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Characterization of Multifunctional Nanosystems Based on the Avidin-Nucleic Acid Interaction As Signal Enhancers in Immuno-Detection

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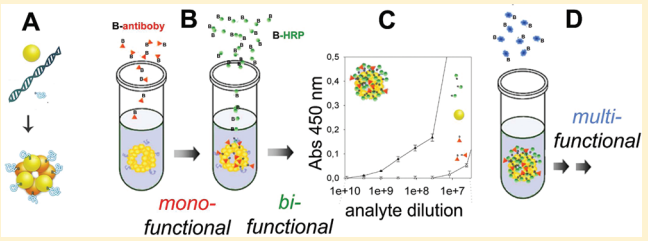
Supporting Information

ABSTRACT: The Avidin-Nucleic-Acids-Nano-Assembly (ANANAS) is a kind of soft poly avidin nanoparticle originating from the high affinity interaction between avidin and the nucleic acids. In this work we investigated the possibility of transforming ANANAS cores into stoichiometrically controlled multifunctional nanoparticles through a "one-pot" procedure, and we measured in a quantitative way their ability to work as reagents for enhanced immunodiagnostic detection. Initially, we measured the ANANAS loading capability for biotinylated proteins of different nature. About 200 molecules of biotin-horseradish-peroxidase (40KDa b-HRP) and 60 molecules of biotin-immunoglobulin-G (150KDa b-IgG) could be accommodated onto each nanoparticle, showing that steric limitations dictate the number of loadable entities. Stoichiometrically controlled functional assemblies were generated by mixing core particles with subsaturating amounts of b-HRP and b-IgG. When applied as detection reagents in an Enzyme-Linked-ImmunoSorbed-Assay (ELISA), these assemblies were up to two-orders of magnitude more sensitive than commercial HRP-based reagents. Assemblies of different composition displayed different efficacy, indicating that the system functionality can be fine-tuned. Within-assay variability (CV%), measured to assess if the assembly procedure is reproducible, was within 10%. Stability experiments demonstrated that the functionalyzed assemblies are stable in solution for more than one week. In principle, any biotinylated function can be loaded onto the core particle, whose high loading capacity and tunability may open the way toward further application in biomedicine.



Avidin and its bacterial analogue streptavidin are well-known proteins extensively used as tools in biomedicine. More precisely, thanks to their property of binding with high affinity ($K_d = 10^{-15}$ M) 4 molecules of biotin, these proteins are used as "molecular bridges" to bring together different chemical entities (provided they have been biotinylated) with applications in different fields, both *in vitro* and in *vivo*.[1−3] One limitation of the avidin−biotin technology is the "low" number of biotin binding sites (BBS) *per* protein, so that only up to 4 biotinylated moieties can be linked together in one assembly. A possibility to overcome this limit is to obtain polymerized forms of the proteins, and several approaches to obtain supramolecular poly(strept)avidin systems have been described. Micron size avidin or streptavidin coated particles have indeed become commercially available, and colloidal polymeric systems have been described in the literature.[4−8] A novel colloidal poly avidin system that has recently been described in the literature is the Avidin-Nucleic-Acid Nano Assembly (ANANAS).[9,10] This poly avidin is obtained by exploiting a stoichiometrically defined double self-assembly process dictated by high affinity interactions[9,10] (Figure 1-**A**). In the process of ANANAS preparation, the high affinity of avidin for the nucleic acids drives the formation of assemblies in which several avidins bind to a nucleic acid filament with precise avidin/DNA base pairs relationship. Even if the protein biotin binding ability is maintained after DNA interaction,[9] the avidin-nucleic acid complex is unusable as such, due to its poor solubility in aqueous buffers. However, if the assembly is obtained using plasmid DNA as the nucleating agent, and controlled amounts of biotinylated hydrophilic polymers (e.g., biotin-poly(ethylene oxide)) to cover a small percentage of the biotin binding sites, buffer-soluble toroid-shaped nanoparticles (Ø ∼ 100 nm) are formed,[10] in which most of the biotin binding ability is maintained. A unique property of this system is that, for a given nucleating DNA, the number of protein units/particle is precisely defined (1 avidin/14 bp[9]) as opposed to poly avidin systems obtained by chemical cross-linking whose composition is defined by statistical rather than high affinity rules. Also the surface protection strategy relies on a high affinity interaction, so that the composition and

