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Detection of *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Legionella pneumophila* in Water Using a Flow-Through Chemiluminescence Microarray Readout System

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Fast, sensitive, and especially, multianalyte test systems are currently of high interest for the monitoring and quality control of drinking water, since traditional microbiological methods are labor intensive and can take days until a response is achieved. In this study, the first flow-through chemiluminescence microarray was developed and characterized for the rapid and simultaneous detection of *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Legionella pneumophila* in water samples using a semiautomated readout system. Therefore, antibody microarrays were produced on poly(ethylene glycol)-modified glass substrates by means of a contact arrayer. For capturing bacteria, species-specific polyclonal antibodies were used. Cell recognition was carried out by binding of species-specific biotinylated antibodies. Chemiluminescence detection was accomplished by a streptavidin-horseradish peroxidase (HRP) catalyzed reaction of luminol and hydrogen peroxide. The chemiluminescence reaction that occurred was recorded by a sensitive charge-coupled device (CCD) camera. The overall assay time was 13 min, enabling a fast sample analysis. In multianalyte experiments, the detection limits were 3×10^6, 1×10^5, and 3×10^3 cells/mL for *S. typhimurium*, *L. pneumophila*, and *E. coli* O157:H7, respectively. Quantification of samples was possible in a wide concentration range with good recoveries. The presented system is well suited for quick and automatic water analysis.

Water monitoring and quality control play an important role in human health care. Besides pesticides, pharmaceuticals, and toxins, pathogenic microorganisms are the most dangerous water contaminants, which have to be detected and identified quickly in order to prevent an outbreak of water-borne diseases.[1] Water control is even of greater interest for military defense since pathogenic bacteria can be considered as possible biological warfare agents.[2] Emerging and known water-borne pathogenic bacteria are, among others, *Escherichia coli* O157:H7, *Campylobacter*, *Legionella pneumophila*, and *Salmonella*.[3,4]

In order to enable quick and early interventions after a contamination event, fast, sensitive, and especially multianalyte screening methods that can be integrated in automated systems are needed. Sensitivity, selectivity, reliability, and assay time are major limitations for most detection methods. Many pathogens have very low infectious doses, e.g., the doses for *E. coli* O157:H7 and *Salmonella* are as low as 10 cells, and these bacteria often exist within complex biological matrixes. In this way false-negative test results may arise.[2]

Until now, mainly microbiological methods have been applied for routine detection of bacteria, which include selective preenrichment steps through cultivation and a number of serological and biochemical tests for identification.[5,6] These tests usually provide for reliable and robust results (no false-positives), and only viable cells are detected. However, they are very time-consuming (e.g 18 h for *E. coli*; 10 days for *Legionella*) and labor-intensive. The number of samples for routine detection is limited. These methods are obviously not suited for the monitoring of drinking water.[7]

Other common tools for analysis of bacteria are based on biological recognition elements like nucleic acids and antibodies. Amplification of small amounts of genetic material is performed by the polymerase chain reaction (PCR). This technique is very sensitive ($10-10^3$ cfu) and specific but often requires intensive sample prepurification and skilled technical staff.[8,9] Immunological methods like enzyme-linked immunosorbent assays (ELISA) use specific antibody−antigen interactions for quantification. Herein the sensitivity of the method mainly depends on the affinity of the used antibodies. Reported detection limits are in order of 10^5

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Table 1. LODs, Linear Ranges, and Assay Times for the Detection of *E. coli* O157:H7 and *S. typhimurium* in Defined Matrixes[a]

technique	bacterium	matrix	time	linear range	LOD	ref
			(A) Nonflow-Through Microarray Readout Systems			
FS	*E. coli* O157:H7	PBS	~2 h 30 min	$(3 \times 10^6)-(9 \times 10^7)$ cells/mL	3×10^6 cells/mL	14
FM	*E. coli* O157:H7	culture	not quoted	10^4-10^8 cfu/mL	10^2 cfu/mL	15
CL	*E. coli* O157:H7	food	1 h	10^4-10^6 cfu/mL	10^4-10^5cfu/mL	20
	S. typhimurium	food	1 h	10^4-10^6 cfu/mL	10^4-10^5cfu/mL	20
	parallel detection of four different bacteria					
TMB	*E. coli* O157:H7	PBS	1−1.5 h	not quoted	5×10^3 cfu/mL	19
			(B) Flow-Through Microarray Readout Systems			
TIRF	*E. coli* O157:H7	food	30 min	$10^4-(5 \times 10^4)$ cfu/mL	5×10^3 cfu/mL	18
TIRF	*S. typhimurium*	food	15 min	$(8 \times 10^4)-(4 \times 10^5)$ cfu/mL	8×10^4 cfu/mL	17
TIRF	parallel detection of bacteria, viruses, proteins, toxins					16

[a] The used techniques are classified in (A) nonflow-through and (B) flow-through readout systems.

cfu/mL.[10] As a result, sample preconcentration steps like microfiltration and immunomagnetic separation become necessary for the detection of single cells. For the detection of microorganisms, sandwich ELISA formats are most common. This format is based on two separate recognition steps, which leads to high test specifity.[11] For the detection step, reporter molecules labeled with fluorescent dyes or enzymes are used.

Highly specific and sensitive antibody−antigen reactions are popular molecular recognition elements on microarray platforms.[12] The microarray technology represents a powerful analytical tool for the rapid and parallel detection of multiple analytes on a single device. In addition, microarrays have the potential to be integrated in automated fluidic systems for screening complex samples in high throughput.[13]

For the detection of microorganisms, some antibody microarray fluorescence readout systems that already have been reported use standard fluorescence detection methods like fluorescence scanners (FS)[14] and fluorescence microscopes (FM).[15] Moreover, new fluorescence techniques such as ultrasensitive total internal reflection fluorescence (TIRF) have been also applied.[16–18] The latter uses an evanescent field for excitation of a planar waveguide. In this way, ultrasensitive detection becomes possible. The TIRF technique is integrated in the NRL array biosensor, a portable flow-through microarray readout system with automated reagent supply that is capable of detecting up to nine target analytes simultaneously.[16]

Some classical sandwich ELISA approaches on antibody microarrays use the enzyme horseradish peroxidase (HRP) and colorimetric (e.g., 3,3',5,5'-tetramethylbenzidine (TMB))[19] or chemiluminescence (CL)[20] substrates. Chemiluminescence signals are detected on the spot by charge-coupled device (CCD) cameras. Advantages of chemiluminescence detection include high sensitivity and simple design of sensor setup, since no external light source or optics are needed. Moreover, matrix effects usually play a subordinate role.[21] Detection limits, linear ranges, and assay times of the indirect techniques listed above are summarized in defined matrixes for the detection of *E. coli* O157:H7 and *S. typhimurium* in Table 1. The techniques are divided in flow-through and nonflow-through systems. To our knowledge, *Legionella pneumophila* has only been detected with the label-free surface plasmon resonance (SPR) technique.[22] In single analyte experiments, a lower detection limit of 10^5 cells/mL was achieved.[23]

