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Development of Rapid On-site Immunoassays for the Detection of Antibiotic Residues in Foodstuffs

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Für meine Familie

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ABBREVIATIONS

AgNPRs	Silver nanoprisms
ALP	Alkaline phosphatase
AuNPs	Gold nanoparticles
AuNRs	Gold nanorods
BSA	Bovine serum albumin
CCD	Charge coupled device
CLIA	Chemiluminescence immunoassay
DCC	N, N-dicyclohexylcarbodiimide
EFSA	European food safety authority
ECIA	Electrochemical immunoassay
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EU	European union
FAO	Food and agriculture organization
FIA	Fluorescence immunoassay
FITC	Fluoresceinisothiocyanate
GC	Gas chromatography
GlcOx	Glucose oxidase
HRP	Horseradish peroxidase
LC	Liquid chromatography
LFA	Lateral flow assay
LSPR	Localized surface plasmon resonance
MCR 3	Munich chip reader
MNPs	Magnetic nanoparticles
MRLs	Maximum residue limits
MS	Mass-spectrometry
NC	Nitrocellulose

NHS	N-hydroxysuccinimide
NPs	Nanoparticles
PBS	Phosphate buffered saline
PC	Polycarbonate
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PMMA	Polymethylmethacrylate
PNPP	P-nitrophenyl phosphate
PS	Polystyrene
TMB	3,3',5,5'-tetramethylbenzidine
WHO	World health organization
1F7	Anti-cloxacillin antibody (produced in our laboratory)

I. INTRODUCTION

1. Immunoassays for Antibiotic Residues

1.1. Antibiotic residues in foodstuffs

The finding of antibiotic active drugs can be traced back to the 20's of the last century. In 1928, Alexander Fleming accidentally discovered the first naturally occurring antibiotic, penicillin (DEMAIN und ELANDER, 1999). Today, antibiotics can be categorized into natural, semi-synthetic, and synthetic types based on their origins. They are widely used in veterinary medicine to treat animal diseases, prevent microbial infections, and improve product yield (CERNIGLIA und KOTARSKI, 1999; TOLLEFSON und MILLER, 2000; GRAVE et al., 2006). On the other side, antibiotics, as the most important and most frequently used group of veterinary drugs, are of great concern to regulatory agencies and consumers.

Various antibiotic families are used in veterinary medicine:

- β-Lactam antibiotics
- Aminoglycosides
- Tetracyclines
- Macrolides
- Pleuromutilins
- Lincosamides
- Quinolones
- Diaminopyrimidines
- Chloramphenicol and its analogues
- Ionophores

Sulfonamides, representing a group of synthetic bacteriostatic compounds, are also popular drugs because of their wide spectrum of antibacterial activity.

The improper or prolonged use of antibiotics can result in the appearance of residues in products of animal origins, such as milk, honey, meat, liver, kidney, eggs, etc. (LEE et al., 2001). The presence of antibiotic residues in animal products has a negative impact on public health. Particularly drug induced allergy,

disruption of gut microbiome, and increased occurrence of strains of bacteria resistant to antibiotics are among the most relevant risks (BOISSEAU, 1993; KELKAR und LI, 2001). Although most antibiotics are not toxic at their level of residue, some of them may exhibit toxicological effects and are responsible for triggering intense immune reaction (BORRIE und BARRETT, 1961). In addition, residues of antibiotics may contribute to the promotion of resistant bacteria, and moreover, food-producing animals may act as carriers of resistant bacteria and cause diseases (AARESTRUP et al., 2008; AARESTRUP et al., 2010). Therefore, much attention has been paid in recent years to the problem that the treatment of food-producing animals possibly increases the frequency of bacterial strains resistant to antibiotics (LAXMINARAYAN et al., 2013). Apart from this, antibiotic residues in raw milk also represent a technological hazard for the dairy sector (MITCHELL et al., 1998), because starter cultures for producing cheese, yoghurt, or other dairy products are extremely sensitive to some antibiotics, particularly penicillin, and thus can be inhibited in the case of processing antibiotic contaminated milk.

In order to control antibiotic residues, the World Health Organization (WHO), Food and Agriculture Organization (FAO), and the European Union (EU) have established a series of strict measures. For example, maximum residue limits (MRLs) for veterinary drugs, which are allowed to occur in foodstuffs have been set within the EU (EUROPEAN COMMISSION, 2010). Consequently, there is an increasing demand for efficient and exact analytical methods applicable to monitor antibiotic residues in edible animal products, in order to protect human health and avoid negative influence on food industry.

Microbial inhibition assays are the classical method to determine antibiotic residues in foodstuffs (BABINGTON et al., 2012). Until now, they are still widely used as a qualitative, simple, and rapid screening method. Main advantages of this assay are its cost-effectiveness and the simple-to-operate feature. Thus, a large number of commercial tests have been developed for screening the antibiotic residues in foodstuffs (KANTIANI et al., 2009). However, there are also some outstanding weaknesses of this method, which include the lack of specificity, non-quantitative output, and long incubation times (COMPANYO et al., 2009; DE BRABANDER et al., 2009). For quantitative analysis, instrument-based analysis methods are regularly used, such as liquid chromatography (LC) and gas

chromatography (GC). Most often, these methods are combined with mass-spectrometric (MS) detectors. These technologies are highly sensitive, specific, and reliable, and therefore represent nowadays the gold standard for quantitative determination of antibiotic residues (HOLSTEGE et al., 2002; KANTIANI et al., 2010). However, the advanced analytical equipment is expensive and relies on well-trained operators. Besides, most often complicated sample pre-treatment and time-consuming operating steps are required. As a result, the advanced instrument-based analysis cannot be extensively used for on-site detection or in resource-poor regions.

1.2. Immunoassays for antibiotic residues detection

Immunoassays have been employed in a wide range of methods to detect a number of compounds in the field of medicine, environment, and food analysis (SAMARAJEEWA et al., 1991; SADIK und VAN EMON, 1996; HARTMANN et al., 2009). The core principle of immunoassay is the specific recognition of an antigen by a specific antibody. Figure 1 illustrates some procedures and principles involved in the generation of immunoassays. The commonly used immunoassay formats for antibiotic detection in foodstuffs are enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CLIA), fluorescence immunoassay (FIA), electrochemical immunoassay (ECIA), immunochromatographic assays, and other advanced immunosensors.

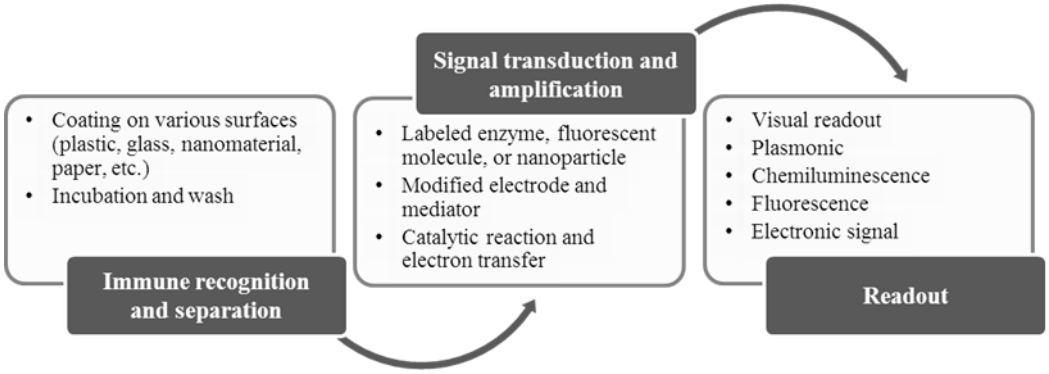

Figure 1: Schematic representation of basic immunoassay steps and principles used for detection of veterinary antibiotic drug residues.

In immunoassays, target recognition during immunoreaction is the initial step. In this phase, antibodies are a key factor for a successful immune recognition and to a large extent dictate the performance of an assay. Various types of antibodies have been used for sensing of antibiotics in food, such as polyclonal, monoclonal,

and recombinant antibodies (GAZZAZ et al., 1992; DILLON et al., 2003; CHEN et al., 2009). By applying antibodies with high quality, the demand on high sensitivity, high specificity, and low cross-reactivity in immunoassays can be fulfilled. In the second step, best described as signal transduction and amplification, labeled immunoreagents play an important role. For this purpose, antibodies or antigens are labeled with enzymes, fluorescent molecules, or nanoparticles, enabling the transformation of binding kinetics to detectable signals. A broad panel of commercial conjugates can be used directly in immunoassays, such as horseradish peroxidase (HRP), alkaline phosphatase (ALP), or glucose oxidase (GlcOx) labeled secondary antibodies. Alternatively, fluorescent compounds and functionalized nanoparticles can also be employed for the establishment of immunoassays (BEYZAVI et al., 1987; HAGE, 1995). In addition, more and more new signal-transduction pathways were also intensively investigated in recent years allowing a more sensitive and straightforward detection. In the final assay step, readout techniques are dependent on the previous steps. Colorimetric, fluorescent, luminescent, and electronic signals can be quantitatively determined by specific detectors or suitable particles enable a qualitative readout by the naked eye. Traditional ELISA methods are based on enzyme-catalyzed (HRP or ALP) reaction resulting in the generation of an optical signal. By using chromogens such as tetramethylbenzidine (TMB) or p-nitrophenyl phosphate (PNPP) the terminal measurement of absorbances by a UV-vis photometer is enabled. In CLIA luminol/hydrogen peroxide is used as a standard substrate. In electrochemical immunoassays, potentiometric, capacitive, and amperometric detectors characterized by a broad linear range and low detection limit have been used for detection (RICCI et al., 2007).

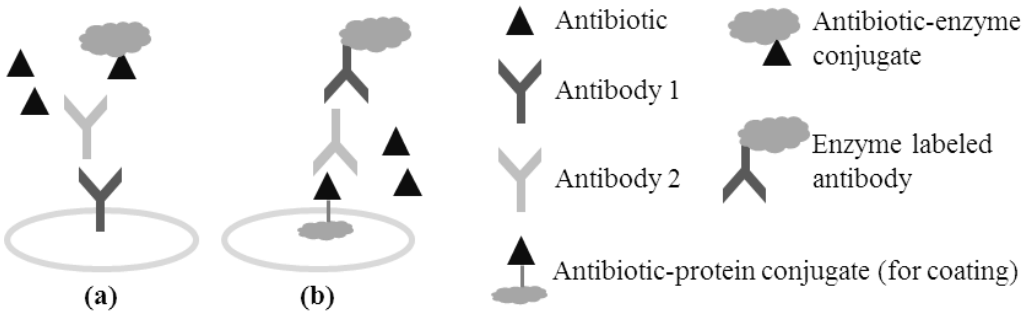

Figure 2: Two different typical formats of ELISAs for antibiotics determination: (a) direct competitive assay format; (b) indirect competitive assay format.

Immunoassays have been extensively used in the field of food analysis for antibiotics determination (SAMARAJEEWA et al., 1991). Because antibiotics are small molecular analytes, a competitive format of ELISA is typically used (Fig. 2). In competitive immunoassays, antibiotic conjugates or antibody are first immobilized. After the competition reaction, signal is quantitatively produced by enzyme. A major issue of traditional immunoassay is the involvement of several time-consuming operating steps and consumption of relatively high quantities of expensive reagents. The total incubation time for most of the ELISAs using 96-well plates is more than 4 hours. Taking into account the lengthy coating step, the whole assay takes 1 day.

The main challenge of applying immunoassays for antibiotics determination is to develop simpler and more efficient methods for on-site analysis. Therefore, recent researches focused on the integration of advanced materials and other techniques into the conventional immunoassay system. The following section focuses on two typical emerging technologies, namely microfluidics and nanotechnology.

2. Immunoassays using Microfluidic Systems

Microfluidics, as a new advanced technology, with significant advantages, can positively influence the performance of immunoassays in several aspects (LIN et al., 2010). The advantages include less reagents consumed, shorter assay time expended, sensitivity improved, reliability increased, and higher throughput achieved. In recent years, microfluidic has been employed in various immunoassays, and it turned out that direct, competitive, or sandwich assay types are compatible with the microfluidic format (HENARES et al., 2008; GUO et al., 2015). Microfluidic technology enables researchers from different fields to perform experiments in micrometer dimensioned channels. As a benefit of miniaturization, the ratio of surface area to volume is much higher and chemical interactions between the surface and solution are more effective. In addition, pump systems linked with software can precisely and automatically control the movement of fluids. Accordingly, microfluidic immunoassay devices offer the possibility to integrate other micro-analytical units like valve, mixer, detector, etc., which can significantly improve the analytical performance towards a multi-analyte and user-friendly analysis system (CHOI et al., 2006; SUAREZ et al., 2009). Table 1 summarizes recent publications on the application of microfluidics in immunoassays in the field of food safety.