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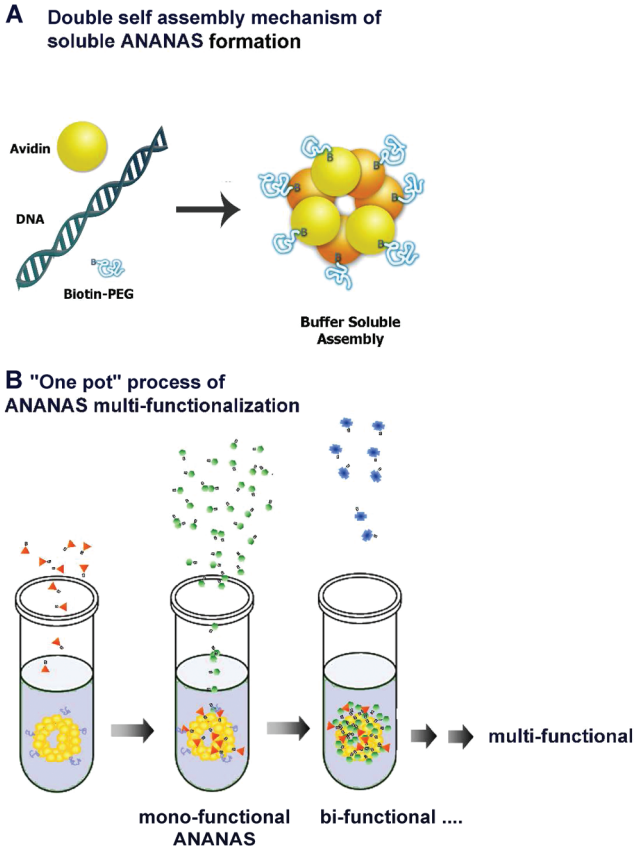

Figure 1. Diagram (not in scale) representing A) the soluble core ANANAS double self-assembly formation B) the general principle of the "one pot" multifunctionalization process.

stoichiometry of the buffer-soluble particle remains finely defined.

Although the composition and the number of biotin binding sites in the core ANANAS particle can be calculated from the stoichiometry of the assembly reagents, the number of BBS truly available for binding biotin derivatives has not been shown yet, and so has the influence of steric or chemical constraints in the particle loading capability. On the other hand, knowing the loading capability of the assembly would allow formulating multifunctional assemblies with stoichiometric precision "in one pot" (Figure 1-**B**), namely without the need for purification steps. In fact, the high affinity between avidin and biotin would ensure quantitative assembly as long as no excess of biotinylating agent is present in solution. It has to be pointed out that more than one composition of solution stable assembly can be obtained, depending on the size and shape of the hydrophilic polymer used for surface protection,[10] so the number of available BBS may vary with each formulation.

In this work we characterized a nanoparticle (NP) preparation which was obtained using a 4.7 Kbp plasmid as the nucleating DNA and a biotin-poly(ethylene oxide) derivative of 5000 MW (b-PEO)[10] as the surface protecting agent. We measured the nanoparticle loading capability for biotin as such, and when linked to macromolecular derivatives of different molecular weight: α-mouse-IgG antibody (b-α-M-IgG, 150KDa) and horseradish peroxidase (b-HRP, 40 KDa). In a second phase, we measured the potentials of the platform for analytical application: we assembled in "one pot" core NPs with different subsaturating amounts b-HRP and b-IgG at controlled relative molar ratios, and we tested their functionality as detection systems in a model Enzyme-Linked ImmunoSorbed Assay (ELISA). The ELISA signal generated by preassembled functionalyzed nanoparticles, or by using the individual components added sequentially in two- and three-step detection protocols, was compared to that generated by HRP-based reagents commercially available. The stability over time of the functionalized assemblies was also monitored. Reproducibility parameters in the detection assay were measured and used as indicators of the constancy of the assembly process.

2. EXPERIMENTAL SECTION

2.1. Materials and Instrumentation. Goat antimouse immunoglobulin-G (α-M-IgG) and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from KPL (Gaithersburg, MD, USA). The ABC Vectastain (antimouse IgG) kit was from Vector Laboratories (Burlingame, CA, USA). HorseRadish Peroxidase (HRP) was from Sigma-Aldrich (St. Louis, MO, USA). The α-mouse-IgG-HRP conjugate (AP124P Goat anti Mouse IgG H&L) perox conjugate) was purchased from Millipore (Billerica, MA, USA). Ninety-six-well High binding Corning EIA/RIA plates were from Sigma Aldrich. Antihuman recombinant growth hormone (h-rGH) mouse serum was kindly provided by Dr. C. Ferracini (University of Padova, Italy). This serum was obtained by immunizing balb-C mice with a Keyhole Lymphet Hemocyanin-hGH conjugate (KLH-h-rGH). Plasmid pEGFP-C1, 4.7 kb (used for the ANANAS core preparation), was from Clontech. . Avidin was purchased from BELOVO chemical (Belgium). The 5KDa biotin-poly(oxyethyleneoxide) (b-PEO), used for soluble ANANAS preparation, was prepared according to Pignatto et al.[10] Nanoparticle protein-free dilution buffer (DB) was kindly provided by ANANAS nanotech (Padova, Italy). Fast Permeation Liquid Chromatography (FPLC) analyses were performed using an AKTA purifier 10 (GE Healthcare) apparatus, equipped with a UV−vis detector. Microplate absorbance was measured with a Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA,USA). UV spectra were recorded on a Varian Cary 50 UV−vis spectrophotometer (Varian Inc. Palo Alto, CA,USA). Dynamic light scattering measurements were performed using a Malvern Zetasizer NANO ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom).

