Love Wave Fully Integrated Lab-on-chip Platform for Food Pathogen Detection - LOVE-FOOD

(Contract No 317742 – Starting Date: 1 September 2012)

Deliverable 7.3

Report on whole bacteria preselection based on magnetic particles or micronanostructured surfaces

(re-named from: Report on whole bacteria detection protocol based on magnetic particles or micronanostructured surfaces preselection)

Due date:	31st August 2014
Date of submission:	3rd November 2014
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DELIVERABLE SUMMARY SHEET

Project Number	:	317742
Project Acronym	:	LOVE-FOOD
Title	:	LOVE Wave Fully Integrated Lab-on-Chip Platform for FOOD-Pathogen Detection
Deliverable	:	D7.3
Partners Contributed	:	UniPardubice, NCSR-D, Curie
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Classification	:	RE

DOCUMENT HISTORY

Date	Version	Description
20/09/2014	1	Draft produced by Z. Bilkova
27/10/2014	2	2nd version with input from K. Tsougeni and E. Gogolides
28/10/2014	-	Coordinator requests for some changes
31/10/2014	3	New version by K. tsougeni and E. Gogolides
2/11/2014	4	Final draft by Z. Bilkova submitted to coordinator
3/11/2014	3	Coordinator approves and submits to EC officer

Table of Contents

Executive Summary

This deliverable D7.3 originated from the WP 7, Task 7.3 - Development of whole bacteria bioassays. It is focused on the specific preselection of target bacteria by their immunocapturing using Ab-functionalized surfaces (magnetic beads or nanostructured surfaces). Detailed information regarding the choice of the best specific antibodies and development of the immunosorbents were thoroughly described in the frame of WP3 – Task 3.3 (D3.4) and 3.4 (D3.5). The aim of this deliverable is to summarize the steps achieved in bacteria capturing, integrate the developed technology to the LOVE-FOOD system and evaluate its efficiency. This task is going to be finished in M36 resulting in direct detection of captured bacteria on acoustic Love wave SAW chip. Close cooperation between UniPardubice developing the magnetic beads-based bioassay, NCSR-D working on the system using nanostructured surfaces and FORTH was essential.

Main Text

1. Introduction

The specific preselection together with the preconcentration of target bacteria in the sample to be analyzed belong to the most important actions of the whole LOVE-FOOD system. However at the same time it is an extremely tricky step – the loss of target bacteria results in decreased sensitivity and can lead to the failure of the whole system. Therefore, very efficient and specific bacteria capturing must be performed. **Immunoextraction** based on the reaction between specific antibodies and target analyte is often used for this purpose and was applied also in this project.

The success of the system lies in the quality of used **antibodies**, zero or minimum **nonspecific binding** of bacteria on the non-functionalized surfaces and high **surface area to volume ratio** during the capturing bringing high probability of interactions between antibodies and bacteria. All these aspects were deeply considered during the development of both protocols (magnetic beads-based and nanostructured surfaces-based approach).

2. Development of the protocol for the specific preselection of bacteria using Ab-functionalized magnetic particles

The approach based on the use of specific Ab-functionalized magnetic particles for capturing of target analyte is called **direct immunomagnetic separation (IMS)** (schematically represented on Fig. 1). The protocol for biofunctionalization of magnetic particles and following IMS of *Salmonella* was developed and optimized at UniPardubice in batch arrangement. To do this standard 2mL polypropylene microtubes and magnetic separators with permanent magnets were utilized. Commercial magnetic particles “Dynabeads anti-Salmonella” (cat. No. 71002 from Dynal, Applied Biosystems) and the IMS protocol suggested by their manufacturer were used as a control of the developed LOVE-FOOD bioassay.

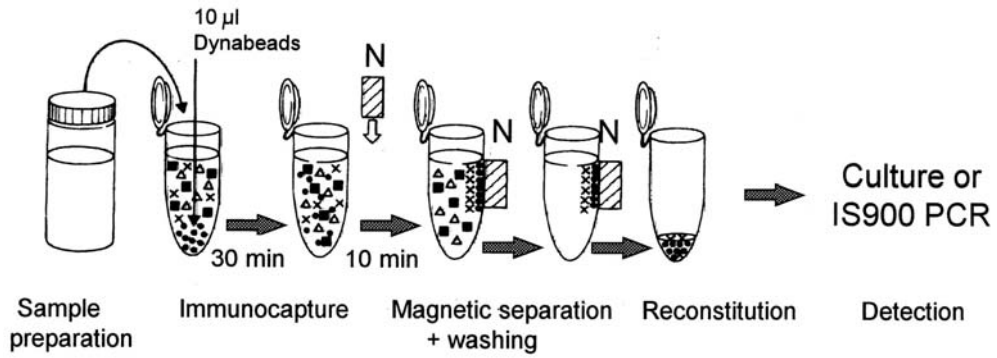

Figure 1: Schematic diagram of the IMS procedure. *(Appl. Environ. Microbiol. September 1998 64 (9), 3153-3158).*

