SCIENTIFIC REPORT

regarding the implementation of the project between December 2013 - December 2014

Title of the project: "DETECTION AND IDENTIFICATION OF BIOMOLECULES OF MEDICAL INTEREST BY USING MAGNETIC AND OPTIC PROCESSES"

Phase II. Functionalization of (a) magnetic microparticles and non-magnetic nanoparticles with antibodies and oligonucleotides to be used by the bio-barcode method; (b) obtaining of experimental data concerning the capacity of the bio-barcode method to detect and identify very small concentrations of target biomolecules by using fluorescent microscopy.

Activity II.1. Preparation of magnetic microparticles functionalized with receptor antibodies.

Antibodies can be used in different bioassays either as bioreceptors towards a specific antigen or as targets. To be used as bioreceptors, the antibodies should be immobilized on different solid supports. The quality of the assay is significantly influenced by the solid supports since the latter dictate not only the efficiency of the strength of the antibody's binding, but also the specific or non-specific binding and antibody's accessibility to the antigen.

Immobilization of the antibodies is further complicated by several chemical and physical properties of the proteins. Thus, there is not a method for protein multiplication as in the case of the nucleic acids (through polymerase chain reaction - PCR) and, also, the proteins are (i) much more complex from the structural and chemical standpoint and (ii) more heterogeneous than nucleic acids.

Therefore, it is difficult to define the general binding and detection strategies able to non-discriminate proteins. Also, contrary to the nucleic acids, proteins reduce their biochemical activity due to denaturation, dehydration and oxidation.

Generally, the functionalization chemistry applied for high area surfaces is available also for polymeric or metallic microparticles coated/uncoated by specific ligands.

<u>Functionalization of magnetic microparticles with anti-IgG antibodies</u>

In this activity of the project, there were used magnetic microparticles coated with polymer and carboxylic groups to bind IgG antibodies (anti-goat IgG) produced in rabbit

against the goat immunoglobulin G (IgG).

Since one of the most used method to functionalize with antibodies the magnetic microparticles coated with polymer and COOH groups is based on 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) approach that allow the binding of the microparticles to amino groups of the antibodies, the anti-goat IgG were immobilized by using this method.

The coupling method is described below:

1. The microparticles, the coupling and the washing buffer were heated at 22 °C (RT).
2. 0.5 ml microparticle suspension (about 22 mg microparticles) was transferred into an Eppendorf tube.
3. The microparticles were centrifuged 15 minutes at 1000 G and re-suspended in 0.4 ml coupling buffer.
4. After centrifuging again 15 minutes at 1000 G, the microparticles were re-suspended into 0.17 ml coupling buffer.
5. Just before use, a carbodiimide solution (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), 10 mg in 50 µL coupling buffer, was prepared.
6. 20 µL from the solution prepared before (EDAC) was added into the microparticles suspension under stirring.
7. After 15 minutes of activation, 120 µg of anti-goat IgG (60 µL from 2 mg/ml IgG solution) were added in 0.25 ml coupling buffer.
8. The solution was slightly stirred at room temperature for 60 min, then centrifuged 15 minutes at 1000 G.
9. Finally, the microparticles were re-suspended in 0.4 ml washing buffer.

Activity II.2. Preparation of non-magnetic nanoparticles functionalized with receptor antibodies and oligonucleotides.

In this activity, gold nanoparticles prepared in the previous activity of the project were used. Two types of functionalized nanoparticles were prepared: (i) gold nanoparticles coated with anti-goat antibodies and two types of oligonucleotides, and (ii) gold nanoparticles coated with oligonucleotides only (Fig. 1).