Table 1: Representative microfluidic chip-based immunosensors

Analytes	**Sample**	**Antibody**	**Detection signal**	**Ref.**
Antibiotics (6)	PBS	pAb/mAb	Optical Response Units	(LI et al., 2016)
Antibiotics (10)	Milk	mAb	Chemiluminescence	(KNECHT et al., 2004)
Antibiotics (4)	Honey	mAb	Chemiluminescence	(WUTZ et al., 2011)
Citrinin	Rice	mAb	Amperometric	(DIDAR et al., 2012)
Aflatoxin M_1	Milk	mAb	Electrochemical	(DILLON et al., 2003)
Testosterone	Serum and urine	mAb	Chemiluminescence	(WANG et al., 2016)

As a new advanced technology, microfluidics offer many advantages but, surprisingly, only a few of these technologies are commercially available, while most of them remain at the stage of proof-of-concept or academic papers. In order

to make a major impact on the commercial market, knowledge from different fields of science and engineering need to be combined and integrated into completely functional systems. The following present a brief summary of the three fundamental challenges, namely:

- Materials and surfaces
- Samples and reagents
- Signal and detection

2.1. Materials and surfaces

Due to microscale environment in microfluidic system, binding capacity of the applied materials and a robust surface modification are key factors for the fabrication of microfluidic immunoassay devices. A comparison of the characteristics of commonly used material properties is listed in Table 2.

Table 2: Comparison of three commonly used materials in microfluidic immunoassays.

	Silicon	**Glass**	**Polymers**
Substrate costs	High	Middle	Low
Fabrication costs	High	Low	Low
Surface modification	Well-developed	Well-developed	Well-developed
Optical transparent	No	Yes	Yes
Mass production	Yes	Yes	Yes

Silicon, glass, and polymers are used most commonly in microfluidic immunoassay chips. Silicon, as an intensively studied material, can be easily etched and processed into any geometrical microstructure. However, silicon is not optically transparent in the wavelength ranges (GARDENIERS et al., 2003), which are typically used for optical detection in bioanalysis systems. Therefore, silicon has been increasingly replaced by glass and polymer materials. Glass as a low cost material is characterized by outstanding optical properties and can be used in the UV/visible region. Therefore, it is compatible with most of the signal transduction and readout steps commonly employed in bioassay systems. Nevertheless, for the micro-fabrication of glass-based microfluidic structures, wet etching method is always required, which is very expensive and cannot fulfill the demand on high aspect ratio (channel depth/channel width) in microchannels

fabrication (BANGE et al., 2005). Due to the relatively low cost, high reproducibility, and simple fabrication process, polymers are alternative materials to silicon and glass (SIA und WHITESIDES, 2003). Several polymer substrates have been used for microfluidic immunoassays, such as polymethylmethacrylate (PMMA), polycarbonate (PC), polystyrene (PS), and polydimethylsiloxane (PDMS), the most extensively used material for immunoassay applications. PDMS is an elastomeric polymer that can be flexibly and rapidly fabricated under normal laboratory conditions and ensures the sealing of microchannels. Due to its chemical properties $(C_2H_6OSi)_n$ PMDS forms a hydrophobic surface, and it enhances the non-covalent interaction between material surface and the immunoreagents used for coating purposes (WANG et al., 2016; YU et al., 2016).

In order to further increase adsorption and other features of microfluidic chips, various modification methods have been investigated (ZHOU et al., 2012). For example, polyethylene glycol (PEG) is the most widely used chemical for modification, not only for changing the hydrophilic properties on the surface of microfluidic chips, but also for acting as a linker to enable further chemical modification (KLOTH et al., 2009b; LILLEHOJ et al., 2010). Besides that, activation of microfluidic chip surfaces by functional groups such as epoxy and aldehyde enhance specific adsorption, robustness, and regenerability (YAKOVLEVA et al., 2002; MEYER et al., 2015).

2.2. Samples and reagents

In general, all immunoassay steps, such as blocking, washing, mixing, immunoreaction, incubation, and signal generation, are carried out in multi-functional designed chips. As antibodies, enzymes, conjugates, and other reagents used in immunoassay are expensive, miniaturization of microfluidic systems can remarkably reduce consumption of these reagents and lead to low-consumption and -cost detection compared to conventional 96-well-based immunoassays.

In order to bring in and transport reagent/sample within the microfluidic system, many strategies have been described, such as capillary force, pressure, centrifugation, and electrokinetical driven approaches (GAO et al., 2005; HOSOKAWA et al., 2006; KNAUER et al., 2012; CZILWIK et al., 2015).

Similar to conventional immunoassays, the first process step is the attachment of antigens or antibodies to modified or unmodified surfaces of devices. On the

surface of microchannels this coating step is based on hydrophobic interactions or covalent binding. Recently, microbeads such as magnetic, polymer, and silica beads, which are used as a binding platform and dramatically increase the surface area, were implemented inside microfluidic systems (LEE et al., 2009; LEE et al., 2011; CZILWIK et al., 2015).

2.3. Signals and detection

Similar to traditional immunoassays, microfluidic chip-based platforms also need a label to amplify and conduct the signal. The current signal detection strategies used in microfluidic chips can be classified in two groups: optical and electrochemical methods. Generally, electrochemical methods are ideal to be incorporated into the microfluidic devices because of the small size of electrode (DE ANGELIS et al., 2007). Wang and his colleagues (ARAZ et al., 2013) developed an immunosensor based on an electrochemical detector, which was characterized by an improved analytical sensitivity. Moreover, by the use of an array of microelectrodes (DILLON et al., 2003), the performance of an electrochemical immunosensor for toxin determination was significantly enhanced in terms of shorter response time, greater sensitivity, and lower signal-to-noise ratio. However, compared with the electrochemical immunosensors, optical methods can be more directly adapted to current immunoassay platforms and are used more frequently nowadays (HENARES et al., 2008). The most commonly used optical detection techniques in microfluidic-based immunoassays are based on fluorescence and chemiluminescence because of their excellent sensitivity and applicability in microscale. Therefore, we mainly focus on optical methods in this section.

Chemiluminescent detection methods are the preferred optical readout due to the high sensitivity, fast response, and their compatibility with conventional immunoassay systems. Luminol is the commonly used substrate for the chemiluminescent reaction. As the result of the chemical reaction (Fig. 3a), the emission of light (hv) allows detection by a charge coupled device (CCD) camera without any extra light source (MARQUETTE und BLUM, 2006). In addition, fluorescence detection has also been integrated in microfluidic chips. Fluorescence intensity can be the result of the activity of an enzyme labeled immunoreagent. For instance, HRP-labeled antibodies oxidize the fluorescent

substrate (Amplex Red) and the measured signal is directly (sandwich EIA) or indirectly (competitive assays) proportional to the analyte concentration (Fig. 3b) (ENDO et al., 2005). In contrast, a more direct and time-saving approach is based on the use of antibodies directly labeled with fluorescent dyes such as the commercially available Alexa series, Cy series, and fluorescein isothiocyanate (FITC). In general, fluorescence signal can only be induced by applying an excitation source. Therefore, the detector system in a microfluidic device has to integrate both excitation and emission units that makes the whole microfluidic system more complicated (GUO et al., 2015).

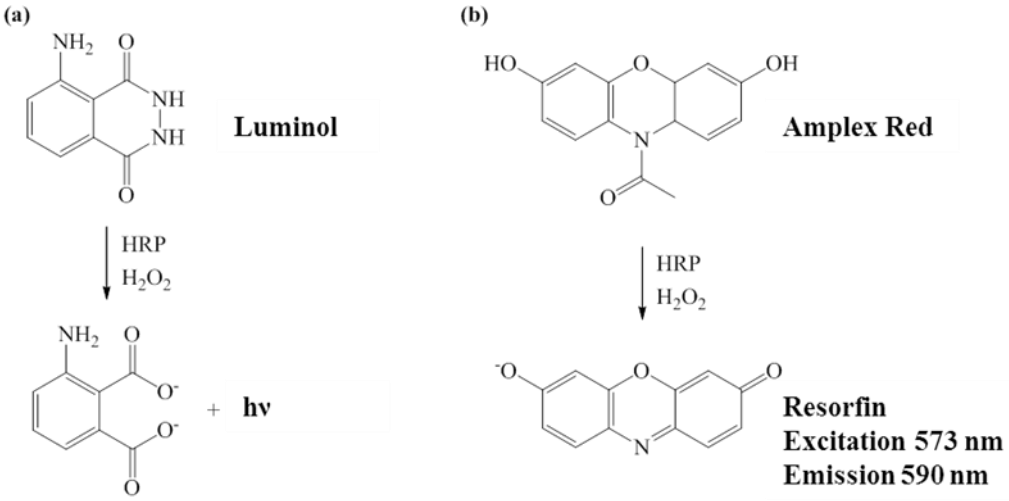

Figure 3: Enzyme-catalyzed activation of substrates used in CLIA and FIA: (a) Chemiluminescent reaction of luminol in the presence of hydrogen peroxide and HRP (b) HRP catalyzes the reaction of Amplex Red resulting in the formation of a fluorescent dye.

2.4. Microfluidic chip-based immunosensor for antibiotics detection

Some of recent studies on microfluidics- and immune reaction-based detection of antibiotics have focused on the multiplex determination of different classes of antibiotics in a single assay (HAASNOOT et al., 2003; KLOTH et al., 2009a; SUAREZ et al., 2009; WUTZ et al., 2011). For this purpose, microfluidic technology is ideal as it enables the integration of a large number of various antibodies within one test. For instance, HAASNOOT et al. (2003) and HUTH et al. (2002) described the parallel detection of four antibiotics based on commercially available microfluidic systems (Biacore 3000 system and Parallux processor). In order to break through the limit of parallelization degree, Munich Chip Reader (MCR 3) was developed for simultaneous detection of a broad range

of different antibiotics (KLOTH et al., 2009b). Eventually, the parallel analysis of 13 different antibiotics in milk was realized on one single immunochip, the assay was additionally characterized by an extraordinary short assay time of 6 min. These multi-antibiotic detection systems are fully self-contained, which means that immunoassay processing can be carried out automatically in the chip-based system.

3. Nanotechnology-based Immunoassays

A lot of nanoscaled materials, such as metal nanoparticles (NPs), semiconductor NPs, carbon nanomaterials, and hybrid nanostructures are increasingly used and, thus, shed a new light on traditional immunoassays (LEI und JU, 2012). Due to the immanent versatility of possible surface modifications and excellent optical properties, nanomaterials are becoming a powerful tool in immunoassay. In the following section, we will focus on the integration of nanomaterials into immunoassays, under two different aspects: (1) nanomaterials serve as carriers for signal amplification and (2) nanomaterials serve as optical signals.

3.1. Nanomaterials serving as carriers for signal amplification

Because of good biocompatibility and easy functionalization with biomolecules, NPs are excellent candidates for approaches aiming at the enhancement of performance of immunoassays. Among the described NPs, gold nanoparticles (AuNPs) are most widely used as biomolecule-functionalized carriers in immunoassays. An important feature is that the size, shape, and surface chemistry of AuNPs can be well controlled by various highly developed synthetic methods. Moreover, the surface of AuNPs can be easily modified with antibodies and other proteins due to electrostatic adsorption of the molecules in a non-covalent binding manner (MAIER et al., 2008; LIU et al., 2010). In an approach described by GAO et al. (2013), AuNPs were simultaneously labeled with catalase and polyclonal antibodies, thus improving the sensitivity about 20-fold compared to a conventional ELISA method.

Magnetic nanoparticles (MNPs) have also been used to improve the performance of immunoassays. Due to the unique magnetic properties, MNPs can be easily and efficiently separated in a magnetic field after conjugation processes. Modification of MNPs with proteins is always carried out using covalent attachment (BEVERIDGE et al., 2011; WANG et al., 2014). The advantages of covalent binding over non-covalent binding lay in the more stable conjugation and the higher coating density. In order to achieve a stable linkage between MNPs surface and the conjugating ligands, various reactions have been applied, such as amine-carboxyl coupling as well as click chemistry (LIU et al., 2013).

3.2. Nanomaterials serving as optical signals

Comparing to bulk materials, nanoparticles are perfect candidates for designing

colorimetric immunoassays due to their optical properties. For instance, well-dispersed spherical gold colloid is red-colored, a feature that can be used directly for naked eye readout. This colorimetric signal of nanomaterials is caused by localized surface plasmon resonance (LSPR) (ENGLEBIENNE, 1998) and the color of nanoparticle colloids highly depend on their morphology, particle size, and interparticle separation distances, which makes nanoparticles excellent signal transducers. Additionally, these optical properties enable extremely sensitive measurements and have been intensively applied in establishing novel colorimetric immunoassays (CHEN et al., 2011; BABINGTON et al., 2012).

3.2.1. Nanomaterials directly used as color labels

Due to the unique surface plasmon resonance effects, AuNPs show a red color, which is widely employed in the generation of immunochemical strip tests. In conventional 96-well-based ELISA, multiple steps including adding, washing, and signal generation are time-consuming processes, while in lateral flow assays (LFA) the whole detection takes only a few minutes. Figure 4 illustrates the typical LFA antibiotic detection principle.