After the IMS it was necessary to find a proper method for quantification of bacteria captured by the magnetic immunosorbents. The method of the first choice was standard **microbiological plating and cultivation** on nutrient agar medium. Although this method is widely accepted and applied for evaluation of the capture efficiency after IMS, the results obtained by this method can be underestimated. This approach works on assumption that 1 colony is a product of 1 single bacteria. Nevertheless when 2 or even more bacteria are captured with 1 magnetic particle they grow in single colony as well. Therefore in order to quantify the exact number of captured cells of *Salmonella* **ELISA** directly on magnetic particles was developed and optimized. ELISA method showed to be very efficient and precise for quantification of 10^4 and more bacteria captured per 0.5 mg of particles (see Figure 2, red part). Unfortunately for the concentrations from 10^1 to10^3 cells per 0.5 mg of immunosorbents it proved to be inaccurate and not reproducible. For this reason the IMS protocol was optimized and evaluated using the "gold standard" – **microbiological plating**.

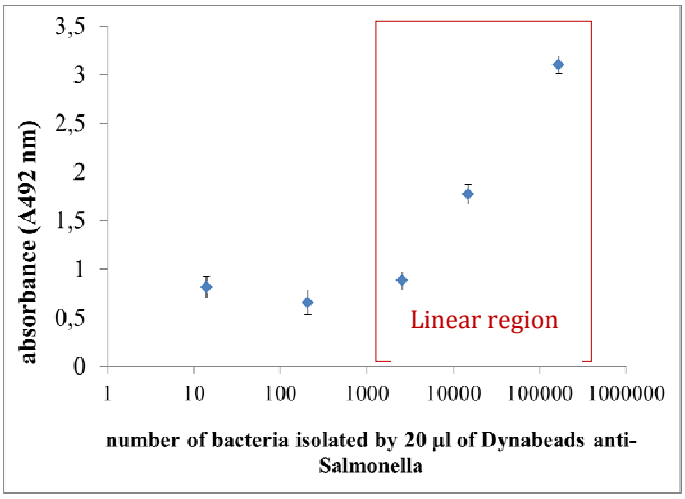

Figure 2: Quantification of *Salmonella* after IMS using ELISA on-beads.

2.1 The optimized protocol for IMS in batch

The best results of IMS of *Salmonella* in batch were achieved with the following protocol:

- 0.5 mg of Ab-functionalized particles (protocol for the biofunctionalization of magnetic particles was thoroughly described in D3.4) were 1x washed with 1mL of PBS with 0.1% BSA (bovine serum albumin) and 3x with 1mL of sterile PBS-T (10mM PBS containing 0.05% Tween 20).
- 0.5 mg of pre-washed particles were mixed in sterile tubes with 1 mL of sample to be analyzed (typically suspension of *Salmonella* Typhimurium in PBS-T in the concentration between 10^1 to 10^4 cells/mL).
- Particles were gently mixed with the bacterial suspension and incubated for 10 min under room temperature and rotation.
- The tubes were placed in magnetic separators and supernatants were removed.
- 1mL of PBS-T for washing was added to each tube; the tubes were capped and placed on the rotator for next 10 minutes.
- Previous step was repeated 1x.
- The supernatants were removed; the complexes of magnetic beads-bacteria were resuspended in 100 µL of PBS-T.
- The resuspended complexes were plated on Petri dishes with nutrient agar and cultivated overnight in the thermostat under 37 °C.

- After 24 h of cultivation the number of colonies on Petri dishes was counted.

By this optimized protocol capture efficiencies from **70 up to 95%** were achieved, depending on the initial concentration of bacteria (for more results see D3.4).

