Optimized Avidin Nucleic Acid Nanoassemblies by a Tailored PEGylation Strategy and Their Application as Molecular Amplifiers in Detection

Mauro Pignatto, Nicola Realdon, and Margherita Morpurgo*

Department of Pharmaceutical Sciences, University of Padova, Via Marzolo, 5, 35131 Padova, Italy. Received January 22, 2010; Revised Manuscript Received April 15, 2010

Avidin was recently found to display the ability to interact with high affinity with nucleic acids. In this work, we investigated how this property is affected by the protein modification with poly(ethylene glycol) (PEG). More precisely, we studied the influence of the size and geometry of the polymer and of the mode of anchorage to the protein surface. To this end, we synthesized five PEG derivatives capable of PEGylating avidin either through covalent attachment to its lysine primary amines or by exploiting its biotin binding pockets. Several differently PEGylated avidin derivatives were then obtained, which were later tested for their affinity for plasmid DNA by means of the electrophoretic mobility assay. The results show that covalent PEGylation reduces the affinity for DNA in a dose-dependent manner, whereas PEG anchoring through the biotin binding sites does not, even when bulky and high MW biotin-PEG derivatives are used. We then investigated how the size and molecular weight of the biotin-PEG affects the solubility and stability of avidin-nucleic acid nanoassemblies in physiological buffer. Among the biotin-PEG derivatives synthesized in this work, the branched forms were more efficient in protecting particle surface and preventing their aggregation. Full nanoparticle solubility was achieved by saturating 30% of the biotin binding sites with a 2 × 5 kDa branched derivative. the optimized avidin nucleic acid nanoassemblies (ANANAS) were employed in a model analytical test where they showed at least 40-fold higher efficiency than monomeric avidin in recognizing biotinylated surface immobilized IgGs. The results pave the way toward the application of this novel nanosystem in biomedicine.

INTRODUCTION

Avidin is a tetrameric glycoprotein known mainly for its ability to bind to four molecules of biotin with very high affinity ($K_d \approx 10^{-15}$ M), a property that forms the basis for its use as a molecular tool in a large number of biotechnological applications (the avidin−biotin technology) (*1*−*5*). The most common applications are in research and diagnostics, in the functionalization of surfaces, and in drug delivery for the targeting of drugs or diagnostic elements to defined locations in the body after parenteral administration (*6*−*10*). However, the classical avidin−biotin technology is somehow limited by the maximum number (four) of biotins that can be brought together by the individual avidin molecule which forms the central nucleus of the system. Having a core unit with greater biotin loading capacity can improve the potential of this technology, and this can be obtained by joining together several avidin molecules into a single unit (a "poly-avidin unit"). To this end, several approaches for obtaining polyavidins are described in the literature. The strategies most commonly adopted are based on the coating of micro- or nanospheres (polymer or metal-based) with several avidin molecules by exploiting nonspecific adsorption processes. Alternatively, several avidin units can be united either by chemical cross-linking or by using polybiotinylated spacers as bridging functions (*11*−*13*). All of these methods share the common drawbacks of leading to polydisperse systems with some degree of avidin inactivation. In practical terms, avidin inactivation is translated into a diminished total biotin binding capacity, whereas polydispersivity is translated into poly avidin assemblies with statistically rather than precisely defined properties. In addition, in most cases the components used for the assembly (namely, the chemical linkers or the polymeric particle cores) are not of natural origin or are potentially toxic, and this limits their use in in vivo biomedical applications.

Another possibility to obtain polyavidin particles is by taking advantage of an additional property of avidin that has been recently brought to light, namely, its ability to bind the nucleic acids with high affinity (*14*). In fact, secondary to this interaction, avidin self-assembles onto the DNA molecule in an organized manner, giving rise to stable agglomerates where the nucleic acid is coated by avidin molecules in a precisely defined stoichiometric ratio. This binding is stable at high dilutions and in physiological buffers and apparently does not directly involve the biotin binding site, since the affinity for DNA is higher in the presence of biotin (*14*).

However, the practical exploitation of these assemblies depends on the possibility to obtain them in the form of reproducible, solution stable and poorly polydispersed particles. In fact, from the macroscopic point of view, the avidin−nucleic acid assemblies assume various shapes and geometries depending on the conditions in which they are obtained (*14*). For example, by mixing avidin and nucleic acids in a buffered aqueous environment, the assembly process gives rise to agglomerates of large size (>1 μm), which are poorly soluble, highly polydispersed, and of undefined geometry, and therefore unusable from the practical viewpoint. Conversely, it was shown that, in a salt-free environment and under specific conditions of concentration and ratio of nucleic acids to protein, nanoparticulate structures of toroid or rod shape are obtained, in which a single nucleic acid molecule is surrounded by several avidin molecules. In this case, the nanoassemblies are poorly polydispersed and their size depends solely on the type and length of the nucleic acid used (*14*). However, when these nanoparticles are transferred into a buffered solution, they undergo a rapid process of aggregation subsequent to which polydispersed macro-aggregates are again obtained, unusable for practical purposes. Therefore, in order to exploit the avidin nucleic acid

* E-mail: margherita.morpurgo@unipd.it, Phone: (+39)-049-827-5339; Fax: (+39)-049-827-5366.

10.1021/bc100044u
Published on Web 06/08/2010

nanoassemblies (ANANAS) for practical application, it is necessary to find a way to prevent their aggregation in physiological buffers. One possibility is to protect their surface with the use of hydrophilic polymers, for example, poly(ethylene glycol) (PEG). The modification of bioactive elements with PEG is a well-known technology used since the 1970s (*15, 16*) to biocompatilize protein drugs and protect them from undesired nonspecific interactions. The same strategy is also used to modify the surface of nanoparticles to improve their solution properties and/or their pharmacokinetic and immunogenic profiles (*17*−*19*), as is the case of the commercial doxorubicin liposome formulation DOXIL (or Caelyx) or of the fluorescent quantum dots (*20*). However, it is known that the type and amount of PEG linked to the surface is a critical point. For example, in the case of proteins, an excessive amount can impair the biological activity; on the other hand, if the amount is too low, it may not be sufficient to improve the pharmacokinetic or pharmacodynamic properties of either proteins or nanoparticles or to prevent them from nonspecific interactions. Therefore, the amount of PEG, the size and shape of the polymer, as well as the chemistry of conjugation are all important variables that need to be optimized (*21*−*23*).

In the case of avidin, two strategies can be adopted to link the PEG chains. On one hand, the polymer can be linked to the protein by classic covalent coupling. On the other hand, it can be attached to the protein by exploiting its biotin binding sites. In any case, the size, geometry, and amount of polymer must be optimized not to affect the twofold protein biological activity, namely, its ability to bind with high affinity both biotin and DNA. In this work, we first investigated the effect of PEG grafting on the ability of avidin to bind the nucleic acids. More precisely, we tested the influence of the amount of PEG and of the mode of grafting to the protein surface, covalent versus the biotin bridging. We then studied the influence of the size, the geometry, and the amount of PEG added to the surface of the nanoparticles on their aggregation in physiological buffer. The results clearly show the importance of identifying the best strategy of surface modification to achieve optimal results. The applicability of the optimized surface-protected nanoparticles in a model analytical assay demonstrate for the first time the potentialities of the avidin−nucleic acid nanoassemblies as novel tools in biomedicine.

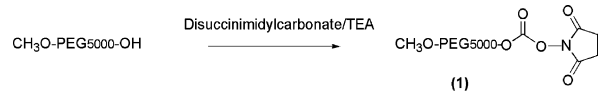
EXPERIMENTAL PROCEDURES

Materials and Instrumentation. Glycosylated chicken egg avidin was obtained from Belovo Chemicals, Belgium. Plasmid pEGFP-C1, 4.7 kb, was from Clontech. Water was Milli-Q or double-distilled (dd-H_2O) grade.

O-Methyl-poly(ethylene glycol) (2 kDa and 5 kDa, methyl-PEG2000-OH and methyl-PEG5000-OH), *O*-(2-aminoethyl)-*O*′-methyl-poly(ethylene glycol) (5 kDa, methyl-PEG5000-NH_2), α-biotin-ω-carboxy-succinimidyl ester poly(ethylene glycol) (5 kDa, biotin-PEG5000-CO-OSu, 5 kDa) was purchased from IRIS Biotech Gmbh (Marktredwitz, Germany), biotinamidocaproate *N*-hydroxysuccinimidyl ester and *N*,*N*′-di-Boc-L-lysine hydroxysuccinimidyl ester, horseradish peroxidase (Tpe VI), and all other reagents were purchased from Sigma-Aldrich (St. Louis, MI, USA); normal goat IgG was purchased from KPL (Gaithersburg, MA, USA); biotin-AlexaFluor^{546} was from Molecular probes (Invitrogen Corporation, USA). All other chemicals were from Sigma-Aldrich.

