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(54) NANOASSEMBLED COMPLEXES OF NUCLEIC ACIDS, AVIDIN AND POLYMERS, AND PREPARATION THEREOF

NANOTECHNISCH ZUSAMMENGESETZTE KOMPLEXE VON NUKLEINSÄUREN, AVIDIN UND POLYMEREN, SOWIE HERSTELLUNG DAVON

COMPLEXES NANO-ASSEMBLÉS D'ACIDES NUCLÉIQUES, D'AVIDINE ET DE POLYMÈRES, ET LEUR PRÉPARATION

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Description

Field of the invention

[0001] The present invention relates to new nanoassembled complexes (also hereinafter known as nanocomplexes or nanoassemblies) and more specifically to nanoassemblies of nucleic acids, avidin and polymers, to their use in the biotechnological field and nanomedicine and to their preparation.

State of the art

[0002] Avidin is a tetrameric glycoprotein known mainly for its ability to bind to four molecules of biotin with very high affinity (K_d~10^{-15} M). From the practical viewpoint, the avidin property of a high and multiple affinity for biotin forms the basis for its use as a molecular instrument in a large number of biotechnological applications (avidin-biotin technology) (Wilchek M and Bayer EA, Analytical Biochemistry. 1988, 171:1-32; Wilchek M and Bayer EA, Methods Enzymol. 1990, 184: 14-45). In respect of this property, avidin can serve as a molecular bridge to stably link together different biological or chemical units, provided that these latter are covalently bound to one molecule of biotin.

[0003] The most common applications of avidin-biotin technology are for analytical purposes, more precisely for detection and quantification systems which are usually based on the ability to link an antibody, or any other molecule having high affinity towards the analyte (ligand/antigen), to a marker system (a fluorophore, an enzyme able to emit light/colour, a radionuclide etc.); other applications include surface functionalization with specific chemical/biochemical entities, being a procedure which is often conducted by using the molecular bridge formed from the avidin-biotin complex; another application is for targeting drugs or diagnostic elements, administered by parenteral means, towards specific sites in the body (Goldenberg DM, Sharkey RM, Paganelli G, Barbet J, and Chatal JF, J. Clin. Oncol. 2006, 24: 823-834).

[0004] One of the main drawbacks of classic avidin-biotin technology is the maximum number of biotins, namely four, that can be joined to a single avidin molecule, which forms the central nucleus of the system. The possibility to have a central nucleus able to bind a greater number of biotin molecules to itself enables the system potentiality to be theoretically increased.

[0005] This increased capability can be achieved by joining together several avidin molecules into a single unit (defined herein as a poly-avidin unit). In this regard the literature describes various technological approaches for obtaining said polyavidin nuclei. The strategies commonly adopted and currently available are based on coating the surfaces of micro- or nano-spheres (consisting of different polymers such as polystyrene or metals, such as gold) with several avidin molecules, or on the chemical "polymerization" of avidin by covalent crosslinking.

[0006] Strategies currently available for forming poly-avidin units are hence based either on chemical synthesis processes aimed at the formation of covalent bonds between avidin units, or on non-specific adsorption processes which lead to avidin molecules adhering to the surfaces of polymer or metal nuclei. However, all these systems have certain disadvantages in common. In particular, the poly-avidins thus obtained are always characterized by: a) a certain degree of polydispersivity depending on the method for obtaining them: b) a partial loss of avidin activity. In practical terms, inactivation of avidin translates into a reduced capacity for binding with biotin (and hence with any other biotinylated ligand), whereas polydispersivity translates into products whose properties are statistically defined and are hence not highly defined.

[0007] Another common disadvantage of poly-avidins obtained by means of the aforesaid methods is that they cannot be used in certain biomedical environments as the materials used for their assembly (e.g. linkers for chemical polymerization, or polymer or metal central nuclei for non-specific adsorption) are either not of natural origin or are not always biocompatible and therefore potentially toxic. The poly-avidins obtained by these methods can thus present toxicological risks related to the elements comprising them and this limits their applicability in pharmaceutical/diagnostic environments when *in vivo* contact of the avidin assembly with human or animal tissue is envisaged.

[0008] Recently, an additional property specific to avidin has been brought to light, this being its capacity to bind to nucleic acids with high affinity (Morpurgo M, Radu A, Bayer EA, and Wilchek M, Journal of Molecular Recognition. 2004, 17: 558-566). Said binding results from a high affinity interaction which also involves specific regions of the protein but does not involve directly the biotin binding site. Subsequently to this interaction, avidin self-assemblies onto DNA in an organized manner, giving rise to stoichiometrically defined agglomerates. Within them, the nucleic acid is coated by avidin molecules in a stoichiometric ratio of avidin to the nucleic acid base pairs equal to 18 $\pm$ 4. These complexes are stable at high dilutions ([DNA] = 10pM) and in the presence of electrolytes in solution.

[0009] Given the stability of the interaction under physiological conditions, the aforesaid assemblies can in effect be described as poly-avidins, similar in part to those already mentioned. The assemblies are stable, are composed only of elements of biological and biodegradable origin, and the ability of avidin, contained within them, to bind to biotin remains intact.

[0010] However, the practical benefits of these poly-avidin systems as instruments for improving the performance of

the classic avidin-biotin system depend on being able to obtain them in the form of reproducible and poorly polydispersed, discrete aggregates of defined colloidal size. From the macroscopic viewpoint the avidin-nucleic acid assemblies are seen to assume various shapes and geometries depending on the conditions in which they are found. For example, by mixing avidin and nucleic acids in a buffered aqueous environment, agglomerates of large size are obtained (» 1 micron), which are highly polydispersed and of undefined geometry and indeed unusable from the practical viewpoint. Conversely, 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 can be 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. However, these latter arrangements, which are already described in the literature (Morpurgo M et al. 2004 *ref. cit*.), are stable and isolatable in aqueous salt-free solution; in the presence of electrolytes they undergo a rapid process of aggregation subsequent to which polydispersed macro-aggregates are again obtained but actually unusable for practical purposes.

[0011] Since any general analytical or biomedical application of the avidin/biotin system comprises biorecognition reactions in saline aqueous environment, the avidin and nucleic acid complexes described above have no practical use because they are unable to exist as discrete and stable entities under the required buffered conditions.

[0012] In any event, aggregation is a general problem common to many small sized particles, particularly when they fall within the colloidal range (< 1 micron - nanoparticles). Aggregation depends on particle surface characteristics (charge type and density, hydrophobicity, hydrophilicity, etc.) and on the type of medium in which they are suspended (inorganic solvent, aqueous solvent, type of buffer, ionic strength, pH, etc.); various technical solutions can be employed to avoid or slow down aggregation.

[0013] Should the suspension medium be an aqueous solution, the most commonly adopted strategy is to use hydrophilic polymers which are covalently bound or adsorbed onto the particle surface so as to partially or completely conceal it from the surrounding environment. A steric hindrance and an enthalpic gain are thus created which prevent the particles from interacting irreversibly with each other. For example, hydrophilic polymers are used to protect the surface of liposomal nanoparticles (Cattel L, Ceruti M, et al. Tumori, 2003, 89:237-249) used as carriers of antitumour drugs to be administered by parenteral means.

[0014] The effectiveness of hydrophilic polymers in preventing non-specific 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) grafting density (Jeon SI, Lee JH et al. J. Colloidal and Interface Sci, 1991, 142: 149-158; Jeon SI and Andrade JD J. Colloidal and Interface Sci. 1991, 142: 159-166; Sofia SJ, Premnath V et al. Macromolecules 1998, 31: 5059-5070). For each system therefore, the same efficacy of aggregation prevention is achievable by varying one, or the other or both the aforesaid parameters.

[0015] Xiong May P. et al. (Xiong May P. et al in Bioconjugate Chemistry , 2007, 18, 746-753) disclose nanoparticles of PEG-avidin/biotin-polyetyleneimine (PEI) complexes for gene delivery, where PEG is covalently to avidin through lysine ε-chains of avidin. The complex 2PEG-avidin /biotin-PE18 forms salt-stable particles under physiologic conditions with a minimum of two 2PEG-avidin molecules bound per polymer chain. The presence of PEI was demonstrated to be fundamental for the nanocomplexes activity pursued by the authors. In fact, in the paper supplementary information nanoassemblies composed of pGL3 DNA and avidin covalenty PEGylated are also described. These PEG-avidin particles were shown to be stable in salt (tested for 20 mins only) but no stability tests upon dilution have been carried out to demonstrate if the affinity for DNA had changed upon covalent PEG attachment but it is shown that these particles failed to work as delivery systems.

[0016] It should be noted that each system, whether surface or nanoparticulate, is characterised by distinctive properties (chemical, angle of curvature, etc.) and so the efficacy of surface protection must be calibrated each time in order to optimize the effects. As aforecited, various parameters are taken into account during optimization and include type of polymer, its length and attachment density, and not least, the grafting method (Owens DE and Peppas NA Int. J. Pharm. 2006, 307: 93-102). Consequently, the results obtained with a determined particle system are not directly transferable to another one and as such, the information already described in the literature is not directly applicable to nanoparticulate systems consisting of avidin and nucleic acids. The surface protection aspect of these systems is therefore described for the first time within the scope of this invention. One aspect of the present invention is to obtain nanoparticles consisting of nucleic acids and avidin which are stable in an aqueous/saline environment.

[0017] A further aspect of the present invention is that said stable systems are able to recognize other biotinylated elements, in that they themselves possess pharmacological activity, or are able to recognize third elements (for example a receptor) or are able to generate signals by themselves or in combination with other reagents in solution (for example fluorescence, colour, radioactivity, photons.)

Summary

[0018] The nanoassembled complexes provided by the inventors fulfil the aforementioned purposes, as they allow

the previously reported drawbacks derived from the known technologies of the art to be overcome.

[0019] In particular, the obtained nanoassembled complexes are highly defined from the qualitative and quantitative composition viewpoint and stable even in the presence of electrolytes.

[0020] In a first aspect the object of the present invention are nanoassembled complexes comprising a nucleus obtained by means of high affinity interaction between one or more avidin tetrameric units and one or more nucleic acid molecules, wherein said nucleus is stabilized by a biotinylated surface protecting agent, represented by the general formula (I)

$$NB_nAv_y(B\text{-}X_a\text{-}PA_b)_z \quad (I)$$

wherein:

- NB are the single nuclobases of a single or double stranded nucleic acid;
- Av is an avidin tetrameric unit;
- B-X_a-PA_b is the biotinylated surface protecting agent in which PA is a polymer unit having at least one or two functionalizable residues of which one binds, by a covalent bond either directly or through a spacer X, to a biotin residue B by means of carboxyl functional group of said residue B;

- *n* is a number varying from 16 to 10,000,000;
- *y* is an integer equal to or greater than (≥) 1 and being relative to *n* can vary from (0.0001)•*n* to (0.0454)•*n*. If a value comprised in the range (0.0001-0.0454)•*n* is less than (<) 1, then *y* is equal to (=) 1;
- *z* is an integer equal to or greater than (≥) 1 and being relative to *y* can vary from (0.02)•*y* to (4)•*y*. If a value comprised in the range (0.02-4)•*y* is less than (<) 1, then *z* is equal to (=) 1;
- a is a number varying from 0 to 50;
- *b* is a number varying from 1 to 128.