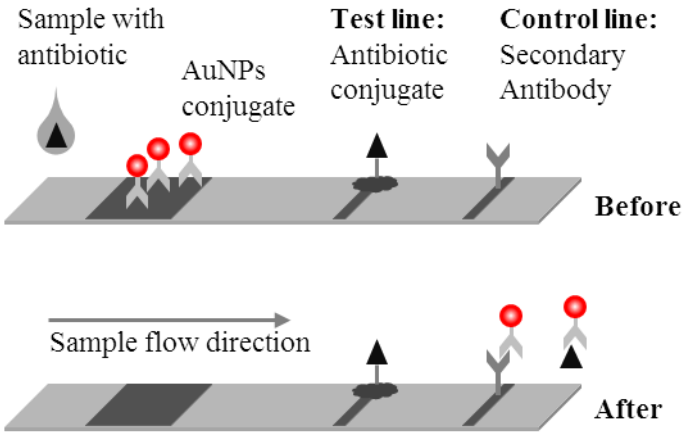

Figure 4: Schematic illustration of the typical LFA antibiotic detection principle.

In LFA, AuNPs-antibody-conjugates are pre-immobilized on the sample pad. While testing a liquid sample or extract, the AuNPs-antibody conjugate is mobilized and migrates with the liquid driven by capillary forces. If the sample contains no antibiotic, the conjugate binds to a pre-immobilized antigen-conjugate (test line). Thus, free antibodies concentrate on the test line and the binding leads to the formation of a highly visible line color due to the aggregated AuNPs. Overall, in the LFA, optical properties of AuNPs allow the generation of a

colorimetric signal at low concentration of AuNPs without an enzyme-mediated amplification step. Until now, LFA strips are one of the most successfully commercially developed devices and have a broad range of applications in on-site detection, such as antibiotics, toxins, and foodborne pathogens (HENARES et al., 2008; CHEN et al., 2009; XU et al., 2015). In addition, to further enhance the sensitivity of the LFA, several different signal amplifying strategies are proposed. For instance, the color of lines on LFA strips was further enhanced by the deposition of silver ions on the AuNPs (YU et al., 2015). Compared with the red color of AuNPs in traditional LFAs, the black lines caused by silver staining can be easier identified from the white background of the nitrocellulose (NC) membrane and enable a 2 to 2.5-folds improvement of sensitivity.

3.2.2. Inter-particle separation distances-based immunosensor system

The optical properties of nanoparticles are highly dependent on their inter-particle separation. For example, the color of gold colloid shows a pronounced shift from red to blue, when well dispersed particles become aggregated. The color changing caused by AuNPs aggregation enables the naked-eye readout and can be further quantitatively determined by photometer (Fig. 5). Hence, this approach has been widely applied for colorimetric immunoassays.

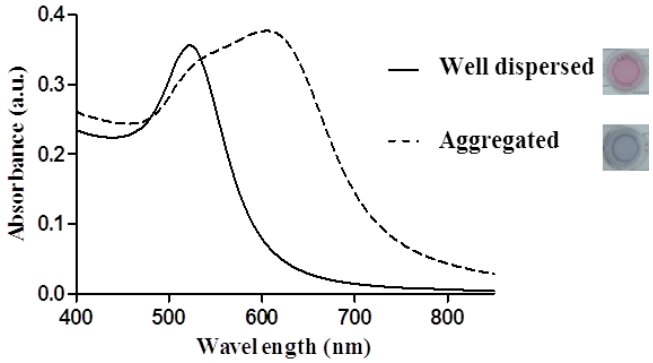

Figure 5: The UV-vis spectra of well dispersed and aggregated 20 nm AuNPs.

In order to implement inter-particle distance-based mechanism in conventional ELISA, various enzyme triggered AuNPs aggregation approaches were designed. For instance, acetylcholinesterase can catalyze the hydrolysis of acetylthiocholine to thiocholine, which is able to induce aggregation of AuNPs through gold-thiol interaction and electrostatic attraction (WANG et al., 2009; LIU et al., 2013). HRP and ALP, both representing typically labeling enzymes employed in

commercial ELISA kits, are also combined with AuNPs aggregation (XIANYU et al., 2014; XIANYU et al., 2015). Aptamers, as novel target recognition elements, showed some advantages when combined with AuNPs according to CHEN et al. (2013) and HE et al. (2013). In the presence of aptamers on the AuNPs surface, the stability of AuNPs was enhanced which prevented the occurrence of salt-induced aggregation. When the applied aptamers reacted with antibiotics, their conformation changed which resulted in AuNPs aggregation and allowed colorimetric detection.

3.2.3. Morphology-dependent immunoassay

Nanoparticles in colloids with different sizes and shapes show attractive optical properties. Therefore, changing the morphology of nanostructures has a great effect on the color of nanoparticle colloid. Recently, some colorimetric immunoassay studies focused on this phenomenon and novel morphology-dependent signaling mechanisms were developed (ZHENG und JIANG, 2016). Immunoassays using morphology-based signaling could follow two paths: (1) etching of nanoparticles and (2) growth of nanoparticles. In these approaches, bound enzyme catalyzes the conversion of the substrate to a reducing or oxidizing agent. As a result, nanomaterials are etched or grow into different shape, size, or structure.

Hydrogen peroxide is a powerful oxidizing agent, which can be generated by oxidation of glucose in the presence of glucose oxidase (GlcOx). The generated hydrogen peroxide can be used as an etchant to dissolve triangular silver nanoprisms (AgNPRs) into plate-like structures (ZHANG et al., 2011). Moreover, the etching caused a blue-shift in the absorbance peak from 680 nm to 580 nm and could therefore be applied to ELISA systems for ultrasensitive and quantitative detection (Fig. 6a) (LIANG et al., 2015). Another example is that during the etching process gold nanorods (AuNRs) were shortened (length: 65 nm → 50 nm → 42 nm → 26 nm) which resulted in a multi-color response (Color: orange → green → blue → pink) of the sandwich ELISA (Fig. 6b) (MA et al., 2016). For this purpose, the authors used catalase-labeled antibodies for decomposition of H_2O_2. Fenton's reaction was utilized to produce hydroxyl radicals, which acts as a more effective oxidant to etch the AuNRs (Fig. 6b).

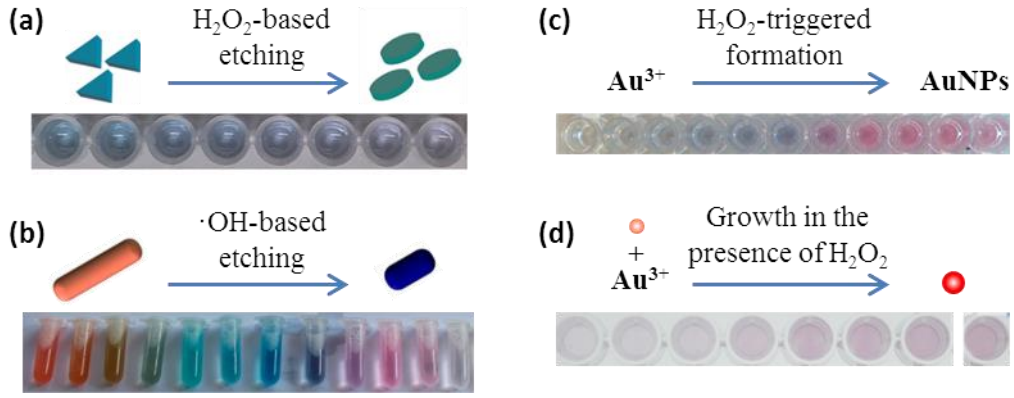

Figure 6: Variants of morphology-dependent immunoassays: (a) GlcOx-catalysed etching of AgNPRs. Reprinted from Biosensors and Bioelectronics, 69, LIANG et al., Silver nanoprism etching-based plasmonic ELISA for the high sensitive detection of prostate-specific antigen, 128-34, Copyright 2015 with permission from Elsevier; (b) hydroxyl radical caused AuNRs etching. Reprinted with permission from MA et al., 2016. Copyright 2016 American Chemical Society; (c) Catalase-mediated formation of AuNPs. Reprinted by permission from Macmillan Publishers Ltd: Nature Nanotechnology (DE LA RICA und STEVENS, 2012), copyright 2012 (d) GlcOx-based growth of small AuNPs. (YU et al., 2017)

In contrast to etching-based immunoassays, formation or growth of nanoparticles were also used to develop sensitive colorimetric methods (Fig. 6c and d). For instance, H_2O_2 can mediate the formation of gold nanoparticles. In the AuNPs growth approach, H_2O_2 acts as a reducing agent ($HAuCl_4 + H_2O_2 \rightarrow Au^0 + O_2$) (ZAYATS et al., 2005) and formation of Au^0 on the AuNPs surfaces results in the growth of small AuNPs. The absorbance of small AuNPs is much lower than the enlarged AuNPs. During the growth of AuNPs, colorless-to-purple change (Fig. 6d) can be easily readout with naked eyes and show a significant increase of absorbance.

4. Scope of the Thesis

The aim of this doctoral thesis was to improve the performance of conventional EIAs by integration of microfluidic and nanotechnologic principles. A cloxacillin specific EIA was used as a model for the detection of antibiotic residues in food.

The specific topics studied were:

1. Develop a straightforward fabrication of microfluidic chips and establish a microfluidic chip-based immunoassay platform.
2. Develop an AuNPs growth-based signal generation system and enable a naked-eye read-out for further on-site detection.
3. Combine these two novel techniques with current EIAs, using an indirect competitive assay format.
4. Evaluate the applicability of these two methods for the detection of cloxacillin in contaminated poultry and bovine samples.

II. PUBLICATIONS

1. Publication 1

Microfluidic Chip-based Immunoassay for Reliable Detection of Cloxacillin in Poultry

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ABSTRACT: By combining a chemiluminescence immunoassay with microfluidic chip technology a simple assay was established for the sensitive detection of cloxacillin in poultry samples. The chip used in this approach was composed of an upper microchannel layer and a lower flat base layer. Each of the formed microchannels has a volume of 18 µL, thus enabling a considerable reduction of reagent consumption as well as sample size needed for analysis by the indirect competitive immunoassay. To obtain labeled antigens for coating, the active ester method was employed to couple cloxacillin to glucose oxidase (GlcOx). The immobilized GlcOx-cloxacillin conjugate on the base layer competed with residues of cloxacillin in samples for binding sites of the monoclonal antibodies (mAb) against cloxacillin. Horseradish peroxidase-labeled anti-mouse IgG antibodies and chemiluminescent substrate were used for signal generation. Under optimized conditions, the IC_{50} and the limit of detection (LOD) of the assay were at 96.5 ± 9.37 ng/mL and 0.92 ± 0.07 ng/mL, respectively, and thus well below the maximum residue level of 300 ng/mL set by the European Commission. Recoveries for spiked chicken and duck samples were in the range from 94% to 118% with relative standard deviations lower than 11%. The results demonstrate that the microfluidic chip-based immunoassay can be used as a rapid and reliable platform for determination of cloxacillin in poultry.

Keywords: Microfluidic chips; Chemiluminescence; Immunoassay; Cloxacillin; Poultry

1. Introduction

Antibiotics are widely used to treat animal diseases, prevent microbial infections, and improve product yield. Cloxacillin is a member of the beta-lactam antibiotics family, which is used for the therapy and prevention of bacterial infections in farming practices. However, improper or prolonged use of cloxacillin in animals can cause accumulation in food-producing animals (Cerniglia and Kotarski 1999). These residues can cause not only a risk to human health due to allergic reactions and induction of antibiotic resistance in pathogenic bacteria, but also problems related to the environment, such as persistence of pharmaceuticals in soil and water (Awad et al. 2014; Lee et al., 2001; Shryock 1999). To avoid potential safety problems caused by cloxacillin residues in foodstuffs, the European Union (EU) has established maximum residue limits (MRLs) (European Commission 2010). The MRL for cloxacillin is 30 ng/mL in milk and 300 ng/mL in muscle. Therefore, in order to monitor cloxacillin residues, it is essential to develop sensitive and effective analytical methods.