At our institute, an automated chemiluminescence microarray readout system for the rapid and simultaneous analysis of a variety of different analytes in fluid samples has already been developed.[24–26] In this sensor system, multiple ELISAs are performed simultaneously on a microarray platform. A horseradish peroxidase catalyzed chemiluminescence reaction is used for detection. For the quantification of microorganisms in water, an array-based chemiluminescence sandwich ELISA was developed. Herein, species-specific antibodies were immobilized on poly(ethylene glycol) (PEG)-modified glass surfaces presenting activated ester groups for covalent antibody coupling. In a recent paper, we have demonstrated that these functional PEG surfaces are highly suitable for antibody microarray applications.[27] Microorganisms, captured on such an array substrate were detected via specific

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secondary antibodies marked with biotin. After addition of horseradish peroxidase labeled streptavidin and chemiluminescence substrates, light was emitted where cells were immobilized. The presented flow-through microarray readout system is characterized by a short assay time (cell detection, 13 min) and a simple and user-friendly construction. Furthermore, the system offers the possibility to be integrated in inline monitoring systems. In this way, an automated sample preconcentration and prepurification unit can be coupled previous to the detection system. Such a monitoring setup should meet most of the requirements for an early warning system for water quality control with respect to bacteria. These demands include automatization, rapid response, and screening for a number of contaminants in parallel experiments with appropriate sensitivity.[7]

EXPERIMENTAL SECTION

Chemicals, Reagents, and Materials. Standard chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany) or VWR (Darmstadt, Germany). 3-Glycidyloxypropyltrimethoxysilane (GOPTS) was supplied by Fluka/Sigma-Aldrich (Taufkirchen, Germany). Dimethylaminopyridine (DMAP), di(*N*-succinimidyl)-carbonate (DSC), anhydrous dimethylformamide (DMF), triethylamine (TEA), Pluronic F127, trehalose, and casein were obtained from Sigma-Aldrich. Streptavidin labeled with horseradish peroxidase was purchased from Axxora (Lörrach, Germany). The chemiluminescence substrate SuperSignal ELISA Femto Maximum Sensitivity Kit was purchased from Pierce (Rockford, IL). Hellmanex II was obtained from Hellma (Müllheim, Germany). Phosphate buffered saline (PBS) containing 145 mM NaCl, 10 mM KH_2PO_4, and 70 mM K_2HPO_4, adjusted to pH 7.6 and was used as the buffer solution. PBS containing 2% casein (w/v) was applied as blocking solution. The running buffer consisted of 0.5% casein (w/v) in PBS (pH 7.6). For spotting, PBS with a final concentration of 10% trehalose (w/v) and 0.005% Pluronic F127 (w/v) was used. All buffers and solutions were prepared freshly prior to each experiment.

Menzel glass slides (26 mm × 76 mm × 1 mm) were purchased from Roth (Karlsruhe, Germany). Activation of the glass slides and cleaning steps were performed in plastic containers from Roth. Microtiter plates (384-well) used as substrates for the spotting solutions were obtained from VWR (BD FalconTM, reference 353265, natural polypropylene, flat bottom, nonsterile, Lot 06291155, low binding).

Diamino PEG 2000 (DAPEG, 2000 g/mol) was kindly provided as a gift by Huntsman Holland (Rozenburg, The Netherlands).

Antibodies and Antigens. Heat-inactivated *Escherichia coli* O157:H7 cells were purchased from Kirkegaard & Perry Laboratories Inc. (KPL), (Gaithersburg, MD). Heat-inactivated *Salmonella typhimurium* ATCC 14028 cells were a gift from the Instituto Zooprofilattico Sperimentale Dell'Abruzzo e del Molise "G. Caporale" (Teramo, Italy). Autoclaved *Legionella pneumophila* cells were a gift from the Max-Pettenkofer-Institut (München, Germany).

Polyclonal antibodies against *E. coli* produced in goat were purchased from Virostat (Portland, ME) and antibodies-online GmbH (Aachen, Germany). Goat anti-*E. coli* O157:H7 and goat anti-*Salmonella* were obtained from KPL (Gaithersburg, MD), rabbit anti-*Legionella pneumophila* from Biodesign (Saco, ME). Biotinylated antibodies against *E. coli*, *Salmonella*, and *Legionella* all produced in rabbit were purchased from Virostat (Portland, ME). Antihorseradish peroxidase developed in rabbit was obtained from Sigma-Aldrich (Taufkirchen, Germany).

Surface Chemistry for Antibody Microarrays. In order to remove glass dust and organic contaminants, glass slides were shaken overnight in a solution of 2% Hellmanex in water. The slides were washed with Millipore water and treated with a freshly prepared 1:1 mixture of 37% HCl and methanol for 1 h by shaking. After the slides were washed with Millipore water, they were shaken for 1 h in 99−100% H_2SO_4. The activated slides were cleaned with Millipore water and dried in a N_2 stream and in a drying cabinet at 70 °C for 15 min consecutively.

After activation, the slides were directly silanized with GOPTS by a sandwich technique for 1 h at room temperature in glass chambers. For creating the sandwiches, 600 μL of GOPTS were given to an activated slide that was covered with a second glass slide. The slides were separated in pure ethanol and were cleaned successively in ethanol, methanol, and ethanol by sonication for 15 min. Afterward, they were dried under a N_2 stream and in a drying cabinet at 70 °C for 15 min. The silanized glass slides were stored in vacuo at room temperature until further modification.

In order to minimize background signals a poly(ethylene glycol) diamine (DAPEG) film was built up on the silanized slides. PEG terminated surfaces are known to prevent the nonspecific binding of proteins and cells.[28] Furthermore, the PEG molecules (2000 Da) act as long cross-linkers between the solid substrate and the immobilized antibodies, which contributes to a good antibody exposure to the antigen. PEG layers were prepared by delivering approximately 1 mL of liquid DAPEG on a silanized glass slide and covering the coated slide with another silanized slide. The sandwiches were baked overnight in an oven at 100 °C. The slides were separated in Millipore water and were sonicated two times in Millipore water for 15 min. Finally, the slides were dried under a N_2 stream and in a drying cabinet for 15 min at 70 °C. The DAPEG-coated slides were stored in vacuo at room temperature.

For covalent immobilization of antibodies via free amines, the slides were further modified by reaction with DSC to obtain a *N*-hydroxysuccinimide (NHS) activated surface. For NHS activation, the sandwich technique was applied again. An amount of 600 μL of a solution of 40 mg of DSC, 2 mg of DMAP, and 63 μL of triethylamine in water-free DMF were dispensed on one DAPEG coated slide, which was then covered by a second one. The sandwiches were incubated for 4 h at room temperature in glass chambers. The sandwiches were separated in a chamber containing methanol and were cleaned twice with methanol by sonication for 6 min each time. Finally, the activated slides were dried under N_2 and stored in vacuo until printing.

