SUPPLEMENTARY INFORMATION

Characterization of multi-functional nanosystems based on the avidin-nucleic acid interaction as signal enhancers in immuno-detection

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1. Biotinylation of antibody and peroxidase

Goat α-M-IgG and HRP were biotinylated using a biotin-*N*-hydroxysuccinimide derivative according to standard bioconjugation protocols[1]. Unbound biotin was removed by ultrafiltration (Millipore PVDF membranes, cut-off 30KDa) or gel permeation chromatography eluting the sample in PBS buffer using a Superose 6™ column (GE Healthcare) on a FPLC system. The mean number of biotin:protein in the products was 3.6 and 1.0 for antibody and HRP, respectively, as determined by the hydroxyphenylazobenzoic acid (HABA) assay[2].

2. Synthesis and characterization of core nanoparticles

Nanoparticles were synthesized with the support of ANANAS Nanotech (Padova, Italy). Briefly, assemblies were generated by mixing avidin, plasmid DNA and b-PEO (5KDa) in water at optimized molar ratios on the basis of Pignatto[3]. After stabilization, they were purified from excess avidin by ultrafiltration (Millipore PVDF membranes, cut-off 100KDa)[4] or gel permeation chromatography eluting the sample in 10mM phosphate, 150 mM NaCl, pH 7.4 buffer (PBS) using a Sepharose 6-FF column on a FPLC system (AKTA purifier, GE Healthcare) (3ml/min, detection 220 nm). The resulting nanoparticles are about 115 nm in size (intensity weighted dynamic light scattering) and contain about 340 avidins each and a theoretical number of biotin biding sites = 1020. The eluted particles (100-150 μg/ml in avidin) were stored in DB buffer at 4°C or at –20°C upon dilution with glycerol (50:50) until further use.

3. Calculation of the ANANAS loading capability for biotinylated proteins

ANANAS and biotinylated α-M-IgG (b-αM-IgG) or biotinylated HRP (b-HRP) were mixed in PBS at room temperature at increasing molar ratios, as described as described in the methods section and were analyzed by gel permeation chromatography. The area under the curve (220 nm) of the peak corresponding to the particles (18.5 ml) was plotted against the b-protein:NP molar ratio in solution. The generated curves (figure 2 in the paper) showed a linear behavior at low b-protein:NP molar ratios, reaching a plateau at higher values. The amount of bound protein in the functional assemblies was calculated by averaging the 18.5 ml peak AUC values of the samples prepared at the highest protein:NP ratios (i.e. b-αM-IgG:NP = 120, 160 and 200 and b-HRP:NP = 250 and 300- corresponding to the *plateau* zone of each curve) and comparing it to that of the sub-saturated samples in which quantitative binding had occurred. More precisely, the AUC values obtained at lower protein:NP ratios (up to b-αM-IgG:NP ≤ 40:1 and b-HRP:NP ≤ 120) were fitted linearly *vs* the protein:NP value. The AUC value of the peak eluting at 18.5 ml of each mixture was then inputted in the generated linear equation to obtain the corresponding "calculated protein load" (**Table S-1**). The maximal loading for each of the two biotinylated proteins was obtained by averaging the "calculated protein load" of the samples at *plateu (*b-HRP:NP = 120, 160, 200 and b-αM-IgG:NP = 250 and 300).

In order to confirm the colloidal stability of the assembly after loading and chromatography, and to measure the influence of increasing amounts of biotinylated proteins on its size, the peak eluting at 18.5 ml was collected and analyzed by dynamic light scattering (DLS). The intensity-weighted mean nanoparticle size of all b-αM-IgG/NP samples is reported in **table S-1**.

Table S-1. **Table Title.** Molar ratio between b-αM-IgG-Ab or b-HRP and ANANAS in solution and resulting assembly composition and size. The size, measured by dynamic light scattering, is expressed as the mean diameter calculated on scattering light intensity +/- the Gaussian dispersion value (not stdv), namely the amplitude of the Gaussian population.

protein:NP in solution (mol:mol)	Calculated b-αMIgG:NP molar ratio in the assembly	b-αMIgG/NP assembly diameter (nm)	Calculated b-HRP:NP molar ratio in the assembly (*)
0	0.00	116.3 ± 39.8	0
5	5.6	118.7 ± 37.1	nd
10	10.7	134.7 ± 49.2	nd
20	18.4	127.6 ± 44.8	25
30	29.2	128.5 ± 34.8	nd
40	37.1	130.9 ± 40.6	87.6
80	50.8	129.3 ± 38.0	nd
120	64.1	141.4 ± 54.9	nd
160	55.4	226.3 ± 138.0	nd
200	63.2	199.1 ± 94.9	166.2
250	nd	nd	203.2
300	nd	nd	189.5

(*) The size of the b-HRP/NP samples could not be measured due to the HRP interference at 403 nm with the DLS instrument laser.

4. Robustness of the assembly process (assessment of detection CV%)

In order to calculate the analytical CV% of the one-, two- and three-steps detection methods, the same mouse antiserum was analyzed in different days, each time using freshly prepared b-αM-IgG/NP (two-step) or b-αM-IgG/NP/b-HRP (one-step) assembly solutions, or the individual reagents in the three-step procedure. CV% (N =10) was determined from the mouse serum dilution that gave 25%, 50% or 75% of the maximum signal. In general, a CV% value equal or below 10% is considered acceptable, the smaller the value, the more reproducible the system is considered. All

of the ANANAS based analytical procedures lead to acceptable CV values (**Table S-2**).

Table S-2. Table Title. Within-assay precision (N=10) values obtained by using the one-, two- or three-step detection methods. The CV% calculation was based on the anti-serum dilution factor that gave rise to 25%, 50% and 75% of the maximum signal intensity. The overall CV% was obtained averaging the above three values.

1-step			
% of signal maximum	Mean of dilution factor	Mean StDv	CV%
75%	9.5E-07	4.5E-08	4.7
50%	4.8E-07	3.8E-08	7.9
25%	2.6E-07	2.9E-09	1.1
All signal % (overall CV%)			4.59+/- 3.4
2-step			
% of signal maximum	Mean of dilution factor	Mean StDv	CV%
75%	6.0E-07	4.3E-08	7.0
50%	3.4E-07	2.8E-08	8.4
25%	1.6E-07	2.5E-08	16.0
All signal % (overall CV%)			10.48+/- 4.8
3-step			
% of signal maximum	Mean of dilution factor	Mean StDv	CV%
75%	1.4E-07	7.6E-09	5.4
50%	8.3E-08	1.2E-08	14.5
25%	3.4E-08	3.6E-09	10.8
All signal % (overall CV%)			10.22+/- 4.6

References

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