Controlling ligand surface density on streptavidin-magnetic particles by a simple, rapid and reliable chemiluminescent test

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ABSTRACT. The use of functionalized magnetic particles is increasing thanks to their benefits, simplifying the analytical process, and great promising results obtained in a wide range of applications. Particularly, streptavidin-coated magnetic beads offer the possibility of a rapid and very efficient grafting of biomolecules. Unfortunately, current methods to control and compute such grafting process are cumbersome and scarce. Herein, we have developed a simple, rapid and reliable chemiluminescent assay to control the grafting rate of the functionalized magnetic beads. The power of the assay also relies on its ability to predict the required amount of ligands to obtain a precise grafting rate. In addition, results were correlated with a more general parameter in material functionalization characterization like the surface ligand density. Finally, the assay was validated for a wide variety of biotinylated biomolecules sizes, ranging from small molecules (around 200 Da) to antibodies (around 150 kDa). This approach will allow a precise quantification and prediction of the functionalization of the magnetic particles that is of enormous importance for quality control in many applications.

KEYWORDS: Streptavidin-magnetic particles, grafting rate, ligand surface density, biotin-HRP, chemiluminescence

INTRODUCTION

Controlling the grafting rate and the ligand surface density is a key parameter for further bio-applications of functionalized particles. The number of molecules per particle is a vital piece of information largely pursued because will enable to the users the real control and design of quantitative *in vitro* and *in vivo* experiments[1]. This interest relies on the importance of these ligands in the final properties of the particles such as stability, reactivity, toxicity as well as *in vivo* (*i.e.* biodistribution, cellular uptake, clearance pathway, formation of the protein corona, etc.) or environmental behavior of the particles[2-4]. Indeed, ligand density and the control of the surface reactive functions are also used, among others, as quality control parameters in the development of nano-therapeutic products[5,6].

Despite the relevance of this kind of study, only few works are completely devoted to this end and sometimes this information is missed or not valorized, as it should be. Inductively coupled plasma mass spectrometry (ICP-MS)[7-10] and optical emission spectrometry (ICP-OES)[11], nuclear magnetic resonance spectroscopy (NMR)[12], infrared spectroscopy (IR)[6], microscale thermogravimetry analysis (TGA)[13], X-ray photoelectron spectroscopy (XPS)[14], and colorimetric or fluorimetric assays[8,15], have been employed so far to characterize the particle surface coverage. In parallel, modeling approaches or theoretical calculations have been also developed either to confirm the experimental results and to predict the behavior or properties of the particle by modifying the ligand density or the environment of exposure[16,17]. Nevertheless, some of these strategies require sophisticated and expensive instrumentation, specialized user, and hard data treatment processes. Thus, simple, easy to use, and general approaches to achieve this important information are currently highly demanded.

Regarding the specific interaction of biotin-streptavidin, the strongest non-covalent interaction that exists, a few approaches have been developed to characterize the biotin-binding capacity of streptavidin-coated supports[18-20]. All these works are focused on the total number of available binding sites onto the support instead of on the grafting rate or the saturation rate by a given molecule. Finally, it should be noted that no general approach to evaluate and control the grafting rate of a streptavidin-coated support applicable to a wide range of biotinylated ligands sizes has been developed.

Herein we propose a rapid, simple, low-sample consumption and reliable chemiluminescent test to predict and control the grafting rates of streptavidin-magnetic beads (MB) and the results have been correlated with a more general characterization parameter such as the ligand surface density. MB are selected as a model support since their use in bio-applications is nowadays increasing due to their benefits simplifying the analytical process (e.g. purification steps, time of analysis, etc) and the great promising results in a wide range of applications[21,22].

The robustness of the proposed methodology has been validated with a range of biotinylated biomolecules with different molecular weights and also tested for its predictive power by grafting with unknown biotinylated proteins.

MATERIAL AND METHODS

Chemicals and reagents

Magnetic beads Dynabeads™ M-280 Streptavidin and magnetic supports DynaMag™-5 Magnet and DynaMag™-96 Side Skirted Magnet were purchased from Invitrogen by Thermo Fisher Scientific (Eugene, OR, USA). Corning® Costar® 96-well White assay plates (high

binding and polystyrene flat bottom) were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Bovine Serum Albumin fraction IV (BSA) and Dulbecco's Phosphate Buffered Saline (PBS) were obtained from Eurobio (Les Ulis, France). Tween 20 and 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP) were purchased from Sigma-Aldrich. Biotinylated peroxidase (bt-HRP) and Super Signal® West Pico Chemiluminescent Substrate (containing Stable Peroxide solution and Luminol Enhancer solution) were obtained from Thermo Fisher Scientific.

Biotin and N-Biotinyl-NH$(PEG)_{11}$-COOH (bt-polymer) were obtained from Sigma-Aldrich. Biotinylated TNFα (bt-TNFα), biotinylated $VEGF_{165}$ (bt-$VEGF_{165}$), biotinylated Goat IgG (bt-Goat IgG) and biotinylated anti-Human Endoglin/CD105 Antibody (bt-anti hEndoglin IgG) were from R&D Systems (Bio-techne, Abingdon, UK). Biotinylated Bovine Serum Albumin (bt-BSA) was from Thermo Fisher Scientific and biotinylated oligonucleotide 80-mer was synthesized by Eurofins Genomics (Ebersberg, Germany). Finally, biotinylated $VEGF_{95}$ (bt-$VEGF_{95}$) and biotinylated peptides A and B (AC-14 and LW146-2, respectively) were homemade.