2.2. ANANAS Loading with Biotin and Biotinylated Proteins. Preparation of ANANAS and biotinylated proteins is summarized in the Supporting Information.

2.2.1. Loading for Biotin. The number of binding sites (BBS) available for biotin was measured *via* the "inverted" HABA assay.[11] In this assay, a known amount of nanoparticles (NP) (0.5 mL of 120 μg/mL) was mixed with a known theoretical excess (2 equiv) of biotin. Nanoparticles were removed from solution by 1 h ultracentrifugation (16600 g), and the free biotin remaining in solution was quantified from after mixing with an equal volume of an avidin/HABA complex[11] solution (avidin = 1 mg/mL) and measuring the absorbance at 500 nm. The number of BBS/NP was computed by subtracting the amount of free biotin from the total initially added.

2.2.2. Loading for Biotinylated Proteins. ANANAS and biotinylated α-M-IgG (b-αM-IgG) or biotinylated HRP (b-HRP) were mixed in PBS at room temperature while gently vortexing. The final volume of the mixtures was adjusted with PBS in order to have avidin concentration equal to 24 μg/mL. In the

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final solution, b-αM-IgG concentration varied between 0.8 to 31 μg/mL and b-HRP varied between 0.46 to 13.7 μg/mL. The b-protein:nanoparticle molar ratio was between 5 and 200 for b-IgG and between 10 and 300 for b-HRP. A control solution without biotinylated proteins was prepared by diluting the particles with PBS buffer only. Solutions were left at 4 °C for 2 h and were then analyzed by size exclusion chromatography (column: Sepharose 6 FF column; eluent PBS; flow: 3 mL/min; detection Abs 220 nm). The number of protein molecules bound/particle was determined from the increase in the area under the curve (AUC) of the peak corresponding to the nanoassembly (eluting at the column void volume of 18.5 mL) as compared to that of the protein-free control sample. The peak corresponding to the nanoparticles was also collected and analyzed by dynamic light scattering.

2.3. ELISA. *2.3.1. Plate Preparation.* Human recombinant growth hormone (h-rGH, 50 μL, 4 μg/mL in 0.05 M carbonate buffer pH 9.6) was adsorbed to each well of the microplate. The microplate was kept at 4 °C overnight and washed 4 times with PBS + 0.05 (w/v) Tween 20 (PBST, 300 μL/well). Wells were blocked by adding a bovine serum albumin solution (BSA) 3% in PBST (300 μL, 1 h, room temperature). After washing, serial dilutions of mouse anti-GH antiserum in PBST + BSA 0.1% (dilutions from 1:4.50 × 10^5 to 1:2.66 × 10^{10}, 50 μL/well) were added and incubated for 1 h at room temperature. After washing and drying, microplates were sealed and stored until further use.

2.3.2. Detection Protocols. Five different procedures were compared

2.3.2.a. ANANAS-Three-Step. The microplate was incubated with b-αM-IgG diluted in PBST+BSA 0.1%, (50 μL/well, 30′, RT). After washing, ANANAS, diluted in DB buffer + BSA0.1%, was added (8 μg/mL in avidin, 50 μL/well, 60′, RT), followed, after rinsing, by b-HRP (50 μL/well, 2 μg/mL, 45′, RT). After washing, color development was carried out with TMB (50 μL/well); the reaction was stopped after 20 min by adding 50 μL of 0.5N H_2SO_4 and well absorbance was measured at 450 nm.

2.3.2.b. ANANAS-Two-Step. Preassemblies composed of ANANAS together with b-αM-IgG ("ANANAS/b-αM-IgG") were prepared in solution by mixing the particles and the biotinylated antibody in DB buffer+BSA0.1%. Several assemblies were obtained by varying the ANANAS:antibody molar ratio in solution between 1:1 and 1:50. Preassemblies were then added to the microplate (8 μg/mL in avidin, 50 μL, 60′, RT). After washing, b-HRP was added (50 μL/well, 2 μg/mL, 45′, RT). Color development was carried out as previously described.

2.3.2.c. ANANAS-One-Step. A preassembly composed of ANANAS together with b-αM-IgG and b-HRP ("b-αM-IgG/ANANAS/b-HRP") was prepared in solution in DB buffer +BSA0.1%, by mixing the ANANAS with the above reagents at relative molar ratios of 10:1:200. The b-αM-IgG/ANANAS/b-HRP assembly was then incubated in the microplate (8 μg/mL in avidin 50 μL, 60′, RT). After washing, color development was carried out as previously described.

2.3.2.d. Commercial HRP Based Antimouse Detection Systems. The Vectastain ABC (antimouse IgG) kit and α-mouse IgG-HRP conjugate were used following manufacturer's guidelines. TMB colorimetric reaction was carried out as above-described.

