(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 December 2016 (01.12.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/189141 A1

(51) International Patent Classification:
G01N 33/543 (2006.01) *B01D 15/38* (2006.01)

(21) International Application Number:
PCT/EP2016/062021

(22) International Filing Date:
27 May 2016 (27.05.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
15169505.3 27 May 2015 (27.05.2015) EP

(71) Applicants: **UNIVERSITAT AUTONOMA DE BARCELONA** [ES/ES]; Edifici A, Campus universitari s/n, 08193 Bellaterra (Cerdanyola del Valles) (ES). **UNIVERSIDADE ESTADUAL PAULISTA JULIO DE MESQUITA FILHO** [BR/BR]; Rua Quirino de Andrade, 215, 01049-010 Sao Paulo (BR).

(72) Inventors: **PIVIDORI GURGO, Maria Isabel**; UAB, Edifici C - Facultat de Ciències, Departament de Química, Àrea de Química Analítica, Carrer del Til.lers, Campus universitari s/n, 08193 Bellaterra (Cerdanyola del Valles) (ES). **TABOADA SOTOMAYOR, Pilar**; Rua prof Francisco Degni, 55, Quitandinha, Araraquara, 14800 060 Sao Paulo (BR).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2016/189141 A1

(54) **Title**: METHOD FOR THE DETERMINATION OF TARGETS OF BIOTINYLATED MOLECULES

(57) **Abstract**: The present invention relates to a method for the determination of targets of biotinylated molecules. The method is based on an assay using magnetic molecular imprinted polymers (MIP), wherein biotin is used as template. The method shows a very low limit of detection and may be used for the determination of biotin and further targets of biotinylated molecules in complex samples including clinical samples, foods and environmental samples, for example.

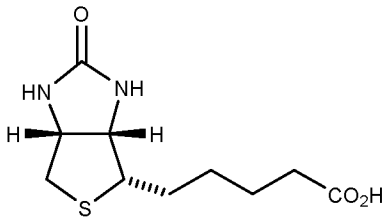
METHOD FOR THE DETERMINATION OF TARGETS OF BIOTINYLATED MOLECULES

Technical Field

The present invention relates to method for the immobilization and determination of targets of biotinylated molecules, for example biotin, which allows direct and indirect separation and purification under magnetic actuation of a wide range of target molecules (e.g. nucleic acids, antibodies, proteins, lectins, carbohydrates, among others).

Technical Background

Biotin is 5-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl]pentanoic acid, which shows the following chemical structure:



Biotin is also called vitamin H, vitamin B_7 or vitamin B_8. It is part of the B complex group of vitamins, which help the body to convert food (carbohydrates) into fuel (glucose), which is used to produce energy. These B vitamins also help the body to metabolize fats and protein. It is disclosed that B vitamins are needed for healthy skin, hair, eyes, and liver, and that they also help the nervous system function properly.

Biotin is a water-soluble vitamin, which is not stored by the body. However, bacteria in the intestine can make biotin. It is also available in small amounts a number of foods, for example, brewer's yeast; cooked eggs, especially egg yolk; sardines; nuts (almonds, peanuts, pecans, walnuts) and nut butters; soybeans; other legumes (beans, blackeye peas); whole grains; cauliflower; bananas; and mushrooms.

Symptoms of biotin deficiency include hair loss, dry scaly skin, cracking in the corners of the mouth (called cheilitis), swollen and painful tongue that is magenta in colour (glossitis), dry eyes, loss of appetite, fatigue, insomnia, and depression.

A variety of assays for biotin have been disclosed in the prior art.

Traditionally, biotin concentration was determined by a microbiological assay based on biotin-requiring microorganisms such as *Lactobacillus arabinosus* and the alga *Amphidinium carterae* as disclosed in Wright *et al.*, Determination of biotin with *Lactobacillus arabinosus*, Proc. Soc. Exp. Biol. Med., 1944, 56, 95-98, and in Carucci,. A.F., *Amphidinium carterae* assay for biotin, 1970, 18A, 379-383.

A spectrophotometric method for determining biotin is disclosed in Neidbala *et al.*, A spectrometric assay for nanogram quantities of biotin and avidin, J. Biochem. Biophys. Methods, 1986, 13, 205-210.

A reciprocal enzyme assay for biotin is disclosed in Bayer *et al.*, A sensitive enzyme assay for biotin, avidin and streptavidin, Anal. Biochem., 1986, 154, 364-370.

In Schray *et al.*, Determination of avidin and biotin by fluorescence polarization, Anal. Chem., 1988, 60, 853-855, it is disclosed a fluorescent polarization method for determining biotin.

However, those methods are either rapid but less sensitive or have a high sensitivity, but require more complicated and time-consuming protocols.

In Chang *et al.*, Determination of biotin concentration by a competitive enzyme-linked immunosorbent assay (ELISA) method, J. Biochem. Biophys. Methods, 1994, 29, 321-329, it is disclosed an assay for determining biotin, which is completed within 6 h with sensitivities of approximately 1 pg/ml for biotin in a simple aqueous medium and 5 pg/ml in complex media like bacterial growth media. The method is based on the measurement of the horseradish peroxidase conjugated to streptavidin (streptavidin-HRP) activities after streptavidin-HRP has reacted with biotin in sample solutions.

In Piletska *et al.*, Biotin –specific synthetic receptors prepared using molecular imprinting, Anal. Chim. Acta, 2004, 504(1), 179-183, it is disclosed new molecularly imprinted polymers (MIPs) specific for biotin prepared from three functional monomers: methacrylic acid (MAA), 2-(trifluoromethyl)acrylic acid (TFAA) and 2-acrylamido-2-methyl-propanesulfonic acid (AMPSA). It is disclosed that the imprinted polymers were photografted to the surface of polystyrene microspheres in water, and that the dissociation constants for all MIPs were 1.4-16.8 nM, which was considered sufficient for an analytical application in assays and separation..

In Kergaravat *et al.*, Biotin determination in food supplements by an electrochemical magneto biosensor, Talanta, 2012, 97, 484-490, it is disclosed

electrochemical magneto biosensor for the rapid determination of biotin food samples. The affinity reaction was performed on streptavidin-modified magnetic microbeads as a solid support in a direct competitive format. The biotinylated horseradish peroxidase enzyme (biotin-HRP) competes with free biotin in the sample for the binding sites of streptavidin on the magnetic microbeads. The modified magnetic beads were then easily captured by a magneto graphite–epoxy composite electrode and the electrochemical signal was based on the enzymatic activity of the HRP enzyme under the addition of H_2O_2 as the substrate and o-phenylendiamine as cosubstrate. The response was electrochemically detected by square wave voltammetry. The limit of detection was $8.4x10^{-8}$ mol/L of biotin (20 µg/L) with a dynamic range from 0.94 to $2.4x10^{-7}$ mol/L. Biotin fortified commercial dietary supplement and infant formula samples were evaluated obtaining good performances in the results. Total time of analysis was 40 min per 20 assays.

In International patent application WO-A-2014/030002 it is disclosed molecularly imprinted polymers (MIP) targeting biotin, a biotin derivative, a biotin analogue or a biotinylated molecules, which are prepared from a polymerisable monomer, which is prepared from i) an isophthalic acid derivative or a pyridine2,6-dicarboxylic acid derivative, ii) a vinyl-2-aminopyridine derivative, and optionally iii) a 2-aminopyridine derivative, to define a recognition cleft for biotin, a biotin derivative, a biotin analogue or a biotinylated molecules. In the document there are no data regarding to the performance of such MIP.

The highly specific interaction of biotin with the proteins avidin and streptavidin is a useful tool in a wide range of detection systems. Avidin and streptavidin binds four moles of biotin per mole of protein. Avidin and streptavidin have similar affinities for biotin, although they are vastly different in other respects. The two proteins have different molecular weights and electrophoretic mobility. The extraordinary affinity of avidin/streptavidin for biotin (K_a = 10^{15} M^{-1}) is the strongest known non-covalent interaction of a protein and ligand and allows biotin-containing molecules in a complex mixture to be discretely bound with avidin conjugates.

Avidin is a protein originally isolated from chicken egg white. It is also found in the tissues of birds. Avidin is tetrameric with four identical subunits having a combined molecular weight of about 67,000. The bond formation between biotin and avidin is very rapid and, once formed, is unaffected by wide extremes of pH, temperature, organic solvents and other denaturing agents. While free avidin is

inactivated at 85"C, the avidin-biotin complex can withstand brief exposures to 132° C. This complex is not significantly affected by pH values between 2 and 13 or by concentrations of guanidine hydrochloride up to 8 M at neutral pH's.

Streptavidin is a biotin-binding protein isolated from culture broth of *Streptomyces avidinii*. Streptavidin binds four moles of biotin per mole of protein. This corresponds to 16.5-18 mg of biotin bound per gram of streptavidin. This tetrameric protein was originally characterized as having a molecular weight of 60000 daltons.

Some applications in which the strept(avidin)-biotin interaction has been used include ELISA; immunohistochemical staining; Western, Northern and Southern blotting; immunoprecipitation; cell-surface labelling; affinity purification; and fluorescence-activated cell sorting (FACS).