For determination of cloxacillin and other beta-lactam antibiotics residues in foodstuffs, chromatographic methods such as liquid chromatography (LC) most often combined with mass-spectrometric (MS) detectors are regularly used (Camara et al. 2013; Perez et al. 1997), while for screening purposes microbial inhibition tests as well as enzyme immunoassays have been developed and extensively studied (Babington et al. 2012; Strasser et al. 2003). Nowadays, LC-MS/MS is widely used as a classical analytical method for quantitative determination of residues. However, these detection methods require both advanced instruments and well-trained personnel. Furthermore, complicated sample pretreatment steps are always needed. Conventional immunoassays are economical and highly sensitive, but experienced personnel are still mostly required. Therefore, it is desirable to develop an easy-to-perform, cheap, and highly sensitive method for the analysis of antibiotics in foodstuffs. A major problem of traditional immunoassay is the involvement of several time-consuming operating steps and consumption of relatively high quantities of expensive reagents. Many studies have attempted to develop a simple and rapid method for on-site analysis. Immunochromatographic assays possess several advantages over traditional immunoassays, such as low-cost, simple procedure, and rapid detection (Taranova et al. 2013). Nevertheless, due to the qualitative or,

at best, semi-quantitative output of immunochromatographic analysis a quantification of residues is not achievable. As an alternative immunochemical technique, an automated multi-analyte detection system based on a regenerable immunochip has been described representing a powerful tool for high-throughput screening of antibiotic residues in milk (Kloth et al. 2009); however, the sophisticated equipment needed, limits its broad application. Hence, it still remains a great challenge to develop high-efficient, sensitive, and simple immunoassay variants. Microfluidics is an emerging technology with significant advantages that can positively influence the performance of immunoassays in several aspects, such as decreased consumption of reagents, shortened assay time, and improved sensitivity. Microfluidic systems have been established as a new platform (Whitesides 2006) and are widely used in clinical diagnosis, bioanalysis, and genetic analysis (Chen et al. 2011; Lee et al. 2003; Pan et al. 2010; Shamsi et al. 2014). Recently, several sandwich ELISAs for high-molecular proteins were successfully combined with microfluidic systems (Fan et al. 2014; Fu et al. 2008; Li et al. 2016). However, the detection of small molecules such as antibiotics utilizing competitive immunoassay has not been investigated on this kind of chips. Additionally, none of the present systems was focused on food analysis.

Herein, we report the details of the development of a sensitive, indirect competitive chemiluminescence assay based on microfluidic immunosensing. By applying the microfluidic system, the amount of reagents and samples needed for analysis could be considerably diminished to the microliter level. To our best knowledge, this is the first microfluidic chip-based indirect competitive immunoassay platform reported for analyzing cloxacillin residues in poultry.

2. Materials and Methods

2.1 Chemicals

N,N-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), dimethylformamide (DMF), glucose oxidase, and cloxacillin were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Bovine serum albumin (BSA) was purchased from Amresco Inc. (Solon, U.S.A.). Silicone elastomer kit (PDMS base agent and curing agent) used as the polydimethylsiloxane (PDMS) layer, was purchased from Dow Corning Inc. (Midland, U.S.A.). Chemiluminescent substrate reagent kits were obtained from Millipore (Billerica, U.S.A.). Goat anti-

mouse IgG labeled with HRP was purchased from Jackson ImmunoResearch (West Grove, U.S.A.). Anti-cloxacillin antibodies (1F7) were generated in a previous study (Märtlbauer et al. 1994). All the other reagents required for the experiments were of analytical grade and used as received.

2.2 Synthesis of GlcOx-cloxacillin conjugates via DCC/NHS coupling

Cloxacillin was conjugated to GlcOx using the active ester method, according to an earlier described procedure (Usleber et al. 1994). Briefly, 2.46 mg cloxacillin, 11.06 mg DCC, and 3.09 mg NHS were dissolved in 0.2 mL DMF and incubated for 16 h by stirring in dark at room temperature to form active esters. 10.0 mg GlcOx were mixed with 2 mL $NaHCO_3$ (0.13 M in H_2O). The cloxacillin solution was then added dropwise to GlcOx solution and the mixture was stirred for 2 h at room temperature. The conjugates were purified by dialysis for 72 h against 3 changes of each 5 L PBS. The concentration of the GlcOx-cloxacillin was characterized by UV-Vis spectroscopy at $\lambda = 280$ nm (Specord 200 Plus, Analytik Jena, Germany).

2.3 Preparation of microfluidic chips

In this study, microfluidic chips were prepared as previously described (He et al. 2015; Zhang et al. 2015) with slight modifications. Briefly, the PDMS base agent and curing agent from Silicone elastomer kit were mixed 11:1 (v:v). Subsequently the mixture was poured into the mould and dried at 80 ^{o}C for 45 min on a heating plate. After cooling at ambient temperature, the cured microfluidic channel layer (60 mm × 60 mm × 5 mm) was carefully removed from the mould.

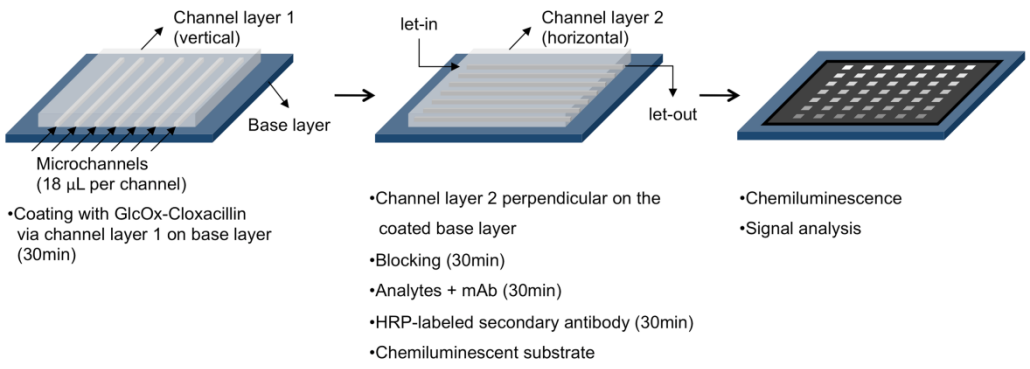

Fig. 1 Schematic illustration of microfluidic chip-based immunoassay for determination of cloxacillin in poultry

2.4 Microfluidic chip-based immunoassay

Coating: as shown in Figure 1, the channel layer was placed onto the base layer to form 7 microchannels with inlets and outlets. 18 μL reagents for each channel could be injected from inlets into the channels and removed from outlets by a micropipette. First, GlcOx-cloxacillin conjugates were delivered on base layer via microchannels and coated for 30 min. After that, unadsorbed GlcOx-cloxacillin conjugates were pipetted out from outlets. Subsequently, the upper channel layer was taken off and a new channel layer was placed perpendicularly onto the coated base layer. The free binding sites on the base layer were blocked with 3% BSA in PBS for 30 min and the solvents were removed from the outlets, followed by a washing procedure with 0.05% Tween-20 in phosphate buffered saline (PBST).

Assay: for the competitive reaction, cloxacillin of varying concentrations was mixed 1:1 (v:v) with 1F7. After incubation for 30 min, the solutions were injected into the microchannel chip and left for another 30 min. Unbound antibody and free antigen were then removed by washing the chip three times with PBST. Then, goat anti-mouse IgG labeled with HRP (1:200 in PBS) was added and incubated for 30 min. After that, the channels were washed and the second channel layer was taken off. The chemiluminescent substrate reagent from Millipore was added upon the base layer. All the incubations were carried out at room temperature. The chemiluminescence images were captured by a self-designed imager, signal intensity was analyzed by ChemiScope Analysis, and expressed as gray value (GV) (Fan et al. 2014).

2.5 Evaluation and optimization of microfluidic chip-based immunoassay

To investigate the influence of the coating hapten and antibody concentrations on the assay characteristics, checkerboard titrations were performed using different dilutions of coating antigen (40, 20, 10, 5, 2.5, and 1.25 μg/mL) and anti-cloxacillin mAb (20, 10, 5, 2.5, 1.25, 0.625, and 0.313 μg/mL) in the absence and presence of cloxacillin as competitor (10 ng/mL) in buffer solution (Figure 2). The %inhibition value was used to evaluate the sensitivity (Eq. 1).

$$\%\text{inhibition} = 100 - \frac{\text{GV in presence of competitor}}{\text{GV in absence of competitor}} \cdot 100 \quad \text{(Eq. 1)}$$

Standard calibration curves under optimized conditions were established and obtained by plotting GV against the cloxacillin concentration. The calibration

curve was analyzed with a four-parameter logistic equation using Prism 5 (GraphPad Software, La Jolla, USA). The limit of detection (LOD) was defined by a signal-to-noise ratio of 3.

2.6 Sample pretreatment

In order to obtain an effective and feasible sample preparation, which can be performed without use of expensive equipment or chemicals, meat juice was used throughout the whole study. Previous studies have shown that meat juice is a suitable matrix for the analysis of antibiotic residues and results were comparable to those obtained for extracts of muscle samples (Lamar and Petz 2007; Schneider et al. 2010). Poultry meat was purchased from local retail shops. Meat juice was obtained by freezing the poultry at -20 ^{o}C and defrosting at 4 ^{o}C followed by a juice collection using a funnel. The meat juices were spiked with cloxacillin at concentrations corresponding to 50%, 100%, and 150% of the MRL. Meat juice without cloxacillin was used as a negative control. The spiked meat juice was extracted with methanol-water (80/20, v/v) by vigorous vortex mixing for 4 min and the homogenate was centrifuged at 5000 × g for 10 min. The supernatant was collected for further use. Reproducibility of the assay was determined by three independent experiments.

3 Results and Discussion

3.1 Characterization of GlcOx-cloxacillin conjugation

GlcOx was used as carrier protein for microtiter plate coating due to its excellent properties, such as, high solubility, perfect stability, and easy coupling with haptens by common methods (Strasser et al. 2003). The GlcOx-cloxacillin conjugation was confirmed by UV-Vis spectroscopy. The results (Figure 2) show that the carrier has its main peak at 280 nm, while the hapten forms a shoulder at 250 nm and a small peak at 330 nm. In the UV-Spectrum of the conjugate an additional peak at 330 nm proved that cloxacillin was successfully coupled to GlcOx.

The GlcOx-cloxacillin conjugates showed a high affinity for the PDMS base layer as the conjugates could be successfully immobilized in only 30 min, in neutral buffer solution (PBS), and at room temperature.

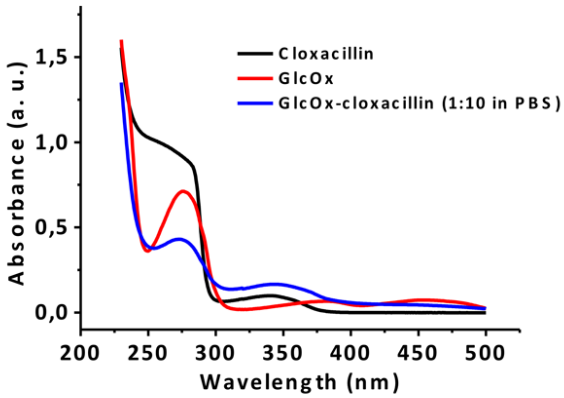

Fig. 2 UV-Vis spectroscopy of coating antigen. GlcOx-cloxacillin (1:10 in PBS buffer) compared to cloxacillin and GlcOx

3.2 Optimization of the microfluidic chip-based immunoassay

The microfluidic platform has been well demonstrated as an efficient strategy for improving efficiency and simplicity of immunoassay (Sun et al. 2014). In this study, the microfluidic chip served as basis for a competitive indirect immunoassay. For this kind of assay it is a well-known fact that the lower the concentrations of the immunoreagents the higher the sensitivity. However, too low measurement signals limit this approach. Therefore, the effects of coating concentration and the antibody dilution were investigated using a checkerboard assay with and without competition to optimize the assay sensitivity. The results of the checkerboard titration are shown in Figure 3. At GlcOx-cloxacillin concentrations of 20 and 40 µg/mL, a sufficient chemiluminescence signal was obtained. As documented in Table 1, the optimal reagent concentrations, reflected by high GV and a high %inhibition value, were found at coating antigen (GlcOx-cloxacillin) and antibody (1F7) concentration of 20 µg/mL and 2.5 µg/mL, respectively.

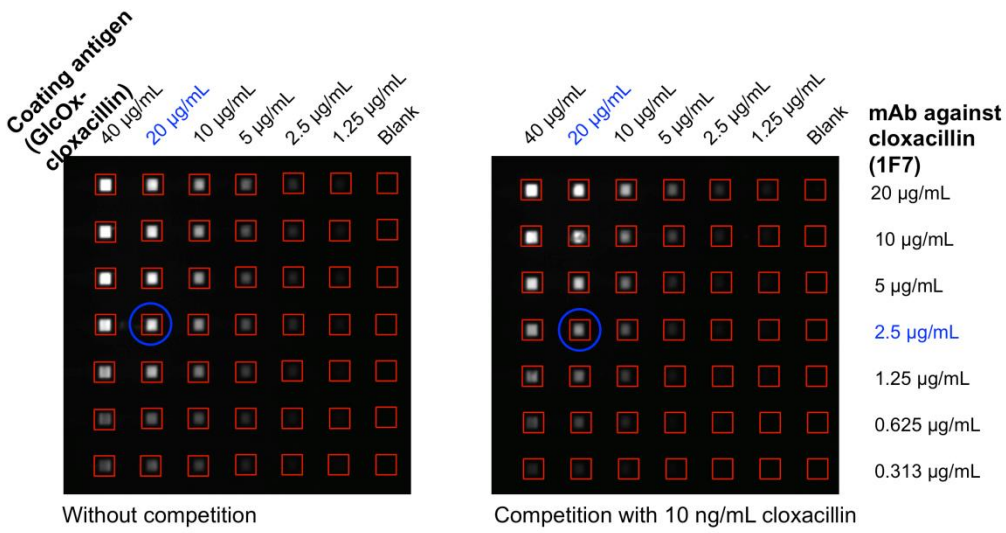

Fig. 3 Results of checkerboard titration with the chemiluminescence immunoassay in combination with the microfluidic chip in buffer solutions. The optimum concentrations of coating antigen and anti-cloxacillin antibody were 20 µg/mL and 2.5 µg/mL, respectively

Table 1 Optimization of GlcOx-cloxacillin conjugate and 1F7 concentrations. The Gray values were determined in the presence and absence of 10 ng/mL cloxacillin. Concentrations of 20 µg/mL for GlcOx-cloxacillin and 2.5 µg/mL for 1F7 were found as the optimized conditions in this experiment (in buffer solutions).

