standards. The CL readout process is illustrated in Fig. 2 as an example of flow-based microarrays.

A limited number of CL microarrays using alkaline phosphatases, luciferase, or β-galactosidase as alternative CL labels for CL microarrays have been described in the literature [7]. Horseradish peroxidase (HRP) is the most widely used enzyme [15]. HRP-conjugated receptors, for example antibodies or streptavidin, bind specifically on each immobilized antigen spot of the CL microarray. The CL substrate luminol reacts with H_2O_2 and hydroxide ions in the presence of HRP as catalyst. Luminol reagents and hydrogen peroxide are mixed before the microarray is incubated for CL measurement. The light emitted by the unstable CL product is related to the diffusion coefficient and CL occurs when HRP is very close to the enzyme label. To obtain sharp, rounded microarray spots the liquid must remain stationary during the measurement. Light is emitted at 425 nm and is imaged over a defined decay time between 1 s and some minutes by use of a digital imaging system, for example a CCD camera. The chemical reaction yields 6-aminophthalate (6-APA) in a singlet excited state that decays to the ground state by emission of photons. The overall quantum yield of the CL reaction is between 0.001 and 0.1 [16] and can be increased by use of luminol analogs and enhancers, for example *p*-iodophenol (PIP) [17], 4-(1-imidazolyl)phenol [18], or other *p*-phenol derivatives [19]. The compounds *p*-phenylphenol and sodium tetraphenyl borate are used as synergistic enhancers [20], and $K_3Fe(CN)_6$ is used as an electron mediator [21]. Costly commercial CL reagent kits are available with defined mixtures of enhancers and stabilizers. Compared with other microarray readout principles, the highest measurement cost is that of the CL reagent. The sensitivity of an HRP-based CL reaction is increased by higher temperatures [22]. Multi-HRP labels are more effective initiators [23, 24]. Rough gold surfaces prepared by pulsed-laser deposition enhance the HRP-dependent CL reaction as a result of optical and catalytic effects [25, 26]. High-reproducibility CL-based multi-analyte methods can be achieved only by use of an invariant CL reaction. Stabilizers are therefore added so the CL reagent can be stored at room temperature in dark vessels for a full working day without loss of CL efficiency (Φ_{CL}) [27]. Reproducibility depends on

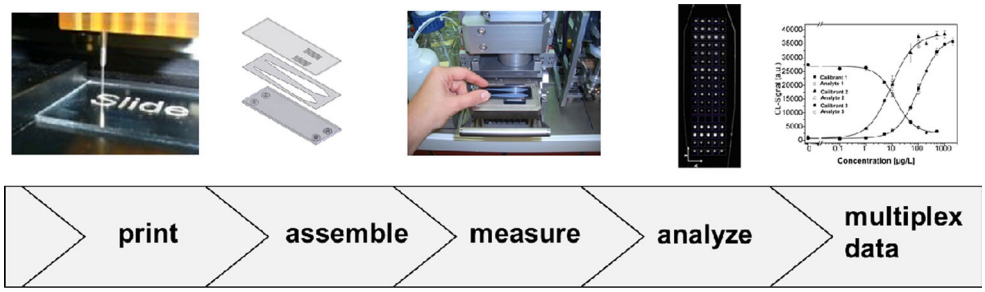

Fig. 1 The procedure for analytical microarrays includes microarray manufacture, measurement techniques for microarray readout, establishment of multi-analyte assays, and processing of multiple sets of quantitative data

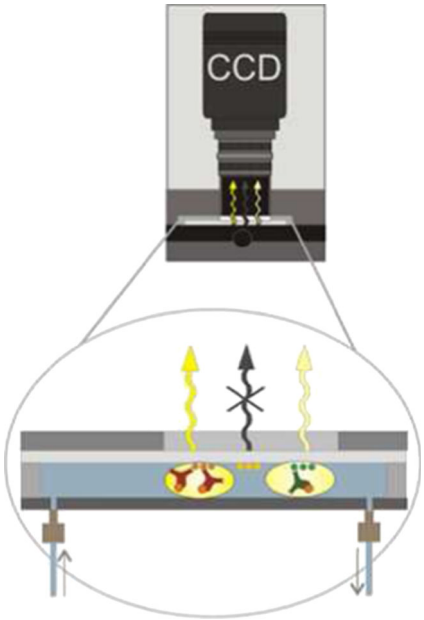

Fig. 2 Principle of CL imaging by measurement of a localized HRP-catalyzed reaction by use of a CCD camera

enzyme activity. Constant temperatures on the microarray chip are therefore necessary for constant enzyme activity and reproducible CL signals. This is especially important if portable measurement systems are developed for use in the field.

Two-dimensional imaging systems for CL microarrays are usually based on CCD camera observation [28, 72]. The sensitivity of these microarrays depends on reaction time, surface chemistry, the sensor chip of the digital camera used, and the construction of the microarray readout system. As for any enzyme reaction, product formation depends on reaction time. If the background signal is zero, greater sensitivity can be achieved by use of a longer incubation time, because of increased light integration by the CCD camera. However, longer integration times increase the background signal caused by dark current noise. Cooled CCD cameras with low dark current are therefore needed. Construction of the CL microarray readout system must be optimized to minimize the number of photons entering the CCD chip from outside. Optical lenses are used to achieve high spatial resolution. However, the efficiency of collection of the photons is limited by optics.

Rapid multiplex on-site analysis for patients (point-of-care diagnostics), suspect persons (forensics), or for quality control of food and water are important research topics in analytical chemistry. Low-cost and miniaturized readout systems are needed with portable and inexpensive instrumentation. The possibility of constructing simple optics for a CL microarray readout encourages research on alternative CL imaging systems. A lensless imaging system for CCD and CMOS sensors has been investigated [29]. A fiber optic taper was placed in contact with the CCD camera, resulting in efficient light collection. The differences between CCD-based lensless imaging and conventional imaging were analyzed on the basis of the detection limit of HRP in solution; LODs were 2.3 and 5.7 pmol L^{-1}, respectively. The sensitivity was lower, LOD 28 pmol L^{-1}, for the CMOS sensor in the lensless configuration. A photodiode array with 32 photodiodes on a 4×8 grid was investigated for an integrated CL microarray readout [30]. Small portable instruments can be built by integration of the readout system on CL microarrays. Limitations are the flexibility of multi-analyte assay design and the cost of disposable components of the microarray chip. Organic photodiodes (OPDs) are a solution for cheap readout layers for hand-held CL-microarray instrumentation [31].

Microarray technology for CL microarray chips

Microarrays are produced by using microdispensing devices to deposit small volumes in the range between picoliters and nanoliters, creating a matrix of spots on a micrometer scale [32]. Besides many other microdispensing principles, contact and ink-jet printers are primarily used [33]. The pins or nozzles are moved very precisely by use of a robot system. Contact printers are more robust than ink-jet printers, but slower. Contact printers are more useful for research applications with small numbers of microarrays. Ink-jet printers are faster but susceptible to clogging of the nozzle. The spotting solution contains the capturing molecules (e.g., antibodies, DNA, or haptens) and supplements for yielding homogeneous signals per spot, possible only if the immobilizing molecules in the droplet at each spot are uniformly dispersed. For proteins, such additives as trahalose and sorbitol are important to prevent dried spots. DNA is dissolved in aqueous solutions, and haptens are dissolved in mixtures of water and organic solvents. Both classes of molecule should dry on the microarray substrate. For the spotting process, defined humidity between 40 and 70 % is important.

The simplest method of immobilization of proteins, for example antibodies, on CL microarrays is by use of adsorption mechanisms. Different supporting material, for example nitrocellulose-coated glass slides [34], polystyrene [35], or other plastic materials [36], are used as substrates for CL microarrays. The advantage of this method is that limited surface chemistry is needed. If sensitivity and reproducibility are not the limiting factors, direct immobilization of capturing agents could be favorable for multiplex assays. Sera or food matrices often produce a CL signal with a large offset [37]. Therefore, intensive surface blocking steps, by use of adsorption, are necessary to minimize unspecific binding of assay reagents and CL labels.

Analytical applications place severe demands on the manufacture of microarray chips. Intra-assay variance is determined by redundant measurements of one analyte species on

one microarray chip. The homogeneous distribution of immobilized molecules on each spot must therefore be characterized and optimized by 2D imaging, for example the method published by Wolter et al. for CL antibody microarrays against HRP [38]. CL signals detected beside the spot area should be minimized to achieve high signal-to-noise ratios. Nonspecific bound CL labels on the surface of the microarray can result in background signals and must be avoided. Therefore, more complex surface chemistry is necessary for CL-based analytical microarrays. An effective link between the substrate and the recognition element should be implemented for each microarray-supporting material. Glass-based microarray chips are silanized with, e.g., 3-glycidyloxypropyltrimethoxysilane (GOPTS) as an initial stage of functionalization [39]. A shielding layer is introduced to minimize nonspecific binding outside the microarray spots. Intensive research on polymer brushes [40], hydrogels [41, 42], and dendrimers [43, 44] has therefore been performed by many research groups. For CL flow-based microarrays, Jeffamine 2000 was found by our group to be the best shielding layer for producing high signal-to-noise ratios. Polyoxypropylenediamine (diamino-PEG) surfaces on glass slides have been shown to be enable signal-to-noise ratios of more than 600:1 for HRP as CL label [38]. Polyoxypropylenediamine is a diamino-PEG consisting of a bifunctional primary amine of average molecular weight approximately 2000 g mol^{-1}. The amine groups of diamino-PEG are located on the secondary carbon atoms at the ends of an aliphatic polyether chain. The terminal amine groups serve as functional groups. The last functionalization step is necessary for cross-linking of recognition elements. Aldehyde, epoxy, or NHS-activated microarray chips are used mainly for the covalent linkage [45, 46]. Diamino-PEG surfaces are either activated by the homobifunctional crosslinker poly(ethylene glycol)diglycidyl ether (diepoxy-PEG) or (*N*,*N*-disuccinimidyl) carbonate (DSC). DSC-activated microarray chips are highly suitable for DNA [47] or antibody [48] immobilization, because of the terminal primary amines present in both types of biomolecule.

Microscope glass slides are the cheapest available substrate material. In contrast with fluorescence microarrays, no background signal from the microscope glass slide substrate can be tolerated. The quality of the glass slides is, therefore, highly significant for the surface chemistry. Another important point is flexible chemical treatment of glass slides by use of layer-by-layer surface chemistry. Different linkers and coating polymers in solvents or water can be chosen for development of optimum surface chemistry. A disadvantage is the cost-intensive manufacturing process by wet-chemical surface chemistry. Regarding production costs of CL microarrays, costs of pure organic solvents and labor are the main factors in the final price. For fully automated fabrication of microarray chips, research on alternative surface chemistry, for example chemical vapor deposition would be necessary [49]. Aluminium oxide and plastic material could be an alternative to glass [50]. However, the possibility of achieving greater sensitivity on glass slides for CL microarray analysis as a result of the high signal-to-noise ratio should not be neglected.