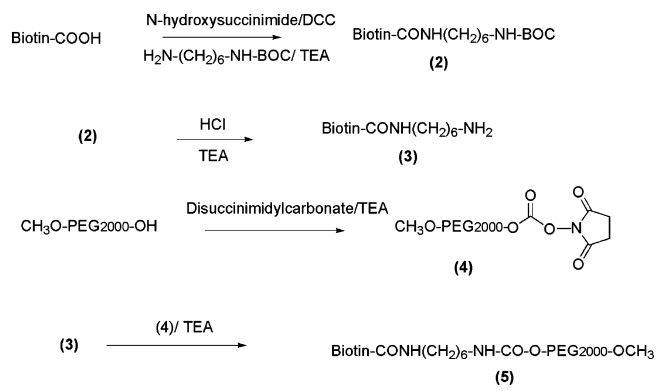
NMR analysis was carried out using a Bruker AMX 300 MHz. Agarose gel electrophoresis analysis was performed using the Biorad GEL DOC XR trans-illuminator; UV spectra were recorded on a Varian cary 50 UV−vis spectrophotometer; fluorescence was determined using a JASCO FP-6200 spectrofluorimeter. Dynamic light scattering measurements were performed using an in-house assembled system as further described in the Experimental Procedures. Biotinyl derivatives were detected by spraying the TLC plate with an acidic dimethylamino cinnamaldehyde solution (*24*); NH-containing compounds were detected using ninhydrin (*25, 26*). Primary amines in solution were detected and quantified by the trinitrobenzenesulfonic acid assay (TNBSA) according to Habeeb (*27, 28*). Biotin in solution was quantified by the 2-(4′-hydroxyazobenzene) benzoic acid (HABA) assay (*29*). PEG in solution was detected and quantified by the iodine method (*28*).

Scheme 1



(1)

Scheme 2



Synthesis of PEG Derivatives. *O-(N-Hydrosuccinimidyl-carbonate)-O′-methyl-poly(ethylene glycol)5000 (**1**, Scheme 1).* A similar procedure as the one described by Miron et al. (*30*) was followed. *O*-Methyl-poly(ethylene glycol)5000 (5 g, 1 mmol) was dried by azeotropic distillation in toluene and then redissolved in an anhydrous mixture of chloroform/toluene (1:3) (40 mL) containing disuccinimidylcarbonate (DSC) (1.02 g; 4 equiv) and triethylamine (TEA) (556 μL; 4 equiv). The reaction was stirred for 12 h at room temperature; the product was precipitated in anhydrous diethyl ether, recovered by filtration, and dried in vacuo. It was then purified by hot−cold crystallization in absolute ethanol, filtered, rinsed with ether, and dried in vacuo.

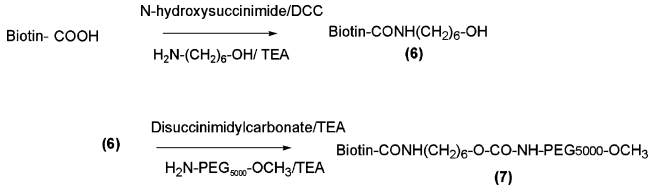
^{1}H NMR (d_6-DMSO, 300 MHz) δ 4.46 and 3.38 ppm (t, 2H, -O-CO-OC$\underline{H}_2$-CH), δ 3.38 ppm (s, 3H, $-CH_2CH_2-O-C\underline{H}_3$-). The degree of conversion was 95%; yield 92%.

*N-BOC-biotinamidohexylamine (**2**, Scheme 2).* Biotin (0.5 g; 2 mmol) was dissolved in dry dimethylformamide (DMF, 12 mL); then, *N*-hydroxysuccinimide (259 mg; 1.1 equiv) and dicyclohexylcarbodiimide (DCCI, 464 mg; 1.1 equiv) were added. The reaction was carried out at room temperature and was monitored by TLC (chloroform/methanol 80/20). After 12 h, the dicyclohexyl urea formed was removed by filtration and *N*-BOC-1,6-diaminohexane (517 mg; 1 equiv) and TEA (286 μL; 1 equiv) were added. After 2 h at room temperature, the product (*N*-BOC-biotinamidohexylamine, compound **2**) was precipitated by adding cold water, it was isolated by filtration and rinsed with cold HCl 0.1 N and anhydrous diethyl ether, dried in vacuo, and the purity assessed by ^{1}H NMR. The degree of conversion was 100%; yield 90%.

^{1}H NMR (d_6-DMSO, 300 MHz) δ 4.29 and 413 ppm (m, 2H, NH-C$\underline{H}$R-C$\underline{H}$R-NH in biotin ring), δ 1.37 ppm (s, 9H, C(C$\underline{H}_3$)$_3$).

Biotinamidohexylamine (3, Scheme 2). Compound **2** was dissolved in methanol saturated with HCl. After 15 min, the solvent was evaporated and the product was redissolved in the

Scheme 3



minimum amount of chloroform, and TEA was added to obtain biotinamidohexylamine as the free base. The insoluble product was isolated by filtration; it was rinsed with chloroform and cold diethyl ether and dried in vacuo. The degree of conversion was 100%; yield 52%.

^{1}H NMR (d_6-DMSO, 300 MHz) of **2**: δ 4.29 and 4.12 ppm (m, 2H, -NH-CHR-CHR-NH in biotin ring), δ 3.03 ppm (m, 2H, -CO-NH-CH$_2$-).

*O-(N-Hydrosuccinimidylcarbonate)-O′-methylpoly(ethylene glycol)2000 (**4**, Scheme 2).* This compound was synthesized through the same protocol as compound **1** (*30*) using methyl-PEG2000 instead of methyl-PEG5000.

^{1}H NMR (d_6-DMSO, 300 MHz) δ 4.46 and 3.38 ppm (t, 2H, -O-CO-OCH$_2$-CH), δ 3.38 ppm (s, 3H -CH$_2$CH$_2$-O-CH$_3$-). The degree of conversion was100%; yield 83%.

*O-Biotinamidohexylamate,O′-methyl-PEG2000 (methyl-PEG2000-biotin, **5**, Scheme 2).* Compound **3** (10 mg; 0.03 mmol) was dissolved in anhydrous dimethylsulfoxyde (DMSO, 1.5 mL), and compound **4** (200 mg; 3 equiv) and TEA (4.2 μL; 1 equiv) were added. The reaction was stirred for 2 h at room temperature, and the product was then precipitated in diethyl ether, filtered, and dried under vacuum. After hot−cold crystallization in absolute ethanol, the product was rinsed with diethyl ether and dried in vacuo. It was analyzed by ^{1}H NMR; the absence of unreacted biotinamidohexylamine was also confirmed by TNBSA assay. Biotin titer in the final product was measured by the HABA assay.

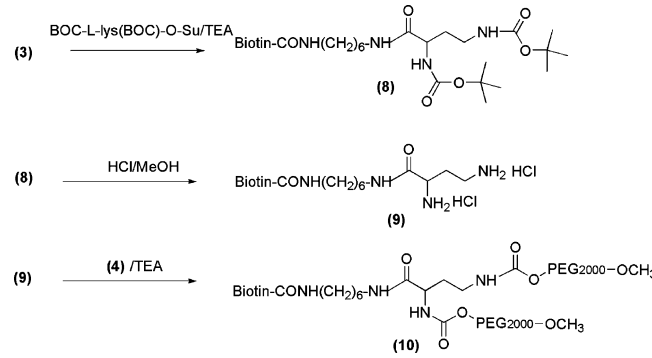
^{1}H NMR (*d*-chloroform, 300 MHz) δ 4.51 and 4.32 ppm (m, 2H, -NH-CHR-CHR-NH in biotin ring); δ 4.04 ppm (m, 2H, -CO-O-NH-CH$_2$-CH$_2$-O-), δ 3.64 ppm (wide s, about 180 -O-CH$_2$-CH$_2$-O-, PEG chain). The degree of amine conversion was 100%; yield 90%.

Biotinamidohexanol (6, Scheme 3). This product was synthesized through a similar procedure as the one followed to obtain compound **2**. 6-Aminohexanol was used instead of *N*-BOC-1,6-diaminohexane. It was isolated upon filtration after precipitation with cold, diluted HCl. It was then rinsed with diethyl ether and dried in vacuo.

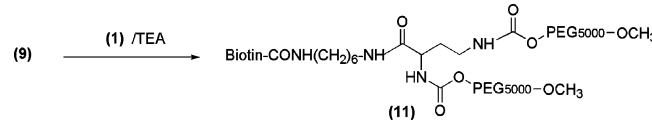
^{1}H NMR (d_6-DMSO, 300 MHz) δ 7.71 ppm (t, 1H, -CH$_2$-OH), δ 4.31 and 4.12 ppm (m, 2H, -NH-CHR-CHR-NH in biotin ring). The degree of biotin conversion was 100%; yield 56%.