Nomenclature

Along this manuscript the following terms will be used to identify different status of beads:

- Non-grafted beads are the naked beads
- Saturated beads are the beads totally covered with any ligand
- X% saturated beads are the beads partially covered with any ligand
- Saturation rate (Sat. rate) is the percentage of bead surface covered by the ligand
- Binding capacity is the inflexion point in the curves of saturation

Grafting of streptavidin-magnetic beads

Dynabeads™ M-280 Streptavidin were functionalized with different biotinylated biomolecules as described in the provider datasheet[23]. Typically for functionalization of 0.12 mg of MB, the beads were re-suspended in the vial (vortexed for 30 s) and then 12 µL of stock solution were transferred to a tube. MB were washed two times with 200 µL of PBS and re-suspended at a concentration of 10 mg/mL in PBS. The calculated amount of the biotinylated molecule in PBS was added to MB and incubated for 1 h at room temperature using gentle rotation (300 rpm). Grafted beads were washed four times with 300 µL of PBS/0.1% BSA (w/v) and dispersed at the initial concentration of MB (10 mg/mL) in PBS/0.1% BSA and stored at 4°C until use.

Consecutive grafting of streptavidin-magnetic beads

The experimental procedure for consecutive grafting of MB was similar to the procedure used for grafting (see previous section). Practically, 12 µL of 10 mg/mL MB were first grafted with different molecules (i.e. biotin; bt-VEGF95; and bt-BSA) at different concentrations. After incubation, supernatants were taken and used to perform a second grafting of other fresh 12 µL of 10 mg/mL MB. After each incubation, MB were washed and stored at 4°C until use.

Procedure of the bt-HRP assay

A white high binding 96-well plate (Costar 3922) was blocked by adding 200 µL/well of blocking buffer (PBS containing 3% BSA) and incubating during 2 h at 37 ºC. The plate was then washed three times with 200 µL/well of PBS/0.1%Tween 20. Afterwards, 20 µL of the desired diluted grafted MB (beads dilution = 1:40) and 100 µL of diluted bt-HRP (15 nM) were added to each well (120 µL final volume). Both dilutions were done in PBS/0.05%Tween 20.

After 1 h incubation at 37 ºC, the plate was washed three times with 200 µL/well PBS/0.1%Tween 20. Then, 100 µL of chemiluminescent substrate (containing a solution of peroxide and luminol enhancer at 1:1, v:v) were added to each well. Immediately after the addition of the chemiluminescent substrate (5 min), chemiluminescent signal was measured on an Infinite F200 Pro Microplate Reader from TECAN (Männedorf, Switzerland). It is noteworthy that MB were fixed at the flat bottom of wells using a 96-well magnet plate both during washing steps and just before measuring the chemiluminescent signal.

For each biotinylated molecule grafted onto MB, the Sat. rate (%) of grafted beads was calculated using the equation 1:

$$\textbf{\textit{Saturation rate}}\ (\%) = \left[\frac{S_{max} - S_i}{S_{max} - S_{sat}}\right] \times \mathbf{100} \qquad \text{Equation 1}$$

where S_i is the measured signal for grafted beads, S_{max} is the maximum signal of the non-grafted beads, and S_{sat} is the signal of 100% saturated beads (obtained with a large excess of ligand, approx. 2 times the binding capacity). All experiments were performed in triplicate, unless otherwise indicated.

RESULTS AND DISCUSSION

Development and optimization of bt-HRP assay

Saturation rate and the number of ligands per particle are critical parameters for the correct design of magnetic particle-based quantitative bioassays. Thus, taking advantage of the use of streptavidin functionalized MB, we have developed an indirect bioassay based in the measurement of the free streptavidin sites onto the beads after conjugation of the target

biotinylated molecules (Figure 1). To do so, biotinylated horseradish peroxidase (bt-HRP) was used and the subsequent addition of its substrate (Luminol + H_2O_2) allows quantifying the free streptavidin sites by chemiluminescence. Non-grafted beads must be used as reference of the total sites available onto the beads.

First of all, some parameters should be optimized: the amount of beads needed to perform the assays, the concentration of bt-HRP and the incubation time. Two first parameters are related and will be optimized to ensure the maximum signal of the assay while reducing the reagents consumption. In the optimization of the amount of beads it is also crucial to adapt the protocol for easy handling of the MB by the final users. Since it has been arbitrary chosen that 20 µL of beads and 100 µL of bt-HRP are added in each well, several beads dilutions have been tested ranging from 1:10 to 1:100. Dilution 1:40 of the stock solution of MB (10 mg/ml) has been selected as the best compromise between easy handling and beads consumption. More diluted solutions are difficult to handle, inducing loses of beads during the washing steps, while more concentrated ones require a large amount of beads and may disrupt the signal measurement. Finally, the selected 1:40 dilution resulted in a consumption of 5 µg of beads per well.

Signal maximum of the assay was then optimized by adjusting the concentration of bt-HRP. For such purpose, a saturation curve of bt-HRP (0 to 365 pmol bt-HRP/mg beads) was built (Figure 2). As observed, a minimum bt-HRP concentration of 90 pmol/mg of beads (4.5 nM; inflexion point of the curve) is required to saturate the beads. In this sense, to ensure the complete saturation of beads and a constant maximum signal (no depletion in bt-HRP in any conditions), we decided to work with a three times more concentrated solution of bt-HRP (300 pmol/mg, 15 nM).

