CLICK CHEMISTRY IMMOBILIZATION OF ANTIBODIES ON POLYMER COATED GOLD NANOPARTICLES

Chiara Finetti[1,4], Laura Sola[1], Margherita Pezzullo, Davide Prosperi[2], Miriam Colombo[2], Benedetta Riva[2], Svetlana Avvakumova[2], Carlo Morasso[3], Silvia Picciolini[3], Marcella Chiari*[1]

[1]Istituto di Chimica del Riconoscimento Molecolare, CNR, Via Mario Bianco 9, 20131, Milano, Italy

[2]NanobioLab, Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, Piazza della Scienza 2, 20126 Milano, Italy

[3]Laboratory of Nanomedicine and Clinical Biophotonics, Centre for Innovation and Technology Transfer, Fondazione Don Carlo Gnocchi ONLUS, Milano, Italy

[4] Università degli Studi di Milano, Dipartimento di Scienze Farmaceutiche, Via Mangiagalli 25, 20133, Milano, Italy.

* Corresponding author: Marcella Chiari, [1]Istituto di Chimica del Riconoscimento Molecolare, CNR, Via Mario Bianco 9, 20131, Milano, Italy,

email: marcella.chiari@icrm.cnr.it, Phone +390228500035

KEYWORDS: gold nanoparticles, label, polymer coating, biosensing

Number of words: 4982

Figure: 6

Table: 1

Abstract

The goal of this work is to develop an innovative approach for the coating of gold nanoparticles (AuNPs) with a synthetic functional copolymer. This stable coating with a thickness of few nanometers provides, at the same time, stabilization and functionalization of the particles. The polymeric coating consists of a backbone of polydimethylacrylamide (DMA) functionalized with an alkyne monomer that allows the binding of azido modified molecules by Cu(I)-catalyzed azide/alkyne 1,3-dipolar cycloaddition (CuAAC, click chemistry). The thin polymer layer on the surface stabilizes the colloidal suspension whereas the alkyne functions pending from the backbone are available for the reaction with azido-modified proteins. The reactivity of the coating is demonstrated by immobilizing an azido modified anti-mouse IgG antibody on the particle surface. This approach for the covalent binding of antibody to a gold-NPs is applied to the development of gold labels in biosensing techniques.

1. Introduction

In the past decade, the use of nanoparticles in biomedical research has significantly expanded[1]. Their application in diagnosis and therapeutics, including among others, chemical sensor[2], magnetic resonance imaging contrast agent[3], drug/gene delivery system[4], and cancer treatment [5] have been extensively reviewed. The immobilization of proteins, DNA or organic molecules on nanoparticles allows the formation of hybrid materials with interesting characteristics[6].

Biomolecules can be immobilized on a nanoparticle either passively through hydrophobic or ionic interactions (physical adsorption), or covalently by a chemical reaction with an activated surface group. Covalent immobilization provides important advantages over physical adsorption, the most important being the higher stability of the bioconjugate[7]. Commonly used strategy for protein immobilization implies the activation of the nanoparticle with a chemical group that reacts with primary amines or carboxylic acids present on the surface of any protein. However, the coating of nano-sized objects is sometimes difficult due to poor stability of the nanoparticles in suspension

during and after the coating process. A convenient way to functionalize the surface of nanoparticles employs functional polymers that, in additon to providing anchoring points to the surface, stabilize the colloidal suspension.

The purpose of this study is to demonstrate that a synthetic functional copolymer, copoly(DMA-PMA-MAPS), recently introduced by our group [8] represents a convenient method to provide stabilization and functionalization of nanoparticles by a robust and user friendly one-step procedure. The polymer belongs to a family of copolymers which have been successfully employed to produce various surface coatings. The key of their success in forming stable coatings of few nanometers, is the combination, on the same chain, of two monomers: *N, N*-dimethylacrylamide (DMA) and γ-methacryloxypropyltrimethoxy silane (MAPS). Furthermore, a number of functional monomers can be introduced by random radical polymerization into this basic structure to confer to the coating specific binding properties. In the version presented here, one of such functional monomers bears an alkyne functionality that promotes the binding of azido modified molecules by Cu(I)-catalyzed azide/alkyne 1,3-dipolar cycloaddition (CuAAC, click chemistry). In this work, the polymer was used for the modification of gold nanoparticles surrounded by a thin silicon oxide layer. The polymer coating stabilizes the colloidal suspension, whereas the alkyne functions pending from the backbone are available for the reaction with azido modified proteins. To demonstrate the functionality of the modified nanoparticles, an anti-mouse IgG antibody, modified with azido groups, was covalently linked to the nanoparticle surface. This surface modification approach is of general applicability in different fields spanning from the functionalization of antibodies, whose use is widespread from clinical diagnosis or disease treatment [9] [10], to the development of labels in biosensing techniques [11] [12]

2. Materials and methods

2.1 Materials

Phosphate-buffer saline (PBS), Tris(hydroxymethyl)aminomethane (Tris), HCl, sodium hydroxide (NaOH), ethanolamine, *N, N*-dimethylformamide (DMF), sodium chloride (NaCl), sodium phosphate

(Na phosphate), bovine serum albumin (BSA), Copper (II) sulfate pentahydrate ($CuSO_4$), L-Ascorbic acid (AAC), tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), Agarose low gelling temperature, ethylenediaminetetraacetic acid (EDTA), boric acid, Tween20, ammonium sulfate ($(NH_4)_2SO_4$), *N,N*-dimethylacrylamide (DMA), γ-methacryloxypropyltrimethoxy silane (MAPS), azoisobutyonitrile (AIBN), goat anti-mouse polyclonal IgG (whole molecule) antibody, were all purchased from Sigma-Aldrich (St.Louis, MO, USA). *N*-acryloyloxy-succinimide (NAS) and 3-trimethylsilyl-prop-2-ynyl methacrylate (protected PMA) were synthesized as reported elsewhere[13 14]. Rabbit anti-bovine beta-lactalbumin was purchased from Jackson Immuno Research (WestGrove, PA, USA); purified anti-mouse CD63 was purchased from BioLegend; azido-PEG_8-*N*-hydroxysuccinimide (N_3-PEG-NHS) ester was purchased from Jena Bioscience (Jena, Germany). Silicon oxide chips with a 100 nm thermal oxide layer were bought from Silicon Valley Microelectronics (SantaClara, CA, USA); 30 KDa centrifugal filters were purchased from Amicon.

