

ON-CHIP ELISA ON MAGNETIC PARTICLES: ISOLATION AND DETECTION OF SPECIFIC ANTIBODIES FROM SERUM

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Abstract

In recent years, microfluidics has shown considerable promise for improving diagnostics and biology research. Certain properties of microfluidic technologies, such as rapid sample processing and the precise control of fluids in an assay, have made them attractive candidates to replace traditional experimental approaches [1]. Here, an example of such application is presented: ELISA that uses magnetic particles as the solid phase (MELISA) implemented into a microfluidic device. The microfluidic Rhombic chamber chip (120 μ l chamber volumes, hydrophilized) in ChipGenie[®] edition-P holder (microfluidic ChipShop, Jena, D) with strong integrated magnets enabling to fix the magnetic particles in reaction chamber and arrange them as a fluidized bed was employed. The behaviour and colloidal stability of 11 various types of superparamagnetic particles varying in composition and diameter (0.5-100 μ m) were compared. Parameters as optimal amount of magnetic particles inside the chamber, rate of aggregation, clogging the chip channels and stability of magnetic cloud inside the chip were evaluated. The microparticles with the best parameters were then coated with specific antigen (chymotrypsin) and traditional MELISA with such bioactive carrier and variously diluted porcine serum was performed in microplate arrangement and simultaneously in microfluidic chip. Only the final step, measurement of sample absorbance was performed off-line in microplate spectrophotometer. Our results repeatedly confirmed that MELISA can be easily adopted for microfluidic version drawing all benefits associated with the miniaturization.

Keywords: ELISA, Microfluidics, Magnetic Particles, Lab-on-Chip

1. INTRODUCTION

The magnetic particles (MPs) have numerous properties that make them suitable for wide-spread use in various applications from imaging to drug delivery: ease of manufacturing, manipulation in fluid and a wide range of commercially available MPs diameters, ranging from nanoparticles (50 nm) to microparticles (up to tens of μ m) [2]. One of the possible applications of MPs is a magnetic solid phase enzyme-linked immunosorbent assay (MELISA). The principle of the method is the same as for commonly used enzyme-linked immunosorbent assay (ELISA) except: the binding ligand is bound on the surface of MPs instead of walls of microtitration plate [3], the 96-well plate magnetic separator is applied for separation of the MPs during washing steps and the microplate-shaker for homogenous dispersion of MPs. The MELISA arrangement brings advantages such as robustness of ligand amount per well compare to inaccuracy of adsorbed ligand in microtitration plate. Moreover, the dispersed MPs have higher interaction with the target ligand in the sample ensuring better and faster kinetics of the reaction [4] and thus higher sensitivity. Therefore this technique is used for example for detection of antibodies that are not detectable with conventional ELISA [5]. Both conventional methods ELISA and MELISA are quite laborious and time-consuming since require numerous washing steps, accurate pipetting, placing on shaker etc. The use of microfluidics to perform sandwich immunoassays permits one to specifically address these drawbacks. The low scale of these devices reduces the volumes and diffusion distances inside the micro-channels, therefore decreasing the duration of the successive incubation periods especially in combination with ligand-coated MPs and easy fluid manipulation [6-8]. The aim of this work was to optimize the MELISA protocol for detection of antibodies for on-chip application. Simple commercial microfluidic set from microfluidic ChipShop was employed. This device

provides easy liquid application, automatic mixing of the MPs and high ratio of used MPs vs. chamber volume. Therefore, this setup enables one to capture specific antibodies on the surface of the magnetic biofunctionalized microparticles that can be (i) eluted and subsequently detected and/or (ii) directly detected by MELISA protocol upon the application requirements. Both MELISA arrangements (in microtitration plate and/or in microfluidic chip) were performed simultaneously under the same conditions and the results were subsequently compared. The preliminary results of on-chip MELISA for detection/isolation of anti-chymotrypsin antibodies are presented in here.