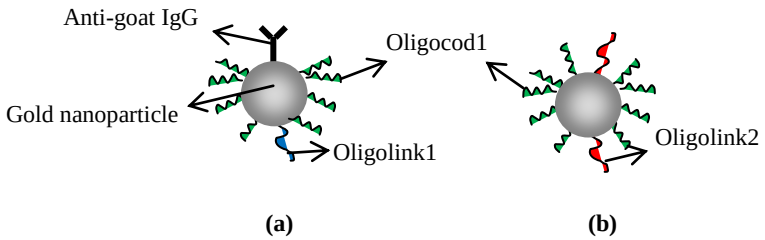

Fig. 1. Gold nanoparticles coated by (a) anti-goat IgG and Oligocod1 and Oligolink1 oligonucleotides and (b) Oligocod1 and Oligolink2.

A. Preparation of gold nanoparticles coated with receptor antibodies (anti-goat IgG)

To functionalize citrate-coated nanoparticles, there were used three types of thiol-ended oligonucleotides (Table 1) presenting an increased affinity for gold surfaces and able to displace the citrate molecules. Another fourth type of oligonucleotides (Oligocod2) were tagged by a fluorophore (AlexaFluor488), being complementary to Oligocod1 immobilized on the gold surfaces.

Tabel 1. Base sequences of the oligonucleotides bound to the gold nanoparticles

Name	Structure
Oligocod1	TAA TTC CGG TTA ATG CGG CCC AAA AAA AAA A [ThiC3];
Oligocod2	GGG CCG CAT TAA CCG GAA TTA[A488];
Oligolink1	CAG CTA GTA TGT TCC GGA ATG TAC TGT TCG GAA AAA AAA AAA AAA AA [ThiC3];
Oligolink2	CCG AAC AGT ACA TTC CGG AAC ATA CTA GCT GAA AAA AAA AAA AAA AA [ThiC3];

Given the fact that the thiol groups are susceptible to oxidation, forming disulfidic (S-S) groups, the thiol-terminated oligonucleotides were rebuilt before using them. Thus, the oligonucleotides were first centrifuged and dispersed in phosphate buffered saline (PBS) ph 7.4. Then, DL-Dithiothreitol (DTT) was added to a final concentration of 100 mM DTT. After 30 minutes incubation at room temperature, a NAP-10 (GE Healtcare - Sigma) column was used to remove the PBS and DTT excess. After reducing with DTT, *Oligocod1* and *Oligolink1* oligonucleotides were used along with anti-goat antibodies to functionalize the first type of oligonucleotides, while the *Oligocod1* and *Oligolink2* were used to functionalize the second type of nanoparticles.

For experiments, 5 ml of colloidal suspension of gold nanoparticles (3.5 nM) were used; the pH was modified to 9 by using Na_2CO_3. Over the colloidal solution, 30 μg anti-goat IgG along with 900 μl *Oligocod1* (20 μg/ml) and 100 μl *OligoLink1* (20 μg/ml) were added, and after stirring 3 hours at room temperature, the solution was overnight kept at 4 °C in the fridge.

Next, 500 μl PBS (0.01 M, pH 7.4) and 5 μl 10 % (w/v) sodium dodecyl sufate (SDS - anionic surfactant) were added, mixing 30 minutes at room temperature.

1 ml NaCl (1.2 M), was gradually added, then 0.5 ml 5 % (w/v) bovine serum albumin, 3 hours later.

The sample was kept in the dark at 4 °C for further use.

Activity II.3. Experimental tests for evaluating the capacity of the bio-barcode method to detect and identify small concentrations of target biomolecules

To detect target antibodies, a fluorescence inverted microscope (Axio Observer D1 Carl Zeiss) was used to measure the AlexaFluor488-modified fluorescent oligonucleotides.

The detection method (Fig. 2) have used magnetic microparticles functionalized with anti-goat IgG and 2 types of gold nanoparticles functionalized with anti-goat IgG and 2 types of oligonucleotides: one type used as receptors for AlexaFluor488-modified fluorescent oligonucleotides and the others were used to bind the 2 different types of gold nanoparticles.