Microcontact Printing of Antibodies. For all microarray immunoassays, capture antibodies were covalently immobilized onto NHS-activated DAPEG-functionalized glass substrates by means of the BioOdyssey Calligrapher miniarrayer from Bio-Rad (München, Germany). This contact printer provides temperature and humidity control. For measurement of *Escherichia coli* O157:H7, pAb *E. coli* from Virostat, pAb *E. coli* from Ab-online, and pAb *E. coli* O157:H7, for *Salmonella typhimurium*, pAb *Salmonella*,

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and for *Legionella pneumophila* cells, pAb *L. pneumophila* were immobilized as capture antibodies. Besides surface chemistry, antibody preparation plays an important role for the fabrication of high-quality antibody microarrays. The antibody has to be kept in an active state. This was achieved by using a spotting buffer containing PBS as well as 0.005% Pluronic F127 and 10% trehalose. Trehalose prevents denaturation caused by spot evaporation. Pluronic F127 is added because large polyethers are known to be strong protein structure stabilizers.[29] The influence of varied trehalose and Pluronic concentrations on antibody microarray production using the contact arrayer has recently been investigated by our group.[27]

The capture antibodies were prepared in spotting buffer and were deposited onto the activated DAPEG surfaces using a TeleChem Stealth SNS9 microspotting pin, which transfers a volume of approximately 1 nL of sample per spot. The humidity of the chamber was set to 50% in order to prevent spot dehydration. The cooling agent regulated by cryostat that cools both the printing area of the metal plate and the antibody source plate was adjusted to 15 °C. The air temperature inside the chamber was 20 °C. Aliquots (35 μL) of the antibody solutions in spotting buffer were transferred to the wells of a 384-well MTP, placed in the microcontact printer.

For measuring dose−response curves, a 7×6 array grid was defined by means of the software "Calligrapher" (Bio-Rad, München, Germany). Each of the seven printed samples were deposited in replicates of six spots with a spacing of 1100 μm. The grid spacing between the different rows was 1500 μm. In the first row, anti-horseradish peroxidase was printed in a final concentration of 0.1 mg/mL in spotting buffer (PBS/10% trehalose/0.005% Pluronic F127) that was used as a positive control for streptavidin-HRP. In the second row, spotting buffer without antibody was printed, which served as a negative control. Then capture antibodies against bacteria were spotted in the following order: 2−2.5 mg/mL goat anti-*E. coli* IgG from Virostat, 2−2.5 mg/mL goat anti-*E. coli* IgG from antibodies online, 0.5 mg/mL goat anti-*E. coli* O157:H7 IgG, 0.5 mg/mL goat anti-*Salmonella* IgG, and 2−2.5 mg/mL rabbit anti-*L. pneumophila* IgG. After printing, the microarrays were stored in Petri dishes containing 100 μL of water at room temperature overnight.

In order to saturate free binding sites of the microarray surface and to reduce background effects, the printed slides were blocked by rigid mixing in 50 mL reagent tubes containing blocking solution (PBS with 2% casein (w/v)). Additionally, the slides were incubated in blocking solution for 4 h by shaking. Eventually, the microarray slides were washed with Millipore water and stored in PBS at 4 °C until use.

Flow-Through Chemiluminescence Readout System and Assay Conduction. The chemiluminescence microarray readout system with an automated reagent supply is a further development of the setup described earlier.[26] Setup modifications include the insert of a hand operated distribution valve and a changeable flow cell. The flow cell was cut out of a silicone foil and holds a volume of 60 μL. This silicone foil was attached to a transparent polycarbonate slide possessing an inlet and an outlet connection. After the microarray was placed on top of the flow cell, the assembly was sealed with a cap by pressure. The pumping parameters of each pump including volumes, speed, intervals, and interruptions can be adjusted with the software Immunomat V7 from Atto-tec GmbH (Siegen, Germany). Since the positions of the selection valve have to be set manually, so far the whole assay protocol can only be executed semiautomatically. For each assay, the chemiluminescence reaction was generated with a 1:1 mixture of "Supersignal ELISA Femto Luminol Enhancer Solution" and "Supersignal ELISA Femto Stable Peroxide Solution". After insertion of the disposable antibody microarray in the flow cell of the flow-through chemiluminescence readout system, all steps of the sandwich ELISA were carried out there.

The general assay protocol for the measurement of cells that was used for all tests is summarized as follows: after a rinsing step with running buffer (PBS with 0.5% casein, 2 mL, 250 μL/s), 1.2 mL of cell solution in PBS was pumped over the chip, whereas the first 600 μL were pumped with a flow rate of 100 μL/s and the rest with a flow rate of 20 μL/s. This was followed by a washing step with running buffer (2 mL, 100 μL/s). Next, the chip was incubated with 1.2 mL of antibody dilution in PBS (typical antibody concentrations were 8 μg/mL). The pumping steps and volumes were carried out analogical to the cell incubation. After an additional washing step with running buffer (6 mL, 100 μL/s), 1.2 mL of a solution of HRP labeled streptavidin in running buffer at a final concentration of 0.1 μg/mL was passed over the chip using again the previously described incubation scheme. Then, the flow cell was washed with running buffer (8 mL, 100 μL/s), and consecutively each 600 μL of the two chemiluminescence substrates were simultaneously pumped into the flow cell at a flow rate of 100 μL/s. Finally, the chemiluminescence signals were integrated for 60 s. The general overall assay time was 13 min. After the measurements, the whole system was thoroughly cleaned with water and a solution of 1% (v/v) Hellmanex in water.

For the determination of dose−response curves in single analyte experiments the following concentrations were measured: 0, 3×10^3, 1×10^4, 3×10^4, 1×10^5, 3×10^5, 1×10^6, 3×10^6, 3×10^7, 3×10^8 cells/mL *E. coli* O157:H7 in PBS; 0, 1×10^5, 3×10^5, 1×10^6, 3×10^6, 1×10^7, 3×10^7, 1×10^8, 3×10^8, 1×10^9, 3×10^9 cells/mL *S. typhimurium* in PBS; 0, 1×10^4, 1×10^5, 3×10^5, 1×10^6, 3×10^6, 1×10^7, 3×10^7, 1×10^8, 1×10^9 cells/mL *L. pneumophila* in PBS. For the parallel detection, dilution series were prepared starting from a stock solution containing 3×10^7 cells/mL *E. coli* O157:H7, 1×10^9 cells/mL *S. typhimurium* and 1×10^9 cells/mL *L. pneumophila* by adding PBS. Accordingly, the following concentrations were measured: 0, 1×10^3, 3×10^3, 1×10^4, 3×10^4, 1×10^5, 3×10^5, 1×10^6, 3×10^6, 1×10^7, 3×10^7 cells/mL *E. coli* O157:H7; 0, 3×10^4, 1×10^5, 3×10^5, 1×10^6, 3×10^6, 1×10^7, 3×10^7, 1×10^8, 3×10^8, 1×10^9 cells/mL *S. typhimurium*; 0, 3×10^4, 1×10^5, 3×10^5, 1×10^6, 3×10^6, 1×10^7, 3×10^7, 1×10^8, 3×10^8, 1×10^9 cells/mL *L. pneumophila*.