[0021] The nanoassembled complexes of the invention are in the form of nanoparticles which are another object of the invention.

[0022] A still further object of the invention is a method for preparing the nanoassembled complexes of general formula (I).

[0023] The advantages achievable with the present invention will become more apparent to an expert of the art from the following detailed description of particular embodiments, given for the purposes of non-limiting illustration, and with reference to the following figures. Also described herein is the use of nanoassembled complexes of formula (I) as means *vitro* and *in vivo* diagnostics, in the field of nanomedicine for targeting and concentrating bioactive molecules towards specific sites in the body, in the field of nanotechnology in general for the localization of molecules onto surfaces, and in any application (biomedical and engineering) that requires a co-localization of several chemical or biological functions of varying natures on a central core, being in its turn present in colloidal suspension or localized onto a surface.

Brief description of the figures

[0024]

Figure 1: the figure shows the size distribution (INTENSITY-weighted-GAUSSIAN Analysis) of the particles of the nanoassembled complexes **A**) Av-pEGFP 3 (sample 1 of examples 1 and 2); **B**) Av-pEGFP 3-B-X_a-PA_b IV-30 (sample 26 of example 2); **C**) Av-GenNB 2-B (sample 31 of example 4); **D**) Av-GenNB 2-B-X_a-PA_b IV-30 (sample 35 of example 4).

Figure 2: the figure shows the kinetics of aggregation in a buffered solution of the different nanoassembled complexes of example 2 as a function of the type of B-X_a-PA_b used and its quantity. The composition of the various formulations are summarized in table 2. **A**: B-X_a-PA_b I, % total occupied biotin binding sites (BBS = Biotin Binding Sites) equal to 0 (•), 20 (○), 30 (■), 40 (□), 50 (▲), 60 (Δ) %; **B**: B-X_a-PA_b IIa, % of occupied BBS equal to 0 (•), 20 (○), 30 (■), 40 (□) 50 (▲), 60 (Δ) %; **C**: B-X_a-PA_b IIb, % of occupied BBS equal to 0 (•), 20 (○) 30 (■), 40 (□), 50 (▲), 60 (Δ) %; **D**: B-X*a*-PA_b III, % of occupied BBS equal to 0 (•), 20 (○), 30 (■), 40 (□), 50 (▲), 60 (Δ) % ; **E**: B-X_a-PA_b IV, % of occupied BBS equal to 0 (•), 20 (○), 30 (■), 40 (□), 50 (▲), 60 (Δ) %.

Figure 3: the figure shows fluorescent microscope images of membranes used in an assay, with dot blot fluorescent detection, comparing avidin in monomeric form and in nanocomplexed form with nucleic acid. Incubation was carried out using avidin-biotin-Alexa solutions at 1.3 μg/ml. **A1**: monomeric avidin (sample 38 example 5); **A2**: Av-pEGFP 1.5 B-X_a-PA_b IV-25 (sample 39 example 5); **A3**: Av-pEGFP 0.75 B-X_a-PA_b IV-25 (sample 40 example 5).

Figure 4: the figure shows fluorescent microscope images of membranes used in a further assay, with dot blot fluorescent detection, comparing avidin in monomeric form and in nanocomplexed form with nucleic acid. Incubation

was carried out using avidin-biotin-Alexa solutions at 5 μg/ml in monomeric form (sample 38 of examples 5 and 6) and in nanoassembly form (sample 41 example 6).

Figure 5: the figure shows the comparison of detecting efficiency of avidin in monomeric (o) and nanocomplexed (•) form with nucleic acid, in dot blot with enzyme (HRP)-linked detection system. Spot detection was achieved upon incubation with biotin-HRP and development with DAB substrate of membranes previously incubated with avidin solutions at 5 μg/ml in monomeric form (sample 38 of examples 5 and 6 and 7) and in nanoassembly form (sample 42 example 7).

Detailed description of the invention

[0025] The invention described hereinafter relates to the obtaining of nanoassembled complexes in the form of nanoparticles comprising a nucleus of polyavidin, obtained by the nucleation of several avidin units onto one or more nucleic acid molecules, then stabilized by the presence of surface protecting agents so as to be able to remain as discrete and stable entities in saline aqueous solution and free from further non-specific interactions.

[0026] With the nanoassembled complexes of the present invention, discrete nanoparticles are obtained which are stabilized against risks of: a) aggregation in aqueous saline environments and b) non-specific interactions with other molecules in solution, by virtue of the presence of protective elements on their surface.

[0027] Said protective elements are themselves present on the particle surfaces in controlled and highly defined quantities. Moreover, surface protection according to the preparative method developed by the inventors takes place without destroying the nucleic acid-avidin self-assembled complex and without modifying the total capability of assembled avidins for binding to biotin (i.e. without modifying biotin binding sites).

[0028] The size of these nanoparticles can be established from the length of the nucleic acid which is the assembling nucleus of more avidin units, and accordingly, particles characterized by different sizes and different charges on the avidin can be obtained by suitably varying the size of the nucleating nucleic acid (NA).

[0029] The characteristics of said particles are precisely defined and their properties can be modulated by the user by varying:

a) the type and size of the nucleating NA;
b) the ratio between avidin and nucleic acid bases;
c) the nature and quantity of the protecting agent present on the surface.

[0030] For the purposes of the present invention the compounds object of the same are nanoassembled complexes comprising a nucleus obtained by nucleation secondary to a high affinity interaction of several avidin units onto one or more nucleic acid molecules, and stabilized by a biotinylated surface protecting agent, represented by the general formula (I)

$$NB_nAv_y(B\text{-}X_a\text{-}PA_b)_z \quad (I)$$

wherein:

- NB are the single nuclobases of a single or double stranded nucleic acid;
- Av is an avidin tetrameric unit;
- B-X_a-PA_b is the biotinylated surface protecting agent in which PA is a polymer unit having at least one or two functionalizable residues of which one binds, by a covalent bond either directly or through a spacer X, to a biotin residue B by means of its carboxyl functional group;
- *n* is a number varying from 16 to 10,000,000;
- *y* is an integer equal to or greater than (≥) 1 and being relative to *n* can vary from (0.0001)•*n* to (0.0454)•*n*. If a value comprised in the range (0.0001-0.0454)•*n* is less than (<) 1, then *y* is equal to (=) 1;
- *z* is an integer equal to or greater than (≥) 1 and being relative to *y* can vary from (0.02)•*y* to (4)•*y*. If a value comprised in the range (0.02-4)•*y* is less than (<) 1, then z is equal to (=) 1;
- a is a number varying from 0 to 50 and is preferably comprised from 0 to 10;
- *b* is a number varying from 1 to 128.

[0031] If *z* is less than 4, and hence the biotin binding sites present on the nucleus NB_nAv_y are not saturated by binding with biotin B of the protecting agent (B-X_a-PA_b), the nanocomplexes of the invention can bind additional biotinylated compounds, different from the protecting agent, onto said binding sites. Consequently, NB means a nucleic acid consisting of a number of nucleobases (NB) equal to *n*, with *n* varying from 16 and 10,000,000, referring to the total number of bases, irrespective of whether the nucleic acid is single or double stranded. Preferably the nucleic acid consists of a

base number varying from 30 to 100,000 and more preferably the base number is from 3,000 to 50,000.

[0032] Therefore, the term nucleic acid refers equally to:

i) any sequence of a single stranded (ss) or double stranded (ds) deoxyribonucleic acid (DNA) polymer;
ii) any sequence of a ribonucleic acid (RNA) polymer in single stranded form or hybridized with a RNA or a complementary DNA chain;
iii) a sequence, in accordance with the above points, in which a part of or all the bases have been chemically modified.

[0033] Moreover, the usable nucleic acid for the nanoassembled complexes of formula (I) can be in linear or circular form, in a relaxed, coiled or supercoiled state.

[0034] With reference to the term avidin, avidin is defined as being derived from chicken eggs or another similar source (eggs of birds in general) or from recombinant technology, either in glycosylated or deglycosylated form. Also included are other chemically or genetically modified avidin forms, provided they can assemble onto a single or double stranded nucleic acid as previously established,

[0035] In view of the relationship between the number *n* of NB and the number *y* of avidin units self-assembling onto the nucleic acid, *y* is preferably comprised from (0.0001)•*n* to (0.0357)•*n* and more preferably comprised from (0.01)•*n* to (0.0357)•*n*. For example, if *n* =10,000, *y* can vary from 10 to 357, preferably being from 100 to 357. If instead *n* = 100,000, *y* is comprised from 10 to 3,570 and is preferably from 1,000 to 3,570.

[0036] In addition, with reference to the biotinylated surface protecting agent B-X_a-PA_b:

- B means biotin;
- PA means preferably a linear unit of a hydrophilic polymer of any molecular weight capable of binding to biotin by a covalent bond, either directly or through a spacer X, by means of the biotin carboxyl group. If PA has two functionizable residues, the second of said residues is free or protected by protecting groups know to an expert of the art, for example a methoxyl group.

[0037] If *b* is greater than 1, and hence PA represents a hydrophilic polymer consisting of several polymers units, these latter are joined together by a further ligand having a number of functionalites equal to or greater than 3 (≥ 3) of which one binds to the spacer X or to biotin B and the remaining other functional groups bind to the polymer units PA;
- X is a spacer consisting of a bifunctional molecule of general formula (II)

Y-R-Y' (II)

wherein:

Y, Y' being the same or different from each other are -COO-; -NH -,-O-; SO_2-; -S-; -SO-; -CO-; -COS-; -NH-CO-; -NH-COO; HN-SO-NH- ;
R can be an alkyl, an alkenyl, an alkinyl, a cycloalkyl, or an aryl with a carbon atom number comprised from 1 to 20 and preferably from 5 to 20, also optionally substituted.

[0038] Therefore, the bond between the spacer X and biotin B and that between the spacer X and the hydrophilic polymer PA can be indiscriminately an amide bond, an amino bond, a carbamide bond, an ester bond, a ketone bond, an ether bond, a thioester bond, a thioether bond, an urea bond, a thiourea, sulphonic or sulphoxide bond.

[0039] In view of the relationship between the number *y* of avidin units and the number *z* of biotinylated surface protecting agent B-X_a-PA_b units, *z* is comprised from (0.02)•*y* to (4)•*y*, and preferably is comprised from (0.4)•*y* to (4)•*y*.

[0040] For example: in a particle with *n* = 10,000 and *y* = 357 (0.0357•*n*), *z* varies from 7 to 1,429, and preferably from 143 to 1,429; in the case of a particle with *n* = 10,000 and *y* = 100, z varies from 2 to 400 and, more preferably, from 40 to 400; in the case of a particle with *n* = 50,000 and *y* = 1,786 (= 0.0357•*n*), *z* varies from 36 to 7,143 and, more preferably, from 714 to 7,143; in the case of a particle with *n* = 50,000 and *y* = 500 (*y* = 0.01•*n*), *z* varies from 10 to 2,000, and more preferably from 200 to 2,000.

