

"Fluorescent nanoparticles"

The invention relates to fluorescent nanoparticles with particular suitability as in vivo diagnostic aid, especially as contrast agent for discriminating between different tissue types and claims the priority of European patent application 05 025 022.4, reference being made to the contents thereof.

10 In a large number of disorders, a diagnosis which is as early and informative as possible is crucially important for the choice and the harmonization and implementation of the necessary medical procedures. This applies in particular to a large number of tumor types, for whose determination and therapy (including possible sections) discrimination between healthy and carcinogenic tissue is essential. Accordingly, the recovery or even the survival of a patient depends crucially on whether and how well the treating and/or operating clinician can distinguish between different tissue types.

In the past, to improve diagnosis and the medical procedures, contrast agents with whose aid it is possible to visualize functions and structures in the body by imaging methods have been developed. These methods are used inter alia for targeted detection of cancer-associated cell alterations.

30 Thus, for example, Hsu et al. (2004) ("A far-red fluorescent contrast agent to image epidermal growth factor receptor expression", Photochemistry and Photobiology, 79 (3): 272-279) have developed a molecular-specific contrast agent based on an organic fluorophore as marker for early carcinogenic transformation. In this case, the tumor-associated

overexpression of the epidermal growth factor receptor (EGFR) is utilized for identifying altered tissue in the mouth via a red fluorescent anti-EGFR antibody conjugate (Alexa660).

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A general disadvantage of organic fluorophores is that they are metabolized in the body, with the fluorochrome being degraded or inactivated. The metabolization thus counteracts the high labeling intensity which is necessary for diagnosis. As the residence time of the organic fluorophore in vivo increases, this problem intensifies and represents a considerable difficulty, especially in the labeling of cells in deeper tissue layers.

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In addition, organic fluorophores which emit at longer wavelengths in particular have the disadvantage that their quantum yield is reduced by the chemical conjugation process. Moreover, organic fluorophores are very susceptible to photobleaching, which may even after brief irradiation lead to a substantial loss of fluorescence. A contrast agent based on these fluorophores thus has a fluorescence strength and stability with prolonged excitation time which are too low to be suitable for the detection/labeling of cells in deeper tissue layers ("deep tissue imaging"). Thus, it is evident from the study by Hsu et al. (2004) that the Alexa660 conjugates exhibit a maximum depth of penetration of 0.5 mm, so that detection of the fluorescence is no longer a reasonable possibility.

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A further known possibility for the fluorescent labeling of cellular alterations consists of using so-called quantum dots (QDs), which are fluorescent nanoparticles a few nanometers in size whose core consists of semiconductor materials such as CdSe, CdTe,

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InP or the like.

- However, when the known QDs are used in biological systems they show the so-called blinking phenomenon, i.e. the nanoparticles alternate between a fluorescent and a nonfluorescent state. This phenomenon makes the quantum dots useless in particular for in vivo application. In addition, the "blinking" may also indicate a disintegration of the nanoparticle core, by means of which toxic cadmium may be released into the body. This is particularly disadvantageous because the quantum dots accumulate in the body, for example in the liver or the spleen.
- The present invention is accordingly based on the object of providing fluorescent nanoparticles which exhibit particular suitability for use as diagnostic aid, in particular as contrast agent in vivo.
- This subject is achieved as set forth in the main claim. The dependent claims and separate independent claims relate to advantageous embodiments. The nanoparticles of the invention can be employed both in vitro and in vivo for the specific labeling of selected biological structures or functions. In particular, the nanoparticles selected here can serve as in vivo contrast agents for assisting medical interventions, especially surgical interventions.
- The nanoparticles of the invention comprise at least three structures, specifically an inorganic core, which is sheathed by a passivating layer which then in turn carries specific ligands, it also being possible for the specific ligands to be part of the passivating layer. These lead to the specific binding of the nanoparticles to the target molecule (target) of the

biological system. The inorganic core of the nanoparticles of the invention with the passivating layer surrounding it has a hydrodynamic diameter of not more than 15, preferably not more than 10 nm. 5 thermodynamic diameters of not more than 8 nm or not more than 5 nm are particularly preferred.