Marquette and Blum have mentioned that, besides fluorescence, CL microarrays have greater sensitivity and dynamic ranges than colorimetric and label-free multiplex immunoassays [6]. For example, a study was conducted to compare the sensitivity of CL microarrays on PEGylated microarray glass slides with 96-well polystyrene plates and nitrocellulose test strips for colorimetric readout. The same antigens were immobilized on each substrate. An antibody-capturing assay was conducted for an antibody against infectious diseases in swine sera. CL microarrays were shown to be more sensitivity and have wider dynamic ranges than ELISA or lineblot immunoassays (LIAs), as shown in Fig. 3 [51].

CL microarray analysis techniques and applications

Depending on the analysis, either segmented CL microarrays or flow-based CL microarrays are used. Static-incubated CL microarray analysis uses open reaction wells. Reagents are placed on top of the CL microarray. Between assay processes, intensive washing steps needed to reduce background signals. The equilibrium state should be achieved in each reaction vessel for each incubation process. This is the time-limiting process in static incubated CL microarrays. Microarrays are imaged either through the CL reagent solution or from beneath the well. Open systems, for example CL microarrays in microwell plates, have the potential for high-throughput

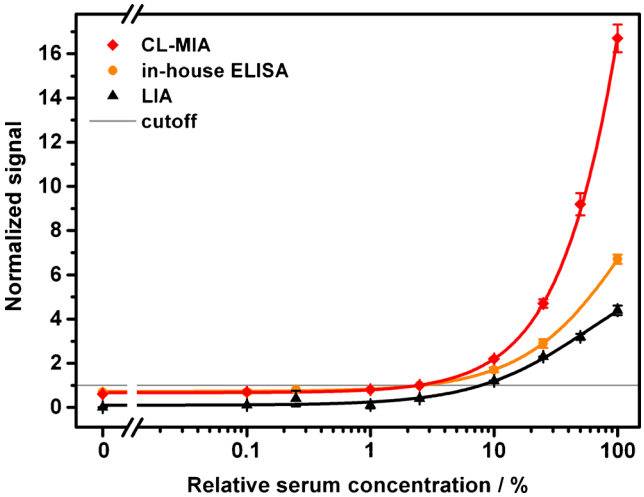

Fig. 3 Comparison of antibody-capturing immunoassay based on CL-MIA on diepoxy-PEG-activated, diamino-PEG-coated glass slides, LIA on nitrocellulose test strips, and colorimetric ELISA on 96-well polystyrene plates

analysis [16] if fluid handling is performed in parallel by use of relatively large pipetting robots (Fig. 4).

Static MIAs need a longer incubation time than flow-based microarrays because equilibrium must be achieved in each well. For rapid analysis in flow-based configurations, only a short incubation is performed in solution and at the spot. Measurements are conducted without reaching an equilibrium state, only possible with automated processes because the velocity and volumes should be recorded very accurately. A stop–flow mode could be chosen if a longer reaction time is needed [53]. In both configurations, endpoint measurements, only, are possible, because washing steps are necessary between the affinity reaction on the microarray and the CL readout.

In general, any test format suitable for single-analyte applications can be converted for use with multi-analyte CL microarray assays. Assay formats are discussed for flow-based microarrays because applications are published for all of the test formats. Small organic molecules are quantified by competitive MIA. The analyte itself or a derivative is immobilized. Small molecule microarrays have the advantage that regenerable MIAs can be performed, reducing the cost per microarray chip because each CL microarray can be calibrated and used for several measurements. Protein-conjugated haptens are immobilized, however, so the microarray is non-regenerable and useable for a single measurement only.

Directly competitive MIAs use immobilized antibodies and labeled hapten derivatives. The antibody microarrays are single-use microarrays. The advantage is that the immobilization procedure for each analyte is not changed, and data processing is easier. However, for automated and continuous analysis of samples, regenerable CL microarrays are preferable.

Antibody capture or indirect non-competitive MIA is a common assay format for quantification of antibodies in blood serum [54]. This format is transferred to multiplex microarray analysis by production of antigen microarrays. For this format, species-specific HRP-conjugated secondary antibodies are needed.

Analytes that contain more than one binding site for antibody detection (e.g., microorganisms and proteins) are quantified by sandwich immunoassays. This assay format is transferred to multiplex microarray analysis by creation of antibody microarrays. The capturing antibodies are immobilized on the microarray chip to bind the analytes selectively. Detection antibodies are used to create a sandwich of antibodies and analytes on each spot. Detection antibodies are directly conjugated to HRP or biotinylated antibodies are used, requiring a second assay step with streptavidin-labeled HRP.

For molecular biological multi-analyte detection, quantitative analysis of DNA is performed by using oligonucleotide microarrays and the NAMA principle. DNA probes are immobilized for hybridization of the complementary DNA of labeled single-stranded target DNA sequences. Hybridization products on each spot of the microarray are detected by antibodies against the label used.

Static-incubated MIAs

Static-incubated CL-MIAs on flat microarray substrates were introduced by Joos et al. for multi-analyte detection in clinical diagnostics [55]. Eighteen autoantigens were immobilized by ink-jet microdispensing on nitrocellulose, poly-L-lysine, and aldehyde-coated glass slides. Microarrays on nitrocellulose provided the most sensitive assay; the lowest detectable amount of microdispensed antigen was 8 fg per 0.25 nL, and the detection limit was approximately 0.2 μmol L^{-1}.

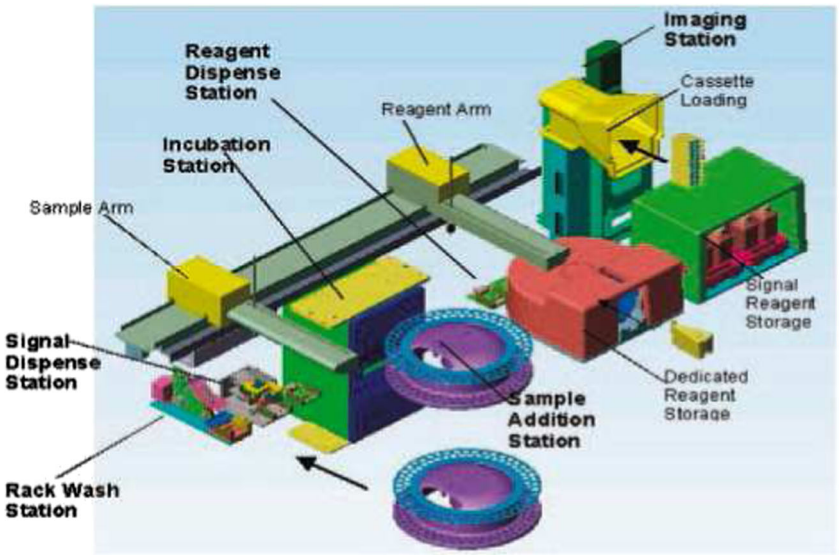

Fig. 4 Evidence biochip array analyzer (reprinted from Ref. [52])

However, dispensing such small volumes in routine diagnostic laboratories is not practicable. A sandwich MIA on nitrocellulose membranes was evaluated for detection of 24 cytokines in the serum of a patient [56]. Hybond membranes could be used to detect cytokines down to 5 pg mL^{-1}. The sensitivity was lower and the detection range greater than for colorimetric ELISAs as references. In another study, a poly(vinylidene difluoride) (PVDF) membrane was used for immobilization of capture antibodies dissolved in methanol [57]. Thirty-five cytokines were quantified in cell lysates and tissue lysates. The author mentioned that nitrocellulose membranes produce high background signals when cytokines are analyzed in cell or tissue lysates. With PVDF membranes, the lowest detectable concentration was 0.1 pg mL^{-1} MIP-1δ, which was a factor of 20 better than for conventional ELISA technology. The detection limit of heat-killed pathogenic bacteria was similar for sandwich MIAs and conventional sandwich ELISAs [58]. The author stated that poly-L-lysine-coated glass slides generated lower background signals than nitrocellulose-coated glass slides. Use of flat microarray slides is not preferable for practical analyses because the incubation and washing steps are conducted manually in an undefined setup. Because these procedures typically result in poor reproducibility, static-incubated MIAs in wells or tubes were studied. A filtration-assisted microarray in 96-well plates was introduced for screening of autoimmune antibodies [59]. The wells contained nitrocellulose and/or cellulose acetate membranes in the bottom of the wells. Mass transport limitations should be overcome by filtration-assisted processing of MIAs. The indirect antibody-capturing MIA was processed by use of vacuum pumps. The antibodies in sera were sucked into the membrane filter. Protein microarrays were read with a CCD camera from the underside of the 96-well plate after an assay time of 95 min. The results obtained for the filtration-assisted indirect antibody-capturing MIA were in good agreement with conventional ELISA. Moody et al. used the bottom of 96-well polystyrene plates for a sandwich MIA with nine capture antibodies against cytokines for high-throughput screening of anti-inflammatory compounds [60]. Further applications of the CL microarrays in 96-well plates have been reported for serological diagnosis of autoantibodies from rheumatoid diseases [61] and from *M. bovis* infections in cattle [62] by use of indirect antibody-capturing MIAs. In another study, six cytokine biomarkers were analyzed by use of a sandwich MIA in 96-well plates. The method was quantitative and reproducible, with an intra and inter-assay accuracy between 70 % and 130 %, and assay precision of <30 % [63]. Sandwich MIA and conventional ELISA were compared. High correlation coefficients (>0.9) were obtained for five measured analytes. A lower correlation coefficient was obtained for one analyte. Cross reactivity between antibody pairs and proteins other than the target proteins were responsible for lowering the correlation coefficient. This result confirms that measurement costs and time can be saved by multi-analyte approaches. However, intensive research on a suitable group of antibodies with low cross-reactivity in multiplex immunoassays is needed. Therefore, greater investment of both time and money is necessary for development of multiplex immunoassays compared with assays for single-analyte measurements.

A sandwich CL-MIA for detection of *E. coli* O157:H7, *Y. enterocolitica*, *S. typhimurium*, and *L. monocytogenes* in food was performed in a 96-multiwell plate with four subwells in each well [35]. The standards for calibration and the samples were measured simultaneously. Bovine meat and fecal samples were spiked with bacteria. The samples were cultivated for 9 h before the CL sandwich MIA was performed. Four pathogenic bacteria were quantified in parallel, with a limit of quantification of the order of 10^4–10^5 CFU mL^{-1}. Incubation time both for the analytes and for the HRP-labeled detection antibody was 30 min. Intensive washing after the incubation steps, for an unspecified time, was needed. This CL-MIA was limited to four analyses. The advantage was that one-quarter of the amount of reagents was consumed compared with conventional single-analyte ELISA.