Reagent	Concentration (µg/mL)	Cloxacillin[a]		%Inhibition
		0 ng/mL	10 ng/mL	
GlcOx-Cloxacillin (at 1F7 = 2.5 µg/mL)	40	7344	5846	20.4%
	20	6742	5393	20.0%
	10	5539	4697	15.2%
	5	4669	4184	10.4%
	2.5	4248	4002	5.8%
1F7 (at GlcOx-cloxacillin = 20 µg/mL)	10	6767	6727	0.6%
	5	6798	6207	8.7%
	2.5	6742	5394	20.0%
	1.3	6001	5005	16.6%
	0.6	4986	4478	10.2%

[a]: Gray values

3.3 Analytical performance of the microfluidic chip-based immunoassay

Under optimized assay conditions, a standard curve for cloxacillin was prepared (Figure 4). In order to compensate for possible food matrix effects, standard curves were performed in PBS buffer containing 1% BSA. The standard concentrations ranged from 10000 to 0 ng/mL. The standard curve was obtained by plotting GV against the logarithm of the cloxacillin concentration. With increasing cloxacillin concentration, the GV decreased correspondingly. According to the triplicate test results, the assay is characterized by an extreme wide working range from 5 ng/mL to 1000 ng/mL. The IC_{50} value was at 96.5 ± 9.37 ng/mL and LOD was 0.92 ± 0.07 ng/mL.

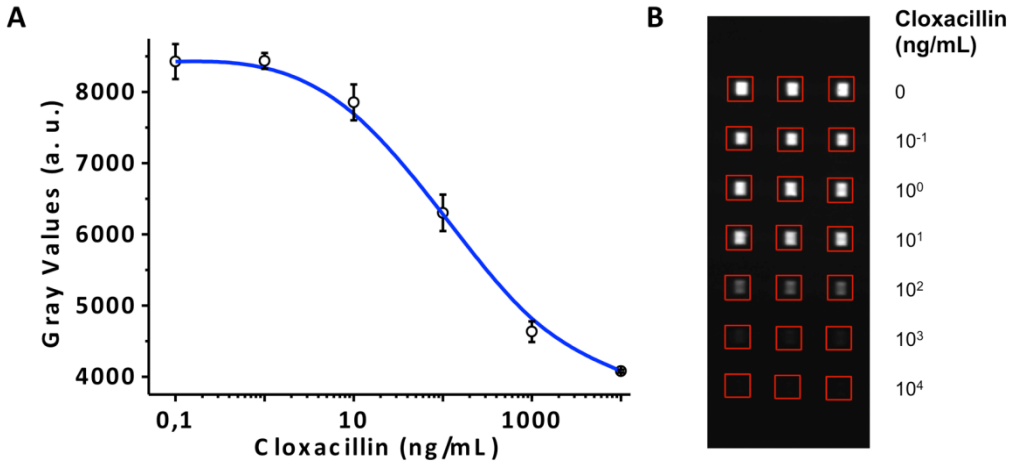

Fig. 4 Quantitative assay of cloxacillin using microfluidic chips. Assay performed in 1% BSA in PBS (n = 3). (A) Standard curve for the determination of cloxacillin (IC_{50} = 96.5 ± 9.37 ng/mL). (B) After inhibition of the assay by applying varying amounts of cloxacillin, the gray values changed gradually from gray to black

The applicability of this microfluidic chip-based immunosensor for the detection of cloxacillin in contaminated poultry was evaluated. Chicken and duck samples were spiked at levels of 0.5, 1, and 1.5 × MRL. Because of the complexity of the sample matrix, an extraction step was required. As shown in Table 2, the sample pretreatment enabled recoveries ranging from 95.2% to 117.7% with CVs of 3.6% to 6.7% in chicken and 94.0% to 116.8% with CVs of 3.5% to 10.5% in duck samples. This is comparable to the results of cloxacillin detection in poultry samples described by previous studies (Berendsen et al. 2013; Macarov et al. 2012).

Table 2 Recoveries of cloxacillin from spiked chicken and duck samples at different concentration levels (n = 3).

Matrix	Added (ng/mL)		Found (ng/mL)	Recovery (%)	Coefficient of variation (%)
Chicken	150	0.5 × MRL	176.5 ± 9.6	117.7	5.5
	300	1 × MRL	285.6 ± 19.2	95.2	6.7
	450	1.5 × MRL	460.1 ± 16.5	102.2	3.6
Duck	150	0.5 × MRL	146.9 ± 13.4	97.9	9.1
	300	1 × MRL	282.1 ± 29.5	94.0	10.5
	450	1.5 × MRL	525.7 ± 18.3	116.8	3.5

4 Conclusion

In this work, we have developed a straightforward and rapid method for the quantitative detection of cloxacillin. Unlike conventional ELISAs, which generally depend on the 96-well plate and TMB/hydrogen peroxide, the presented assay is based on a microfluidic chip and chemiluminescence. This assay format utilizes a very small sample size (18 µL per channel) and is characterized by a detection limit well below the current MRL. Furthermore, the established method is applicable for the quantification of cloxacillin in poultry samples. Therefore, it has great potential for the application in screening of cloxacillin in different food samples. Additionally, the assay format could be easily adapted to test for other residues and contaminants.

Compliance with ethical standards

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Conflict of interest: Wenbo Yu was financially supported by Zhonghechuang Investment Co., Ltd, China and Wuhan PriCells Biomedical Technology Co., Ltd, China. Yiping Chen and Xingyu Jiang received research grants by the CAS/SAFEA international partnership Program for Creative Research Teams. All other authors declare that they have no conflict of interest.

Research involving human participants and/or animals: Not applicable

Informed consent: Not applicable

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2. Publication 2

A Gold Nanoparticles Growth-based Immunoassay for Detection of Antibiotic Residues

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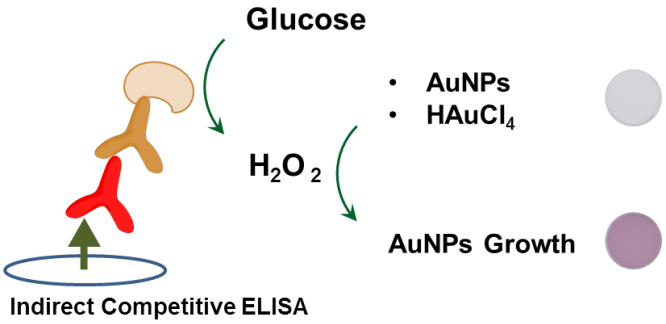
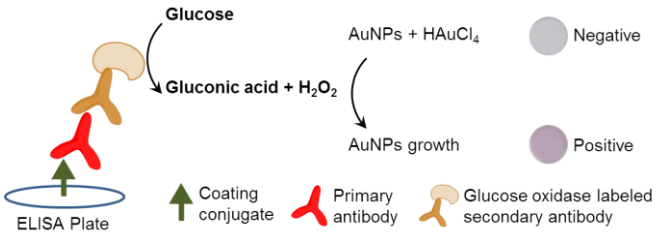
ABSTRACT: A quantitative immunosensor for determination of cloxacillin in foodstuffs was developed by combining glucose oxidase controlled growth of gold nanoparticles (AuNPs) with an indirect competitive enzyme-linked immunosorbent assay. The AuNPs growth-based approach allowed quick visualization of the results with the naked eye when the concentration of cloxacillin exceeded 11 ng mL^{-1}.



The frequent use of veterinary drugs in animal husbandry can result in occurrence of antibiotic residues in drinking water and foodstuff,[1] which can have serious adverse effects for consumers. The currently available quantitative analytical methods, such as chromatographic detection,[2, 3] require both sophisticated instruments and skilled technicians that limit their broad application. Enzyme-linked immunosorbent assays (ELISA) have been extensively used in the field of medical, environmental, and food analysis, because they are easy to operate and highly sensitive. However, essential equipment such as microplate reader or microarray system is not suitable for resource-poor regions or on-site detection.[4, 5] By contrast, advances in nanotechnology improve the efficiency of analytical chemistry in several aspects, such as excellent optical properties,[6] use of common methods for biomolecules conjugation,[7, 8] and high assay sensitivity.[9-11] In the past decade, researchers have developed a variety of enhanced plasmonic immunoassays combined with nanoscale materials.[12] However, previously established nanomaterial-based immunosensor strategies[13-15] cannot directly be applied to current commercial ELISA platforms and suffer from several drawbacks: (a) biomolecules have to be labelled in house because commercial conjugates are not available; (b) steps for modification of nanoparticles are time-consuming; (c) signal generation is often based on expensive materials.



Scheme 1 Schematic diagram of the plasmonic indirect competitive ELISA based on GlcOx-mediated 7 nm AuNPs growth.

Hence, it still remains a great challenge to develop low-cost assays, which are compatible with current standard commercial ELISA-based platforms. Here, we report a glucose oxidase (GlcOx)-mediated nanocrystals growth that is completely compatible with conventional ELISA formats (Scheme 1). GlcOx, used in this approach due to its low-cost and high catalytic efficiency, is one of the most frequently used enzymes for clinical screening, e.g. determination of glucose.[16-18] Basically, by means of GlcOx, H_2O_2 is generated through the oxidation of glucose. The obtained H_2O_2 acts as reductant for the formation of Au_0 from

$HAuCl_4$ in the presence of 7 nm AuNPs. This process leads to the growth of AuNPs and results in a colour change of the solution visible by the naked eye.[19-21] Since the H_2O_2 generation is controlled by enzyme activity, the absorbance of the enlarged AuNPs in solution is GlcOx concentration-dependent. Unlike previous nanomaterial-based immunosensors, which are always based on state change of AuNPs from dispersion to aggregation and a concomitant red-to-blue colour change,[22-24] in this approach, the enhanced purple colour is dependent on 7 nm AuNPs growth and can be easily readout with a standard ELISA reader or the naked eye. A similar assay principle has been successfully used in a sandwich assay to detect high molecular weight cancer biomarkers at the attomolar range.[15] However, time-consuming steps for nanoparticles functionalization and expensive chemicals for synthesis were required. Here, we describe a less sophisticated version of this principle for the detection of a low molecular weight antibiotic compound in a common indirect ELISA system. Particularly the time-consuming synthesis of antibody-GlcOx-magnetic beads was avoided. Furthermore, the properties of the 7 nm AuNPs (15 nM) prepared in our lab were similar to commercial 5 nm AuNPs[15] but less expensive. The presented method could be generally applicable to other residues and contaminants because (a) preparation of AuNPs requires only a few steps and the particles can be applied without any further functionalization; (b) the AuNPs growth-based signal generation by GlcOx is cheap and requires no toxic chemicals; and (c) the developed immunosensor accommodates all conventional ELISA platforms.

As a proof of principle, we demonstrated the applicability of this approach for determination of the antibiotic residue cloxacillin. Commercial GlcOx-labelled anti-mouse IgG antibodies were employed for H_2O_2 generation and subsequent induction of growth of AuNPs. The absorbance increase was measured by a conventional ELISA plate reader at 562 nm. We first checked whether GlcOx is capable to initiate AuNPs growth. Glucose used as a substrate was incubated with varying concentrations of GlcOx for 1 h at 37 ^{o}C. H_2O_2, as the GlcOx catalytic reaction product, stimulated the growth of 7 nm Au seeds through reducing $HAuCl_4$ and formation of Au_0 on the AuNPs surfaces ($HAuCl_4 + H_2O_2 \rightarrow Au_0 + O_2$).[25] This growth process could be quantified measuring the peak absorbance of the UV-vis spectrum. We employed various concentrations of GlcOx ranging from 3.1 mU mL^{-1} to 200 mU mL^{-1} to gradually catalyze the generation of H_2O_2.

