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(54) Title: METAL(III)-POLYPEPTIDE COMPLEXES

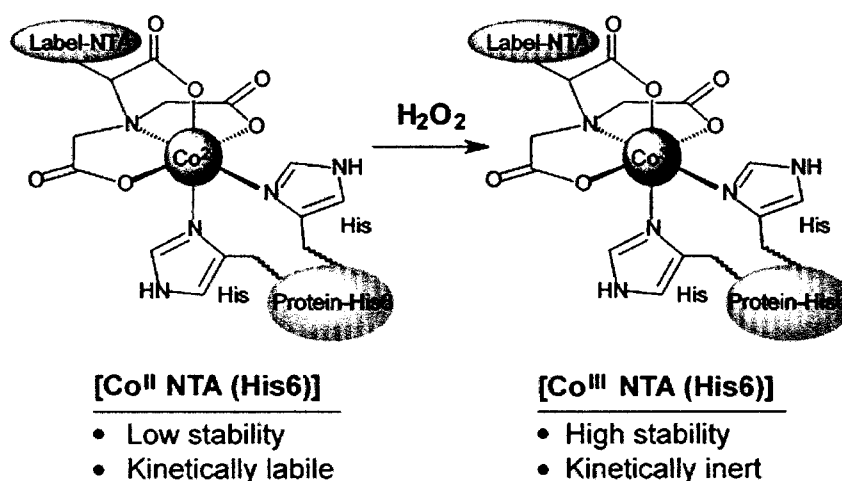


Figure 1

(57) Abstract: The present invention refers to a stable complex comprising a trivalent metal cation, a polypeptide with a plurality of histidine residues and a chelator, its manufacturing process and its use in medical and diagnostic applications as well as for labelling proteins. Further, the invention relates to solid phases to which such complexes are bound.

Metal(III)-polypeptide complexes

Description

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The present invention refers to a stable complex comprising a trivalent metal cation, a polypeptide with a plurality of histidine residues and a chelator, its manufacturing process and its use in medical and diagnostic applications as well as for labelling proteins. Further, the invention relates to solid phases to which such complexes are bound.

While the Ni^{2+} mediated interaction between the hexahistidine tag (His6-tag) and the nitrilotriacetic acid (NTA) was initially developed for the purification of recombinant proteins this chemistry has gone far beyond (Porath et al. Nature **1975**, 258, 598–599; Block et al.; Chapter 27 - Immobilized-Metal Affinity Chromatography (IMAC): A Review; 1st ed., Elsevier Inc., **2009**; Vol. 463, pp. 439–473). The small size of the tag and the site specific interaction under the mild conditions that do not interfere with the native activity of the protein combined with the large library of existing His-tagged proteins allowed for numerous applications and is proof of the value and flexibility of this system.

Just a few examples of what has been achieved with this technology are the specific immobilization of proteins on protein chips (Rusmini et al. **2007**, 8, 1775–1789; Valiokaset al.; Chembiochem **2006**, 7, 1325–1329), the incorporation of active proteins to nanomaterials (Boeneman et al. *J. Am. Chem. Soc* **2010**, 132, 5975–5977; Kim et al. *Anal Biochem* **2008**, 379, 124–126) and surfaces (Grunwald et al. *Proc Natl Acad Sci USA* **2010**, 107, 6146–6151; Kang et al. *Langmuir* **2007**, 23, 6281–6288), the labelling of proteins with fluorophores (Guignet et al; JACS 2006, 128, 418–419; Soh et al.; Sensors **2008**, 8, 1004–1024; Guignet et al.; Nat Biotech 2004, 22, 440–444; Kamoto et al.; Chemistry **2008**, 14, 8004–8012) and the specific conjugation of biomolecules with proteins (Gavutis et al.; J. Am. Chem. Soc

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1994, 116, 8485–8491; Pires et al; *Biomac.* **2011**, 12, 2429–2433).

In the [Ni^{II} NTA (His6-tag)] complex the Ni²⁺ is in an octahedral coordination environment, with 4 coordinating groups coming from NTA and two from the His6-tag. Similarly, other divalent ions such as Co²⁺, Cu²⁺ and Zn²⁺ can also be used as mediator ions between the His6-tag and NTA (Block et al.). The few limitations of this technology are that the affinity between the NTA and the His6-tag protein mediated by Ni²⁺ or the other above listed ions is usually only in the micromolar range, which is not tight enough for some applications (Rusmini et al.). Secondly, these complexes are kinetically labile, thus undergo rapid ligand exchange and chelators such as imidazole and EDTA quickly disturb the complex. While this reversible character is favorable for some applications such as protein purification, it is not if a permanent interaction is desired. To date the addition of multiple NTA groups and longer His-tags has been proposed to increase the affinity (Lata et al.; *JACS* **2006**, 128, 2365–2372; Kapanidis et al. J.; *Am. Chem. Soc* **2001**, 123, 12123–12125; Strunk et al.; *Bioconjug Chem* **2009**, 20, 41–46). The photo-chemical formation of covalent bonds after the complex is formed (Hintersteiner et al.; *Chembiochem* **2008**, 9, 1391–1395; Meredith et al.; *Bioconjug Chem* **2004**, 15, 969–982) or other less specific reactions have been proposed to achieve a permanent interaction (Chevalier et al., *Langmuir* **2010**, 26, 14707–14715; Mateo et al.; *Biotechnol. Bioeng.* **2001**, 76, 269–276), but these approaches require complex synthesis or/and are unspecific. US 5,439,829 discloses the immobilization of a biologically active molecule having a chelator group to a solid support containing a transition metal ion. The oxidation state of the metal ion is changed to provide a kinetically inert oxidation state.

Thus, it was desirable to overcome these limitations by forming a more stable and kinetically inert interaction between a polypeptide with a plurality of histidine residues and a chelator group.

It was surprisingly found that such stable complexes can be obtained by

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using trivalent metal cations, particularly kinetically inert trivalent metal cations, as coordination center.

Thus in a first aspect, the present invention relates to a complex comprising
5 a trivalent metal cation M^{3+} and at least two ligands, wherein a first ligand is a polypeptide with a plurality of histidine residues, and a second ligand is a chelator having at least three coordination groups and optionally another functional moiety. The polypeptide and the chelator, thus, form a chelate complex with the metal cation M^{3+} .

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The metal cation is preferably stable in the oxidation state +3 and at least in one of the oxidation states +2 or +1, preferably in the oxidation state +2. Thus, in a preferred embodiment the metal cation M^{3+} is selected from transition metals or rare earth metals such as Co^{3+} , Cr^{3+} or Fe^{3+} , more
15 preferably Co^{3+} .

The complexes according to the invention have several advantages:

1. M^{3+} complexes have significantly higher formation constants compared to
20 M^{2+} complexes in similar coordination environments,
2. M^{3+} complexes are exchange-inert as they undergo only very slow ligand exchange in their primary coordination sphere, and
3. M^{3+} complexes are relatively stable in their oxidation state, even in the presence of reducing agents.

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Preferably, the chelator according to the present invention is an amino-polycarboxylic acid, particularly having 1, 2, 3, 4, 5 or more carboxylic acid groups. Both, amino and carboxylic acid groups of the chelators may act as coordination groups.

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In a preferred embodiment, the chelator is selected from ethylene diamino tetraacetic acid (EDTA), nitrilo triacetic acid (NTA), iminodiacetic acid (IDA), ethylene glycol tetraacetic acid (EGTA), diethylene triamino pentaacetic acid

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(DPTA), and triethylene tetramine-N,N,N',N',N'',N''-hexa-acetic acid (TTHA), tris carboxymethyl ethylene diamine (TED), 1-aceto-1,4,7-triazacyclononane (Tacn) or derivatives thereof, particularly NTA. In this context the term “derivative” refers to compounds having the same lead structure, but may be substituted by further chemically reactive groups.

The chelator preferably bears another functional moiety. This functional moiety is preferably different from the coordination group(s) and may be selected from a labelling moiety, e.g. spectroscopically active moiety or a radio-active moiety, such as dyes or a fluorescent group, a pharmaceutically and/or biologically active moiety, such as a drug, e.g. a cytotoxic or cytostatic drug. The labelling moiety is preferably configured such that it can be determined by conventional detection methods known in the art (e.g. NMR-, fluorescence-, IR-, UV-spectroscopy, etc.).

The polypeptide with a plurality of histidine residues of the present invention is particularly an extracellular polypeptide. The polypeptide is preferably a recombinant polypeptide, particularly a recombinant His-tagged polypeptide which preferably comprises at least 4, preferably at least 6 consecutive histidine residues.

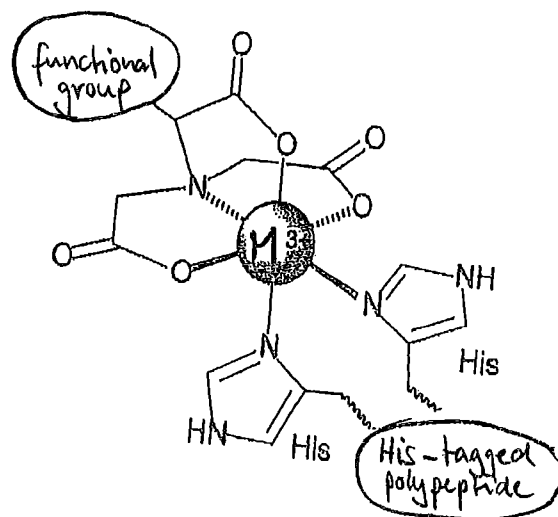
In another preferred embodiment the polypeptide comprises a spaced histidine tag with at least four histidine residues in a sequence $[H_n S_m]_k$, wherein H is histidine, S is an amino acid residue different from histidine, selected from glycine and/or serine and/or threonine, n is in each case independently 1-4, m is in each case independently 0-6, and k is 2-6, preferably 2-5. The spaced histidine tag may have a regular sequence, i.e. n and m have in each occurrence the same value, or an irregular sequence, i.e. n and m may have different values. A larger number of histidines within a polyhistidine tag may increase binding strength and specificity for a M^{3+} chelate matrix. Too many consecutive histidines, however, may lower expression levels and solubility of recombinant proteins, e.g. proteins recombinantly expressed in E. coli. These problems can be overcome by

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interrupting continuous runs of histidines with short spaces, comprising glycine, serine or threonine, i.e. with a spaced histidine tag.

In a preferred embodiment, the histidine sequence is located at the N- and/or
5 at the C-terminus or is inserted into the sequence of the polypeptide.

In a very preferred embodiment, the present invention provides a structure $[M^{3+} \text{NTA}(\text{His-tagged polypeptide})]$ according to the following formula:



10 Preferably, in $[M^{3+} \text{NTA}(\text{His-tagged polypeptide})]$ the functional group is a labelling group and/or M is Co.

In another aspect, the present invention refers to a method of preparing the metal complexes as described above. The method comprises the steps of
15 (i) providing a complex comprising a divalent metal cation M^{2+} and at least two ligands, wherein a first ligand is a polypeptide with a plurality of histidine residues, and a second ligand is a chelator having at least three coordination groups and optionally another functional moiety,
(ii) oxidizing the divalent cation of the metal complex in step (i) using an oxidizing agent or photochemical means, and optionally
20 (iii) separating the complex comprising a trivalent metal cation M^{3+} and at least two ligands, wherein a first ligand is a polypeptide with a plurality of histidine residues, and a second ligand is a chelator having at least three

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coordination groups and optionally another functional moiety from the mixture obtained in step (ii).

Because the formation of M^{3+} complexes is usually slow, this indirect
5 preparation where the complex with the exchange-labile divalent ion is formed and then the metal center is oxidized *in situ*, is preferred.

The divalent cation of the complex provided in step (i) is preferably a transition metal or rare earth metal, such as Co^{2+} , Cr^{2+} , Fe^{2+} , particularly Co^{2+} .

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The oxidizing step (ii) must be adapted such that the divalent cation of the metal complex is converted to the respective trivalent cation M^{3+} . As oxidizing agents, peroxides such as hydrogen peroxide, hypochlorites, periodates and nicotinamide adenine dinucleotide (NAD) may be used. Preferably,
15 peroxides and even more preferably hydrogen peroxide is used as an oxidizing agent.

