Discrimination of Different Foodborne Pathogens onto Carbohydrate Microarrays Using Surface Plasmon Resonance Imaging

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Abstract: Food safety is a public health challenge. Devices allowing early, fast, label-free and *in situ* detection of bacteria are of great interest to prevent outbreaks. *Listeria monocytogenes*, *Salmonella* spp. and *Escherichia coli* O157:H7 are foodborne pathogens which were responsible of 60% of the hospitalizations in the USA in 2011. In this study, we conceived a carbohydrate microarray in order to detect and discriminate these three food pathogenic bacteria. In less than 10 hours, from an initial bacterial suspension of 100 bacteria per mL, Surface Plasmon Resonance imaging allowed the detection and the discrimination of these bacteria while they were growing and interacting specifically with the carbohydrate microarray. Moreover, this device is easily regenerable and can be re-used: it is probably a promising tool to early detect bacteria in food.

1 INTRODUCTION

Foodborne illness is one of the major public health problems. The estimation of hospitalizations and deaths caused by pathogenic food pathogens is a challenge. However, Centers for Disease Control and Prevention estimate that 9.4 million episodes of foodborne illnesses occurred in the USA in 2011. For example, 35% of hospitalizations (*i.e* 19,336 hospitalizations) and 28% of deaths were caused by *Salmonella* spp., 19% of deaths were caused by *Listeria monocytogenes* and *Escherichia coli* O157 outbreaks contribute to 4% of hospitalizations in 2011 (Scallan, 2011). Then these three pathogenic strains are part of the top five pathogens contributing to foodborne diseases resulting in hospitalizations and/or deaths. Conventional methods to detect bacteria in food are reliable but time-consuming. The need for bacterial cultures is one of the major disadvantages of these techniques allowing the spread of epidemics. For example, detection of *L. monocytogenes* require one week with standards methods (Velusamy, 2010). During this period, consumers are not warned.

Since the last decade, the development of fast, sensitive and reliable techniques has emerged. To this aim, nanotechnology has already improved early nanodiagnostics based on selective capture of bacteria using nanoparticles (Tallury, 2010), fluorescent nanoparticles, quantum dots (Gilmartin, 2012), bioconjugated magnetic particles (Chu, 2013), etc) or microengines modified with lectins (Campuzano, 2012). However, these different methods require complex functionalized probes and a robust chemistry for surface functionalization. Moreover, they consist at least in a two-step process, the first one being the capture of bacteria and the second one being the detection of bacteria-nanoparticles interactions. For example, bacteria-magnetic nanoparticles are easy to remove from the sample by magnetic separation techniques but they still need to be identified using other classical techniques such as MALDI-MS spectrometry, flow cytometry (Cellular analysis and detection using surface plasmon resonance techniques, 2013), fluorescence or scanning electron microscopy (Gu, 2006). The disadvantage of these techniques is that they do not allow a real-time and *in situ* monitoring of the bacteria-probe interactions. To solve this

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problem, label-free and real-time cutting edge methodologies have been proposed such as Quartz Crystal Microbalance (Chu, 2013). However, this detection technique requires a high number of bacteria and a uniformly functionalized surface difficult to obtain in general. Microcantilevers have also been used in order to detect bacteria in a complex sample (Mader et al., 2012).

Surface Plasmon Resonance (SPR) has emerged as a very well-adapted method for the detection of analytes onto microarrays (Abadian, 2014, Safina, 2012). This technique, based on refractive index changes, monitors the interactions occurring between biomolecules grafted on a biochip and target molecules within the sample. SPR imaging (SPRi) collects the reflected light with a CCD camera. Futhermore, it allows label-free detection in real-time of up to one hundred images and SPRi signals simultaneously within one biochip (Bouguelia, 2013).

Our team has developed a new method called the Culture-Capture-Measure (CCM) (Bouguelia, 2013, Mondani, 2014). It consists in following *in situ* the bacterial growth in a medium by SPRi. It allows detecting bacteria in one step from an initial concentration of 20 Colony Forming Units (CFU) per mL. In these works, antibodies were used as probes and grafted on gold surfaces. Antibodies microarrays are efficient to detect bacteria (Delehanty, 2002, Gao, 2010) but their cost and fragility (dehydration and denaturation sensitivity, single use) make them difficult to use on an industrial scale. In this study, antibodies were replaced by carbohydrates. Carbohydrates provide interesting alternatives to antibodies in order to overcome their limitations. They are less expensive, easily-chemically-modified than antibodies. They usually allow regeneration of the biochip because their interactions are known to be low-energetic. Besides, bacteria are known to interact with carbohydrates by different ways: through particular lectins produced on the outer cell wall, named adhesins (Bierne, 2007, Chessa, 2009, Cowart, 1990) and also by non-specific bindings such as interactions with membrane transporters or electrostatic interactions (Audfray, 2013, Mader, 2012). This underlines the great potential of the use of carbohydrates as probes for bacteria detection and interactions.