Once these parameters were fixed, we evaluated the influence of incubation time. Thus, we saturated beads with bt-HRP at four different incubation times: 30, 60, 90 and 120 min. Despite no significant differences in the chemiluminescent signal were observed, the shorter incubation times led to less precise signal with a CV of 8% while for the rest of assayed times the CV remains constant at 4%. Therefore 1 hour was selected as the minimum incubation time required for a robust maximum signal.

Grafting yield and Stability

In order to achieve a precise amount of biomolecules grafted on the beads, the yield of grafting of MB with various molecules was evaluated. For this purpose, consecutive grafting procedures of MB with biotinylated molecules were carried out. After the first grafting, the supernatant was recovered and used again in a fresh MB suspension. This allows the molecules that have not been bound during the first grafting procedure to graft during the second grafting procedure. The Sat. rate of MB of each step was estimated with the bt-HRP assay.

Figure 3 shows percentages of saturation after the two grafting procedures of MB for three model molecules (biotin, bt-$VEGF_{95}$ and bt-BSA) at different concentrations. As observed in Figure 3a, before reaching the total saturation of beads (600 pmol biotin/mg of beads), biotin molecules were totally bound onto the MB during the first grafting. Indeed, the Sat. rate were negligible after the second grafting procedure. In addition, it is interesting to note that with 800 pmol of biotin, corresponding to 200 pmol above the saturation, the resulting second Sat. rate (40%) is the same than the Sat. rate obtained with 200 pmol of biotin in the first grafting, indicating a yield of grafting of 100%.

In the case of bigger molecules, such as bt-$VEGF_{95}$ (MW = 28 kDa, Figure 3b) and bt-BSA (MW = 66 kDa, Figure 3c), a small amount of ligand remained unbound after the first grafting as indicated by the small percentage of saturation observed after the second grafting at concentrations below the total saturation. For example, at 200 pmol/ mg of beads of bt-$VEGF_{95}$, 15% of Sat. rate was obtained during the second grafting. The same behavior is observed for bt-BSA. Thus, for bt-$VEGF_{95}$ and bt-BSA the yield of the grafting of MB was assessed around 80%. The observed difference in term of yield of grafting between biotin and bigger biotinylated molecules may be due to biotin accessibility. Indeed, we can assume that biotin covalently bounded to a folded protein may be less available because of steric hindrance.

On the other hand, the possibility of displacement of the biotin or other biotinylated molecule grafted onto MB by bt-HRP molecules during the incubation of grafted beads was evaluated. For that purpose, bt-HRP assay was carried out in both non-grafted MB and biotin-saturated beads (900 pmol biotin/mg beads) using a range of different concentrations of bt-HRP (from 0.05 nM to 18 nM). As observed in Figure S1 (see in the Supporting Information), biotin-saturated beads exhibited the same behavior at any bt-HRP concentration assessed with a mean Sat. rate of 100%, showing CV below 4%. Additionally, Sat. rate of MB grafted with biotin at different concentration (from 0 to 900 pmol biotin/mg beads) was assessed at four different bt-HRP concentrations (2.5, 5, 10 and 15 nM, Figure S2). As observed, there are no differences in the saturation obtained for each biotin-grafted beads with regard to bt-HRP concentrations used for incubation. Therefore, taking all of the above into account, we have corroborated that no displacement of grafted molecules onto beads takes place during the 1h incubation time with the bt-HRP, even at high concentration (15 nM). These results are consistent with the high affinity constant of the streptavidin-biotin interaction[24].

Beads saturation and molecular weights

Once determined the yield of grafting and assessed the robustness of the assay regarding displacement by bt-HRP, the grafting of MB with different biotinylated molecules at different molecular weights was evaluated. Moreover, the Sat. rate obtained by the assay was correlated to the ligand surface density.

First, a set of different biotinylated molecules that covered a wide range of molecular weights was selected to assess the grafting of MB. In addition, these molecules also present different chemical structures (*i.e.* small organic molecules, peptides, proteins and antibodies). Biotin (244 Da) was selected as the smallest molecule since free biotin molecules should occupy all available streptavidin sites on the surface of the MB in the saturation condition, whereas a biotinylated antibody of 150 kDa (immunoglobulin G, IgG) was selected as the biggest molecule to be evaluated. Different biotinylated molecules such as bt-peptide A (1951 Da), bt-$VEGF_{95}$ (28 000 Da), bt-$VEGF_{165}$ (40 000 Da) and bt-BSA (66 000 Da) were also used to explore the predictive power of the bt-HRP assay.

Figure S3 displays the Sat. rate of grafted MB for different biotinylated molecules on a wide range of concentrations (0 to 1000 pmol molecule/mg beads). As it can be observed, the 100% of saturation of MB was reached at different concentrations depending on the biotinylated molecule and, particularly, depending on its molecular weight. In particular, the Sat. rate profile for all biotinylated molecules exhibits two well-differentiated ranges: one range of linearity in which the saturation of MB increases as a function of the concentration of the molecule until reaching the 100% of saturation and another flat and constant range in which the concentration of molecule used for the grafting leads to the total saturation of beads (Figures 4 and S4). The

concentration range assayed in the bt-HRP assay, the saturation concentration as well as the linear range for each biotinylated molecule are all collected in Table 1.