3. RESULTS AND DISCUSSION

3.1. Loading Capability for Biotin and Biotinylated Proteins. *3.1.1. Biotin.* The number of BBS/NP was measured *via* the "inverted" HABA assay. Previous observations suggested that the interaction of avidin with DNA involves a site in the protein that is different from that used for binding biotin. However, this fact had only been demonstrated through the indirect observation that the strength of the avidin/DNA interaction is higher in the presence of biotin,[9,10] the number of biotins interacting with the macromolecularized protein not having been being quantified yet. Indeed, both the assembly entanglement leading to the toroidal conformation[9,10] and the presence of the solubilizing polymer cloud[9,10] may reduce the effective surface available for anchoring biotinylated entities. The number of BBS/NP measured was 973 ± 23. This number is close to the theoretical number of BBS calculated from the stoichiometry of the assembly preparation (1020, see the Supporting Information) and demonstrates that the majority of the BBS in the macromolecularized avidin remains accessible to the low MW biotin molecule.

3.1.2. Biotin Immunoglobulin-G and Biotin-HRP. Loading of ANANAS with biotinylated macromolcules was carried out by chromatography titration. Figure 2 shows the increase of the

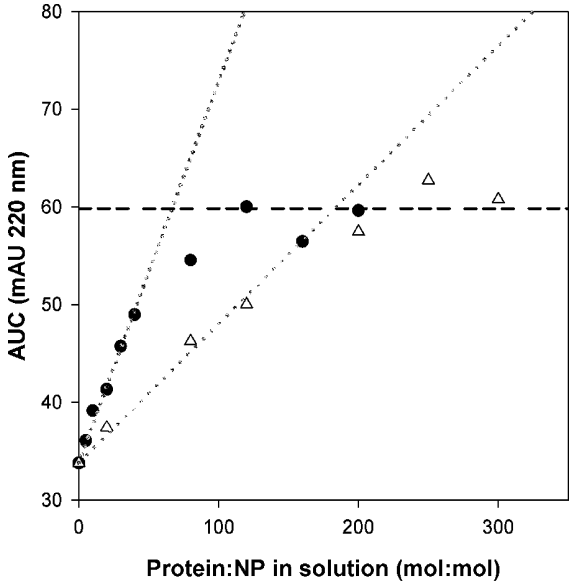

Figure 2. A) Area Under Curve (AUC) of the peak eluting at 18.5 mL in the size exclusion chromatography analysis of antibody-nanoparticle assemblies prepared at increasing b-αM-IgG:NP (●) or b-HRP:NP (Δ) molar ratios. Analysis was performed on Sepharose 6 FF column, using PBS as elution buffer.

area under the curve (AUC) of the peak eluting at 18.5 mL, corresponding to the nanoparticles retention volume, as a function of the protein:particle (b-αM-IgG:NP or b-HRP:NP) molar ratio in solution.

In both cases, the AUC increased linearly with the protein:NP until it reached a *plateau*. A peak eluting at about 35.5−40 mL, corresponding to the unbound biotinylated proteins, was detected at high protein:NP values, the intensity of which increased linearly with the increase of protein concentration in solution. This peak started to appear at different protein:NP values depending on the assembly under investigation, namely at b-αM-IgG:NP ≥ 80 and at b-HRP:NP

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≥ 200. This indicates that at protein:NP below 80 for the b-αM-IgG/NP assembly, and below 200 for the b-HRP/NP one, the biotinylated protein was quantitatively captured by the ANANAS. A precise estimation of the total amount of bound b-HRP and b-αM-IgG was obtained by a mathematical fit of data of Figure 2 (Supporting Information). Values of 61 ± 4 b-αM-IgG:NP and 196 ± 10 b-HRP:NP were obtained, which are much lower than the total number of BBS available (973 ± 23, see above). In fact, considering the degree of IgG and HRP biotinylation (biotin:protein = 3.6 and 1 for b-αM-IgG and b-HRP, respectively), the amount of biotins present on the 61 b-αM-IgG for the 196 b-HRPs corresponds to about 22% and 20% of the total BBS available. We therefore conclude that the maximum number of molecules that can be brought to the surface of the ANANAS is dictated by steric constrains, and only a limited number of macromolecules can fit, this limit depending on their molecular weight and hydrodynamic radius (IgG MW 150 KDa and hydrodynamic radius of about 5.5 nm, foot print ∼100 nm^2; HRP MW 44 KDa, hydrodynamic radius = 3 nm, foot-print ∼ 30 nm^2). Interestingly, b-HRP and b-aM-IgG loading data converge in indicating a total NP surface area available for linking macromolecules of about 6000 nm^2.