[0041] In the nanoassembled complexes of formula (I) of the present invention, the polymer units PA are biocompatible and preferably hydrophilic polymers and are known polymers (Owens DE and Peppas NA 2006 *ref. cit.*) in which the polymer unit PA has a molecular weight preferably comprised from 400 to 40,000 and more preferably from 1,000 to 20,000. Said polymer units are preferably selected from the group consisting of polyethylene oxide or polyethylene glycol (PEO or PEG) also optionally substituted, a copolymer of polyoxyethylene and polyoxypropylene (PEO-PPO), polyvinylpyrrolidone (PVP), polyacryloylmorpholine (PacM), a polyoxamine, a polylactide (PLA), a polyglycolide (PLG), a copolymer of lactic acid and glycolic acid (PLGA).

[0042] More preferably the polymer PA is a substituted polyoxyethylene (PEO) and is therefore characterized by the

following formula (III):

$$-(CR^1R^2CR^3R^4O)_m- \quad (III)$$

where:

R^1, R^2, R^3 and R^4 can be independently equal to hydrogen, alkyl, cycloalkyl, aryl, alkenyl, alkinyl, alcoxyl, thioalkoxy, aryloxy and thioaryloxy
m is an integer from 2 to 900.

[0043] If the polymer consists of several polymer units, and these are bound together by a polyfunctional ligand with functionality equal to or greater than 3 (≥ 3), said ligand can be lysine, glutamic acid, aspartic acid, cysteine, a dendrimer. The term "dendrimer" means a symmetrical macromolecular compound consisting of branches repeated around a central core consisting of a smaller molecule or a polymeric nucleus. The functional groups present outside the dendrimer, whose number depends on its number of branches, are themselves functionalizable with other molecules including, for example, PA polymers.

[0044] Furthermore, if the polymer unit PA is bifunctional, it can further covalently bind, through a second free functional group, to a compound suitable for the uses pursued with the nanoassembled complex, and in particular compounds selected from ligands, sugars, chromophores or fluorophores, drugs, chelating agents for radionuclides, peptides, antibodies, proteins, enzymes and the like.

[0045] The preparation of the nanoassembled complexes of the invention comprises three successive steps in aqueous solutions: in the first step nanoparticles consisting of only avidin and nucleic acid are obtained, constituting the central nucleus of the complexes of the invention. The two subsequent steps comprise optionally preparing the biotinylated surface protecting agent B-X_a-PA_b but mainly adding said surface protecting agent to the nucleic acid-avidin nanoparticles obtained in the first step.

[0046] Therefore, the method for preparing the nanoassembled complexes of general formula $NB_nAV_y(B\text{-}X_a\text{-}PA_b)_z$ (I) comprises at least the steps of:

a) preparing the self-assembled primary nucleus NB_nAv_y by mixing avidin Av with nucleic acid in predefined stoichiometric molar ratios of avidin to nucleobases;
b) mixing the biotinylated surface protecting agent B-X_a-PA_b with the previously obtained primary nucleus.

[0047] Optionally, preparation of the nanoassemblies of the invention can also comprise preparation of the biotinylated surface protecting agent B-X_a-PA_b.

[0048] The first step is undertaken by mixing, under stirring, the solutions of avidin and nucleic acid, preferably both in salt-free water. In this first step the molar ratios of avidin to nucleobases NB is within the range from 0.44 to 0.0001 and preferably from 0.133 to 0.0044, and more preferably 0.044. The reagents are mixed under continuous stirring at a temperature from 0 to 50°C for a time between 1 and 600 seconds.

[0049] The biotinylated surface protecting agent B-X_a-PA_b is prepared by synthesis or, if commercially available, is purchased. Preparation of B-X_a-PA_b by synthesis involves conjugating the biotin molecule to the polymer PA_b by chemical means, using classical bioconjugation techniques known to any expert of the art. Subsequently, the previously prepared or purchased biotinylated surface protecting agent B-X_a-PA_b is added in a stoichiometrically controlled quantity relative to the concentration of biotin binding sites present in the solution, which are themselves relative to the avidin concentration. The molar ratios of avidin: B-X_a-PA_b are hence comprised between 4 and 0.02.

[0050] Addition of the biotinylated surface protecting agent B-X_a-PA_b is also carried out under stirring in aqueous solutions at a controlled temperature from 0 to 50°C for a time between 1 and 120 minutes.

[0051] Moreover, the nanoassembled complexes of the invention can be prepared by a method in which steps a) and b) are substantially inverted, hence the preparation method can comprise:

a) adding the biotinylated surface protecting agent B-X_a-PA_b to the avidin in pre-defined stoichiometric molar ratios of biotin to avidin;
b) adding nucleic acid to the conjugate $Av_y(B\text{-}X_a\text{-}PA_b)_z$ obtained in the preceding step in pre-defined stoichiometric molar ratios of avidin to nucleobases.

[0052] The preparation conditions are the same as those previously described for the first method.

[0053] If necessary, as well as the aforementioned steps, whether the nanoassembled complexes are prepared by the first or second process, the preparation method can further comprise the purification of the particles from any monomeric avidin eventually present in the solution as a residue of the first step. Purification can be undertaken after

either step a) or step b).

[0054] Purification of the nanoassembled complexes from any monomeric avidin present in solution can be carried out by known methods, for example ultrafiltration or size exclusion chromatography. In the case of ultrafiltration, suitable systems are used, characterized by a cut-off equal to or greater than (≥) 100 kDa. In the case of size exclusion chromatography, chromatographic media are used which are suitable for retaining protein molecules of sizes up to (<) 200 kDa.

[0055] If the biotin binding sites present on the avidin of the nucleus are not saturated by the biotinylated surface protecting agent B-X_a-PA_b the preparation method can also comprise a further optional step of adding additional biotinylated compounds equal or different each other.

[0056] With the previously described preparation methods, nanoassembled complexes having the features of nanoparticles of any size can be obtained. In particular, said nanoassembled complexes are in form of nanoparticles of at least 10 nm in size and preferably from 50 to 1,000 nm in size.

[0057] The use of nanoassembled complexes in nanoparticulate form herein described extends to all currently known applications of the avidin-biotin system, for which they act as "amplification" systems. Examples of these applications include their use as: a) detection means in *in vitro* diagnostics; b) amplifiers in the localization and patterning of molecules on surfaces (for example microarrays, protein chips and DNA); c) instruments for *in vivo* diagnostics; d) systems for active or passive targeting of drugs.

[0058] The use will depend on the nature of the biotinylated compounds which can further be introduced onto nanoassembly surfaces through biotin binding sites present on the avidin and not saturated by binding with biotin of the protecting agent B-X_a-PA_b.

Experimental part

[0059] Some examples of the preparation of the nanoassembly compounds of the invention and their characterization will be given hereinafter by way of non-limiting illustration.

[0060] In particular, the nanoassembly compounds obtained by the previously described preparation were characterized by:

a) their size, using light scattering and electronic microscopy techniques;
b) the degree of dispersion, using light scattering;
c) the number of biotin binding sites available for introducing additional biotinylated functions. This assessment was carried out using the HABA assay, as described in the literature (Green NM *Biochem. J.* 1965, 94: 23C-24C);
d) the speed of aggregation in a buffered medium, using light scattering techniques;
e) their stability to freezing and thawing, and to lyophilization, using light scattering techniques.

Example 1: Preparation and characterization of nanoassemblies obtained with plasmid DNA and avidin in different molar ratios without addition of a surface protector

[0061] The complexes were prepared by mixing aqueous solutions of avidin (Av, Belovo, Belgium) and nucleic acid (pNM, plasmid p-EGFP C1 (Clonetech#6084-1) (4.7 Kb)) in varying molar ratios as given in table 1 below.

[0062] The solutions were left to equilibrate for one hour at 0°C in an ice bath, and after centrifugation (15,000 rpm for 5 minutes), the sizes of the nanoassemblies in solution were analyzed by light scattering using an instrument system consisting of a Spectra Physics Stabilite 2017 laser, a Pacific Scientific "Nicomp 370 Computing Autocorrelator" and a system for temperature controlling the samples.

Table 1. Molar ratio of avidin: plasmid poly-nucleic acid and dimensional characteristics of the particles in deionized water

Sample	Avidin:Nucleobase (NB) in preparation solution (y:n)	Avidin:plasmid (pNB) in solution	Mean diameter of nanoassemblies in solution (nm)
1- Av-pEGFP 3.0	0.125:1	1175:1	106±33
2- Av-pEGFP 2.0	0.0833:1	783:1	124±51
3- Av-pEGFP 1.5	0.0625:1	587:1	144±55
4- Av-pEGFP 0.5	0.0208:1	196:1	148±68

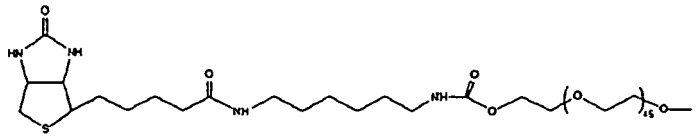
[0063] The size distribution measured on the first sample is given in fig 1. From the figure said assembly can be seen

to be characterized by a moderate polydispersivity. The data in table 1 also show that the sizes and polydispersivity of the nanoassembly increase as the *y*/*n* ratio decreases, indicating that as this value decreases, the degree of condensation of the nucleic acid molecule in the assembly is less. The size variation as *y*/*n* varies is however limited to within the values of about 70 to 200 nm.