*O-Biotinamidohexanolamate,O′-methyl-poly(ethylene glycol)5000 (methyl-PEG5000-biotin) (**7**, Scheme 3).* Compound **6** (150 mg; 0.43 mmol) was dissolved in anhydrous DMF (5 mL) and DSC (243 mg; 2.2 equiv) and TEA (150 μL; 2.5 equiv) were then added. After stirring overnight at room temperature, the solution was mixed with *O*-(2-aminoethyl)-*O′*-methyl-poly(ethylene glycol) (5 kDa, 500 mg; 0.23 equiv) previously dehydrated by azeotropic distillation in toluene. The reaction was monitored by TLC (chloroform/methanol 80/20) until the polymer amino groups were no longer detectable by ninhydrin assay. The product was extracted in chloroform, washed with diluted HCl, then with diluted Na_2CO_3, and, finally, with water. The organic phase was dried over $MgSO_4$, concentrated by rotavapor, and the product precipitated in anhydrous cold diethyl ether, filtered, and dried in vacuo. It was analyzed by ^{1}H NMR; its biotin titer was also confirmed by HABA assay.

Scheme 4



Scheme 5



^{1}H NMR (*d*-chloroform, 300 MHz) Biotin-mPEG5000 δ 4.53 and 4.35 ppm (m, 2H, -NH-CHR-CHR-NH in biotin ring), δ 4.03 ppm (m, 2H, -CO-O-NH-CH$_2$-CH$_2$-O-), δ 3.63 ppm wide s, about 450 -O-CH$_2$-CH$_2$-O-, PEG chain). The degree of amine modification was 100%, yield 82%.

*Biotinamidohexylamido-L-lys·(HCl)$_2$ (**9**, Scheme 4).* Compound **3** (203 mg, 0.594 mmol) was dissolved in of 4 mL of dry DMF. This solution was added of 290 mg of *N,N′*-di-Boc-L-lysine hydroxysuccinimide ester (0.653 mmol, 1.1 equiv), previously dissolved in 3 mL of dry DMF, followed by 82.6 μL of TEA (0.594 mmol, 1 equiv). The reaction was stirred for one hour at room temperature. The product (**8**) was precipitated by the addition of cold, dry diethyl ether, then washed with cold aqueous diluted Na_2CO_3 in H_2O, the diluted citric acid in H_2O, and finally water. It was then redissolved in HCl-saturated chloroform to remove the BOC group. The reaction was monitored by TLC, and after 30 min, biotin-L-lys·(HCl)$_2$ (**9**) was precipitated by the addition of diethyl ether; it was then isolated by filtration and dried in vacuo.

^{1}H NMR (*d*-chloroform, 300 MHz) δ 4.30 and 4.14 ppm (m, 2H, -NH-CHR-CHR-NH in biotin ring), δ 3.74 ppm (m, 1H, CO-CHR-NH$_2$·HC in lysine). The degree of biotinamidohexylamine conversion was 100%; yield 47%.

*Biotinamidohexylamido-L-lys·(methyl-PEG2000)$_2$ (biotin-L-lys-(methyl-PEG2000)$_2$, **10**, Scheme 4).* Compound **9** (8.2 mg, 0.015 mmol) was dissolved in anhydrous dimethylsulfoxide (DMSO) (1.5 mL). Compound **4** was added in several aliquots, each time followed by the same number of equivalents of TEA. One hour after each addition, a small portion of the solution was tested for primary amine content by TNBSA assay. This was repeated until no more free amines could be detected. Due to the large excess of compound **4** used, the final mixture was composed of the desired product together with the hydrolyzed excess of methyl-PEG2000-OH. The PEG mixture was isolated by diethyl ether precipitation and purified by hot−cold crystallization in absolute ethanol, filtered, and dried in vacuo. The amount of biotin-L-lys-(methyl-PEG2000)$_2$ in the final powder was determined on the basis of its biotin titer using the HABA assay. The absence of unreacted primary amines was verified by TNBSA assay. The degree of amine coupling was 100%, PEG product recovery 80%.

*Biotinamidohexylamido-L-lys·(methyl-PEG5000)$_2$ (**11**, Scheme 5).* Compound **9** (13.2 mg, 0.024 mmol) was dissolved in anhydrous DMSO (5 mL), followed by Compound **1** and TEA in a similar fashion as described above for compound **10**. In

Table 1. Properties of Covalent Methyl-PEG5000−Avidin Conjugates

sample name	PEG/avidin in the reaction (mol/mol)	mean number PEG molecules per protein
avidin-$(mPEG5000)_1$	20	1.2
avidin-$(mPEG5000)_{2.1}$	40	2.1
avidin-$(mPEG5000)_{3.6}$	80	3.6

this case, the final mixture was composed of the desired product together with the hydrolyzed excess of mPEG−OH5000. It was isolated by ether precipitation and purified by hot−cold crystallization in absolute ethanol, filtered, and dried under vacuum. The amount of the biotin-L-lys-$(methyl\text{-}PEG5000)_2$ in the mixture was determined on the basis of its biotin titer using the HABA assay. The absence of unreacted primary amines was verified by TNBSA assay.

The degree of amine coupling was 100%, PEG product recovery 87%.

PEGylation of Avidin. *Covalent PEGylation.* We obtained three avidin−PEG covalent conjugates by mixing the protein (10 mg/mL), dissolved in 0.1 M borate pH 8.0, with different amounts of **1**. The polymer/avidin molar ratio in the reaction mixtures was 20, 40, and 80 (15 mg, 30 mg, and 60 mg of compound **1** every milliliter of avidin solution). Each solution was gently mixed for 2 h at room temperature, and the products were purified by ultrafiltration using a polyethersulfone filter membrane (cutoff 30 kDa). Removal of unbound PEG was verified by testing the eluate by iodine assay. Protein concentration in the final solution was determined by UV absorbance ($E_{0.1\%}$ at 280 nm = 1.54). The mean number of polymer chains bound to the protein was determined by both TNBSA and iodine assays. The properties of the resulting products are summarized in Table 1.

PEGylation via Biotin Bridge. PEGylated avidins at controlled PEG/avidin ratios were obtained using compounds **5**, **7**, **10**, and **11** by exploiting the high affinity of avidin for the biotin moiety. Avidin was dissolved in PBS buffer and the biotin-PEG derivative was then added at the desired biotin/avidin molar ratio. We assumed that all of the biotin residues added to the solution would stably bind to avidin. No purification was necessary.

Other Biotinylated Proteins. *Biotin-PEG-Horseradish Peroxidase.* Horseradish peroxidase type VI (HRP) was dissolved in 0.1 borate buffer pH 8.0 (1 mg/1 mL) and biotin-PEG5000-CO-OSu (1.2 mg) was added (PEG/HRP molar ratio = 10). The solution was gently mixed for 1 h at room temperature. The product was purified by ultrafiltration using a polyethersulfone filter membrane (MWCO 30 kDa). HRP concentration in the final solution was determined by UV absorbance ($E_{0.1\%}$ at 403 nm = 2.5). The mean number of biotin−PEG chains bound to the protein was determined by both HABA and "iodine" assays using standard curves obtained with the corresponding polymer. The PEG/HRP molar ratio in the final product was 1.2:1.

Biotin-Immunoglobulin G (IgG). Normal goat IgG was dissolved in 10 mM phosphate, 150 mM NaCl, pH 7.4 (PBS) buffer at 3 mg/mL and added of 2.7 μL of a DMSO solution of biotinamidocaproate *N*-hydroxysuccinimidyl ester (5 mg/mL, biotin/IgG = 1.5:1). The solution was gently mixed for 1 h at room temperature. The product was purified from low-molecular-weight side products by gel filtration using a Sephadex G25 resin. The degree of biotinylation was determined by means of the HABA assay after trypsin digestion according to standard procedures. The biotin/IgG molar ratio in the final product was 1.3:1.

Interaction of the PEGylated Avidins with Plasmid DNA. The ability of avidin and of its PEGylated derivatives to interact with plasmid DNA was assessed by electrophoretic mobility assay (EMSA) on an agarose gel (*14*). Unmodified avidin (with or without biotin), covalently PEGylated avidin (with or without biotin), and avidin previously saturated with different amounts of selected biotin-PEGs or biotin-PEG-peroxidase were tested. Experiments were carried out by mixing constant amounts of a DNA in PBS to equal volumes of avidin solutions (in PBS) at increasing concentration according to the desired molar (or charge) ratios. The final DNA concentration in all mixtures was 6.25 μg/mL. Twenty-four microliter aliquots of each final solution were then loaded on 0.8% agarose gels and run for about 60 min at 80 mV voltage. DNA in the gel was visualized by fluorescence (ethidium bromide) using a GEL DOC XR trans-illuminator (Biorad), and band fluorescence intensity was quantified with *ImageJ* software. For quantitative analysis, the time of exposure to UV light was controlled in order to prevent signal saturation. The linearity of fluorescence intensity versus DNA concentration was verified by means of calibration curves built in parallel to each experiment (serial dilutions from 6.25 μg/mL to 24 ng/mL dissolved in the same buffer).