Data Processing. The background originating from the CCD dark signal was recorded before measurement and was substracted from each image prior to evaluation. The signal intensities of each spot were integrated over seven pixels, and the mean value as well as the standard deviation of the integrals were determined for the six spot replicates per antibody. These calculations were also applied for pixels located on the chip background. The mean background integrals were then substracted from the mean spot integrals. Dose−response curves were obtained by plotting these

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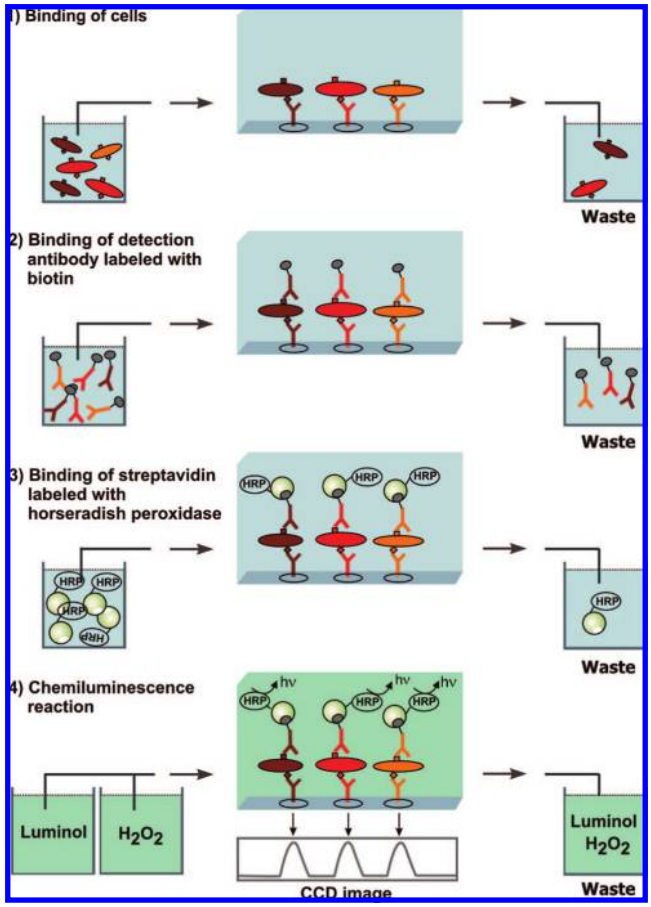

Figure 1. Scheme of the assay principle illustrated for the detection of three different bacteria on the antibody microarray surface.

background corrected chemiluminescence signals in a double-logarithmic scale against different concentrations of bacteria. The standard errors of the values are indicated in the diagrams as error bars. The limit of detection (LOD) was determined to be the concentration where S/N = 3 was reached, whereas the S and N values also represented integrated CL signals. For the determination of the linear ranges of the curves, the range of concentrations that best fitted the linear equation $y = mx + b$ were specified. The linear ranges were also plotted in a double-logarithmic scale. Cross-reactivities were calculated by correlating the mean spot CL signal integrals of the unspecific antibodies with the mean spot CL signal integrals of the specific antibody. The values are given in percent.

RESULTS AND DISCUSSION

Detection of Microorganisms on an Antibody Microarray Platform. For the quantification of bacteria, chemiluminescence sandwich ELISAs were performed on antibody microarray surfaces. Cells were captured by specific antibodies, which had been covalently immobilized on PEG modified glass substrates. All assay steps including consecutive addition of bacterial cells (Figure 1, step 1), species-specific biotinylated antibodies (Figure 1, step 2), horseradish peroxidase labeled streptavidin (Figure 1, step 3) and chemiluminescence substrates (Figure 1, step 4) were conducted with the flow-through chemiluminescence readout system.

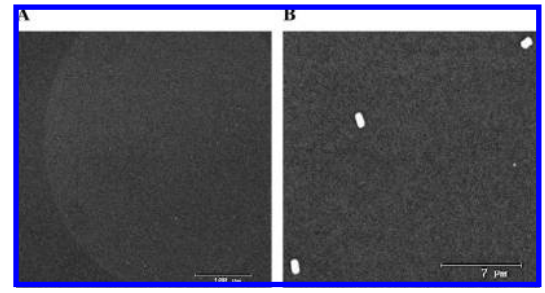

Figure 2. (A) SEM image of *E. coli* O157:H7 cells bound to anti-*E. coli* O157:H7 at a definite spot on the microarray surface. The small lightened points represent cells. The spot diameter is approximately 450 μm. (B) SEM image of immobilized cells with a resolution of a few micrometers.

In order to examine if whole cells and not only cell fragments could be immobilized on the antibody microarrays through antibody binding, the microarrays were examined both with a light optical microscope and a scanning electron microscope (SEM) (Figure 2) after cell detection. In a first experiment 3.6×10^7 heat-killed *E. coli* O157:H7 cells were measured with the flow-through chemiluminescence microarray readout system using an 4×6 anti-*E. coli* O157:H7 microarray. After washing and drying, the slide was examined under the light microscope. Accumulations of *E. coli* O157:H7 cells were observed exactly on these positions of the chip surface where anti-*E. coli* O157:H7 antibodies had been immobilized. The spots exhibited an average diameter of about 450 μm. In contrast, the average spot diameter determined by counting lightened pixels on the CCD images was significantly larger, namely, 566 μm. On CCD images, the spot diameters generally increase with rising chemiluminescence signals. In this case, spot sizes can be determined more precisely with the direct optical method. Apart from spot sizes, cell numbers on the spot regions could also be evaluated. On the 4×6 spot replicates, an average of 51 ± 5 *E. coli* O157:H7 cells were counted per spot, resulting in a total number of approximately 1.2×10^3 cells bound to the chip surface. In conclusion, only 0.003% of the *E. coli* O157:H7 positive control passed over the microarray surface and captured by the specific antibodies were intact cells. Since heat inactivated cells were measured, a whole pool of antigens including cell fragments that were not visible due to their small size may also have been recognized. Furthermore, the incubation time of the cells on the surface accounted for only 50 s. For the detection of intact cells usually much longer incubation times are needed (data not shown). Consequently, a key issue is to choose one of the two possible strategies: detecting intact cells that usually require relatively long incubation times or inactivating cells before measurement and detecting a large pool of antigens consisting of cells, cell fragments, and proteins, within a relatively short assay time. For a fast screening of pathogenic bacteria, the second approach would be more relevant. If a response is achieved, other detection techniques can be additionally applied in order to get information on pathogenicity.

The chip could also be examined with a scanning electron microscope after blotting with silver. In Figure 2A, a part of a spot presenting *E. coli* O157:H7 cells is illustrated. Figure 2B depicts three single cells from this spot region. The cells are rod shaped and are 1 μm wide by 2 μm long.