As it can be observed, the saturation of MB using the smallest molecule (biotin) was obtained using a concentration of 605 ± 10 pmol biotin/mg beads, while using the biggest one (bt-Goat IgG) was obtained with a concentration of 70 ± 2 pmol bt-GoatIgG/mg beads. Interestingly, these results are in perfect agreement with those reported for typical binding capacities of one mg of commercial MB[23]: between 650 and 900 pmol/mg for free biotin and ~10 μg/mg (~67 pmol/mg) for a biotinylated antibody.

Considering all the biotinylated molecules whose molecular weights vary from 200 Da to 150 kDa, we can confirm that the saturation concentration is correlated to the molecular weight (Figures 4 and S4, and Table 1). In other words, the amount of biotinylated molecule which is necessary to saturate MB decreases as the molecular weight of the molecule increases likely due to steric hindrance effects.

Determination of the ligand surface density on streptavidin-magnetic beads

The proposed bt-HRP assay allows evaluating the different behaviors and Sat. rate obtained when grafting MB. However, percentage of saturation of beads is a relative parameter. The ligand surface density (σ) is a stronger factor as it is an absolute parameter. Such ligand surface density on the MB was determined using the equation 2, which is based on the equation already reported by Elzey *et al.*[11] and Ndolomingo *et al.*[15], considering that yield of grafting is 100%:

$$\boldsymbol{\sigma_{calc}} = \left(\frac{\boldsymbol{m_{molecule}}}{\boldsymbol{m_{bead}}}\right)\left(\frac{\boldsymbol{N_A\,\rho_{bead}\,d_{bead}}}{\boldsymbol{6\,MW_{molecule}}}\right) \qquad \text{Equation 2}$$

where σ_{calc} is the calculated ligand surface density; $m_{molecule}$ and m_{bead} are the biotinylated molecule mass and the magnetic bead mass, respectively; N_A is the Avogadro constant; ρ_{bead} and d_{bead} are the density and the diameter of the spherical MB, respectively; and $MW_{molecule}$ is the molecular weight of the biotinylated molecule used in the grafting.

Consequently, we were able to establish the correlation between the ligand surface density and the Sat. rate of these grafted beads. In this context, Figure S5 shows linear relationships that relate the Sat. rate of beads (until achieving the 100% of the bead saturation) with the ligand surface density for different concentrations of each biotinylated molecule. Furthermore, the ligand surface density at 100% of saturation of beads was calculated for the free biotin and the five biotinylated molecules assessed. Ligand surface densities obtained in terms of number of molecules/μm^2 were the following: $266 \cdot 10^3 \pm 4 \cdot 10^3$ for biotin (244 Da), $234 \cdot 10^3 \pm 6 \cdot 10^3$ for bt-peptide A (1951 Da), $169 \cdot 10^3 \pm 6 \cdot 10^3$ for bt-VEGF$_{95}$ (28 kDa), $76 \cdot 10^3 \pm 2 \cdot 10^3$ for bt-VEGF$_{165}$ (40 kDa), $38 \cdot 10^3 \pm 2 \cdot 10^3$ for bt-BSA (66 kDa), and $27 \cdot 10^3 \pm 4 \cdot 10^3$ for bt-Goat IgG (150 kDa). These results not only evidence the different surface packing of molecules, but also confirm the fact that the ligand surface density on MB decreases as the molecular weight of biotinylated molecules increases, leading to a steric saturation of beads as the molecule size is higher. It is also interesting to note that evolution of ligand surface density with molecular weight is not linear (see Figure S-6 in the Supporting Information) confirming the importance of the volume occupied by the molecule and consequently steric hindrance.

Control and validation of the developed methodology

In previous sections, we have demonstrated that the developed bt-HRP assay allows establishing linear relationships between the Sat. rate of MB or ligand surface density with

concentrations of biotinylated molecules used for the grafting of beads. In this context, it would be interesting to verify if those linear relationships can be used to predict the Sat. rate of grafted beads with any other biotinylated molecules within the molecular range assayed.

In a first step, two others biotinylated molecules, a bt-peptide (bt-peptide B) of 1895 Da and a bt-IgG (bt-anti hEndoglin IgG) of 150 kDa, were selected. The idea was to prove that we are able to estimate the necessary concentration to obtain a desired percentage of saturation of beads based on the previous experimental results obtained for similar molecules (respectively bt-peptide A and bt-Goat IgG). Figures 4f and S4b were respectively used to determine the required concentrations of each molecule to obtain 30%, 50% and 100% saturation of MB.

Table 2 collects interpolated and experimentally obtained saturation results at different concentrations. As can be seen, the Sat. rate experimentally obtained by the bt-HRP assay for both biotinylated molecules fits very well with the saturation percentages interpolated from the linear equations for each molecular weight.

In a second step, we estimated the robustness of our predictive assay with a biotinylated molecule with any molecular weight between 0.2 and 150 kDa. Indeed, using results from Figures 4 and S4, a correlation between concentration of biotinylated molecules and molecular weight can be established. Specifically, two linear relationships ($C_{molecule}$ function of $MW_{molecule}$) for achieving half-saturation (50%) and saturation (100%) of the MB were obtained (Figure 5). Adequate correlation coefficient is obtained in the range between biotin (244 Da) and bt-BSA (66 kDa). The bt-Goat IgG (150 kDa) was excluded due to its huge difference in molecular weight in comparison to the other biotinylated molecules and the bt-HRP. However, the linear relationship of the bt-Goat IgG (Figure 4f) could be used to determine the necessary

concentration of any bt-antibody to obtain the desired saturation of beads with this type of molecule.