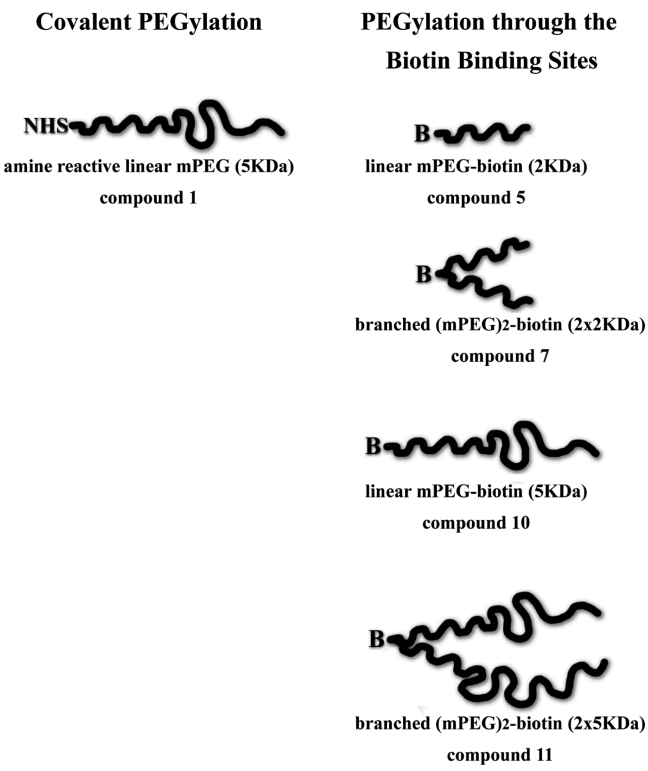
Preparation of Nanoassembled Avidin−Nucleic Acid Complexes. DNA (430 μg/mL) and avidin (20 μg/mL) were dissolved in double-distilled water (dd-H_2O) and cooled in an ice bucket. Equal volumes of each solution were then mixed while vortexing. Vortexing was maintained for 10 s after mixing, and then samples were kept in an ice bucket until further use.

Aggregation Studies. Nanoassembly aggregation was tested by following the increase in time of the average particles size via dynamic light scattering measurements (DLS). These experiments were carried out with core nanoparticles obtained as described above and upon addition of different amounts of each of the biotin-PEG synthesized. DLS measurements were carried out on samples after preparation in dd-H_2O and after the addition of 0.1 vol of 10× PBS buffer. Measurements were repeated every 5−10 min for up to 2 h or until precipitation occurred.

Light scattering measurements were performed using an in-house assembled system. Samples (300 μL) were placed in thin-walled cylindrical borosilicate glass cuvettes (1 cm diameter) and placed in a vat filled with toluene as the index matching fluid. During the course of the measurements, the vat temperature was kept at 25 °C. The light source was a Spectra Physics Stabilite 2017 laser (λ = 632.8 nm), and photons scattered by the sample were collected by a photomultiplier tube mounted on the goniometer arm at 90° to the direction of the incident radiation. The photoelectron count-time autocorrelation function was measured with a Pacific Scientific NICOMP 370 correlator. Applying the Stokes−Einstein equation to the translation diffusion coefficients provides an intensity-weighted distribution of hydrodynamic sizes (*31*).

Optimized Nanoassemblies in a Model Analytical Test. Nanoassemblies were obtained in dd H_2O at charge ratio of ±3 as previously described (calculated on the basis of the number of basic and acid residues on avidin that give rise to a net positive charge of 24 at pH 7.4 (*14*)), using a stock avidin solution where 2% of the total biotin binding sites had been saturated with biotin-AlexaFluor^{546}. Compound **11** was then added in order to cover 30% of the total biotin binding sites. After 1 h, the excess of avidin unbound to the DNA was removed using a cross-flow filtration cartridge (Vivaspin 50, Sartorius Stedim Biotech S.A., Aubagne Cedex, France) with a membrane with 100 kDa cutoff and water as the flowthrough buffer. The amount of avidin in the eluting buffer was quantified through the AlexaFluor^{546} fluorescent signal (λ_{exc} 556; λ_{emiss} 573). The retained solution containing the nanoassembled avidin was analyzed by dynamic light scattering and used in a dot blot assay described as follows.

Scheme 6



Various amount of biotinylated goat IgG (99 ng to 0.02 ng in 0.2 μL) were spotted on a nitrocellulose membranes, which were then soaked 60 min in a blocking solution (10% skimmed milk in PBS). Avidin as a monomer or in the form of the ANANAS assembly was added to the block solution to a final concentration of 4 μg/mL. After 60 min, the membranes were thoroughly washed with PBS and incubated for 60 min with biotin-PEG-HRP dissolved in 10% skim milk/PBS (4 μg/mL). After washing, detection was carried out by adding a 3,3′-diaminobenzidine (DAB)/H_2O_2 solution in PBS (0.03% and 0.015%, respectively). The reaction was stopped after 10 min by washing the membrane twice with dd-H_2O. The intensity of the signal generated on the membrane was quantified after air drying upon recording the membrane image with a digital camera and using the NIH *ImageJ* software.

RESULTS

Synthesis of the PEG Derivatives for Avidin PEGylation. PEGylation of avidin was achieved either through covalent bonding at the protein primary amines or through the addition of biotinylated PEGs. All PEGylating derivatives were synthesized within this work. More precisely (Scheme 6), we obtained one amine reactive derivative of methyl-PEG having 5000 Da MW (**1**) that was used for covalent coupling and four biotin-PEG derivatives (compounds **5**, **7**, **10**, **11**) (Schemes 2−5) with different sizes (from 2000 to 10 000 Da) and geometry (linear or branched) for high-affinity attachment through the biotin binding pockets. In all biotin derivatives, a 6 carbon atom spacer between biotin and the polymer chain was introduced to maximize the interaction with the protein (*32*).

The PEG reagent for covalent modification (**1**) was synthesized according to a protocol already described in the literature (*30*). The same compound was also used as an intermediate for the preparation of two of the four biotin-PEG derivatives. The latter were obtained either (a) by generating primary amine-containing derivatives of biotin (compounds **3** or **9**), which were reacted with an excess of amine-reactive PEG, or (b) by synthesizing an amine-reactive derivative of biotin (DSC derivative of compound **6**) which was reacted with biotin-PEG-amine. All syntheses have been carried out in such a way to guarantee the final product would not contain any biotin derivative of low or intermediate molecular weight. In fact, this was fundamental for later experiments, which required the preparation of avidin/biotin-PEG complexes with polymer/protein molar ratios precisely defined. All product mixtures were therefore characterized for their biotin and primary amine titers using the HABA and TNBSA assays, respectively. The composition of the four product mixtures is shown in Table 2.

In order to achieve full biotin modification through the first synthetic strategy, (compounds **5**, **10**, and **11**), excess of amine-reactive PEGs had to be used. As a consequence, the final reaction products were composed of mixtures of the desired biotinylated-PEG together with the excess of hydrolyzed polymer reagent. Even if this impurity is inert and does not interfere with the avidin−DNA interaction, the exact composition of the product had to be precisely determined.

In the case of the linear derivatives, two distinct synthetic strategies were adopted, which lead to derivatives with an inverted chemistry within the six-carbon spacer between the biotin and PEG moieties. The choice of using the two strategies was made in order to compare them in terms of reaction efficacy and cost. We assumed that small differences in this region of the conjugate would have no influence on the final properties of the PEGylated avidin. As opposed to the first synthetic strategy described, quantitative PEG biotinylation was achieved by reacting the amine-PEG with an excess of amine-reactive biotin. The excess of low-molecular-weight biotin derivative was removed through the washing steps, and the final product (compound **7**) was composed of the sole biotin-PEG. This strategy was indeed more straightforward than the previous one. However, it required the use of methyl-PEG-amine, which is more expensive than the hydroxyl derivative.

Effect of PEGylation on the Affinity of Avidin for Plasmid DNA. The interaction of DNA with any DNA binding agent can be followed by gel retardation assay (EMSA). The assay is based on the rationale that the electrophoresis mobility of a biomolecule in a nondenaturing gel depends on both its charge and molecular weight. If a DNA-binding agent is added to the DNA prior to its loading on the gel, the electrophoresis mobility of DNA will vary depending on the size and charge of the resulting complex. In the assay, the binding agent is mixed with DNA at different molar ratios and loaded in the gel. Qualitative and quantitative information on the interaction can be obtained by measuring the intensity of the band relative to the unbound (free) DNA. Quantitative information may also be obtained from the intensity and position of the band(s) relative to the bound DNA.

