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

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

Deliverable 3.4

Report on biofunctionalization of magnetic particles and their characterization (M24)

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Executive Summary

The present deliverable deals with the biofunctionalization of commercial magnetic particles with specific anti-Salmonella antibodies and arose in the frame of WP3 as a result of the Task 3.3 (Biofunctionalization of commercial and innovative particles). It logically follows the previous deliverables D3.3 (Characterized magnetic particles suitable for LOC application), which was focused on the characterization of magnetic particles and their selection from the point of view of their subsequent integration into LoC. The ProMag particles from Bangs Laboratories showed the highest binding capacity. Therefore, the development of the first generation of specific anti-Salmonella immunosorbent was aimed at these magnetic particles.

The immobilization of specific antibodies on the surface of magnetic spheres had been an essential step in the course of development of selective and efficient magnetic immunosorbent, which was thereafter implemented into the magnetically stabilized fluidized bed developed by Partner 2, Institute Curie.

Different anti-Salmonella antibodies were tested from the point of view of their immunoreactivity with the cells of *Salmonella*. The monoclonal anti-LPS core antibodies from MyBiosource showed excellent immunoreactivity and minimum unspecific reactions with related bacteria (e.g. *Citrobacter*). Nevertheless after their immobilization on ProMag particles very low capture efficiencies of *Salmonella* were achieved. Therefore the polyclonal anti-Salmonella antibodies from the KPL Company were also immobilized on the Promag particles. With this polyclonal immunosorbent capture efficiencies from 70 up to 95 % depending on the initial concentration of *Salmonella* were attained.

Main Text

1. Introduction

High quality magnetic microparticles covered with specific antibodies, known as an immunosorbent, are a key prerequisite to performing successful immunomagnetic separation (IMS). Thus, the selection of the proper antibodies with the desired specificity as well as sensitivity towards the target antigen is an essential initial step in the preparation of such an immunosorbent. There are usually several antibodies of the same specificity available from different suppliers in the market, so the quality of antibodies often needs to be compared and then the right one for a particular application selected. Then, the best immobilization strategy leading to desired orientation of antibodies on the surface of magnetic particles has to be carefully chosen and usually optimized for the particular purpose. Introducing the spacer arms or the additional polymers during the biofunctionalization has to be considered as well.

2. Choice of anti-Salmonella antibodies

The antibodies can be evaluated from various points of view – purity, source, presence of glycosylation and affinity or immunoreactivity. These parameters can be significantly affected by the method of manufacture and storage conditions.

For testing the immunoreactivity between different commercial anti-Salmonella antibodies (their list is summarized in Table 1) and the cells of *Salmonella* Typhimurium, whole-cell dot-blot method was performed.

Table 1: List of commercial anti-Salmonella antibodies selected for the LOVE-FOOD project.

Clonality	Host animal	Isotype	Immunogen	Supplier	Form
polyclonal	goat	ND	Various strains of *Salmonella*	KPL	lyophilized
	rabbit	ND	Mixture of *S*. Enteritidis, *S*. Typhimurium, *S.* Heidelburg	Virostat	0.01 M PBS pH 7.2 with 0.1% NaN_3
monoclonal	mouse	IgG2a	LPS core of *Salmonella*	GenWay Biotech	
	mouse	IgG2a	LPS core of *Salmonella*	MyBiosource	

It was found out that both tested polyclonal antibodies immunospecifically reacted with the cells of *Salmonella* Typhimurium, low cross-reactivity with *Escherichia coli* and *Citrobacter braakii* was

observed as well, as illustrated in Figure 1. In case of monoclonal anti-LPS core antibodies from MyBiosource, the immunoreactivity with Salmonella typhimurium was excellent and no unspecific reaction with related bacteria was detected. In contrast, the monoclonal GenWay did bind to the cells of Salmonella typhimurium with very low intensity.