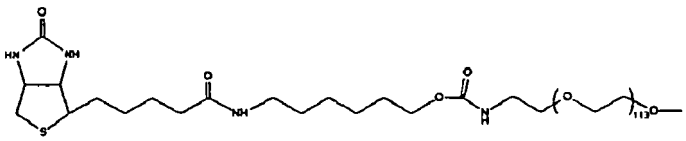
Example 2: Preparation and characterization of nanoassemblies obtained with plasmid DNA, avidin and surface protecting agents

[0064] Different quantities of the various surface protecting agents (B-X_a-PA_b) were added to sample 1 Av-pEGFP 3, prepared as described in example 1, using B-X_a-PA_b: avidin molar ratios varying between 0 and 2.4 as shown in table 2. Five different B-X_a-PA_b agents were used (I, IIa, IIb, III and IV), whose chemical formulas are given as follows:

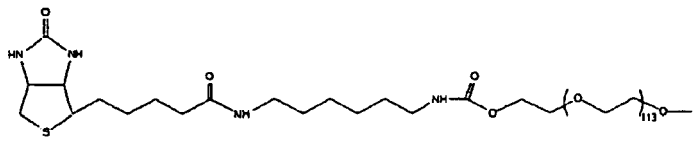
Biotin-CO-NH-(CH2)6-NH-O-CO-mPEG2000 (B-PA I)



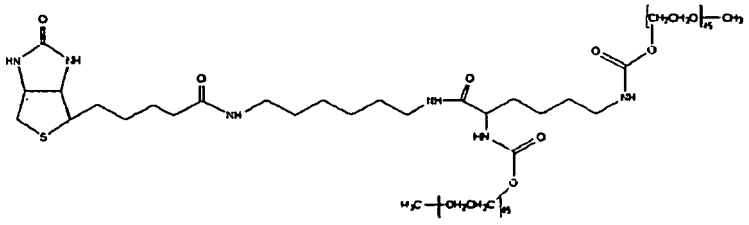
Biotin-CO-NH-(CH_2)$_6$-O-CO-NH-mPEG5000 (B-PA IIa)



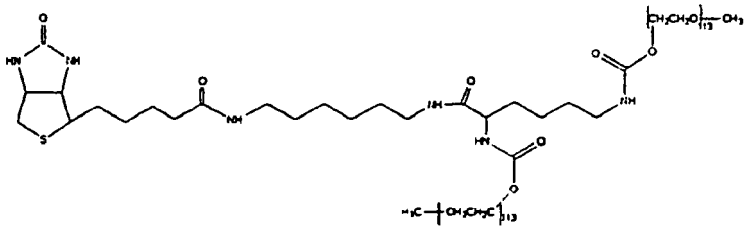
Biotin-CO-NH-(CH2)6-NH-O-CO-mPEG5000 (B-PA IIb)



Biotin-CO-NH-(CH_2)$_6$-NH-Lys-(mPEG2000)$_2$ (B-PA III)



Biotin-CO-NH-(CH_2)$_6$-NH-Lys-(mPEG5000)$_2$ (B-PA IV)



[0065] Said protecting agents B-X_a-PA_b were synthesized and characterized as described below.

[0066] B-X_a-PA_b I: was obtained by condensing the 6-amino-n-hexylamide of biotin with the N-hydroxysuccinimidyl carbonate of monomethoxy polyethylene glycol 2,000 (Monfardini C, Schiavon O et al. Bioconjugate Chemistry 1995, 6: 62-69).

[0067] B-X_a-PA_b IIa: was obtained by condensing α-amino, ω-methoxy-polyethylene glycol 5,000 (Fluka cat#06679) with the N-hydroxysuccinimidyl carbonate of biotinyl-n-hexanolamide (Morpurgo M, Bayer EA et al. J. Biochem. Biophys. Meth. 1999, 38: 17-28).

[0068] B-X_a-PA_b IIb: was obtained in a similar manner to 8-X_a-PA_b I using monomethoxy polyethylene glycol 5,000 instead of 2,000.

[0069] B-X_a-PA_b III: was obtained by condensing the N-hydroxysuccinimidyl carbonate of monomethoxy polyethylene glycol 2,000 with the amino groups of the amide of 2,6 diaminohexanoic acid and with biotinyl-n-hexyldiamine (2,6-diamino-hexanoic acid (6-biotinylamidohexyl)-amide).

[0070] 8-X_a-PA_b IV: was obtained in a similar manner to B-X_a-PA_b III using monomethoxy polyethylene glycol 5,000 instead of 2,000.

[0071] The dimensions of the final nanoassembled complexes in the assembling solutions were measured by light scattering, as described in example 1. The size results are summarized in table 2 and figure 1.

Table 2. Composition of the assembling solutions and dimensional characteristics of the relative nanoassemblies described in example 2.

Sample	Type of B-X_a-PA_b	% occupied BBS	B-X_a-PA_b: avidin (z/y)	Mean diameter (nm)
1- Av-pEGFP 3 (ex.1)	None	0	0	106±33
5-Av-pEGFP3 - B-X_a-PA_b I-20	Biotin-mPEG2000 (I)	20	0.8	88 ±20
6-Av-pEGFP3 - B-X_a-PA_b I-30	idem	30	1.2	96±33
7-Av-pEGFP3-B - X_a-PA_b I-40	idem	40	1.6	85±25
8-Av-pEGFP3 - B-X_a-PA_b I-50	idem	50	2.0	88 ±26
9- Av-pEGFP3 - B-X_a PA_b I-60	idem	60	2.4	92±23
10- Av-pEGFP3 - B-X_a-PA_b IIa-20	Biotin-mPEG5000 (IIa)	20	0.8	91±31
11-Av-pEGFP3 - B-X_a-PA_b IIa-30	idem	30	1.2	87±21
12- Av-pEGFP3 - B-X_a-PA_b IIa-40	idem	40	1.6	90±26
13- Av-pEGFP3 - B-X_a-PA_b IIa-50	idem	50	2.0	88±32
14- Av-pEGFP3 - B-X_a-PA_b IIa-60	idem	60	2.4	96±34
15- Av-pEGFP3 - B-X_a-PA_b IIb-20	Biotin-mPEG5000 (IIb)	20	0.8	111±28
16- Av-pEGFP3 - B-X_a-PA_b IIb-30	idem	30	1.2	109±39
17- Av-pEGFP3 - B-X_a-PA_b IIb-40	idem	40	1.6	112±14
18- Av-pEGFP3 - B-X_a-PA_b IIb-50	idem	50	2.0	115±28

(continued)

Sample	Type of B-X_a-PA_b	% occupied BBS	B-X_a-PA_b: avidin (z/y)	Mean diameter (nm)
19- Av-pEGFP3 - B-X_a-PA_b IIb-60	idem	60	2.4	116±36
20- Av-pEGFP3 - B-X_a-PA_b III -20	Biotin-Lys-$(mPEG2000)_2$ (III)	20	0.8	91±28
21- Av-pEGFP3 - B-X_a-PA_b III-30	idem	30	1.2	91±22
22- Av-pEGFP3 - B-X_a-PA_b III-40	idem	40	1.6	90±27
23- Av-pEGFP3 - B-X_a-PA_b III-50	idem	50	2.0	93 ±32
24- Av-pEGFP3 - B-X_a -PA_b III-60	idem	60	2.4	96±31
25- Av-pEGFP3 - B-X_a-PA_b IV-20	Biotin-Lys-$(mPEG5000)_2$ (IV)	20	0.8	101 ±41
26- Av-pEGFP3 - B-X_a-PA_b IV-30	idem	30	1.2	101±20
27- Av-pEGFP3 - B-X_a-PA_b IV-40	idem	40	1.6	100±24
28- Av-pEGFP3 - B-X_a-PA_b IV-50	idem	50	2.0	101±20
29- Av-pEGFP3 - B-X_a-PA_b IV-60	idem	60	2.4	100±34

[0072] All the samples, initially prepared in salt- and ion-free water, were then diluted in PBS buffer and their aggregation rate was measured by light scattering. The aggregation kinetics are shown in figure 2. It can be seen from the figure that when the B-X_a-PA_bs are introduced onto the surfaces of the nanoassemblies they slow aggregation of the latter, in a salt-containing environment, until they inhibit it completely. The protective efficacy of each B-X_a-PA_b increases with increasing surface concentration of B-X_a-PA_b. The protective efficacy also depends on the type of B-X_a-PA_b, with B-X_a-PA_b IV and B-X_a-PA_b IIb being the most effective of all those tested.

Example 3: Preparation and characterization of nanoassemblies obtained with plasmid DNA and avidin, and purification by ultrafiltration.

[0073] Alexa-Fluor546-biocytin (Molecular probes # A12923) was added to sample 1 Av-pEGFP 3, prepared as described in example 1, in a quantity equal to that needed to saturate 2% of total biotin binding sites. The prepared product was subjected to various ultrafiltration steps using Vivaspin 100K PES membranes (Sartorius, 100,000 Da cut-off) so as to enable monomeric but not nanoassembled avidin to pass through. The avidin concentration in the supernatant and in the filtrate was determined by fluorescence, based on the signal of the Alexa-Fluor546 fluorophore. The supernatant obtained after four ultrafiltration steps was analyzed by light scattering. The avidin:NB ratio in the nanoparticulate system was calculated from the avidin concentration present therein with the assumption that the DNA present was the same as that present prior to ultrafiltration.

Table 3. Composition of the solution containing nanoparticles before and after their purification expressed as avidin: nucleobase ratio *(y/n)*

Sample	Avidin:Nucleobase (NA) (y/n)	Size (nm)
1- Av-pEGFP 3 (ex. 1) before purification	0.125:1	106±33

(continued)

Sample	Avidin:Nucleobase (NA) (y/n)	Size (nm)
1- Av-pEGFP 3 (ex. 1) after purification	0.0375:1	152±68

[0074] From the results given in the table it is apparent that ultrafiltration treatment is able to remove excess monomeric avidin introduced in the preparative stage. Particle sizes are found to be slightly larger than those recorded before purification. This difference (not statistically relevant) is probably ascribable to the lower level of DNA packing recorded as the *y*/*n* ratio in solution decreases, as already described in example 1.

Example 4. Preparation and characterization of nanoassemblies obtained with genomic DNA and avidin in different molar ratios, with and without addition of surface protector.

[0075] The nanocomplexes were prepared by mixing aqueous solutions of avidin (Av, Belovo, Belgium) and fragmented bacterial genomic nucleic acid (Gen pNB, Sigma cat #D1760) (average size about 16-24Kb) in a variable molar ratio (see table 4). The solutions were left for one hour at 0ºC in an ice bath and after centrifugation (15,000 rpm for 5 minutes) the dimensions of the nanoassemblies in solution (table 4) were analyzed by light scattering as described in example 1.

Table 4. Molar ratio of avidin:genomic nucleic acid: B-X_a-PA_b and dimensional characteristics of the particles in deionized water

Sample	Avidin:Nucteobase (NB) in solution	Avidin:nucleic acid (Gen pNB) in solution	B-X_a-PA_b:Av	Mean diameter of nanoassemblies in solution (nm)
30- Av-GenpNB 3	0.125:1	5000:1	0	132±75
31- Av-GenpNB 2	0.0833:1	3333:1	0	133±37
32- Av-GenpNB 1.5	0.0625:1	2500:1	0	160±23
33- Av-GenpNB 0.75	0.0312:1	1666:1	0	140±18
34- Av-GenpNB 3-B-X_a-PA_b IV 30	0.125:1	5000:1	1.2	nd
35- Av-GenpNB 2- B-X_a-PA_b IV 30	0.0833:1	3333:1	1.2	107±50
36- Av-GenpNB 1.5 B- X_a-PA_b IV 30	0.0625:1	2500:1	1.2	112±62
37- Av-GenpNB 0.75-B-X_a-PA_b IV 30	0.0312:1	1666:1	1.2	205±79

Example 5: First comparison of efficiency of avidin in monomeric form and in nanocomplexed form with nucleic acid, in dot blot fluorescent detection

[0076] A biotinylated antibody (anti-hPSMA) was immobilized by spotting (1μl) onto nitrocellulose membranes. The membranes were blocked by immersing into PBS containing 2% w/v of BSA (PBS/BSA) then treated with solutions containing avidin (1.3 μg/ml in PBS/BSA), with previously added biotin-Alexa-Fluor® in a quantity so as to saturate 25% of total biotin binding sites. The avidin in said solutions was used in the monomeric or nanoassembled form (table 5).