In this work, the assay was used to estimate the relative affinity of avidin differently PEGylated either through covalent attachment to its surface lysines or by adding stoichiometrically controlled

Table 2. Biotin Titer, Primary Amine, and Unmodified Methyl-PEG Content in 1 g of Biotin-mPEG Compounds 1, 7, 10, and 11

	PEGylated biotins (moles × 10^{-5})	primary amines (moles ×10^{-5})	unmodified mPEG (moles ×10^{-4})
Methyl-PEG2000-biotin (**5**)	13.10	0	3.50
Methyl-PEG5000-biotin (**7**)	16.50	0	0
Biotin-L-lys-(methyl-PEG2000)$_2$ (**10**)	4.83	0	3.96
Biotin-L-lys-(methyl-PEG5000)$_2$ (**11**)	1.57	0	1.67

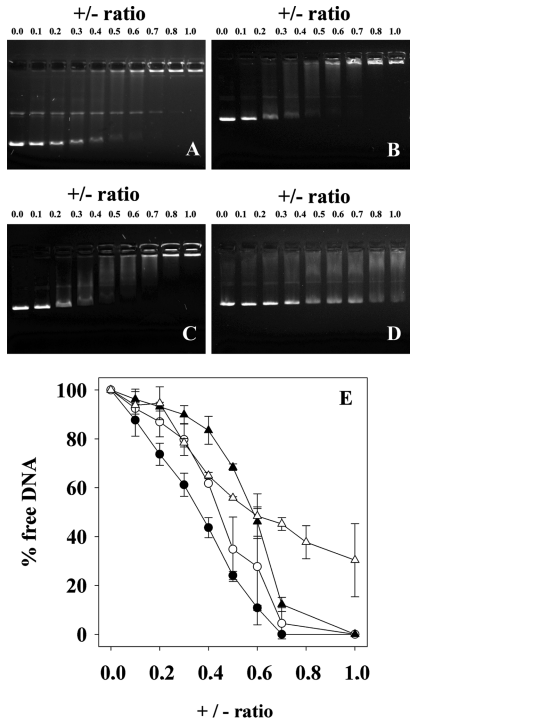


Figure 1. Electrophoretic mobility assay of plasmid DNA upon interaction with avidin covalently modified with increasing amounts of methyl-PEG5000 (see methods): (A) native avidin; (B) methyl-PEG 5000/avidin = 1.2; (C) methyl-PEG 5000/avidin = 2; (D) methyl-PEG 5000/avidin = 3.6; (E) binding curves obtained measuring the intensity of band relative to the free DNA; experiments were carried out in duplicate: (•) native avidin; (°) methyl-PEG 5000/avidin = 1.2; (▲) methyl-PEG 5000/avidin = 2; (Δ) methyl-PEG 5000/avidin = 3.6.

amounts of biotinylated-PEGs. The experiments were performed on agarose gels, and the amount of free DNA in each sample was quantified by measuring the fluorescence intensity of the ethidium bromide-stained bands. Binding curves were derived as a function of the protein/plasmid molar ratio. Since avidin is a basic protein characterized by a large excess of positively charged residues (12 acid vs 17 basic amino acids + 1α-NH_2/subunit), the same data can be plotted by substituting the protein/plasmid molar ratio with the corresponding (±) charge ratio, a parameter that is commonly used when evaluating DNA binding proteins that have a positive charge in physiological buffer.

Figure 1 shows the images of the DNA electrophoretic mobility assay carried out with avidin covalently modified with increasing amounts of methyl-PEG5000. The binding curves obtained from the quantitative analysis of band intensity are also reported. The results indicate that, when PEG is covalently bound to avidin, its affinity for DNA decreases in a dose-dependent manner. At one PEG per avidin, the loss of affinity is of minor intensity; however, at higher polymer loading it becomes dramatic.

In order to evaluate the effect of the mode of PEGylation, avidins with the same total PEG load attached either through covalent binding or via biotin bridge were tested in parallel. Figure 2A and B shows the results of the EMSA carried out with avidin in its native form or PEGylated with 1 or 4 molecules of methyl-PEG5000 attached either covalently (compound **1**) or through the biotin bridge (compound **7**).

PEGylation through biotin bridging has no impact on the affinity for DNA that remains totally preserved. Since it is known that the affinity of avidin for plasmid DNA increases upon addition of biotin (*14*), the covalently PEGylated avidins were also tested upon addition of free biotin at equimolar amounts as the PEG molecules covalently bound. The loss in affinity caused by covalent attachment is only partially reversed by the presence of biotin.

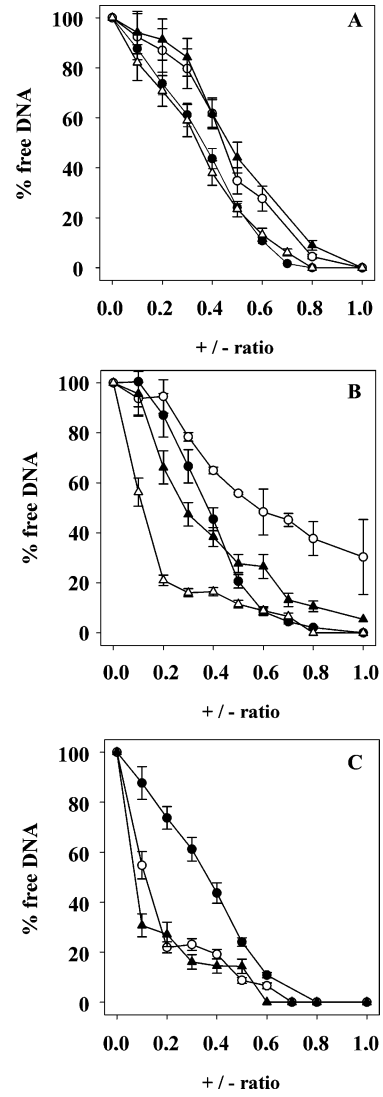


Figure 2. Electrophoretic mobility assay of plasmid DNA upon interaction with avidin differently PEGylated through the covalent or the biotin-bridge strategy. Binding curves obtained by measuring the intensity of band relative to the free DNA. (A) Low degree of PEGylation using linear 5000 MW polymers (•) native avidin; (○) covalent PEGylation with 1.2 methyl-PEG5000; (▲) covalent PEGylation with 1.2 methyl-PEG5000 + 1 biotin; (Δ) PEGylation with 1 linear biotin-PEG5000 (compound **7**). (B) High degree of PEGylation using linear 5000 MW polymers: (•) native avidin + 4 free biotins; (○) covalent PEGylation with 3.6 methyl-PEG5000; (▲) PEGylation with 3.6 methyl-PEG5000 + 4 free biotins; (Δ) PEGylation with 4 biotin-PEG5000 (compound **7**). (C) Very high degree of PEGylation using bulky biotin-PEG derivatives: (•) native avidin + 4 free biotins; (○) PEGylation with 3 molecules of biotin-PEG5000-HRP; (▲) PEGylation with 4 molecules of compound **11**. All experiments were carried out in duplicate.

Figure 2C shows the results of the EMSA carried out using avidin PEGylated to a large extent with highly bulky biotin derivatives, namely, compound **11** and biotinylated-PEG-HRP. Interestingly, in this case the affinity for DNA seems higher than that of native avidin.

Effect of the Amount of PEG on the Size of the Nanossemblies and on Their Aggregation Behavior. Avidin-plasmid DNA nanoassemblies were obtained in double-distilled water as described by Morpurgo et al. (*14*). This procedure leads to discrete nanoparticles of defined sizes (about 100 nm) and composed of individual DNA filaments covered by several avidin molecules. Even though the interaction between the protein and DNA is not affected by the presence of salts or buffers, when the nanoparticles are transferred to a buffered

Table 3. Size of the Avidin-DNA Nanoassemblies in Water as a Function of Type and Amount of Biotin-PEG Used for Their Surface Coating

	mean diameter (nm)			
% BBS occupied	methyl-PEG2000-biotin (**5**)	biotin-L-lys-(methyl-PEG2000)$_2$ (**7**)	methyl-PEG5000-biotin (**10**)	biotin-L-lys-(methyl-PEG5000)$_2$ (**11**)
no biotin-PEG	97 ± 10.7	97 ± 10.7	97 ± 10.7	97 ± 10.7
20	88.5 ± 0.7	101 ± 14.8	95.5 ± 6.4	93.5 ± 9.2
30	93 ± 2.8	99 ± 11.3	93.5 ± 7.8	95 ± 7.1
40	86 ± 1.4	90 ± 11.3	94 ± 5.7	99.5 ± 0.7
50	88.5 ± 0.7	91 ± 2.8	93.5 ± 7.8	100 ± 1.4
60	91.5 ± 0.7	109 ± 19.1	99 ± 4.2	98 ± 2.8

solution they undergo rapid aggregation. This phenomenon can be followed by measuring the size of the nanoassemblies at regular time intervals after buffer addition.

Since anchorage through the biotin binding sites is the only PEGylation strategy that does not alter the protein DNA binding affinity, the effect of the amount of PEG on the aggregation of avidin-plasmid DNA assemblies has been investigated with the biotin-PEG derivatives only.