The GlcOx-catalyzed oxidation was carried out at 37 ^{o}C for 1 h with a fixed glucose concentration of 10 mM in H_2O. By monitoring the colour and UV-vis spectrum of the growth solution, a qualitatively and quantitatively characterization of the 7 nm AuNPs growth processes was achieved (Figure S1 in the ESI). The colour change was detectable by the naked-eye, when the concentration of GlcOx was between 6.3 mU mL^{-1} to 25 mU mL^{-1} (Figure 1). The colour intensity increases remarkably as the GlcOx concentration was between 25 mU mL^{-1} to 200 mU mL^{-1}. This light to dark red colour change was quantified by plotting the absorbance at 562 nm versus different concentrations of GlcOx. The calibration curves (Figure 1 inset) indicated a good linear response (R^2 = 0.9896) in the range from 0 mU mL^{-1} to 50 mU mL^{-1} GlcOx. Additional, kinetic measurements showed a significant increase in absorbance at 562 nm for the first 30 min and indicated saturation between 40 and 60 min (Figure 2). As control experiment, we tested the response of 7 nm AuNPs and $HAuCl_4$ without GlcOx and glucose. Even after 40 min, no noticeable AuNPs growth was obtained. In addition, neither 7 nm AuNPs nor $HAuCl_4$ alone reacted with H_2O_2, formed after incubation of GlcOx with glucose.

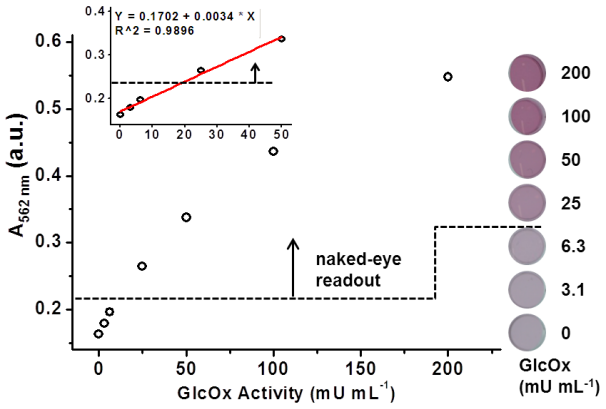


Figure 1 AuNPs growth-based colorimetric nanosensor for GlcOx sensing in the presence of glucose, AuNPs seed, and $HAuCl_4$ (incubation for 40 min). $A_{562\ nm}$ values were plotted against different concentrations of GlcOx. Photograph of different solutions was taken at 40 min.

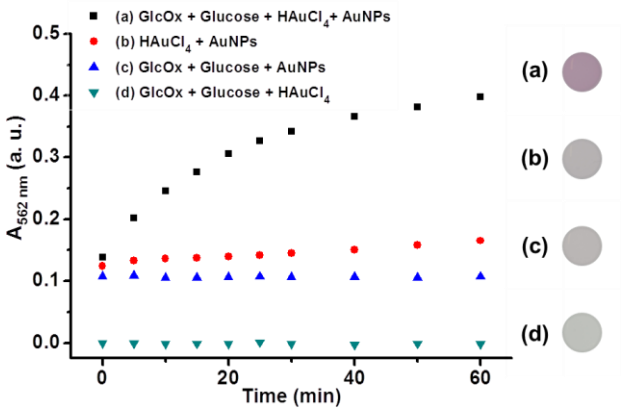


Figure 2 The kinetic process of the AuNPs growth in the presence of GlcOx, glucose, 7 nm AuNPs or $HAuCl_4$. Photograph of different solutions at 40 min.

The GlcOx-mediated 7 nm AuNPs growth was subsequently integrated into an indirect competitive ELISA for cloxacillin detection (Figure 3a). GlcOx-conjugated cloxacillin acted as the coating antigen and competed with residues of cloxacillin in PBS or sample for binding of the primary monoclonal antibody 1F7. GlcOx, used for preparing the coating conjugate, was enzymatically inactive and showed a negligible effect on AuNPs growth. After the competitive reaction, commercial GlcOx labelled goat anti-mouse IgG is used as secondary antibody for H_2O_2 generation in the presence of glucose. Compared to the horseradish peroxidase (HRP)/3,3′,5,5′-tetramethylbenzidine (TMB) signal generation mechanism in conventional ELISA, the AuNPs growth-based approach is more cost-effective and user-friendly (Table S1 in the ESI). Although both of the labelled secondary antibodies are commercially available, costs for the used enzyme and substrate in the developed system are significant lower. Additionally, TMB is potentially toxic and stop solution is essential, while the signal based on AuNPs growth is stable without any quencher (Figure 2).

Using indirect competitive ELISA, standard curves were prepared in a concentration range from 0.4 to 300 ng mL^{-1}. With increasing cloxacillin concentration, binding of primary antibody and GlcOx labelled secondary antibody decreased correspondingly and the H_2O_2-stimulated growth of 7 nm AuNPs was reduced (Figure 3b). At cloxacillin concentrations above 11 ng mL^{-1}, the decrease in colour intensity could be clearly distinguished by the naked eye.

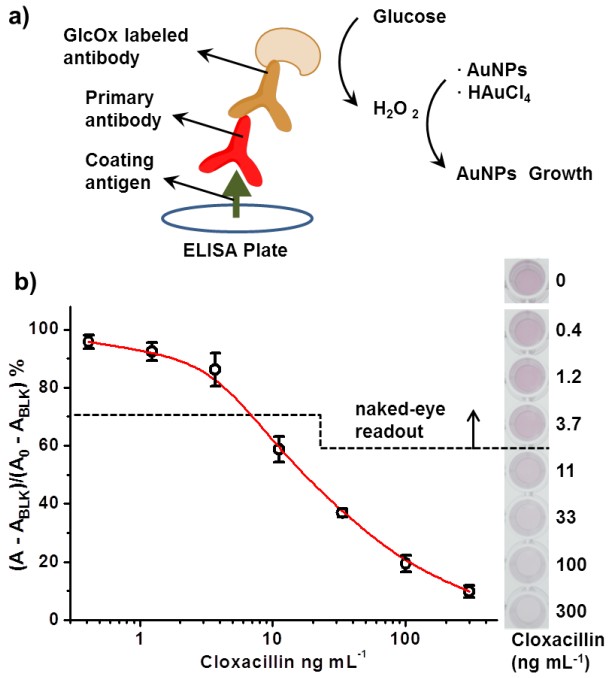


Figure 3 AuNPs growth-based plasmonic ELISA for cloxacillin determination: (a) schematic illustration of indirect competitive ELISA and (b) Inhibition curve for quantitative determination of cloxacillin using glucose oxidase labelled antibody and the corresponding photograph at different cloxacillin concentrations for naked-eye readout.

Furthermore, colour formation was quantified by absorbance measurement at 562 nm with an ELISA plate reader. We employed the relative absorbance $(A-A_{BLK})/(A_0-A_{BLK})\%$ to quantitatively evaluate the purple colour of the AuNPs solutions (A, A_{BLK}, and A_0 are absorbance in the presence of varying concentrations of cloxacillin, in the absence of GlcOx conjugated secondary antibody, and at 0 ng mL^{-1} of cloxacillin, respectively). The calibration curve showed a working range from approximately 4 to 150 ng mL^{-1} corresponding to relative absorbance values of 85% and 15%, respectively. The limit of detection, defined by a signal-to-noise ratio of 3, was determined to be 3.4 ± 0.4 ng mL^{-1} in PBS. The IC_{50} value was at 16.9 ± 1.9 ng mL^{-1}. The analytical characteristics of the developed AuNPs growth-based immunosensor and other previously reported immunoassays are listed in Table S2. In this study, antibiotics were detected in bovine muscle, which is a much more complicated matrix than PBS or milk. Furthermore, only a quick pre-treatment was required for the bovine samples. The data indicated that the AuNPs growth-based assay showed a satisfactory analytical performance for cloxacillin determination, and more important, the approach

allowed even a naked eye readout when the concentration of cloxacillin was above 11 ng mL^{-1}.

To demonstrate the applicability of the GlcOx-mediated AuNPs growth-based plasmonic immunoassay for the detection of antibiotic residues in foodstuffs, we used cloxacillin contaminated bovine muscle samples as a model. Bovine muscle samples were spiked with cloxacillin at concentrations of 150, 300, and 600 ng mL^{-1}. Despite the complexity of this food matrix only a quick sample pre-treatment was required. The samples were homogenized in PBS buffer, filtrated, and then applied to the assay. According to the competitive assay format, negative bovine muscle samples generated the highest signal. The gradually reduced colour intensity obtained for the positive samples could be distinguished by naked eye, which renders the assay suitable for on-site detection even in resource-constrained areas (Figure 4). To quantitatively analyse the concentration of cloxacillin in bovine muscle samples, the absorbance at 562 nm could be determined on a conventional ELISA reader. Under these conditions, the recovery rates for extracts containing 150, 300, and 600 ng cloxacillin per mL ranged from 93.8 to 105.9% with CVs of 10.1 to 14.3% (Table S3 in the ESI). These results indicated that the developed AuNPs growth based immunoassay could be successfully applied not only in buffer solutions but also for the quantitative measurement of cloxacillin in a complex food matrix.

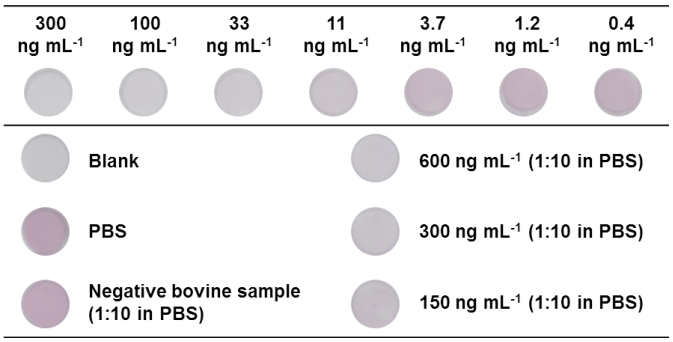


Figure 4 Colour intensity of AuNPs solutions is enhanced as the concentration of cloxacillin is decreased. Blank: in the absence of GlcOx labelled secondary antibody. PBS and negative bovine sample: in the presence of GlcOx labelled secondary antibody to stimulate AuNPs growth reaction. Detection of cloxacillin contaminated bovine muscle sample and readout with the naked eye.

Conclusions

In summary, we have designed a detection system for antibiotic residues in foodstuffs by introducing plasmonic AuNPs growth-based signal generation into a competitive immunoassay format. The colour development enables a naked-eye readout, making this approach useful for on-site detection or in resource-constrained areas. Furthermore, the colour intensity can be quantitatively assayed by measuring the absorbance with a high-throughput plate reader. The established immunoassay utilizes the conventional ELISA format but GlcOx as enzyme label, which together with glucose and AuNPs represents a stable and low-cost alternative to existing commercial signal generation systems. The presented method could be generally applicable to the detection of low molecular weight compounds. Therefore, we anticipate that it will become valuable in future for prompt and on-site detection in the field of food control, clinical diagnosis, and environmental monitoring.

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Supporting Information for

A Gold Nanoparticles Growth-based Immunoassay for Detection of Antibiotic Residues

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EXPERIMENTAL SECTION

Materials and chemicals. Gold (III) chloride hydrate, cloxacillin, D-glucose, sodium borohydride, trisodium citrate dehydrate, glucose oxidase (GlcOx), and casein sodium salt were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Goat anti-mouse IgG labeled with GlcOx was purchased from Abcam (Cambridge, UK). GlcOx-cloxacillin conjugates and monoclonal antibodies against cloxacillin (mAb 1F7) were produced in our laboratory as described.[1,2] All the other reagents required for the experiments were of analytical grade and used as received.

Gold seed solution. Gold seed solution was produced using the previously published method.[3] Briefly, 23.0 µmol gold (III) chloride hydrate and 26.4 µmol trisodium citrate dehydrate were dissolved in 100 mL deionized water. Then, 20 mL of the mixture were transferred into a sterile centrifugation tube, 600 µL freshly made 0.01 M $NaBH_4$ were added and the tube was inverted 3 times. To allow the borohydride to react completely, the reaction mixture was left at room temperature for 4 hours and stored at 4 ^oC until use. The final concentration was 15 nM and the nanoparticles had a diameter of 7 nm.

Identification of GlcOx-mediated sensing. GlcOx was serially diluted with deionized water to reach a final concentration ranging from 200 to 0 mU mL^{-1} in cuvettes. Then, 200 µL glucose (10 mM in H_2O) were mixed with the GlcOx solutions at a 2:1 volume ratio and the mixtures were incubated at 37 ^oC for 1 h. Subsequently, 150 µL $HAuCl_4$ (1.25 mM in H_2O) and 150 µL gold seed solution were added into each cuvette. This solution was incubated at ambient temperature for another 40 min for the AuNPs growth reaction. The photographs were taken by a digital camera (D7000, Nikon, Japan) on a light table (SlimLine S, Rex, Germany). The spectrum from 400 to 800 nm of the AuNPs solutions was recorded by UV-Vis spectroscopy (Specord 200 Plus, Analytik Jena, Germany).