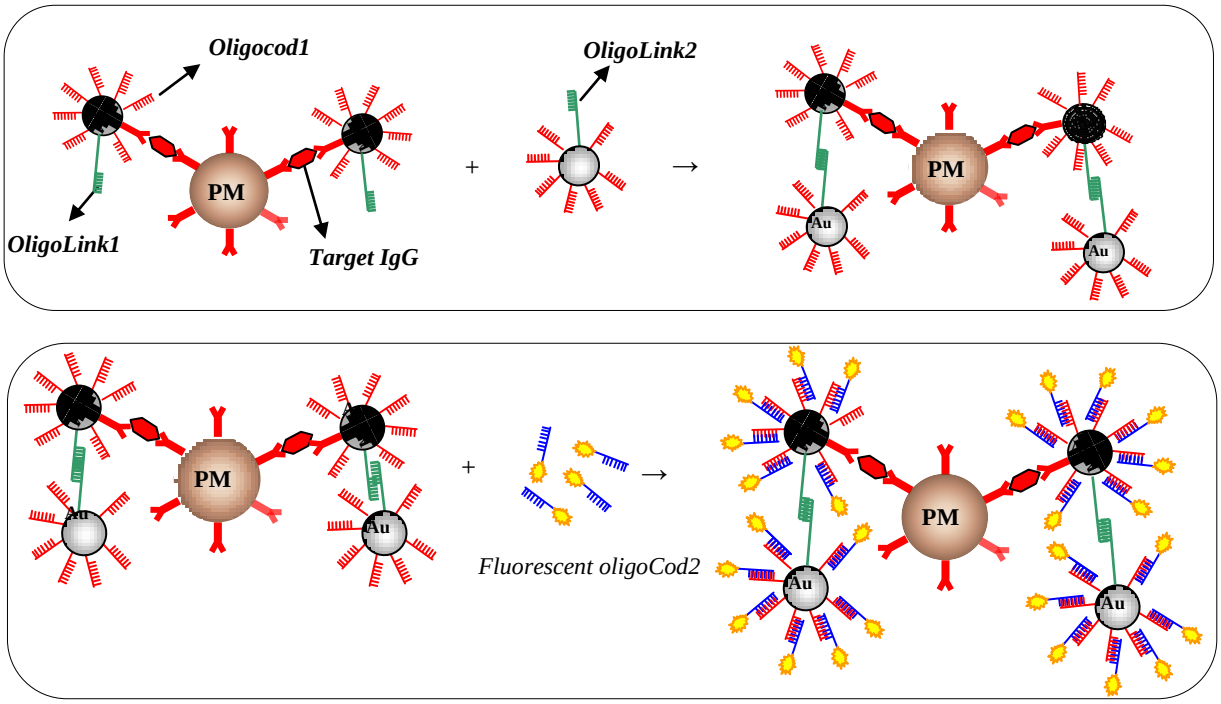

Fig. 2. The detection principle

After binding the goat IgG to anti-goat IgG (in PBS 7.4), the microparticles have been magnetically separated by using a permanent magnet NdFeB. Then, the first type of gold nanoparticles was added and incubated at room temperature. Thereafter, the formed complexes were magnetically separated.

After washing with PBS, the second type of gold nanoparticles were added and bound to the first series of gold nanoparticles through the complementary oligonucleotides oligolink1 and oligolink2. The new formed complexes were magnetically separated and washed with PBS.

After adding the fluorescent oligocod2 oligonucleotides, followed by magnetic separation and washing with PBS, a solution of dithiothreitol 100 mM was added into the particle

suspension to remove the oligonucleotides from the gold surfaces. After magnetic separation of the magnetic microparticles, the supernatant (containing the released fluorescent oligonucleotides) was investigated by using the fluorescent microscope. The samples were dispersed between 2 transparent lamella when they were measured by fluorescence microscopy.

The intensity of the fluorescence was calculated from the images (Fig. 3) obtained through fluorescence microscopy by using a software for image processing and analysis [1], ImageJ (http://imagej.nih.gov/ij/).