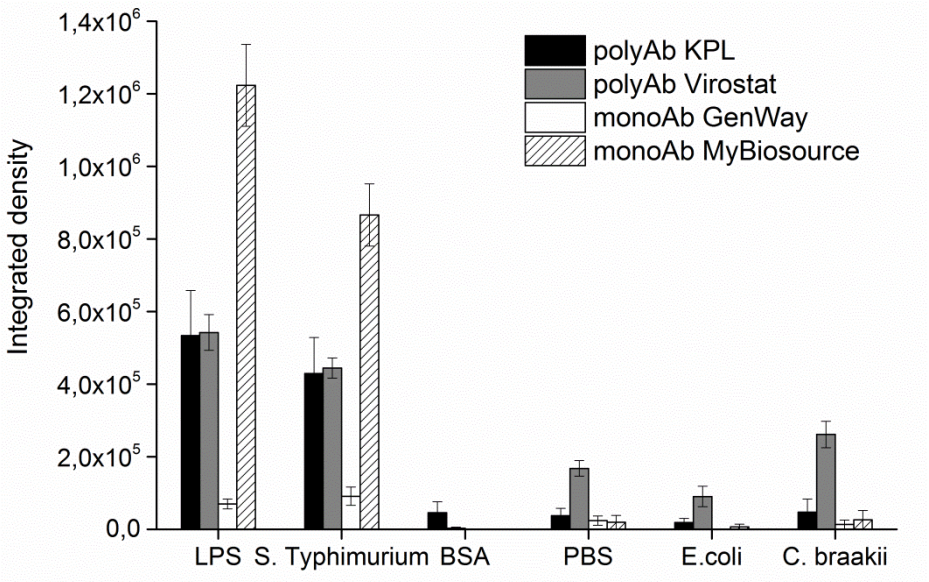

Figure 1: Integrated density as an expression of immunoreactivity of anti-Salmonella antibodies with bacterial cells, lipopolysaccharide (LPS) and reaction blanks (phosphate buffered saline, PBS; bovine serum albumin, BSA).

Based on these results monoclonal antibodies from MyBiosource seemed to be the best from the list of tested antibodies and were thus applied for biofunctionalization of ProMag magnetic particles.

3. Immobilization of anti-Salmonella antibodies on magnetic particles

3.1 Choice of the coupling method for immobilization of anti-Salmonella antibodies

There are different possibilities regarding the immobilization of antibodies on the surface of magnetic particles. The most widely used approach for bioconjugation reactions leading to the formation of covalent bonds uses of ***N*-substituted carbodiimides**. These reagents can react with the carboxylic groups to form a highly reactive derivative and subsequently with primary amines to form a stable peptide bond. The advantage of this approach lies in the high yields of bioconjugations, while its disadvantage is the random orientation of antibodies on the carrier. Immobilization of antibodies trough their conserved **carbohydrate moieties** located in the Fc fragment of IgG has been found to increase the steric accessibility of the immunospecific sites on the Fab fragments. This approach leads to the pre-defined orientation of antibodies on the surface

of carrier and therefore assures the steric availability of the antibody binding site for the reaction with the ligand. In this project both mentioned approaches were applied.

3.2 Immobilization of MyBiosource antibodies on the ProMag particles

First, monoclonal anti-Salmonella antibodies from the MyBiosource Company were immobilized on the ProMag particles by the carbodiimide coupling strategy. The amount of antibodies for biobunctionalization of 1 mg of ProMag particles was optimized and evaluated by the SDS-PAGE analysis followed by silver staining. It was found that immobilization of 25 µg of anti-Salmonella antibodies per 1 mg of carrier is sufficient, leading to high binding efficiency and minimum Ab loss. The optimized protocol for this coupling reaction is the following:

- 1 mg of ProMag magnetic particles was washed 4x with 1 mL of 0.1M MES buffer, pH 5.0.
- 7.5 mg of sulfo-NHS and 1.25 mg of EDC dissolved in 0.5 mL 0.1M MES buffer, pH 5.0, were successively added to pre-washed particles.
- The reaction suspension was stirred gently for 10 minutes at room temperature.
- Supernatant was removed and particles were washed 1x with 1 mL of 0.1M MES buffer, pH 5.0.
- 25 µg of antibodies was added to washed particles and the reaction mixture was incubated overnight at 4°C with gentle mixing.
- Thereafter, the immunosorbent was washed 3x with 1 mL of MES buffer, pH 5.0, then 2x with 1mL of 1M NaCl with stirring for 10 min to remove all unreacted or non-specifically adsorbed molecules and 5x with 1 mL of PBS buffer with 1% BSA.

As soon as the protocol for biofunctionalization of ProMag particles with the MyBiosource antibodies was optimized and the successful coupling was confirmed, Immuno-Magnetic-Separation (IMS) of *Salmonella* in batch followed. However, the results of IMS were repeatedly unsatisfactory. From the initial suspension of bacteria with approx. 1300 cells only 8 cells were captured resulting in 6% capture efficiency (according to the results from plating). After that, different immobilization protocols together with different particles were tried in order to prepare magnetic immunosorbents of high separation efficient carrying the MyBiosource antibodies (summarized in Table 2). Prepared immunosorbents were used for IMS of *Salmonella* Typhimurium in batch.

Table 2: Summary of different particles and conjugation methods which were applied for immobilization of the monoclonal anti-Salmonella MyBiosource antibodies.