The oxidation of the metal cation M^{2+} may also be performed via photo-chemical means.

20

The separation step (iii) can be preferably performed via chromatography. Preferred separation may be carried out on conventional Ni-NTA columns. The inventors have found that the complexes according to the present invention have lowered affinity towards Ni-NTA columns which, hence,
25 allows for the separation of the complexes of the invention from non-oxidized complexes and from un-modified protein: the stable complexes of the invention have no affinity to the Ni-NTA column, whereas un-modified polypeptide bind to the Ni-NTA column and also polypeptide in non-oxidized M^{2+} -polypeptide complexes may exchange with NTA ligands.

30

A further aspect of the present invention relates to a solid phase having a chelator covalently bound thereto, wherein the chelator moiety and at least one polypeptide having a plurality of histidine residues are coordinated to a

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trivalent cation M^{3+} .

In accordance to the present invention, thus, solid phases may be prepared which exhibit several different polypeptides stably complexed on one
5 common carrier.

Preferred chelators are as described above. Particularly, the chelator is an aminopolycarboxylic acid which is covalently bound to the solid phase via at least one of the carboxylic acid groups or the amino groups.

10 The solid phase carrier is preferably selected from the group consisting of metals, such as titanium or steel, metal oxides such as silica or titanium dioxide, glass, carbohydrates, such as agarose, sepharose, or cellulose, magnetic beads and plastics, such as polystyrene.

15 The solid phase carrier may be in any conventional form. Preferably, the solid phase carrier is in form of spheres, nanospheres, beads, quantum dots, and prosthetics.

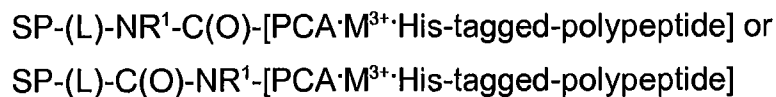
20 The solid phase carrier is preferably functionalized by amino-groups, carboxylic acid groups and/or activated esters, such as NHS-esters. The functionalized solid phase carrier usually allows a simple coupling reaction with at least one of the chelators' functional groups. The above described coupling reaction may be accelerated by conventional methods known to the
25 skilled person, e.g. by base or acid catalysed esterification/amidation or carbodiimide mediated esterification. Hence, in a preferred embodiment the chelator is bound to the solid phase carrier via an amide- or ester-linkage. In a preferred embodiment, the solid phase carrier bears no more free functionalization groups after reaction with the chelator.

30 The trivalent metal cation M^{3+} is preferably as described above.

In a preferred embodiment, the solid phase of the present invention has the

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following structure:



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where SP is the solid phase

R¹ is hydrogen or an C₁₋₆-alkyl,

(L) is an optional linker such as C₁₋₆-alkyl, polyethyleneglycol, and

PCA is a residue of an aminopolycarboxylic acid.

10

A further aspect of the present invention refers to a method of labelling a protein having a plurality of histidine residues comprising the steps

15

(i) forming a complex comprising a divalent metal cation M²⁺ and at least two ligands, wherein a first ligand is a polypeptide with a plurality of histidine residues, and a second ligand is a chelator having at least three coordination groups and a labelling moiety, and

(ii) subjecting the complex obtained in step (i) to oxidizing conditions using an oxidizing agent or photo-chemical means, and optionally

20

(iii) separating the complex obtained in step (ii) comprising a trivalent metal cation M³⁺ and at least two ligands, wherein a first ligand is a polypeptide with a plurality of histidine residues, and a second ligand is a chelator having at least three coordination groups and a labelling moiety.

25

The chelator of the complex according to step (i) may correspond to the chelators as described above. It is essential that the chelator has a labelling moiety. The labelling moiety is preferably selected from spectroscopically active agents and radio-active agents, such as dyes or fluorescent groups, coumarin.

30

The polypeptide as used according to the present method may be the same as described above.

The divalent cation comprised in the complex of step (i) may be the same as already described above.

5 The oxidizing agents can be the same as described above.

Further, a separation step (iii) may be carried out via chromatography, particularly in combination with Ni-NTA columns resulting in the above-described advantages.

10

In a preferred embodiment of the present invention, a metal complex, namely $[\text{Co}^{2+}\text{NTA-label (His-tagged polypeptide)}]$ is formed in step (i) which after subjecting the complex to oxidizing conditions result in a metal complex according to $[\text{Co}^{3+}\text{NTA-label (His-tagged polypeptide)}]$.

15

The metal complexes or the solid phases according to the present invention may be used in diagnostic or medical applications.

20

When using the metal complexes or the solid phases of the invention in medical application, the chelator preferably exhibits another functional moiety, e.g. a pharmaceutically and/or biologically active agent as described above.

25

When using the metal complexes or the solid phases in diagnostic applications, the chelator preferably exhibits a labelling group or/and the solid phase carrier is preferably suitable to be detected by conventional means, such as NMR-, fluorescence-, IR-, or UV-spectroscopy).

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The metal complexes or the solid phases according to the invention are particularly suitable for *in vivo* targeting. That is, the polypeptide comprised in the complexes may be selected such that it selectively interacts with specific targets, such as proteins, tissues, etc. within the body, particularly the human body, after administration. Thus, the concentration of the complex

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or the solid phase increases selectively at the site of interest.

5 The pharmaceutically or/and biologically active agent comprised in the complex or the solid phase may then be cleaved from the complex or the solid phase in a hydrolytically or enzymatically or pH-dependent manner (depending on the target environment). In another embodiment, the agent may be released upon reduction of the reducing environment of the cell. The complexes or the solid phases of the invention, thus, allow a target-specific release of a pharmaceutically or/and biologically active agent, e.g. a drug.

10

In another embodiment, the complex or the solid phase preferably exhibiting a labelling moiety or/and a radio-active solid phase carrier may be used in radiotherapy. In such case the complex or the solid phase is transported to the target of interest. External radiation may then activate and/or stimulate the labelling moiety and/or the radio-active solid phase carrier selectively at the site of interest.

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The solid phases according to the present invention may further be used for culturing cells. In one embodiment the solid phases allow cell culturing of different cells on one common solid phase carrier. The latter embodiment may ideally be exercised on solid phases having bound complexes with different polypeptides.

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The above invention is illustrated by the following examples.

25

Figures:

Figure 1 Comparison of [Co^{II} NTA His6] and [Co^{III} NTA His6]

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Figure 2 Control experiments for GFP-His6 binding onto Ni-NTA column. The addition of 10 mM H₂O₂ or up to 800 µM Co²⁺-NTA does not affect the binding of GFP-His6 to the Ni-NTA column.

Figure 3A FPLC analysis of GFP-His6 (20 μ M) with coumarin-NTA and Co^{2+} with H_2O_2 (10 mM). Each sample was first passed over a desalting column.

Figure 3B The second peak (between 15 ml and 30 ml) after the Ni-NTA purification from a labeling reaction of His6-GFP (80 μ M) and [Co^{II} coumarin-NTA] (200 μ M) incubated with H_2O_2 (10 mM) was concentrated. a) The UV-VIS spectra of the complex (second peak), His6-GFP (94 μ M) and coumarin-NTA (130 μ M) were recorded. In the complex His6-GFP and coumarin-NTA, concentrations are calculated to be 96 μ M and 91 μ M, respectively. Thus, the formed complex is approximately 1:1. b) The concentrated sample was run on a size exclusion column (CV=25 ml, fraction size 1 ml) using detection at 280 nm, 400 nm and 490 nm. Afterwards, all the fractions were also analyzed for their GFP and coumarin fluorescence. In the chromatogram, signal from the coumarin and GFP were only detected at the same time. Thus, the Co^{3+} mediated binding of coumarin-NTA to His6-GFP is stable.

Figure 4 Agarose beads loaded with Co^{2+} and His6-GFP were incubated with various concentrations of hydrogen peroxide for 1 hour. Then the beads were treated with 250 mM imidazole and the eluted His6-GFP was quantified. His6-GFP bound to Co^{2+} centers is eluted while His6-GFP bound to Co^{3+} centers is not. Point at the left in the graph is a sample not treated with H_2O_2 .

Figure 5 Agarose beads loaded with Co^{2+} and His6-GFP were incubated with various concentrations of H_2O_2 . The oxidation of Co^{2+} to Co^{3+} can be observed by the color change of the beads from pink to purple.

Figure 6 UV-VIS spectra of different Co^{2+} and Co^{3+} complexes with NTA in the absence and presence of His6-peptide and imidazole. The absorption maximum is slightly blue shifted when additional nitrogen donor ligands are added to [Co^{III} NTA] ($\lambda_{\text{max}}=554$ nm) to form [Co^{III} NTA(His6-peptide)] ($\lambda_{\text{max}}=542$ nm) and [Co^{III} NTA(Imidazole) $_2$] ($\lambda_{\text{max}}=543$ nm).

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Figure 7 Kinetic data analysis of GFP-His6 dissociation from Co^{3+} -NTA on Agarose beads. A) Measurement of GFP-His6 in the beads over 16 days. B) The data in A) was fitted to first order kinetics under the assumption that the dissociation of GFP-His6 is a dissociative reaction. $k_{\text{off}} = 0.0972 (\pm 0.0119) \text{ M}^{-1} \text{ day}^{-1} = 1.12 (\pm 0.14) \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$. C) NTA agarose beads with different immobilized His6-tagged proteins at Co^{2+} (lane 1, 3, 5, 7, 9) and Co^{3+} (2, 4, 6, 8, 10) centers were incubated with 250 mM imidazole and the protein in the solution above the beads was analyzed by SDS-PAGE gel. Lane 1-2: His6-GFP (29 kDa), lane 3-4: His6-TEV (26 kDa), lane 5-6: His6-SUMO1 (11 kDa), lane 7-8: His6-transportin (100 kDa), lane 9-10: His6-MBP (43 kDa). While the His6-tagged proteins bound to Co^{2+} centers elute from the beads, the proteins at Co^{3+} centers stayed immobilized.

Figure 8 Chemical reactivity of $[\text{Co}^{\text{III}} \text{NTA}(\text{His6-GFP})]$. NTA beads with immobilized His6-GFP at Co^{2+} and Co^{3+} centers were incubated with imidazole (250 mM), the chelators NTA and EDTA (25 mM) and the reducing agents cysteamine, DTT, TCEP and ascorbate (1 mM) in combination with 250 mM imidazole for 1 hour and the eluted His6-GFP was analyzed. Due to the kinetic inertness of the Co^{3+} centers the His6-GFP is not eluted.

Figure 9A Chemical reactivity of the immobilized His6-GFP. 250 mM imidazole, 25 mM NTA and EDTA and 1 mM of the reducing agents in combination with 250 mM imidazole were used. Agarose beads loaded with His6-GFP with Co^{2+} and Co^{3+} centers are treated with the chelators imidazole (250 mM), NTA and EDTA (25 mM) or various reducing agents (1 mM) commonly used in protein chemistry in combination with imidazole (250 mM) for 24 hours. Due to the kinetic inertness of the cobalt(III) centers only very little His6-GFP is eluted.

Figure 9B Chemical reactivity of immobilized His6-GFP on Co^{3+} -NTA agarose beads. The amount of His6-GFP in the supernatant, which eluted from the NTA agarose beads, was quantified by a) measuring the GFP fluorescence after 1 hour, 1 day and 3 days, and SDS-PAGE gel after b) 1

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hour and c) 1 day. NTA agarose beads with immobilized His6-GFP at Co^{2+} (1, 3, 5, 7) and Co^{3+} (2, 4, 6, 8) centers were incubated 1-2) with 250 mM imidazole, 3-4) in 100 mM pH 3.5 acetate buffer, 5-6) with 100 mM ascorbate in the presence of 250 mM imidazole and 7-8) with 100 mM TCEP in the presence of 250 mM imidazole. As can be observed both from the GFP fluorescence and in the SDS-PAGE gel, 100 mM ascorbate can almost entirely reduce the Co^{3+} centers, therefore the His6-GFP can be eluted with imidazole. While 100 mM TCEP can partially reduce the Co^{3+} centers, acidification of the Co^{3+} beads does not lead to protein elution.