In order to validate the potential of these linear correlations, two biotinylated molecules were assessed: a small bt-polymer (844 Da) and the bt-TNFα protein (51 kDa). Required concentrations to achieve 50% and 100% of saturation with these two molecules were predicted simply using their corresponding molecular weights and previously obtained equations (Figure 5). As shown in Table 3, the feasibility of this methodology was demonstrated as the Sat. rate obtained experimentally by the bt-HRP assay carried out using the calculated concentrations, were statistically undistinguishable from the percentages of saturation intended, that is, 50% and 100% of saturation.

Finally, a biotinylated single stranded oligonucleotide of 80 nucleotides (~25 kDa) was also evaluated. Again, required concentrations of bt-oligonucleotide to obtain 50% and 100% of saturation were determined (185 and 410 pmol/mg beads, respectively). However, percentages of saturation obtained experimentally (Table 3) were both around 100 %. In this case experimental and intended values are not in agreement with the predictive values of the assay. Nevertheless, these results are corroborated by the binding capacity announced in the commercial datasheet (200 pmol/mg beads)[23], showing the performance of the test to characterize the grafting process. Particular behavior may be explained by the fact that DNA is a highly negatively charged molecule which can induce additional repulsions on the surface of the MB. In addition, it has also been reported that the binding capacity for oligonucleotides is inversely related to their molecule size (number of bases), being reduced for large DNA fragments[25].

CONCLUSIONS

In response to the need of controlling particle functionalization in terms of stoichiometry, saturation and ligand surface density, we present a new rapid, easy and cost-effective assay for the determination of the saturation grade of MB. This saturation rate can be easily correlated with a more general characterization parameter such as surface ligand density. Of course, in order to fully characterize the functionalized particle, it would be also recommended to evaluate the ligand functionality after grafting by a complementary test (e.g. immunoassay). Please note that both parameters (ligand density and ligand function) are related since saturation rate may influence the ligand functionality (i.e. crowded ligand at the surface may have negative impact on its functionality). Of course, such relationship should be studied for each specific case as it is species specific. However, it should be mentioned here that there are many examples in the bibliography where such functionalized particles are grafted with different molecules while keeping their activity (i.e. recognition capability).REF

In addition of this control issue, it is remarkable to emphasize the predictive power of the assay that allows proceeding easily to controlled beads grafting with any biotinylated molecule in a molecular range from 1 to 150 kDa, except for the special case of DNA. These control and predictive features will help the users to efficiently design the functionalized beads for further applications. Nevertheless, the predictive model was built using smaill molecules, linear peptides and polymers and globular proteins (antibodies, enzymes…). Of course, complementary studies with different types of biotinylated molecules (e.g. fibrillar proteins) could be performed in order to further generalize the predictive value of the assay.

Finally, this assay can be considered as a general approach for the saturation rate determination of any streptavidin functionalized particle or support by adapting the protocol. For example, centrifugation or ultrafiltration can be employed as purification steps when non-magnetic

particles are used. In the case that nanoparticles show native optical properties, spectral interferences (energy transfer, spectral overlapping, etc) should be considered and the methodology accordingly modified (e.g. change of the enzyme substrate).

FIGURES

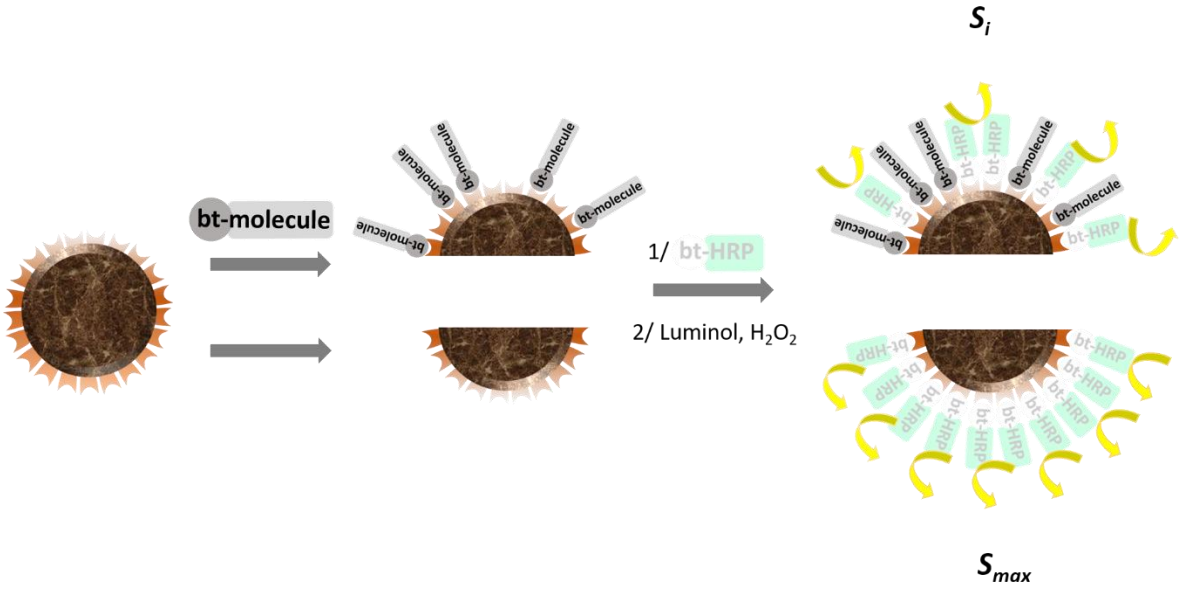

Figure 1. Schematic representation of the streptavidin MB grafting process and the bt-HRP chemiluminescent test.