We chose seven simple mono- and di-saccharides for the conception of the microarray: glucose (Glc), galactose (Gal), mannose (Man), fucose (Fuc), maltose (Malt), *N*-acetylglucosamine (GlcNAc), and sialic acid (Neu5Ac). First, pyrrole-carbohydrate conjugates were synthetized and then grafted onto gold surfaces by coelectropolymerization. The microarray functionalization was then characterized by lectin-carbohydrate recognitions. Finally, bacterial detection was evaluated by SPRi onto the carbohydrate microarray and monitored by the CCM method.

This biochip allows the detection and the clear discrimination of the three bacterial strains tested: *E. coli* O157:H7, *L. monocytogenes* subserotype 1/2a and *S. enteritidis*.

2 MATERIALS & METHODS

2.1 Pyrrole-Carbohydrate Conjugates Syntheses

2.1.1 Reactants

Monosaccharides, maltose, lithium bromide, sodium hydride, allyl bromide, dimethylformamide, acetonitrile, cysteamine, phosphate buffer, manganese(II) chloride tetrahydrate, sodium chloride, and sodium hydroxide were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Pyrrole was purchased from Acros Organics (Geel, Belgium), calcium(II) chloride from Merck (Darmstadt, Germany).

2.1.2 Syntheses

According to the previously reported procedure (Fort et al., 2005), pyrrole-carbohydrate conjugates have been prepared from the corresponding allyle glycoside by UV-promoted radical addition of cysteamine followed by reaction with a pentafluorophenyl-activated pyrrole ester. Allyl glycosides have been prepared as reported in the literature: allyl β-D-glucopyranoside and allyl β-D-galactopyranoside (Lin, 2010), allyl α-D-mannopyranoside (Nishida, 2004), allyl β-maltoside (Takeo, 1987), allyl α-D-fucopyranoside (Vermeer, 2001), allyl α–D–N-acetyl-neuraminic acid (Roy, 1990), allyl 2-acetamido-2-deoxy-beta-D-glucopyranoside (Vauzeilles, 2001).

The different conjugates were isolated in 30-50% yield after purification by reverse phase Solid Phase Extraction and characterized by ^{1}H, ^{13}C NMR and MS spectrometry. Final solid products were stored at 4°C.

2.2 Fabrication of a Carbohydrate Microarray

Aliquots of pyrrole-carbohydrate conjugates were dissolved in spotting buffer (50 mM phosphate buffer, pH 6.8, 50 mM NaCl and 10% glycerol) containing 20 mM pyrrole. The concentration of pyrrole-carbohydrate conjugate in the mixture was 10 mM.

SPRi biochips were purchased from Horiba Scientific (Chilly-Mazarin, France) and are made of a high index glass prism covered with a 2 nm-thick chromium and a 50nm-thick gold layer used as a working electrode (about 2 cm^2).

Coelectropolymerization of pyrrole and pyrrole-carbohydrate conjugates on the biochips was carried out in an automatized pipette tip (diameter 500µm) filled with the solution to be polymerized and containing a platinum wire used as a counter electrode. The pipette tip was moved at the vicinity of the gold layer of the SPR biochip, till an electrical contact was applied between the working (gold surface) and counter (platinum wire) electrodes. The polymerization on the prism gold layer was performed with a 100 ms electric pulse at a 2.0 V bias independently of any reference electrode. After microarraying, biochips were copiously washed with water, dried and stored in air at 4°C (Mercey, 2008). Each biochip was arrayed with quadruplicates of pyrrole-carbohydrate conjugates. Moreover, four spots of polypyrrole (Ppy) deprived of any carbohydrates were also deposited onto the gold surface to assess nonspecific SPR response.

2.3 SPRi Setup

Signal measurements were performed using a SPRi-Lab+TM system (Horiba Scientific, Chilly-Mazarin, France). Using this commercial software, regions of interest (ROI) corresponding to individual spots on the biochips were defined (diameter 260 µm). The SPRi signal was monitored with a CCD camera, and reflectivity changes (ΔR) of each ROI were followed and plotted upon time.

2.4 Characterization of the Carbohydrate Microarray

Functionalized biochips were characterized by SPR measurements after injection of different lectins already known to interact specifically with carbohydrates (Chang, 2011, Safina, 2012) in phosphate buffer at 25°C.