2. EXPERIMENTAL

2.1. Magnetic particles

Magnetic bead cellulose MT200 with hydroxyl functional group, size 80-100 μm (Iontosorb, Ústí n. Labem, CZ, EU); magnetic hypercrosslinked poly(styrene-co-divinylbenzene) microspheres (HMP-S-MG4) with sulphite functional group, size 5.7 μm [9] and magnetic poly(glycidyl methacrylate) albumin-coated microspheres with carboxyl and/or amino functional group, size 4.5 μm (PGMA-ALB) [10] were obtained from Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague, CZ, EU; Micromer microparticles with carboxyl or amino functional group, size 3 μm and 4 μm (Micromod, Rochester, NY); Dynabeads with carboxyl functional group, size 1 μm or 2.8 μm (Thermo Fisher Scientific, Waltham, MA); SiMAG particles with amino functional group, size 0.5 μm or 1 μm (Chemicell, Berlin, D) and Sera-Mag® Speed Beads with carboxyl functional group, size 0.771 μm or 0.816 μm (Seradyne, Indianapolis, IN).

2.2. Microfluidic device

The microfluidic Rhombic chamber chip (120 μl chamber volumes, hydrophilized) contains two microfluidic chambers with two inlets and two outlets, the dimensions are mentioned in **Fig. 1a**. The chip fits into ChipGenie® edition-P holder (microfluidic ChipShop, Jena, D, see **Fig. 1b**) with two strong integrated magnets enabling fix the magnetic particles in reaction chamber and arrange them as a fluidized bed. The magnets inside the chip holder move forward and backward below the microfluidic chamber and keep the fluidized bed moving left and right thus efficient mixing inside the chip is ensured.

2.3. MELISA in microtitration plate

Suspension of magnetic biofunctionalized carrier was added into microtitration plate (25 $\mu\text{g}/\text{well}$) in sample buffer (PBS pH 7.4 with 0.05% Tween and 0.1% BSA). 100 μl of porcine hyperimmune serum prediluted by the sample buffer in range 1:30,000-1:120,000 was added and 1h incubation at RT under agitation followed. Subsequently, the particles were three times washed by PBS pH 7.4 with 0.05% Tween and 5% BSA using the magnetic separator for 96 well plates (MagnetoPURE96, Chemicell, Berlin, D). Then, 100 μl of goat anti-porcine IgG marked with HRP (Sigma-Aldrich, St. Louis, MO) diluted in ratio 1: 16,000 in bicarbonate buffer pH 9.5 with 0.05% Tween and 0.1% BSA was added and incubation for 1h at RT under agitation followed. The particles were washed by PBS pH 7.4 with 0.05% Tween and 5% BSA three times and then two times with PBS pH 7.4. After that, 100 μl of substrate solution (0.003% H_2O_2 in 0.1M acetate buffer pH 5.5 with 0.1 mg/ml of substrate TMB from Immunotech Beckman Coulter, Marseille, F) was added per well. The incubation took 15 min at RT in dark. The reaction was stopped by adding 50 μl 1M H_2SO_4 and the absorbance was measured at 450 nm in microplate spectrophotometer (LabSystems Multiskan RC, Thermo Fisher Scientific, Waltham, MA).

2.4. On-chip MELISA

The suspension of magnetic biofunctionalized carrier (1 mg in sample buffer) was added into microfluidic chamber via orthogonally placed syringe underneath the inlet of the chip. The magnet was switched on. Other steps were similar to 2.3. except the washing step (continuous injection of 300 μl) and final off-line step (adding 50 μl 1M H_2SO_4 and microplate spectrophotometer measurement).

3. RESULTS AND DISCUSSION

3.1. On-chip behaviour and properties of various magnetic particles

Magnetic particles are made of different materials, undergo various surface modifications (pegylation, polymerization, insertion of functional groups and/or bridges enabling efficient binding of target ligands) and therefore have diverse behaviour and colloidal stability inside the microfluidic chip (**Fig. 1**). In this work, eleven superparamagnetic particles varying in material, structure and diameter (0.5-100 μm) were compared. Initially, aliquot of 10 mg particles was taken from the stock solution and applied one by one (milligram) into the chip. Parameters as optimal amount of magnetic particles inside the chamber (in other words how much of the beads can be applied inside the chamber) and the maximal capacity without losing the magnetic particles were evaluated. In addition, rate of particles aggregation, clogging the chip channels by the aggregates, stability of the fluidized bed (leaking the particles out of the chip) and speed of the cloud shift (how fast the fluidized bed moves from the inlet to the outlet when the magnet is moving left and right) were observed.