In order to evaluate and quantitate the effect of biotin-PEGylation on aggregation, the four biotin-PEGs were added to the particles at precisely defined polymer/protein ratios in order to cover increasing percentages of their biotin binding sites (between 20% and 60% of the total amount). The size of the particles was initially measured in dd-H_2O and then at regular times after mixing with the buffer solution. As seen in Table 3 and Figure 3, the presence of biotin-PEG does not affect the particle's size in dd-H_2O but reduces their aggregation behavior in a dose-dependent manner. The four biotin-PEG derivatives behave differently: the two linear ones slow down the aggregation in a dose-dependent manner, but at the concentrations tested, only the highest-molecular-weight one (5 KDa) was capable of preventing it completely, namely, when 60% of the biotin binding sites were occupied. The branched biotin-PEGs were more effective than the linear ones. However, in the case of the lower MW derivative (MW = 2 KDa × 2), the size of the particles increased up to about 200 nm in ~10 min independently of the amount of polymer added. After this initial phase, the aggregation process slowed down significantly, and at the highest polymer concentration investigated, it stopped completely. In the case of the branched high MW derivative (5 KDa × 2), aggregation was detectable only when low amounts of PEG were added: 30% biotin binding sites (BBS) coverage was enough to stabilize the particles indefinitely. In fact, the size of the particles was re-analyzed 24 h after their preparation and it remained unchanged.

Application of the Optimized Assemblies in Detection. The potential usefulness of the optimized assembly prepared upon protecting the particle surface with 30% of the BBS with compound **11** was assessed by means of a simple analytical test. This was carried out after removing the excess of free avidin resulting from the preparation process. Purification was carried out by tangential flow filtration (TFF) using 100 kDa cutoff filters, and the efficiency of the purification step was measured through two parameters: (a) the amount of protein recovered and (b) the size of the particles in the retained solution.

The time necessary to run the purification was about 1 h, with 500−700 mL total filtered volume. After purification, avidin recovery was quantitative, as calculated from the UV absorbance of the filtered and retained solutions. Dynamic light scattering analysis of the retained sample (intensity-weighted Nicomp distribution) indicated the presence of two different particle populations, one of 130 nm size and the other of 600−800 nm. The first one, representing 96% of the total number, had about the same size of the nanoassemblies before TFF, whereas the second population was of larger size. This suggests that that, upon removal of the excess of avidin, either aggregation occurs partially or the DNA loosens its condensed architecture and unrolls, at least to some extent, into a nonglobular shape. If this were the case, the particles might still exist as individual and discrete objects, but the DLS technique would not be the best tool to analyze their size, as the algorithm it uses is based on the assumption that the particles are of spherical geometry. Only the sample ultrastructure analysis could clarify which phenomenon underlies the change of sample size registered after purification. However, from a practical viewpoint the fact that total avidin recovery was

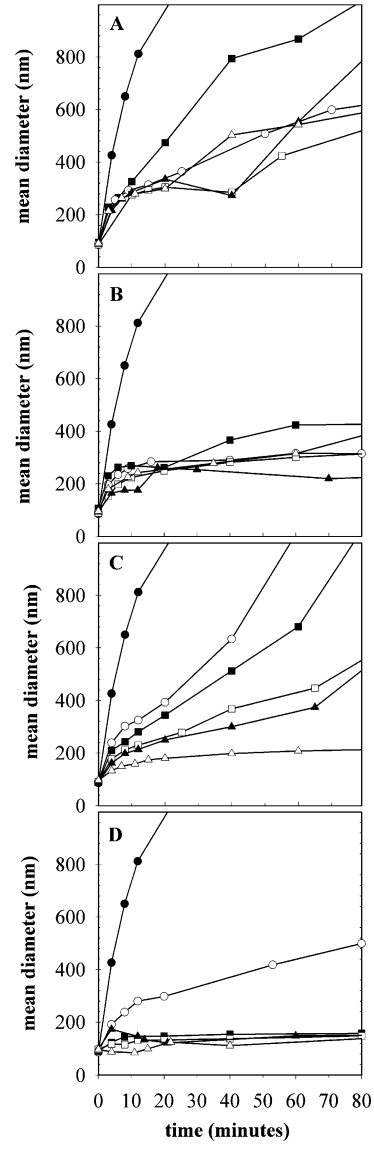


Figure 3. Aggregation in PBS buffer of avidin-DNA nanoassemblies PEGylated through the use of different amounts of the four biotin-PEG polymers. (A) methyl-PEG2000-biotin (**5**); (B) biotin-L-lys-(methyl-PEG2000)$_2$ (**10**), (C) methyl-PEG5000-biotin (**7**), or (D) biotin-L-lys-(methyl-PEG5000)$_2$ (**11**). Biotin-PEG derivatives were added in order to occupy increasing percentages of the total biotin binding sites (BBS) available: 0% (•), 20% (○), 30% (■), 40% (□), 50% (▲), and 60% (Δ). Standard deviations have been omitted for clarity purposes, as they increased together with the increase of particle's size.

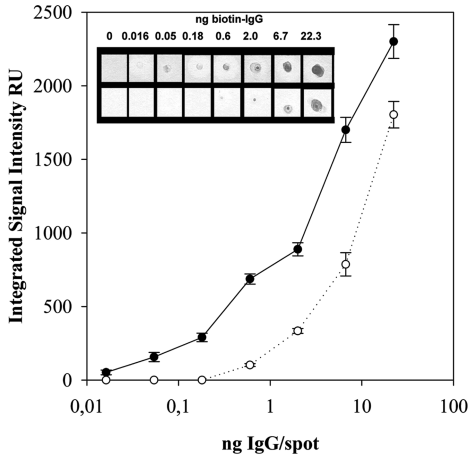


Figure 4. Integrated signal of biotin-HRP/DAB based dot-blot on a nitrocellulose membrane spotted with a biotinylated model IgG and incubated with monomer (○) or nanoassembled (•) avidin. The DAB substrate generated spot intensities were quantified with *ImageJ* software. The assay was carried out in duplicate.

achieved indicates that, even if in a larger size, the purified product remains in solution and can be used as an analytical tool in solution assays.

The results of the first analytical test are shown in Figure 4. In the test, different amounts of a biotinylated sample protein were spotted on a nitrocellulose membrane and detected upon adding the nanoassemblies followed by biotinylated-HRP and development using DAB substrate. In order to evaluate if the nanoassemblies were capable of improving the performance of the classical avidin−biotin system, the assay was carried out in parallel with monomer avidin. Figure 4 shows the intensity of the signal generated upon recognition of decreasing amounts of a biotinylated protein spotted on a nitrocellulose membrane. Both signal intensity and onset of detection improved when using the purified nanoassemblies as compared to monomer avidin. Signal intensity was higher at all analyte concentrations tested. In addition, the limit of detection (LOD) observed with the nanoassemblies was lower by more than 40-fold than that with monomer avidin: in fact, monomer avidin failed to detect the biotin-analyte below 0.6 ng/spot, whereas the nanoassemblies gave rise to a positive signal even when the biotinylated IgG was as low as 0.016 ng/spot.

DISCUSSION

The idea of exploiting for biomedical applications a double self-assembly process driven by nature-dictated affinities is a fascinating one and was the driving force behind this work. However, the applicability of the ordered polyavidin nanoassemblies generated from the high-affinity interaction of avidin with the nucleic acids depends primarily on the possibility of generating solution-stable nanoassemblies capable of remaining as such when diluted in physiological buffers. In this respect, the nature-driven process moves in the opposite direction to the one required by applicative science: the avidin-nucleic acid assemblies are insoluble in buffer solution, and this is probably because of the natural role of the interaction which is presumably to segregate undesired exogenous DNA when it enters the egg (*14*). In any case, aggregation is a common phenomenon occurring with nanosized particles, largely related to the large surface area exposed with its peculiar characteristics of charge type and density, and hydrophobicity−hydrophilicity balance. Hydrophilic polymers, covalently bound or adsorbed onto the particle surface, are often used to avoid or slow down aggregation of otherwise solution-unstable nanoparticles (*20, 33*). Their effectiveness in preventing nonspecific interactions between different surfaces (and hence also between nanoparticles) to which they are attached is related to two parameters: (a) polymer chain length and (b) its grafting density (*17, 34*−*36*). For each system, the same efficacy of aggregation prevention is achievable by varying each or both of the above parameters. Despite the common general rules dictating the efficacy of hydrophilic polymers surface protection, each individual nanosystem, being characterized by unique size, surface properties, and curvature angle, has to be optimized individually. In the case of the avidin-nucleic acid assemblies, an additional property that needs to be taken into consideration is the fact that the particles exist only secondary to a high-affinity phenomenon whose likelihood depends on the ability to preserve the DNA binding site on the protein surface. Avidin covalent PEGylation has been described by some authors, whose main objective was to increase the protein in vivo half-life and to reduce its immunogenicity. In those works, it was shown that the protein ability to bind biotin is affected by covalent modification in a dose-dependent manner (*37*) unless a site protection strategy is adopted to prevent it (*38*). However, the effect of covalent PEGylation on the more recently discovered property of avidin of binding the nucleic acids has not yet been described. This second property is the key element that drives the formation of the avidin-nucleic acids described here, because a diminished or lost affinity would impair the assemblies stability and therefore their applicability.

