DEVELOPMENT OF FLUORESCENCE TECHNIQUES FOR THE EVALUATION OF ANTIBODY CONJUGATION TO MICROSPHERES

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In the last years, the development of new strategies for a more sensitive and accurate analysis of biomarker molecules in health, food and environmental samples has been very promoted. In a biosensor, a biological target and a ligand bind in a reaction that is collected as signal to a transducer using different technologies (optics, electrochemistry, magnetism, etc...). The use of the physical properties of micro and nanoparticles in the development of biosensors has represented a great advantage. In some cases, magnetic particles are used as carriers of antibodies to immunomagnetic separation in complex samples. In other cases, particles labelled with fluorochromes could be detected in a detection system by their fluorescent signal.

Antibody conjugation to particles can be obtain by adsorption (at the isoelectric point of the antibody via electrostatic interaction), by direct covalent linkage between the surface of particle and the antibody, or by using bridge molecules (like streptavidin-avidin complex). In covalent linkage, optimal bioconjugation would involve the stable attachment of the antibody through his Fc region to surface particle leaving the antigen-binding site Fab region full functional. This work evaluate different methods for determinate the antibody conjugation efficiency on particle surface.

Materials and methods: In this study we have worked with Dynabeads® M-270 of Invitrogen, 2,80μm size, mainly for two reasons:

- We were looking for surface-modified microspheres and for a strong attachment of antibodys to them, and carboxyl-modified microspheres meet these requirements. As it is extensively defended in literature, activation of carboxyl-modified microspheres can be performed with a carbodiimide followed by coupling of an amine containing ligand, resulting in a stable amide bond between the bead and the ligand,
- We were interested to work with magnetic microspheres, knowing that magnetic particles have been utilized extensively in diagnostics and other research applications for the capture of biomolecules because they confer a number of benefits, including ease of separation.

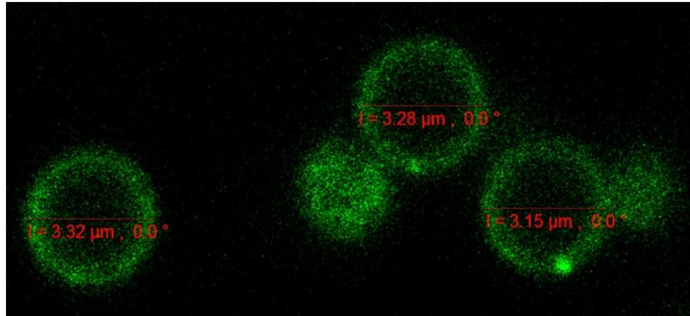
Selected antibody to be linked to the beads was a mouse monoclonal. For the visualization of conjugation yields of a mouse antibody to these microspheres, we have used different techniques based on fluorescence phenomenon:

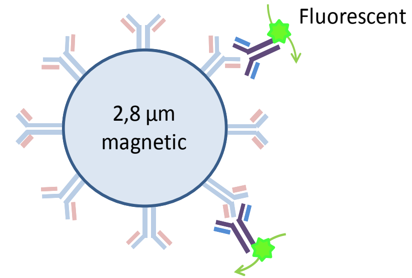
- Fluorescence microscopy (confocal microscopy): a non-quantitative technique that allows visualization of the distribution of the antibody through the microsphere.
- Flow cytometry: a quantitative technique that lets users to know information about the physical and chemical structure of each individual particle present in the sample, and extrapolate it to the entire sample as a mean value of fluorescence emitted for it. At least, 20.000 beads per single determination were measured.
- Immunofluorescence techniques: two quantitative techniques have been evaluated. The first one allows us to quantify the antibody that is chemically linked to a fluorophore and that has not been linked to the microsphere, and the second one measures the fluorescent antibody that has been linked to the beads.

To evaluate the mouse antibody conjugation efficiency on 2,8μm particle surface an anti-mouse Alexa Fluor 488 antibody has been used.

Results and discussion:

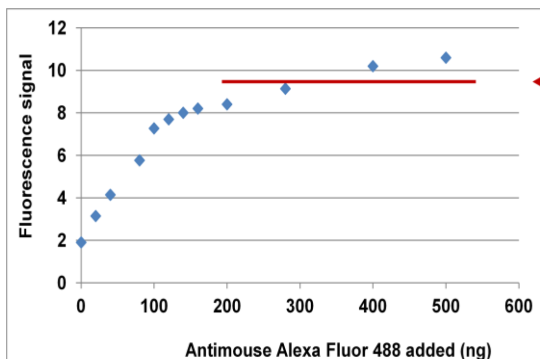
Confocal microscopy:





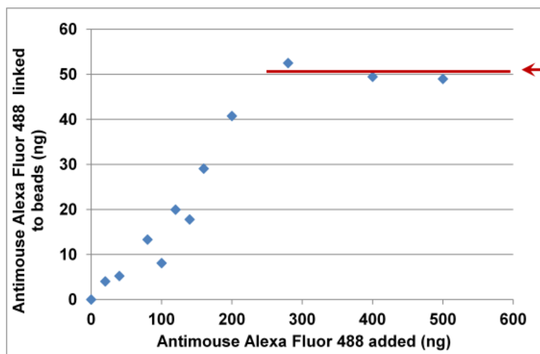
Visually it was confirmed the linkage of mouse antibody to the beads through the union of this antibody to an antimouse Alexa Fluor 488 antibody.

Flow cytometry:



As it was expected, as much as antimouse Alexa Fluor 488 was added to conjugated beads, higher was the fluoresecence found in the sample, until achieved a maximum in which there are no free mouse antibody on the bead to be linked with. Nevertheless, since we didn't have standards of these beads conjugated with the same antibody in different concentrations, using this result, we couldn´t determine the number of molecules of mouse antibody linked to each bead.

Immunofluorescence techniques:



Taking into account the number of beads in the sample and maximum of antimouse Alexa Fluor 488 linked to them, we concluded that the number of molecules of mouse antibody that were linked to each 2,8µm microsphere was $3*10^5$. This result was achieved analyzing both, the antimouse Alexa Fluor 488 that has not been linked to the microsphere, and the antimouse Alexa Fluor 488 that has been linked to the beads.

Although the number of molecules of mouse antibody that can be bound to the beads was quantified, the main objective of this research was to determine the number of them that maintained their functionality after being linked to the microspheres. Further assays are under development in order to achieve this goal using an indirect method: an antibody that is chemically linked to a fluorophore is linked to a target molecule that previously was bound to the mouse antibody present in the beads.