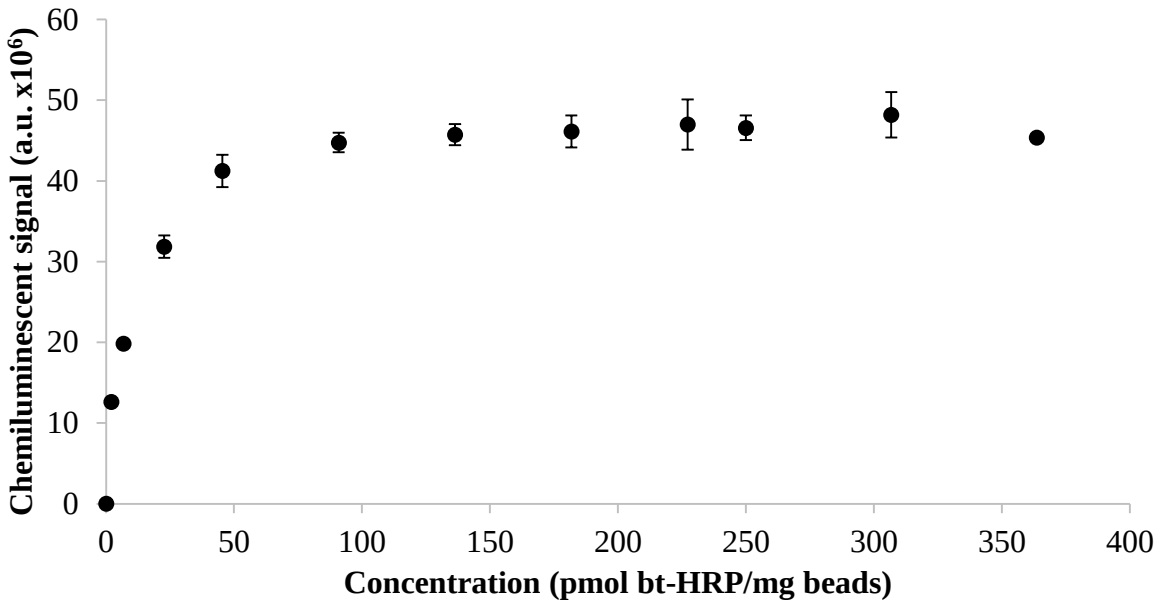

Figure 2: Saturation curve of streptavidin MB with bt-HRP

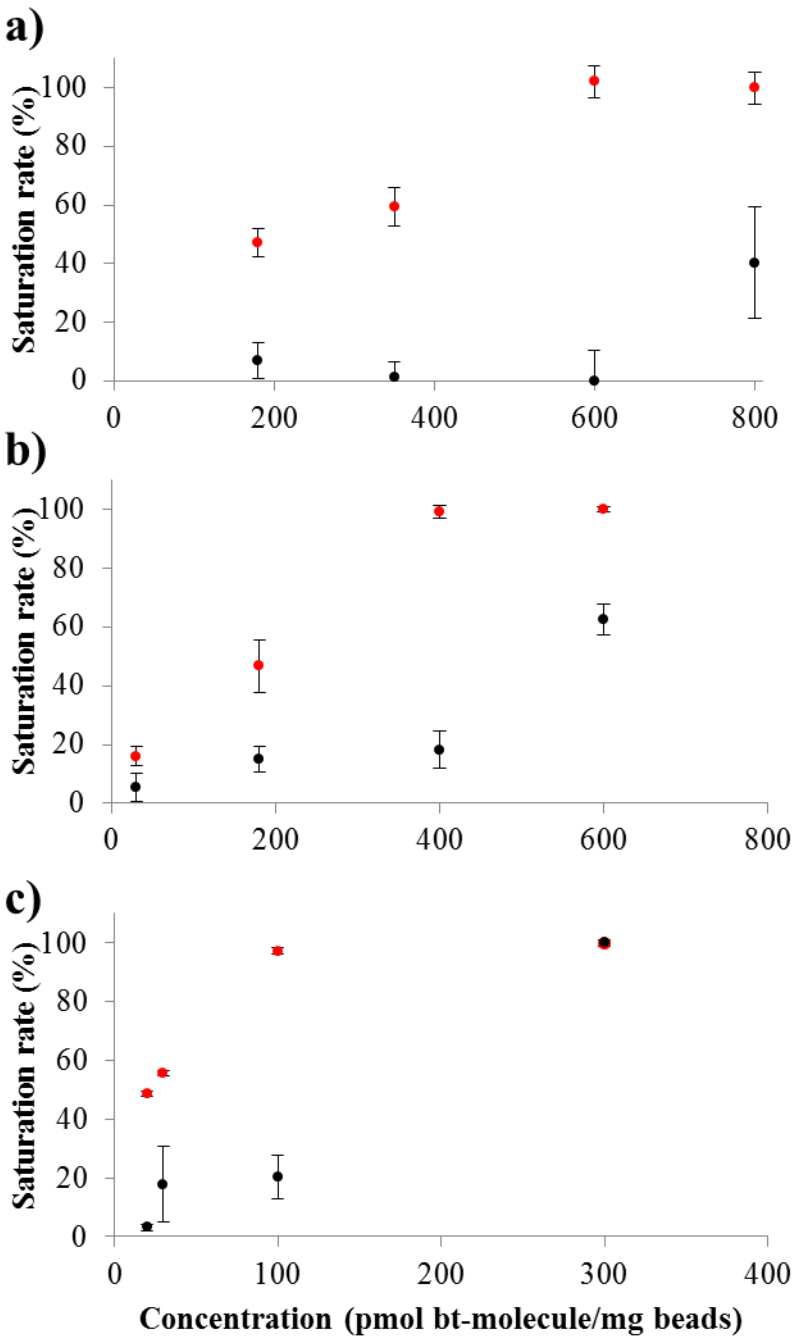

Figure 3. Saturation rate (%) obtained after grafting (red points) and second grafting (black points) of MB with different concentrations of a) biotin, b) bt-$VEGF_{95}$ and c) bt-BSA. *n*=4, uncertainties correspond to 1SD.