Microarrays in single reagent tubes instead of multiwell plates have been investigated for CL-MIA [64]. Antibodies were immobilized with the highest efficiency on epoxy-coated glass substrates. Biotoxins, inactivated bacteria, and viruses in a sandwich format for multiplex analysis of biowarfare agents were quantified in 1.5 h. The CL signal was generated after incubation with streptavidin-poly-HRP. Washing and pipetting were performed manually. Incubation was performed with a horizontal tube shaker. The detection limits for viruses ($TCID_{50}$ means 50 % tissue culture infection dose), bacteria, and protein biotoxin were 6×10^2–5×10^6 $TCID_{50}$ mL^{-1}, 5×10^3–2×10^6 CFU mL^{-1}, and 0.1–0.2 ng mL^{-1}, respectively. Microarrays in tubes were an alternative to microwell plates for static-incubated CL-MIAs. The limitation was practicability, because of the lack of a fully automated process.

A disposable screen-printed microarray chip has been developed for point-of-care (POC) diagnostics [65]. Myoglobin, cardiac troponin I, C-reactive protein, and brain natriuretic peptide were analyzed by use of a sandwich CL-MIA in 25 min. Dynamic ranges of 0.5–50, 0.1–120, 0.2–20, and 0.67–67 mg L^{-1} were obtained for C-reactive protein, myoglobin, cardiac troponin I, and brain natriuretic peptide, respectively. The capture antibodies were electrochemically grafted on to the screen-printed microarray by electroaddressing of the diazotized aniline derivatives. Nanostructured gold was used as the conducting material, enhancing the CL signal [38]. The screen-printed microarray chip was limited to a maximum six spots. Automated processing was not discussed, even though automated processing is especially important for the POC diagnostics.

The first commercially available automated CL microarray analysis was the *Evidence Biochip Array Analyser* from Randox Laboratories (Belfast, UK). The system was developed initially for the clinical diagnostics market. The product comprises a fully automated dispensing station for reagent supply, washing solutions, and sample introduction [52]. Antibody microarrays, mainly, were performed in static-incubated sandwich or direct CL-MIA test formats. The single-use plastic carrier holder contains 3×3 microarray chips. On each chip, 5×5 spots are microdispensed by ink-jet technology. The capture antibodies are immobilized on an alumina substrate activated with a mixture of GOPTS and *N*-ethyldiisopropylamine. A sandwich MIA was designed for analysis of 12 cytokines. The assay process included incubation of the analytes for 1 h on a thermoshaker, followed by overnight incubation. After washing, the detection antibodies were also incubated for 1 h. The LOD was between 0.12 pg mL^{-1} for IL-2 and 2.12 pg mL^{-1} for IL-4 [66]. A wide range of multiplex applications has been published, including screening for 20 anthelmintic drugs in milk and muscle samples [67], drugs of abuse in meconium specimens [68], and nitrofuran metabolites [69] and sulfonamides [70, 71] in honey. Small molecules were analyzed in 100 min by direct competitive MIAs. The Randox *Evidence Investigator* used was a non-automated microarray analysis system. Reagent dispensing and washing steps were performed manually. All results are listed in Table 1. Static-incubated CL microarrays can easily be run in parallel for high sample throughput. Lengthy incubation and washing steps are the disadvantage of this technology.

Analysis based on CL flow-based microarrays

Use of CL imaging as a detection technique for flow-based antibody microarrays was first described by Weller and Niessner [72]. Flow channels on the microarray are processed with fluidic systems by use of pumps and valves. With flow-based microarrays, research on automated and rapid multiplex analysis has led to the concept of portable, decentralized, multiplex microarray analysis as the next generation of applications suitable for research in analytical chemistry.

The first instrumentation for processing CL flow-based microarrays by use of a fluidic system with tubing, pumps, and valves was designed for detection of small organic molecules by indirect or direct competitive MIAs [72]. The microarray chip was called a parallel affinity sensor array (PASA). The PASA system consisted of six syringe pumps, a set of tubing, an autosampler, and a dark chamber for readout of the microarray chip by use of a CCD camera. Triacines, trinitrotoluene (TNT), and fluorescein were NHS-activated before being microdispensed on *N*-(2-aminoethyl)-3-amimopropylmethyldimethoxysilane-coated microscope glass slides. The principle of regeneration of immobilized stable chemical molecules was introduced for the first time for CL microarrays. Calibration for atrazine and TNT was performed on one microarray chip, resulting in detection limits of 0.04 μg L^{-1} and 0.13 μg L^{-1}, respectively. Total assay time was 29 min, including the regeneration step. The PASA system was also convenient for multiplex diagnosis of allergies. A total of 24 preparations of recombinant or purified allergens were immobilized on GOPTS-coated glass microarray chips for screening of allergen-specific IgE. The antibody-capturing CL-MIA was performed by static incubation during flow-based CL analysis in less than 1 h [73, 74]. Detection limits between 0.16 and 1.9 μg L^{-1} were achieved for different allergen-specific IgEs.

The same group has developed a second-generation automated CL microarray analysis system [75]. The so-called Immunomat consists of eight syringe pumps, a set of tubing, and a CL-detection system. The flow cell comprised a glass carrier with inlet and outlet connections and a silicone seal that contained the cut-out flow channel. The Immunomat has shown, for the first time, that very fast indirect competitive MIAs are possible with a flow-based configuration. Ten antibiotics in milk were quantified in 5 min. Detection limits ranged from 0.12 μg L^{-1} (cephapirin) to 32 μg L^{-1} (neomycin). Each hapten was conjugated to proteins before immobilization on GOPTS-coated glass slides. In this manner, the immunogens for antibody production could be utilized directly for protein microarray production. The disadvantage was that the indirect competitive MIA was not regenerable and, therefore, the cost of quantification of antibiotics in milk was high, and the analysis with several chips was found to be better. The same instrumentation was used for quantification of bacteria by sandwich MIA. A shielding layer for poly(ethylene glycol)-coated glass slides was introduced for CL antibody microarrays and resulted in high signal-to-noise-ratios [38]. Heat-inactivated *S. typhimurium*, *L. pneumophila* Sg 1, and *E. coli* O157:H7 were quantified in parallel in 13 min with detection limits of 3×10^6 cells mL^{-1}, 1×10^5 cells mL^{-1}, and 3×10^3 cells mL^{-1}, respectively [48]. For quantification of viable *E. coli* cells, a stop-flow process was performed on the Immunomat [52]. The method, with alternation of resting volume elements and elements for forward pumping, was more effective than continuous-flow approaches for analysis of bacteria. Flow-based microarrays have the advantage that the assay processes can be adapted very easily to the association and dissociation constants of the affinity reactions between antibody and antigen. For this approach, optimum conditions were 30 pumping cycles with a volume of 20 μL (one-third of the flow cell of the microarray chip) and a break of 108 s for every pumping cycle. With these conditions, the sandwich MIA achieved a detection limit of 4×10^5 cells mL^{-1} for living *E. coli* cells. When the same antibodies were used, a conventional CL-ELISA on microwell plates had a detection limit of 5×10^6 cells mL^{-1} and an assay time of 3 h [76].

Table 1 Applications of static-incubated, CL-based antibody microarrays

Analyte	Matrix	Analysis system	LOD	Assay time[a]	Ref.
E. coli O157:H7, *Y. enterolica*, *S. typhimurium*, *L. monocytogenes*	Meat, human fecal matter	Sandwich MIA, multiwell plate with four subwells	10^4 –10^5 CFU mL^{-1}	60 min	[35]
Biotoxin (ricin, SEB), viruses (Venezuelan equine encephalitis virus, Yellow fever virus, St. Louis encephalitis virus, West Nile virus, Orthopox virus), bacteria (*Y. pestis*, *B. meditensis*, *B. mallei*, *E. coli* O157:H7, *F. tularensis*)	Buffer (PBS+0.1 % FCS+0.01 % Tween)	Sandwich MIA, array tube (AT), AT reader (CLONDIAG)	Viruses: 6×10^2–5×10^6 $TCID_{50}$ mL^{-1} Bacteria: 5×10^3–2×10^6 CFU mL^{-1} Biotoxin: 0.1–0.2 ng mL^{-1}	1.5 h	[64]
Cytokine: IL-2, IL-4, IL-6, IL-8, IL-10, TNFα, IL-1α, IL-1β, MCP-1, EGF	Human serum	Sandwich MIA, *Randox Evidence Analyzer*	ng L^{-1}-range	2000 results h^{-1}	[66]
Anthelmintic drugs: aminobenzimidazoles (aminomebendazole, albendazole 2-aminosulfone, aminoflubendazole), avermectins (emamectin benzoate, eprinomectin, abamectin, ivermectin, doramectin), benzimidazoles (albendazole sulfone, albendazole, albendazole sulfoxide, oxibendazole, oxfendazole, flubendazole), thiabendazoles (cambendazole, thiabendazole, and 5-hydroxythiabendazole), triclabendazoles (ketotriclabendazole, triclabendazole, triclabendazole sulfoxide)	Milk, tissue	Sandwich MIA, *Randox Evidence Analyzer*	0.3 ppb–2.0 ppb in milk 0.15 ppb–6.5 ppb in tissue	100 min	[67]
Drugs of abuse: (+)-amphetamine, barbiturates, benzoylecgonine, oxazepam, lorazepam, methadone, (+)-methamphetamine, morphine, PCP, (−)-9-carboxy-11-nor-Δ^9-THC	Meconium extract	Direct competitive MIA, *Randox Evidence Investigator*	Cutoff: 10–100 ng g^{-1}	100 min	[68]
Nitrofuran metabolites: AHD, AOZ, AMOZ, SEM	Honey	Direct competitive MIA, *Randox Evidence Investigator*	Below 1 μg kg^{-1}	100 min	[69]
Sulfonamides: sulfodiazine, sulfadimethoxine, sulfakinoxaline, sulfhametazine, sulfatiazol, sulfisoxazol, sulfapyridine, sulfamerazine, sulfametoxipiridazine, sulfachlorpyridazine, sulfametizol, sulfadoxin	Honey	Direct competitive MIA, *Randox Evidence Investigator*	0.3–7.5 μg kg^{-1}	100 min	[70, 71]

[a] Assay time without dispensing time of analytes and reagents and time for washing steps

However, the total analysis time, 67 min, was greater than that for analysis of heat-inactivated bacterial cells, discussed above. The principle was applied to quantification of *L. pneumophila* Sg1 in bioaerosols [77]. A detection limit of 1×10^3 cells mL^{-1} was achieved for purified water, a hundred-fold increase in sensitivity. For bioaerosol measurements, bacteria were nebulized in a simple aerosol chamber then collected by use of a wetted wall cyclone Coriolis μ. This combination of impingement and multiplex microarray analysis was characterized by use of bioaerosols containing inactivated *L. pneumophila* Sg1. The detection limit obtained experimentally for heat-inactivated *L. pneumophila* Sg1 was 4×10^3 cells m^{-3}.