Since biotin is a relatively small molecule, it can be conjugated to many proteins (peptides, antibodies, enzymes, receptors) and other biomolecules including nucleic acids, lectins, among others, without significantly altering their biological activity. The biomolecule can be reacted with several molecules of biotin that, in turn, can each bind a molecule of avidin. This greatly increases the sensitivity of many assay procedures.

The valeric acid side chain of the biotin molecule can be derivatized to incorporate various reactive groups that are used to attach biotin to other molecules. Using these reactive groups, biotin can be easily attached to most proteins and other molecules. Biotinylation reagents are available for targeting a variety of functional groups, including primary amines, sulfhydryl groups, carbohydrates and carboxyl groups.

The most frequently used biotinylation reagents, *N*-hydroxysuccinimide (NHS) esters and *N*-hydroxysulfosuccinimide (sulfo-NHS) esters, react with primary amines. The functional groups available on the surface of the protein to be biotinylated may not be known. However, with most proteins, it is safe to assume that primary amines are available and accessible for biotinylation. The likelihood that primary amines are present increases as molecular weight increases.

Antibodies are biotinylated more often than any other class of proteins and it is advantageous to be biotinylated in a manner that will maintain immunological reactivity.

To take advantages of this interaction for separation, purification, of biotinylated biomolecules, as well as the downstream application, there are a lot of

commercial available avidin/streptavidin modified solid supports, including magnetic particles, for example, Streptavidin MagBeads (GenScript), Dynabeads® (Dynal), hyBeads® Streptavidin (Hyglos), Adembeads (Ademtech), or modified microplates (Pierce). The main drawback of this support is that as the avidin/streptavidin should be kept refrigerated.

Although there are several available methods for the determination of biotin, there remains a need to provide an alternative method for the detection of biotin in samples with lower detection limit and simplicity of the protocol and for the determination of further targets of biotinylated molecules.

Object of the invention

The object of the present invention is a method for the determination of targets of biotinylated molecules, in particular, a method for the determination of biotin.

Figures

Figure 1

In figure 1 it is shown a specific embodiment of a method for preparing magnetic biotin imprinted polymer particles.

Figure 2

In figure 2 it is represented schematically the method for the determination of biotin as disclosed in Examples 1 and 6.

Figure 3

In figure 3 it is represented the raw data for the competition assay in two steps performed by the incubation of biotin-HRP followed by the incubation of biotin ranging from 0.2 pg/mL to 1.9 ng/mL, as disclosed in Example 1. Error bar illustrates the standard deviation for the samples (n=4). Only one replicate was rejected among 56 replicates. In the X-axis it is represented the concentration of biotin in ng/mL and in the Y-axis the absorbance read at 450 nm.

Figure 4

In figure 4 it is represented the fitted curve adjusted to a nonlinear regression (Sigmoidal dose-response with variable slope) of the raw data for the

competition assay in two steps, as disclosed in Example 1, according to y ={(A−B)/[1 + 10 exp((log C−log X)x D)]}+ B, where A is the maximal absorbance at 450 nm, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, X is the biotin concentration in ng/mL and D is the slope at the inflection point of the sigmoid curve. LOD values were obtained as 90% of A value. The competition assay in two steps was, performed by the incubation of biotin-HRP, followed by the incubation of biotin ranging from 0.2 pg/mL to 1.9 ng/mL. Error bar illustrates the standard deviation for the samples (n=4). Only one replicate was rejected among 56 replicates, being R^2 = 0.9782. In the X-axis it is represented as the logarithm of the biotin concentration (ng/ mL) and in the Y-axis it is the normalized optical signal (Absorbance at 450 nm).

Figure 5

In figure 5 it is represented schematically the method for the separation and purification of biotinylated biomolecules

Figure 6

In figure 6 it is represented schematically the method for immobilization and determination of biotin-HRP as biotinylated biomolecule, as disclosed in Examples 2 and 4.

Figure 7

In figure 7 it is represented the fitted curve adjusted to a nonlinear regression (one site-binding/hyperbola) of the raw data for the separation and detection of biotin-HRP in one step from 6.125 to 100 ng/mL as disclosed in Example 2. Error bar illustrates the standard deviation for the samples (n=3). In the X-axis it is represented the concentration of biotin-HRP expressed in ng/mL, and in the Y-axis it is represented the absorbance at 450 nm using de magnetic-MIP (▲), as disclosed in Example 2. The results obtained using non-imprinted polymers (NIP,■) under analogous conditions are also shown.

Figure 8

In figure 8 it is represented schematically the characterization of the binding of biotinylated biomolecules on the magnetic-MIP by confocal microscopy.

Panel A shows the binding on the magnetic-MIP of Atto 665-biotin, while panel B shows the binding of a G4 biotinylated dendrimer, followed by the incubation with streptavidin-Cy5. In both cases, the characterization was performed by confocal microscopy, as disclosed in Example 3.

Figure 9

In figure 9 it is represented the characterization of the binding of biotinylated biomolecules on the magnetic-MIP by confocal microscopy as disclosed in Example 3. The binding on the magnetic-MIP of Atto 665-biotin is shown in panels B and C, as well as the negative control (panel A). The binding of a G4 biotinylated dendrimer, followed by the incubation with streptavidin-Cy5 is shown in panels E and F, as well as the negative control (panel D).

Figure 10

In figure 10 it is represented the magnetic actuation of the magnetic-MIP in TRIS buffer at t=0 (panel A), after 5 seconds (panel B) and after 30 seconds (Panel C). It is observed that the magnetic-MIP is highly magnetizable.

Figure 11

In figure 11 it is represented a schematic procedure for the magneto-actuated immunoassay based on magnetic-MIP for the detection of double-tagged DNA, as disclosed in Example 5.

Figure 12

In figure 12 it is represented the fitted curve adjusted to a nonlinear regression (one site-binding/hyperbola) of the raw data for the separation and detection of *E. coli* double tagged DNA amplicon in one step from 0.4 to 207 ng/mL as disclosed in Example 5. Error bar illustrates the standard deviation for the samples (n=3). In the X-axis it is represented the concentration of *E. coli* double tagged DNA amplicon expressed in ng/mL, and in the Y-axis it is represented the absorbance at 450 nm using de magnetic-MIP (■), as disclosed in Example 5. The results obtained using non-imprinted polymers (NIP, ▲,) under analogous conditions are also shown.

Detailed Description of the Invention

The object of the present invention is a method for the determination of targets of biotinylated molecules, which comprises the step of immobilizing a biotinylated molecule by incubation of magnetic biotin imprinted polymer particles with such biotinylated molecule.

Surprisingly, the authors of the invention have developed magnetic biotin imprinted polymer particles, which show a significant immobilization capacity of biotinylated molecules, giving rise to a cheaper and a robust method (it is not required to be stored at 4° C) with high binding capacity for the separation and purification under magnetic actuation of a wide range of biotinylated molecules (e.g. nucleic acids, antibodies, enzymes, proteins, lectins, dyes, dendrimers), and their downstream application including determination of their specific targets. In the case of biotinylated-HRP, the method allows the detection of that molecule with a limit of detection of 1.054 ng/mL in a fast way (30 minutes) by means of an immunoassay (ELISA type). Moreover, the method allows the determination of biotin with a significantly low limit of detection of 0.857 pg/mL and in short time (1 h) by means of a competitive immunoassay (ELISA type). In the case of the determination of *E. coli* double tagged DNA amplicon, the method allows the detection of DNA with a limit of detection of 1.28 ng/mL. The magnetic-MIP is highly magnetizable, as shown in Figure 10.

In the present description, as well as in the claims, the singular forms “a” or “one” include also the reference to the plural unless the context clearly indicates otherwise.

In the present invention the wording “determination of targets” means the quantitative or qualitative determination of targets, in short “quantification of targets”.

Biotinylated molecules

Biotinylation is defined usually as the process of incorporating biotin into biological material, that is covalently attaching biotin to a protein, nucleic acid or other biological molecule. Biotinylation is rapid, specific and is unlikely to perturb the natural function of the molecule due to the small size of biotin.

Within the scope of this invention, a biotinylated molecule is a molecule that contains biotin attached covalently. The method of the invention is suitable for determining targets of any biotinylated molecule.

In a preferred embodiment, the immobilization takes place incubating a biotinylated molecule selected from the group consisting of: biotin-enzyme conjugate, preferably biotin-HRP, biotinylated DNA, biotinylated RNA, biotinylated oligonucleotides, biotinylated antibodies, biotinylated peptides, biotinylated proteins, biotinylated dyes, biotinylated dendrimers and biotinylated lectins.

Biotinylated molecules are available commercially, for example, through Thermo Fisher Scientific, Merck-Millipore, Sigma-Aldrich or SeraCare, or they may be prepared using biotinylation methods well known in the scientific literature, for example, Kay *et al.*, High-throughput Biotinylation of Proteins, Methods Mol. Biol., 2009, 496, 185-196; or Huang *et al.*, Binding of Biotinylated DNA to Streptavidin-Coated Polystyrene Latex: Effects of Chain Length and Particle Size, Anal. Biochem., 1996, 237, 115-122.

Targets of biotinylated molecules

The targets of biotinylated molecules are of different nature, depending on the biotinylated molecules. In the context of the present invention, biotin is considered the target of a biotin-enzyme conjugate, preferably the target of biotin-HRP conjugate (biotin-HRP).