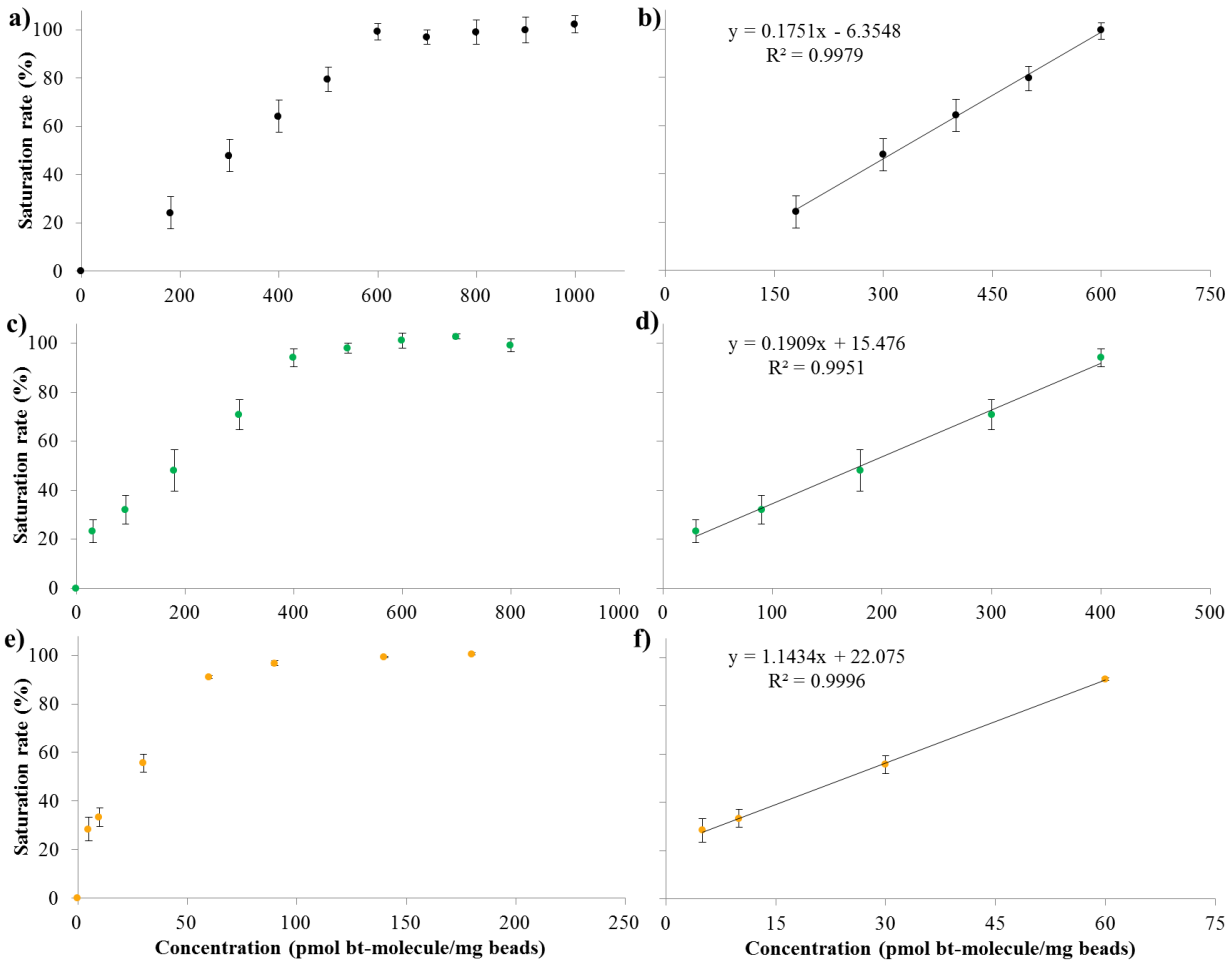

Figure 4. Saturation curves (a,c,e) and linear relationships (b,d,f) of the grafted MB at different concentrations of biotinylated molecules: a,b) biotin (black); c,d) bt-$VEGF_{95}$ (green); and e,f) bt-Goat IgG (orange). *n*=3, uncertainties correspond to 1SD. (continues in Figure S-4 in the Supporting Information)