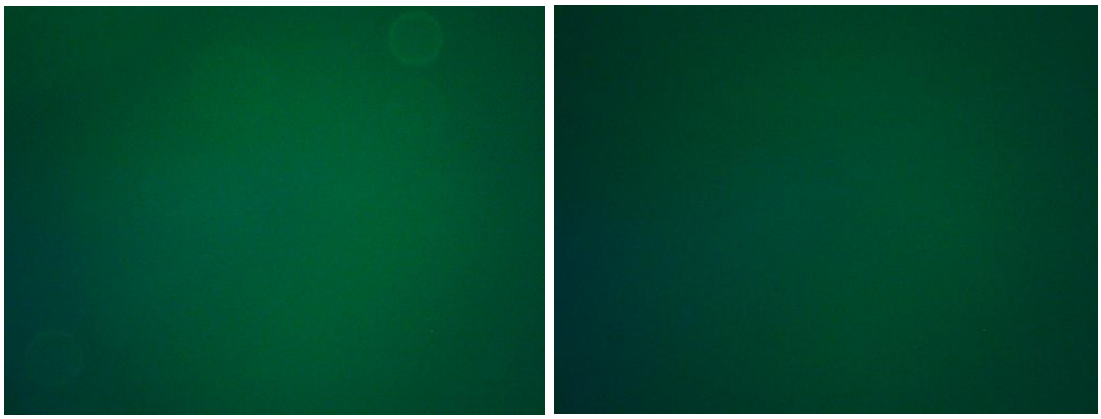

Fig. 3. Fluorescence images for fluorescent oligocod2 oligonucleotides from two different samples

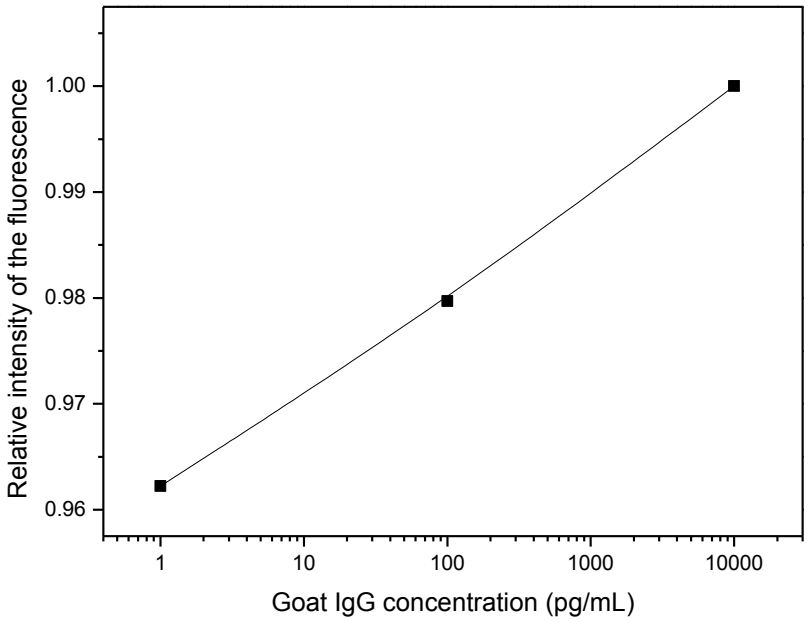

Fig.4. The dependence of the relative intensity of the fluorescence on the concentration of the goat IgG (target immunoglobulin)

The dependence of the relative intensity of the fluorescence on the concentration of

the goat IgG (1 pg/mL, 10^2 pg/mL si 10^4 pg/mL) was linear on a logarithmic scale (Fig. 4), the method allowing detection of target biomolecules in low concentration.

In the next phase of the project, according to the Realization Plan, the limit of the detection will be also established.

In conclusion, all the activities and objectives proposed for this phase of the project were successfully fulfilled.

Reference

1. Tony J. Collins, ImageJ for microscopy, BioTechniques, 2007, 43:S25-S30, doi:10.2144/000112517.

Project Director,
Dr. Dumitru-Daniel Herea