Figure 9C Stability of immobilized His6-GFP on Co^{3+} -NTA agarose beads in cell culture. NTA-agarose beads with His6-GFP immobilized at Ni^{2+} , Co^{2+} and Co^{3+} centers were placed into cultures of adherent REF52 cell cultures for 1, 6 and 24 hours before the beads were taken out with the medium and 250 mM imidazole was added. The amount of His6-GFP in the supernatant, which eluted from the NTA agarose beads, was quantified by measuring the GFP fluorescence. Even after 24 hours in cell culture, the His6-GFP immobilized at the Co^{3+} centers is still bound to the beads.

Figure 10 His6-GFP (20 μM) treated with various amounts of $[\text{Co}^{\text{II}}\text{NTA}]$ and 10 mM H_2O_2 for 1 hour is passed over a Ni-NTA column, eluted with a linear imidazole gradient and then the GFP fluorescence in each fraction was measured. His6-GFP conjugates with one (B) or two (A) NTA- Co^{3+} units could be separated from the His6-GFP (C).

Figure 11 The distribution of GFP-His6 species in the presence of various amounts of $[\text{Co-NTA}]$.

Figure 12 MALDI-TOF analysis of His6-GFP under the reaction conditions used for protein labeling. a) His6-GFP (20 μM), b) with 800 μM $[\text{Co}^{\text{II}}\text{NTA}]$ before and c) after 1 hour H_2O_2 (10 mM) treatment. d) His6-GFP under reaction conditions where Fenton reactions takes place. (200 μM Co^{2+} , 4.6 mM ascorbate and 10 mM H_2O_2). The theoretical MW of GFP with His6-

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tag and without His-tag is 29621 and 28567 g/mol, respectively. In d), a shoulder peak is observed due to the cleavage of the His6-tag by Fenton reactions, but this is not observed in the other sample despite the presence of [Co^{II} NTA] and H₂O₂.

5

Examples

Experimental Details

10 Expression and purification of His6-GFP

The His6-GFP expression plasmid (Addgene # 29663) {Pedelacq, 2005, r02241} was transformed into BL21(DE3) *E.coli* cells. A 10 ml overnight pre-culture started from single colony was added to 1 L of fresh autoclaved LB medium with 50 mg kanamycin. The cells were grown to OD₆₀₀ = 0.6 at 37 °C at 250 rpm and then protein expression was induced with 0.5 mM IPTG and the cells were grown for another 4 hours. Cells were harvested by centrifugation at 5000 rpm for 10 min. The cell pellet was suspended into 20 ml buffer A (50 mM Tris-HCl [pH 7.4], 300 mM NaCl). The cells were lysed by sonication and the lysate was cleared by centrifugation at 12000 rpm for 30 min followed by filtration through a 0.45 µm filter. The lysate was incubated with 2 ml of Ni-NTA Agarose beads for 1 hour. Then, the beads were washed with 50 ml buffer C (Buffer A with 25 mM imidazole) and the protein was eluted with 10 ml buffer B (Buffer A with 250 mM imidazole). The purified GFP-His6 was dialyzed against 2 L buffer A twice for at least 6 hours.

25

Study of Co³⁺ mediated GFP-His6 binding to NTA beads

Preparation of Co²⁺-NTA beads with His6-GFP. 1.75 ml of Ni-NTA Agarose resin (Novagen, 2.39x10⁹ beads/ml, 5.3x10⁷ NTA groups/bead) were treated with the flowing solutions in the given order and between each step the bead suspension was centrifuged for 1 min at 2000 rpm to sediment the beads and decant the liquid: 15 ml Mili-Q water, 5 ml 0.1 M EDTA (pH 7.5), 3 x buffer A,

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2 ml 0.1 M CoCl_2 , 15 ml buffer B, 3xbuffer A. Then the Co^{2+} loaded NTA beads were incubated with an equal volume of 10 μM GFP-His6 solution in buffer A. Complex $[\text{Co}^{\text{II}} \text{NTA His6}]$ and $[\text{Co}^{\text{III}} \text{NTA His6}]$ (without bead) is shown in Figure 1.

5

Optimization of H_2O_2 treatment for the oxidation to Co^{3+}

100 μl aliquots of the above described bead suspension was added to 1.5 ml tubes for each experiment. Then 50 μl of a H_2O_2 solution in buffer A was added to each tube to result in 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 mM H_2O_2 final concentration and the beads were reacted for 1 hour. Then 50 μl of a 1 M imidazole stock solution (final imidazole concentration=250 mM) was added to each tube and 100 μl of each solution was analyzed for GFP fluorescence ($\lambda_{\text{ex}} = 480 \text{ nm}$, $\lambda_{\text{em}} = 510 \text{ nm}$) S2 (TECAN, infinite 2000 plate reader). The solutions were added back to the beads after the fluorescence measurements and the fluorescence was measured again after 1, 3, 7, and 16 days to analyze the release of GFP-His6 from Co^{3+} -NTA beads over time. The initial rate of His6-GFP release from the beads was fitted to 1st order kinetics using the data from the samples which were treated with 5, 10 and 20 mM H_2O_2 (Figures 7A) and 7B)).

20

To show the generality of the presented concept, aliquots of Co^{2+} -NTA agarose beads, prepared as described above, were incubated with an equal volume of various His6-tagged protein solutions (10 μM). 100 μl aliquots of these beads were incubated with 20 mM H_2O_2 for 1 hour before imidazole was added to a final concentration of 250 mM. The solution above the beads was analyzed for eluted protein by SDS-PAGE gel (Invitrogen, NuPage Bis-Tris 4-12 %) (Figure 7C)).

25

Chemical Stability Measurements

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To obtain Co^{3+} -NTA beads with His6-GFP, the above described beads with immobilized His6-GFP on Co^{2+} -NTA beads were reacted with 20 mM H_2O_2 for

- 16 -

1 hour. Then the excess H_2O_2 was removed by repeated washing of the beads with buffer A. Twice the bead volume of buffer A was then added to the beads and 150 μl aliquots of this suspension are used in each experiment. For comparison an equal number of Co^{2+} -NTA with GFP-His6 in an equal volume of buffer A was used as well. 50 μl of the appropriate chelators (final concentrations: 250 mM imidazole, 25 mM NTA and 25 mM EDTA) or reducing agents (final concentration: 1mM) in combination with 250 mM imidazole was then added to the beads and let to react for 1 hour. 100 μl of the solution above the beads was then analyzed for the His6-GFP fluorescence with the same settings as above. The solutions were added back to the beads after the measurements and analyzed 24 hours later again (Figure 9A). Further, equal amounts of Co^{2+} -NTA and Co^{3+} -NTA beads were treated with 100 mM ascorbate, TCEP and DTT in the presence of 250 mM imidazole or put into pH 3.5 acetate buffer. The amount of eluted protein after 1 hour and 1 day was quantified by His6-GFP fluorescence, where possible, and SDS-PAGE gels (Invitrogen, NuPage Bis-Tris 4-12%) (Figure 9B).

Stability of His6-GFP immobilized at Co^{3+} -NTA centers in cell culture

REF52 cells were kept under standard cell culture conditions in 10% FBS-DMEM medium in 48 well cell culture plates. To a 70% confluent REF52 culture, equal amounts of His6-GFP immobilized onto NTA agarose beads at Ni^{2+} , Co^{2+} and Co^{3+} centers in DMEM were added (250 μl DMEM with 50 μl bead volume). The medium with the beads was removed from the culture plate after 1, 6 and 24 hours, 250 mM imidazole was added and the His6-GFP fluorescence in the supernatant was measured. The fluorescence intensity measured for the Ni^{2+} beads was set to 100% (Figure 9C).

UV-Vis spectra of Co^{2+} and Co^{3+} complexes

Different complexes were obtained by mixing the ligands in the corresponding stoichiometry with 1.5 mM CoCl_2 in buffer A to obtain

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[Co^{II}(NTA)], [Co^{II}(NTA)(Imidazole)₂] and [Co^{II}(NTA)(His6-peptide)]. His6-peptide was purchased from Innovagen, with an acetyl functionalization on the N-terminus. To obtain the Co³⁺ versions of these complexes 20 mM H₂O₂ was added to these solutions and reacted for 1 hour. The UV-Vis spectra of these complexes were recorded and the maxima were determined.

Separation of different His6-GFP NTA bound forms by Ni-NTA columns Co³⁺-mediated labelling of His6-GFP with NTA

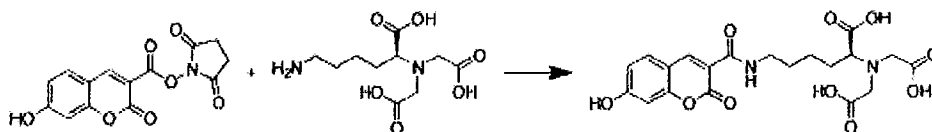
250 µl of 20 µM His6-GFP with various amounts of [Co^{II}NTA] (0-800 µM) is incubated with 10 mM H₂O₂ for 1 hour before it is applied to a 5 ml Ni-NTA column connected to a FPLC system (AKTA Purifier). As controls GFP-His6 alone and GFP-His6 in the presence of 800 µM [Co^{II}NTA] is also analyzed in the same way as the H₂O₂ treated samples. The column is run with 2.5 ml/min flow and 1.5 ml fractions are collected. The column is first washed with 10 ml buffer A and then a linear S3 imidazole gradient up to 100 mM imidazole over 50 ml is used to elute different His6-GFP species. Then 200 µl from each fraction is analyzed for His6-GFP fluorescence as above. The distribution of the peaks was analyzed by integrating the area of each peak and setting the total area under the peaks to 100% (Figure 2). To ensure that His6-GFP is not damaged by Fenton reactions in the presence of [Co^{II} NTA] (800 µM) before and after incubation with 10 mM H₂O₂ for 1 hour, these samples are analyzed by MALDI-TOF. As a positive control, His6-GFP (20 µM) was incubated with 200 µM Co²⁺, 4.6 mM ascorbate and 10 mM H₂O₂, a conditions where Fenton reactions are known to take place (Figure 12).

Synthesis of coumarin-NTA

7-hydroxycoumarin-3-carboxylic acid N-succinimidyl ester (5 mg, 16.5 nmol) was dissolved in 100 µl DMF and added dropwise to a solution of N_α,N_α-bis(carboxymethyl)-L-lysine hydrate (8.6 mg, 33 nmol) in 0.3 M HEPES pH 7.4. The reaction was stirred for 30 min and the purified by C18 reverse phase column (30 % acetonitrile 0.1 % TFA, flow rate). The purified

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compound was lyophilized and the product was characterized by MALDI-TOF and ^1H -NMR. Molecular formula: $\text{C}_{20}\text{H}_{22}\text{N}_2\text{O}_{10}$ Calculated: 450.13 g/mol Found: 451.26 g/mol



Co^{3+} mediated labelling of His6-GFP with coumarin-NTA

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250 μl of 20 μM His6-GFP with various amounts of $[\text{Co}^{\text{II}}\text{NTA-coumarin}]$ (0-400 μM) is incubated with 10 mM H_2O_2 for 1 hour before it is applied to a HiTrap Desalting column (GE Bio-sciences, CV 5 x 5ml, Buffer A with 2.5 ml/min, fraction size 1 ml over 30 ml). The fraction with the highest His6-GFP fluorescence (Fraction 9) is then applied to the Ni-NTA column and run with the same gradient as described above. The fluorescence of each fraction is recorded both for the His6-GFP and coumarin-NTA ($\lambda_{\text{ex}} = 386 \text{ nm}$, $\lambda_{\text{em}} = 448 \text{ nm}$) signal and plotted (Figure 3A). In a large scale reaction, 500 μl of 80 μM His6-GFP and 200 μM $[\text{Co}^{\text{II}} \text{NTA-coumarin}]$ were incubated with 10 mM H_2O_2 for 1 hour, then the mixture was purified by Ni-NTA column and the second peak was concentrated with a 10 kDa centrifugal concentrating device. The UV-VIS spectrum of the concentrated second peak was recorded and the His6-GFP ($\epsilon_{400} = 16000 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{485} = 83300 \text{ M}^{-1}\text{cm}^{-1}$) and coumarin-NTA ($\epsilon_{400} = 36000 \text{ M}^{-1}\text{cm}^{-1}$) concentrations in the sample were calculated (Figure 3B, a)).