On the basis of the Immunomat system, a new fluidic setup for flow-based DNA microarrays was created [47]. In a first proof-of-principle study, the general detection limit of a hybridization assay on CL flow-based oligonucleotide microarrays was characterized. The surface chemistry was optimized for diamino-PEG-coated microarray chips. NHS-activated surfaces were identified as the most promising substrate. After optimization of the surface chemistry and microcontact printing of DNA probes, synthesized biotinylated 25-mer oligonucleotides were hybridized on DNA microarrays by use of a flow-injection system. A detection limit of 500 fmol L^{-1} (40 oligonucleotides mL^{-1}) was achieved by CL imaging in 15 min. When a microarray flow cell and CL imaging with a CCD were used, the system was faster and more sensitive than other published CL oligonucleotide-microarray analysis methods [78–80]. In a second step, it was necessary to study the analysis of PCR products. These amplified DNA sequences are typically longer (100–1000 bp) and double-stranded. Sensitivity and analysis time of the hybridization assay on the CL microarrays were, therefore, degraded. For quantitative analysis of PCR products, the cycling was stopped at the reaction point, when the spread between the different starting amounts of target DNA had reached a maximum. A combination of the so-called stopped PCR and CL oligonucleotide microarrays was shown to generate quantitative results for PCR products. Single-stranded PCR products were generated by boiling and subsequent cooling on ice. A flow-based hybridization assay was performed in 7 h, because the hybridization reaction on the oligonucleotide microarrays had to be repeated 20 times. The incubation time needed was similar to that of automated hybridization instrumentation for DNA microarray analysis [81–83]. The advantage of the flow-injection-based analysis was the direct coupling of the detection unit to the oligonucleotide microarray. The detection limit for the gene uidA (β-galactosidase) of *E. coli* was 1.1×10^5 copies mL^{-1} [47]. A process scheme for accessing single-stranded DNA quantitatively after stopped PCR was established to reduce the assay time. Three waterborne pathogenic bacteria, *E. coli* O157:H7, *S. enterica*, and *C. jejuni*, were used as model organisms for this approach [84]. The procedure started with a dilution series of the heat-inactivated bacterial cells in water. The bacterial cells were lysed in a PCR cycling instrument at 95 °C for 15 min before the PCR was started. The amplification was stopped at the logarithmic phase of the PCR. Single-stranded DNA was generated by magnetic nanoparticle separation (MNS). A reverse primer labeled with biotin for MNS and a forward primer labeled with digoxigenin (DIG) were used. The DIG-labeled single-stranded PCR products were quantified on oligonucleotide microarray chips. Quantification was conducted by use of a flow-based CL microarray readout system. Total assay time was 3.5 h, and the detection limits determined on CL oligonucleotide microarrays were 136, 500, and 1 cell mL^{-1} for *E. coli* O157:H7, *S. enterica*, and *C. jejuni*, respectively. These detection limits are comparable with those of SYBR green-based assays analyzed with a real-time PCR device. The assay time was halved and a process suitable for integration with automated multiplex microarray analysis was developed.

Rapid analytical methods need high flow rates, resulting in a high pressure drop inside the microarray chip. The microarrays on microscope glass slides were sealed by a closure head. This sealing principle was not reproducible, and the optical lens was contaminated with reagents. For this reason, a three-layer concept for the microarray chips was designed for easy buildup (Fig. 5). This microarray chip consists of three inexpensive basic material components: microarrays on modified glass slides, microfluidic channels on double-sided adhesive foil, and a plastic carrier with inlet and outlet ports (Fig. 5a). The closed microarray chip could be stored easily, was transportable, and could be connected directly to the flow system by establishing pressure on the O-rings (Fig. 5b). The CL signal was recorded through a transparent microarray substrate, and the imaging system was on the top.

Automated processing of flow-based CL microarrays has been performed on the MCR3 (microarray chip reader, third generation). The initial design of the MCR3 microarray analysis technique was dedicated to flow-injection-based, multi-analyte, regenerable, indirect competitive CL immunoassays [85]. The concept of the MCR3 was suitable for portable stand-alone equipment in which all of the necessary reagents are stored for one working day. Therefore, running, regeneration, cleaning, and storage buffers were connected inside the instrumentation for automated processing with three syringe pumps, three six-port distribution valves, and four separate turning valves. One 25-mL syringe and one 50-mL syringe were filled with a cocktail of detection antibodies and the HRP-labeled secondary antibody conjugates, respectively. Multi-analyte samples were injected via a syringe-based loading unit, containing single-use 1-mL syringes, connected to the flow-injection system. The pumps, valves, and CCD were controlled via Labview-based software (National Instruments, USA).

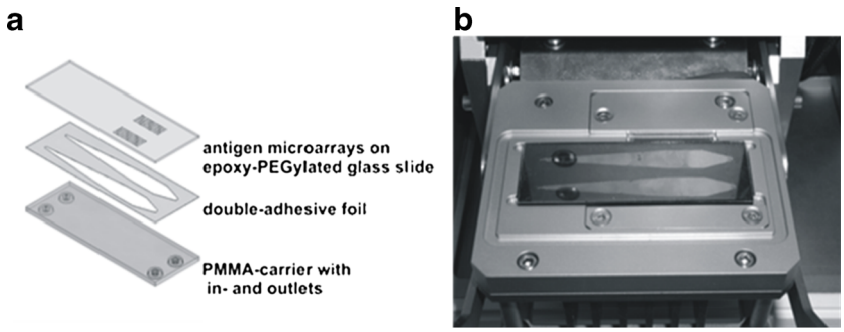

Fig. 5 Three-layer principle of the microarray chip (**a**) and insertion in the loading unit for flow-based microarray chips (**b**)

The first application was a multi-analyte immunoassay for quantification of 13 antibiotics in raw milk by use of a regenerable hapten microarray [86]. Antibiotics of five antibiotic classes (sulfonamides, β-lactams, aminoglycosides, fluoroquinolones, and polyketides) were quantified simultaneously in 6 min by performing an indirect competitive MIA. The flow-based microarray chip consisted of two flow channels, one for measurement and the other for regeneration. The microscope glass slides were coated with diamino-PEG and were subsequently activated by use of diepoxy-PEG. The antibiotics were immobilized directly by microcontact printing without further use of linking agents. The microarray chip and plastic carrier were assembled with adhesive foil. By internal calibration and use of regeneration cycles, the raw milk was automatically analyzed without changing the microarray chip during a working day. One microarray chip could be used for at least 50 measurements. Detection limits were between 0.05 μg L^{-1} (tetracyclin) and 135 μg L^{-1} (neomycin). Regenerability was limited, because of signal losses during measurement. Possible reasons were overloading of immobilized antibiotics, chemical changes in the structure of the antibiotics during the assay processes, or inefficient recovery of antibodies. Despite these problems in regenerability, results from antibiotic microarray analysis and the conventional microbial inhibition test were comparable. The microarray chip reader (MCR3, Fig. 6) was further developed for routine screening of antibiotics in raw milk.

In a proof-of-principle study, identification and quantification of antibiotic derivatives in honey was shown to be possible by use of regenerable antibiotic microarrays in combination with the MCR3 [87]. Because of the need for longer washing steps to minimize cross-contamination of honey components, total assay time increased to 14 min, including regeneration and washing of the MCR3. The indirect competitive MIA method enabled rapid analysis of the four antibiotics without purification or extraction steps. As a result of the high viscosity of the honey, the samples were diluted 1:10, leading to reduced sensitivity compared with the other foods, for example milk. Regeneration of the microarray chip was possible for 40 cycles. Multi-analyte calibration curves were obtained for the antibiotics enrofloxacin, sulfadiazine, sulfamethazine, and streptomycin, and detection limits were between 4.2 μg kg^{-1} (enrofloxacin) and 192.6 μg kg^{-1} (sulfadiazine). Spiking experiments with the same microarray chip revealed recovery was adequate within the dynamic ranges of the calibration curves of enrofloxacin (92 %), sulfamethazine (130 %), sulfadiazine (89 %), and streptomycin (93 %).

It was shown that the concept of regenerable hapten microarrays could be transferred to other small molecules and to other matrices. Ochratoxin A (OTA) was quantified in green coffee extracts by indirect competitive MIA in 12 min, including measurement and surface regeneration [88]. A peptide–OTA conjugate was synthesized for covalent immobilization on NHS-activated, diamino-PEG-coated glass microscope slides. The analytes were extracted with 20:80, (*v*/*v*) methanol–PBS solution before measurement on the MCR3. The limit of quantification for the extracted OTA was 0.03 μg L^{-1}. Signal losses observed during regeneration resulted in overestimation of recovery.

The concept of regenerable hapten microarrays was used for rapid multiplex quantification of mycotoxins in cereals [89]. Aflatoxins (AFB1s), OTA, deoxynivalenol (DON), and fumonisins (FB1s) were analyzed on the MCR by means of an

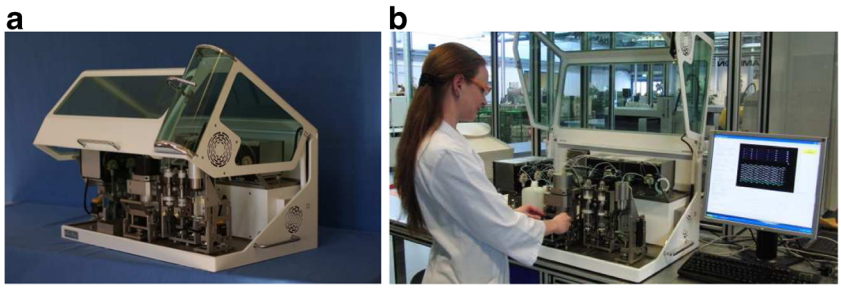

Fig. 6 Image of the MCR3 instrument for microarray analysis (**a**) and use of the instrument for analysis of raw milk in a routine control laboratory (**b**)

indirect competitive MIA. Total assay time was 19 min, including extraction, extract dilution, measurement, and surface regeneration. Diamino-PEG-coated glass slides were used directly for microarray production. NHS-activated carboxyl groups were introduced into the mycotoxins before production of the microarray by contact printing. The mycotoxin derivatives were, therefore, covalently immobilized, and the microarray chip was not completely activated. Intensive regeneration studies demonstrated regenerability over 50 measurements with minimum signal losses—between 96 % and 88 %. Detection limits between 0.06 μg L^{-1} (OTA) and 9.9 μg L^{-1} (FB1) were obtained. Recovery was between 55 and 80 % for water-soluble mycotoxins (FB1 and DON) and from 82–132 % for more nonpolar substances (AFB1 and OTA).