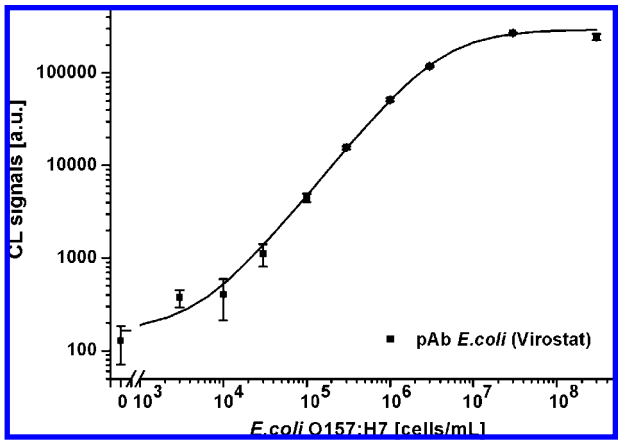

Figure 3. Dose−response curve ($n = 6$) for *Escherichia coli* O157:H7 (pAb *E. coli* (Virostat)).

Detection of Microorganisms in Individual Experiments. At the beginning, dose−response curves for *E. coli* O157:H7, *S. typhimurium*, and *L. pneumophila* were measured in single analyte assays on antibody microarrays, each containing six replicate spots ($n = 6$) of pAb *E. coli* O157:H7, pAb *E. coli* (from Virostat and Ab-online), pAb *Salmonella*, and pAb *L. pneumophila*. For detecting *E. coli* O157:H7, two different polyclonal antibodies against common *E. coli* antigens were deployed as capture antibodies in addition to the serotype-specific antibody. In order to determine the slide-to-slide reproducibility, two independent microarrays were measured for each analyte concentration. In Figure 3, an array dose−response curve for *Escherichia coli* is exemplified for the detection of heat-killed bacteria using the array-based chemiluminescence readout system. The curves show a wide linear range and reach a plateau at very high concentrations.

The biotinylated detection antibodies against *E. coli* and *Salmonella* showed no nonspecific binding to any of the immobilized capture antibodies. The LOD for these bacteria were determined to be the concentration corresponding to S(CL integral)/N(CL integral) = 3. In contrast, the biotinylated detection antibody against *Legionella* showed nonspecific binding to pAb *E. coli* (Virostat), pAb *E. coli* (Ab-online), and pAb *L. pneumophila*, resulting in average CL signal integrals 9−11 times higher than the corresponding background values. For *L. pneumophila*, the calculated mean CL signal integral resulting from the nonspecific binding event was subtracted from the average integrals obtained for measurement of relatively low concentrations of *Legionella*. Subsequently, the limit of detection was defined as the concentration corresponding to 3 times the background noise. The nonspecific binding event was only considered in the determination of the LOD because its contribution to the signal intensities at higher concentrations is not clear. Since more and more cells are bound to the capture antibodies, this effect may be reduced. For the higher concentrations, nonspecific binding events of cells to the microarray surface probably have a higher impact on the signal intensities than the nonspecific binding of the detection antibody.

The linear ranges of the dose−response curves were determined to be the concentration ranges that fitted the linear equation $y = mx + b$ best. The linear ranges of the assays are illustrated in Figure 4A−C. All sandwich ELISAs performed under flow conditions on the antibody microarray surface showed linear signal to

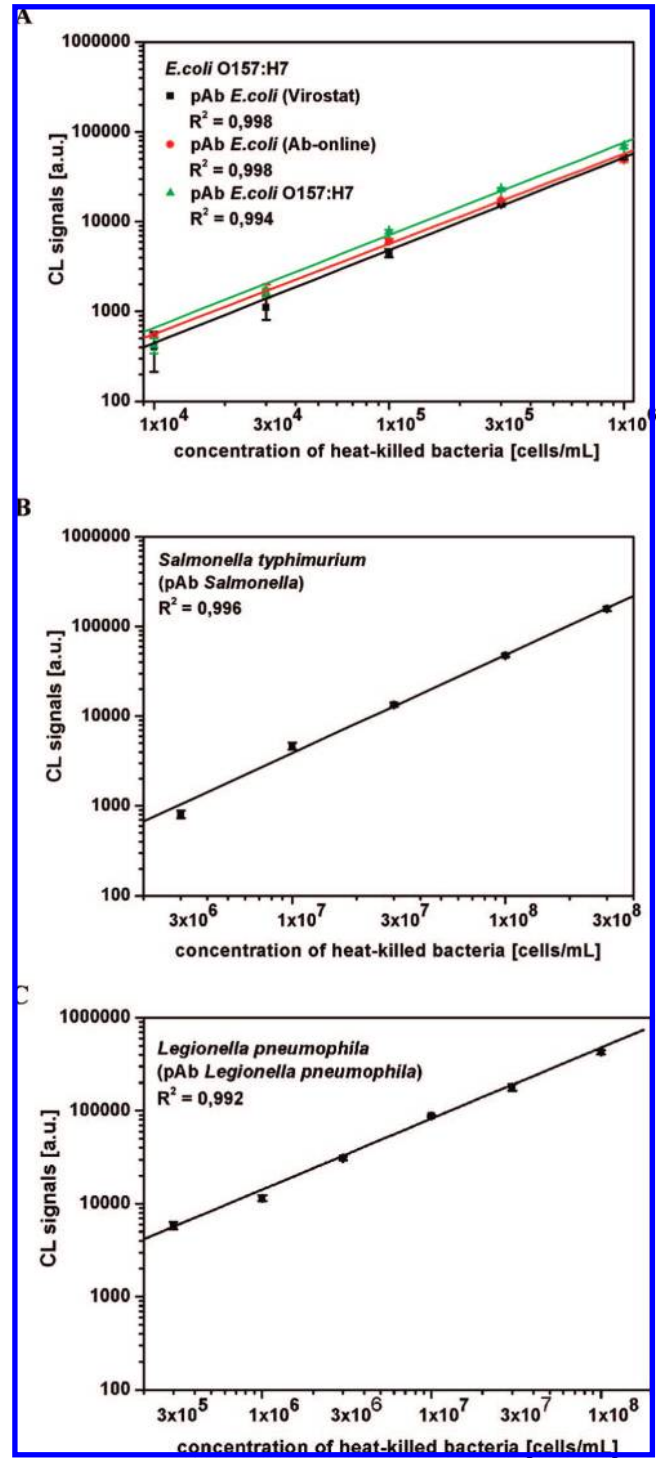

Figure 4. Linear ranges for the quantification of (A) *E. coli* O157:H7 (pAb *E. coli* (Virostat (■)), pAb *E. coli* (Ab-online (●)), pAb *E. coli* O157:H7 (▲)), (B) *S. typhimurium* (pAb *Salmonella* (■)) and (C) *L. pneumophila* (pAb *Legionella pneumophila* (■)). The intra-assay standard deviations ($n = 6$) are indicated as error bars.

concentration dependencies that covered at least 2 orders of magnitude with very high R^2. At analyte concentrations above the linear ranges, the curves began to plateau. In Table 2, the calculated LODs, linear ranges, as well as inter- and intraassay standard deviations for the measurement of *E. coli* O157:H7, *S. typhimurium*, and *L. pneumophila* with the corresponding antibodies are listed. The LODs, as well as the linear response ranges