Sample preparation. Bovine muscle samples obtained from local retail shops were used to demonstrate the applicability of the approach for cloxacillin determination. Exactly 2.00 g of bovine muscle samples were homogenized in 10 mL PBS by using BMT-20-S tubes containing stainless steel balls and an Ultra-Turrax Tube driver (Ika, Germany) at 6000 rpm for 10 s. The homogenates were spiked with cloxacillin at concentrations of 150, 300, and 600 ng mL^{-1}. After that,

the spiked homogenates were mixed for another 30 s at 6000 rpm, filtered by medium fast filter paper (Grade 597, Whatmann, UK), and directly analyzed by immunoassay without any further clean-up.

Plasmonic ELISA for food samples. For the determination of cloxacillin in bovine muscle, microtiter plates were coated with GlcOx-cloxacillin (2 µg mL^{-1} in PBS; 100 µL per well) at room temperature overnight. After washing for 3 times with wash solution (0.85% NaCl containing 0.025% Tween-20 in H_2O), the microtiter plate was blocked with 3% casein in PBS for 30 min. Subsequently, various concentrations of cloxacillin (diluted in PBS) or samples (50 µL) were added together with 50 µL mAb 1F7 (0.5 µg mL^{-1} in PBS) and incubated for 1 h. Not adsorbed antibody and antigen were then removed by washing 4 times with wash solution. Then, GlcOx labeled goat anti-mouse IgG from Abcam was employed as secondary antibody (1:125 diluted in PBS; 100 µL per well) and incubated for 1 h at room temperature. Subsequently, the plate was carefully washed 4 times with wash solution and another 4 times with deionized water. Then, 100 µL glucose (10 mM in H_2O) were added into each well and incubated at 37 ^{o}C for 1 h. For the colorimetric detection, 50 µL gold seed solution and 50 µL $HAuCl_4$ (1.25 mM in H_2O) were added into each well and kept to react at ambient temperature for 40 min. Quantitative measurements were obtained with an high-throughput ELISA Reader (Sunrise RC, Tecan, Germany). The calibration curve was analyzed with a four-parameter logistic equation using Prism 5 (GraphPad Software, La Jolla, USA).

SUPPLEMENTARY FIGURE AND TABLE

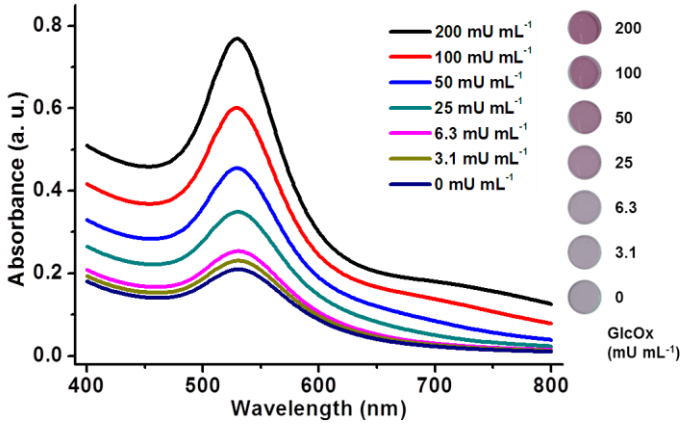

Figure S1 UV-vis spectra of AuNPs growth-based colorimetric nanosensor using varying GlcOx concentrations (0 – 200 mU mL^{-1}). Measurements were performed after incubating the enzyme in the presence of AuNPs and $HAuCl_4$ for 40 min. Photograph of different solutions was taken at 40 min.

Table S1 Comparison between the developed GlcOx/AuNPs/$HAuCl_4$-based signal generation mechanism to the most commonly used HRP/TMB-based mechanism

		HRP/TMB-based mechanism	**GlcOx/AuNPs/$HAuCl_4$-based mechanism**
Enzyme	Protein	Horseradish peroxidase	Glucose oxidase
	Price	175 Euro per 10 KU	47.8 Euro per 10 KU
	Secondary antibody conjugate	Commercial available	Commercial available
Substrate	Chemicals	3,3',5,5'-tetramethylbenzidine	AuNPs and HAuCl4
	Price for 100 mL substrate solution	1.08 Euro	0.69 Euro
	Stability	Need stop solution	Stable signal
	Toxicity	Potentially toxic	Not toxic

Table S2 Comparison of the performances of various immunoassay variants for cloxacillin detection

Platform	**Signal readout**	**Antibody**	**LOD (ng mL^{-1})**	**Sample**	**Ref.**
Biosensor BIAcoreTM	Optical Response Units	Polyclonal Antibody	60	PBS	[4]
Automated microarray	Chemiluminescence	Monoclonal antibody	0.29	Milk	[5]
ELISA Plate	Colorimetric (3,3',5,5'-tetramethylbenzidine)	Monoclonal antibody	6.3	Milk	[6]
ELISA Plate	Colorimetric (3,3',5,5'-tetramethylbenzidine)	Polyclonal antibody	4	Milk	[7]
ELISA Plate	Colorimetric (AuNPs)	Monoclonal antibody	3.4	Bovine muscle	This work

Table S3 Recovery of cloxacillin from artificially contaminated bovine muscle samples at different concentration levels (n = 5)

Added	**Found**			
(ng mL^{-1})	**Mean (ng mL^{-1})**	**±SD [a] (ng mL^{-1})**	**Recovery (%)**	**CV [b] (%)**
150	158.8	16.0	105.9	10.1
300	294.2	36.5	98.1	12.4
600	562.7	80.5	93.8	14.3

[a]: standard deviation

[b]: coefficient of variation

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III. DISCUSSION

Antibiotics are widely used for treating and preventing bacterial infections in food animals. At the global scale, the antibiotics consumption in 2010 was approximately 63,151 tons. Germany was one of the five countries with the largest shares (approx. 3%) of global consumption of antibiotics in veterinary medicine, while China was with 23% the largest consumer (VAN BOECKEL et al., 2015). According to some statistical models, global antibiotics consumption is expected to reach more than 105,000 tons by 2030. The frequent use of antibiotics in food animal production can result in occurrence of antibiotic residues in animal products, which have been reported to be harmful to humans, causing health problems such as allergic reactions and antibiotic-resistance (LEE et al., 2001). Therefore, MRLs were set by the European Commission for residues of antibiotics as well as other veterinary drugs in food-producing animals. According to the statistical data presented in 2013 by the European Food Safety Authority (EFSA), 0.21% of the collected samples were over the set antibiotic concentration limits (EFSA, 2015). The percentages of non-compliant samples in four typical animal products at the European level are presented in Fig. 7.

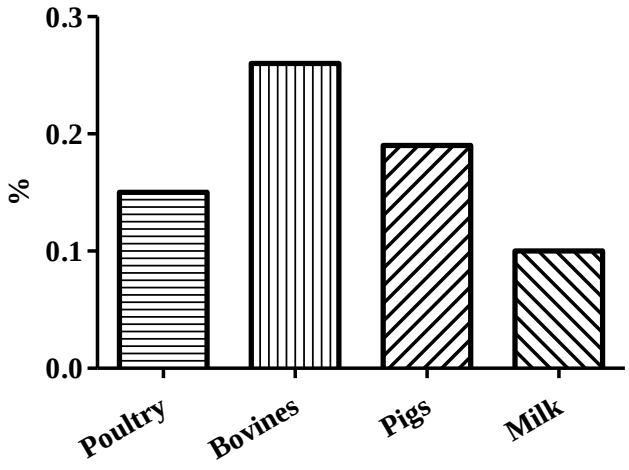

Figure 7: Percentage of non-compliant samples in 4 typical animal products in European countries (EFSA, 2015)

Immunoassays have been intensively used for antibiotic residues determination due to their high sensitivity, simplicity, and cost effectiveness. In addition, there is an ongoing process to further improve the performance of EIAs by the integration

of new techniques. Two of the most representative technologies, microfluidics and nanomaterials have the potential to catalyze the development of novel immunosensors. However, up to now, these approaches have mainly focused on improving the detection limits of the assays. The papers reviewed in the introduction section have got into “endless ultralow level”, detection limits in the picomolar, femtomolar, or even zeptomolar range have been realized. The pursuit of ultrahigh sensitivity is commonly seen in not only food analysis but also clinical diagnosis, but the developed methods are not always practical. For example, α-fetoprotein and prostate-specific antigen are typically used as model cancer markers for the development of high sensitive immunosensors, but they are naturally present in blood at certain concentrations (ng/mL level) (DE ANGELIS et al., 2007). In food control, detection systems for antibiotics may not require ultralow sensitivities, because MRLs are always set at µg/kg levels. Basically, antibiotic residues, whose concentrations are below the set MRLs, are regarded as acceptable and allowed to occur in foodstuffs. Thus, development of methods that are easy-to-perform and can be combined with current platforms might be more important than ultralow detection methods, which are not practical due to error-prone handling steps and the use of luxury materials. We herein suggest that alternative approaches based on commonly available reagents and robust/mass-producible materials, are worth pursuing more broadly in the field of food analysis.

In this thesis, conventional immunoassays for antibiotics detection were combined with microfluidic devices and nanomaterials. The developed methods were presented in two papers, and their feasibility and practicability were discussed. The approaches described in the papers well adapt to current ELISA platforms. In both publications, cloxacillin was used as a model antibiotic due to the availability of both a high-affinity monoclonal antibody and well-established conjugation procedures for this β-lactam antibiotic. Moreover, in previous studies this mAb has also been integrated in other automated immunosensors in our lab (KNECHT et al., 2004; KLOTH et al., 2009a). Under an immunochemical view, antibiotics are haptens due to their low molecular weights, and thus competitive immunoassays are applied for quantitative detection. Therefore, both in the microfluidic chip-based method and the nanoparticles growth-based immunosensor, indirect competitive ELISA for cloxacillin determination was

used. In detail, GlcOx-cloxacillin conjugates were synthesized via N,N'-dicyclohexylcarbodiimide/N-hydroxysuccinimide (DCC/NHS) coupling (USLEBER et al., 1994) and used to coat the surface of PDMS base layer (microfluidic-based method) or microtiter plate (AuNPs growth-based method). The competition step was carried out by simultaneous addition of mAb 1F7 and analyte (cloxacillin standard or sample extract) into microchannels or 96-well plates. Due to the competitive assay principle, the obtained signal intensity is indirect proportional to the analyte concentration, i.e. the higher the test signal the lower the analyte concentration. In the signal transduction step, two different commercially available enzyme-labeled secondary antibodies, i.e. HRP labeled anti-mouse IgG and GlcOx-labeled anti-mouse IgG, were employed for the generation of a chemiluminescent signal or the growth of AuNPs. In the microfluidic chip-based assay, the chemiluminescent signal results from the reaction of luminol/hydrogen peroxide, while in AuNPs growth-based immunosensor the increase in color intensity is based on enlargement of AuNPs, triggered by the glucose oxidase generated H_2O_2 (Fig. 8).

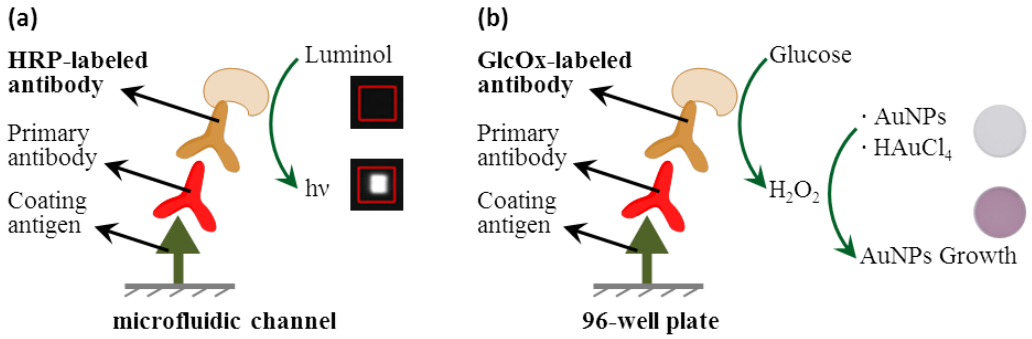

Figure 8: Indirect competitive ELISA format used in (a) microfluidic chip- and (b) AuNPs growth-based immunosensors

By combining the microfluidic technology with traditional immunoassays, the performances were significantly improved in several aspects. Unlike previous conventional ELISAs in our lab, which were generally carried out on the 96-well plate, the chip-based assays were performed in PDMS microchannels. The base layer and channel layer 1 were used for coating and channel layer 2 for the following immune reactions (channel layer 1 and 2 were perpendicular on the base layer). Each channel layer has 7 microchannels and the 7 × 7 arrayed format of images were generated by luminol/hydrogen peroxide and captured by a CCD

camera. Compared with other microarray-based technologies (KNECHT et al., 2004), in which surface functionalization and spotting processes by complex equipment are essential, in this approach PDMS was used directly for coating without further time-consuming activation. Generally, hapten conjugates were dissolved in PBS for coating because β-lactam antibiotics are hydrolyzed at higher pH values. An attractive feature of the coating in microchannels is that GlcOx-cloxacillin could be immobilized in only 30 min in this neutral buffer solution, while on 96-well plates it usually takes at least several hours. According to other studies on microfluidic-based immunoassays, coating density and detection sensitivity could be improved by covalent binding of the protein-conjugate on a functionalized surface (DIDAR et al., 2012; LI et al., 2016). Nevertheless, the complicated and time-consuming chemical reactions and the use of large amount of chemical reagents make these approaches not suitable for mass fabrication and low-cost detection.