Table 5. Compositional characteristics of the detecting avidin solutions used in the dot blot fluorescent assay

Sample	Form of avidin	Avidin :Nucleobase (NB) (y/n)	B-X_a-PA_b:Avidin (z/y)
38- Av.	Monomeric	-	1
39- Av-pEGFP 1.5 B-X_a-PA_b IV 25	Nanoparticulate	0.0625	1

(continued)

Sample	Form of avidin	Avidin :Nucleobase (NB) (y/n)	B-X_a-PA_b:Avidin (z/y)
40- Av-pEGFP 0.75 B-X_a-PA_b IV 25	Nanoparticulate	0.0312	1

[0077] After 2 hours of incubation at ambient temperature, the membranes were washed with PBS and visualized with a fluorescence microscope (figure 3). It can be seen from the figure that nanoassembled avidin is more effective at detecting the immobilized sample on the membrane.

Example 6. Second comparison of efficiency of avidin in monomeric form and in nanocomplexed form with nucleic acid, in dot blot fluorescent detection

[0078] Varying quantities of biotinylated BSA (100, 50, 20, 10, 5, 2 ng of protein corresponding respectively to 10, 5, 2, 1, 0.5 and 0.2 pmoles of biotin/spot) were immobilized by spotting (0.1μl) onto nitrocellulose membranes. The membranes were blocked by immersing into PBS containing 2% w/v of BSA (PBS/BSA) then treated with solutions containing avidin (5 μg/ml in PBS/BSA), with previously added biotin-Alexa-Fluor® in a quantity so as to saturate 40% of total biotin binding sites. The avidin in said solutions was used in the monomeric or nanoassembly form (table 6).

Table 6. Compositional characteristics of the detecting solutions used in the 2nd dot blot fluorescent assay

Sample	Form of avidin	Avidin:Nucleobase (NB) (y/n)	B-X_a-PA_b :Avidin (z/y)
38- Av.	Monomeric	-	1
41- Av-pEGFP 0.5 B-X_a-PA_b IV 25	Nanoparticulate	0.0208	1

[0079] After 2 hours of incubation at ambient temperature, the membranes were washed with PBS then visualized with a fluorescence microscope (figure 4). It can be seen from the figure that the detection limit using monomeric avidin is equal to 1 pmole of biotin, whereas when avidin is used in the nanoparticulate form, biotin is visible even in quantities equal to or less than 0.2 pmoles. The detection limit with the nanoassembly system was not achieved in this experiment.

Example 7. Stability to freezing/thawing of the nanoassemblies in the absence and presence of B-X_a-PA_b agents

[0080] The nanoassembly samples obtained with genomic DNA as given in example 4 were subjected to a freeze-thaw cycle. The size measurements of the particles present in solution after thawing were compared to those of the same preparations before treatment. The results are shown in table 7.

Table 7. Dimensional characteristics of the nanoassemblies before and after freezing/thawing

Sample	Mean diameter of nanoassemblies in solution (nm) before freezing	Mean diameter of nanoassemblies in solution (nm) after freezing and thawing
30- Av-GenpNB 3	132±75	>1000
31- Av-GenpNB 2	133±37	>1000
32- Av-GenpNB 1.5	160±23	>1000
33- Av-GenpNB 0.75	140±18	>1000
34- Av-GenpNB 3-B-X_a-PA_b IV 30	Nd	143±75
35- Av-GenpNB 2- B-X_a-PA_b IV 30	107±50	210±147
36- Av-GenpNB 1.5 B-X_a-PA_b IV 30	112±62	193±155
37- Av-GenpNB 0.75- B-X_a-PA_b IV 30	205±79	129±55

[0081] It can be deduced from the results that the particles devoid of protection agent are not resistant to the freeze-thaw process, subsequent to which they aggregate irreversibly. When instead the protecting agent B-X_a-PA_b is present on

the surface, aggregation is inhibited.

Example 8. Comparison of efficiency of avidin in monomeric form and in nanocomplexed form with nucleic acid, in an enzyme-linked detection system

[0082] Varying quantities of biotinylated-IgG (IgG-B) (0.054, 0.18, 0.6, 2.0, 6.7 and 22.3 ng of protein were immobilized by spotting (0.5μl) onto nitrocellulose membranes. The membranes were blocked by immersing into PBS containing 2% w/v of BSA (PBS/BSA) then treated with solutions containing avidin (5 μg/ml in PBS/BSA), The avidin in said solutions was used in the monomeric or nanoassembly form (table 7). After 1 hour of incubation at ambient temperature, the membranes were washed with PBS and incubated (1 h) with biotin-horseradish peroxidase (Sigma-Aldrich, 4 μg/ml in PBS/BSA). Membrane development was carried out with diaminobenzidine (DAB). Spot density was analyzed through the ImageJ software and translated into the graph of figure 5. It can be seen from the figure that the detection limit using monomeric avidin is equal to 0.6 ng of IgG-B, whereas when avidin is used in the nanoparticulate form, IgG is visible even in quantities equal to or less than 0.054 ng. The detection limit with the nanoassembly system was not achieved in this experiment.

Table 7. Compositional characteristics of the detecting avidin solutions used in the enzyme-linked dot blot assay

Sample	Form of avidin	Avidin:Nucleobase (NB) (y/n)	B-X_a-PA_b:Avidin (z/y)
38- Av.	Monomeric	-	1
42- Av-pEGFP 0.95 B-X_a-PA_b IV 25	Nanoparticulate	0.0396	1

Claims

1. Nanoassembled complexes comprising a nucleus obtained by means of high affinity interaction between one or more avidin tetrameric units and one or more nucleic acid molecules, wherein said nucleus is stabilized by a biotinylated surface protecting agent, represented by the general formula (I)

$$NB_nAv_y(B\text{-}X_a\text{-}PA_b)_z \qquad (I)$$

wherein:

- NB are the single nucleobases of a single or double stranded nucleic acid;
- Av is an avidin tetrameric unit;
- B-X_a-PA_b is the biotinylated surface protecting agent in which PA is a polymer unit having at least one or two functionalizable residues of which one binds, by a covalent bond either directly or through a spacer X, to a biotin residue B by means of carboxyl functional group of said residue B;
- n is a number varying from 16 to 10,000,000;
- y is an integer equal to or greater than 1 and being relative to n is comprised from $(0.0001)\cdot n$ to $(0.0454)\cdot n$ with the proviso that if a value comprised in the range $(0.0001\text{-}0.0454)\cdot$n is less than 1, then *y* is equal to 1;
- *z* is an integer equal to or greater than 1 and being relative to *y* is comprised from $(0.02)\cdot y$ to $(4)\cdot y$ with the proviso that if a value comprised in the range $(0.02\text{-}4)\cdot y$ is less than 1, then *z* is equal to 1;
- *a* is a number comprised from 0 to 50;
- *b* is a number comprised from 1 to 128.

2. Nanoassembled complexes according to claim 1, wherein *n* is comprised from 30 to 100,000.

3. Nanoassembled complexes according to claim 1, wherein *y* is comprised from $(0.0001)\cdot n$ to $(0.0357)\cdot n$.

4. Nanoassembled complexes according to claim 1, wherein z is comprised from $(0.4)\cdot y$ to $(4)\cdot y$.

5. Nanoassembled complexes according to claim 1, wherein a is comprised from 0 to 10.

6. Nanoassembled complexes according to claim 1, wherein the single or double stranded nucleic acid is selected

from the group consisting of any sequence of a single or double stranded deoxyribonucleic acid (DNA) polymer, any sequence of a ribonucleic acid (RNA) polymer in single stranded form or hybridized with a RNA or a complementary DNA chain and a sequence thereof in which a part of or all the bases have been chemically modified.

7. Nanoassembled complexes according to claim 1, wherein the polymer unit PA is selected from the group consisting of polyethylene oxide or polyethylene glycol (PEO or PEG) optionally substituted, a polyoxyethylene and polyoxypropylene copolymer (PEO-PPO), polyvinylpyrrolidone (PVP), polyacryloylmorpholine (PacM), a polyoxamine, a polylactide (PLA), a polyglycolide (PLG), a copolymer of lactic acid and glycolic acid (PLGA).

8. Nanoassembled complexes according to claim 7, wherein the polymer unit PA has a molecular weight comprised from 400 to 40,000.

9. Nanoassembled complexes according to claim 7, wherein the polymer unit PA is a substituted polyoxyethylene (PEO) represented by the formula (III)

$$-(CR^1R^2CR^3R^4O)_m- \qquad \text{(III)}$$

wherein:

R^1, R^2, R^3 and R^4 can be independently equal to hydrogen, alkyl, cycloalkyl, aryl, alkenyl, alkinyl, alcoxyl, thioalkoxy, aryloxy and thioaryloxy
m is an integer from 2 to 900.

10. Nanoassembled complexes according to claim 1, wherein if *b* is other than 1 the polymer units PA are bound together by a further polyfunctional ligand having at least 3 functional groups selected from the group consisting of lysine, glutamic acid, aspartic acid, cysteine and a dendrimer, of which one binds to biotin B directly or indirectly through a spacer X and the remaining functional groups bind to the polymer units PA.

11. Nanoassembled complexes according to claim 1, wherein if the polymer unit PA has two functionalizable residues, the second of said residues is free or protected by a protecting group or covalently bound to a compound selected from ligands, sugars, chromophores or fluorophores, drugs, chelating agents for radionuclides, antibodies, peptides, proteins and enzymes.

12. Nanoassembled complexes according to claim 1, wherein the spacer X is a bifunctional compound represented by the general formula (II)

$$Y\text{-}R\text{-}Y' \qquad \text{(II)}$$

wherein:

- Y, Y' being the same or different from each other are -COO-; -NH -; O-; SO_2-; -S-; -SO-; -CO-; -COS-; -NH-CO-; -NH-COO; HN-SO-NH-;
- R can be an alkyl, an alkenyl, an alkinyl, a cycloalkyl, or an aryl, with a carbon atom number of from 1 to 20 optionally substituted.

13. Nanoassembled complexes according to any one of the preceding claims, wherein z is less than 4, and wherein they further comprise biotinylated compounds different from the protecting agent B-X_a-PA_b and being the same as or different from each other.

14. Nanoassembled complexes according to claims 1-13 for use in the biotechnological field or in nanomedicine.

15. Nanoassembled complexes according to claim 14 for use as a detection means for *in vitro* and *in vivo* diagnostics.

16. Nanoassembled complexes according to claim 14 for use as an amplification means for the localization and patterning of surface molecules.