Particles	Immobilization method	Captured cells (%)*
ProMAG-Carboxyl (no additional functionalization)	• Non-oriented immobilization. Two-step carbodiimide method.	• 0.6 %
Dynabeads MyOne-Carboxyl , Dynabeads M270 Carboxyl, SiMAG-COOH, Micromer-COOH (no additional functionalization)	• Non-oriented immobilization. Two-step carbodiimide method.	• 0.8 %
Dynabeads-Amine coated with Hyaluronic Acid	• Non-oriented immobilization through the HA. Two-step carbodiimide method.	• 17.0 %
SiMAG-Hydrazide	• Oriented immobilization. Oxidation of carbohydrates (IgG).	• 0 %
ProMAG-Carboxyl coated with PEG (Hydrazide-PEG-Hydrazide)	• Oriented immobilization. Oxidation of carbohydrates (IgG).	• 2.2 %

*... compared to the number of bacteria in the initial suspension

Although the monoclonal MyBiosource antibodies showed excellent immunoreactivity and specificity in the dot blot experiment (see 2), after their immobilization on different types of magnetic particles the capture efficiency of *Salmonella* was very low and typically did not exceed 2 %. Therefore polyclonal anti-Salmonella antibodies from the KPL Company were immobilized on the ProMag particles and the modified immunosorbent was applied for IMS (see 3.3).

3.3 Immobilization of KPL antibodies on the ProMag particles

The same protocol which was used for the immobilization of MyBiosource antibodies on the ProMag particles (see 3.2) was applied for the biofunctionalization of particles with the KPL antibodies. The amount of antibodies in the immobilization mixture was also optimized (0 - 25 – 50 – 75 – 100 μg of antibodies per 1 mg of particles). Prepared immunosorbents were immediately used for IMS in batch (results are summarized Figure 2). The capture efficiency of all immunosorbents **exceeded 89 %.** When higher density of bacterial suspension (thousands of cells) was used for the immunomagnetic separation, capture efficiencies (percentage of captured cells vs. the concentration of the initial suspension) slightly decreased (down to 50 - 60%).

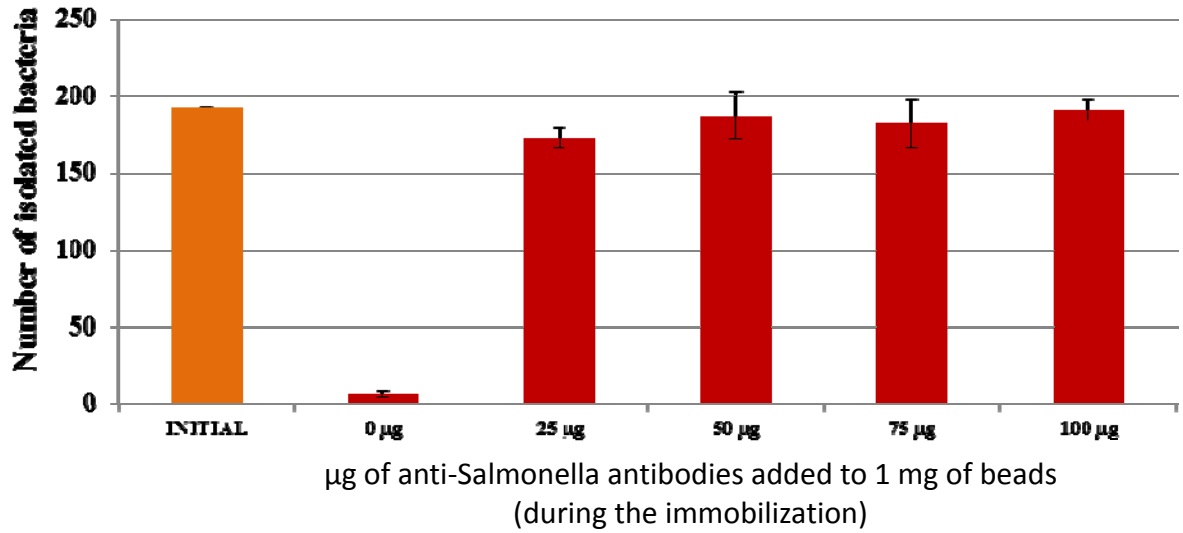

Figure 2: Number of bacteria captured using the newly prepared anti-Salmonella immunosorbents (polyclonal KPL antibodies, ProMag magnetic particles).

This immunosorbent proved to be highly efficient (especially for IMS of bacteria from suspension containing 100-500 bacteria), colloidaly stable, monodisperse and highly magnetically susceptible. Its development has been completed. The following paragraph summarizes its characteristics:

- diameter: 0.88 µm
- density of particles: 1.33g/cm^3
- type of magnetic microspheres: polymer
- uniformity: monodisperse
- specificity of the Ab: polyclonal, affinity purified from serum pool of goats immunized with *Salmonella* serotypes of group A, B, C, D, E
- capture efficiency: 50 - 98 % (depending on the number of cells used for IMS)

Conclusions

Task 3.4 was successfully finished in time leading to the development of highly efficient immunosorbent for Salmonella detection. The immunosorbent was also implemented into magnetically stabilized fluidized bed developed by Institute Curie. Results from microfluidic chip supported the results from batch wise arrangement, excellent behavior together high capture efficiencies of prepared immunosorbent were recorded.

Even if the Tasks 3.3 as well as 3.4 were completed additional detailed characterization of immunosorbents is still in progress in order to obtain the most accurate and reliable information.