The colloidal property of the b-α-MigG/NP assemblies eluting from gel chromatography was confirmed by dynamic light scattering (DLS) measurements (see also the Supporting Information). Only a minimal size increase occurred until b-α-MIgG:NP = 80. Within this ratio range, the size registered increased from the initial 116 nm to about 130 nm, consistently with solution stable discrete particles on top of which an IgG monolayer is being gradually built. A major size increase occurred by further increasing the b-αM-IgG:NP ratio. The size registered at b-αM-IgG:NP = 120 was about 140 nm, while a greater change was observed at b-αM-IgG:NP = 180 and 200, when the average particle diameter was about 200 nm. At these high ratios, particles were also more polydisperse. Considering the fact that the maximum particle load calculated from the chromatography data was about 61 b-αM-IgG:NP, the increase in size observed in the formulations at higher b-αM-IgG content (especially at 160 and 200 b-αM-IgG:NP in the mix) is probably due to some interparticle aggregation. In fact, the b-αM-IgGs used here had more than 1 biotin:molecule. Since steric constraints and not the availability of biotin biding sites is the limiting factor for IgG particle load, it is likely that at low b-αM-IgG:NP ratio there are enough biotin biding sites to host all of the biotins in proximity of the particle surface; at high b-αM-IgG:NP ratios, the larger amount of biotins available is capable of interparticle bridging. Even if this phenomenon is limited, since the aggregates still retain colloidal size, it clearly shows that it is useless and "detrimental" to add in solution more than the maximum amount of b-protein the particles can accommodate. On one hand, this would be unproductive in terms of final particle avidity for the target; on the other hand, it would represent a useless waste of biotinylated protein and the final product would have to be purified from the excess prior to its use.

3.2. "One-Pot" Assemblies: Composition and Function. *3.2.1. The Model System.* These experiments were carried out in order to verify if preloaded particles were able to work as functional entities and if their activity could be modulated by varying the relative molar ratio of the bioactive components in the assembly solution. To this end, we designed a simple immunodiagnostic (ELISA) test in which functional assemblies could act as analyte-detecting reagents. The model test (Figure 3), which was based on reagents already available in the laboratory, was designed to permit a direct comparison of

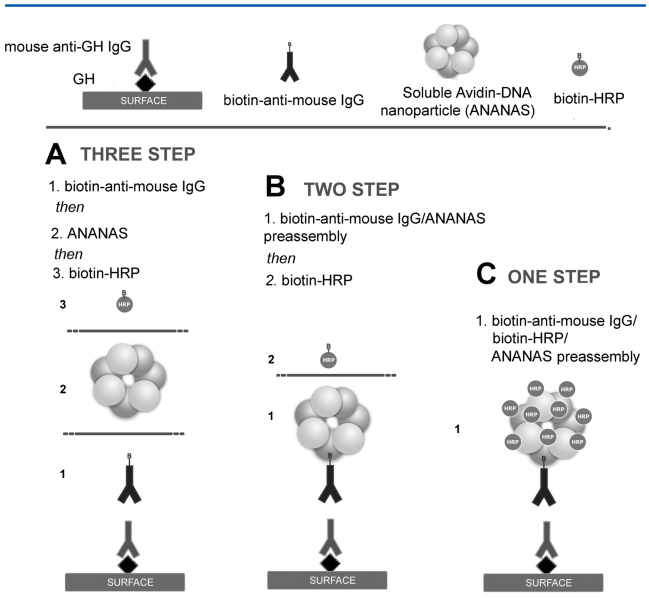

Figure 3. Diagram (not in scale) describing the three ANANAS based procedures to detect anti-h-rGH mouse antibodies.

the activity of assemblies of different composition by the intensity of the detection signal generated.

In this model test, the "analytes" are mouse anti-h-rGH IgGs from h-rGH immunized mouse serum. Serial dilutions of these IgGs were immobilized by immunosorption onto h-rGH coated ELISA microwells. Detection of the immunocaptured mouse IgGs was then carried out using the differently functionalyzed ANANAS. More precisely, the ANANAS-based reagents were used in three different protocols (Figure 3): a) a "three-step" one, where the three reagents were added to the wells one after the other in separate steps, b) a "two-step" one, in which a preassembly composed of ANANAS + b-αM-IgG was generated in solution and added to the wells, followed in the second step by b-HRP, and c) a "one-step" one in which a preassembly composed of ANANAS + b-HRP + b-αM-IgG was prepared in solution and then added directly in one step to the microplate wells. A positive signal in the "one- and two-step" protocols indicates that the reagents mixed in solution had assembled into active particles. Commercial monomeric and supramolecular HRP-based detection systems were also tested in parallel as controls, namely an HRP-antimouse IgG conjugate and the vectatstain "ABC" complex. The latter is a mixture composed of biotinylated-HRP with avidin at optimized molar ratio, which is recommended when enhanced sensitivity is needed.[12−14]

3.2.2. Effect on ANANAS Functionality of the Reagents Molar Ratio in the Preassembly Solution. A polyavidin supramolecular assembly should be able to couple a single biorecognition event to a high a number of signal generating moieties, the number of which depends on its loading capability. In principle, the high number of BBS should also permit to modulate the amplification behavior. Therefore, experiments were carried out to verify a) whether active assemblies form in solution and b) if their properties could be tuned by varying the solution component's molar ratios. We thus generated different b-αM-IgG:NP mixtures by mixing in

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solution b-αM-IgG with ANANAS at increasing molar ratios (between 5 to 40 b-αM-IgG:NP). The ratios selected were all within the subsaturating range measured in previous experiments so that no purification was necessary prior to addition to the ELISA wells. The detection performance of all mixtures was then tested in the two-step detection. The results are shown in Figure 4.