Further targets of biotinylated molecules are: complementary DNA for a biotinylated DNA, antigen for a biotinylated antibody, substrate for a biotinylated enzyme, cofactor for a biotinylated enzyme, glycoprotein for a biotinylated lectin, and glycolipid for a biotinylated lectin.

Magnetic biotin imprinted polymer particles

A molecularly imprinted polymer (MIP) is a polymer that has been processed using the molecular imprinting technique which leaves cavities in polymer matrix with affinity to a chosen "template" molecule. The process usually involves initiating the polymerization of functional monomers in the presence of a template molecule that is extracted afterwards, thus leaving complementary cavities behind. These polymers have affinity for the original molecule. Published works on the topic date to the 1930s.

The molecular imprinting process can be carried out either by means of covalent molecular imprinting, which involves the preparation of a polymerizable derivative of the template, or non-covalent molecular imprinting, as disclosed in Diaz-

Garcia *et al.*, Chapter 2. Molecularly Imprinted Polymers for Optical Sensing Devices, in R. Narayanaswamy, O. S. Wolfbeis, Optical Sensors. Industrial Environmental and Diagnostic Applications, Springer-Verlag, Berlin, 2004 [ISBN 978-3-642-07421-9].

In the present invention it is preferably used a non-covalent imprinting, which relies on the self-assembly of functional monomers around the template (biotin) in the pre-polymerization mixture in a way that maximizes the binding interactions between the two species.

Magnetic biotin imprinted polymer particles are particles, which comprise a magnetic particle as a core and a polymeric shell where the biotin is imprinted.

Magnetic particles may be prepared according to well-known procedures disclosed in the literature, for example, according to the procedure disclosed in Thach *et al.*, Size Controlled Magnetite Nanoparticles and Their Drug Loading Ability, J. Korean Phys. Soc., 2008, 52(5), 1332-1335.

A suitable process for preparing magnetic particles includes co-precipitation of a Fe^{2+}/Fe^{3+} mixed solution prepared from iron salts, for example, sulfates or chlorides, with an aqueous solution of ammonia, being the molar ratio $Fe^{2+}:Fe^{3+}$ = 1:2. After the reaction is it obtained a black precipitate of magnetite Fe_3O_4, which can be separated from the soluble substances by magnetic decantation. Magnetite particles obtained according to this process show an average diameter of about 15 nm.

In the method of the invention the magnetic particle is preferably a magnetite particle.

Magnetic biotin imprinted polymer particles may be prepared according to well-known procedures disclosed in the literature, for example, according to the procedure disclosed in the International patent application WO-A-96/37527 or in the US patent US6316235. Polymerization can take place in bulk, in suspension, in emulsion or it can be core-shell polymerization. Preferably it is used a core-shell polymerization on the magnetite magnetic particles.

The polymeric shell on the magnetic particles is prepared by a process comprising the following steps:

a) mixing biotin with one or more functional monomers in a porogenic solvent,
b) polymerizing the mixture of step a) with one or more crosslinking monomers in the presence of an initiator and magnetic particles, and
c) extracting the biotin from the polymeric shell by an extraction solvent.

The polymeric shell on the magnetic particles can be prepared by well-known polymerization techniques using reactant monomers capable of acting as functional monomers in polymerization, and reactant monomers capable of acting as crosslinking monomers in polymerization.

The functional monomer suitable for preparing the polymeric shell is selected from the group consisting of acrylic acid, methacrylic acid, acrylic acid C_1-C_6 alkyl esters, methacrylic acid C_1-C_6 alkyl esters, 4-vinylbenzoic acid, 4-ethylstyrene, acrylamide, methacrylamide, 2-acrylamido-2-methylpropane sulfonic acid (AMPS®), vinylsuccinic acid, vinylpyrrolidone, 2-vinylpyridine, 4-vinylpyridine, and mixtures thereof. Preferably it is used acrylic acid as functional monomer.

The crosslinking monomer suitable for preparing the polymeric shell is selected from the group consisting of *o*-divinylbenzene, *m*-divinylbenzene, *p*-divinylbenzene, ethylene glycol diacrylate, di(ethylene glycol) diacrylate, tetra(ethylene glycol) diacrylate, ethylene glycol dimethacrylate, di(ethylene glycol) dimethacrylate, tri(ethylene glycol) dimethacrylate, *N*,*N*-methylenebisacrylamide, *N*,*N*-(1,2-dihydroxyethylene)bisacrylamide, *N*-(1-hydroxy-2,2-dimethoxyethyl)acrylamide, *N*,*N'*-phenylenediacrylamide, trimethylolpropane trimethacrylate, pentaerythritol triacrylate, pentaerythritol tetraacrylate, *N*,*O*-bisacryloyl-L-phenylalaninol, 3,5-bisacrylamidobenzoic acid, 2,6-bisacrylamidopyridine, and mixtures thereof. Preferably it is used ethylene glycol dimethacrylate as crosslinking monomer.

The initiator for the polymerization reaction is selected from the group consisting of 2,2'-azobisisobutyronitrile (AIBN), 4,4-azobis(4-cyanovaleric acid), 1,1'-azobis(cyclohexanecarbonitrile), , *tert*-amyl peroxybenzoate, benzoyl peroxide, 2,2-bis(*tert*-butylperoxy)butane, 1,1-bis(tert-butylperoxy)-cyclohexane, *tert*-butyl peroxide, cumene hydroperoxide, potassium persulfate, and 2,4- pentanedione peroxide. Preferably the initiator is AIBN.

The polymerization takes place usually in the presence of a porogenic solvent, which is selected from the group consisting of ethanol, methanol, decanol, dioxane, tetrahydrofurane, acetone, acetonitrile, and mixtures thereof. Preferably it is used ethanol as porogenic solvent.

The ratio biotin:functional monomer is comprised usually between 1:1 to 1:8, preferably between 1:2 to 1:6, more preferably between 1:3 to 1:5, and more preferably it is 1:4.

In a preferred embodiment, biotin and the functional monomer are maintained at least 4 hours, preferably 6 hours, more preferably at least hours, and more preferably at least 12 hours before adding the magnetic particles.

In a preferred embodiment, magnetic particles are previously functionalized in order to be polymerized with the functional monomers and the crosslinking monomers in the polymerization step as disclosed, for example in Kong *et al.*, Synthesis and characterization of the core–shell magnetic molecularly imprinted polymers (Fe_3O_4@MIPs) adsorbents for effective extraction and determination of sulfonamides in the poultry feed, J. Chromatogr. A, 2012, 1245, 8-16.

In a preferred embodiment the magnetic biotin imprinted polymer particles are prepared by a process comprising the following steps:

a) preparing magnetite particles from co-precipitation of a Fe^{2+}/Fe^{3+} mixed solution,

b) reacting magnetite particles with tetraethoxysilane,

c) reacting the hydroxyl-modified magnetite particles prepared in step 2) with a (meth)acrylic group containing silanizating agent, preferably methacryloxypropyltrimethyloxysilane,

d) polymerizing the silanized magnetite particles prepared in step 3) with a functional monomer, preferably acrylic acid, in the presence of biotin, a crosslinking monomer, preferably ethylene glycol dimethacrylate, a radical initiator, preferably AIBN, and a porogenic solvent, preferably ethanol, and

e) extracting the particles prepared in step 4) with a solvent comprising a C_1-C_4 alkyl alcohol, preferably methanol, and acetic acid, and more preferably in a methanol: acetic acid ratio = 9:1 (v/v).

The magnetic biotin imprinted particles are preferably dried at 40° C under vacuum.

The ratio biotin:functional monomer is comprised usually between 1:1 to 1:8, preferably between 1:2 to 1:6, more preferably between 1:3 to 1:5, and more preferably it is 1:4.

In a preferred embodiment, biotin and the functional monomer are maintained at least 4 hours, preferably 6 hours, more preferably at least hours, and more preferably at least 12 hours before adding the magnetic particles, eventually silanized magnetic particles.

The reaction of magnetite particles with tetraethoxy silane (TEOS), also named tetraethyl orthosilicate, modifies the surface of the particles introducing hydroxyl groups. Those particles coated with TEOS retain the spherical shape and show an average diameter comprised between 200 nm and 565 nm. Those coated particles are further silanized with an unsaturated silanizing agent. Among the silanizing agents methacryloxypropyltrimethyloxysilane, also named 3-(trimethoxy-silyl)propyl methacrylate, is preferred.

The silanized magnetite particles contain an unsaturated functional group, which is suitable to be polymerized with one or more functional monomers, i.e. α,β-unsaturated monomers, under radical conditions in the presence of one or more cross-linking monomers and biotin, which is the template molecule to be imprinted in the polymer particles. The magnetic biotin imprinted polymer particles prepared according to the process of the invention are spherical-like particles showing some aggregations and an average diameter comprised of 990 nm and a large surface area according to BET analysis: 119.97 m^2/g.

Incubation with biotinylated molecules and determination of the target thereof

In an embodiment the method for the determination of the target of a biotinylated molecule, according to the invention, comprises the following steps:

1) immobilizing a biotinylated molecule by incubation of magnetic biotin imprinted polymer particles with a biotinylated molecule,
2) optionally incubating the particles of step 1) with the target of the biotinylated molecule, and optionally with a labelled probe,
3) optionally carrying out an enzymatic reaction with the incubated particles, and
4) reading out.