2.2 Synthesis of poly(DMA-NAS-MAPS) and Poly(DMA-PMA-MAPS)

The polymers, poly(DMA-NAS-MAPS) and poly(DMA-PMA-MAPS) were synthesized as reported in [15] and [8]. The copolymers were obtained by free radical polymerization of DMA and MAPS with NAS or protected PMA. The total monomer concentration in the feed was 20% w/v, while the molar fraction of the three monomers was, in both polymer, 97%, 1% for DMA and MAPS and 2% for NAS or protected PMA.

Briefly, for the synthesis of poly(DMA-NAS-MAPS), the three monomers were diluted in anhydrous tetrahydrofuran, together with a thermocatalyst (AIBN); the reaction flask was heated to 65°C to initiate the polymerization process and after two hours the polymer was precipitated in petroleum ether and collected as a white powder.

The synthetic process of poly(DMA-PMA-MAPS) required two separate steps: a) the synthesis of polymer which contains 3-trimethylsilanyl-prop-2-yn methacrylate, a protected form of prop-2-ynyl prop-2-enoate (PMA) and b) removal of the protective trimethylsilane groups. The first step was

similar to that used for the synthesis of poly(DMA-NAS-MAPS), while the deprotection was achieved by dissolving the polymer in a basic solution containing potassium carbonate. After a 3 hours reaction, the polymer was dialyzed and lyophilized [8].

2.3 Synthesis of Silica Gold Nanoparticles

A solution (200 mL) of tetrachloroauric(III) acid (0.01% w/v) in water was heated to reflux. Next, 700 μL of trisodium citrate (2%) were added to the solution and left under stirring at 100° C for a few minutes until the appearance of a deep red color indicated the formation of the nanoparticles. The suspension was left under stirring at 100° C for further 20 minutes and then slowly cooled down to room temperature.

A very thin layer of silicon oxide was grown on the surface according to the protocol described by Li et al [16]. Thirty mL of a gold nanoparticles suspension were mixed with 400 μL of APTES (1 mM) and left under stirring. After 15 minutes, 3.2 mL of a sodium silicate solution (0.54% w/v) acidified until the pH was <11 were added to the gold nanoparticles. The suspension was left under stirring for 3 minutes at room temperature and for 35 minutes in a water bath at 70°C.

To stop the reaction the gold nanoparticles were moved in a bath of water at 4°C. At last, gold nanoparticles were centrifuged at 3000g for 20 minutes at 15°C and resuspended in water.

2.4 Nanoparticles coating

Poly(DMA-PMA-MAPS) was dissolved in DI water to a final concentration of 1%; to this solution, a suspension of AuNPs (0.1 mg/mL) was added and the mixture was gently stirred for 1 h in the dark on a shaker. To remove the polymer excess, the AuNPs suspension was washed two times with DI water by centrifugation (10 min at 7000 RPM). After the second washing cycle, the supernatant was discarded and replaced by sodium phosphate buffer (pH 7.4, 50mM) and the suspension stored at 4 °C.

2.5 Antibody derivatization

An anti-mouse IgG antibody was dissolved in PBS to a final concentration of 5mg/mL; to this solution, Azido-PEG_8-NHS ester was added to a final concentration of 0.5 mM. The mixture was stirred for 2 h at room temperature. Once the reaction was completed, the azido modified IgG was washed three times by centrifugation on 30 KDa centrifugal filters (10 min at 7000 rpm) to remove residual traces of unreacted azido ester.

2.6 Functionalization of Gold Nanoparticles

Polymer coated AuNPs were spinned down and resuspended in Na phospate buffer (50mM pH 7.4); to this suspension, the azido modified antibody (1 mg/mL), $CuSO_4$ (100 μM), THPTA (400 μM) and ascorbic acid (6.25mM) were added. The mixture was then stirred overnight. The antibody-AuNPs suspension was washed three times by centrifugation (10 min at 7000 rpm) to remove residual traces of unreacted azido antibody. To prove that the covalent binding was promoted by the $CuSO_4$/THPTA/AAC assisted click reaction, the same procedure was performed without addition of the click catalysts. In this case, the anti-mouse antibody could only be adsorbed on the polymer coated AuNPs.

2.7 Nanoparticles Characterization

2.7.1 Morphology

Transmission electron microscopy Transmission electron microscopy (TEM) images of AuNPs were obtained on a "FEI Tecnai G" Spirit BioTWIN microscope (Oregon, USA) operating at 120 kV. The samples were prepared by evaporating a drop of nanoparticles onto carbon-coated copper grid (200 mesh) and allowing it to dry on the air. Nanoparticles were sonicated prior to analysis. The histograms of the particle size distribution and the average particle diameter were obtained by measuring about 150-200 particles by using Measure IT Olympus Software.

2.7.2 Particle size and ζ-potential analyses

Dynamic light scattering (DLS) measurements were performed at 173° with a Zetasizer Nano ZS ZEN3600 from Malvern Instruments Ltd (Worcestershire, UK) working at 4 mW of a He-Ne laser (λ = 632.8 nm). A disposable cuvette with 1 cm optical path length was used for the measurements. The samples were prepared by dilution with Milli-Q water containing 1 mM citrate. Each sample was allowed to equilibrate for 30 sec prior to starting measurement. The measurements were performed at 25°C. The calculations of hydrodynamic diameter were performed using Mie scattering theory, considering absolute viscosity and refractive index values of the medium to be 0.8872 cP and 1.334, respectively. The results are reported in Intensity. ζ-potential measurements were elaborated on the same instrument by electrophoretic light scattering; ζ-potential values were automatically calculated from electrophoretic mobility using Zetasizer Software (Malvern Instruments Ltd, Malvern, UK). A viscosity of 0.8872 cP, a dielectric constant of 78.5 and a Henry function of 1.5 were used for the calculations. All measurements were performed in triplicate and the average values were calculated.

2.7.3 Gel electrophoresis

In order to characterize the particles after each derivatization step, 100 µL of functionalized and non-functionalized gold NPs were loaded on a 0.7% agarose gel in 0.5X Tris-borate-EDTA buffer pH 8.8 (TBE: 67 mM Tris(hydroxymethyl)aminomethane, 37 mM boric acid, 1.6 mM EDTA). The separation was run for 60 min at a constant voltage of 100 V.