17. Nanoassembled complexes according to claim 14 for use as a means for targeting drugs.

18. Nanoparticles comprising nanoassembled complexes according to any of the claims 1-17.

19. Nanoparticles according to the preceding claim having a dimension of at least 10 nm and up to 1,000 nm.

20. Method for preparing the nanoassembled complexes according to claim 1 comprising at least the steps of:

a) preparing the self-assembled primary nucleus NB_nAv_y by mixing avidin Av with the nucleic acid in stoichiometrically predefined molar ratios of nucleobases NB to avidin Av; and
b) mixing the biotinylated surface protecting agent B-X_a-PA_b with the primary nucleus NB_nAv_y obtained in step a), the latter being added in stoichiometrically predefined molar ratios of avidin Av to the biotin B of B-X_a-PA_b; or

a) preparing the conjugated compound $Av_y(B\text{-}X_a\text{-}PA_b)_z$ by mixing avidin Av with the surface protecting agent B-X_a-PA_b in stoichiometrically predefined molar ratios of the biotin B of B-X_a-PA_b to avidin Av; and
b) mixing the conjugated compound $Av_y(B\text{-}X_a\text{-}PA_b)_z$ obtained in step a) with nucleic acid added in stoichiometrically predefined molar ratios of avidin Av to nucleobases NB;

wherein the mixing of nucleobases with avidin, free or conjugated, is carried out in salt-free aqueous solutions at a temperature from 0ºC to 50ºC and the stoichiometrically predefined molar ratios of nucleobases NB to avidin are comprised from 0.44 to 0.0001.

21. Method for preparing the nanoassembled complexes according to claim 20, wherein addition of the surface protecting agent B-X_a-PA_b is carried out in aqueous solutions at a temperature from 0º to 50ºC and the stoichiometrically predefined molar ratios of avidin to B-X_a-PA_b are comprised from 4 to 0.02.

22. Method for preparing the nanoassembled complexes according to claim 20 comprising the additional step of purifying the compound obtained in step a) or the nanoassembled complex obtained in step b) from monomeric avidin.

Patentansprüche

1. Nanoassemblierte Komplexe umfassend einen durch hochaffine Wechselwirkung zwischen einer oder mehreren Avidin-Tetramereinheiten und einem oder mehreren Nukleinsäuremolekülen erhaltenen Kern, wobei der Kern durch ein biotinyliertes Oberflächenschutzmittel stabilisiert ist, weclehs durch die allgemeine Formel (I)

$$NB_nAv_y(B\text{-}X_a\text{-}PA_b)_z \qquad (I),$$

dargestellt wird,
wobei:

- NB die einzelnen Nukleobasen einer einzel- oder doppelsträngigen Nukleinsäure sind;
- Av eine Avidin-Tetramereinheit ist;
- B-X_a-PA_b das biotinylierte Oberflächenschutzmittel ist, in welchem PA eine Polymereinheit mit wenigstens einem oder zwei funktionalisierbaren Resten ist, von denen einer durch eine kovalente Bindung entweder direkt oder durch einen Spacer X an einen Biotinrest B durch eine Carboxylfunktionalität von dem Rest B bindet, ist;
- n eine Zahl von 16 bis 10.000.000 ist;
- y eine ganze Zahl von gleich oder größer als 1 ist und bezogen auf n von $(0{,}0001)\cdot n$ bis $(0{,}0454)\cdot n$ beträgt mit der Bedingung, dass, wenn ein Wert in dem Bereich $(0{,}0001\text{-}0{,}0454)\cdot n$. kleiner als 1 ist, y dann gleich 1 ist;
- z eine ganze Zahl von gleich oder größer als 1 ist und bezogen auf y von $(0{,}02)\cdot y$ bis $(4)\cdot y$ beträgt mit der Bedingung, dass, wenn ein Wert in dem Bereich $(0{,}02\text{-}4)\cdot y$ kleiner als 1 ist, z dann gleich 1 ist;
- a eine Zahl von 0 bis 50 ist;
- b eine Zahl von 1 bis 128 ist.

2. Nanoassemblierte Komplexe nach Anspruch 1, wobei n von 30 bis 100.000 beträgt.

3. Nanoassemblierte Komplexe nach Anspruch 1, wobei y von $(0{,}0001)\cdot n$ bis $(0{,}0357)\cdot n$ beträgt.

4. Nanoassemblierte Komplexe nach Anspruch 1, wobei z von $(0{,}4)\cdot y$ bis $(4)\cdot y$ beträgt.

5. Nanoassemblierte Komplexe nach Anspruch 1, wobei a von 0 bis 10 beträgt.

6. Nanoassemblierte Komplexe nach Anspruch 1, wobei die einzel- oder doppelsträngige Nukleinsäure aus der Gruppe bestehend aus einer beliebigen Sequenz eines einzel- oder doppelsträngigen Desoxyribonukleinsäure-(DNS)-Polymers, einer beliebigen Sequenz eines Ribonukleinsäure-(RNS)-Polymers in einzelsträngiger Form oder hybridisiert mit einer RNS oder einer komplementären DNS-Kette und einer Sequenz davon, in welcher ein Teil oder alle der Basen chemisch modifiziert worden sind, ausgewählt ist.

7. Nanoassemblierte Komplexe nach Anspruch 1, wobei die Polymereinheit PA aus der Gruppe bestehend aus Polyethylenoxid oder Polyethylenglykol (PEO oder PEG), welche optional substituiert ist, einem Polyoxyethylen- und Polyoxypropylencopolymer (PEO-PPO), Polyvinylpyrrolidon (PVP), Polyacryloylmorpholin (PacM), einem Polyoxamin, einem Polylactid (PLA), einem Polyglycolid (PLG), einem Copolymer aus Milchsäure und Glykolsäure (PLGA) ausgewählt ist.

8. Nanoassemblierte Komplexe nach Anspruch 7, wobei die Polymereinheit PA ein Molekulargewicht von 400 bis 40.000 aufweist.

9. Nanoassemblierte Komplexe nach Anspruch 7, wobei die Polymereinheit PA ein substituiertes Polyoxyethylen (PEO) ist, welches durch die Formel (III)

$$-(CR^1R^2CR^3R^4O)_m- \qquad \text{(III)},$$

dargestellt ist,
wobei:

R^1, R^2, R^3 und R^4 unabhängig voneinander gleich Wasserstoff, Alkyl, Cycloalkyl, Aryl, Alkenyl, Akinyl, Alkoxyl, Thioalkoxy, Aryloxy und Thioaryloxy sein können,
m eine ganze Zahl von 2 bis 900 ist.

10. Nanoassemblierte Komplexe nach Anspruch 1, wobei, wenn b von 1 verschieden ist, die Polymereinheiten PA durch einen weiteren polyfunktionalen Liganden mit wenigstens 3 funktionellen Gruppen ausgewählt aus der Gruppe bestehend aus Lysin, Glutaminsäure, Asparaginsäure, Cystein und einen Dendrimer, von denen eine an Biotin B direkt oder indirekt durch einen Spacer X bindet und die verbleibenden funktionellen Gruppen an die Polymereinheiten PA binden, miteinander verbunden sind.

11. Nanoassemblierte Komplexe nach Anspruch 1, wobei, wenn die Polymereinheit PA zwei funktionalisierbare Reste aufweist, der zweite der Reste frei ist oder durch eine Schutzgruppe geschützt ist oder kovalent an eine Verbindung ausgewählt aus Liganden, Zuckern, Chromophoren oder Fluorophoren, Arzneimitteln, Chelatisierungsmitteln für Radionuklide, Antikörpern, Peptiden, Proteinen und Enzymen ist.

12. Nanoassemblierte Komplexe nach Anspruch 1, wobei der Spacer X eine bifunktionelle Verbindung, dargestellt durch die allgemeine Formel (II)

$$Y\text{-}R\text{-}Y' \qquad \text{(II)},$$

ist,
wobei:

- Y, Y' gleich oder voneinander verschieden sind und jeder - COO-; -NH-; -O-; SO_2-; -S-; -SO-; -CO-; -COS-; -NH-CO-; -NH-COO-; HN-SO-NH- ist;
- R ein Alkyl, ein Alkenyl, ein Alkinyl, ein Cycloalkyl oder ein Aryl mit einer Anzahl von Kohlenstoffatomen von 1 bis 20, das optional substituiert ist, sein kann.

13. Nanoassemblierte Komplexe nach einem der vorhergehenden Ansprüche, wobei z kleiner als 4 ist und wobei diese des Weiteren biotinylierte Verbindungen, welche von dem Schutzmittel B-X_a-PA_b verschieden und gleich oder voneinander verschieden sind, enthalten.

14. Nanoassemblierte Komplexe nach den Ansprüchen 1-13 zur Verwendung auf dem biotechnologischen Gebiet oder

in der Nanomedizin.

15. Nanoassemblierte Komplexe nach Anspruch 14 zur Verwendung als ein Detektionsmittel für die in *vitro*- und in vivo-Diagnostik.

16. Nanoassemblierte Komplexe nach Anspruch 14 zur Verwendung als ein Verstärkungsmittel für die Lokalisierung und Musterbildung von Oberflächenmolekülen.

17. Nanoassemblierte Komplexe nach Anspruch 14 zur Verwendung als Mittel zum Targeting von Arzneimitteln.

18. Nanopartikel umfassend nanoassemblierte Komplexe nach einem der Ansprüche 1-17.

19. Nanopartikel nach dem vorhergehenden Anspruch mit einer Dimension von wenigstens 10 nm und bis zu 1.000 nm.

20. Verfahren zum Herstellen von nanoassemblierten Komplexen nach Anspruch 1 umfassend wenigstens die Schritte:

a) Herstellen des selbstassemblierten primären Kerns NB_nAv_y durch Mischen von Avidin Av mit der Nukleinsäure in stöchiometrisch vorgegebenen molaren Verhältnissen der Nukleobasen NB zu Avidin Av; und
b) Mischen des biotinylierten Oberflächenschutzmittels B-X_a-PA_b mit dem in dem Schritt a) erhaltenen primären Kern NB_nAv_y, wobei der letztere in stöchiometrisch vorgegebenen molaren Verhältnissen von Avidin Av zu dem Biotin B von B-X_a-PA_b zugegeben wird; oder

a) Herstellen der konjugierten Verbindung Av_y(B-X_a-PA_b) durch Mischen von Avidin Av mit dem Oberflächenschutzmittel B-X_a-PA_b in stöchiometrisch vorgegebenen molaren Verhältnissen des Biotins B von B-X_a-PA_b zu Avidin Av; und
b) Mischen der in dem Schritt a) erhaltenen konjugierten Verbindung Av_y(B-X_a-PA_b)$_z$ mit Nukleinsäure, welche in stöchiometrisch vorgegebenen molaren Verhältnissen von Avidin Av zu Nukleobasen NB zugegeben wird;

wobei das Mischen von Nukleobasen mit Avidin, frei oder konjugiert, in salzfreien wässrigen Lösungen bei einer Temperatur von 0°C bis 50°C durchgeführt wird und die stöchiometrisch vorgegebenen molaren Verhältnisse der Nukleobasen NB zu Avidin von 0,44 bis 0,0001 betragen.

21. Verfahren zum Herstellen der nanoassemblierten Komplexe nach Anspruch 20, wobei die Zugabe des Oberflächenschutzmittels B-X_a-PA_b in wässrigen Lösungen bei einer Temperatur von 0° bis 50°C durchgeführt wird und die stöchiometrisch vorgegebenen molaren Verhältnisse von Avidin zu B-X_a-PA_b von 4 bis 0,02 betragen.