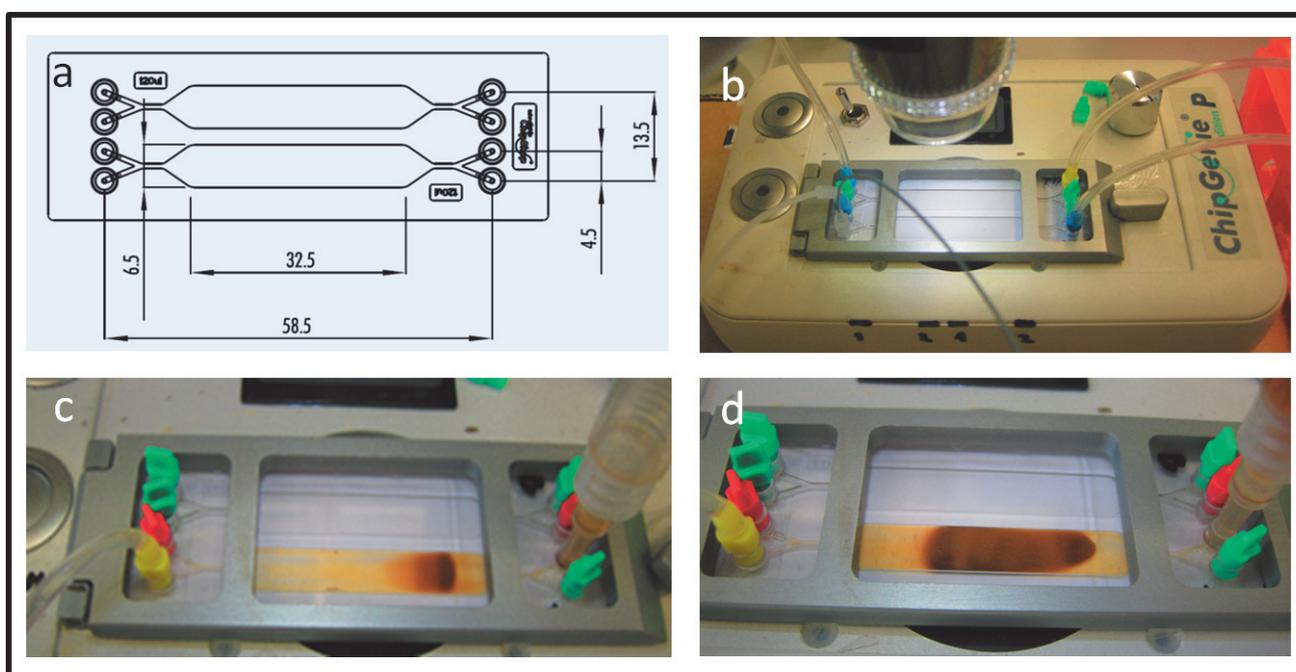


Fig. 1 (a) The microfluidic Rhombic chamber chip (120 μl chamber volume, hydrophilized); (b) ChipGenie® edition-P chip holder (microfluidic ChipShop, Jena, D). (c) Microfluidic chamber filled with 1 mg and/or (d) 5 mg of superparamagnetic microparticles (PGMA-ALB, 4.5 μm in PBS pH 7.4) forming a fluidized bed

Table 1 summarizes these observations. The MPs that have the optimal parameters, such as wide working range of amount of the applied particles, low/no leaking of the particles from the chip, no aggregation of the particles, no clogging in the microfluidic channels, and that moreover had slow shift of the fluidized bed are highlighted in the table (grey) and are overall evaluated as (+). Other magnetic beads suffered especially from leaking from the chip in spite of small amount of particles inserted, also from aggregation inside the chamber and fast shift of fluidized bed caused by moving the magnets underneath the chip forward and backward. Generally, small particles (size 0.5-1.0 μm) leaked out of the chip from the beginning as well as the biggest one (size 80-100 μm). Probably, this is due to the low iron oxide content inside each of the small particle. In case of the big particles, we suppose that the weight vs. size ratio decrease the stability inside the chamber and the particle kinetics export them out of the chip. The particles of size 2.8 to 4.5 μm performed the highest stability and were evaluated as the most suitable for this microfluidic device.