2.7.4 Optical properties

The optical properties of AuNPs, polymer coated AuNPs and AuNPs functionalized with antibody were assessed by UV visible spectrometry (Spectrophotometer VP-650, Jasco). Before collecting UV-Vis spectra (400-700 nm) all sample solutions were sonicated for few seconds in order to minimize aggregation. The Plasmon band shift due to the polymer layers deposited on AuNPs and to

the subsequent antibody linking was evaluated keeping in consideration the maximum absorption peak of bare AuNPs is 525nm. The concentration of the AuNPs suspension is expressed in optical density (OD), measured at the maximum absorption peak (525nm).

2.8 Bioassay

2.8.1 Glass slide coating procedure

The coating procedure of a glass slide with poly(DMA-NAS-MAPS) is described elsewhere [17]. Briefly, silicon slides bearing a 100 nm oxide layer were activated by an oxygen plasma treatment and then immersed in a 0.8 M ammonium sulfate solution containing poly(DMA-NAS-MAPS) at 1% w/v concentration for 30 minutes. The chips were rinsed with water, dried with a nitrogen stream and finally cured under vacuum at 80°C.

2.8.2 Microarray experiments

To demonstrate the binding between the antibody and AuNPs, an anti-CD63 mouse antibody, and an anti-β-Lactoglobulin rabbit antibody (negative control) were patterned on two silicon chips coated with poly(DMA-NAS-MAPS) by means of a SciFlexArrayer S5 spotter from Scienion (Berlin, Germany). Both, the mouse anti-CD63 capture antibody (20 replicates) and the rabbit anti-β-lactoglobulin antibody (5 replicates), dissolved in PBS, were spotted at a concentration of 1 mg/mL. In the experimental conditions used, the volume of the spotted drop was 400 pL. The chips were placed in a humid chamber immediately after the spotting and stored overnight at room temperature. After the immobilization, the residual active esters on the chip were blocked by immersing the chips in a solution of 50 mM ethanolamine in 0.1 M TRIS/HCl, pH 9, for 1 h at room temperature; the chips were then rinsed with DI water and dried with a nitrogen stream.

One chip was incubated overnight in a humid chamber with a AuNP-labeled anti-mouse antibody (OD=0.05) in PBS in static incubation conditions. The chip was washed with the washing buffer (Tris/HCl 50 mM pH 9, NaCl 0.25 M, Tween 20 0.05% v/v) for 10 min under stirring and finally

rinsed with MilliQ water. A second chip was incubated with the same anti-mouse antibody reacted with AuNPs in the absence the click catalyst. Both chips were imaged with the SP-IRIS instrument to detect individual particles of AuNPs bound to the capture surface antibody

2.8.3 Optical Detection

The Single Particle Interferometric Reflectance Imaging System (SP-IRIS) instrument (NexGen Arrays) is a prototype instrument for the detection of individual particles on surface. The instrument uses one discrete LED wavelength (525 nm) to illuminate the sensor's surface using a high magnification objective to detect and count nanoparticles of known materials located on the SiO_2 surface; the principles are thoroughly illustrated elsewhere[17] [18]. Briefly, this modality of IRIS enhances the contrast of a single nanoparticle on a bilayered substrate by interfering the scattered field produced by the nanoparticle on the substrate surface with the reflected field generated by the buried $Si{-}SiO_2$ interface of the IRIS chip. The CCD camera senses the individual nanoparticles on the IRIS chip as point objects, which are processed to extract size information.

3. Results and discussion

3.1 Coating procedure

The attachment of proteins, and other biomolecules, to nanoparticles is of critical interest in the development of medical products and biosensors using nanoparticles. Different strategies of surface GNP modification and functionalization are reviewed in [19]. Among the various approaches, thiolated PEGs, modified with a carboxyl group and activated via EDC/NHS reaction, are widely used for the immobilization of proteins. The coating strategy, based on the adsorption of these polymers, is simple and effective in suppressing particles aggregation, however it suffers from some drawbacks. The procedure requires two steps: polymer adsorption and activation of carboxyl group

to provide N-hydroxysuccinimide (NHS) ester terminal group. PEG chains have only one functional group per chain, making it difficult to achieve a high immobilization density. Most importantly, the coupling reaction is often performed in buffered aqueous solutions near physiological pH (pH 6 to pH 9). Under these conditions, the hydrolysis of the ester group competes with the amidization process, potentially degrading the efficiency of the coupling chemistry[20]. Last but not least, the reaction between active esters and amino groups is not regiospecific and does not allow the controlled and oriented immobilization of the protein. To overcome most of these drawbacks while mantaining the robustness and easy of operation of the coating process, a polymeric coating that allows immobilization of chemically modified proteins by Click Chemistry is used in this work. Recently, we have introduced a copolymer with an alkyne functional monomer. This copolymer made of *N,N*-dimethylacrylamide (DMA), [3-(methacryloyl-oxy) propyl] trimethoxysilyl (MAPS) and prop-2-ynyl prop-2-enoate (PMA) was designed to bind azido modified biomolecules via Cupper(I) catalyzed Huisgen 1,3-dipolar cicloaddition (CuAAC, *click chemistry*) and was successfully used for the immobilization of glycans in microarray[8] .

Our group has a long tradition in the development of DMA based copolymers. The first polymer of the series, the poly(DMA-NAS-MAPS), copolymerized with *N*-acryloyloxy succinimide (NAS) and MAPS, was introduced to form, in few minutes, a stable functional coating on microarray glass slides[15]. This polymer is extremely versatile and a wide number of functional monomers can be added to the basic structure of poly(DMA-MAPS) by random radical polymerization, in order to confer to it specific properties; examples of various monomers that have been used include active ester, oxyrane[21] and ionizable groups [22].The members of this polymer family adhere to a variety of different materials including glass, silicon oxide[17], gold[23], PDMS and thermoplastics[24] by a combination of a chemi- and physisorption mechanism. Thanks to the MAPS monomer that promotes silanol condensation with hydroxyl groups introduced onto the surface by an oxygen plasma treatement, the weak non-covalent interactions between the AuNPs surface and the DMA segment are reinforced leading to the formation of an extremely stable nanometric layer. We demonstrate that one of the