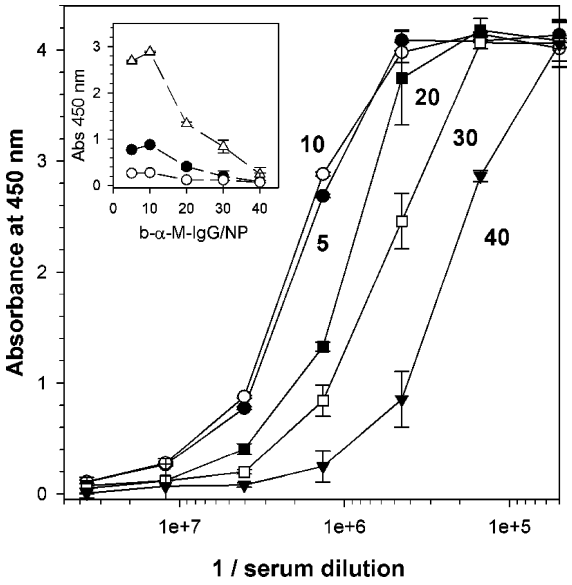

Figure 4. Detection of anti h-rGH serum in an ELISA assay performed using preassembled b-αMIgG/ANANAS particles at different b-IgG:NP ratio and used in a two-step detection protocol. A) signal intensity vs serum dilution generated by b-αMIgG/ANANAS assemblies obtained at 5 (●), 10 (o), 20 (■), 30 (□), and 40 (▼) b-αMIgG:NP ratios; ***Indent***: signal generated at selected anti-GH mouse antisera dilutions, namely $1/1.22 \times 10^7$ (Δ), $1/3.65 \times 10^7$ (●), and $1/1.09 \times 10^8$ (o) as a function of b-αMIgG:NP ratio. TMB color development was allowed for 20 min before acid blocking. Bars indicate the standard deviation of three experiments run in parallel.

All solution mixtures were capable of generating dose/response curves demonstrating that functional assemblies (capable of recognizing both the biotinylated analyte and b-HRP) had formed at all b-αM-IgG:NP ratios tested. Detection performance was strongly influenced by the solution composition. The best performance was observed at b-αM-IgG:NP = 10:1. At both higher and lower antibody payloads, the signal generated was lower. When compared to the 10:1 assembly, the signal generating efficiency varied between 14% (40:1) to 95% (5:1). The differences observed can be explained by the fact that the ability of the assembly to work as a detecting reagent results from a compromise between its avidity for the target and steric constrains. Avidity, which is more important at low analyte concentration, increases with the NPs antibody paylod;[15] steric factors act in the opposite direction, since higher antibody loads reduce the particle availability for the signal generating molecules (b-HRP). From a general general point of view, this result confirms that the behavior of an ANANAS functional assembly can be tuned by controlling its components solution composition (or the reagents' relative molar ratio).

The assembly generated for the "two-step" detection protocol contained only one kind of bioactive moiety. In order to evaluate if active multifunctional assemblies could also be generated, we premixed the ANANAS with both b-αM-IgG and b-HRP, and we evaluated the assembly ability to work as a detection reagent in the "one-step" protocol. Based on the results of particle loading and of the two-step detection experiments, the b-αM-IgG:NP:b-HRP molar ratio selected for this experiment was 10:1:200. The ability of this functional assembly to work in the model ELISA test was compared to that of the two-step 10:1 assembly and to that of the individual reagents added stepwise (three-step).

All ANANAS-based systems were efficient in detecting mouse IgGs (Figure 5), proving that also bifunctional ANANAS assemblies can be successfully generated in solution.

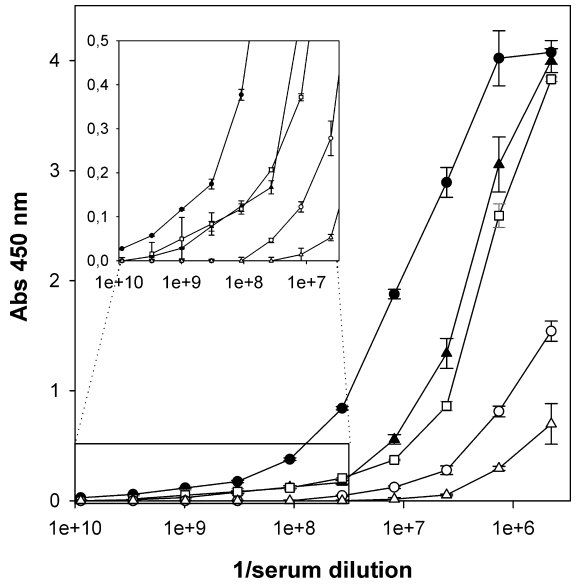

Figure 5. ELISA detection of serial dilutions of immunosorbed anti h-rGH mouse serum IgGs. Detection was performed using commercial Vectastain ABC system (o), antimouse IgG-HRP conjugate (Δ) or ANANAS based systems using the 3 (●), 2 (▲), or 1 (□) step procedures.