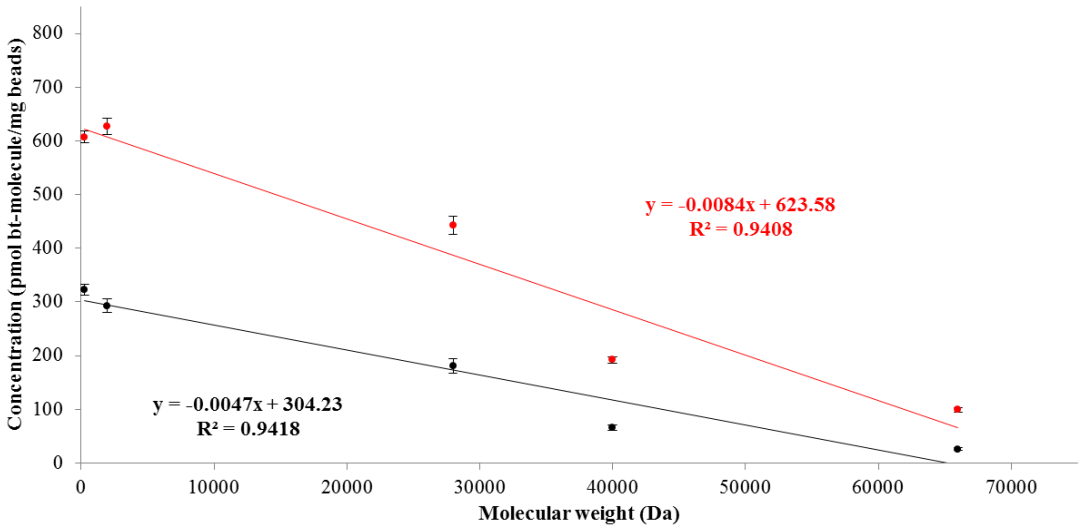

Figure 5. Linear relationship obtained when the influence of the molecular weight of the molecule used for the bead grafting on the concentration of the biotinylated molecule was studied for achieving the 50% (black) and 100% (red) of the bead saturation. *n*=3, uncertainty corresponds to 1SD.

TABLES.

Table 1. Concentration range assayed, linear range and saturation concentration obtained in the bt-HRP assay for various biotinylated molecules with different molecular weights. *n*=3, uncertainties correspond to 1SD.

Biotinylated molecule	**MW (kDa)**	**Concentration range assayed (pmol/mg beads)**	**Linear range (pmol/mg beads)**	**Saturation concentration (pmol/mg beads)**
biotin	0.244	0 - 1000	180 - 600	605 ± 10
bt-peptide A	1.951	0 - 900	90 - 600	625 ± 15
bt-VEGF$_{95}$	28	0 - 800	30 - 400	440 ± 15
bt-VEGF$_{165}$	40	0 - 500	10 - 180	190 ± 5
bt-BSA	66	0 - 600	30 - 90	100 ± 5
bt-Goat IgG	150	0 - 180	5 - 60	70 ± 2

Table 2. Saturation rate (%) intended and experimentally obtained for two biotinylated molecules (bt-peptide B and bt-anti hEndoglin IgG) using the bt-HRP assay and concentrations interpolated by means of equations of linear relationships obtained for bt-peptide A (1951 Da) and bt-Goat IgG (150 kDa), respectively. *n*=3, uncertainties correspond to 1SD.

Biotinylated molecule	**MW (kDa)**	**Concentration interpolated * (pmol/mg beads)**	**Sat. rate intended (%)**	**Sat. rate obtained (%)**
bt-peptide B	1.895	160	30	34 ± 7
		295	50	58 ± 4
		625	100	98 ± 1
bt-IgG (bt-anti hEndoglin IgG)	150	5	30	26 ± 4
		25	50	52 ± 4
		70	100	99 ± 1

*Equation used for bt-peptide B: $Sat.\ rate = 0.1496 \cdot [bt\text{-}peptide] + 6.135$.

Equation used for bt-anti hEndoglin IgG: $Sat.\ rate = 1.1434 \cdot [bt\text{-}IgG] + 22.075$.

Table 3. Saturation rate (%) intended and experimentally obtained for three biotinylated molecules (bt-polymer, bt-TNFα protein and bt-oligonucleotide) using the bt-HRP assay and concentrations interpolated by means of equations of linear relationships relating molecular weights (MW) of biotinylated molecules with concentrations of these molecules for achieving 50% and 100% of the bead saturation. *n*=3, uncertainties correspond to 1SD.

Biotinylated molecule	**MW (kDa)**	**Concentration interpolated * (pmol/mg beads)**	**Sat. rate intended (%)**	**Sat. rate obtained (%)**
bt-polymer	0.844	300	50	51 ± 6
		615	100	96 ± 7
bt-TNFα protein	51	65	50	48 ± 14
		195	100	102 ± 4
bt-oligonucleotide	~25 (80 nucleotides)	185	50	100 ± 1
		410	100	100 ± 2

*Equation used for achieving 50% of the bead saturation: $[bt\text{-}molecule] = -0.0047 \cdot MW + 304.23$.

Equation used for achieving 100% of the bead saturation: $[bt\text{-}molecule] = -0.0084 \cdot MW + 623.58$.

ASSOCIATED CONTENT

Supporting Information. Supplementary figures non-essential but informative

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