22. Verfahren zum Herstellen der nanoassemblierten Komplexe nach Anspruch 20, welches den zusätzlichen Schritt des Reinigens der in Schritt a) erhaltenen Verbindung oder des in Schritt b) erhaltenen nanoassemblierten Komplexes von monomerem Avidin umfasst.

Revendications

1. Complexes nano-assemblés comprenant un noyau obtenu au moyen d'une interaction à haute affinité entre un ou plusieurs motifs tétramères d'avidine et une ou plusieurs molécules d'acide nucléique, ledit noyau étant stabilisé par un agent de protection de surface biotinylé représenté par la formule générale (I)

$$NB_nAv_y(B\text{-}X_a\text{-}PA_b)_z \qquad (I)$$

dans laquelle :

- NB représente les nucléobases individuelles d'un acide nucléique monocaténaire ou bicaténaire ;
- Av est un motif tétramère d'avidine ;
- B-X_a-PA_b est l'agent de protection de surface biotinylé dans lequel PA est un motif polymère contenant au moins un ou deux résidus fonctionnalisables parmi lesquels un est lié par une liaison covalente, soit directement, soit par l'intermédiaire d'un espaceur X, à un résidu B de biotine au moyen d'un groupe fonctionnel carboxyle dudit résidu B ;

- *n* est un nombre variant de 16 à 10 000 000 ;
- *y* est un nombre entier supérieur ou égal à 1 et, par rapport à *n*, est compris entre (0,0001)·*n* et (0,0454)·*n*, à condition que si une valeur comprise dans la plage (0,0001-0,0454)·*n* est inférieure à 1, y soit égal à 1 ;
- *z* est un nombre entier supérieur ou égal à 1 et, par rapport à *y*, est compris entre (0,02)·*y* et (4)·*y*, à condition que si une valeur comprise dans la plage (0,02-4)·*y* est inférieure à 1, *z* soit égal à 1 ;
- *a* est un nombre compris entre 0 et 50 ;
- *b* est un nombre compris entre 1 et 128.

2. Complexes nano-assemblés selon la revendication 1, dans lesquels *n* est compris entre 30 et 100 000.

3. Complexes nano-assemblés selon la revendication 1, dans lesquels *y* est compris entre (0,0001)·*n* et (0,0357)·*n*.

4. Complexes nano-assemblés selon la revendication 1, dans lesquels *z* est compris entre (0,4)·*y* et (4)·*y*.

5. Complexes nano-assemblés selon la revendication 1, dans lesquels a est compris entre 0 et 10.

6. Complexes nano-assemblés selon la revendication 1, dans lesquels l'acide nucléique monocaténaire ou bicaténaire est choisi dans le groupe constitué de n'importe quelle séquence d'un polymère d'acide désoxyribonucléique monocaténaire ou bicaténaire (ADN), de n'importe quelle séquence d'un polymère d'acide ribonucléique (ARN) sous forme monocaténaire ou hybridé avec un ARN ou une chaîne d'ADN complémentaire et d'une séquence de celles-ci dans laquelle une partie ou la totalité des bases a été chimiquement modifiée.

7. Complexes nano-assemblés selon la revendication 1, dans lesquels le motif polymère PA est choisi dans le groupe constitué d'un poly(oxyde d'éthylène) ou d'un polyéthylène glycol (PEO ou PEG) facultativement substitué, d'un copolymère de polyoxyéthylène et de polyoxypropylène (PEO-PPO), d'une polyvinylpyrrolidone (PVP), d'une polyacryloylmorpholine (PacM), d'une polyoxamine, d'un polylactide (PLA), d'un polyglycolide (PLG), d'un copolymère d'acide lactique et d'acide glycolique (PLGA).

8. Complexes nano-assemblés selon la revendication 7, dans lesquels le motif polymère PA possède un poids moléculaire compris entre 400 et 40 000.

9. Complexes nano-assemblés selon la revendication 7, dans lesquels le motif polymère PA est un polyoxyéthylène (PEO) substitué représenté par la formule (III)

$$-(CR^1R^2CR^3R^4O)_m- \qquad \text{(III)}$$

dans laquelle :

R^1, R^2, R^3 et R^4 peuvent représenter indépendamment un hydrogène, un alkyle, un cycloalkyle, un aryle, un alcényle, un alcynyle, un alcoxyle, un thioalcoxy, un aryloxy et un thioaryloxy
m est un nombre entier allant de 2 à 900.

10. Complexes nano-assemblés selon la revendication 1, dans lesquels si b est différent de 1, les motifs polymères PA sont liés les uns aux autres au moyen d'un ligand polyfonctionnel supplémentaire contenant au moins 3 groupes fonctionnels choisis dans le groupe constitué de la lysine, de l'acide glutamique, de l'acide aspartique, de la cystéine et d'un dendrimère, parmi lesquels un est lié à une biotine B directement ou indirectement par l'intermédiaire d'un espaceur X et les groupes fonctionnels restants sont liés aux motifs polymères PA.

11. Complexes nano-assemblés selon la revendication 1, dans lesquels si le motif polymère PA contient deux résidus fonctionnalisables, le second desdits résidus est libre ou protégé par un groupe protecteur ou lié par liaison covalente à un composé choisi parmi les ligands, les sucres, les chromophores ou les fluorophores, les médicaments, les agents de chélation pour radionucléides, les anticorps, les peptides, les protéines et les enzymes.

12. Complexes nano-assemblés selon la revendication 1, dans lesquels l'espaceur X est un composé bifonctionnel représenté par la formule générale (II)

$$Y\text{-}R\text{-}Y' \qquad \text{(II)}$$

dans laquelle :

- Y, Y' sont identiques ou différents et sont -COO- ; -NH- ; -O- ; SO_2- ; -S- ; -SO- ; -CO- ; -COS- ; -NH-CO- ; -NH-COO- ; HN-SO-NH- ;
- R peut être un alkyle, un alcényle, un alcynyle, un cycloalkyle ou un aryle, contenant 1 à 20 atomes de carbone facultativement substitués.

13. Complexes nano-assemblés selon l'une quelconque des revendications précédentes, dans lesquels z est inférieure à 4, et dans lesquels ils comprennent en outre des composés biotinylés qui sont différents de l'agent de protection $B\text{-}X_a\text{-}PA_b$ et qui sont identiques les uns aux autres ou différents les uns des autres.

14. Complexes nano-assemblés selon les revendications 1 à 13, pour une utilisation dans le domaine de la biotechnologie ou en nanomédecine.

15. Complexes nano-assemblés selon la revendication 14, pour une utilisation comme moyen de détection pour un diagnostic *in vitro* et *in vivo.*

16. Complexes nano-assemblés selon la revendication 14, pour une utilisation comme moyen d'amplification pour la localisation et la typification de molécules surface.

17. Complexes nano-assemblés selon la revendication 14, pour une utilisation comme moyen pour vectoriser les médicaments.

18. Nanoparticules comprenant les complexes nano-assemblés selon l'une quelconque des revendications 1 à 17.

19. Nanoparticules selon la revendication précédente, dont la dimension est d'au moins 10 nm et d'au plus 1 000 nm.

20. Procédé de préparation des complexes nano-assemblés selon la revendication 1, comprenant au moins les étapes suivantes :

a) la préparation du noyau primaire auto-assemblé NB_nAv_y par le mélange d'avidine Av avec l'acide nucléique à des rapports molaires stoechiométriques prédéfinis entre les nucléobases NB et l'avidine Av ; et
b) le mélange de l'agent de protection de surface biotinylé $B\text{-}X_a\text{-}PA_b$ avec le noyau primaire NB_nAv_y obtenu dans l'étape a), ce dernier étant ajouté à des rapports molaires stoechiométriques prédéfinis entre l'avidine Av et la biotine B de $B\text{-}X_a\text{-}PA_b$; ou

a) la préparation du composé conjugué $Av_y(B\text{-}X_a\text{-}PA_b)_z$ par le mélange d'avidine Av avec l'agent de protection de surface $B\text{-}X_a\text{-}PA_b$ à des rapports molaires stoechiométriques prédéfinis entre la biotine B de $B\text{-}X_a\text{-}PA_b$ et l'avidine Av ; et
b) le mélange du composé conjugué $Av_y(B\text{-}X_a\text{-}PA_b)_z$ obtenu dans l'étape a) avec un acide nucléique ajouté à des rapports molaires stoechiométriques prédéfinis entre l'avidine Av et les nucléobases NB ;

dans lequel le mélange des nucléobases avec l'avidine, libre ou conjuguée, est réalisé dans des solutions aqueuses exemptes de sel à une température de 0 °C à 50 °C et les rapports molaires stoechiométriques prédéfinis entre les nucléobases NB et l'avidine sont compris entre 0,44 et 0,0001.

21. Procédé de préparation des complexes nano-assemblés selon la revendication 20, dans lequel l'ajout de l'agent protecteur de surface $B\text{-}X_a\text{-}PA_b$ est réalisé dans des solutions aqueuses à une température de 0 °C à 50 °C et les rapports molaires stoechiométriques prédéfinis entre l'avidine et $B\text{-}X_a\text{-}PA_b$ sont compris entre 4 et 0,02.

22. Procédé de préparation des complexes nano-assemblés selon la revendication 20, comprenant l'étape supplémentaire de séparation par purification du composé obtenu dans l'étape a) ou du complexe nano-assemblé obtenu dans l'étape b) de l'avidine monomère.