members of this polymer family, the poly(DMA-PMA-MAPS), coats 45 nm gold nanoparticles, prepared according to the commonly used citrate methods developed by Turkevich [25] and Frens[26]. In order to promote a better adhesion of the polymer, a very thin layer of silica has been grown on top of gold. The silica shell has been obtained thanks to the condensation of sodium silicate on the surface of gold nanoparticles previously treated with APTES as described by Li et al [16]. A careful study of the reaction parameters (time, pH and temperature) was required to keep the silica layer very thin to not compromise the optical properties of the nanoparticles. We selected an alkyne polymer to allow bio-conjugation via click chemistry to particles that are stabilized and functionalized by a single-step process. Although the polymer is not new, its use in the context of nanoparticles poses new challenges. Given the mechanism of coating formation, which entails an adsorption step followed by silanol condensation, the polymer adsorption is usually performed in highly concentrated ammonium sulphate solution to reduce polymer solubility and force its interaction with the surface. The use of salts is incompatible with nanoparticles which would aggregate and precipitate during the coating. Also the condensation step at high temperature, which normally increases the binding strength of the polymer film, is incompatible with a colloidal suspension. We demonstrate in this work that the functional PDMA copolymer has a strong affinity to the inorganic core and quickly and effectively replaces the original citrate molecules. Even when the coating process is carried out in sub-optimal conditions the polymer chains wrap around the particles, forming a film dense enough to confer high stability to the colloidal solution.

The derivatization process is depicted in Figure 1. In the first step, AuNPs with a silica shell are coated with the linear polymer copoly(DMA-PMA-MAPS) simply by adding the copolymer to the aqueous particle suspension, as detailed in section 5 (Figure 1a). The presence of alkyne functionalities on the external polymer layer allows the covalent binding of azido-PEG modified IgGs on the AuNPs surface via CuAAC reaction. The CuAAC process is an example of a click chemistry reaction that is easy to perform, giving rise to the intended products in very high yields with little or no byproducts: it works well under many conditions, and is not affected by the presence of functional

groups other than those being connected to each other. Since the conjugation requires the presence on the biomolecules of functional groups that are not naturally present, the process works well for DNA or peptides that are easily functionalized during their synthesis. For proteins, the introduction of clickable functionalities is more challenging. An enzymatic approach, commercialized under the trade name of Site-Click™ by Thermo-Fisher, allows simple site-selective attachment of an azido moiety to the heavy chain N-linked glycans—far from the antigen-binding domain. If used in conjunction to our alkyne modified polymer this approach provides en effective means of orienting antibodies on the surface of gold nanoparticles. In this work, the amino groups of an anti-mouse IgG antibody are reacted with an Azido-PEG_8-NHS ester, in order to introduce clickable functionalities on the antibody, as schematically reported in Figure 1. The molar-ratio of PEG-ester and antibody determines the degree of substitution. Even though we can not claim that the immobilization is oriented as the reaction between PEg-succinimidyl ester and protein amino groups is not regioselective, still the process is advantageous over other types of immobilization since it allows to control the degree of azide insertion thus limiting the point of contact between the protein and the surface. In the limiting case, the immobilization may involve only one azido group, leaving the antibody accessible to the antigen in solution. This would never be possible with standard NHS chemistry.

The successful formation of the coating was confirmed by subjecting to electrophoresis samples of AuNPs, at each stage of the functionalization, in an agarose gel. Uncoated gold nanoparticles (Figure 2a, lane 1) have a different mobility compared to polymer-modified particles (lane 2). The higher electrophoretic mobility of uncoated gold NPs is due to the high density of negative charges on their surface. On the contrary, the charges on the coated particles are shielded by the polymer layer, causing a significant mobility reduction. The antibody functionalization of AuNPs was confirmed by the difference of electrophoretic profile of polymer coated NPs (Figure 2b, lane 1) and antibody coated NPs (Figure 2b, lane 3). The covalent binding of the antibody to the surface was confirmed by the electrophoretic behavior of AuNPs incubated with the antibody in the absence of the click chemistry

reagents (THPTA/CuSO4 and ascorbic acid). In this case, as shown in Figure 2b, lane 2, no mobility shift was observed, indicating that no immobilization of antibody was obtained and confirming that the shift observed in lane 3 is not due to non-specific antibody adsorption onto the nanoparticle surface.

3.2 Surface characterization

The surface modification was monitored after each step with a number of state-of-the-art analytical techniques including UV spectroscopy, Dynamic Light Scattering (DLS), zeta potential measurements, Trasmission Electron Microscopy (TEM), as well as with functional tests on particles behavior.

3.2.1 UV spectroscopy, zeta potential and dynamic light scattering measurements

In the UV spectra of Figure 3, surface plasmon band shifts resulting from the formation of copolymer and antibody layers are shown. The deposition of each layer on the surface causes small changes in the local refractive index of the material that induce shifts of the plasmon band.

Furthermore, ζ-potential measurements of coated AuNPs confirmed the presence of the polymer layer. As shown in Table 1, a marked change of ζ-potential was found for particles coated with the copolymer compared to that of naked ones.

The AuNPs hydrodynamic diameter was measured after polymer and antibody functionalization by means of DLS analyses (Table 1). Also in this case, significative changes in the hydrodynamic diameter were detected after each modification step, demonstrating the success of the functionalization.

3.2.2 Stability of the nanoparticles

The transmission electron microscopy (TEM) images reported in Figure 4 show that the nanoparticle quality and morphology is maintained after each step of the functionalization: nanoparticles stability is not compromised neither by polymer coating nor by the binding of the antibody.

We have investigated the stability of Ab coated AuNPs and the results show that the coating is essential to protect nanoparticles from the pH dependent aggregation. The stabilization is due to steric stabilization rather than electrostatic repulsion. In fact, when AuNPs are uncoated, a marked color change, indicative of aggregation, is clearly detectable at high and low pH values as well as at high salt concentration. On the contrary, when the polymer is grafted on AuNPs surface, the pH and ionic strength do not affect the suspension stability and the original color is maintained (Figure 5). The protection induced by the polymer coating makes AuNPs more suitable for biological applications, where high ionic strength and pH changes are currently present.