The concentrated sample was also applied to a HiTrap Desalting column (GE Bio-sciences, CV 5 x 5 ml, Buffer A with 2.5 ml/min, fraction size 1 ml over 30 ml) using detection at 280 nm, 400 nm and 490 nm. Further, the fluorescence of each fraction was measured for both His6-GFP and coumarin-NTA (Figure 3B, b)).

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Example 1: Co^{3+} complex with NTA and His6 protein $[\text{Co}^{3+}\text{NTA}(\text{His6-GFP})]$

His-6-GFP (N-terminal His6-tagged green fluorescent protein) was

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immobilized onto Co^{2+} loaded agarose-NTA beads and aliquots of these beads were incubated with various amounts of H_2O_2 for 1 hour. Subsequently 250 mM imidazole were added to each sample and the GFP fluorescence in the solution above the beads was measured (Figure 4) While His6-GFP bound to Co^{2+} centers is eluted, the protein bound to exchange inert Co^{3+} centers is not. As is observed in these experiments with increasing H_2O_2 concentrations less and less proteins is eluted and above 10 mM H_2O_2 concentration very little protein is eluted. The oxidation of Co^{2+} to Co^{3+} on the beads with H_2O_2 can also be observed by the color change of the beads from pink to purple (Figure 5) Similarly, in solution when the model complex, $[\text{Co}^{\text{II}} \text{NTA}(\text{His6-peptide})]$ is oxidized with H_2O_2 a characteristic d-d transitions peak for Co^{3+} is observed in the UV-VIS spectrum at 542 nm (Figure 6) This peak is blue shifted compared to $[\text{Co}^{\text{III}} \text{NTA}]$ ($\lambda_{\text{max}} = 554 \text{ nm}$) as expected with increasing number of nitrogen donor ligands and the similar to $[\text{Co}^{\text{III}} \text{NTA}(\text{Imidazole})_2]$ ($\lambda_{\text{max}} = 543 \text{ nm}$).

Example 2: Kinetic inertness of the Co^{3+} mediated interaction between NTA and His6-tag

The kinetic inertness of the Co^{3+} mediated interaction between NTA and His6-tag is evaluated. For this, the amount of His6-GFP, which elutes from the beads described above via the ligand exchange reaction with imidazole, is monitored over 16 days (Figure 7). The initial dissociation rate is fitted to first order reaction kinetics and the rate constant is $1.12 (\pm 0.14) \times 10^{-6} \text{ s}^{-1}$. Thus the $[\text{Co}^{\text{III}} \text{NTA}(\text{His6-peptide})]$ complex has a half-life of 7.1 days at room temperature in the presence of 250 mM imidazole where the homologues Co^{2+} complex is transient.

Example 3: Ligand Exchange and Reduction of Co^{3+} mediated interaction between NTA and His6-tag

The Co^{3+} mediated interaction between NTA and His6-tag is also very inert towards ligand exchange with strong chelators and reduction. To

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demonstrate this aliquots of agarose beads with immobilized [Co^{II} NTA(His6-GFP)] and [Co^{III} NTA(His6-GFP)] were incubated with either the strong chelators EDTA and NTA (25 mM) or with commonly used reducing agents in protein chemistry; DTT, TCEP, cysteamine and ascorbate (1 mM) in combination with 250 mM imidazole. After 1 and 24 hours incubation the amount of His6-GFP eluted from the beads was measured (Figures 8 and 9) When His6-GFP was bound to Co³⁺ centers no significant increase in the eluted His6-GFP was observed upon incubation with the tested chelators or reducing agents, while all His6-GFP bound to Co²⁺ beads is eluted under the same conditions and the strong chelators remove the Co²⁺ from the beads (as can be observed by the change color of the beads from pink to white). Thus the [Co^{III} NTA(His6-GFP)] complex is both inert towards the disruption by strong chelators and the reduction back to Co²⁺.

Example 4: Purification over Ni-NTA column

The [Co^{III} NTA(His6-GFP)] complexes are so inert towards ligand exchange that they can even be passed over a Ni-NTA column without disturbing the complexes and we observed that these complexes have a lower affinity towards this column than His6-tagged protein alone. This can be explained by the fact that some of the histidines in the His6-tag are permanently coordinated to the Co³⁺ center and are no longer available to interact with the Ni-NTA in the column. In protein labelling with small molecules this circumvents an important issue since labelling efficiencies are not 100%. While it is easy to separate the unreacted small molecule from the protein by size exclusion chromatography or dialysis, the separation of the labelled protein from the unlabelled one is far more challenging and in most cases is not possible. Further the slow ligand exchange rates also avoid label exchange, a problem with non-covalent reversible labelling. To demonstrate this samples with 20 µM His6-GFP and various amounts of [Co^{II} NTA] were treated with 10 mM H₂O₂ for 1 hour, then applied to a Ni-NTA column and the protein is eluted with a linear imidazole gradient. We observed 3 well-separated peaks, which have different affinities to the column (Figure 10);

- 21 -

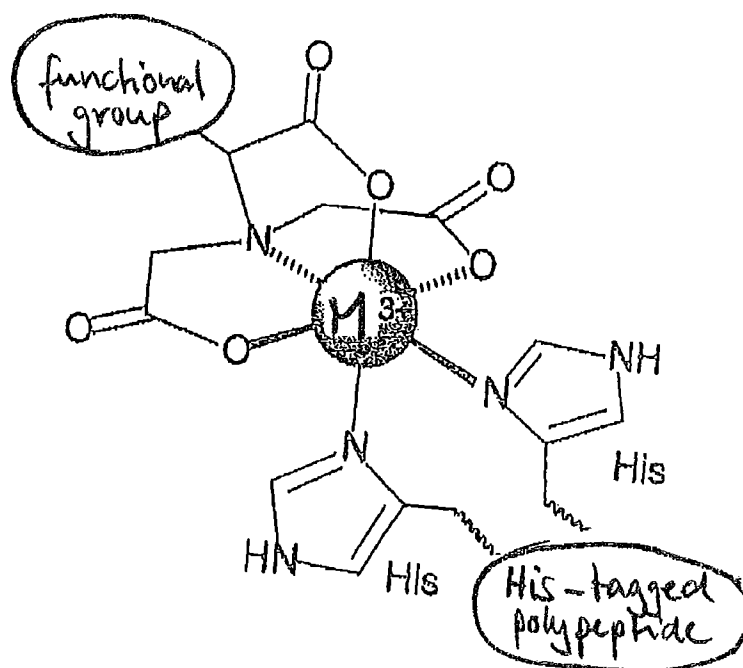
peak A and B have lower affinity to the column with two and one NTA attached to the His6-tag via a Co^{3+} respectively and peak C is unmodified His6-GFP. The distribution of the peaks changed depending on the $[\text{Co}^{\text{II}} \text{NTA}]$ concentration initially used; the higher the concentration the larger peak A with two NTA units bound is (Figure 11). In control experiments we showed that the addition of 10 mM H_2O_2 or 800 μM of $[\text{Co}^{\text{II}} \text{NTA}]$ to His6-GFP has no effect on the elution profile (Figure 2). Similarly, the fluorophore coumarin-NTA was used to label His6-GFP via the Co^{3+} interaction with similar results (Figure 3). Thus, taking advantage of the slow ligand exchange rates of the Co^{3+} center labelled and unlabelled His6-tagged proteins can be separated from each other.

Claims

1. A complex comprising a trivalent metal cation M^{3+} and at least two
5 ligands, wherein a first ligand is a polypeptide with a plurality of
histidine residues, and a second ligand is a chelator having at least
three coordination groups and optionally another functional moiety.
2. The complex according to claim 1, wherein the chelator is an
10 aminopolycarboxylic acid, particularly having 1, 2, 3, 4, 5 or more
carboxylic acid groups.
3. The complex according to claim 1 or 2, wherein the chelator is selected
15 from ethylene diamino tetraacetic acid (EDTA), nitrilo triacetic acid
(NTA), iminodiacetic acid (IDA), ethylene glycol tetraacetic acid
(EGTA), diethylene triamino pentaacetic acid (DPTA), and triethylene
tetramine-N,N,N',N',N'',N''-hexa-acetic acid (TTHA), tris carboxymethyl
ethylene diamine (TED), 1-aceto-1,4,7-triazacyclononane (Tcn) or
derivatives thereof, particularly NTA.
- 20 4. The complex according to any of claims 1-3, wherein the other
functional moiety is selected from labelling moieties, e.g.
spectroscopically active agents and radio-active agents, such as dyes
or a fluorescent group and pharmaceutically and/or biologically active
25 moieties, such as cytotoxic and cytostatic agents.
5. The complex according to any of claims 1-4, wherein the trivalent cation
 M^{3+} is selected from transition metals or rare earth metals, such as
 Co^{3+} , Cr^{3+} , Fe^{3+} , particularly Co^{3+} .
- 30 6. The complex according to any of claims 1-5, wherein the polypeptide is
a recombinant polypeptide and particularly comprises at least 4,
preferably at least 6 consecutive histidine residues.

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7. The complex according to any of claims 1-6, wherein the polypeptide comprises at least 4 histidine residues in a sequence $[H_nS_m]_k$ wherein S is a spacer amino acid residue, n is in each case independently 1-4, m is in each case independently 0-6, and k is 2-6.
8. The complex according to any of claims 1-7, wherein the histidine sequence is at the N- and/or at the C-terminus or inserted into the sequence of the polypeptide.
9. The complex according to any of claims 1-8 having the structure $[M^{3+}NTA(\text{His-tagged polypeptide})]$:



$[M^{3+} NTA (\text{His-tagged polypeptide})]$

10. Method of preparing a metal complex according to any of claims 1-9 comprising the steps:

(i) providing a complex comprising a divalent metal cation M^{2+} and at least two ligands, wherein a first ligand is a polypeptide with a plurality of histidine residues, and a second ligand is a chelator having at least three coordination groups and optionally another functional moiety,

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- (ii) oxidizing the divalent cation of the metal complex in step (i) using an oxidizing agent or photo-chemical means, and optionally
- (iii) separating the complex comprising a trivalent metal cation M^{3+} and at least two ligands, wherein a first ligand is a polypeptide with a plurality of histidine residues, and a second ligand is a chelator having at least three coordination groups and optionally another functional moiety from the mixture obtained in step (ii).
- 5
11. The method according to claim 10, wherein the divalent cation is selected from transition metals or rare earth metals, such as Co^{2+} , Cr^{2+} , Fe^{2+} , particularly Co^{2+} .
- 10
12. The method according to claim 10 or 11, wherein the oxidizing agent is selected from peroxides, such as hydrogen peroxide, hypochlorites, periodates, and nicotinamide adenine dinucleotide (NAD), particularly peroxides.
- 15
13. The method according to any of claims 10-12, wherein the separation step (iii) is carried out via chromatography, particularly using Ni-NTA columns.
- 20
14. Solid phase having a chelator covalently bound thereto, wherein the chelator moiety and at least one polypeptide having a plurality of histidine residues are coordinated to a trivalent cation M^{3+} .
- 25
15. Solid phase according to claim 14, wherein the chelator is an aminopolycarboxylic acid which is covalently bound to the solid phase via at least one of the carboxylic acid groups or the amino groups, wherein the chelator and at least one polypeptide having a plurality of histidine residues being coordinated to a trivalent metal cation M^{3+} .
- 30
16. Solid phase according to claim 14 or 15, wherein the solid phase is selected from the group consisting of metals, such as titanium or steel,

- 25 -

metal oxides such as silica or titanium dioxide, glass, carbohydrates, such as agarose, sepharose, or cellulose, magnetic beads and plastics, such as polystyrene.

5 17. Solid phase according to any of claims 14-16, wherein the solid phase is functionalized by amino-groups, carboxylic acid groups and/or activated esters, such as NHS-esters.

10 18. Solid phase according to any of claims 14-17, wherein the chelator is bound to the solid phase via an amide- or ester-linkage.