Table 2. Linear Ranges, LODs, and Intra-($n = 6$)/Interassay ($n = 2$) Standard Deviations for the Detection of *E. coli* O157:H7, *S. typhimurium*, and *L. pneumophila* in Individual Experiments Using the Corresponding Antibodies

				standard deviation [%]	
bacterium	antibodies	linear range [cells/mL]	LOD [cells/mL]	intra-assay	interassay
E. coli O157:H7	pAb *E. coli* (Virostat) pAb *E. coli* biotin	$(1 \times 10^4) - (1 \times 10^6)$ $R^2 = 0.998$	1×10^4	10 ± 9	28 ± 23
E. coli O157:H7	pAb *E. coli* (Ab-online) pAb *E. coli* biotin	$(1 \times 10^4) - (1 \times 10^6)$ $R^2 = 0.998$	1×10^4	6 ± 3	26 ± 22
E. coli O157:H7	pAb *E. coli* O157:H7 pAb *E. coli* biotin	$(1 \times 10^4) - (1 \times 10^6)$ $R^2 = 0.994$	1×10^4	7 ± 3	20 ± 16
S. typhimurium	pAb *Salmonella* pAb *Salmonella* biotin	$(3 \times 10^6) - (3 \times 10^8)$ $R^2 = 0.996$	3×10^6	7 ± 4	17 ± 9
L. pneumophila	pAb *L. pneumophila* pAb *Legionella* biotin	$(3 \times 10^5) - (1 \times 10^8)$ $R^2 = 0.992$	1×10^5	7 ± 3	13 ± 8

achieved for the detection of *E. coli* O157:H7 were comparable to those reported for nonflow-through chemiluminescence detection methods and for highly sensitive internal reflection fluorescence sandwich assays performed with flow-through systems (see Table 1). The sandwich ELISA presented in this study is characterized by a short assay time of maximum 13 min in buffer, thus providing for fast response with appropriate sensitivity. The assay time is comparable to other flow-through microarray systems (see Table 1). The two tested serotype-unspecific anti-*E. coli* capture antibodies showed the same sensitivities and are therefore likewise applicable for the measurement of all kinds of *E. coli* species.

For the detection of *S. typhimurium*, the sensitivity was significantly lower. In this case, capture antibodies with higher affinities to the antigen are needed in the future. Nevertheless, quantitative measurements of *Salmonella* were possible in a wide concentration range.

The sensitivity achieved for the detection of *L. pneumophila* lies in the range generally observed for ELISAs. However to our knowledge, this is the first study presenting a sandwich ELISA on an antibody microarray platform for the detection of *L. pneumophila*. Detecting *Legionella* cells by a flow-through device within a short assay time is not trivial, since these cells reach up to 10 μm in length resulting in a correspondingly low diffusion coefficient. In conclusion, the LOD achieved in this experiment can be considered quite good.

The intra-assay standard deviations for all three bacteria were calculated from the average CL signal variations among the six replicate spots at each concentration detected. These intra-assay standard deviations were 10% at most, thus leading to the performance of highly reproducible sandwich assays on the chip surface. In contrast, the slide-to-slide reproducibilities were significantly lower. This can probably be traced back to the fact that, until now, the sandwich assay protocol for the chemiluminescence readout system can only be conducted semiautomated, since the positions of the selection valve have to be set manually. In this way, incubation times can vary, leading to lower/higher CL signal intensities. However, by integration of the selection valve in an automated process, the interslide reproducibility should increase dramatically. By a complete automatization of the whole readout system, the LODs should also be enhanced. Something that's worthy of remark is that the serotype-specific antibody against *E. coli* O157:H7 shows lower interslide variations than the antibodies that bind all kinds of *E. coli* species. Consequently, by applying antibodies that are more selective, assay reproducibility can be improved, too. Another factor that has to be taken into consideration is varying surface properties. However, we could already demonstrate that the applied surface chemistry provides highly reproducible antibody microarrays.[27]

In summary, it could be demonstrated that the flow-through antibody microarray is particularly suitable for the quantitative detection of bacteria in single analyte experiments within a short assay time.

Cross-Reactivity Studies and Signal-to-Noise Ratios. In order to enable the quantification of multiple analytes in one sample, cross-reactivities of capture antibodies toward unspecific antigens have to be excluded. Since the dose−response curves for single analytes were measured in the presence of capture antibodies directed against other species, information on cross-reactivity could also be obtained from these experiments. In this context, it was interesting to know whether it was possible to directly determine cross-reactivities on the chip surface by detecting only one bacterial concentration too.

Regarding *E. coli* O157:H7, nonspecific binding to pAb *Salmonella* and pAb *L. pneumophila* was neglectable (Figure 5A). In the range of $(10^5) - (3 \times 10^8)$ *E. coli* O157:H7 cells/mL, the cross-reactivity values (referring to the average spot CL signal integrals of pAb *E. coli* O157:H7 at each concentration measured) remained relatively constant (Table 3). Cross-reactivity of *S. typhimurium* to any of the other specific antibodies in the detected cell concentration range from (3×10^7) to (1×10^9) cells/mL could be neglected as well (Figure 5B). In this case, the observations were similar to these made with *E. coli* O157:H7. Within a certain range, the cross-reactivity values were relatively constant (Table 3). In conclusion, measurement of a single cell concentration would be sufficient within the specified test ranges in order to get cross-reactivity information of all used antibodies immobilized on the chip platform. Nonspecific binding of *L. pneumophila* cells to antibodies not specific to *Legionella* was not registrated (Table 3). In this case, only nonspecific binding of the detection antibody to the immobilized capture antibodies pAb *E. coli* (from Virostat and Ab-online) and pAb *L. pneumophila* was observed. An explanation could be that sugar residues located in the F_c-domain of the capture antibodies are recognized by some of the polyclonal detection antibodies.

The background signals increased slightly with rising bacterial concentration as a consequence of nonspecific binding of bacterial cells to the chip surface. In Figure 6, the calculated signal-to-noise ratios for the detection of *E. coli* O157:H7, *S. typhimurium*, and *L. pneumophila* are plotted in a double-logarithmic scale against

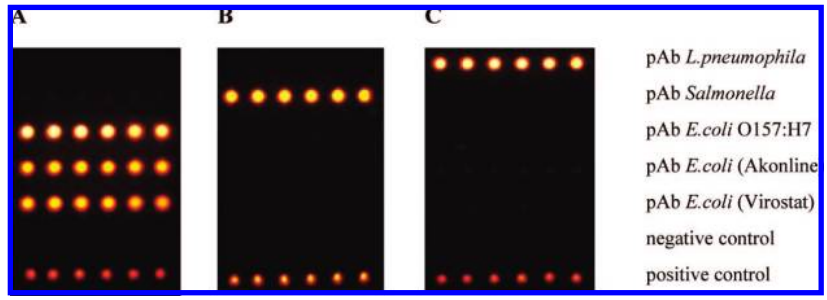

Figure 5. Chemiluminescence signals for the detection of bacterial cells illustrated in miscolors (signal integration: 60 s). The capture antibody positions are listed aside ($n = 6$). The positive control (anti-HRP) showed binding of streptavidin-HRP in all assays. (A) Detection of 3×10^8 *E. coli* O157:H7/mL. Spot signals were only observed where antibodies against *E. coli* had been immobilized. (B) Detection of 1×10^9 *S. typhimurium*/mL. Signals could only be registered at anti-*Salmonella* spot regions. (C) Measurement of 1×10^8 *L. pneumophila*/mL. Spot signals were observed where anti-*L. pneumophila* had been immobilized. Signals obtained for anti-*E. coli* resulted from nonspecific binding of biotinylated pAb *Legionella* to the immobilized antibodies.