2.4.1 Lectins

UEA I (Ulex Europaeus Agglutinin-I) from *Ulex europaeus*, PNA (Peanut Agglutinin) from *Arachis hypogaea,* WGA (Wheat Germ Agglutinin) from *Triticum vulgaris* and concanavalin A (ConA) from *Canavalia ensiformis* (jack bean) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

2.4.2 SPRi Monitoring of Lectin - Carbohydrate Recognitions

The concentrations used were: 500 nM of PNA (specific for Gal), 280 nM of UEAI (specific for Fuc), 50 nM of WGA (specific for GlcNAc, Neu5Ac) and 1 µM of ConA in the presence of 1 mM Ca^{2+} and Mn^{2+} (specific for Man, Malt).

SPRi signals (not shown in this study) proved the efficient grafting and the accessibility of the grafted sugars by revealing an increase in the reflectivity variation corresponding to the recognition of specific spots by lectins.

2.5 Detection of Bacterial Growth by SPRi

2.5.1 Bacterial Suspensions

E. coli O157:H7 CIP 105917 strain, *L. monocytogenes* subserotype 1/2a and *S. enterica* subserotype *enteritidis* were purchased from the Institut Pasteur (Paris, France).

Bacterial cells were grown overnight at 37 °C in a Tryptic Soy Broth medium (TSB, Saint-Quentin-Fallavier, France). Overnight grown bacterial cultures – corresponding to 10^8-10^9 CFU.mL^{-1} – were used for SPRi experiments after serial dilutions (up to 10^2 CFU.mL^{-1}). Bacterial counting was carried out either by McFarland turbidity measurements (Densimat apparatus, BioMerieux, Marcy l'Etoile, France) or colony counting after plating and culture on solid media Tryptic Soy Agar (TSA, Saint-Quentin-Fallavier, France). For colony counting, the counting of 4 plates was averaged and standard deviation was calculated.

2.5.2 Monitoring of Bacterial Kinetics using SPRi

The freshly bacterial suspension of 100 CFU.mL^{-1} in TSB medium was deposited in the thermalized (37°C) SPRi chamber (1.6 mL) onto the carbohydrate microarray. SPRi kinetic experiment of

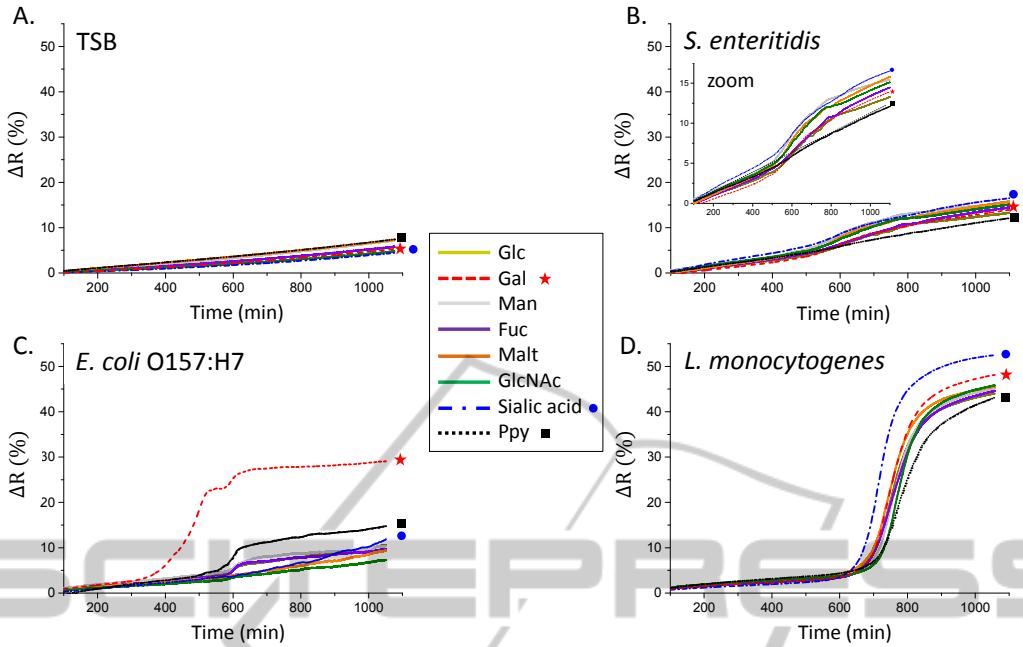

Figure 1: SPRi signals obtained from the spots of the carbohydrate microarray, after deposition of the bacterial suspension at 10^2 CFU.mL^{-1} in TSB; in the case of: A. buffer (control), B. *S. enteritidis*, C. *E. coli* O157:H7, and D. *L. monocytogenes*.

Table 1: Detection times at the inflection point of SPRi signals obtained after deposition of the bacterial suspension at 10^2 CFU.mL^{-1} in TSB. Data were obtained from the SPRi signal averages of three independent runs.