In a preferred embodiment, the target of the biotinylated molecule is biotin.

The method for the determination of a target of a biotinylated molecule, according to the invention, comprises preferably the following steps:

1) immobilizing a biotinylated molecule by incubation of magnetic biotin imprinted polymer particles with a biotinylated molecule,
2) incubating the particles of step 1) with the target of the biotinylated molecule and optionally with a labeled probe,

3) optionally carrying out an enzymatic reaction with the incubated particles, and
4) reading out.

After incubation step preferably it is carried out the steps of applying a magnetic field and washing of the particles by removing the supernatant.

The labeled probe is mainly a complementary labeled DNA, the specific labeled antibody against the antigen, or any other labeled biomolecules able to specifically interact with the target of the biotinylated molecule.

The label of the labelled probe can be a fluorescent dye, an enzyme, or a radioactive molecule. The optional enzymatic reaction is performed only if the label is an enzyme. The reading out can be optical, electrochemical, fluorescent, chemiluminescent, or radioactive.

In a preferred embodiment of the invention, the incubation takes place with biotinylated molecules selected from the group consisting of biotinylated DNA, biotinylated RNA, biotinylated oligonucleotides, biotinylated antigens, biotinylated antibodies, biotinylated peptides, biotinylated proteins, biotin-enzyme conjugates, biotinylated dyes, biotinylated dendrimers and biotinylated lectins. In a more preferred embodiment the incubation takes place with a biotin-enzyme conjugate, preferably with biotin-HRP.

The method of the present invention is also suitable for the determination of biotinylated biomolecules, which comprises:

1) immobilizing a biotinylated molecule by incubation of magnetic biotin imprinted polymer particles with a biotinylated molecule,
2) optionally carrying out an enzymatic reaction with the incubated particles, and
3) reading out.

The biotinylated molecule in this instance is labelled with a fluorescent dye, an enzyme, or a radioactive molecule. The optional enzymatic reaction is performed only if the label is an enzyme. The reading out can be optical, electrochemical, fluorescent, chemiluminescent, or radioactive. After the incubation step preferably it is carried out the steps of applying a magnetic field and washing of the particles by removing the supernatant.

In a preferred embodiment of the present invention, the method refers to the determination of the target of biotinylated DNA, which comprises:

1) immobilizing a biotinylated molecule by incubation of magnetic biotin imprinted polymer particles with a biotinylated molecule, which is biotinylated capture DNA,
2) incubating the particles of step 1) with the target of the biotinylated DNA, which is the complementary DNA, and a labeled DNA probe,
3) optionally carrying out an enzymatic reaction with the incubated particles, and
4) reading out.

Biotinylated capture DNA is defined as DNA labeled with biotin.

After the incubation step preferably it is carried out the steps of applying a magnetic field and washing of the particles by removing the supernatant.

The label of the labeled probe can be a fluorescent dye, an enzyme, or a radioactive molecule. The optional enzymatic reaction is performed only if the label is an enzyme. The reading out can be optical, electrochemical, fluorescent, chemiluminescent, or radioactive.

In another preferred embodiment of the present invention, the method refers to the determination of the target of a biotinylated antibody, which comprises:

1) immobilizing a biotinylated molecule by incubation of magnetic biotin imprinted polymer particles with a biotinylated molecule, which is biotinylated capture antibody,
2) incubating the particles of step 1) with the target of the labeled antibody, which is the antigen, and a labeled antibody.
3) optionally carrying out an enzymatic reaction with the incubated particles, and
4) reading out.

After the incubation step preferably it is carried out the steps of applying a magnetic field and washing of the particles by removing the supernatant.

The label of the labeled antibody can be a fluorescent dye, an enzyme, or a radioactive molecule. The optional enzymatic reaction is performed only if the label is an enzyme. The reading out can be optical, electrochemical, fluorescent, chemiluminescent, or radioactive.

In another preferred embodiment of the present invention, the method refers to the determination of the target of a biotinylated enzyme, which is a substrate of the enzyme, which comprises:

1) immobilizing a biotinylated molecule by incubation of magnetic biotin imprinted polymer particles with a biotinylated molecule, which is a biotinylated enzyme,

2) incubating the particles of step 1) with the substrate and the cofactor of the biotinylated enzyme in an enzymatic reaction, and
3) reading out.

After the incubation step preferably it is carried out the steps of applying a magnetic field and washing of the particles by removing the supernatant.

The reading out can be optical, electrochemical, fluorescent, chemiluminescent, or radioactive.

In another preferred embodiment of the present invention, the method refers to the determination of the target of a biotinylated enzyme, which is a cofactor or inhibitor of the enzyme, which comprises:

1) immobilizing a biotinylated molecule by incubation of magnetic biotin imprinted polymer particles with a biotinylated molecule, which is a biotinylated enzyme,
2) incubating the particles of step 1) with the substrate and the cofactor or inhibitor of the biotinylated enzyme in an enzymatic reaction, and
3) reading out.

After the incubation step preferably it is carried out the steps of applying a magnetic field and washing of the particles by removing the supernatant.

The optional enzymatic reaction is performed only if the label is an enzyme. The reading out can be optical, electrochemical, fluorescent, chemiluminescent, or radioactive.

In a preferred embodiment of the present invention, the method refers to the determination of biotin by a competitive approach, which comprises:

1) immobilizing a biotinylated molecule by incubation of magnetic biotin imprinted polymer particles with a biotinylated molecule,
2) incubating the particles of step 1) with biotin to perform the competition,
3) optionally carrying out an enzymatic reaction with the incubated particles, and
4) reading out.

The biotinylated molecule in this instance is labeled with a fluorescent dye, an enzyme, or a radioactive molecule. The optional enzymatic reaction is performed only if the label is an enzyme. The reading out can be optical, electrochemical, fluorescent, chemiluminescent, or radioactive.

After the incubation step preferably it is carried out the steps of applying a magnetic field and washing of the particles by removing the supernatant.

In a more preferred embodiment, the process of the present invention is a method for the determination of biotin, which comprises:

1) immobilizing a biotinylated molecule by incubation of magnetic biotin imprinted polymer particles with a biotinylated molecule, which is biotin-HRP conjugate,
2) incubating the particles of step 1) with a sample comprising biotin,
3) carrying out an enzymatic reaction with the incubated particles, and
4) optical reading out.

After the incubation step preferably it is carried out the steps of applying a magnetic field and washing of the particles by removing the supernatant.

Surprisingly, the authors of the invention have developed a competitive immunoassay (ELISA type) for detecting biotin with a limit of detection of 0.857 pg/mL, which is a significantly low limit of detection and being very fast (1 h). In the case of the determination of *E. coli* double tagged DNA amplicon, the method allows the detection of DNA with a limit of detection of 1.28 ng/mL. In the case of biotinylated-HRP, the method allows the detection of that molecule with a limit of detection of 1.054 ng/mL in a fast way (30 minutes).

Incubation with biotinylated molecules

In the method of the invention for the determination of the target of a biotinylated molecule, the magnetic biotin imprinted polymer particles are incubated with biotinylated molecules. In the incubation process, biotinylated molecules are captured by the magnetic biotin imprinted polymer particles occupying the binding sites of the polymer.

Biotinylated molecules are obtained by biotinylation of the molecule by covalent attachment of biotin, using commercial available kits, for instance EZ-LinkTM NHS-PEG4 Biotinylation Kit, EZ-Link BMCC-Biotin, among many others (Pierce).

The incubation takes usually place in a microplate, for example, a polypropylene microtiter plate available, for example, through the companies Corning or Eppendorf or in Eppendorf® tubes.

The incubation process is carried out usually at room temperature, and for a period of time from 10 min to 45 min, preferably 30 min. After the incubation a 96-well magnet plate separator or a magnet for Eppendorf® tubes is usually positioned under the microtiter plate or Eppendorf® tubes until pellet formation on the bottom corner, followed by supernatant separation.

The incubation process takes place usually in the presence of a buffer, for example, citrate, PBS, borate, or TRIS. Preferably it is used citrate or TRIS.

PBS is phosphate buffered saline, an aqueous buffer solution, which comprises disodium hydrogen phosphate, dipotassium hydrogen phosphate, sodium chloride and potassium chloride.

TRIS buffer is a buffer of tris(tris(hydroxymethyl)aminomethane, also known as tromethamine or trometamol, showing a pK_a of 8.07 at 25° C and having an effective pH range between 6.5 and 9.0.

The incubation process takes place generally at a pH comprised from 6.0 to 9.0, preferably from 6.4 to 8.4, and more preferably at pH 6.4, 7.4 or 8.4 In a preferred embodiment, the incubation takes place either at pH 6.4 or 7.4 using citrate buffer or at pH 7.4 or 8.4 using TRIS buffer; in a yet more preferred embodiment the incubation takes place at pH 7.4 using either citrate or TRIS buffer.

Incubation with biotinylated DNA and oligonucleotides

In the method of the invention for the determination of the target of a biotinylated DNA or biotinylated oligonucleotides, the magnetic biotin imprinted polymer particles are incubated with biotinylated-DNA or biotinylated oligonucleotides. In the incubation process, the biotinylated DNA molecules are captured by the magnetic biotin imprinted polymer particles occupying the binding sites of the polymer.