3.3. Assemblies As Signal Amplifiers in Diagnostic Application. The dose/response curves of Figure 5 were quantitatively compared for a) the signal intensity generated at each analyte concentration and b) the "signal onset", defined as the maximum dilution at which the registered positive signal was at least double than its standard deviation (N = 8). The results are summarized in Table 1. The signal developed introducing ANANAS nanoparticles in the detection protocol was always considerably greater than that produced by the two commercial reagents. This result is of particular interest considering that the ABC system is already a signal enhancer (thanks to its supramolecular property) and further confirms that the avidin of the ANANAS works in an assembled format. The best analytical performance was achieved with the three-step method which showed two- and almost three-orders of magnitude sensitivity increase with respect to the ABC system and the IgG-HRP conjugate, respectively (calculated from the ratio of the two onset values). Detection using the two types of prefunctionalyzed ANANAS was less sensitive than that of the three-step protocol. This may be related to steric reasons that dictate the total number of effective b-HRP molecules that can be bound to a single ANANAS core. It should be noted that the effect of steric constrains is more relevant at low analyte titer, namely when the average spatial distance between individual

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Table 1. Signal Onset of ELISA Assays Performed Using Different Detection Systems and Detection Sensitivity with Respect to ABC and HRP-Antibody Conjugate Commercial Systems

detection system	signal onset (serum dil. factor)	sensivity enhancement vs ABC	sensivity enhancement vs HRP-conjugate
ANANAS 3 step	8.9×10^9	242.7	726.0
ANANAS 2 step	3.0×10^9	80.9	242.0
ANANAS 1 step	1.09×10^8	3.0	9.0
Antimouse ABC	3.65×10^7	**1.0**	3.0
Antibody-HRP conjugate	1.22×10^7	0.3	**1.0**

surface immunosorbed analytes is larger than the foot-print of the detection system. In this situation, only a small fraction of the ANANAS particle surface is occupied by the analyte, while the free one remains available for b-HRP. While in the three-step procedure all of the remaining space is theoretically available, in the preassembled complexes of the one- and two-step procedures, a defined surface area of the ANANAS has already been taken by default by the 10 molecules of b-αM-IgG added in solution.

The one- and two-step detection systems displayed similar dose/response curves. However, the true analytical performance - as indicated by the maximum analyte serum dilution giving rise to a signal that is twice its standard deviation - was better for the two-step procedure (Table 1). In fact, the one-step signal was characterized by larger standard deviation (especially at low analye concentrations), thus increasing the onset value.

3.4. Stability of the Functional Assemblies in Solution. The lower analytical performance displayed by the monofunctional nanoparticles (Table 1) is mostly due to the higher standard deviation in the signal generated at low analyte concentration. This could be due to some sort of instability of this kind of assembly. In order to evaulate this hypothesis, a detailed investigation over the preassembled nanoparticles stability in solution was carried out. Solutions containing b-αM-IgG/NP (molar ratio = 10:1) and b-αM-IgG/NP/b-HRP (molar ratio = 10:1:200) assemblies were prepared and tested (in the two- and one- step protocols, respectively) the same day of preparation (day 0) or after 3 and 7 days of storage at 4 °C. Figure 6 shows the dose/response curves obtained at each time point. The 450 nm reading of the no-analyte wells (indents in Figure 6) was also monitored. This value comprises the plate background absorbance (about 0.05 units) and the noise generated by the detecting reagent. High readings in these wells indicate either nonspecific interaction between the detection system and the plate coating and/or, in the case of a nanoparticle based detection system, colloidal instability of the same, due to partial or total aggregation and precipitation. The dose/response profiles (subtracted from the no-analyte well reading) generated by the mono- and bifunctionalyzed nanoparticles remained similar after storage. The curves generated by the 2-step detection at day 0, 3, and 7 are almost superimposable, whereas higher variability for the bifunctional assembly can be observed. The difference between two assemblies is more evident from the no-analyte well readings

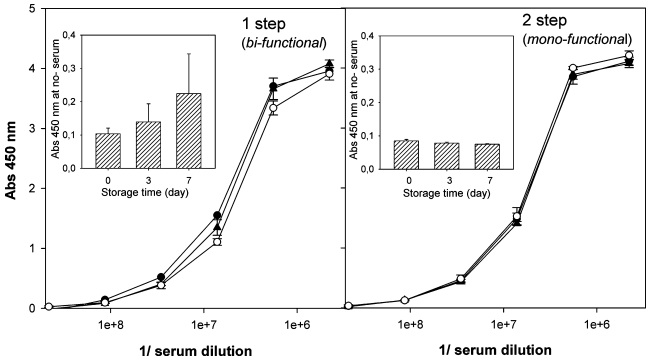

Figure 6. Stability of preassemblies in solution. ELISA detection dose/response curves generated by mono- and bifunctional assemblies used the same day of their preparation (o) or after 3 (▲) and 7 (●) days of storage at 4 °C. Each curve is the result of a test run in duplicate.