3.2.3 Application of antibody labeled nanoparticles in biosensing

The new functionalization approach described here might find application in several research fields including therapeutic drug delivery, diagnostics and photodynamic therapy. However, we focused mostly on application in bio-sensing. In this work, we have tested the activity of gold immobilized antibodies in an innovative biosensing technology, called Interferometric Reflectance Imaging System (IRIS). This technology is based on well-known principles of light interference, and it was previously applied for rapid detection of large virus particles[17] . This platform is also suitable for detection of single molecules in a sandwich assay format. The sensor surface, coated with a functional polymer and arrayed with specific detection probes (i.e antibodies, aptamers, and nucleic acid oligos), captures one or more targets from the solution. The surface immobilized target is then recognized by a secondary probe labeled with a gold nanoparticle of 40 nm. Gold nanoparticle labels with their high optical signal are individually counted by the SP-IRIS. A successful outcome of an SP-IRIS experiment not only indicates that the particles are properly functionalized with active antibodies but also that they and are not aggregated. In particular, we have immobilized on the surface an anti-CD63

antibody, which was specifically recognized by an AuNP labeled anti-mouse polyclonal IgG. In Figure 6a (top right), the gold particles counted by the SP-IRIS software are shown as red circles. The histogram in Figure 6b reports the number of particles per mm^2 found in different sensing experiments. In the absence of THPTA/CuSO4 and ascorbic acid during AuNPs functionalization, the click chemistry reaction does not occur and, as a consequence, the secondary antibody is not labeled. In fact, the density of particles detected by SP-IRIS in this case (bottom right) is close to that of the control experiment where a non correlated antibody (images on the right) is spotted. These experiments confirm that the particles are detected on the surface only when the conjugation reaction is carried out in the presence of click chemistry catalysts demonstrating the covalent character of the conjugation.

4. Conclusions

In this study, we described a simple and reliable method to coat AuNPs with a copolymer which stabilizes the colloidal suspension of nanoparticles in several conditions and facilitate the coupling with antibodies by means of a Cu(I)-catalyzed click reaction. The nanoparticles coating has been characterized by different complementary techniques. A gold coated anti-CD63 antibody, was used as label in an innovative bioassay based on single particle counting. The successful binding of the anti-mouse-IgG antibody shows that the labeled antibody is fully functional. The proposed method can be used to label antibodies in a number of different applications.

References

(1) Bawarski, W. E.; Chidlowsky, E.; Bharali, D. J.; Mousa, S. A. Emerging Nanopharmaceuticals. *Nanomedicine: Nanotechnology, Biology, and Medicine*. 2008, pp 273–282.

(2) Howes, P. D.; Chandrawati, R.; Stevens, M. M. Bionanotechnology. Colloidal Nanoparticles as Advanced Biological Sensors. *Science* **2014**, *346* (6205), 1247390.

(3) Artemov, D.; Mori, N.; Okollie, B.; Bhujwalla, Z. M. MR Molecular Imaging of the Her-2/neu Receptor in Breast Cancer Cells Using Targeted Iron Oxide Nanoparticles. *Magn. Reson. Med.* **2003**, *49*, 403–408.

(4) Forbes, Z. G.; Yellen, B. B.; Barbee, K. A.; Friedman, G. An Approach to Targeted Drug Delivery Based on Uniform Magnetic Fields. *IEEE Trans. Magn.* **2003**, *39*.

(5) Fortina, P.; Kricka, L. J.; Graves, D. J.; Park, J.; Hyslop, T.; Tam, F.; Halas, N.; Surrey, S.; Waldman, S. A. Applications of Nanoparticles to Diagnostics and Therapeutics in Colorectal Cancer. *Trends Biotechnol.* **2007**, *25* (4), 145–152.

(6) Katz, E.; Willner, I. Integrated Nanoparticle-Biomolecule Hybrid Systems: Synthesis, Properties, and Applications. *Angew. Chemie Int. Ed.* **2004**, *43* (45), 6042–6108.

(7) Wilson, D. S.; Nock, S. Functional Protein Microarrays. *Curr. Opin. Chem. Biol.* **2002**, *6*, 81–85.

(8) Zilio, C.; Bernardi, A.; Palmioli, A.; Salina, M.; Tagliabue, G.; Buscaglia, M.; Consonni, R.; Chiari, M. New “clickable” Polymeric Coating for Glycan Microarrays. *Sensors Actuators B Chem.* **2015**, *215*, 412–420.

(9) Maggon, K. Monoclonal Antibody “Gold Rush”. *Curr. Med. Chem.* **2007**, *14*, 1978–1987.

(10) Baptista, P.; Pereira, E.; Eaton, P.; Doria, G.; Miranda, A.; Gomes, I.; Quaresma, P.; Franco, R. Gold Nanoparticles for the Development of Clinical Diagnosis Methods. *Anal. Bioanal.*

Chem. **2008**, *391*, 943–950.

(11) Buijs, J.; Lichtenbelt, J. W. T.; Norde, W.; Lyklema, J. Adsorption of Monoclonal IgGs and Their F(ab′)2 Fragments onto Polymeric Surfaces. *Colloids and Surfaces B: Biointerfaces*. 1995, pp 11–23.

(12) Radwan, S. H.; Azzazy, H. M. E. Gold Nanoparticles for Molecular Diagnostics. *Expert Rev. Mol. Diagn.* **2009**, *9*, 511–524.

(13) Mammen, M.; Dahmann, G.; Whitesides, G. M. Effective Inhibitors of Hemagglutination by Influenza Virus Synthesized from Polymers Having Active Ester Groups. Insight into Mechanism of Inhibition. *J. Med. Chem.* **1995**, *38* (21), 4179–4190.

(14) Ladmiral, V.; Mantovani, G.; Clarkson, G. J.; Cauet, S.; Irwin, J. L.; Haddleton, D. M. Synthesis of Neoglycopolymers by a Combination of "Click Chemistry" and Living Radical Polymerization. *J. Am. Chem. Soc.* **2006**, *128* (14), 4823–4830.

(15) Pirri, G.; Damin, F.; Chiari, M.; Bontempi, E.; Depero, L. E. Characterization of A Polymeric Adsorbed Coating for DNA Microarray Glass Slides. *Anal. Chem.* **2004**, *76* (5), 1352–1358.

(16) Li, J. F.; Tian, X. D.; Li, S. B.; Anema, J. R.; Yang, Z. L.; Ding, Y.; Wu, Y. F.; Zeng, Y. M.; Chen, Q. Z.; Ren, B.; Wang, Z. L.; Tian, Z.Q. Surface Analysis Using Shell-Isolated Nanoparticle-Enhanced Raman Spectroscopy. *Nat. Protoc.* **2013**, *8* (1), 52–65.

(17) Daaboul, G. G.; Yurt, a.; Zhang, X.; Hwang, G. M.; Goldberg, B. B.; Ünlü, M. S. High-Throughput Detection and Sizing of Individual Low-Index Nanoparticles and Viruses for Pathogen Identification. *Nano Lett.* **2010**, *10*, 4727–4731.