Biotinylated DNA and oligonucleotides is a common molecule readily available from different sources, for example, Roche, Sigma-Aldrich, Thermo Scientific.

Biotinylated DNA can be also obtained by biotinylation of the 3’ or 5’ end of DNA or oligonucleotides, i.e. covalent attachment of biotin to the nucleic acid, using commercial available kits, for instance Thermo Scientific Pierce Biotin 3' End DNA Labeling Kit for tagging single-stranded DNA primers with biotin. The DNA biotinylation procedure uses terminal deoxynucleotidyl transferase (TdT) to catalyze non-template-directed nucleotide incorporation onto the 3'-OH end of single-stranded DNA. TdT exhibits a substrate preference of single-stranded DNA, but it will label duplex DNA with 3' overhangs and blunt duplexes, albeit with a lower efficiency.

Biotinylated DNA can be also obtained by amplification of a DNA template by Polymerase Chain Reaction with biotinylated primers or by biotinylated desoxynuclotides or retrotranscription of RNA using biotinylated polydT.

The incubation and separation takes usually place as disclosed above for the Incubation with biotinylated molecules.

Incubation with biotinylated antibodies

In the method of the invention for the determination of the target of a biotinylated antibody, the magnetic biotin imprinted polymer particles are incubated with a biotinylated antibody. In the incubation process, biotin-antibody conjugate molecules are captured by the magnetic biotin imprinted polymer particles occupying the binding sites of the polymer.

Biotin-antibody conjugate is obtained by biotinylation of an antibody by covalent attachment of biotin to the enzyme, using commercial available kits, for instance EZ-Link® NHS-PEG4 Biotinylation Kit, EZ-Link® BMCC-Biotin, among many others (Pierce).

It is also a common reagent readily available from different sources, for example, Abcam, Sigma-Aldrich, Life Technologies, or Bio-Rad.

The incubation and separation takes usually place as disclosed above for the Incubation with biotinylated molecules.

Incubation with biotin-lectin conjugate

In the method of the invention for the determination of the target (glycoprotein or glycolipid) of a biotinylated lectin, the magnetic biotin imprinted polymer particles are incubated with biotin-lectin conjugate. In the incubation process, biotin-lectin conjugate molecules are captured by the magnetic biotin imprinted polymer particles occupying the binding sites of the polymer.

Biotin-lectin conjugate is obtained by biotinylation of the lectin, for instance Concanavalin A, by covalent attachment of biotin to the lectin, using commercial available kits, for instance, EZ-Link® NHS-PEG4 Biotinylation Kit (Pierce).

Lectin is a common substance readily available from different sources, for example, Sigma-Aldrich, among others.

The incubation and separation takes usually place as disclosed above for the Incubation with biotinylated molecules.

Incubation with biotin-enzyme conjugate

In the method of the invention for the determination of biotin, the magnetic biotin imprinted polymer particles are incubated with biotin-enzyme conjugate, preferably biotin-HRP. In the incubation process, biotin-enzyme conjugate molecules are captured by the magnetic biotin imprinted polymer particles occupying the binding sites of the polymer.

Biotin-enzyme conjugate is obtained by biotinylation of the enzyme, for example, horseradish peroxidase (HRP), alkaline phosphates, glucose oxidase, i.e. covalent attachment of biotin to the enzyme, using commercial available kits, for instance, EZ-Link® NHS-PEG4 Biotinylation Kit (Pierce).

Biotin-HRP is a common enzyme readily available from different sources, for example, Sigma-Aldrich, Life Technologies, or Bio-Rad.

The incubation and separation takes usually place as disclosed above for the Incubation with biotinylated molecules.

Incubation with biotin-dye conjugates

In the method of the invention for the determination of dyes, the magnetic biotin imprinted polymer particles are incubated with biotin-dye conjugate. In the incubation process, biotin-dye conjugate molecules are captured by the magnetic biotin imprinted polymer particles occupying the binding sites of the polymer.

Biotin-dye conjugate is obtained by biotinylation of the dye, for instance fluorescein, rodhamine, Cy3, Cy5, atto dyes by covalent attachment of biotin to the dye, using commercial available kits, for instance, EZ-Link® NHS-PEG4 Biotinylation Kit (Pierce).

Biotin-dye conjugates are also common substances readily available from different sources, for example, Sigma-Aldrich, among others.

The incubation and separation takes usually place as disclosed above for the Incubation with biotinylated molecules.

Incubation with biotin-dendrimer conjugates

In the method of the invention for the determination of dendrimers, the magnetic biotin imprinted polymer particles are incubated with biotin-dendrimer conjugate. In the incubation process, biotin-dendrimer conjugate molecules are captured by the magnetic biotin imprinted polymer particles occupying the binding sites of the polymer.

Biotin-dendrimer conjugate is obtained by biotinylation of the dendrimer, by covalent attachment of biotin to the dye, using commercial available kits, for instance, EZ-Link® NHS-PEG4 Biotinylation Kit (Pierce).

Dendrimers are common substances readily available from different sources, for example, Sigma-Aldrich, among others.

The incubation and separation takes usually place as disclosed above for the Incubation with biotinylated molecules.

Washing

After the incubation of magnetic biotin imprinted polymer particles with biotin-molecule conjugate, being the molecule, for example, DNA, oligonucleotides, enzymes, lectins, antibodies, dyes, dendrimers, the microplate or Eppendorf® tube is washed usually in the presence of a buffer, for example, citrate, PBS, borate, or TRIS. Preferably it is used citrate or TRIS. The washing process takes place generally at a pH comprised from 6.0 to 9.0, preferably from 6.4 to 8.4, and more preferably at pH 6.4, 7.4 or 8.4. In a preferred embodiment, the washing takes place either at pH 6.4 or 7.4 using citrate buffer or at pH 7.4 or 8.4 using TRIS buffer; in a yet more preferred embodiment the incubation takes place at pH 7.4 using either citrate or TRIS buffer. Usually the microplate Eppendorf® tube is washed with 100 to 400 µL of PBS, preferably 100 µL, usually for 5 minutes at room temperature.

After the incubation a 96-well magnet plate separator or a magnet for Eppendorf® tubes is usually positioned under the microtiter plate or Eppendorf® tubes until pellet formation on the bottom corner, followed by supernatant separation.

Incubation with the target of the biotinylated molecule

In the method of the invention, after the washing, the microplate or the Eppendorf® tubes containing magnetic imprinted polymer particles bound to the biotinylated capture biomolecules is incubated with samples containing the target of the biotinylated molecule, i.e. complementary DNA, antigen, substrate of the enzyme, cofactor of the enzyme, glycoprotein or glycolipid, and optionally with a labelled molecule with a fluorescent dye, enzyme, radioactive probe, among others, for their determination.

In a preferred embodiment of the present invention, i.e. the method for the determination of biotin, after the washing, the microplate containing magnetic biotin

imprinted polymer particles is incubated with samples comprising biotin, for example, for 30 minutes at room temperature and washed, for example, for 5 minutes.

In the incubation process with samples comprising biotin, some of the sites previously occupied by the biotin-HRP conjugates are substituted by biotin in a competitive assay.

Enzymatic reaction and readout

To determine the content of target molecules in the samples when the label is an enzyme, for example, HRP, it is used for example a chromogenic assay.

Chromogenic assays result in a colored reaction product that absorbs light in the visible range.

In the method of the invention, the horseradish peroxidase is reacted with a substrate, which is transformed in a colored product. The concentration of the colored product, determined by optical reading at a specific wavelength can be used for determining the amount of biotin in the sample.

The most common substrates that produce soluble reaction products with HRP conjugates are 3,3',5,5'-tetramethylbenzidine (TMB), 2,2'-azino-di [3-ethylbenzthiazoline] sulfonate (ABTS), and o-phenylenediamine (OPD).

In the method of the invention TMB is preferably used. TMB is a highly sensitive substrate and due to its rapid reaction rate, it is ideally suited for on-line kinetic analysis. It produces a blue color measurable at a wavelength of 650 nm. TMB can also be used in endpoint assays by stopping the reaction with phosphoric acid or sulfuric acid. A yellow reaction product is formed upon acidification that is measurable at 450 nm.

The concentration of biotin in the sample is obtained from a curve of absorbance unit vs. concentration generated by standard solutions containing biotin, as shown in Example 1.

In other cases, when the label is not an enzyme, the direct optical, electrochemical, fluorescent, chemiluminescent, or radioactive readout can be performed. Confocal microscopy is also suitable therefor, in particular for the qualitative determination, as shown in Example 3.

Performance of the method

The application of the method of the invention to samples containing different amounts of biotin-HRP shows surprisingly that the limit of detection for the determination of biotin-HRP is as low as 1.054 ng/mL, which is a significantly low limit of detection and being very fast (30 min). The authors of the invention have developed a cheaper and a robust method, which does not require to be stored at 4° C with high binding capacity for the separation and purification under magnetic actuation of biotin-HRP or biotinylated molecules (e.g. nucleic acids, antibodies, enzymes, proteins, lectins, dyes, or dendrimers), and their downstream application including the sensitive determination of their specific targets.

In the case of the determination of double tagged DNA amplicon from *E. coli*, the method allows the detection of DNA with a limit of detection of 1.28 ng/mL, which is a significantly low limit of detection and being very fast (30 min).