Multiplex microarray analysis on the MCR3 has been shown to be suitable for analysis of phycotoxins in shellfish [90]. Saxitoxin (STX), ocadoic acid (OA), and domoic acid (DA) were directly immobilized on epoxy-activated diamino-PEG-modified glass surfaces. The possibility of direct immobilization of the phycotoxins on epoxy-activated microarray chips was an advantage, because of the limited concentrations available for chemical derivatization. Three highly relevant marine toxins were quantified simultaneously on regenerable hapten microarrays. The bound anti-phycotoxin antibodies were regenerated over 25 consecutive measurements. Regeneration experiments revealed a constant reduction for OA and DA (3.1 % for DA and 1.5 % for OA per cycle). Because an exponential decrease observed for STX would result in overestimation of recovery, a mathematical correction factor (B/B_0) was introduced, using the blank directly measured before each sample and the third measured blank (zero standards). Average recoveries of 86.2 % for DA, 102.5 % for OA, and 61.6 % for STX were determined. Except for STX, signal losses by regeneration of the phycotoxin microarray could be corrected mathematically. The multi-analyte calibration LODs for DA, OA and STX were 0.5 μg L^{-1}, 1.0 μg L^{-1}, and 0.4 μg L^{-1}, respectively. The overall assay time was 20 min, including 5.5 min for the regeneration step. Fast and automated analysis could be performed by use of a simple extraction procedure which would be manageable for field analysis. Limitations include lack of availability of antibodies for all analogs of the phycotoxins.

For multiplex biosecurity analysis, simultaneous detection of chemical toxins, for example STX, and protein toxins (e.g., staphylococcal enterotoxin B (SEB) and ricin) is important. However, small molecules and proteins are quantified separately by competitive MIA and sandwich MIA. Combined analysis of low (<1000 Da) and high-molecular-weight (>1000 Da) toxins can be achieved by use of anti-idiotypic antibodies for such chemical toxins as STX. Therefore, an antibody against the antigen-binding site is used that is specific for STX. The multiplex microarray analysis was run on the MCR3 system. STX was detected by use of an indirect competitive MIA. Ricin and SEB were identified by use of a sandwich MIA. The challenge was combination of both MIA processes in one measurement. A mixture of detection antibodies against STX, ricin, and SEB was mixed in-line in an incubation loop by parallel injection and pushed over the microarray chip. Standard sandwich MIA procedures were used for sequential incubation of the antibody microarray with analytes and detection antibodies. It was shown that both assay principles could be combined on the MCR3 [91]. A detection limit of 2.3 μg L^{-1} was obtained for STX, similar to that described previously for regenerable indirect competitive MIA. The LODs for ricin and SEB were 2.9 μg L^{-1} and 0.1 μg L^{-1}, respectively.

Regenerable microarrays for biotoxin analysis were introduced for ricin [92]. This lectin binds specifically to terminal D-galactose and lactose. By preparation of glycosylated microarray chips, regeneration cycles could be implemented for ricin analysis. 6-Azidohexyl lactose was immobilized on an alkyne silane surface by use of click chemistry. The immobilized carbohydrate captured the B-chain of ricin. The A-chain of ricin was detected by use of an antibody. With this regenerable sandwich glyco-immunoassay format, ricin could be quantified in 13 min on the MCR3 with a detection limit of 80 ng mL^{-1}.

As a proof-of-concept study, antibodies against the emerging zoonotic pathogen hepatitis E virus (HEV) and enteropathogenic *Yersinia spp.* were analyzed in parallel by use of immobilized recombinant antigens (rAgs) of HEV genotypes 1 and 3 and Yersinia outer protein D (YopD) on epoxy-activated diamino-PEG-coated microarray chips [51]. In this study, sera from slaughtered pigs were tested on the MCR3 for detection of anti-HEV and anti-Yersinia IgG in parallel. The assay for detection of antibodies in sera was an indirect method. Swine serum diluted 1:100 (1 mL) was pumped over the antigen microarray chip. The captured antibodies were detected by HRP-conjugated polyclonal anti-swine antibodies. Multiplex screening of serum samples for emerging zoonotic infectious diseases could be performed in 9 min on the MCR3.

Use of antibodies for flow-based CL-MIA applications can differ from that for static-incubated CL-MIA. For flow-based MIAs, highly reactive antibodies are needed. High dissociation constants result in wash-off effects, and lower CL signals are observed. Against this background, antibodies should be screened by use of the same analytical techniques. Efficient hybridoma screening with the MCR3 has been reported. Polyclonal anti-mouse antibodies were coated on NHS-activated diamino-PEG-coated glass slides. The supernatant of hybridoma cells was immobilized on microarrays by contact printing. Screening was performed with HRP-labeled analytes, for example, aflatoxin B2 [93] or benzo[*a*]pyrene [94]. Rapid analysis times (5 min), reduced work load, and greater

automation were advantages compared with conventional hybridoma screening on multiwell plates.

Quantification of PCR products was transferred to the MCR3 system to perform CL-based NAMA. The capability for multi-analyte quantification was examined by parallel analysis of bacteriophage MS2 (RNA virus), bacteriophage PhiX174 (DNA virus), and the human pathogen adenovirus (DNA virus, hAdV2) [22]. The MCR3 system was modified for oligonucleotide microarray analysis. The CL microarray imaging unit was equipped with a Peltier heater to optimize the selectivity of hybridization reactions on oligonucleotide microarrays and to reduce the secondary structures of single-stranded PCR products. The efficiency and selectivity of hybridization reactions was increased. The increase of temperature was important mainly for the hybridization reaction. In addition, the activity of subsequent antibody and CL reactions was also increased. As a consequence, higher CL signals and lower cross-reactivity of PCR products were observed. Multi-analyte calibration experiments for dilution series of active viruses in tap water were examined. The DNA/RNA extraction and PCR reaction were conducted separately for each virus. The PCR products were combined in one sample before MNS was performed. Multiplex analysis of the single-stranded DIG-labeled PCR products was performed on the MCR3 system. The total analysis time for DNA/RNA extraction, cDNA synthesis for the RNA viruses MS2, PCR, single strand separation, and oligonucleotide microarray analysis was 4–4.5 h. Detection limits were 6.6×10^5 GU mL^{-1} for MS2, 5.3×10^3 GU mL^{-1} for PhiX174, and 1.5×10^2 GU mL^{-1} for hAdV2 at 40 °C. In conclusion, multiplex quantification of PCR products was possible on the MCR3 system. No multiplex PCR was performed. To perform single PCRs the sample must be divided into smaller volumes depending on the amount of virus present. Further investigations are necessary, for example nucleic acid amplification on microarray chips to compete with multiplex real-time PCR applications.

A microfluidic microarray chip with an antibody-capturing format has been developed for allergen diagnostics [95]. The microfluidic cartridge consisted of a PMMA chip holder containing the fluidic connections to the pumping system, an O-ring joint, the microfluidic channels in SU-8 polymer, a protein microarray on the PDMS substrate, and a bottom PMMA chip holder. For rapid analysis of allergen-specific antibodies in serum samples, a dynamic incubation time of 6 min (flow rate 50 μL min^{-1}; 300 μL sample volume) was found to be the best. The detection limit was higher than for conventional ELISA. Similar detection limits were achieved by use of a higher sample volume (1.2 mL) and a lower flow rate (20 μL min^{-1}). Similar to the ELISA, analysis time was 60 min. The so-called "macromolecules to PDMS transfer" method has been used for direct modification of PDMS surfaces for protein microarrays. Beads or proteins were spotted and dried on a 3D master coated with Sylgard 184, cured, and recovered after peeling off as spots of beads or proteins entrapped at the surface of the bar PDMS [96, 97].

An automated CL microarray analysis system was developed for detection of autoantibodies causing such autoimmune diseases as rheumatoid arthritis, multiple sclerosis, and autoimmune diabetes [98]. The autoantigens were photoimmobilized on polystyrene chips by microdispensing mixtures of the autoantigens, the polymer poly(ethylene glycol) methacrylate, and a photoreactive crosslinker (4,4 -diazidostilbene-2,2 -disulfonic acid disodium salt; BIS). A transparent PDMS microfluidic chip was attached to the microspotted polystyrene plate. The instrumentation consisted of a CCD camera, a syringe pump, two additional six-port distribution valves, and a personal computer for processing the antibody-capturing MIA with the flow-based microarrays. Seven autoantigens were detected in 1:100-diluted serum in 30 min. Strong correlations (0.83–0.97) were obtained between results from conventional ELISA and microarray assays.

An automated flow-based system has been developed for processing a ten-channel capillary as a multiplex immunodetector [99]. A sandwich MIA was performed in ten parallel arranged glass capillaries for detection of *E. coli* O157:H7, SEB, and bacteriophage M13 in 29 min. The polyclonal capture antibodies were immobilized on the silanized glass capillaries. CL signals from each capillary were detected by use of a multianode photomultiplier array. Limits of detection were 0.1 mg L^{-1} for SEB, 10^4 CFU mL^{-1} for *E. coli* O157:H7, and 5×10^5 PFU mL^{-1} for bacteriophage M13.

A multichannel sandwich CL-MIA on single-use ABS plastic chips was evaluated for parallel calibration and quantification of pathogenic bacteria [36]. Capture antibodies were immobilized adsorptively in the microchannels of the acrylonitrile–butadiene–styrene (ABS) substrate by contact printing. The sandwich MIA was performed with a multi-channel peristaltic pump. Five multiplex standard concentrations and one multi-analyte sample were measured in parallel. Total assay time for measurement and calibration was 18 min. The detection limits were 2×10^4 cells mL^{-1} for *E. coli* O157:H7, 2×10^7 cells mL^{-1} for *S. typhimurium*, and 8×10^4 cells mL^{-1} for *L. pneumophila* Sg1. The high detection limits were a result of high background signals, because the channels of the ABS substrate were blocked with protein solution (BSA) only. Multi-channel microarrays were seen as a means of increasing the throughput of flow-based microarrays. However, a complicated fluidic system is desired for full automation of parallel MIA processes.

Portable microarray analysis has been developed for personalized diagnostics [29]. A multichannel microarray chip was created by use of the three-layer principle. Three channels and bore holes for the inlet and the outlet were cut in a polycarbonate plastic carrier to form the microfluidics. Antibodies or NH_2-labeled oligonucleotides were immobilized by

microdispensing on to GOPTS-coated glass slides. The microarray chip was sealed by use of adhesive foil containing the same channel structure as the microfluidic carrier. The CL signal was detected by lensless imaging from the underside of the glass slide. A syringe pump was used for the flow-based MIA process. Digoxigenin-labeled PCR products (1 μL) were quantified by NAMA in 33 min. Target DNA of parvovirus B19 DNA could be detected down to 0.05 μmol L^{-1}.