19. Solid phase according to any of claims 14-18 having the structure
 SP-(L)-NR¹-C(O)-[PCA·M³⁺·His-tagged polypeptide] or
 SP-(L)-C(O)-NR¹-[PCA·M³⁺·His-tagged-polypeptide]

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wherein SP is the solid phase;

R¹ is hydrogen or an C₁₋₆-alkyl,

(L) is an optional linker such as C₁₋₆-alkyl, polyethyleneglycol, and

PCA is a residue of an aminopolycarboxylic acid.

20

20. Solid phase according to any of claims 14-19, wherein the amino polycarboxylic acid is selected from ethylene diamino tetraacetic acid (EDTA), nitrilo triacetic acid (NTA), iminodiacetic acid (IDA), ethylene glycol tetraacetic acid (EGTA), diethylene triamino pentaacetic acid (DPTA), and triethylene tetramine-N,N,N',N',N'',N''-hexa-acetic acid (TTHA), tris carboxymethyl ethylene diamine (TED), 1-aceto-1,4,7-triazacyclononane (Tacr) or derivatives thereof, particularly NTA.

25

21. Solid phase according to any of claims 14-20, wherein M³⁺ is selected from the group consisting of transition metals or rare earth metals, such as Co³⁺, Cr³⁺, Fe³⁺, particularly Co³⁺.

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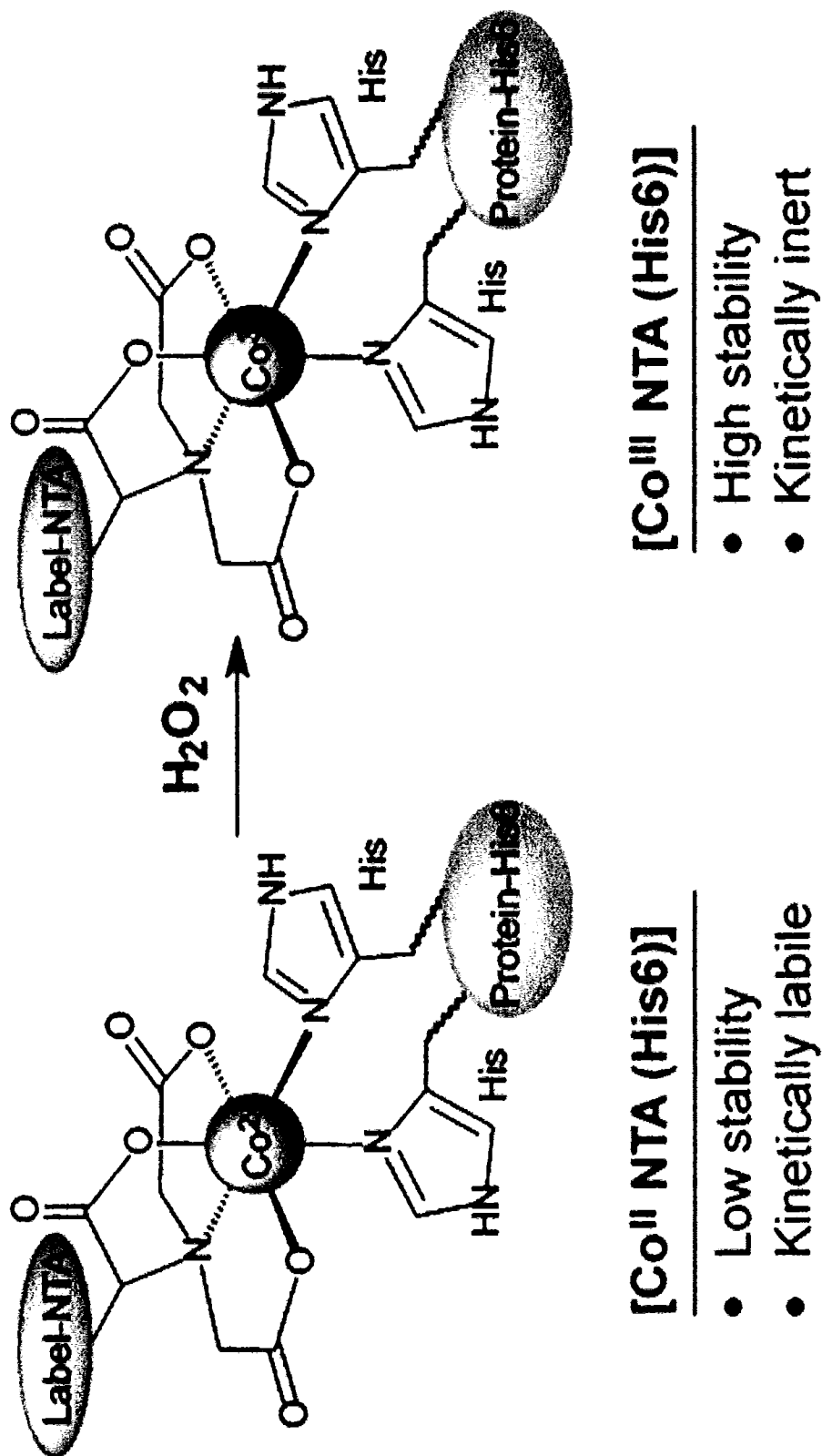
22. Method of labelling a protein having a plurality of histidine residues comprising the steps
- (i) forming a complex comprising a divalent metal cation M^{2+} and at least two ligands, wherein a first ligand is a polypeptide with a plurality of histidine residues, and a second ligand is a chelator having at least three coordination groups and a labelling moiety, and
 - (ii) subjecting the complex obtained in step (i) to oxidizing conditions using an oxidizing agent or photo-chemical means, and optionally
 - (iii) separating the complex comprising a trivalent metal cation M^{3+} and at least two ligands, wherein a first ligand is a polypeptide with a plurality of histidine residues, and a second ligand is a chelator having at least three coordination groups and a labelling moiety from the mixture obtained in step (ii).
23. The method according to claim 22, wherein the chelator is an aminopolycarboxylic acid, particularly having 1, 2, 3, 4, 5 or more carboxylic acid groups.
24. The method according to claim 22 or 23, wherein the chelator is selected from ethylene diamino tetraacetic acid (EDTA), nitrilo triacetic acid (NTA), iminodiacetic acid (IDA), ethylene glycol tetraacetic acid (EGTA), diethylene triamino pentaacetic acid (DPTA), and triethylene tetramine-N,N,N',N',N'',N''-hexa-acetic acid (TTHA), tris carboxymethyl ethylene diamine (TED), 1-aceto-1,4,7-triazacyclononane (Tacr) or derivatives thereof, particularly NTA.
25. The method according to any of claims 22-24, wherein the labelling moiety is selected from spectroscopically active agents and radio-active agents, such as a dye or a fluorescent group.
26. The method according to any of claims 22-25, wherein the divalent cation M^{2+} is selected from the group consisting of transition metals or rare earth metals such as Co^{2+} , Cr^{2+} , Fe^{2+} , particularly Co^{2+} .

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27. The method according to any of claims 22-26, wherein the metal complex of step (i) is [Co²⁺NTA-label His-tagged polypeptide].
- 5 28. The method according to any of claims 22-27, wherein the oxidizing agent is selected from peroxides, such as hydrogen peroxide, hypochlorites, periodates, and nicotinamide adenine dinucleotide (NAD), particularly peroxides.
- 10 29. The method according to any of claims 22-28, wherein the separation step (iii) is carried out via chromatography, particularly using Ni-NTA columns.
30. Metal complexes according to any of claims 1-9 or solid phases
15 according to any of claims 14-21 for use in diagnostic applications.
31. Metal complexes according to any of claims 1-9 or solid phases according to any of claims 14-21 for use in medicine.
- 20 32. Metal complexes or solid phases according to claims 30 or 31 for *in vivo* targeting.
33. Metal complexes or solid phases according to claims 31 or 32 for target-specific drug release.
- 25 34. Metal complexes or solid phases according to claims 31 or 32 for radiotherapy.
35. Use of solid phases according to any of claim 14-21 for cell culture
30 applications.

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Figure 1



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Figure 2

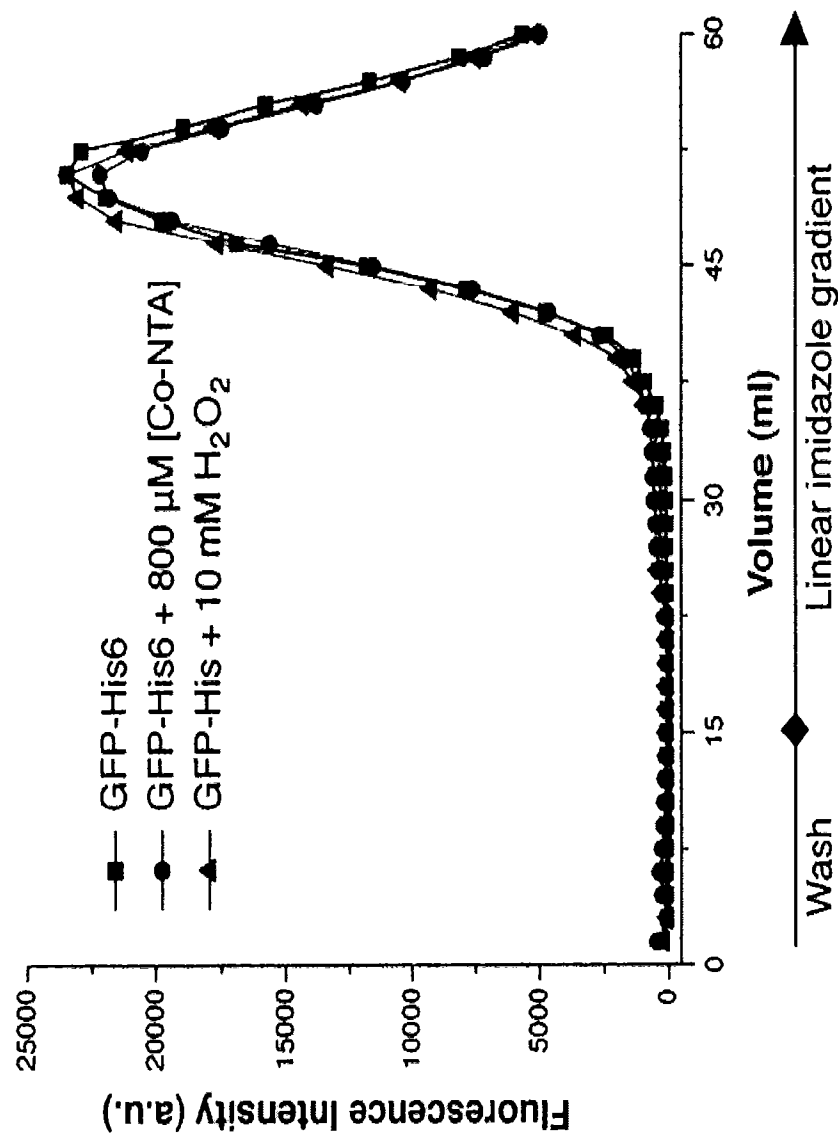
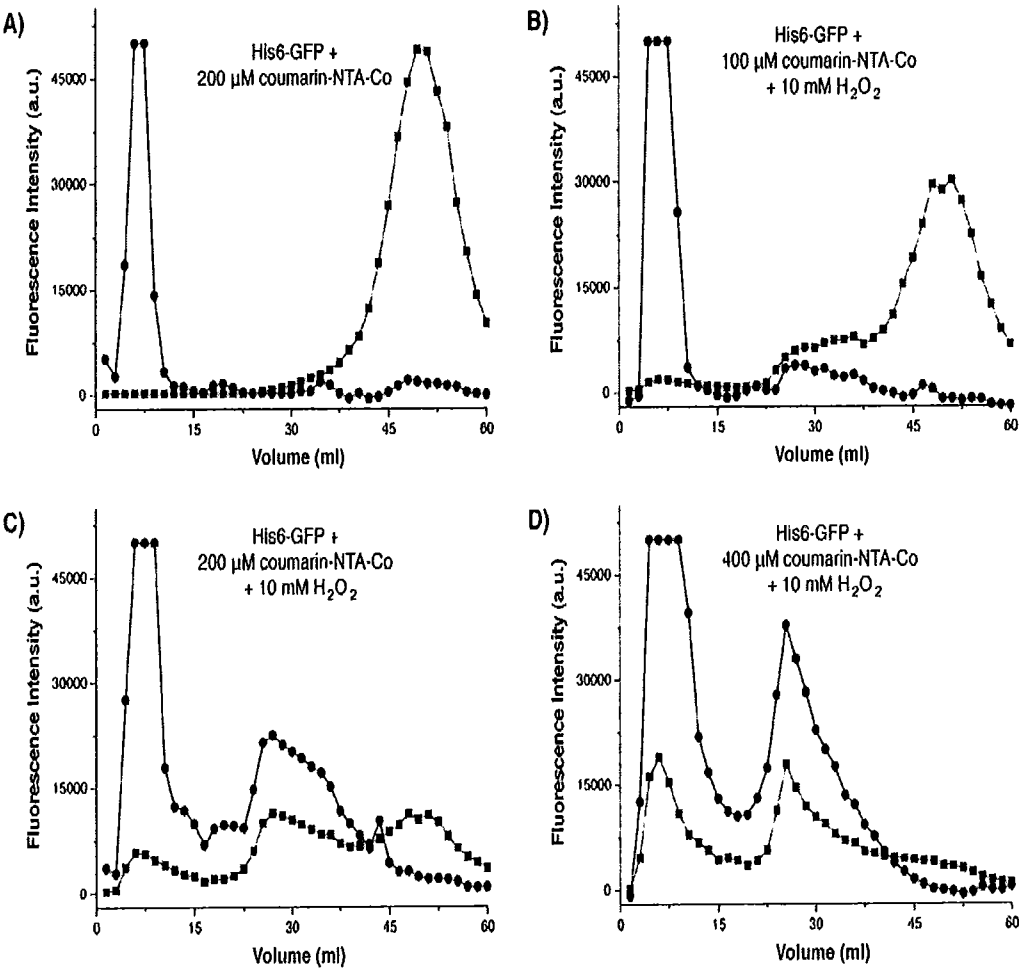
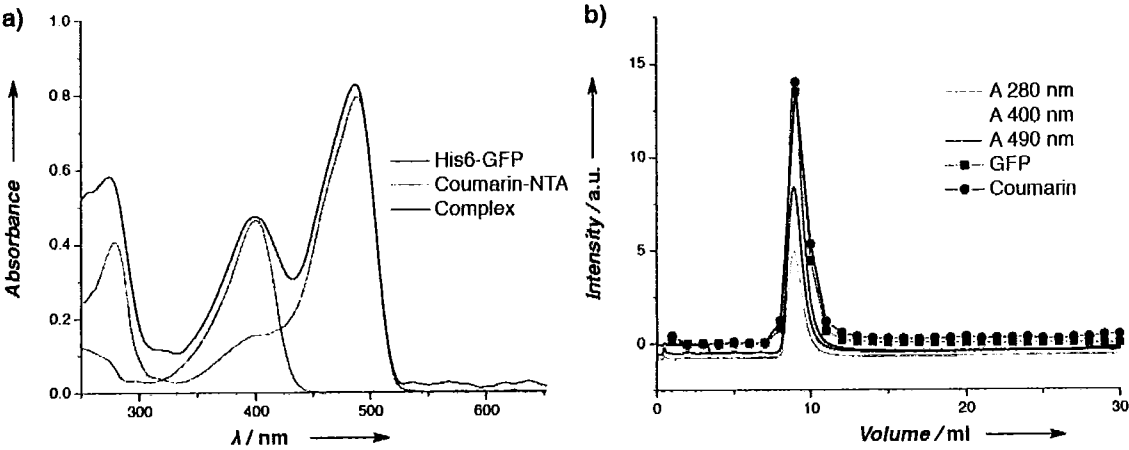