The application of the method of the invention to samples containing different amounts of biotin shows surprisingly that the limit of detection for the determination of biotin is as low as 0.857 pg/mL, which corresponds to $3.51x10^{-12}$ mol/L, which is 5 orders of magnitude more sensitive than the method disclosed in Kergaravat *et al.*, *op.cit.*, based on the biotin determination in food supplements by an electrochemical magneto biosensor, showing a limit of detection with a dynamic range from 0.94 to $2.4x10^{-7}$ mol/L.

Consequently, the method of the invention is suitable for detecting very low amounts of biotin in samples.

Human serum and plasma, as well as foods, such as milk, can be assayed without any pre-treatment, but it is preferably diluted in a suitable buffer such as PBS or Tris buffer.

Next, several examples are provided which are illustrative of the invention but which are not limitative thereof.

Examples

Preparative example 1: Method for preparing magnetic particles

1.72 g of $FeCl_2 \cdot 4H_2O$ and 4.72 g of $FeCl_3 \cdot 6H_2O$ were placed in a flask and were dissolved in 80 mL of water with vigorous stirring under nitrogen. 10 mL of NH_4OH (28 wt. %) was added in system drop by drop, and the reaction was maintained at 80° C for 30 minutes. The black precipitated of Fe_3O_4 was separated with a

permanent magnet and washed through water to remove the unreacted chemicals, and then dried under vacuum. The magnetic particles of Fe_3O_4 were modified further by dispersing 300 mg Fe_3O_4 magnetic particles in 40 mL of ethanol and 4 mL of ultrapure water, followed by ultra-sonication for 15 min and then followed by the addition of 5 mL of NH_4OH and 2 mL of tetraethoxysilane (TEOS). The mixture was left to react for 12 h at the room temperature under stirring. The product was collected by magnetic separation and was washed with ultrapure water and then dried in the vacuum.

In the next step 250 mg of Fe_3O_4 modified with TEOS were dispersed in 50 mL of anhydrous toluene containing 5 mL of 3-metacryloxypropyltrimethoxysilane (MPS), and the mixture was allowed to react for 12 hours under dry nitrogen. After magnetic separation, the product was washed with water and dried under vacuum.

Magnetic particles containing Fe_3O_4 and bearing a 3-metacryloxypropyltrimethoxysilane rest were obtained. They were suitable for further polymerization with functional monomers and crosslinking monomers.

Preparative example 2: Method for preparing biotin magnetic MIP particles

0.8 mmol of acrylic acid, 0.2 mmol of biotin and ethanol (30 mL) were shaken at 25° C for 12 h , then 200 mg of the magnetic particles containing Fe_3O_4 and bearing a 3-metacryloxypropyltrimethoxysilane rest prepared according to the process disclosed in Preparative example 1 were added to the mixture and the shaking was maintained for another 3 hours at 25° C. Afterwards, 4.0 mmol of ethylene glycol dimethacrylate (EGDMA) and 0.05 mmol of azobisisobutyronitrile (AIBN) were added into the system and the mixture was sonicated in a water bath for 5 minutes. After spurge with nitrogen gas for 5 minutes to remove oxygen, system was left at 60° C under nitrogen gas protection for 24 hours. After polymerization, the template molecule was removed by Soxhlet extraction using methanol: acetic acid (9:1, v/v) as eluent, and the eluent was replaced every 12 hours for 4 days.

Magnetic biotin imprinted particles (magnetic-MIP) were obtained. They were used in the method for the determination of biotin as disclosed in Example 1.The same procedure, but just avoiding the addition of biotin as a template, can be also performed for obtaining the magnetic non-imprinted polymer (magnetic-NIP) as a negative control for further studies of non-specific adsorption.

Example 1: Determination of biotin

The schematic representation for the determination of biotin on the magnetic-MIP is shown in Figure 2, while the results are shown in Figure 3 and 4.

3.2 mL of 0.1 M TRIS, 0.15 M NaCl, 0.05 % Tween pH= 7.4 were added to a falcon tube and then 17.6 mg of magnetic-MIP (or the magnetic-NIP, respectively, as a negative control), prepared according to the process disclosed in Preparative example 2, were added to the tube.

After mixing the solution by repeatedly turning the tube over, it was inserted in the magnetic separator until solution became clear (around 2 min). Afterwards the supernatant was removed.

3.2 mL of 0.1 M TRIS, 0.15 M NaCl, 0.05 % Tween pH= 7.4 were added to wash and the previous step was repeated. The washing step was repeated for a second time.

Magnetic-MIP particles were resuspended in 3.2 mL of 0.1 M TRIS, 0.15 M NaCl, 0.05 % Tween pH= 7.4 in order to have a final magnetic-MIP concentration of 5.5 mg/mL.

100 µL of the MAGNETIC-MIP suspension was added to each of 64 wells of a polypropylene microtiter plate, which was placed on 96-well magnet plate separator until solution became clear (around 2 min). The supernatant was removed carefully.

The microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

7 mL of a solution of biotin-HRP (1.5 ng mL-1) was prepared in advance. 100 µL of the solution of biotin-HRP were added to each well and the microplate was inserted on the microtiter plate shaker for 30 min at 750 rpm.

Afterwards, the microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

100 µL of 0.1 M TRIS, 0.15 M NaCl, 0.05 % Tween pH= 7.4 for washing were added. The microplate was placed on the microtiter plate shaker for 5 min at 750 rpm. The microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

Serial dilutions (1/2) of biotin in 0.1 M TRIS, 0.15 M NaCl, 0.05 % Tween pH= 7.4 were prepared on Eppendorf® tubes according to the following concentrations: 1.91 ng/mL; 0.95 ng/mL; 0.48 ng/mL; 0.23 ng/mL; 0.12 ng/mL; 60 pg/mL; 30 pg/mL; 15 pg/mL; 7 pg/mL; 3.7 pg/mL; 1.8 pg/mL; 0.9 pg/mL; and 0.4 pg/mL.

From the Eppendorf® tubes 100 µL of each dilution of biotin in triplicates were taken to the microplate and inserted it on the microtiter plate shaker for 30 min at 750 rpm.

The microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

100 µL of 0.1 M TRIS, 0.15 M NaCl, 0.05 % Tween pH= 7.4 were added for washing. The microplate was inserted on the microtiter plate shaker for 5 min at 750 rpm.

Afterwards, the microplate was placed on 96-well magnet plate separator and the solutions were removed.

To each well 100 µL of a solution of TMB:H_2O_2 (1:1) were added. The microplate was protected from the light and it was placed on the microtiter plate shaker for 30 min at 750 rpm.

To each well were added 100 µL of the H_2SO_4 solution to stop the reaction. The microplate was placed on the microtiter plate shaker for 1 min at 750 rpm.

Finally, the microplate was placed on 96-well magnet plate separator and the supernatants transferred to a polystyrene microtiter plate for the read-out. The microplate was inserted on the microplate reader, and the absorbance was measured at 450 nm, which is the wavelength suitable for measuring the absorbance of the resulting product from the reaction of TMB and the peroxidase (HRP) present in the biotin-HRP conjugate.

The limit of detection calculated for biotin was 3.51×10^{-12} mol/L or 0.857 pg/L.

An analogous method can be used for the determination of biotin in food or clinical samples, for example.

Example 2: Determination of biotin-HRP

The schematic representation for the determination of biotin-HRP on the magnetic-MIP is shown in Figure 6, while the results are shown in Figure 7.

3.2 mL of 0.1 M TRIS, 0.15 M NaCl, 0.05 % Tween pH= 7.4 were added to a falcon tube and then 17.6 mg of MIP (or the NIP, respectively, as a negative control), prepared according to the process disclosed in Preparative example 2, were added to the tube.

After mixing the solution by repeatedly turning the tube over, it was inserted in the magnetic separator until solution became clear (around 2 min). Afterwards the supernatant was removed.

3.2 mL of 0.1 M TRIS, 0.15 M NaCl, 0.05 % Tween pH= 7.4 (or any other of the table 1) were added to wash and the previous step was repeated. The washing step was repeated for a second time.

Magnetic-MIP particles were resuspended in 3.2 mL of 0.1 M TRIS, 0.15 M NaCl, 0.05 % Tween pH= 7.4 (or any other of the table 1) in order to have a final magnetic-MIP concentration of 5.5 mg/mL.

100 µL of the magnetic-MIP suspension was added to each of 64 wells of a polypropylene microtiter plate, which was placed on 96-well magnet plate separator until solution became clear (around 2 min). The supernatant was removed carefully.

The microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

Serial dilutions of biotin-HRP in 0.1 M TRIS, 0.15 M NaCl, 0.05 % Tween pH= 7.4 (or any other of the table 1) buffer solution were prepared on Eppendorf® tubes according to the following concentrations: 6.125 ng/mL; 25.00 ng/mL; 50.00 ng/mL; 75.00 ng/mL; 100.00 ng/mL; and 125.00 ng/mL (1.393180×10^{-10} mol/l; 2.840910×10^{-10} mol/l; 5.681820×10^{-10} mol/l; 1.136360×10^{-10} mol/l; 1.704550×10^{-9} mol/l; 2.272730×10^{-9} mol/l; and 2.840910×10^{-9} mol /l)

From the Eppendorf® tubes 100 µL of each dilution of biotin-HRP in triplicates were taken to the microplate and inserted it on the microtiter plate shaker for 30 min at 750 rpm.