The results show that when PEG is covalently bound to avidin, its affinity for DNA decreases in a dose-dependent manner. At one PEG per avidin the loss of affinity is of minor intensity, however, at higher polymer loading it becomes dramatic. It was shown previously that the affinity for plasmid DNA is higher when the protein biotin binding pockets are occupied by biotin (*14*). Therefore, in order to better compare biotin-PEGylated samples with the covalently PEGylated ones, the latter have been tested also upon addition of 1 equiv of biotin/each covalent PEG chain attached to the protein. Nevertheless, the original affinity was not recovered, indicating that the loss in affinity is nonreversible. Even though covalently PEGylated avidins are still capable of interacting with DNA at the concentrations used in the EMSA assay, the results suggest that the loss of affinity induced is likely to affect the solution stability of the avidin-DNA assemblies at lower concentrations regimes. Data from our laboratory (manuscript in preparation) indicate that the affinity between native avidin and double-stranded DNA is in the nanomolar range, but no information is yet available on the PEGylated protein. It must be pointed out that the agarose gel retardation assays of this work and the aggregation tests have been carried out at relatively high concentrations (micromolar range). Therefore, losses in affinity of 1 or 2 orders of magnitude are likely to remain undetected by both assays, while they might induce instability at lower concentrations, thus reducing the application potential of the assemblies. As a matter of fact, some authors tried, without success, to use covalently PEGylated avidin-nucleic acid assemblies as cell transfecting systems (*39*). In that work, short-term (up to ten minutes) aggregation kinetics had been carried out demonstrating the particle solution stability at the concentration tested. However, the aggregation assays had been carried out at higher concentrations than that later used in the cell assays, so the loss in DNA affinity might have had a negative impact on the biological activity pursued.

On the other hand, PEGylation through biotin bridging has no impact on the affinity for DNA, which remains totally preserved. In reality, the data obtained with avidin PEGylated to a large extent with highly bulky biotin-PEG derivatives (compound **11** and biotinylated-PEG-HRP) indicate an increase in affinity. This is difficult to explain: it could be related to the fact that biotinylated avidin has a higher affinity for DNA that

the apo form. However, higher affinity in the presence of biotin is observable only at ionic strength conditions higher that those used in this work (*14*), and indeed, no difference in affinity was detected upon saturating native avidin with free biotin (Figure 2A vs B). The increase in affinity could therefore be related to an interaction between the PEG chains, but the nature of this phenomenon is yet to be investigated.

In any case, the EMSA data show clearly that the use of the biotin binding sites as the anchoring points for polymer attachment is the best strategy to achieve surface protection of the nanoassemblies. On the basis of these data and taking into account that aggregation studies on covalently PEGylated avidin have been described by other authors (*39*), the effect of the amount of PEG on the aggregation has been investigated here with the biotin derivatives only. In fact, the next questions that needed to be answered were how much and what kind of biotin-PEG should be used. This is not a trivial matter, as the use of too much biotin-PEG would, on one hand, reduce the assemblies cargo capability and, on the other hand, possibly affect their ability to interact with further biotinylated functions. On the contrary, amounts of PEG that are too low would not be enough to protect them from aggregation. The four PEG-biotin derivatives described in this work differed in the biotin/PEG-monomer ratio and/or the polymer geometry: two of them (**1** and **7**) were of linear geometry and two (**10** and **11**) had a Y-shape, similar to the amine-reactive Tween-PEG described by Monfardini (*40*). Monfardini's Y-shaped polymer is capable of inducing higher surface protection than that induced by two individual molecules of linear PEG having half its molecular weight, with the advantage of generating minor protein perturbation and loss of biological activity. Similarly, the Y-shaped biotin-PEGs synthesized in this work (compounds **10** and **11**) were more efficient than the linear ones (compounds **1** and **7**) in preventing sample aggregation. As a matter of fact, total protection from aggregation was achieved by saturating 30% of the total biotin binding sites with the Y-shaped 2 × 5 kDa MW compound or 60% of the BBS with the linear 5 kDa MW one. Therefore, the amount of PEG/surface area necessary to totally prevent the aggregation is the same. However, the use of the branched compound allows the majority (70%) of the original biotin binding sites to remain available for further interactions, and this translates into a more powerful nanoassembly. The results can also be discussed in terms of PEG size and degree of surface coverage as a function of polymer chain distance. According to the literature, the calculated radii of gyration (R_g) of the 2, 4, 5, and 10 kDa PEGs are about 1.7, 2.5, 2.8, and 4.2 nm, respectively (*41*), and the theoretical distances between two adjacent polymer chains necessary to achieve maximal surface protection are, respectively, 3.8, 5.5, 6.1, and 8.9 nm (*34, 35, 42*−*47*). On the basis of the information available from the avidin crystallography data (*48*), the distance between two biotin binding sites on an individual avidin molecule (or between two biotin binding sites in two adjacent avidins) can vary between about 3 and 6 nm, depending on the biotin binding sites considered. Even if the precise information regarding the distance between the biotin binding sites in the nanoassembly can only be obtained by ultrastructure investigation, these numbers suggest that the 2 kDa and the Y-shaped 4 kDa PEGs should not always be able to provide maximal surface protection even at 100% BBS coverage, and this is in agreement with our observations. On the contrary, the 5 kDa and the Y-shaped 10 kDa polymers are large enough to provide maximal protection.

The next questions we wanted to answer were if the optimized and stabilized assemblies were still capable of recognizing biotinylated molecules once immobilized on a surface, and consequently, if they were capable of acting as signal amplifiers with respect to the avidin monomer. This was indeed verified by a simple dot blot assay that demonstrated that the particles are capable of generating a higher signal as compared to monomer avidin and to improve the limit of detection by at least 40-fold. This result demonstrates for the first time that the optimized assemblies can indeed be used as molecular amplifiers in detection, therefore paving the way toward further application where a higher sensitivity such as the one obtained by the classical avidin−biotin technology might be of use.

CONCLUSIONS

The results demonstrate for the first time the possibility of exploiting the avidin-DNA high-affinity interaction to obtain solution-stable nanoassemblies with improved detection capabilities as compared to monomeric avidin. This was achieved by fine-tuning the surface protection strategy of the assemblies so that neither the biotin- nor the DNA-binding properties are impaired upon PEGylation. In fact, covalent PEGylation partially reduces the affinity for DNA, whereas PEG anchoring through the biotin binding sites does not. The full potential of this new system is yet to be explored. However, as compared to other polyavidin systems described in the literature, the ANANAS particles have the additional advantages of (a) being stoichiometrically defined and (b) being composed of biocompatible or biodegradable elements only, and (c) having the full biotin binding potentials preserved. These are fundamental features that allow for envisioning their potential application in further areas of investigation, among which are in vivo diagnostics and drug delivery.

ACKNOWLEDGMENT

This work was funded by the University of Padova 2007 "Progetto di Ateneo" and the Italian Ministry of Education (ex 60% funds). The authors are grateful to Meir Wilchek for his constant support, especially in the early stages of this work.

LITERATURE CITED

(1) Wilchek, M., and Bayer, E. A. (1988) The avidin-biotin complex in bioanalytical applications. *Anal. Biochem. 171*, 1–32.
(2) Wilchek, M., and Bayer, E. A. (1990) Introduction to avidin-biotin technology. *Methods Enzymol. 184*, 5–13.
(3) Wilchek, M., and Bayer, E. A. (1990) Avidin-biotin mediated immunoassays: overview. *Methods Enzymol. 184*, 467–9.
(4) Wilchek, M., and Bayer, E. A. (1990) Applications of avidin-biotin technology: literature survey. *Methods Enzymol. 184*, 14–45.
(5) Wilchek, M., Bayer, E. A., and Livnah, O. (2006) Essentials of biorecognition: the (strept)avidin-biotin system as a model for protein-protein and protein-ligand interaction. *Immunol. Lett. 103*, 27–32.
(6) Goldenberg, D. M., Sharkey, R. M., Paganelli, G., Barbet, J., and Chatal, J. F. (2006) Antibody pretargeting advances cancer radioimmunodetection and radioimmunotherapy. *J. Clin. Oncol. 24*, 823–34.
(7) Mamede, M., Saga, T., Ishimori, T., Higashi, T., Kobayashi, H., Konishi, J., and Brechbiel, M. W. (2002) Radiolabeling of Avidin with very high specific activity for internal radiation therapy of intraperitoneally disseminated tumors. *J. Nucl. Med. 43*, 270p–70p.
(8) Hytonen, V. P., Laitinen, O. H., Grapputo, A., Kettunen, A., Savolainen, J., Kalkkinen, N., Marttila, A. T., Nordlund, H. R., Nyholm, T. K., Paganelli, G., and Kulomaa, M. S. (2003) Characterization of poultry egg-white avidins and their potential as a tool in pretargeting cancer treatment. *Biochem. J. 372*, 219–25.
(9) Chinol, M., De Cobelli, O., Trifiro, G., Scardino, E., Bartolomei, M., Verweij, F., Papi, S., Matei, D. V., and Paganelli, G. (2003) Localization of avidin in superficial bladder cancer: a potentially new approach for radionuclide therapy. *Eur. Urol. 44*, 556–9.