A microfluidic cartridge has been developed for allergen screening. An antibody (IgE)-capturing CL-MIA was performed on flow-based microarrays [100]. The 7 cm×4 cm injection-molded microfluidic cartridge contained an antigen microarray, reagent tanks, a waste tank, and pumps. A computer-controlled array of solenoid valves provided the necessary actuation force for automated processing of reagents and samples in a total assay time of 27 min. Dissolved nitrocellulose was sprayed on to silicone rubber as a substrate for the antigen microarray. Twenty allergen extracts were immobilized by contact printing. The cutoff was estimated to be less than 1 IU mL^{-1} (2.4 ng mL^{-1}).

A miniaturized microarray has been developed for quantitative analysis of three parvovirus B19 DNA genotypes in serum [101]. The disposable microarray chip was based on lateral flow MIA. Liquids were propelled through microfluidic channels by capillary forces. The microfluidic microarray chip consisted of three channels in PDMS coated on to microscope glass slides. DNA probes were immobilized on diamino-PEG (Jeffamine ED-600)-coated and subsequently NHS-activated glass slides. The CL-NAMA was performed in 30 min. The detection limit for B19 target DNA was 650 pmol L^{-1}.

The advantage of flow-based CL microarrays is that heterogeneous reactions are accelerated, and miniaturization of the CL microarray readout systems is possible. The detection system is integrated on the microarray chip. The complete analytical process can therefore be performed automatically by use of computer-controlled pumps and valves in a closed system. The disadvantage is that only sequential assay processes can be performed, and high-throughput measurement is limited. An overview of flow-based CL microarray applications is given in Table 2.

Multi-analyte aspects for CL microarray analysis

Multiplex analytical methods have several advantages over conventional analysis of a single species per sample. The economy of analyzing more than one species per sample is an obvious advantage for commercial analytical laboratories. Reagents, solvents, time, and space can be reduced by multiplexing. Instead of one instrument for one analyte and several instruments for several single analytes, one analysis can be used for several species per sample. Therefore, the cost of laboratory space, personnel, running instrumentation, and consumables is reduced. Multiplex analytical methods can be regarded as "green" analytical chemistry, because use of hazardous solvents and reagents can be reduced [102, 103]. Moreover, the bioanalytical methods of CL microarrays are mainly solvent-free and waste is reduced by reducing the amount of sample and reagent volumes. Detection of small organic molecules by use of MIA test formats requires sample preparation with little or no solvent. Otherwise, the antibodies would be deactivated [104]. Automation and miniaturization of flow-based methods, especially the flow-based CL microarrays discussed above, are seen as a contribution to greener analytical methods [105].

Furthermore, new analytical approaches can be investigated, because combining classes of analytes provides more information for solving a defined analytical problem. By use of multi-analyte methods relevant classes of analytes can be combined to furnish solutions to more generic analytical problems. Examples of applications in food and water safety, environmental monitoring, diagnostics, forensics, toxicology, and biosecurity are discussed below. Multi-analyte approaches are summarized in Table 3.

Food and water safety

Safe food and water is a principal demand for ensuring the high quality of products and the health of humans and animals worldwide. During production, processing, storage, and transport, a variety of hazardous chemicals, toxins, microorganisms and other contaminants can enter the food chain. The chemical and pharmaceutical industry produces a large variety of small organic molecules, for example pharmaceuticals, pesticides, and food and feed supplements. Products of agriculture, for example crops, fruit, or vegetables are treated with these small organic molecules. Animals and humans come into contact either directly by treatment or indirectly by consumption of feed. Residues or metabolites enter the environment by excretion in the urine or feces and are further transformed by microorganisms in the environment or in waste water-treatment plants. Food products of animal origin, for example meat, eggs, milk, and honey could also contain these small organic molecules. In conclusion, many residues and contaminants are present in food and feed and can be accumulated in the environment (water and soil). Small organic molecules could be hazardous to both human and animal health and welfare at significant exposure levels. Therefore, legislation has defined maximum residue levels (MRLs) for many substances. In addition to anthropogenic introduction of chemicals into the food chain and water cycle, natural toxins of plants or microbial toxins, antibiotic-resistant bacteria, pathogenic microorganisms, and pathogenic viruses are

Table 2 Overview of multi-analyte applications with flow-based CL microarray analysis systems

Analyte	Matrix	Analysis system	LOD	Assay time	Ref.
DNA microarray					
E. coli	Purified water	NAMA, recycled, stop-flow, modified Immunomat	1.1×10^5 GU mL^{-1}	7 h	[47]
E. coli O157:H7, *S. enterica, C. jejuni*	Purified water	NAMA, recycled, stop-flow, modified Immunomat	136 cells mL^{-1}, 500 cells mL^{-1}, 1 cell mL^{-1}	3.5 h	[84]
MS2, PhiX174, hAdV2	Tap water	NAMA, continuous flow, MCR3	6.6×10^5 GU mL^{-1}, 5.3×10^3 GU mL^{-1}, 1.5×10^2 GU mL^{-1}	4–4.5 h	[22]
Three genotypes of parvovirus B19	Human serum	NAMA, continuous flow, disposable reaction chip	650 pmol L^{-1}	2 h	[101]
Antibody microarray					
E. coli O157:H7, *S. typhimurium* *L. pneumophila*	Purified water	Sandwich MIA, stop-flow, Immunomat	3×10^3 cells mL^{-1}, 3×10^6 cells mL^{-1}, 1×10^5 cells mL^{-1}	13 min	[48]
E. coli	Purified water	Sandwich MIA, stop-flow, Immunomat	4×10^5 cells mL^{-1}	67 min	[53]
L. pneumophila SG 1	Purified water	Sandwich MIA, stop-flow, Immunomat	1×10^3 cells mL^{-1}	67 min	[77]
E. coli O157:H7, *S. typhimurium, L. pneumophila*	Purified water	Sandwich MIA, continuous flow, multichannel plastic chip	1.8×10^4 cells mL^{-1}, 2.0×10^7 cells mL^{-1}, 7.9×10^4 cells mL^{-1}	18 min	[36]
Ricin, SEB, saxitoxin	Purified water	Sandwich and indirect competitive MIA, continuous flow, MCR3	2.9 μg L^{-1}, 0.1 μg L^{-1}, 2.3 μg L^{-1}	18 min	[91]
Carbohydrate microarray					
Ricin	Purified water	Regenerable chip, sandwich MIA, continuous flow, MCR3	80 ng mL^{-1}	20 min	[92]
Hapten microarray					
Cephapirin, penicillin G, cloxacillin, erythromycin, gentamicin, neomycin, streptomycin sulfadiazine, sulfamethazine, tylosin	Raw milk	Regenerable chip, indirect competitive MIA, stop-flow, PASA	0.12 μg L^{-1} (cephapirin) to 32 μg L^{-1} (neomycin)	5 min	[75]
Ampicillin, cephapirin, cloxacillin, erythromycin, gentamicin, neomycin, norfloxacillin, penicillin G, sulfadiazine, sulfamethazine, streptomycin, tetracycline tylosin	Raw milk	Regenerable chip, indirect competitive MIA, continuous flow, MCR3	Lowest WR: 0.05–43 μg L^{-1} Highest WR: 135–2300 μg L^{-1}	6 min	[86]
Enrofloxacin, streptomycin, sulfamethazine, sulfadiazine	Honey	Regenerable chip, indirect competitive MIA, continuous flow, MCR3	4.2 μg kg^{-1}, 15.9 μg kg^{-1}, 81.5 μg kg^{-1}, 192.6 μg kg^{-1}	8 min	[87]
Ochratoxin A	Green coffee extract	Regenerable chip, indirect competitive MIA, continuous flow, MCR3	0.3 μg L^{-1} (LOQ)	12 min	[88]
Aflatoxins (AFB1), ochratoxin A, dioxinivalenol, fumonisins (FB1)	Methanol–water extract of cereals	Regenerable chip, indirect competitive MIA, continuous flow, MCR3	0.06 μg L^{-1}, 0.07 μg L^{-1}, 2.5 μg L^{-1}, 9.9 μg L^{-1}	19 min	[89]
Okadaic acid, domoic acid, saxitoxin	Shellfish extracts	Regenerable chip, indirect competitive MIA, continuous flow, MCR3	1 μg L^{-1}, 0.5 μg L^{-1}, 0.4 μg L^{-1}	20 min	[90]
Antigen microarray					
IgE against 24 allergens	Human plasma and serum	Indirect MIA, static incubated, PASA	0.35 μg L^{-1} (rBet v1) 0.16 μg L^{-1} (PLA_2) 1.9 μg L^{-1} (Der p1)	1 h	[73, 74]
IgE against 20 allergens	Human plasma and serum	Indirect MIA, continuous flow, microfluidic cartridge	Average LOD 0.535 AU	27 min	[100]
Antibody against HEV and *Yersinia* spp.	Swine serum	Indirect MIA, continuous flow, MCR3	n.d. (no standards available)	8.5 min	[51]

Table 3 Overview of multi-analyte applications and possible multiplex analysis methods