Table 3. Cross-Reactivities of Immobilized Antibodies Against *E. coli* O157:H7, *S. typhimurium*, and *L. pneumophila* Determined in the Specified Bacterial Concentration Range

bacterium (detected concentration range)	antibody	cross-reactivity [%]
E. coli O157:H7 (10^5–(3×10^8) cells/mL)	pAb *E. coli* O157:H7	100
	pAb *E. coli* (Virostat)	67 ± 9
	pAb *E. coli* (Ab-online)	72 ± 9
	pAb *Salmonella*	0.52 ± 0.30
	pAb *L. pneumophila*	0.08 ± 0.06
S. typhimurium ((3×10^7)–(1×10^9) cells/mL)	pAb *E. coli* O157:H7	0.24 ± 0.10
	pAb *E. coli* (Virostat)	0.01 ± 0.08
	pAb *E. coli* (Ab-online)	0.16 ± 0.07
	pAb *Salmonella*	100
	pAb *L. pneumophila*	0.26 ± 0.23
L. pneumophila ((3×10^6)–(1×10^9) cells/mL)	pAb *E. coli* O157:H7	<0.01
	pAb *E. coli* (Virostat)	<0.01
	pAb *E. coli* (Ab-online)	<0.01
	pAb *Salmonella*	<0.01
	pAb *L. pneumophila*	100

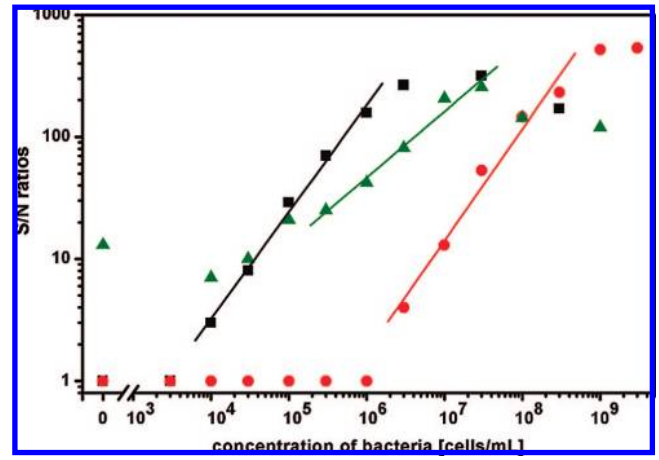

Figure 6. Correlations between the signal-to-noise ratios and the detected concentrations of *E. coli* O157:H7 (black ■); *S. typhimurium* (red ■), and *L. pneumophila* (green ■). Linear ranges are indicated in the plot.

the corresponding bacterial concentrations. The linear ranges of the S/N ratios are indicated in the plot.

Concerning the detection of *E. coli* O157:H7, the S/N ratio increased from 3:1 for 10^4 cells/mL to 315:1 for 3×10^7 cells/mL. However, at a concentration of 3×10^8 cells/mL the S/N ratio declined to 170:1. In this case, the background CL signal integrals were about 8 times higher than the blank values and the signal from the antigen binding event reached the plateau of the dose–response curve. With a focus on the detection of *S. typhimurium*, the background signals on the chip for the highest measured concentration were merely 4 times higher than the background signals of the blank. A maximum S/N ratio of 534:1 was reached at a concentration of 3×10^9 cells/mL. In conclusion, in the case of *S. typhimurium*, nonspecific binding to the microarray surface was not detectable at high concentrations. Regarding the values for *L. pneumophila*, the relatively high S/N ratio for the blank was noticeable. This could be contributed to nonspecific binding of the biotinylated detection antibody to the immobilized antibody against *L. pneumophila*. For *L. pneumophila*, the highest degree of nonspecific binding to the chip surface was observed. The background noise for the detection of concentrations above 3×10^7 cells/mL (S/N = 255:1) was up to 18 times higher than the blank background. Toward a concentration of 1×10^9 cells/mL, the S/N ratio decreased to 120:1. In conclusion, measurement of *E. coli* O157:H7 and *L. pneumophila* cells above 3×10^7 cells/mL is not reasonable. As a result, the working range of the *L. pneumophila* assay had to be reduced to (3×10^5)–(3×10^7) cells/mL. This was considered in Table 4. However, detection of these high cell concentrations should not be relevant with respect to quality control of drinking water.

Parallel Detection. By measurement of dose–response curves for *E. coli* O157:H7, *S. typhimurium*, and *L. pneumophila* in individual experiments, it could be demonstrated that the applied array format and assay protocol were suitable for quantifying cells with adequate sensitivity. In addition, the antibodies immobilized on the microarray surface showed negligible cross-reactivities to bacteria of other species. The next step was to investigate the ability of the antibody microarray to simultaneously detect the three different bacterial strains in the same sample. Therefore, dose–response curves for *E. coli* O157:H7, *S. typhimurium*, and *L. pneumophila* were measured in parallel. Starting from a sample containing 3×10^7 cells/mL *E. coli* O157:H7, 1×10^9 cells/mL *S. typhimurium*, and 1×10^9 cells/mL *L. pneumophila*, a dilution series was prepared. The diluted mixtures were measured subsequently by the readout system. In Table 4, the linear ranges and LODs of the resulting dose–response curves are compared to the values that were obtained in single analyte experiments. For the detection of *S. typhimurium*, *L. pneumophila*,

Table 4. Comparison of Linear Ranges and LODs of the Dose−Response Curves Obtained for Single and for Parallel Detection of *E. coli* O157:H7, *S. typhimurium*, and *L. pneumophila*

		linear range [cells/mL]		LOD [cells/mL]	
bacterium	antibodies	single	multianalyte	single	multianalyte
E. coli O157:H7	pAb *E. coli* (Virostat) pAb *E. coli* biotin	$(1 \times 10^4)-(1 \times 10^6)$ $R^2 = 0.998$	$(3 \times 10^4)-(3 \times 10^6)$ $R^2 = 0.992$	1×10^4	3×10^3
E. coli O157:H7	pAb *E. coli* (Ab-online) pAb *E. coli* biotin	$(1 \times 10^4)-(1 \times 10^6)$ $R^2 = 0.998$	$(3 \times 10^4)-(3 \times 10^6)$ $R^2 = 1.000$	1×10^4	3×10^3
E. coli O157:H7	pAb *E. coli* O157:H7 pAb *E. coli* biotin	$(1 \times 10^4)-(1 \times 10^6)$ $R^2 = 0.994$	$(3 \times 10^4)-(3 \times 10^6)$ $R^2 = 0.992$	1×10^4	1×10^4
S. typhimurium	pAb *Salmonella* pAb *Salmonella* biotin	$(3 \times 10^6)-(3 \times 10^8)$ $(R^2 = 0.996)$	$(3 \times 10^6)-(1 \times 10^9)$ $R^2 = 0.996$	3×10^6	3×10^6
L. pneumophila	pAb *L. pneumophila* pAb *Legionella* biotin	$(3 \times 10^5)-(3 \times 10^7)$ $R^2 = 0.992$	$(1 \times 10^5)-(1 \times 10^7)$ $R^2 = 0.998$	1×10^5	1×10^5

and *E.coli* O157:H7 (pAb *E. coli* O157:H7), the same LODs were reached as for the single analyte detection. Regarding the two serotype-unspecific *E. coli* antibodies, the sensitivity for the detection of *E. coli* O157:H7 was even improved. With a focus on the linear ranges, quantification of *E. coli* O157:H7 is still possible within 2 orders of magnitude, but a slight shift to higher concentrations is observed. In contrast, detection of *L. pneumophila* is possible in a lower concentration range. For quantifying *S. typhimurium*, the linear range is somewhat extended to higher concentrations.