2.2 Integration of the IMS protocol to the microfluidic chip

The IMS was performed in a magnetically stabilized fluidized bed recently developed by Partner 2 – Institut Curie. This is a novel PDMS microfluidic device, in which magnetic particles are confined in a cone-shaped chamber and kept in equilibrium between the drag force imposed by the flow of the liquid solution and the magnetic force acting on the beads due to the presence of an external magnet. This configuration results in a dynamic recirculation of the magnetic particles inside the chamber, in which the magnetic beads in the center of the fluidized bed move in the direction of the flow and change directions at a given point to come to their original position following the side walls of the chamber. This recirculation enables the efficient mixing of the liquid solution with the particles, and enhances the liquid/surface contact. Furthermore, the advantage of this constant-flow system is that, compared with other microfluidic devices, relatively large volumes of sample can be made pass through the system. This allows the selective extraction of a specimen present in the solution if for example specific antibodies are grafted on the surface of the beads, resulting in the enrichment of this specimen in a much smaller volume as compared with the initial sample.

The system had been previously optimized for the extraction of sample proteins. For the immunocapture of living entities such as bacteria a new optimization was then required. In a first time the effect that the presence of bacteria can have in the recirculation of the magnetic beads was studied, as the size of these beads is comparable to the size of the individual bacteria. No significant hinder in the functioning of the system was found. In addition, tests were done to determine the non-specific adsorption of bacteria on the surface of the beads, after which it was determined that a washing step with PBS at a flow rate of 1.5 µL/min and 40 µL was sufficient to remove more than 99% of non-specifically adsorbed entities.

As shown in WP3 Task 3.3 the immunocapture of Salmonella Typhimurium with different magnetic beads and antibodies were studied by partner 4 – Pardubice, resulting in several immunosorbents that could be adapted to the fluidized bed system. Hence, and taking into account the fact that the degree of magnetization and surface chemistry affect the recirculation of the beads, several immunosorbents were

explored for their microfluidic integration. As the ideal quantity of magnetic particles for this device was previously optimized to be approx. 50 µg, the same quantity was used for the LOVE-FOOD system.

2.2.1 The optimized protocol for IMS in microfluidic chip

Three immunosorbents were selected for further capture studies and the following protocol was established for IMS in chip after optimization:

- The entrances of the microfluidic chip were branched to solenoid valves and the output was connected to a Tygon® tube with an internal volume of 100 µL followed by a PEEK tube with an internal diameter of 50 µm and 40 cm in length, being the purpose of this last one to impose the global resistance of the system.
- The chip was prefilled with PBS-T and 50 µg of beads were loaded in the main chamber with the help of a small magnet.
- A short flow of PBS-T at 1.5 µL/min and 5 µL was made pass through the chamber to remove any possible debris and homogenize the bead distribution.
- The IMS of Salmonella was performed by connecting the sample reservoir to the chip and keeping a flow rate of 1 µl/min for 50 min (50 µL).
- A washing step was followed with PBS-T at 1.5 µL/min and 40 µL, based on the tests previously described.
- At the end of the experiment the output of the chip was disconnected and magnetic beads with bacteria were flushed away and plated on Petri dishes with nutrient agar. The content of the previously described Tygon® tube was also plated to quantify non- captured bacteria. Finally 50 µL of the initial reservoir were also plated in a third Petri dish for comparison purposes.

The following capture efficiencies were determined for the three analyzed immunosorbents:

- **88.7%** for commercial anti-salmonella Dynabeads® (diameter of x µm)

- **92.3**% for Promag® particles (x µm in diameter) coated with KPL anti-salmonella antibodies

- **77.3**% for Dynabeads® M-270 Carboxylic Acid (2.8 μM in diameter) coated with KPL anti-salmonella antibodies.

3. Development of the protocol for the specific preselection of bacteria using Ab-functionalized micro-nano-structured surfaces (NCSR-D)

Details of this approach have been presented in: a) deliverable 3.5, "Nanotextured surfaces for DNA capture and related report", where all the details of the work can be found, and b) in the 24th month report (WP3), where a detailed summary of the work can be found. Here we only summarize our findings in the context of WP 7.

Fabrication and successful demonstration of a sample preparation module comprising bacteria cell capture have been performed. The module is based on capture of bacteria on plasma nanotextured surfaces and has achieved 100% efficiency for bacteria capture below a certain concentration (10^4 cells/ml). The cell capture microfluidic is composed of 3 parallel microchannels with common input and output ports, which were fabricated by direct lithography and plasma etching on the PMMA substrate, and sealed with lamination film.

We employed two polyclonal and one monoclonal antibodies (from KPL, AbD Serotec and MyBioSource Companies) targeting at the LPS core of the bacteria, prior to the addition of bacteria (Salmonella Typhimurium or E.coli). In order to confirm the attachment or not of the bacteria, fluorescence images after the completion of experiments were obtained. For the experiments we selected Salmonella Typhimurium (strain number 60.62) as aim bacteria strain (provided by Pasteur). Chips were fabricated in NCSR D and experiments with bacteria were conducted at FORTH.