Figure 3

A

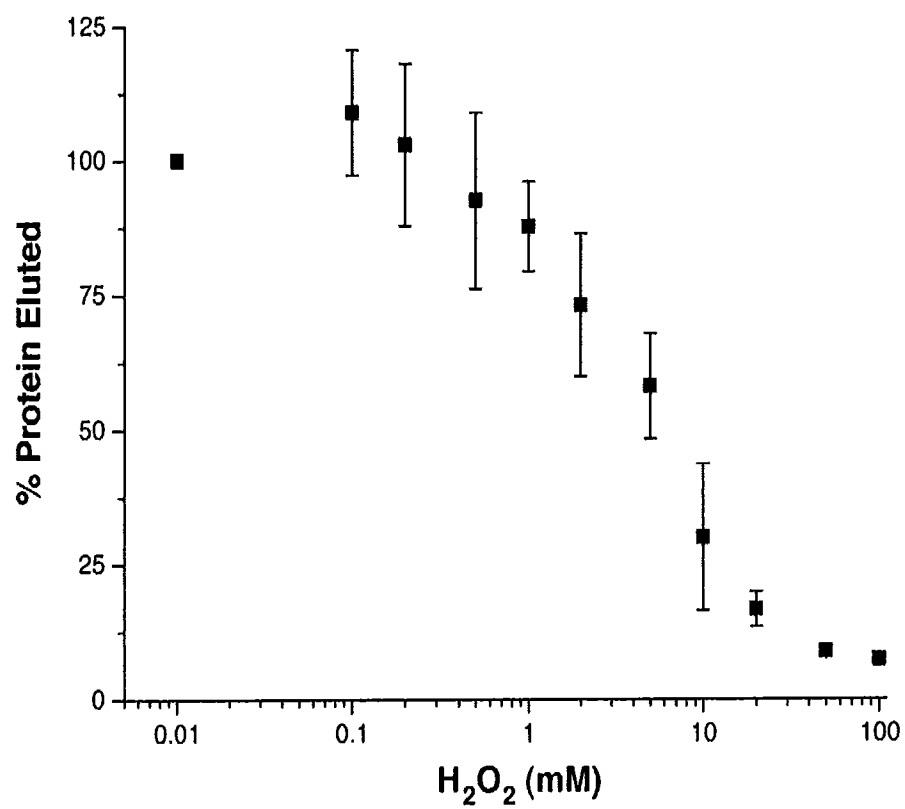


B



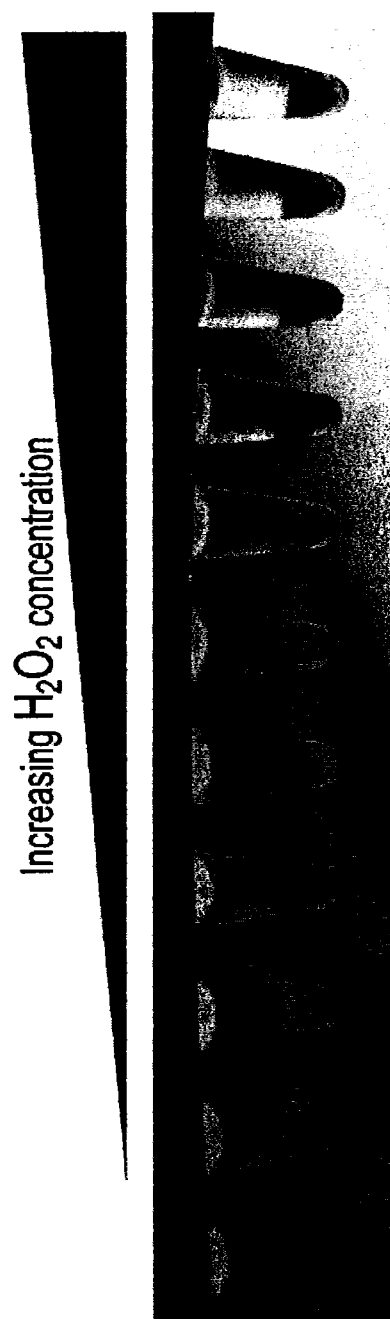
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Figure 4



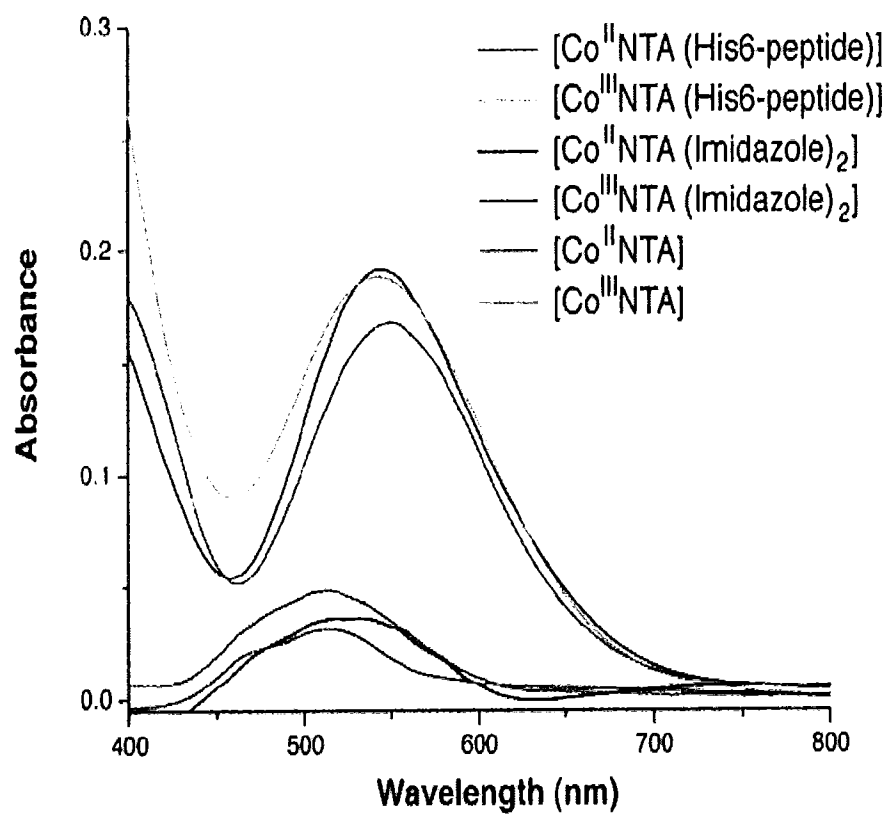
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Figure 5



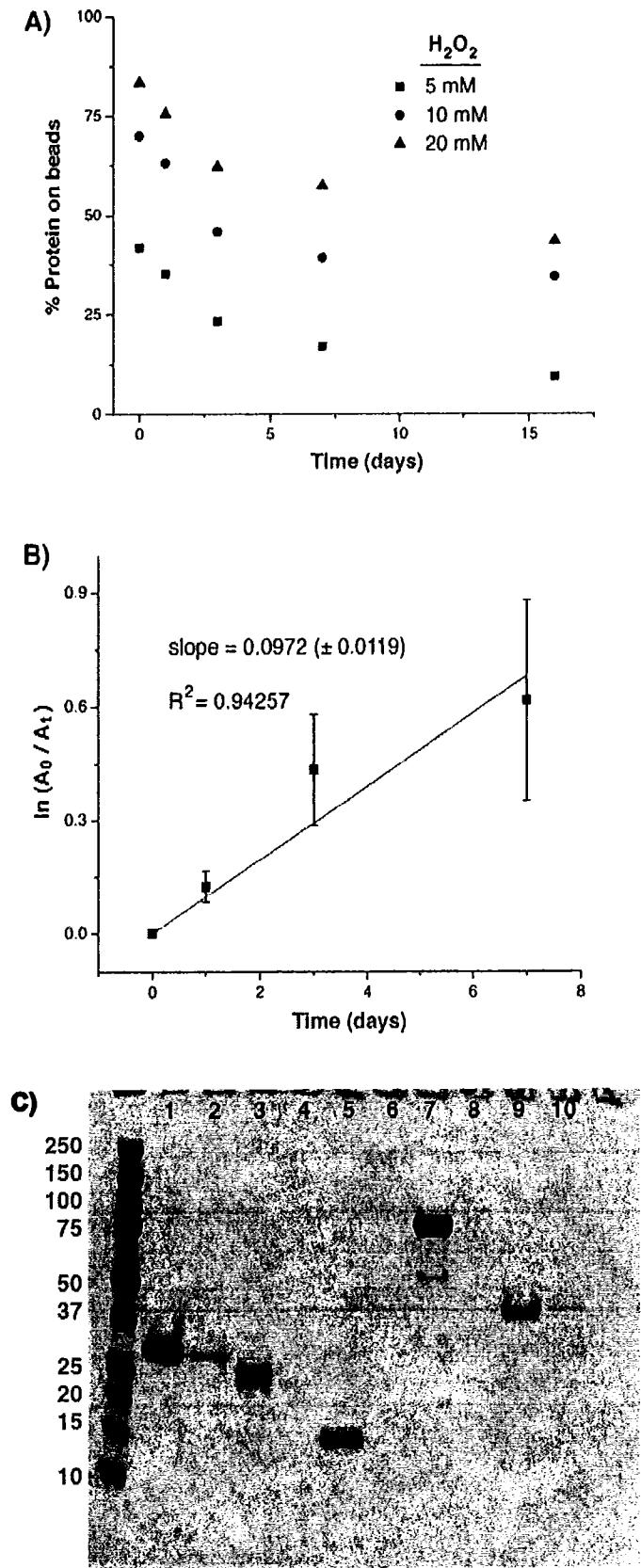
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Figure 6