Analyte classes	Analytes	Fields	Matrix
Small organic molecules			
Phycotoxins	Group of okadaic acid, domoic acid, saxitoxin, azaspiracid, brevetoxin, ciguatera toxin	Food safety, diagnostics, biosecurity	Mussels, fish, human serum
Mycotoxins	Aflatoxins, trichothecenes, fumonisins, ochratoxins, fusarium toxins, ergot alkaloids	Food safety	Crops, cereal and cereal products, milk, coffee, beer, wine, dried fruits, must, nuts, spices, baby food, infant food, feed
Cyanotoxins	Neurotoxins (anatoxin-a, homoanatoxin-a, saxitoxin), hepatotoxins (microcystins, nodularin), cytotoxins (cylindrospermopsin), dermatoxins	Water safety, food safety	Surface water, drinking water, seafood, crops
Antibiotics	Sulfonamides, β-lactams, aminoglycosides, tetracyclines, quinolones, nitrofurans, nitroimidazoles, macrolides, lincosamides, amphenicols, polymyxins, vancomycins	Food safety, water safety, veterinary testing	Milk, honey, animal tissue, egg, feed treated waste water, surface water, drinking water, ground water
Growth promoters	Hormones, β–agonists, steroids, thyrostats, stilbenes	Food safety, veterinary testing, diagnostics, water safety	Animal tissue, egg, serum, urine, treated waste water, surface water, drinking water, ground water
Anthelmintic drugs	Aminomebendazole, avermectins, thiabendazole, triclabendazole	Food safety	Animal tissue, milk, eggs
Other human pharmaceuticals	β-blockers, anti-inflammatory drugs, psychiatric drugs, hormones	Food safety, water safety, forensics, toxicology	Urine, treated waste water
Drugs of abuse	Cannabinoids, cocaine, amphetamines, opioids, methadone, barbiturates, phencyclidine, benzodiazepines	Forensics, diagnostic toxicology	Urine, blood, meconium
Vitamins	Vitamin A, B1, B12, D, C, E, K, niacin, thiamin, riboflavin, biotin, folic acid	Control of food supplements and infant milk	Milk, beverages
Biocides	Pesticides, herbicides, fungicides	Food safety, feed control, water control e.g., banned products	Drinking water, surface water, ground water, soil, honey, fruits, vegetables, beverages
Combustion products	PAHs, PCBs, furans, dioxins,	Food safety, water safety,	Mussels, fish, feed, egg, milk, drinking water, air, soil
Proteins			
Allergens	Food proteins in milk, eggs, fish, peanuts, tree-nuts, sesame seeds, soybeans, celery, cereals, wheat, pollen	Food safety, diagnostics, air control	Extracted food, bioaerosols
Antibodies	Antibodies against infectious diseases, IgE antibodies against allergens, autoantibodies	Diagnostics, food safety	Animal and human serum, blood
Biomarkers	Biomarkers for cytokine network profiling, early diagnosis of cancer, sepsis, acute myocardial infection	Diagnostics, forensics	Blood, animal and human serum
Biotoxins	Abrin, botulinum toxins, cholera toxin, *Clostridium perfringens* toxins, diphtheria toxin, conotoxin, modeccin, ricin, Shiga-like toxin, Staphylococcus enterotoxins, tetanus toxin, verotoxin, viscumin, volkenesin	Food safety, biosecurity	Animal and human serum, blood
Microorganisms and viruses			
Pathogenic bacteria	*Bacillus spp*., *Campylobacter spp.*, *Clostridium spp*., *Cronobacter spp., Enterococcus spp.,* pathogenic *E. coli, Legionella spp., Listeria spp., Mycobacterium spp*., *P. aeruginosa, Salmonella spp., Streptococcus spp*., *Vibrio spp*., *Yersinia spp.,* antibiotic resistant bacteria (e.g., MRSA, VISA, ESBL, VRE, GRE)	Water safety, food safety, diagnostics, biosecurity, air control	Milk, dairy products, vegetables, fruits, drinking water, ground water, waste water, surface water, process water, bioaerosols, blood, serum, urine

Table 3 (continued)

Analyte classes	Analytes	Fields	Matrix
Pathogenic viruses	Adenoviruses, enteroviruses, hepatitis viruses, noroviruses, rotaviruses	Water safety, food safety, diagnostics biosecurity	Milk, dairy products, vegetables, fruits, drinking water, ground water, surface water, waste water, human and animal blood, bioaerosols
Pathogenic parasites	*Cryprosporidia spp.*, *Giardia spp.*	Water safety, diagnostics, biosecurity	Drinking water, surface water, food
Plants and animals			
Food authenticity	Alcoholic beverages, cereals and cereal products, cocoa, coffee, dairy products, fish and fish products, honey, meat and meat products, wine, vegetable oil and fats	Food safety	Extracted plants and animals, beverages
Genetically modified food	Genomic DNA of corn, crops, fruits, soy products vegetables, fruits, GM animals (GM salmon)	Food safety	Extracted plants and animals, extracted food, honey

contaminants in food, feed, or drinking water and in the environment [106, 107]. With regard to food and water safety, acceptance of non-culture methods is increasing for rapid analysis of pathogenic microorganisms by PCR methods or immunoassays [108]. The advantage of multiplex analysis of pathogens is that the quality of food is ensured because false negative results are reduced, and, second, measurement time and costs of reagents are minimized [109]. Reliable quantitative results from nucleic amplification tests will be important for food and water control. Therefore, defined calibration standards of organisms or nucleic acids are needed and validated procedures should be available.

To control and monitor the occurrence of natural or the anthropogenic contaminants, samples are taken at different stages in the food chain and analyzed for a variety of species as recommended by legislation. Liquid chromatography–mass spectrometry is a standard technique for determination of small molecules [110, 111]. The extraction and cleanup processes involve a variety of sample preparation steps [112]. For quantification of several classes of small molecules in food or water, a single method is often not sufficient. The multi-analyte methods LC–MS or LC–MS–MS are laborious and time-consuming for the complex matrices. These cost-intensive instruments are established for routine quality testing in large food-safety institutions or companies only. Microarray-based methods of analysis are, therefore, promising technology for covering the complete range of chemical and microbial species in food and water.

The dairy industry is one of the largest food markets. Because of the worldwide distribution of milk products, securing food safety must be monitored throughout the food chain. Anthropogenic and the natural contaminants from farming side can enter food via feed and water, as a result of poor hygiene, or as a result of veterinarian treatment. Food safety concerns in milk include such pharmaceuticals as hormones [113] and antibiotics [114–116], toxins (e.g. aflatoxin M1 [117], diverse staphylococcus enterotoxins [118, 119], *Bacillus cereus* enterotoxins [120]), and pathogenic bacteria [121]. In addition, concentrations in infant milk of such food supplements as vitamins are regulated [122].

Intensive quality testing of honey is needed because honey is generally regarded as a natural and healthy food product [123]. However, honey bees come in contact with agricultural environment, which is polluted by different sources of contamination. Species for the analytical microarray are mainly antibiotics, pesticides, pathogenic bacteria, and genetically modified organisms [124]. Antibiotics are the main class of contaminants from beekeeping. In European Union (EU) legislation, no MRLs for antibiotics in honey are defined because no residues are allowed. In some European countries, tolerable levels have been set in the last few years, and many honey products are now imported or mixed with honey from other countries outside the EU [125].

Growth-promoting agents, veterinary drugs, and chemical contaminants are either prohibited or MRLs are defined for food-producing animals and animal products [126]. Growth-promoting agents, for example hormones and β-agonists are prohibited. Defined MRLs are regulated for such veterinary drugs as antibacterial substances, including sulfonamides and quinolones, anthelmintics, anticoccidiostats, carbamates, pyrethroids, sedatives, and non-steroidal anti-inflammatory drugs, and for such environmental contaminants as organochlorine and organophosphorus compounds and mycotoxins [127]. Antibodies are available for many of these small organic molecules [128]. Avoidance of sample preparation is not possible for immunoanalytical methods in tissues. Meat juice, blood (serum or plasma), or urine are preferred matrices for rapid testing with analytical microarrays. Emerging pathogenic microorganisms and antibiotic-resistant bacteria are major meat-safety concerns in the 21st century [129]. In, e.g., porcine meat, the pathogens *Campylobacter* spp., *Yersinia* spp., *Salmonella* spp., *Trichinella* spp., hepatitis E virus (HEV), *Taenia* spp., and *Toxoplasma* spp. are of great interest for food safety and public health [130]. These pathogenic bacteria, viruses, and parasites are zoonotic agents. Zoonoses are infectious diseases that can be transmitted from animals, both wild and domestic, to humans [131]. Infected animals produce antibodies against these zoonotic pathogens. Blood sera of, e.g., slaughtered pigs, cows, chickens, and other poultry can easily be analyzed to determine the hygiene status of the animals.

Another aspect is food authenticity. Deliberate mixing of horse meat with ham products has resulted in Europe-wide comprehensive testing of ham products [132, 133]. Identification of different animals in food is possible by NAT [134], and automated systems would reduce costs and analysis time.

Food allergies are an important health problem in industrialized countries. Accidental or undeclared contamination of food products with allergens are a major risk for people suffering from food allergies. Food allergens include celery, cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk and dairy products, mustards, tree-nuts, and sesame seeds [135]. These allergenic foods are detected either by sandwich immunoassays [136] or by NAT methods [137].

Genetically modified organisms (GMOs) in food products must be declared [138]. Molecular methods have been investigated for detection and identification of such GMO products as maize, soybeans, and canola [139].

Monitoring of seafood poisoning is another issue for food safety. Filter-feeding organisms, e.g., shellfish, ingest phycotoxins by direct filtration of affected phytoplankton and accumulate these toxins [140]. Humans are infected by eating contaminated seafood. This problem is global, with a growing number of outbreaks of human poisoning. More than 60,000 human infections by aquatic toxins occur per year, with 1.5 % mortality [141]. The standard method for phycotoxin analysis is the use of mouse bioassays. This toxicological bioassay suffers from lack of sensitivity, no differentiation within the individual toxin groups is possible, and the assays are time-consuming [142]. Initial screening of seafood, directly at the harbor or on a ship, by multiplex microarray analysis, could replace mouse assays or HPLC–MS measurements.

Environmental matrices (soil, water, air)

Environmental matrices, i.e., soil, water, and air, constitute the largest pool of small organic molecules which occur as a result of industrial, agricultural, or medical pollution [143, 144]. These matrices are either accumulated in soil or transported in ground water or surface water by the rain. In addition, the water from waste water-treatment plants flows into surface water [145]. Emission of aerosols is another mechanism of transport. Chemicals that are not completely degraded are present in the environment and could be harmful to wildlife, agriculture, or humans. Organic micropollutants, for example hormones, pharmaceuticals, drugs of abuse, biological transformation products, biocides, sweeteners, and perfluorinated compounds are emerging contaminants in environmental water matrices [146, 147]. For all of these compounds, the antibodies will not be available, and some of the antibodies have been shown to have cross-reactivity with similar small organic molecules that are present at levels that are too high. Therefore, multi-analyte chromatographic methods are regarded as preferable for quantitative analysis. To monitor water-treatment technology and the quality of water itself, rapid and cost-effective methods are needed [148]. The number of analytes must be reduced to important indicator micropollutants, for example antibiotics (e.g., sulfonamides), pharmaceuticals (e.g., diclofenac), herbicides, and fungicides [149]. These indicators can be analyzed quantitatively by CL-MIA.

Pathogenic microorganisms [150, 151] and viruses [152], antibiotic-resistant bacteria [153, 154], and biotoxins [155] are emerging contaminants in water that should be reduced with high efficiency by drinking water-treatment plants because of acute health risks and sometimes even high mortality. Drinking water is either consumed directly or used as process water in industry or in agriculture. Drinking water should not cause illness, and, therefore, hygiene indicators, for example coliforms [156], *E. coli*, and *E. faecalis* are routinely analyzed in drinking water by cultivation methods. Rapid culture-independent methods, for example immunoassays or molecular methods would be accepted as alternative methods because the results would be comparable [157, 158]. Intensive research is needed to increase sensitivity (e.g., one colony-forming unit

of *E. coli* in 100 mL of drinking water and 1 rotavirus in 90 m^3 drinking water [159, 160]) and to enable differentiation between living and the dead microorganisms. NAMA methods with a live or dead marker [161, 162] in combination with effective concentration methods [163] could be a solution for rapid multiplex analysis of drinking water. More frequent measurement with more hygiene indicators would improve the quality of drinking water. Additional pathogenic indicators for drinking water, for example noroviruses, adenoviruses, rotaviruses, enteroviruses, *L. pneumophila*, *P. aeruginosa*, and *C. jejuni*, have been discussed [164].