Afterwards, the microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

100 µL of 0.1 M TRIS, 0.15 M NaCl, 0.05 % Tween pH= 7.4 for washing were added. The microplate was placed on the microtiter plate shaker for 5 min at 750 rpm. The microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

To each well 100 µL of a solution of TMB:H_2O_2 (1:1) were added. The microplate was protected from the light and it was placed on the microtiter plate shaker for 30 min at 750 rpm.

To each well were added 100 µL of the H_2SO_4 solution to stop the reaction. The microplate was placed on the microtiter plate shaker for 1 min at 750 rpm.

Finally, the microplate was placed on 96-well magnet plate separator and the supernatants transferred to a polystyrene microtiter plate for the read-out. The microplate was inserted on the microplate reader, and the absorbance was measured at 450 nm, which is the wavelength suitable for measuring the absorbance of the resulting product from the reaction of TMB and the peroxidase (HRP) present in the biotin-HRP conjugate.

The limit of detection calculated for biotin-HRP was 1.054 ng/mL, which is a significantly low limit of detection and being very fast (30 min). The Kd (dissociation constant) was found to be 5.2995×10^{-10}, while de affinity constant was found to be 1.89×10^{9}.

Example 3: Characterization of the binding of a biotinylated dye and a biotinylated dendrimer by confocal microscopy

The schematic representation for the characterization of the binding of biotinylated biomolecules on the magnetic-MIP by confocal microscopy is shown in Figure 8. Panel A shows the binding on the magnetic-MIP of Atto 665-biotin (Sigma Aldrich), while panel B shows the binding of a G4 biotin dendrimer (dendrimer (obtained by biotinylation of a commercial dendrimer from Sigma Aldrich), followed by the incubation with streptavidin-Cy5 (Life Technologies). In both cases, the characterization was performed by confocal microscopy.

In the first experiment, the magnetic-MIP was attached with Atto 665-biotin (Figure 9, panels B and C). To achieved this 0,2 mg of magnetic MIPs and 1 µL of Atto 665-biotin (1 mg/mL) were incubated in 200 µL TRIS buffer pH 7.4 for 30 min

with shaking at 37° C. A washing step was then performed for 5 min in TRIS buffer pH 7.4. The negative control was performed with the magnetic-MIP without the addition of the conjugate (Figure 9, panel A).

Another experiment was performed by the incubation of the magnetic-MIP with a G4 biotinylated dendrimer, with 96 molecules of biotin per molecule of dendrimer, followed by the incubation with streptavidin-Cy5 (Figure 9, panels E and F). In this approach 10 µL of biotinylated dendrimer (1 mg/mL in 2% DMSO) and 2 mg of magnetic MIPs and were incubated in 200 µL TRIS buffer pH 7.4 for 30 min with shaking at 37° C. The magnetic-MIP was separated from the supernatant with a magnet. After removing the supernatant, 200 µL of a solution of streptavidin-Cy5 (2 µg/mL) was added and the mixture was incubated again for 30 min at 37° C, followed by a washing step. The negative control was similarly performed but avoiding the addition of the biotinylated dendrimer (Figure 9, panel D). In both cases, the images were scanned in two dimensions along x and y axes by using the laser AOTF at a wavelength of 633 nm whereas the emission was captured in the range of 645 to 785 nm.

Example 4: Determination of biotin-HRP

7 mL of PBS 0.1% BSA buffer were added to a falcon tube and then 23.1 mg of MBIP, prepared according to the process disclosed in Preparative example 2, were added to the tube.

After mixing the solution by repeatedly turning the tube over, it was inserted in the magnetic separator until solution became clear (around 2 min). Afterwards the supernatant was removed.

7mL of PBS 0.1% BSA buffer solution were added to wash and the previous step was repeated. The washing step was repeated for a second time.

MBIP particles were resuspended in 7mL of PBS 0.1% BSA buffer in order to have a final MBIP concentration of 3.3 mg/mL.

100 µL of the MBIP suspension was added to each of 64 wells of a polypropylene microtiter plate, which was placed on 96-well magnet plate separator until solution became clear (around 2 min). The supernatant was removed carefully.

The microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

Serial dilutions of biotin-HRP in PBS buffer solution were prepared on Eppendorf® tubes according to the following concentrations: 6.125 ng/mL; 25.00 ng/mL; 50.00 ng/mL; 75.00 ng/mL; 100.00 ng/mL; and 125.00 ng/mL.

From the Eppendorf® tubes 100 µL of each dilution of biotin-HRP in triplicates were taken to the microplate and inserted it on the microtiter plate shaker for 30 min at 750 rpm.

Afterwards, the microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

200 µL of PBS for washing were added. The microplate was placed on the microtiter plate shaker for 5 min at 750 rpm. The microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

To each well 100 µL of a solution of TMB:H_2O_2 (1:1) were added. The microplate was protected from the light and it was placed on the microtiter plate shaker for 30 min at 750 rpm.

To each well were added 100 µL of the H_2SO_4 solution to stop the reaction. The microplate was placed on the microtiter plate shaker for 1 min at 750 rpm.

Finally, the microplate was placed on 96-well magnet plate separator and the supernatants transferred to a polystyrene microtiter plate for the read-out. The microplate was inserted on the microplate reader, and the absorbance was measured at 450 nm, which is the wavelength suitable for measuring the absorbance of the resulting product from the reaction of TMB and the peroxidase (HRP) present in the biotin-HRP conjugate.

Example 5: Determination of double-tagged DNA from *E. coli* O157:H7

The double-tagged DNA from *E. coli* O157:H7 was achieved by PCR performed with a double-tagging set of primers for the amplification of the eaeA (151 bp) gene fragment specific to *E. coli*, being each primer labeled in 5' with biotin (to achieve the immobilization on the magnetic-MIP) and digoxigenin (for the optical readout by using the optical reporter antiDIG-HRP). The detection of the double-tagged DNA amplicon was performed in a magneto-actuated immunoassay, represented

schematically in Figure 11, in 96-well microtiter plates, involving the following steps: (A) One-step incubation with double-tagged amplicon and AntiDigoxigenin-HRP conjugate. In this step, the magnetic-MIP (0,55 mg per well) was incubated with 50 µL of amplicon (ranging from 0 to 206 ng/mL) and 1.35 µg of antiDIG-HRP in TRIS 0.05 % Tween buffer solution pH 7.4 for 30 min with shaking at room temperature. (B) A washing step with 100 µL of TRIS buffer pH 7.4. (C) Optical readout was performed as in Example 2 for biotin-HRP. The results are shown in Figure 12, wherein the absorbance is represented vs the concentration of *E. coli* double-tagged amplicon, expressed in ng/L.

In the case of the determination of *E. coli* double tagged DNA amplicon, the method allows the detection of DNA with a limit of detection of 1.28 ng/mL.

Example 6: Determination of biotin

7 mL of PBS buffer solution were added to a falcon tube and then 23.1 mg of MBIP, prepared according to the process disclosed in Preparative example 2, were added to the tube.

After mixing the solution by repeatedly turning the tube over, it was inserted in the magnetic separator until solution became clear (around 2 min). Afterwards the supernatant was removed.

7mL of PBS buffer solution were added to wash and the previous step was repeated. The washing step was repeated for a second time.

MBIP particles were resuspended in 7mL of PBS buffer solution in order to have a final MBIP concentration of 3.3 mg/mL.

100 µL of the MBIP suspension was added to each of 64 wells of a polypropylene microtiter plate, which was placed on 96-well magnet plate separator until solution became clear (around 2 min). The supernatant was removed carefully.

The microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

7 mL of a solution of biotin-HRP (1.5 ng mL-1) was prepared in advance. 100 µL of the solution of biotin-HRP were added to each well and the microplate was inserted on the microtiter plate shaker for 30 min at 750 rpm.

Afterwards, the microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

200 µL of PBS for washing were added. The microplate was placed on the microtiter plate shaker for 5 min at 750 rpm. The microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

Serial dilutions (1/2) of biotin:PBS buffer solution were prepared on Eppendorf® tubes according to the following concentrations: 1.91 ng/mL; 0.95 ng/mL; 0.48 ng/mL; 0.23 ng/mL; 0.12 ng/mL; 60 pg/mL; 30 pg/mL; 15 pg/mL; 7 pg/mL; 3.7 pg/mL; 1.8 pg/mL; 0.9 pg/mL; and 0.4 pg/mL.

From the Eppendorf® tubes 100 µL of each dilution of biotin in triplicates were taken to the microplate and inserted it on the microtiter plate shaker for 30 min at 750 rpm.

The microplate was placed on 96-well magnet plate separator until solution became clear (around 2 min) and the supernatant was removed carefully.

200 µL of PBS were added for washing. The microplate was inserted on the microtiter plate shaker for 5 min at 750 rpm.

Afterwards, the microplate was placed on 96-well magnet plate separator and the solutions were removed.

To each well 100 µL of a solution of TMB:H_2O_2 (1:1) were added. The microplate was protected from the light and it was placed on the microtiter plate shaker for 30 min at 750 rpm.