Quantification of Samples. In order to validate the presented technique further, spiking experiments were performed and recovery values were determined. These measurements were conducted completely independent from the calibration experiments. This means that all the samples and assay reagents were prepared freshly prior to analysis, and the experiments were performed during different weeks. Moreover, different chip charges were applied.

The first samples were prepared containing both 3×10^4 *E. coli* O157:H7/mL, 1×10^7 *S. typhimurium*/mL, and 10^7 *L. pneumophila*/mL as single analytes as well as in mixture (mixture 1) for parallel detection. These experiments were repeated with samples spiked with 3×10^5 *E. coli* O157:H7/mL, 1×10^8 *S. typhimurium*/mL, and 10^8 *L. pneumophila*/mL; again in mixture (mixture 2) and as single analytes. For the determination of the recovery values of the single analyte detection, the corresponding dose−response curves were used, whereas the multianalyte curves were applied for the evaluation of the parallel detection. Each sample was measured twice using two independent chips.

In Table 5 the given and found concentrations are compared for single as well as for multiple analyte detection. Additionally, the slide deviations ($n = 6$) within the assays are listed. Both the recoveries of the single and the multiple analysis could be judged as quite good, particulary when taking into account that the microarrays and the calibration curves were generated on different days. Especially the found concentrations of *S. typhimurium* were matching the expected values. This could also be observed for the detection of 1×10^7 *L. pneumophila*/mL. Since quantification of *L. pneumophila* above 3×10^7 cells/mL is not reasonable in individual assays and quantification of *L. pneumophila* above 1×10^7 cells/mL is not possible in mixtures, the found values for the detection of 1×10^8 cells/mL are not quoted. Concerning the recoveries of *E. coli* O157:H7, a tendency to overestimate cell concentration was observable in all assays. In both mixtures, *E. coli* O157:H7 was present at low concentrations compared to the other two bacteria species. The concentrations of *Salmonella* as well as of *Legionella* were about 333 times higher, respectively. In this way, the capability of the system to detect a low concentration of one species in the presence of high concentrations of other species was confirmed.

Table 5. Comparison of Given and Found Concentrations for Single as Well as for Multianalyte Detection and the Corresponding Intra-assay ($n = 6$) Standard Deviations

		single detection		parallel detection	
bacteria/capture antibody	given [cells/mL]	found [cells/mL]	[%]	found [cells/mL]	[%]
E. coli O157:H7/ pAb *E. coli* (Virostat)	3×10^4 3×10^5	5×10^4 8×10^5	8.9 1.3	2×10^4 7×10^5	5.6 4.3
E. coli O157:H7/ pAb *E. coli* (Ak-online)	3×10^4 3×10^5	1×10^5 5×10^5	4.5 9.5	4×10^4 3×10^5	13.4 4.5
E. coli O157:H7/ pAb *E. coli* O157:H7	3×10^4 3×10^5	3×10^4 9×10^5	4.1 9.1	4×10^4 5×10^5	7.6 3.0
S. typhimurium pAb *Salmonella*	1×10^7 1×10^8	1×10^7 8×10^7	6.8 2.4	1×10^7 1×10^8	8.4 5.9
L.pneumophila/ pAb *L. pneumophila*	1×10^7 1×10^8	1×10^7 -	6.6 -	1×10^7 -	4.2 -

The mean intra-assay standard deviation for the single as well as the multiple analyte detection only accounted for 6%. With a focus on the interassay standard deviations, in the single analyte experiments a mean standard deviation of only 13% was observed, whereas standard deviations of 27% were obtained in the multianalyte assays. This can be explained due to the fact that the two microarrays that were measured to detect mixture 1 gave highly varying signal intensities, resulting in a mean standard deviation of 42%. In this case, the first of the two measured microarrays showed comparable low signal intensities. In contrast, the mean interassay standard deviation of mixture 2 only accounted for 7%. Poor slide-to-slide reproducibilities are a general problem related with disposable chips. To improve the interassay reproducibility, the whole detection process has to be automated. This will be an important task for future work. Nevertheless, the presented flow-through microarray readout system is suitable for the parallel and quantitative measurement of all three bacteria species in a wide concentration range within only 13 min.

CONCLUSIONS

Chemiluminescence sandwich ELISAs on antibody microarray platforms were developed for the detection of heat-inactivated

pathogenic bacteria such as *E. coli* O157:H7, *S. typhimurium*, and *L. pneumophila*. The sandwich immunoassays performed with the flow-through microarray readout system were very fast, completed after 13 min. In single analyte experiments with sample volumes of 1.2 mL each, the limit of detection for *S. typhimurium* in buffer was 3×10^6 cells/mL, whereas *E. coli* O157:H7 could be measured in concentrations as low as 1×10^4 cells/mL. For *L. pneumophila* a LOD of 1×10^5 cells/mL was achieved. All three bacteria could be detected in a wide linear concentration range covering at least 2 orders of magnitude. Moreover, to our knowledge this is the first study reporting the detection of *Legionella* by a sandwich ELISA on a microarray platform.

In the multianalyte experiments the LODs of the individual assays were confirmed. Regarding the detection of *E. coli* O157:H7, sensitivity was further enhanced by using the two serotype-unspecific antibodies. Consequently, it could be demonstrated that the applied analysis technique enables multianalyte detection of bacteria. Additionally, in recovery experiments it was shown that the system is suitable for quantifying bacteria mixtures.

The linear ranges, as well as the LODs, were comparable to those reported for other microarray immunoassays. However, the legal regulation in Germany requires the detection of 1 single cell per 100 mL of drinking water. In order to meet this demand and to achieve levels of bacteria detectable by the presented technique, sample preenrichment and preconcentration steps will be necessary prior to analysis. Consequently, for the flow-through chemiluminescence microarray readout system to be used in water monitoring, the system will have to be integrated in an inline setup with preenrichment modules, such as microfiltration and immunomagnetic separation. In this way detection times would increase, but the common microbiological enrichment methods still take much longer time and are more laborious. Furthermore for this application, an extension of the detectable microorganism spectrum would be important. The active area of the chip still offers considerable free space for this. Hence, an integration of a variety of additional antibodies is possible on the presented platform.

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