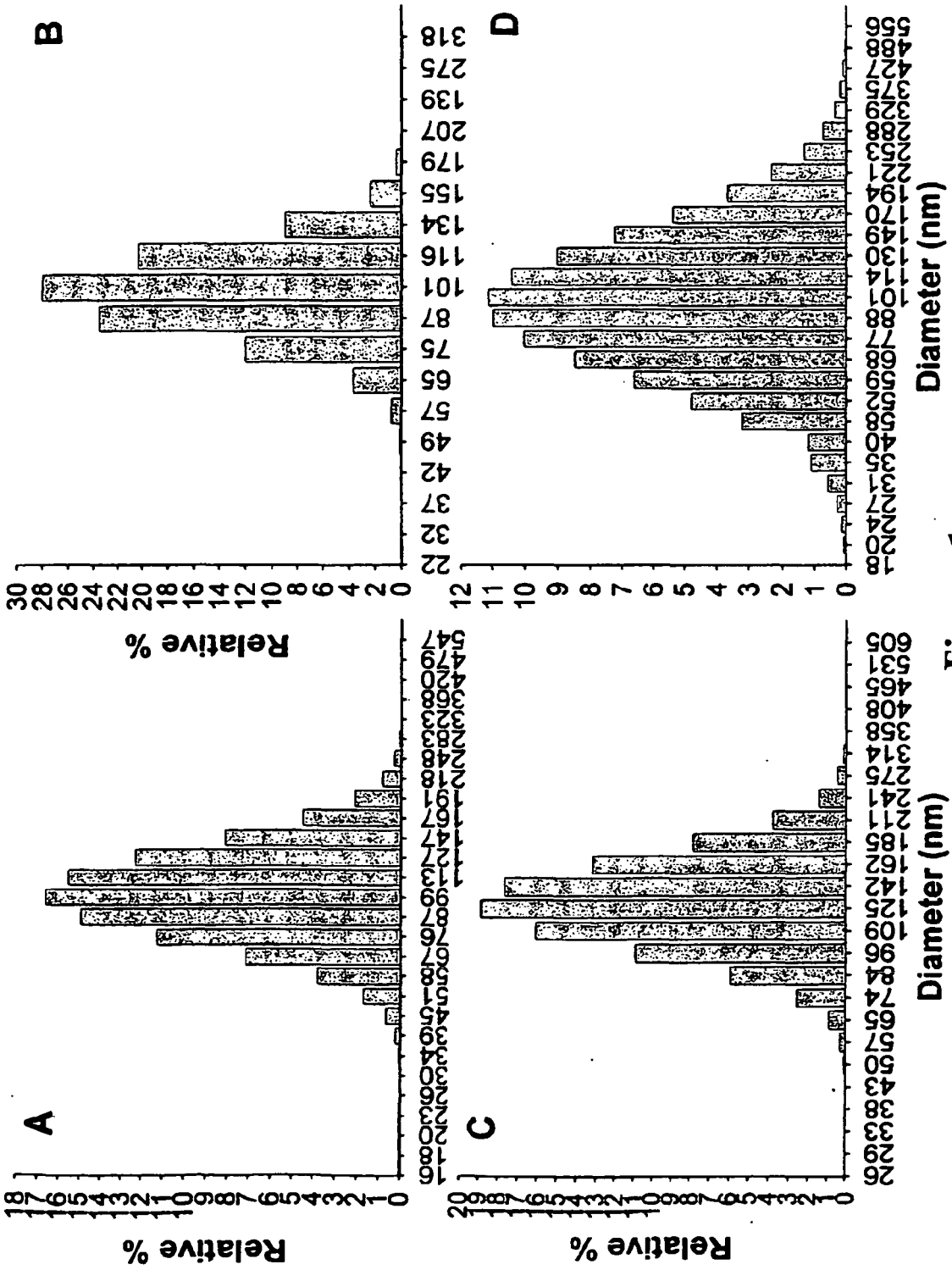


Figure 1

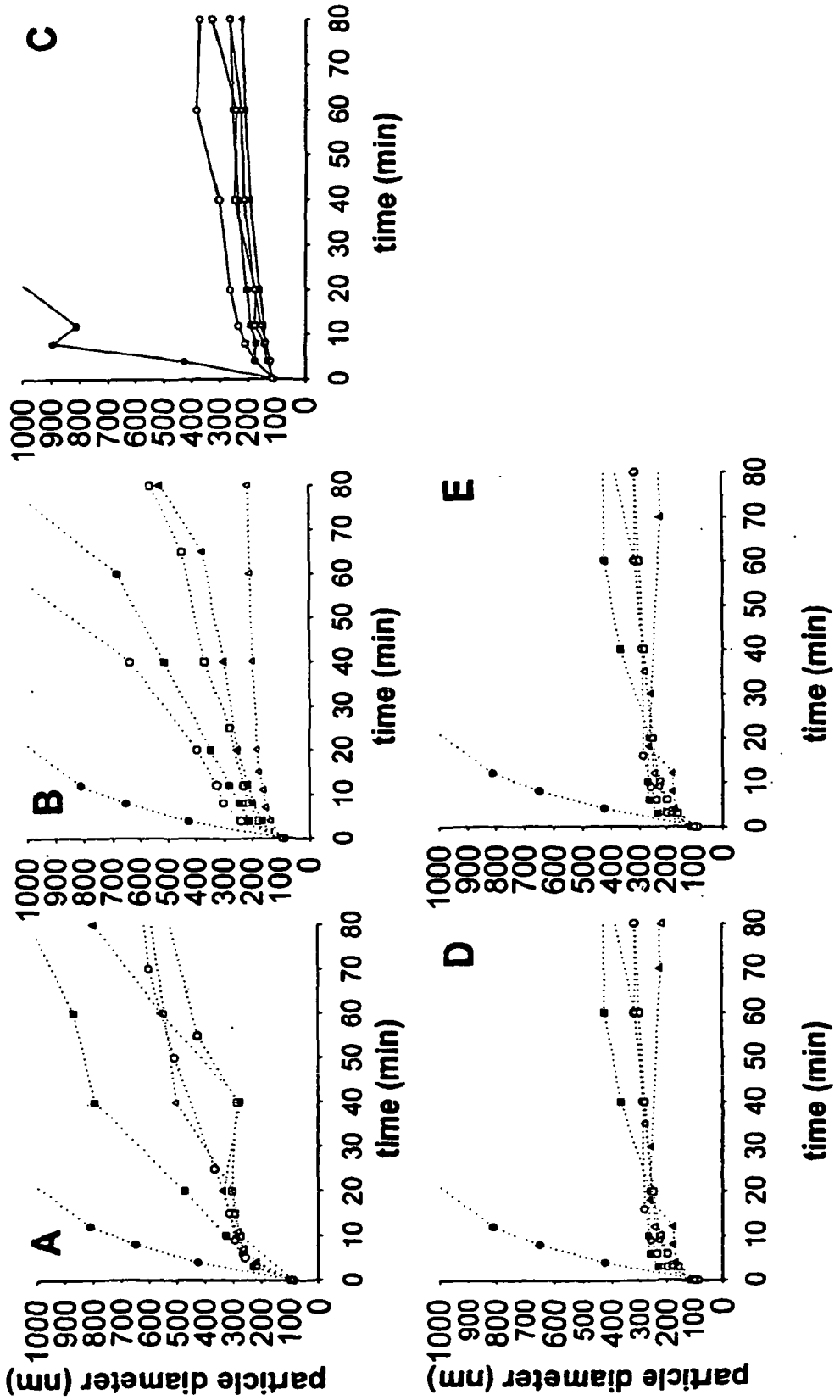

A
B
C
D
E
particle diameter (nm)
time (min)
0
100
200
300
400
500
600
700
800
900
1000
0
10
20
30
40
50
60
70
80

Figure 2

Figure 3

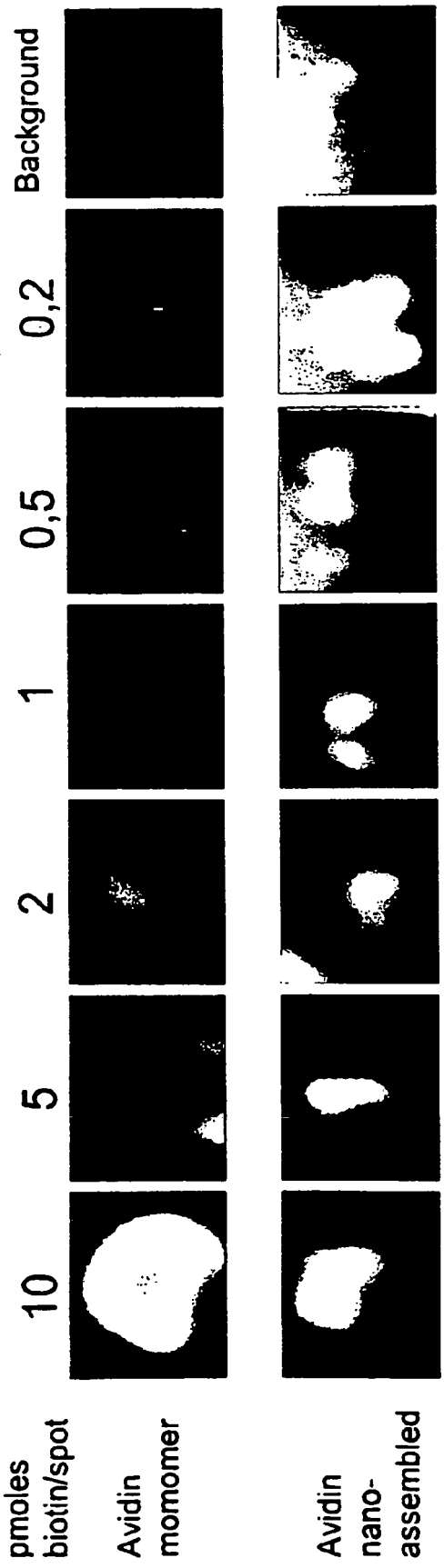

pmoles biotin/spot
10
5
2
1
0,5
0,2
Background
Avidin momomer
Avidin nano-assembled

Figure 4

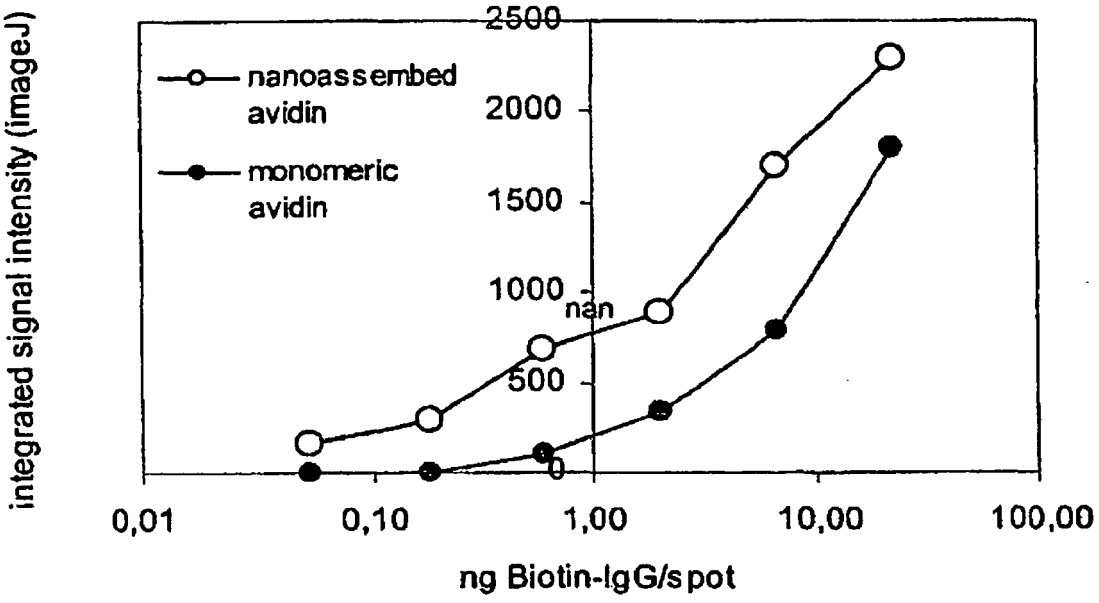

integrated signal intensity (imageJ)
2500
2000
1500
1000
500
0
nan
nanoassembed avidin
monomeric avidin
0,01
0,10
1,00
10,00
100,00
ng Biotin-IgG/spot

Figure 5

REFERENCES CITED IN THE DESCRIPTION

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