Bacteria	*E. coli* O157:H7		*L. monocytogenes*	
Interacting carbohydrate	Gal	Ppy, the others carbohydrates	Sialic Acid	Ppy, the others carbohydrates
Detection Time (min)	529 ± 26	604 ± 9	739 ± 19	783 ± 12

the bacterial growth was realized by Culture-Capture-Measure (CCM) (Bouguelia, 2013, Mondani, 2014). SPRi signals were recorded in real-time during the culture (1100 min) over the biochip.

2.5.3 SPRi Data Treatment

For each experiment, an average of SPRi signal coming from quadruplicates was realized.

SPRi signals (*i.e* ΔR) of the bacterial growth onto the carbohydrate microarray are represented by an inverse tangent function: after smoothing kinetic curves, first order derivative of this function was realized. The maximum of the derivative corresponds to the inflection point of kinetic curve called "detection time".

Finally, all the results present in this paper are the average of at least three independent runs.

3 RESULTS AND DISCUSSION

Using the CCM method developed in our laboratory (Bouguelia, 2013, Mondani, 2014), we monitored the kinetic SPRi signal after bacterial deposition onto the carbohydrate microarray. The curves are represented in Figure 1.

Experimental kinetic curves were similar to standard bacteria growth kinetics occurring in three different stages: first, the lag phase (bacteria adapt to the new medium), then the exponential growth (bacteria divide regularly) and finally the stationary phase (growth stops because of the lack of nutrients and overpopulation). Figure 1.A represent the SPRi signal obtained from the control (*i.e* TSB medium): the linear increase in reflectivity corresponds to the liquid evaporation occurring during the SPRi experiment. The kinetic SPRi curve of *S. enteritidis* (Figure 1.B) presents a low SPRi signal but a shape of bacterial growth for all the carbohydrate-bacteria interactions. On the contrary, Figures 1.C, and 1.D corresponding to *E. coli* O157:H7 and *L. monocytogenes* onto the carbohydrate microarray respectively, show different carbohydrate-bacteria interactions. In the case of *E. coli* O157:H7, the galactose-*E. coli* interaction (red curve) was earlier

detected by SPRi than the others interactions. In the case of *L. monocytogenes*, the SPRi signal earlier detected is the sialic acid-*L. monocytogenes* interaction (dark blue curve). Moreover, the SPRi curve presents two plateaux: the first one may correspond to the classical stationary phase and the second may correspond to the development of the bacterial biofilm.

In this study, the amplitude of the SPRi signal could not be relevant to discriminate the different strains. In the case of S. *enteritidis* and *E. coli* O157:H7, ΔR is less than 20-30%. On the contrary, in the case of *L. monocytogenes*, ΔR is around 50-60%. Indeed, the SPRi signal also depends on the contribution of one bacterium to this SPRi signal. Thus, the ΔR value can not be used as a criterion because of the different shapes and sizes of the three strains. However, it is possible to rationalize the results by defining for each bacterium a "detection time" (Table 1). In the case of *E. coli* O157:H7, the "detection time" of the galactose-*E. coli* interaction is 529 ± 26 min whereas the average "detection time" of the {control, others carbohydrates}-*E. coli* interaction is 604 ± 9 min. Then the time difference between these two "detection times" is 76 ± 32 min. That significates that *E. coli* interact 76 ± 32 minutes earlier with the galactose spots than with the others. In the case of *L. monocytogenes*, the time scale is rather different from the one of *E. coli* O157:H7. The "detection time" of the sialic acid-*L. monocytogenes* interaction is 739 ± 19 min and thus the time difference between this time and the "detection time" of the {control, others carbohydrates}-*L. monocytogenes* interaction is 44 ± 9 minutes.

The observation of the SPRi signals and the quantitative analysis is promising for the further identification and differentiation of the three bacterial strains onto our carbohydrate microarray. This microarray is also regenerable through soft washing (SDS 2% and sodium hydroxide 0.02M) and can be stored at room temperature in air up to 6 months.

4 CONCLUSIONS

This work in progress presents an innovative concept to detect specifically food pathogenic bacteria. Thanks to the Culture-Capture-Measurement methodology developed in our team, bacteria are detected in less than 10 hours, from an initial suspension of 10^2 CFU.mL^{-1}, onto a carbohydrate microarray by SPRi.

We have demonstrated that *S. enteritidis*, *E. coli* O157:H7 and *L. monocytogenes* bacteria interact differently on the carbohydrate microarray (*i.e* different SPRi signals) that is promising to identify these three pathogenic strains. Furthermore, *E. coli* O157:H7 has showed particular interaction with the galactose spots whereas *L. monocytogenes* interacted preferentially with the sialic acid spots.

Then, this cheap, regenerable carbohydrate microarray seems to be a promising tool to detect bacteria in food and a good alternative to the use of expensive, and fragile antibodies. The identification and the differentiation of different strains inside one bacteria specie, in particular *E. coli* and *L. monocytogenes*, are ongoing in our team.

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