(Figure 6 **indents**). The two-step detection displayed low noise over the entire week of experimentation, confirming the stability of the assembly here used. On the contrary, the one-step protocol gave low noise only if used the same day of the preparation, this value increasing upon storage. This result confirms the different colloidal stability of the two assemblies and explains the results of the analytical performance. The more unstable bifunctional assembly generates higher noise, with negative consequences on the detection onset.

The difference among the two functional NPs is probably due to a variation in their surface properties. In fact it is known that colloidal stability depends on several parameters, among which particle surface charge and solvent/buffer composition.[16] Indeed, the dilution buffer (DB) used here had been optimized to maximize the core particle stability.[17] The presence of 10 antibodies in the monofunctional assembly probably does not alter the particle surface properties extensively, as opposed to the full surface coverage reached in the bifunctional system. It is therefore possible that stability of the bifunctional assembly may be improved by optimizing the diluent buffer composition according to the theory of colloidal system stability.[16] The above results also suggest that the differences observed among the mono- and bifunctional assemblies cannot be generalized to any other functionalyzed ANANAS, whose surface properties will depend on both the type of biotinylated entity added and the degree of surface coverage.

3.5. Robustness of the Assembly Process. In order to assess the robustness of the assembly preparation method, we also evaluated the ELISA within-run assay precision (CV%) according to standard analytical validation methods. This parameter, which is commonly used to estimate the reproducibility of an analytical method,[18] was here measured to estimate the variability in the performance of the multifunctional assemblies; in other words, to verify whether the assembly procedure systematically leads to functional nanoparticles with reproducible, therefore predictable, properties. This is an important characteristic in view of any application of functionalyzed particles. The calculated CV% was below or equal to 10% (Supporting Information), demonstrating good reproducibility in the assembly process.

4. CONCLUSIONS

The results indicate that it is feasible and easy to transform a core ANANAS particle into a bioactive multifunctional system with tunable and reproducible properties. The loading

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experiments showed that the maximum number of bioactive moieties that can be loaded onto a single particle is limited by the available particle surface area and not by the total BBS. As a consequence, the particle loading ability is inversely related to the biotinylated moiety molecular weight/size.

From the analytical point of view, the results showed that the optimized functional system is a valid alternative to current supra-molecular[12,19,20] amplification tools in immuno-diagnostics. Two- to three-order sensitivity enhancement was observed with respect to common detection reagents currently on the market. In practical terms, this means that one can use less than 1/100th of biological material for the same analysis and achieve the same results. In this context, the multiple ligand capacity of the particle provides further added value to the performance enhancement. In fact, it is known that multivalent structures possess higher affinity for their target than free ligands alone,[15] and this may be relevant if one were to use biorecognition elements with affinity constant for their target lower than those displayed by full antibody molecules (e.g., peptides, single chain antibodies of antibody fragments).

From a more general point of view, extension of these results can be envisioned to encompass applications different from the analytical one here described. In principle, any kind of biotinylated element can be assembled onto the particle so that the composition of the final assembly can be tuned toward different biological and biotechnological applications. In this respect, the biodegradability and biocompatibility of all nanoparticle components permit envisioning applications that require contact with living cells, both *in vitro* and *in vivo*.[1,21−23] For example, the particles could be loaded with antigens, cytokines, growth factors, cell targeting or internalizing moieties, etc., and administered to cells to induce specific biological responses. Many of the above biomolecules interact with cells in a nonmonomodal dose response relationship, so that the possibility to tune the assembly composition represents an additional advantage.

Finally, it has to be pointed out that even if, in principle, functional assemblies with multiple properties can be easily obtained using this tool, the stability results also indicate that each assembly needs to be regarded individually, since any variation of the core surface may alter the system colloidal stability. Therefore, colloidal stability issues should to be taken into consideration every time a novel functionalyzed system is formulated, especially if obtained upon extensive core surface coverage.

■ ASSOCIATED CONTENT

Supporting Information

The preparation of biotinylated derivatives and ANANAS; how particle loading was calculated from the experimental data; **Table S-1** reports the calculated loadings and dynamic light scattering measurements of all b-protein:NP mixtures; details on analytical CV% calculations: **Table S-2** reports full CV% information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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