In Figure 3, fluorescence images obtained from O_2-plasma-treated PMMA capture modules after immobilization of polyclonal antibody from AbD Serotec are shown for different concentration of bacteria varying from 10^2-10^8 cells/mL. By using the PMMA capture module, the Salmonella cells captured with high efficiency.

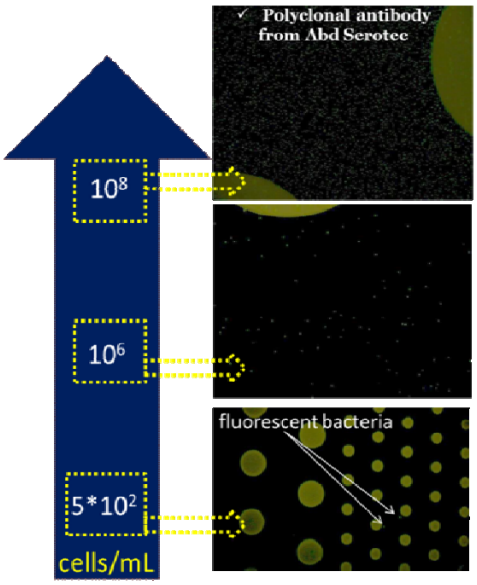

Figure 3: Fluorescence images obtained from O_2-plasma-treated PMMA capture modules after immobilization of anti-LPS antibody from AbD Serotec (100µg/mL) and for different bacteria concentrations ($5*10^2$ – 10^8 cells/mL).

In order to determine the number of bacteria that are captured in the chip in a given population, we counted the bacteria by plating the effluents of the chip on media that supports the growth of the bacteria. For the initial concentrations and the effluents we used serial dilutions, plating and counting of live bacteria in order to determine the number of bacteria in a given population.

In Fig. 4 the capture efficiency calculated after plating of effluents is shown versus the initial bacteria concentration. The capture efficiency was quantified per unit volume of the microfluidic device. If we assume the inlet and outlet concentration of bacteria as C_{in} and C_{out} (in CFU mL^{-1}), respectively, the capture efficiency is derived as

$$capture\ efficiency = \frac{C_{in} - C_{out}}{C_{in}} \cdot 100\%$$

Based on the data the efficiency increases by decreasing the concentration and is approximately 97 ±3 % for concentration 10^5 cells/mL and 100% below that.

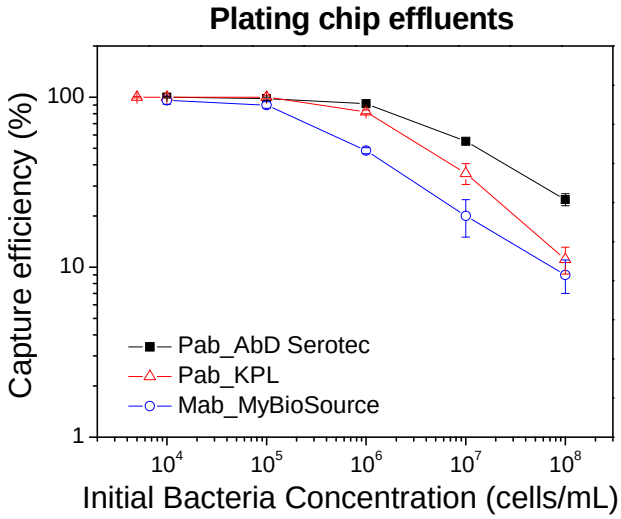

Figure 4: Capture efficiency calculated from plating of chip effluents for three antibodies.

Conclusions

We have proven that preselection of bacteria can be achieved with high efficiency both for Ab-functionalized magnetic particles and nanostructured surfaces. The SAW sensor is presently being tested for detection of bacteria binding directly on the sensor or bound to magnetic particles. If the sensor cannot detect the cell-magnetic particle complex, then bacteria release from the particles or the nanostructure surfaces has to be attempted. Different elution conditions must be tested – low pH, high molarity of sodium chloride, detergents, higher temperature – all these factors support the elution of bacteria after the immunocapturing. Probably the most promising option for elution of bacteria is called "competitive elution" when one incubates the captured cells with an excess of free antibody. This should be a gentle way to release them, but pretty expensive. These entire scenarios will be activated as soon as it is known if the SAW sensor responds to bacteria or bacteria particle complexes.