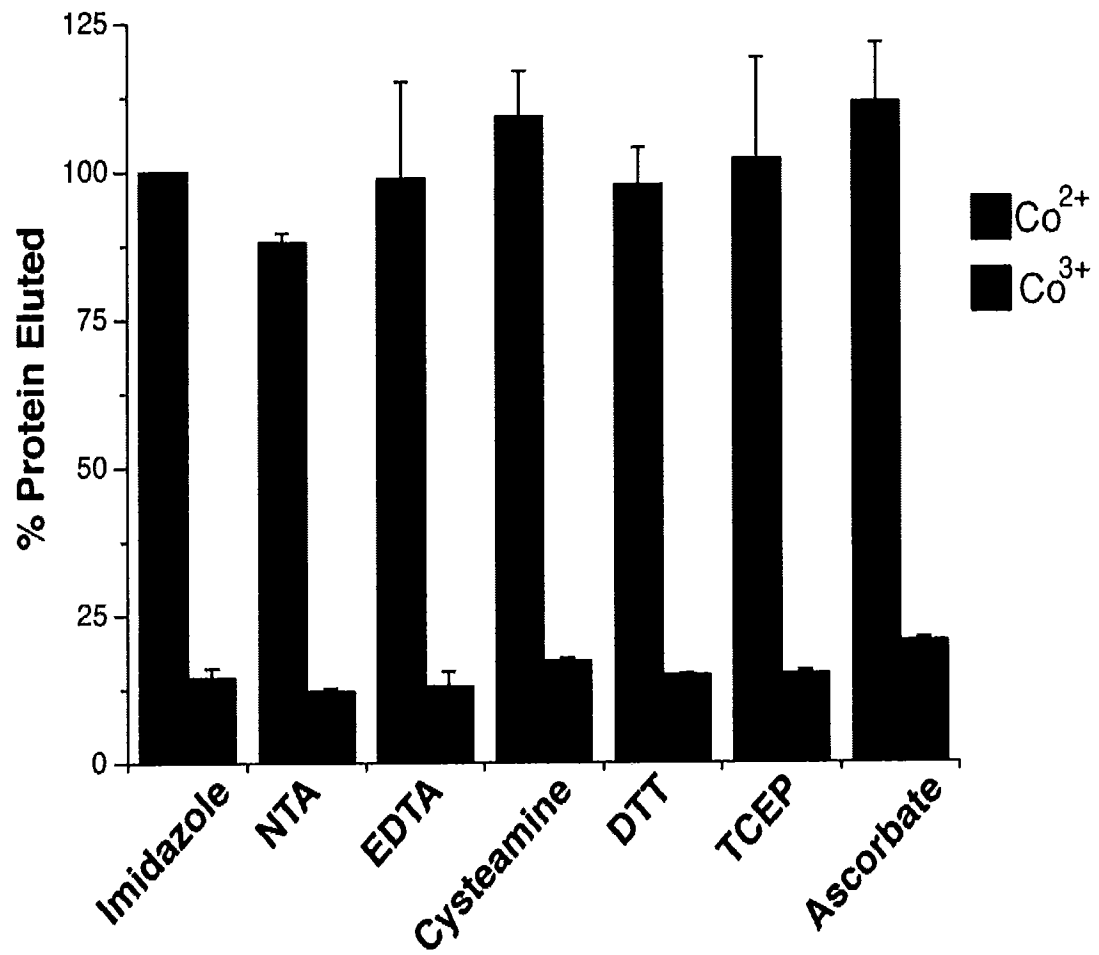
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Figure 7



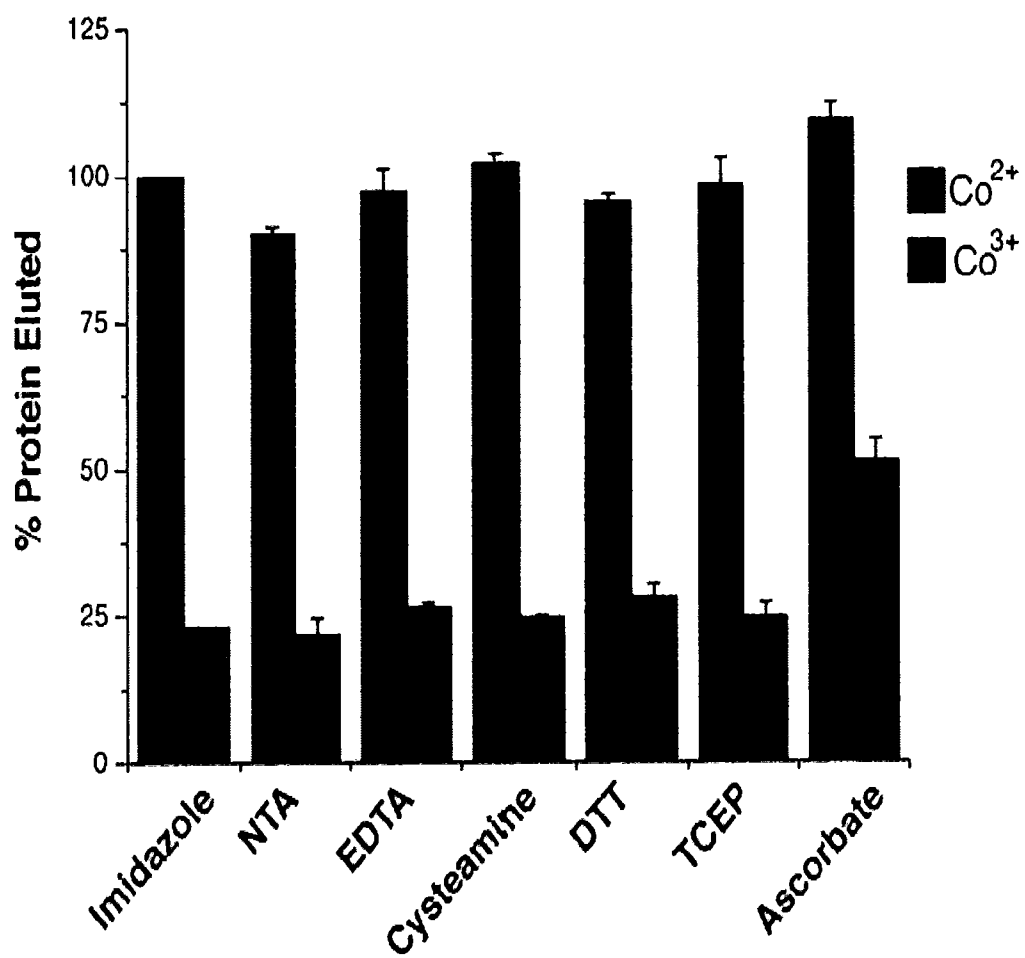
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Figure 8



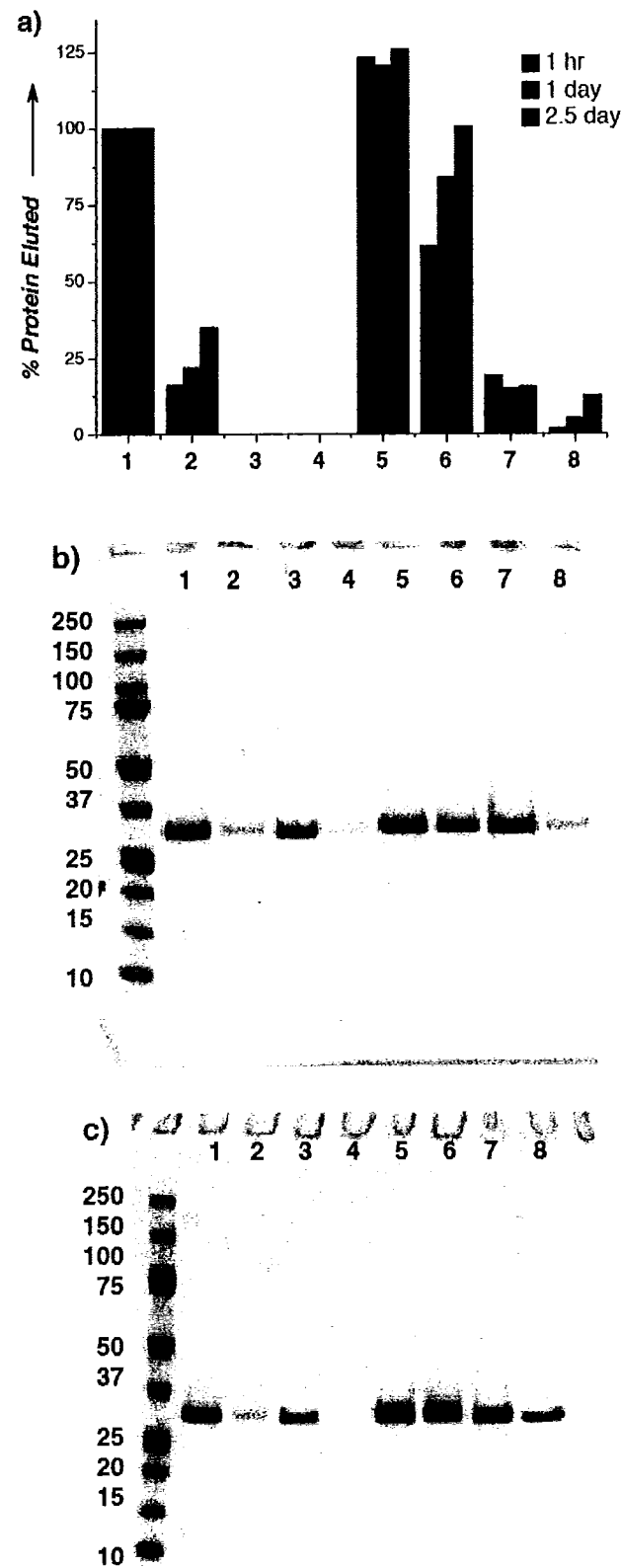
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Figure 9A