Bioaerosols could contain a variety of biological materials, for example pollen, fungal spores, bacteria, or viruses [165]. New quantitative detection methods for pathogens and allergens are an emerging research field in aerosol science [166, 167]. The establishment of multi-analyte methods of quantification for pathogens in bioaerosols would be important for environmental monitoring. A current application is the rapid detection of pathogenic *Legionella spp*. in bioaerosols, because Legionella outbreaks with high mortality have often occurred in recent years because of contamination of artificial aquifers (for instance, by contaminated aerosols from cooling towers [168]). The main pathogen is *L. pneumophila*. Rapid methods of analysis are needed to identify the bioaerosol-producing source. A total of 15 serogroups are known for *L. pneumophila*. Multiplex subtyping on antibody microarrays would save time and the cost of monitoring of airborne *L. pneumophila*. Therefore, research on multiplex microarray analysis for bioaerosol characterization is a promising application.

Diagnostics, forensics, and toxicology

Chemiluminescence immunoassays and nucleic acid assays are important for routine applications in the clinical laboratory [169], and miniaturized analytical systems enable low-cost decentralized diagnostic testing in non-laboratory settings [170]. Several species can be analyzed in blood, plasma, serum, or urine. Research in genomics, proteomics, metabolomics, and other "omics" has identified new biomarkers suitable for multiplex diagnostics [171]. Antigen microarrays are used as high-throughput methods for screening for antibodies that indicate, e.g., autoimmune diseases [171], allergies [172], and infectious diseases. Additional applications for antibody microarrays are cytokine network profiling, sepsis, acute myocardial infarction, and early diagnosis of cancer [173]. Screening for small organic molecules in blood, plasma, serum, or urine is relevant to clinical and forensic toxicology [174, 175]. Rapid screening for drugs of abuse is, for example, important for rapid detection of suspected abusers [176, 177].

Biosecurity

Biosafety entails dealing with biologically active agents in a safe way. Biosecurity is the field that addresses unspecified biological material in, e.g., food, water, air, powder, or other matrices, that can cause death or diseases in humans, animals, or plants. There is a permanent risk that biological agents, for example pathogens and toxins, will be used intentionally or their use will be threatened. Biological weapons, bioterrorist attacks, biocriminal actions, biological disasters, and civil protection for harmful bioagents are scenarios implying public-health preparedness for multiple biological species. Simple, rapid, inexpensive, and automated analytical systems are needed that can be used for biosecurity [178]. In principle, all of the species previously discussed as emerging contaminants in food, water, and air are important for biosecurity. On the basis of several characteristics, for example mortality, infectivity, potential for person-to-person transmission, and dread, the Centers for Disease control (CDC, USA) have prepared a priority list of bioterrorist agents [179]. Critical category A biological agents are *Variola major*, *B. anthracis*, *Y. pestis*, *C. botulinum*, *F. tularensis*, filovirus, and arenavirus. Critical category B biological agents are *C. burnetii*, *Brucella spp.*, *B. mallei*, *B. pseudomallei*, alphaviruses, *R. prowazekii*, biotoxins, for example ricin or SEB, *C. psitacci*, and food safety threats, for example *Salmonella spp*. and *E. coli* O157:H7, or water safety threats, for example *V. cholerae* and *C. parvum.* Critical category C biological agents are emerging-threat agents, for example nipah virus and hantavirus. Microarray-based analysis systems with sandwich MIA or NAMA test formats will be important for biosecurity because parallel monitoring of multiple species reduces costs and the time for preparedness [180].

Concluding remarks

Detection principles, analysis systems, and applications of CL microarrays in analytical chemistry have been reviewed. Two configurations for CL microarrays were compared: static-incubated CL microarrays in wells and tubes and flow-based microarrays. A variety of microarray-based fast assays could be implemented if flow channels were used. Limitations of flow-based CL microarrays include sample throughput for screening applications in routine laboratories with total sample numbers >100 per day. Sequential assay processes should be conducted in parallel on multi-channel microarrays for high-throughput applications. The optical readout is simple and portable. CL microarray analysis systems could be a promising development for the future. Static-incubated CL microarrays are more time-consuming and are not suitable for rapid multi-analyte applications. The performance of both approaches is compared in Table 4.

Table 4 Comparison of the performance of static-incubated and flow-based CL microarray analysis techniques

Performance	Static-incubated	Flow-based
Possibility of miniaturization	–	+++
Possibility of automation	++	+++
Throughput	+++	–
Rapidness	–	+++
Variability in test formats	–	+++
Variability in test processes	–	+++
Sensitivity	+++	+++

The first multiplex microarray analytical techniques that were reported were based on automated assay processes and CL microarray readout. A schematic overview of progress in the development of CL microarray analysis is presented in Fig. 7a. Over the last 15 years the first multiplex applications in analytical chemistry were established with CL microarrays. The main achievements are shown in Fig. 7b. Among requirements for quantitative multiplex analysis, CL microarrays must be reproducibly produced, transportable, active over several months, regenerable for internal calibration, and useable over a full working day. The most important point is that analytical CL microarrays are at least as inexpensive as the cost for one analyte. All analytical process steps in the microarray analysis should be performed automatically and rapidly, data processing of microarray images should be performed by the system, and instrumentation should be, at least, transportable or, even better, portable for decentralized applications.

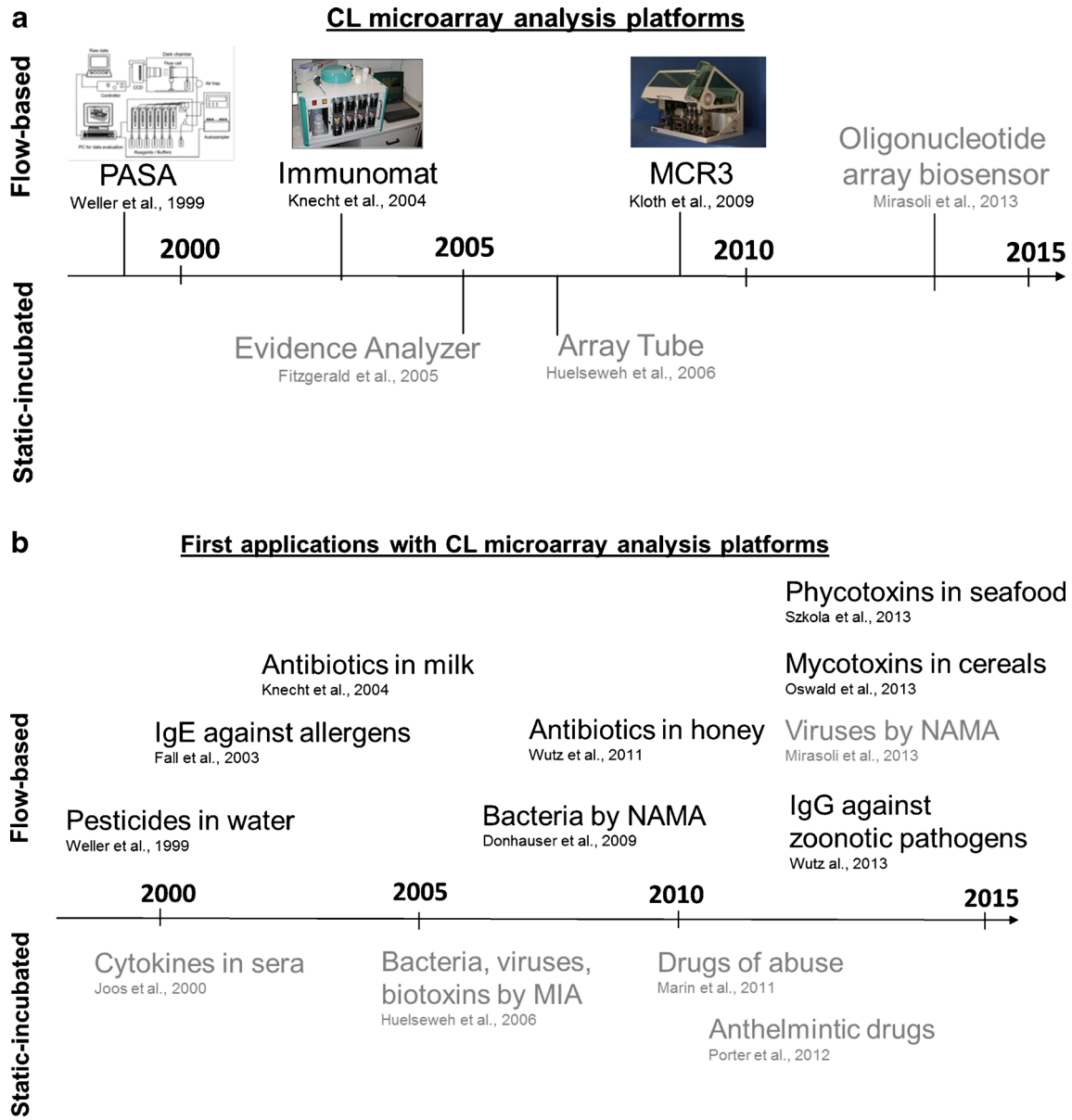

Fig. 7 Achievements in the development of CL microarrays (**a**) and their first applications in analytical chemistry (**b**)

The advantage of CL-MIA in contrast with multi-analyte liquid chromatography-based methods [181] is that little or no sample preparation is needed. Concerning trace analysis with detection limits below the specified analytical performance of CL-MIA, generic enrichment for, e.g., food, water, and blood must be combined as a hyphenated technique [182]. For simple and smart analysis, the sample should be automatically injected for microarray analysis, and quantitative data should be printed after a short while. For many food, water, or environmental samples, in-line coupling of sample preparation and multiplex microarray analysis will be important. In this manner, complex samples of totally different matrices could be analyzed in routine laboratories. NAMA methods have many sample-preparation steps that have to be processed in-line. Automated in-line techniques for molecular methods will increase the acceptance of decentralized applications.

Another point is the availability of sensitive and selective antibodies for multi-analyte immunoassays. Most of the antibodies were not screened by flow-injection assay, and cross-reactivity on microarrays were also not evaluated. Validation studies of multi-analyte immunoassays are more extensive than for conventional single-analyte immunoassays. As a consequence, screening of new selective receptors must be performed by use of the same microarray analysis technique for multiplex microarray analysis. In addition, intensive research on a suitable group of antibodies with low cross-reactivity in multiplex immunoassays is needed. The same applies to multiplex DNA microarray analysis. Evaluation of appropriate nucleic acid primers and probes is also very time-consuming. The design of a multi-analyte application is important. First, the choice of analytes for the multiplex microarray analysis should fulfill one complete analytical task. Second, the selective receptors used must be adjusted to the multi-analyte assay. If these factors are taken into account, multiplex microarray analysis is a powerful technique in analytical chemistry.

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