(18) Yurt, A.; Daaboul, G. G.; Connor, J. H.; Goldberg, B. B.; Selim Ünlü, M. Single Nanoparticle Detectors for Biological Applications. *Nanoscale* **2012**, *4*, 715.

(19) Sperling, R. A.; Parak, W. J. Surface Modification, Functionalization and Bioconjugation of Colloidal Inorganic Nanoparticles. *Philos. Trans. A. Math. Phys. Eng. Sci.* **2010**, *368* (1915),

1333–1383.

(20) Lim, C. Y.; Owens, N. A.; Wampler, R. D.; Ying, Y.; Granger, J. H.; Porter, M. D.; Takahashi, M.; Shimazu, K. Succinimidyl Ester Surface Chemistry: Implications of the Competition between Aminolysis and Hydrolysis on Covalent Protein Immobilization. *Langmuir* **2014**, *30* (43), 12868–12878.

(21) Sola, L.; Chiari, M. Modulation of Electroosmotic Flow in Capillary Electrophoresis Using Functional Polymer Coatings. *J. Chromatogr. A* **2012**, *1270*, 324–329.

(22) Sola, L.; Chiari, M. Tuning Capillary Surface Properties by Charged Polymeric Coatings. *J. Chromatogr. A* **2015**, *1414*, 173–181.

(23) Petti, D.; Torti, a.; Damin, F.; Sola, L.; Rusnati, M.; Albisetti, E.; Bugatti, a.; Bertacco, R.; Chiari, M. Functionalization of Gold Surfaces with copoly(DMA-NAS-MAPS) by Dip Coating: Surface Characterization and Hybridization Tests. *Sensors Actuators, B Chem.* **2014**, *190*, 234–242.

(24) Zilio, C.; Sola, L.; Damin, F.; Faggioni, L.; Chiari, M. Universal Hydrophilic Coating of Thermoplastic Polymers Currently Used in Microfluidics. *Biomed. Microdevices* **2014**, *16* (1), 107–114.

(25) Turkevich, J.; Stevenson, P. C.; Hillier, J. A Study of the Nucleation and Growth Processes in the Synthesis of Colloidal Gold. *Discuss. Faraday Soc.* **1951**, *11*, 55.

(26) FRENS, G. Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Suspensions. *Nature* **1973**, *241* (105), 20–22.

Acknowledgments

This work was partially supported by Regione Lombardia & Fondazione Cariplo through POR-FESR, project MINER (ID 46875467)**.**

Legends

Figure 1. Schematic representation of the surface modification of AuNPs with a functional polymer and consequent derivatization of the polymer with an azido modified antibody

Figure 2. Agarose gel electrophoresis of gold nanoparticles: a) lane 1, uncoated nanoparticles, lane 2, polymer-modified gold nanoparticles; b) lane 1, polymer coated NPs (no conjugation); lane 2, polymer coated NPs treated with antibody in the absence of THPTA/$CuSO_4$ and ascorbic acid (no conjugation); lane 3, polymer coated particles treated with antibody, THPTA/$CuSO_4$ and ascorbic acid (conjugation).

Figure 3. Plasmon band shift due to the polymer and antibody layers deposited on AuNPs.

Figure 4. Transmission Electron Microscopy of individual AuNPs a) before, b) after coating with copoly(DMA-PMA-MAPS) and c) after immobilization of IgG.

Figure 5. Uncoated and polymer coated AuNPs were treated with NaCl, HCl or NaOH. In these conditions uncoated NPs are very unstable and aggregate, causing a red to blue color change (left column). Nanoparticles with the poly(DMA-PMA-MAPS) coating are stable also in harsh conditions (right column).

Figure 6. a) SP-IRIS images of surfaces functionalized with different antibodies, incubated with an anti-mouse antibody conjugated with gold nanoparticles in in the presence (+) or in the absence (-) of THPTA/$CuSO_4$ and ascorbic acid; b) SP-IRIS response (mean ± SD; n=20 of particle number/mm^2) of anti-mouse IgG linked or adsorbed on polymer coated AuNPs.

Table 1 Hydrodinamic diameter and ζ-potential values for uncoated, polymer coated and Ab-linked AuNPs

SAMPLE	Hydrodinamic diameter (nm)	PDI	ζ-potential (mV)
Uncoated AuNPs	47.02 ± 13.52	0.083	-25.1 ± 2.40
Polymer coated AuNPS	131.1 ± 34.93	0.071	-17.6 ± 0.35
Ab linked AuNPs	146.75 ± 87.92	0.403	-14.1 ± 2.26

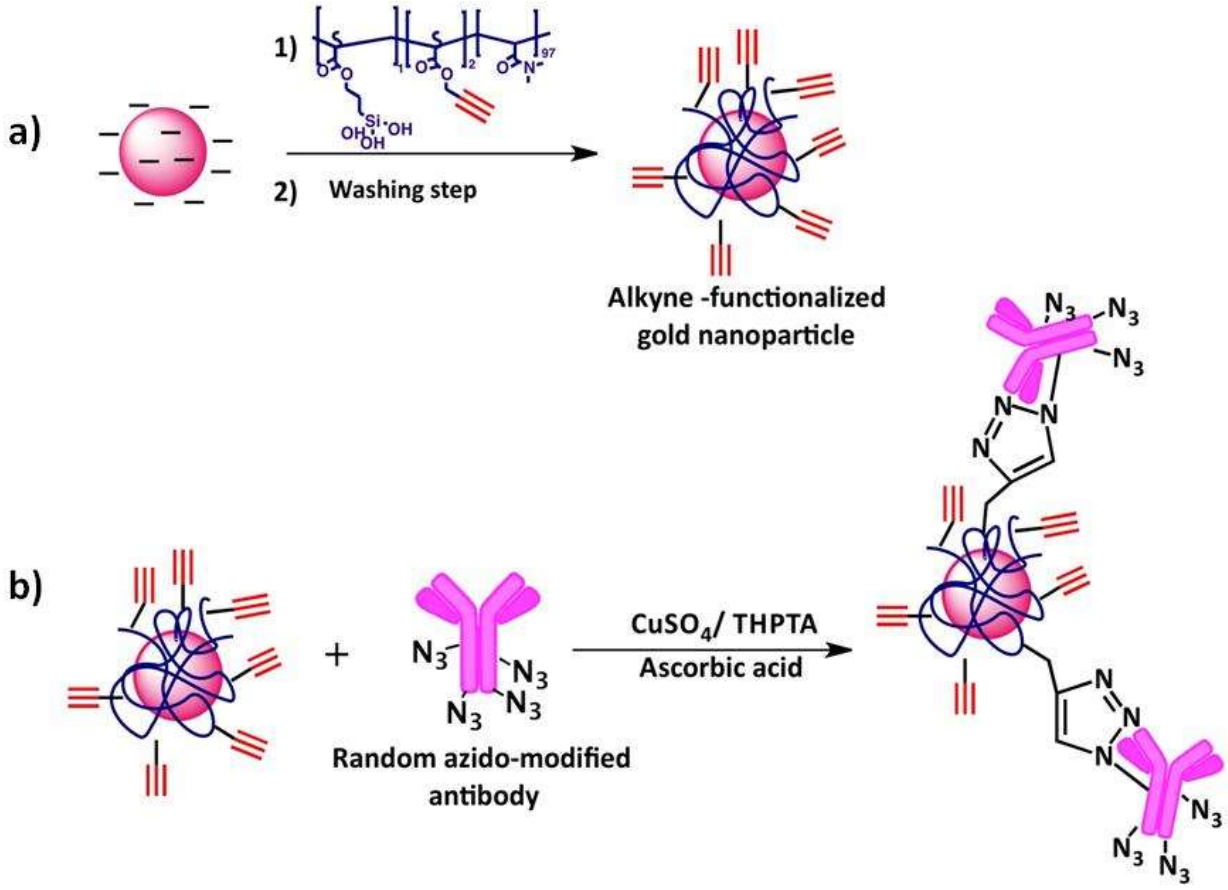
a)
1)
2) Washing step
Alkyne -functionalized
gold nanoparticle
b)
Random azido-modified
antibody
CuSO4/ THPTA
Ascorbic acid