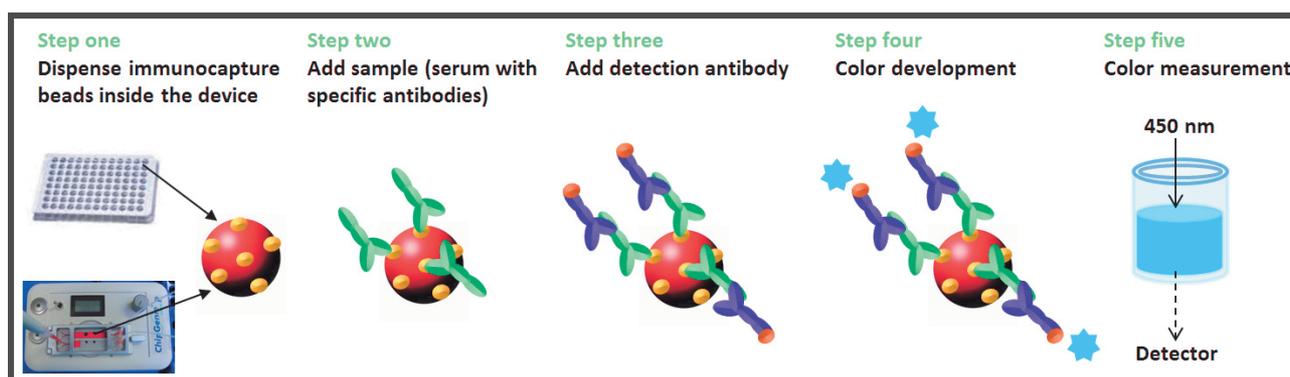
Table 1 Properties of various superparamagnetic particles observed in microfluidic ChipGenie® edition-P Rhombic chip

Name	Size (µm)	Opt. (mg)	Max. (mg)	Particle leaking	Agg.*	Clogg.*	Shift speed	Overall eval.*
SiMAG-NH2	0.5	2-4	6	From the beginning	yes	no	fast	-
Seradyn	0.771	1-2	4	From the beginning	no	no	very fast	-
Seradyn	0.816	1-2	5	From the beginning	yes	no	very fast	-
SiMAG-NH2	1	1-4	10	From the beginning strongly	yes	no	middle	-
Dynabeads	1	1-2	4	From the beginning strongly	no	no	fast	-
Dynabeads	2.8	2-8	10	From 8 mg weakly	no	no	slow	+
Micromer	3	2-5	10	From 5 mg weakly	no	no	middle	+
Micromer	4	2-8	10	From 9 mg weakly	no	no	middle	+
PGMA-ALB	4.5	1-6	10	From 8 mg weakly	no	no	slow	+
HMP-S-MG4	5.7	1-5	9	From the beginning weakly	no	at 9 mg	slow	-
Iontosorb M-OH	80-100	1-2	6	From 3 mg strongly	yes	at 6 mg	fast	-

* Agg. - aggregation; Clogg. - clogging; Eval. - evaluation

3.2. On-chip MELISA

Micromer particles (4 µm in diameter and amino functional group) were selected as the most suitable according to previous experiments for the MELISA application inside the microfluidic chip. The microparticles were coated with specific antigen (chymotrypsin) and such bioactive carrier (1 mg) was applied simultaneously into the reaction chamber of microfluidic chip and into the microtitration plate (see **Fig. 2**). So far, the incubation time was for both arrangements the same although we plan to shorten the incubation time for on-chip application.


Fig. 2 Principle of MELISA (Magnetic Enzyme-Linked Immunosorbent Assay) inside the microtitration plate and/or the microfluidic chip (ChipGenie® edition-P, ChipShop, Jena, D)

The preliminary results from MELISA performed in microtitration plate and in the microfluidic chip are presented in **Fig. 3**. The same protocol for determination of anti-chymotrypsin antibodies in hyperimmune porcine serum was repeated in three subsequent days on always fresh aliquot of the bioactive carrier with immobilized chymotrypsin. The data from the three measurements in microtitration plate and their trend lines are shown in **Fig. 3** (black symbols). The coefficient of determination (R^2) were for day 1 (0.986), for day 2 (0.906) and for day 3 (0.845). The standard deviation between the three measurements in microtitration plate for each serum dilutions were: 1:30,000 (0.057); 1:40,000 (0.120); 1:60,000 (0.081); 1:80,000 (0.065); 1:120,000 (0.029). The

results from the on-chip application, for serum dilution 1:30,000, are also presented in **Fig. 3** (colored symbols) and their values went along the trend of the results from the microtitration plate. The highest values were achieved in the day 2 as well as the lowest values in day 3. The standard deviation among the three subsequent measurements in microfluidic setup was 0.183. Such variability between the measurements were probably due to the technique of the final sample volume uptake from the outlet of the device. We are now about to develop a robust technique which would enable us to reach the more reproducible results.