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Figure 9B



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Figure 9C

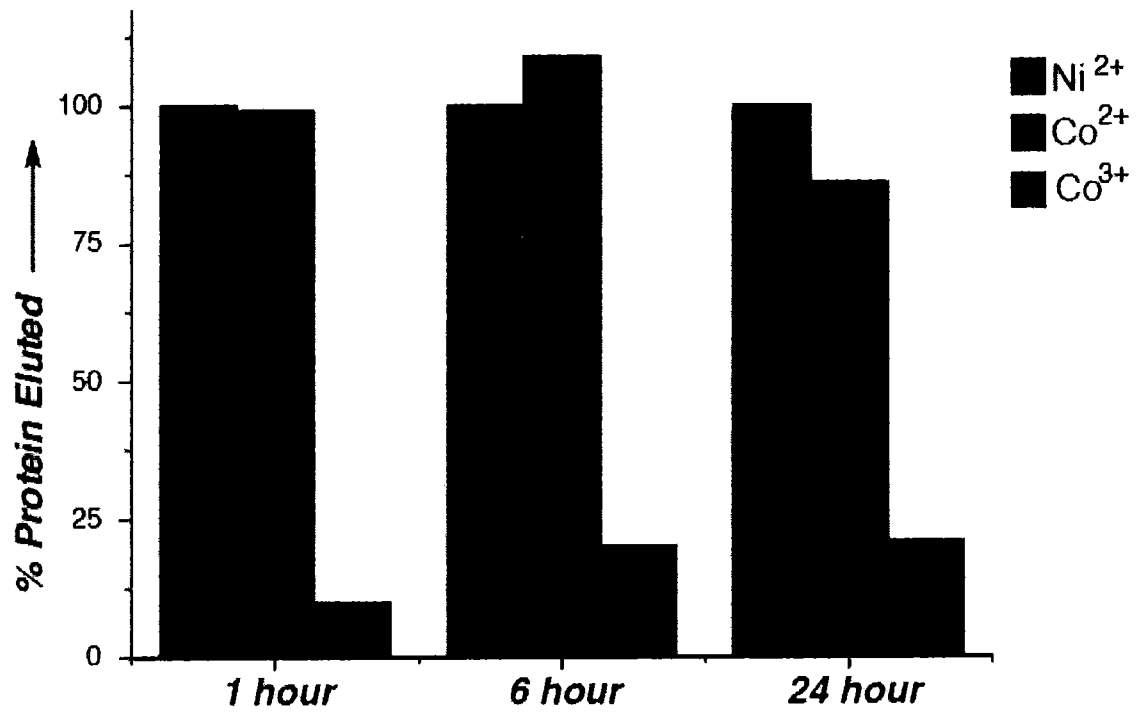
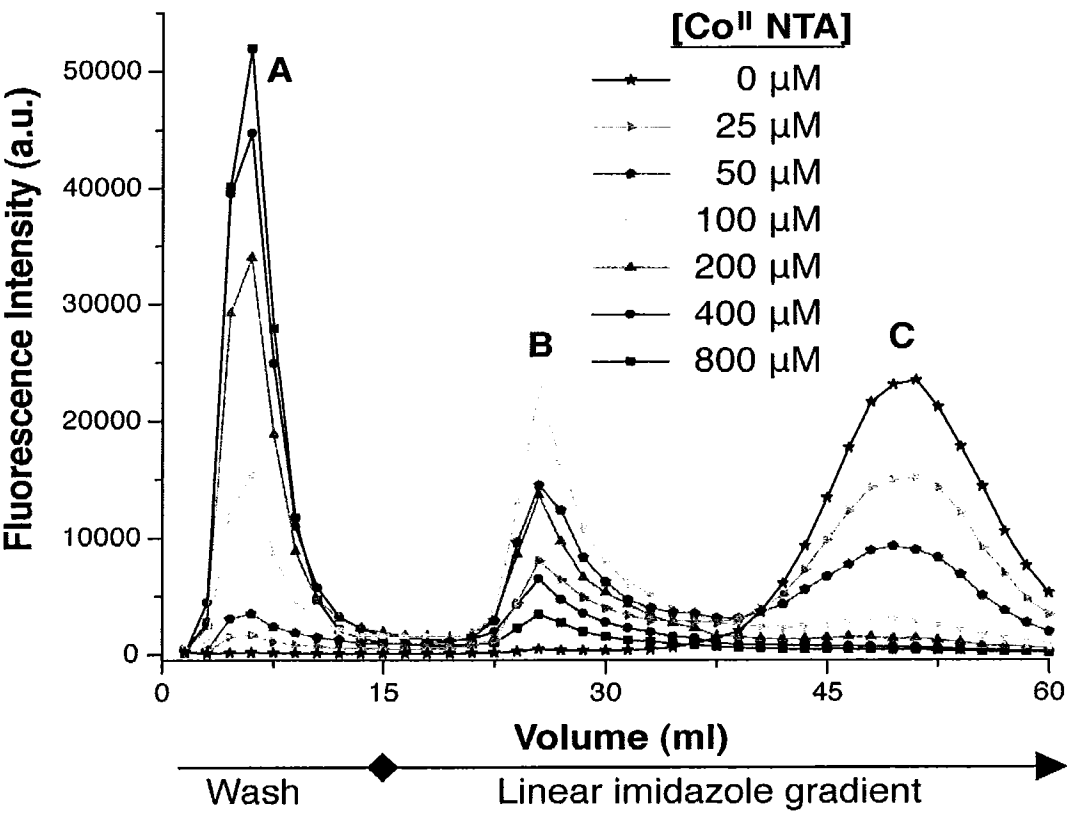


Figure 10



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Figure 11

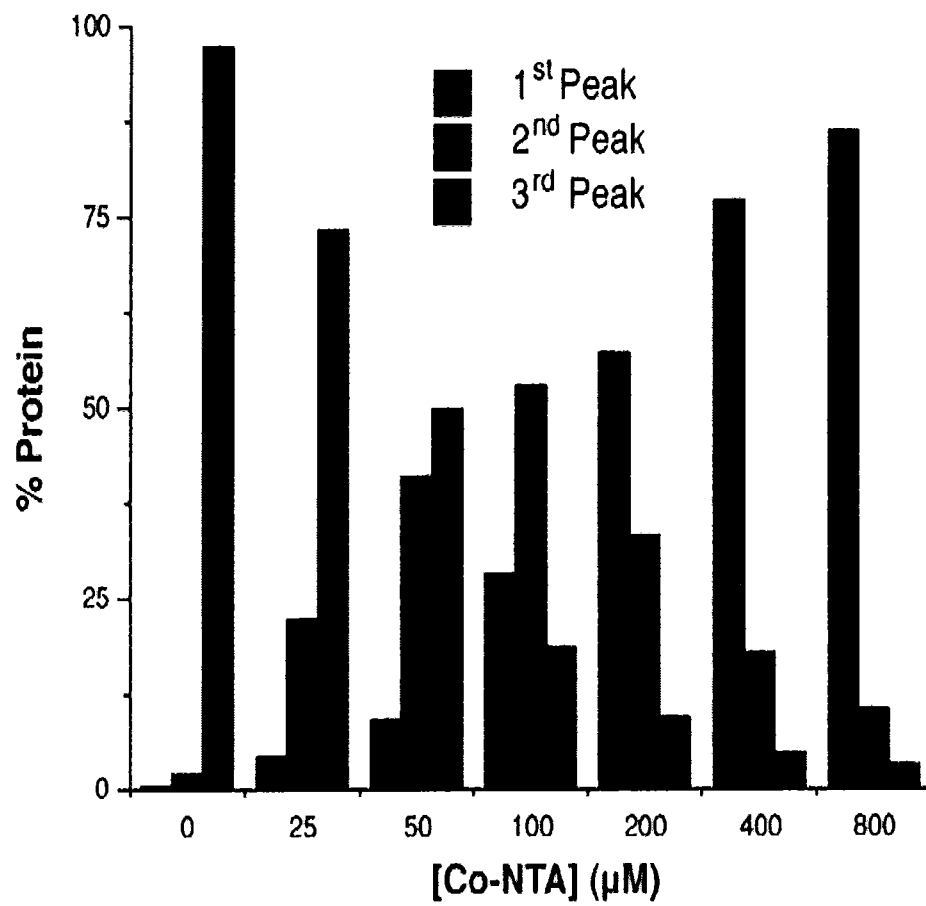
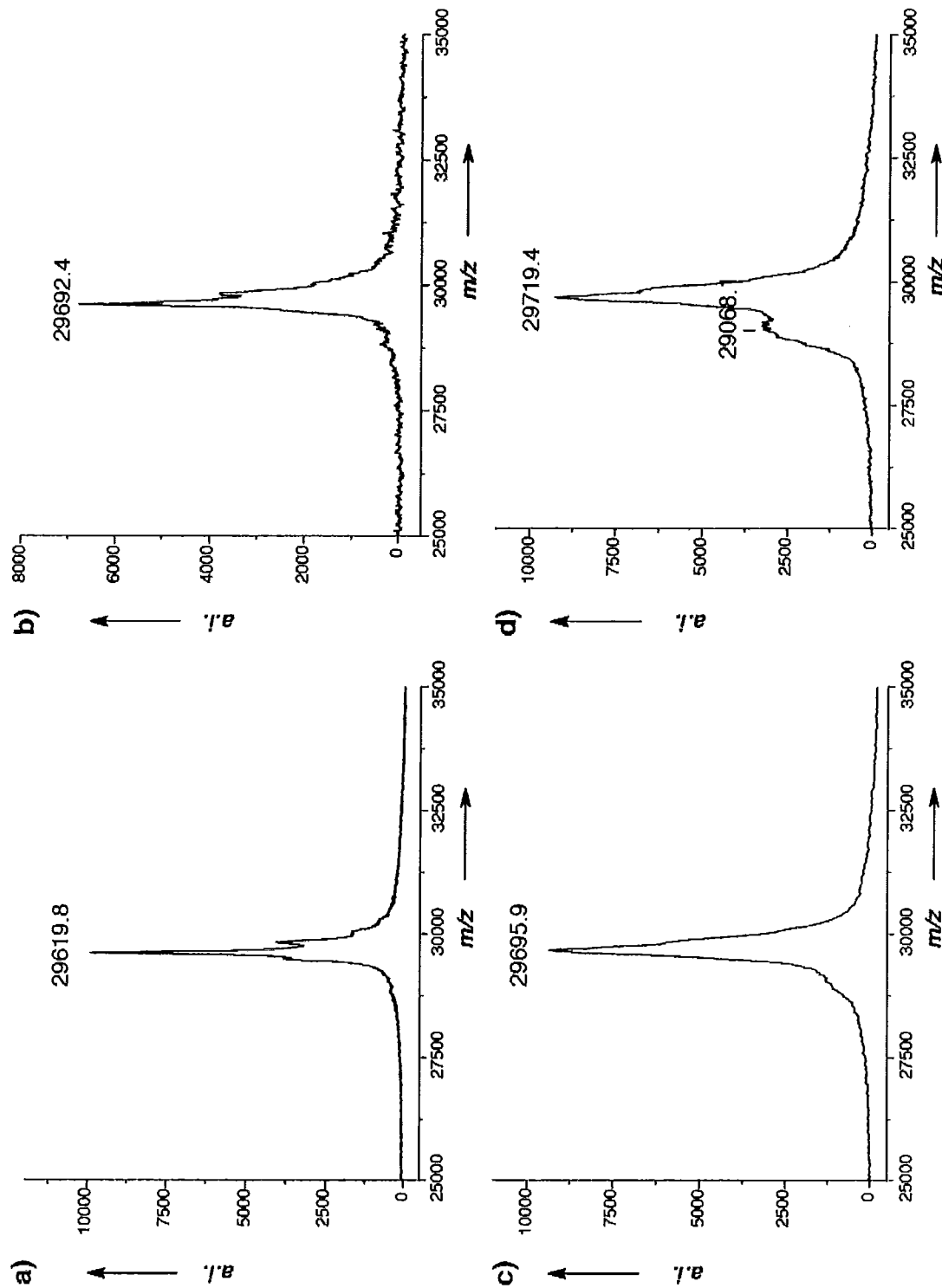


Figure 12



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/073601

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K49/00 A61K31/00 C07F15/06 C07K1/00 C07F15/00
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)
A61K C07F C07K

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/018756 A2 (PFIZER PROD INC [US]; KAROLINSKA INST [SE]; JAKOBSSON PER-JOHAN [SE];) 6 March 2003 (2003-03-06)	1-9, 14-27, 30-35
Y	example 4 page 41, last paragraph - page 42, paragraph 1 claims 40, 45-48, 50-55; example 6 -----	1-29
Y	US 5 439 829 A (ANDERSON LESLIE D [US] ET AL) 8 August 1995 (1995-08-08) cited in the application claim 10; example 4b page 13, paragraph 2 - paragraph 3 column 13, line 23 - line 31 sentence 56, paragraph 1 - sentence 62 ----- -/-	1-29



Further documents are listed in the continuation of Box C.



See patent family annex.

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"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

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Date of the actual completion of the international search

2 December 2013

Date of mailing of the international search report

07/01/2014

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No

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