(10) Laverman, P., Zalipsky, S., Oyen, W. J., Dams, E. T., Storm, G., Mullah, N., Corstens, F. H., and Boerman, O. C. (2000) Improved imaging of infections by avidin-induced clearance of 99mTc-biotin-PEG liposomes. *J. Nucl. Med. 41*, 912–8.
(11) Hsu, S. M., Raine, L., and Fanger, H. (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem. 29*, 577–80.
(12) Li, H., Ha Park, S., Reif, J., LaBean, T., and Yan, H. (2004) DNA-templated self-assembly of protein and nanoparticle linear arrays. *J. Am. Chem. Soc. 126*, 418–19.
(13) Kazuyuki, S., Nobuhiro, H., Hiroki, T., and Satoshi, F. (2004) Complexes containing crosslinked avidin, analytical method with the use of crosslinked avidin and analytical reagents and kits., Iatron Laboratories, Inc., Tokyo (JP), Kikkoman Corporation, Chiba, Japan, U.S. Patent 6,787,325, EP Patent 1052510/B1.
(14) Morpurgo, M., Radu, A., Bayer, E. A., and Wilchek, M. (2004) DNA condensation by high-affinity interaction with avidin. *J. Mol. Recognit. 17*, 558–66.
(15) Abuchowski, A., McCoy, J. R., Palczuk, N. C., van Es, T., and Davis, F. F. (1977) Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem. 252*, 3582–6.
(16) Abuchowski, A., van Es, T., Palczuk, N. C., and Davis, F. F. (1977) Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J. Biol. Chem. 252*, 3578–81.
(17) Torchilin, V. P., Omelyanenko, V. G., Papisov, M. I., Bogdanov, A. A., Jr., Trubetskoy, V. S., Herron, J. N., and Gentry, C. A. (1994) Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. *Biochim. Biophys. Acta 1195*, 11–20.
(18) Immordino, M. L., Dosio, F., and Cattel, L. (2006) Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. *Int. J. Nanomed. 1*, 297–315.
(19) Owens, D. E., 3rd, and Peppas, N. A. (2006) Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *Int. J. Pharm. 307*, 93–102.
(20) Dubertret, B., Skourides, P., Norris, D. J., Noireaux, V., Brivanlou, A. H., and Libchaber, A. (2002) In vivo imaging of quantum dots encapsulated in phospholipid micelles. *Science 298*, 1759–62.
(21) Morpurgo, M., Pasut, G., and Veronese, F. M. (2006) Pegylated proteins as cancer therapeutics in *Delivery of Protein and Peptide Drugs in Cancer* (Torchilin, V., Ed.) Imperial College Press, London.
(22) Zhai, Y., Zhao, Y., Lei, J., Su, Z., and Ma, G. (2009) Enhanced circulation half-life of site-specific PEGylated rhG-CSF: optimization of PEG molecular weight. *J. Biotechnol. 142*, 259–66.
(23) Treetharnmathurot, B., Ovartlarnporn, C., Wungsintaweekul, J., Duncan, R., and Wiwattanapatapee, R. (2008) Effect of PEG molecular weight and linking chemistry on the biological activity and thermal stability of PEGylated trypsin. *Int. J. Pharm. 357*, 252–9.
(24) Renz, C., and Loew, K. (1903) Ueber a-Methyl-Indol. *Ber. Deuts. Chem. Ges. 36*, 4326–30.
(25) Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem. 34*, 595–8.
(26) Harding, V., and Warneford, F. (1916) The ninhidrin reaction with amino-acids and amonium salts. *J. Biol. Chem.* 25.
(27) Habeeb, A. (1966) Determination of free amino groups in proteins by trinitrobenzensulphonic acid. *Anal. Biochem. 14*, 328–36.
(28) Morpurgo, M., and Veronese, F. M. (2004) Conjugates of peptides and proteins to polyethylene glycols. *Methods Mol. Biol. 283*, 45–70.
(29) Green, N. M. (1965) A spectrophotometric assay for avidin and biotin based on binding of dyes by avidin. *Biochem. J. 94*, 23C–24C.
(30) Miron, T., and Wilchek, M. (1993) A simplified method for the preparation of succinimidyl carbonate polyethylene glycol for coupling to proteins. *Bioconjugate Chem. 4*, 568–9.
(31) Finsy, R. (1994) Particle sizing by quasi-elastic light scattering. *Adv. Colloid Interface Sci.* 79–143.
(32) Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968) Selective enzyme purification by affinity chromatography. *Proc. Natl. Acad. Sci. U.S.A. 61*, 636–43.
(33) Cattel, L., Ceruti, M., and Dosio, F. (2003) From conventional to stealth liposomes: a new frontier in cancer chemotherapy. *Tumori 89*, 237–49.
(34) Jeon, S. I., and Andrade, J. D. 1991b Protein surface interactions in the presence of polyethylene oxide 0.2. effect of protein size. *J. Colloid Interface Sci. 142*, 159–66.
(35) Jeon, S. I., Lee, J. H., Andrade, J. D., and Degennes, P. G. 1991a Protein surface interactions in the presence of polyethylene oxide 0.1. simplified theory. *J. Colloid Interface Sci. 142*, 149–58.
(36) Sofia, S. J., Premnath, V., and Merrill, E. W. (1998) Poly(ethylene oxide) grafted to silicon surfaces: Grafting density and protein adsorption. *Macromolecules 31*, 5059–70.
(37) Caliceti, P., Chinol, M., Roldo, M., Veronese, F. M., Semenzato, A., Salmaso, S., and Paganelli, G. (2002) Poly(ethylene glycol)-avidin bioconjugates: suitable candidates for tumor pretargeting. *J. Controlled Release 83*, 97–108.
(38) Salmaso, S., Semenzato, A., Bersania, S., Chinol, M., Paganelli, G., and Caliceti, P. (2005) Preparation and characterization of active site protected poly(ethylene glycol)-avidin bioconjugates. *Biochim. Biophys. Acta Gen. Subj. 1726*, 57–66.
(39) Segura, T., and Shea, L. D. (2002) Surface-tethered DNA complexes for enhanced gene delivery. *Bioconjugate Chem. 13*, 621–9.
(40) Monfardini, C., Schiavon, O., Caliceti, P., Morpurgo, M., Harris, J. M., and Veronese, F. M. (1995) A branched monomethoxypoly(ethylene glycol) for protein modification. *Bioconjugate Chem. 6*, 62–69.
(41) Kenausis, G. L., Voros, J., Elbert, D. L., Huang, N. P., Hofer, R., Ruiz-Taylor, L., Textor, M., Hubbell, J. A., and Spencer, N. D. (2000) Poly(L-lysine)-g-poly(ethylene glycol) layers on metal oxide surfaces: Attachment mechanism and effects of polymer architecture on resistance to protein adsorption. *J. Phys. Chem. B 104*, 3298–309.
(42) Jeon, Y. T., Lee, C. P., and Mariano, P. S. (1991) Radical cyclization reactions of alpha-silyl amine alpha, beta-unsaturated ketone and ester systems promoted by single electron-transfer photosensitization. *J. Am. Chem. Soc. 113*, 8847–63.
(43) Palegrosdemange, C., Simon, E. S., Prime, K. L., and Whitesides, G. M. (1991) Formation of self-assembled monolayers by chemisorption of derivatives of oligo(ethylene glycol) of structure Hs(Ch2)11(Och2ch2)Meta-Oh on Gold. *J. Am. Chem. Soc. 113*, 12–20.
(44) Du, H., Chandaroy, P., and Hui, S. W. (1997) Grafted poly-(ethylene glycol) on lipid surfaces inhibits protein adsorption and cell adhesion. *Biochim. Biophys. Acta 1326*, 236–48.
(45) Lu, H. B., Campbell, C. T., and Castner, D. G. (2000) Attachment of functionalized poly(ethylene glycol) films to gold surfaces. *Langmuir 16*, 1711–18.
(46) Yang, Z. H., Galloway, J. A., and Yu, H. U. (1999) Protein interactions with poly(ethylene glycol) self-assembled monolayers on glass substrates: diffusion and adsorption. *Langmuir 15*, 8405–11.
(47) Harder, P., Buck, M., Zolk, M., Lampert, A., Pertsin, A., and Grunze, M. (1998) Interaction of water with ethylene glycol terminated self-assembled monolayers. *Abstr. Pap. Am. Chem. Soc. 215*, U212–U12.
(48) Livnah, O., Bayer, E. A., Wilchek, M., and Sussman, J. L. (1993) Three-dimensional structures of avidin and the avidin-biotin complex. *Proc. Natl. Acad. Sci. U.S.A. 90*, 5076–80.

BC100044U