Figure 1

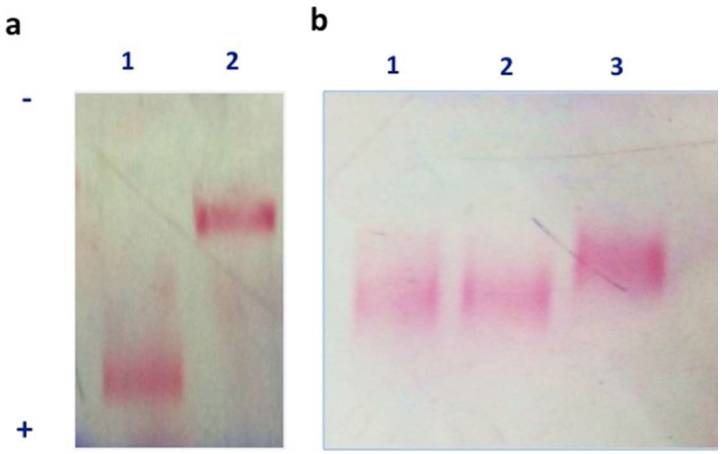

Figure 2

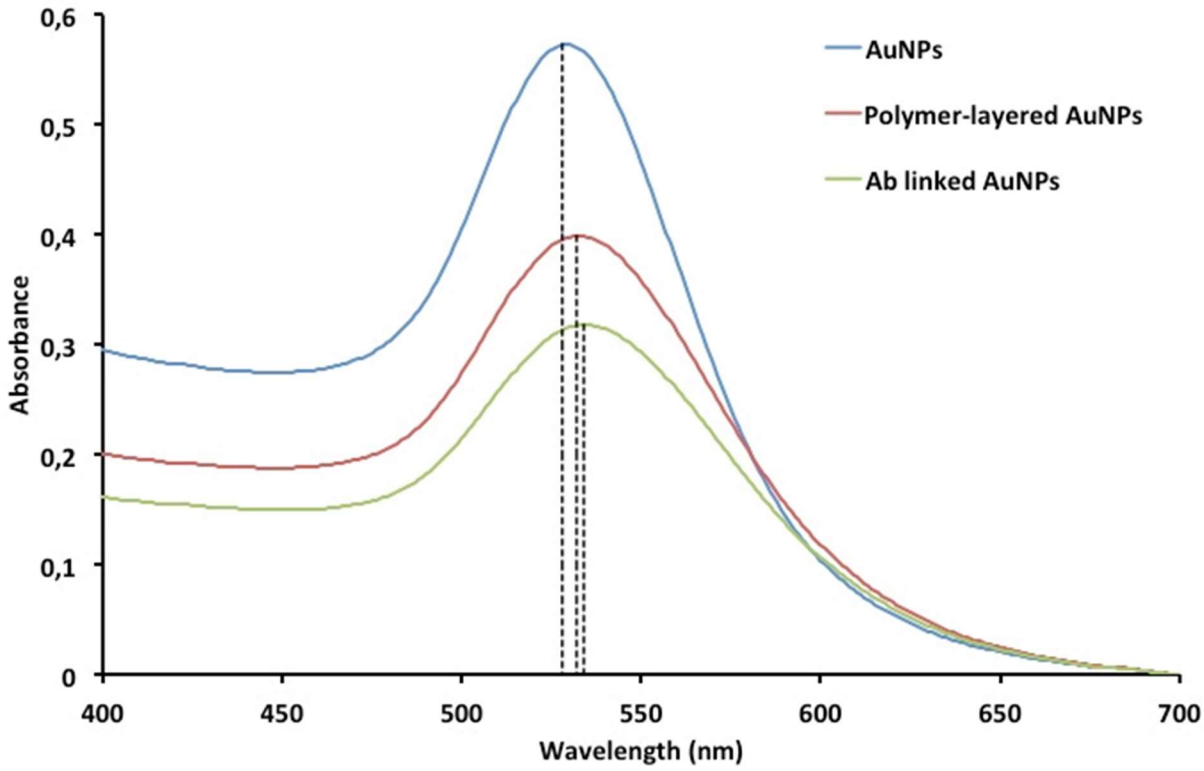
0,6
0,5
0,4
0,3
0,2
0,1
0
Absorbance
400
450
500
550
600
650
700
Wavelength (nm)
AuNPs
Polymer-layered AuNPs
Ab linked AuNPs