Table 3: Comparison of basic features of the developed microfluidic chip-based chemiluminescence immunoasssay with a 96-well-based conventional ELISA

	Microfluidic-based method[1]	**Conventional ELISA[2]**
Assay duration	< 2.5 h	>14 h
Coating time	30 min	>12 h
Incubation time for each step	30 min	1 h
Enzyme-substrate reaction	2 min	20 min
Volume	18 µL per channel	100 µl per well
IC_{50}	96.5 ng/mL (in 1% BSA-PBS)	2.5 ng/mL (in PBS)

[1] YU et al. (2016); [2] DIETRICH et al. (1996)

To demonstrate the feasibility of the assay, inhibition curves under optimized conditions were used to determine the recovery rates of food samples in different matrices, i.e. chick and duck muscles. Meat juice, which is proved as a suitable matrix for antibiotics screening (LAMAR und PETZ, 2007; SCHNEIDER et al., 2010), was used as sample material. Although the developed immunosensor is not as sensitive as the conventional ELISA, the detection limit is still well below the current MRLs. Overall, the assay is characterized by its simplicity, rapid analysis,

and the considerably lower consumption of precious immunoreagents (Tab. 3).

The miniaturization of microfluidics has the potential to dramatically improve the efficiency of immunoassays and is becoming increasingly popular in bioassays (ARAZ et al., 2013). Despite the advantages of microfluidic chip technology, there are still some limitations such as high background, formation of bubbles, and dead volume in microchannels. In our experiment, a relative high background was observed due to non-specific binding of protein on the PDMS surface which negatively influenced the assay sensitivity. The formation of bubbles in microchannels was also observed, which may influence the continuity of the fluids (KOHLHEYER et al., 2008). To enhance the performance of this kind of assay it would be desirable to design novel microfluidic devices as automated and closed system. In that way, it would be possible to realize a robust microfluidic-based on-site immunoassay for sensitive and simultaneous determination of antibiotics. In contrast to other microfluidic systems, specifically developed for multiplex determination (HUTH et al., 2002; HAASNOOT et al., 2003; KLOTH et al., 2009b), the microfluidic chip in our approach could be used for only one target analyte. However, this issue can be handled by novel microchannel designing or/and integration of automated systems in the future.

In the second part of the thesis, a visualized assay based on AuNPs growth for the detection of antibiotics in foodstuffs was developed. As one of the most fascinating nanomaterials, AuNPs have a number of unique physical properties and are widely adopted for biological and chemical analyses (CHEN et al., 2011). In this work, we focused on the development of a straightforward nanomaterial-based immunoassay, which is compatible with current standard commercial ELISA formats.

The used conjugates are all commercially available, further chemicals were not necessary. For H_2O_2-controlled AuNPs growth mechanism, GlcOx-labeled goat anti mouse IgG was employed, which was purchased from Abcam (Cambridge, UK). In principle, GlcOx is much cheaper than HRP and ALP, enzymes commonly used in conventional EIA formats as labels (Tab. 4). However, compared with the traditional enzyme labeled secondary antibodies, the accordingly labeled immunoreagents are more expensive. This may be due to the lack of demand for these conjugates as only few studies have focused on GlcOx-

triggered signal generation mechanism. Moreover, to the best of our knowledge, Abcam is the only provider of GlcOx-labeled secondary antibodies.

Table 4: Comparisons of the costs for enzymes and labeled secondary antibodies used in the conventional ELISA and the AuNPs growth-based method

	Company	**Price**	**Size**
GlcOx	Sigma	47.8 Euro	10 kU
HRP	Sigma	175 Euro	10 kU
ALP	Sigma	203.5 Euro	10 kU
Goat anti-mouse GlcOx	Abcam	219 Euro	500 µL, n/a
Rabbit anti-mouse HRP	Abcam	195 Euro	500 µL, 1 mg
Rabbit anti-mouse ALP	Abcam	195 Euro	1000 µL, 1 mg
Rabbit anti-mouse HRP	Dako	339 Euro	2000 µL, 1.3 mg

The nanoparticles used in the colorimetric immunosensor were straightforwardly synthesized using borohydride as a reductant and citrate as a stabilizer in aqueous solution, which is one of the most commonly applied methods (JANA et al., 2001; XIA et al., 2009). Without extra time-consuming steps for further functionalization, the synthesized AuNPs could be directly used for signal generation and enabled a naked-eye readout. Therefore, the disadvantages of previously described AuNPs growth-based immunoassays have been overcome in our approach, such as complicated AuNPs modification steps and long reaction time for AuNPs enlargement (CAO et al., 2009; WANG et al., 2015). We demonstrated the mechanism of signal generation by GlcOx-mediated AuNPs growth and proved that the intense purple color change caused by AuNPs growth allowed a naked-eye readout. In conventional methods, yellow color is generated by HRP-catalyzed oxidation of TMB. However, the purple color can be more clearly distinguished by the naked eye (Fig. 9). Further on, in the low inhibition range from IC_{10} to IC_{50}, the yellow colors are not distinguishable from each other, while the gradual purple color change from IC_{10} to IC_{90} allows a sensitive naked-eye readout.

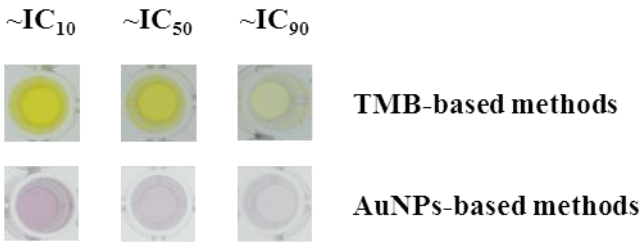

Figure 9: Comparison of color changes in TMB- and AuNPs-based methods

This AuNPs growth-based naked-eye readout is extremely advantageous in resource-constrained areas and the approach is more user-friendly than the TMB-based method because of the potential toxicity of TMB. In addition, the purple color intensity can be quantitatively assayed by measuring the absorbance with a high-throughput ELISA reader. As a further demonstration of the utility of this plasmonic immunoassay in food control, cloxacillin spiked bovine muscle samples were analyzed. The AuNPs growth-based immunoassay can be successfully used not only for the identification of cloxacillin in complex food matrix by the naked eye but also for quantitative measurement by ELISA reader. For quantitative detection, the ideal absorption peak for AuNPs growth solution is at 530 nm (Fig. 10). However, in our study an absorbance at 562 nm was used due to the lacking of corresponding optical filter, and this may have caused a reduction of detection sensitivity.

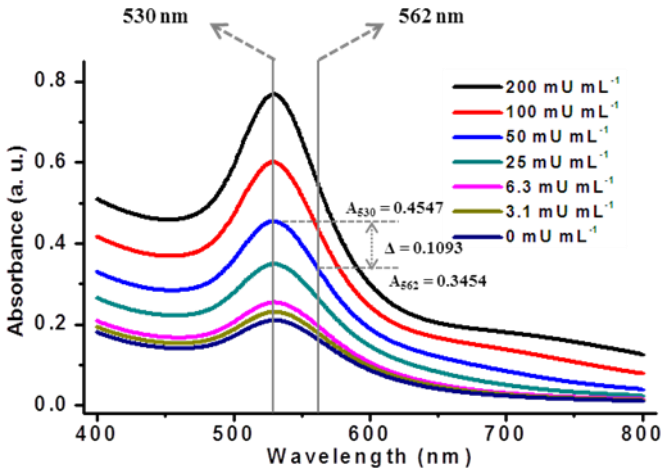

Figure 10: Comparison of the used absorbance (at 562 nm) in the AuNPs growth-based immunoassay and the ideal absorbance (at 530 nm)

In contrast to many other approaches, the GlcOx/Glucose/AuNPs/$HAuCl_4$-based color change is fully compatible with current immunoassay platforms. Even though this colorimetric method represents a promising approach for on-site

detection of antibiotic residues in foodstuffs, some work is still necessary to improve its performance. For instance, the absolute value of background signals was around 0.118, which was caused by absorbance of 7 nm AuNPs. By using AuNPs with smaller sizes, such as 5 nm, which have lower absorbance (XIONG et al., 2015), the issue of background could be minimized.

Overall, this thesis demonstrates the successful application of microfluidic chip- and AuNPs growth-based approaches in antibiotic residues detection in foodstuffs. Due to their practicability and the compatibility with current platforms the developed methods will be valuable in future for other applications enabling rapid, reliable, and on-site detection of residues in food.

IV. SUMMARY

Development of Rapid On-site Immunoassays for the Detection of Antibiotic Residues in Foodstuffs

This thesis describes how the performance of enzyme immunoassays (EIAs) can be improved by microfluidic and nanotechnology. As demonstrated for an EIA enabling the detection of antibiotic residues in food, these two novel techniques can be directly combined with current immunoassay platforms. To realize these alternative test formats, a straightforward fabrication of microfluidic chips was developed and a gold nanoparticles (AuNPs) growth-based colorimetric nanosensor was established. These tools were integrated in an indirect competitive EIA for the analysis of cloxacillin in different food matrices.

In the first part, a microfluidic system was combined with a chemiluminescence immunoassay. Unlike conventional 96-well plate-based EIAs, this immunoassay was performed in polydimethylsiloxane microchannels. Consequently, the microfluidic chip format was characterized by small sample and reagent sizes and short assay times. In the second part, an AuNPs growth-based colorimetric immunosensor was designed. In this immunoassay, AuNPs were used without further modification and the growth-based color change allowed the naked-eye readout of the assay at low analyte concentrations. The established immunoassay is based on a conventional ELISA format but glucose oxidase is used as an enzyme label. Through the oxidation of glucose this enzyme generates H_2O_2, which subsequently induces the growth of AuNPs. The applicability of the developed assays was demonstrated by the analyses of artificially contaminated poultry and bovine meat. In both approaches, sample extracts could be directly analyzed, no complex clean-up procedures were needed. Using cloxacillin as a model antibiotic, good recovery rates (> 90%) were obtained, which demonstrate that the novel immunoassays can be successfully adopted to reliably detect antibiotics in food samples.

V. ZUSAMMENFASSUNG

Entwicklung von schnellen On-site Immuntests zum Nachweis von Antibiotikarückständen in Lebensmitteln

Die vorliegende Dissertation befasst sich mit der Verbesserung der Leistungsfähigkeit konventioneller Enzymimmuntests (EIAs) durch den Einsatz mikrofluidischer und nanotechnologischer Techniken. Wie anhand eines EIAs für den Nachweis von Antibiotika in Lebensmitteln demonstriert werden konnte, können diese neuartigen Techniken direkt mit derzeit gängigen Immuntest-Platformen kombiniert werden. Zur Testentwicklung wurde ein einfaches Verfahren für die Herstellung von Mikrofluidik-Chips bzw. ein auf dem Wachstum von Goldnanopartikeln (AuNPs) basierender colorimetrischer Nanosensor etabliert. Diese analytischen Werkzeuge wurden in einem indirekten kompetitiven EIA integriert, mit dem Cloxacillin-Rückstände in Lebensmitteln detektiert werden können.

Zum einen wurde ein mikrofluidisches System mit einem Chemilumineszenz-Immuntest kombiniert. Im Gegensatz zu herkömmlichen auf 96-Well-Platten basierten EIAs wurde der Test in Polydimethylsiloxan-Mikrokanälen durchgeführt. Bedingt durch das Mikrofluidik-Chip-Format konnten die Tests unter geringem Reagenzienverbrauch in kurzer Zeit durchgeführt werden. Im zweiten Teil wurde ein auf AuNPs Wachstum basierender colorimetrischer Immunsensor entwickelt. In diesem Test werden AuNPs ohne weitere Modifikation eingesetzt, die auf dem Wachstum der AuNPs basierende Farbänderung kann mit bloßen Augen auch bei niedrigen Analyt-Konzentrationen ausgelesen werden. Der entwickelte EIA basiert auf einem konventionellen Ansatz, zur Detektion wurden allerdings Glukoseoxidase-markierte Sekundärantikörper eingesetzt. Durch die Oxidation von Glukose erzeugt dieses Enzym H_2O_2, das anschließend das Wachstum von AuNPs induziert. Die Anwendbarkeit der etablierten Methoden wurde anhand dotierter Fleischproben überprüft. Bei beiden Verfahren konnten die Rohextrakte ohne weitere Aufarbeitung eingesetzt werden. Für das als Modell verwendete Cloxacillin wurden gute Wiederfindungsraten von > 90% erreicht. Dies zeigt, dass mit den neuartigen Immuntests Antibiotikarückstände in Lebensmittelproben zuverlässig nachgewiesen werden können.

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