To each well were added 100 µL of the H_2SO_4 solution to stop the reaction. The microplate was placed on the microtiter plate shaker for 1 min at 750 rpm.

Finally, the microplate was placed on 96-well magnet plate separator and the supernatants transferred to a polystyrene microtiter plate for the read-out. The microplate was inserted on the microplate reader, and the absorbance was measured at 450 nm, which is the wavelength suitable for measuring the absorbance of the resulting product from the reaction of TMB and the peroxidase (HRP) present in the biotin-HRP conjugate.

The limit of detection calculated for biotin was 3.51×10^{-12} mol/L or 0.857 pg/L.

An analogous method can be used for the determination of biotin in food or clinical samples, for example.

CLAIMS

1.- Method for the determination of targets of biotinylated molecules, characterized in that it comprises the step of:

1) immobilizing a biotinylated molecule by incubation of magnetic biotin imprinted polymer particles with such biotinylated molecule.

2.- Method according to claim 1, characterized in that it further comprises:

2) incubating the particles of step 1) with the target of the biotinylated molecule and optionally with a labelled probe,
3) optionally carrying out an enzymatic reaction with the incubated particles, and
4) reading out.

3.- Method according to claim 2, characterized in that after the incubation step it is carried out the steps of applying a magnetic field and washing of the particles by removing the supernatant.

4.- Method according to any of claims 1 to 3, characterized in that the biotinylated molecule is selected from the group consisting of: biotin-enzyme conjugate, biotinylated DNA, biotinylated RNA, biotinylated oligonucleotides, biotinylated antibodies, biotinylated peptides, biotinylated proteins, and biotinylated lectins.

5.- Method according to any of claims 1 to 4, characterized in that the target of the biotinylated molecule is selected from the group consisting of: biotin, complementary DNA for a biotinylated DNA, antigen for a biotinylated antibody, substrate for a biotinylated enzyme, cofactor for a biotinylated enzyme, glycoprotein for a biotinylated lectin, and glycolipid for biotinylated lectin.

6.- Method according to any of claims 1 to 5, characterized in that the label of the labelled probe is a fluorescent dye, an enzyme, or a radioactive molecule.

7.- Method according to claim 5, characterized in that the target of the biotinylated molecule is biotin.

8.- Method according to claim 7, characterized in that it further comprises:

2) incubating the particles of step 1) with a sample comprising biotin,
3) carrying out an enzymatic reaction with the incubated particles, and
4) optical reading out.

9.- Method according to any of claims 1 to 8, characterized in that the magnetic biotin imprinted polymer particles comprise a magnetic particle as a core and a polymeric shell where the biotin is imprinted.

10.- Method according to claim 9, characterized in that the magnetic particle is a magnetite particle.

11.- Method according to claim 9 or 10, characterized in that the polymeric shell on the magnetic particles is prepared by a process comprising the following steps:

a) mixing biotin with one or more functional monomers in a porogenic solvent,
b) polymerizing the mixture of step a) with one or more crosslinking monomers in the presence of an initiator and magnetic particles, and
c) extracting the biotin from the polymeric shell by an extraction solvent.

12.- Method according to claim 11, characterized in that the functional monomer is selected from the group consisting of acrylic acid, methacrylic acid, acrylic acid C_1-C_6 alkyl esters, methacrylic acid C_1-C_6 alkyl esters, 4-vinylbenzoic acid, 4-ethylstyrene, acrylamide, methacrylamide, 2-acrylamido-2-methylpropane sulfonic acid, vinylsuccinic acid, vinylpyrrolidone, 2-vinylpyridine, and 4-vinylpyridine.

13.- Method according to claim 12, characterized in that the functional monomer is acrylic acid.

14.- Method according to any of claims 11 to 13, characterized in that the crosslinking monomer suitable for preparing the polymeric shell is selected from the group consisting of *o*-divinylbenzene, *m*-divinylbenzene, *p*-divinylbenzene, ethylene glycol diacrylate, di(ethylene glycol) diacrylate, tetra(ethylene glycol) diacrylate, ethylene glycol dimethacrylate, di(ethylene glycol) dimethacrylate, tri(ethylene glycol) dimethacrylate, *N*,*N*-methylenebisacrylamide, *N*,*N*-(1,2-dihydroxyethylene)bisacryl-

amide, *N*-(1-hydroxy-2,2-dimethoxyethyl)acrylamide, *N,N'*-phenylenediacrylamide, trimethylolpropane trimethacrylate, pentaerythritol triacrylate, pentaerythritol tetraacrylate, *N,O*-bisacryloyl-L-phenylalaninol, 3,5-bisacrylamidobenzoic acid, 2,6-bisacrylamidopyridine, and mixtures thereof.

15.- Method according to claim 14, characterized in that the crosslinking monomer is ethylene glycol dimethacrylate.

16.- Method according to of claims 11 to 15, characterized in that the porogenic solvent is selected from the group consisting of ethanol, methanol, decanol, dioxane, tetrahydrofurane, acetone, acetonitrile, and mixtures thereof.

17.- Method according to claim 16, characterized in that the porogenic solvent is ethanol.

18.- Method according to of claims 11 to 17, characterized in that the initiator for the polymerization reaction is selected from the group consisting of 2,2'-azobisisobutyronitrile (AIBN), 4,4-azobis(4-cyanovaleric acid), 1,1'-azobis(cyclohexanecarbonitrile), , *tert*-amyl peroxybenzoate, benzoyl peroxide, 2,2-bis(*tert*-butylperoxy)butane, 1,1-bis(tert-butylperoxy)-cyclohexane, *tert*-butyl peroxide, cumene hydroperoxide, potassium persulfate, and 2,4- pentanedione peroxide.

19.- Method according to claim 18, characterized in that the initiator for the polymerization reaction is AIBN.

20.- Method according to any of claims 1 to 19, characterized in that the magnetic biotin imprinted polymer particles are prepared by a process comprising the following steps:

a) preparing magnetite particles from co-precipitation of a Fe^{2+}/Fe^{3+} mixed solution,
b) reacting the magnetite particles prepared in step 1) with tetraethoxysilane,
c) reacting the hydroxyl-modified magnetite particles prepared in step 2) with a (meth)acrylic group containing silanizating agent,

d) polymerizing the silanized magnetite particles prepared in step 3) with a functional monomer, in the presence of biotin, a crosslinking monomer, a radical initiator, a porogenic solvent, and

e) extracting the particles prepared in step 4) with a solvent comprising a C_1-C_4 alkyl alcohol and acetic acid.

21.- Method according to claim 20, characterized in that the silanizating agent is methacryloxypropyltrimethyloxysilane, the functional monomer is acrylic acid, the crosslinking monomer is ethylene glycol dimethacrylate, the radical initiator is AIBN, the porogenic solvent is ethanol, the extracting solvent comprises methanol and acetic acid in a methanol: acetic acid ratio = 9:1 (v/v).

22.- Method according to any of claims 11 to 21, characterized in that the ratio biotin:functional monomer is comprised between 1:1 to 1:8.

23.- Method according to any of claims 11 to 22, characterized in that biotin and the functional monomer are maintained at least 4 hours before adding the magnetic particles.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/062021

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/543 B01D15/38
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N B01D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GB 2 505 249 A (UNIV KENT CANTERBURY [GB]) 26 February 2014 (2014-02-26) examples 1-6 -----	1-23
Y	ELENA PILETSKA ET AL: "Biotin-specific synthetic receptors prepared using molecular imprinting", ANALYTICA CHIMICA ACTA, vol. 504, no. 1, 1 February 2004 (2004-02-01), pages 179-183, XP055088498, ISSN: 0003-2670, DOI: 10.1016/S0003-2670(03)00813-4 the whole document -----	1-23
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 2016	19/07/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pinheiro Vieira, E

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LOUISE ELMLUND ET AL: "Biotin selective polymer nano-films", JOURNAL OF NANOBIOTECHNOLOGY, BIOMED CENTRAL, GB, vol. 12, no. 1, 21 March 2014 (2014-03-21) , page 8, XP021180902, ISSN: 1477-3155, DOI: 10.1186/1477-3155-12-8 figures 1,2	1-23
Y	TOSHIFUMI TAKEUCHI ET AL: "Molecular Imprinting of Biotin Derivatives and Its Application to Competitive Binding Assay Using Nonisotopic Labeled Ligands", ANALYTICAL CHEMISTRY, vol. 72, no. 11, 1 June 2000 (2000-06-01), pages 2418-2422, XP055088497, ISSN: 0003-2700, DOI: 10.1021/ac991357s the whole document	1-23
Y	CN 102 127 182 B (UNIV NANJING MEDICAL) 4 July 2012 (2012-07-04) the whole document	1-23
Y	CN 104 193 875 A (UNIV QILU TECHNOLOGY) 10 December 2014 (2014-12-10) the whole document	1-23
Y	CN 102 827 321 B (UNIV TONGJI) 4 December 2013 (2013-12-04) the whole document	1-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/062021

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2505249 A	26-02-2014	EP 2888246 A2	01-07-2015
		GB 2505249 A	26-02-2014
		US 2015225499 A1	13-08-2015
		WO 2014030002 A2	27-02-2014
CN 102127182 B	04-07-2012	NONE	
CN 104193875 A	10-12-2014	NONE	
CN 102827321 B	04-12-2013	NONE	