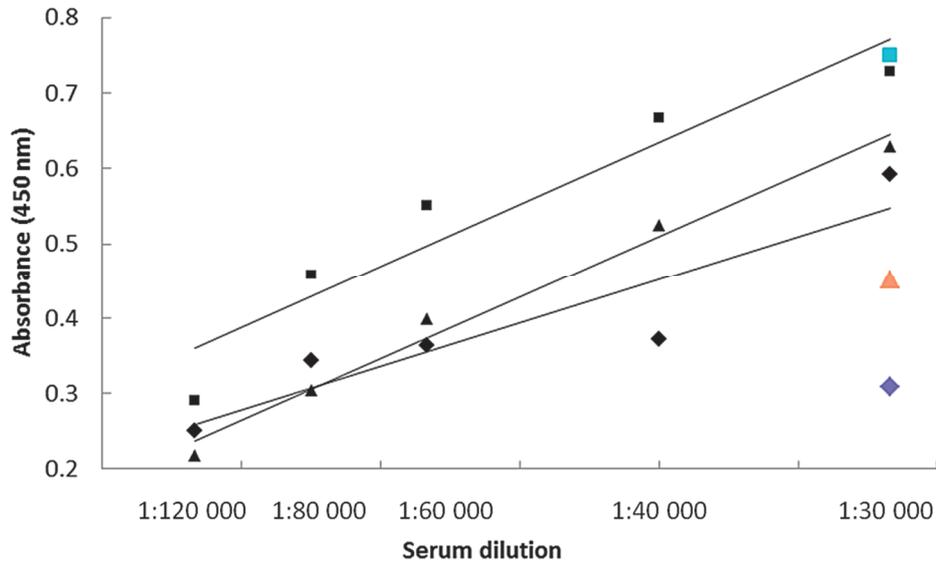


Fig. 3 The results from MELISA determination of anti-chymotrypsin specific antibodies in hyperimmune porcine serum (high titer of specific antibodies to chymotrypsin). The measurement of 5 different serum dilutions was repeated in 3 subsequent days. The black symbols correspond to results from MELISA on microtitration plate and the colour symbols correspond to on-chip MELISA (only for dilution 1:30,000). The day one (triangel), the day two (square), the day three (rhombus)

In these preliminary results 1 mg of magnetic biofunctionalized carrier was employed for fluidized bed formation inside the microfluidic chamber. In the future experiments we would like to use higher amount of particles up to maximal limit for the microfluidic chamber to test the maximal capacity of the device for specific antibody capture and detection via on-chip MELISA. Moreover, we would like to test the stop-flow repetitive sample addition and incubation with the biofunctionalized carrier in on-chip arrangement, preconcentrate the specific antibodies inside the chamber and perform a subsequent elution and collection of the isolated specific antibodies for further analysis or experiments. In the future, we would like to apply the improved and optimized microfluidic setup for anti-amyloid β antibodies isolation/detection from the Ivlg samples since such natural antibodies have potentially neuro-protective effect and therapeutic potential for the treatment of patients with Alzheimer disease [11].

4. CONCLUSION

The on-chip MELISA is an efficient and easy method applied for specific antibody isolation and detection. The preliminary data presented here were obtained with the model system represented by chymotrypsin and the specific anti-chymotrypsin antibodies isolated from hyperimmune porcine serum. The promising results encouraged us in future work that we would like to focus on improving the sample uptake from the chip with the aim to enhance the data reliability and robustness. In addition, modification of the procedure for maximization of the amounts of isolated specific antibodies in minimal volume ensuring low diffusion and

dilution of the target antibodies will be tested. This technique has high potential for automation and parallelization that enables to perform more efficient and thus significantly less time-consuming assay.

ACKNOWLEDGEMENTS

Financial support of the European Union's projects LOVE-FOOD (No. 317742) and NADINE (No. 246513) are gratefully acknowledged as well as the financial support of the student grant of Faculty of Chemical Technology at University of Pardubice No. SGFCHT07/2015.

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