Figure 3

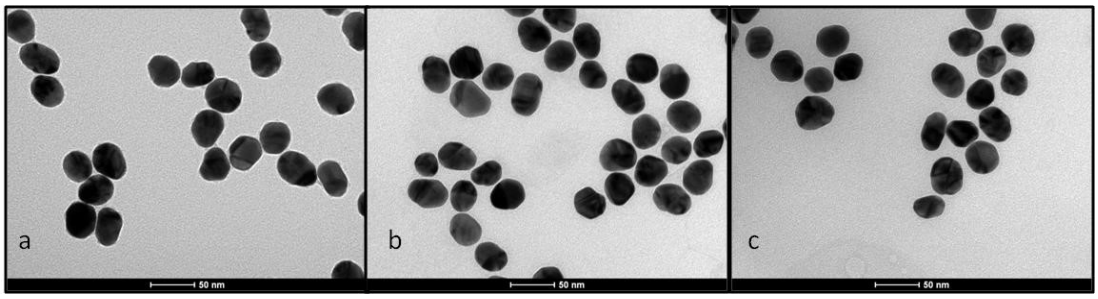

Figure 4

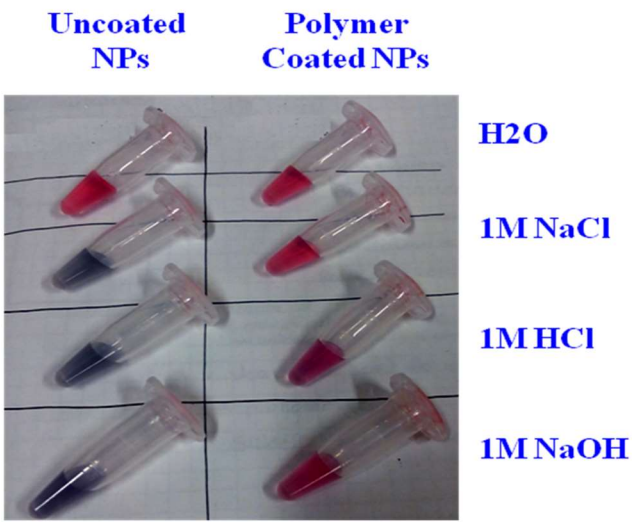

Figure 5

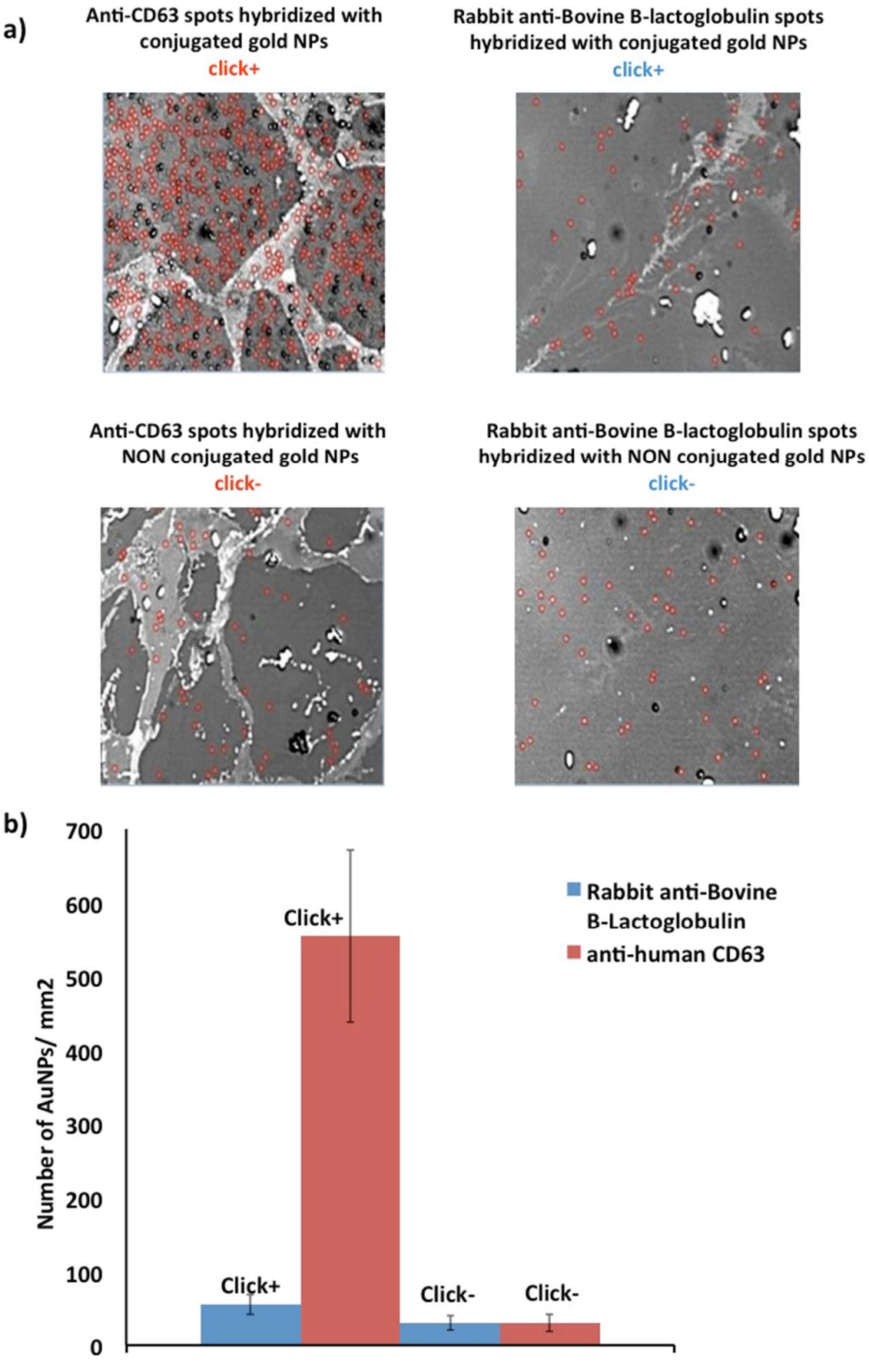
a)
Anti-CD63 spots hybridized with conjugated gold NPs
click+
Rabbit anti-Bovine B-lactoglobulin spots hybridized with conjugated gold NPs
click+
Anti-CD63 spots hybridized with NON conjugated gold NPs
click-
Rabbit anti-Bovine B-lactoglobulin spots hybridized with NON conjugated gold NPs
click-
b)
Number of AuNPs/ mm2
700
600
500
400
300
200
100
0
Click+
Click+
Click-
Click-
Rabbit anti-Bovine B-Lactoglobulin
anti-human CD63

Figure 6

TOC Graphic