The passivating layer has the task in particular of increasing the fluorescence intensity and the chemical 10 and physical stability of the inorganic core. The inorganic cores sheathed by the passivating layer are characterized by a quantum yield of at least 10%, advantageously at least 30, 50 or even 70%. The quantum yield means in this connection the ratio of the amount 15 of light emitted by a sample to the amount of light absorbed by the sample. The passivating layer advantageously has a thickness of not more than 1 nm. The diameter of the passivated core is in this case increased by not more than 2 nm.

20 It is advantageous for the nanoparticles in each case also to be provided with modifiers, in particular to improve the compatibility with the biological environment. The increase in the hydrodynamic radius 25 through the use of modifiers preferably does not exceed 2 nm. The thickness of the passivating layer and of the modifiers depends in the individual case also on the relationships of the two structures to one another and in the relationship to the inorganic core.

30 Owing to the restriction on the size of the nanoparticles of the invention, they are particularly suitable for use as diagnostic aid in the living patient. Thus, the reduction in size results in an 35 increase in the rate of diffusion and in the depth of penetration into tissue. This allows a uniform and

rapid distribution of the nanoparticles in the biological environment, and penetration as far as possible through a tissue (e.g. a tumor) after local administration. The nanoparticles of the invention  
5 likewise allow systemic administration which can also take place by injection. Local administration, e.g. topical administration or intra- or peritumoral administration for the treatment of tumors, is preferred, however.

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Particularly advantageous embodiments of the nanoparticles of the invention have a hydrodynamic diameter of not more than 8, particularly preferably of not more than 4 nm. Nanoparticles with a size of this  
15 order can even be excreted through the kidney, so that their accumulation in the body is distinctly less or zero. The nanoparticles of the invention thus considerably reduce the problem, which is probably associated with the known quantum dots, of long-term  
20 toxicity.

In a further advantageous embodiment, the nanoparticles of the invention emit a fluorescent spectrum between 600 and 700 nm, particularly preferably from 600 to  
25 650 nm, especially preferably 620 to 650 nm. This emission spectrum has the advantage of maximal tissue transmission owing to only low absorption by hemoglobin and other light-absorbing substances in the living system (including water). Light of these wavelengths is  
30 still perceptible by the human eye, so that the treating clinician is able to identify the labeled tissue without further elaborate technical aids for detection (e.g. CCD cameras). This is particularly advantageous when the nanoparticles of the invention  
35 are used as contrast agents during a surgical intervention to discriminate between (for example)

carcinogenic and healthy tissue.

In one embodiment, the nanoparticles which can be employed according to the invention are known  
5 nanoparticles with a core for example composed of CdSe, CdS or CdTe, as are described for example in US 2004/0247861 with reference to scientific publications (see paragraphs [0006]). Reference is also made in this publication to documents concerning the  
10 preparation of the core materials (see [0007]), e.g. to US 6,179,912. Reference is made to these documents in their entirety for the disclosure of the properties of these known nanoparticles and the preparation thereof.

15 It is particularly advantageous if the inorganic core of the nanoparticles of the invention consists essentially of semiconductors. These cores emit, depending on their individual size and/or composition, light in various colors, but all show broad band  
20 absorption in the same region of the light spectrum (UV to VIS region). The excitation and emission spectra lie far apart, because of the high Stokes shift, making simple and simultaneous excitation of different quantum dots possible. They have narrow and symmetric emission  
25 spectra which overlap only slightly or not at all. Further positive properties which are of great importance in particular for the improved depth of penetration and the in vivo labeling are the high quantum yield of up to 80% and the high photostability.

30 Quantum dots, which may represent the inorganic core of the nanoparticles of the invention, are disclosed in WO2005/001889. According to this, an inorganic core composed of an alloy of at least two semiconductors  
35 which are either homogeneously dispersed or else for which a concentration gradient is present in each case

within the alloy is involved. Reference is made to WO2005/001889 cited above concerning the disclosure of the nature and the preparation of these quantum dots. The cores may differ in size by 5% in each case.

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Accordingly, the inorganic core of the nanoparticles of the invention may comprise an alloy of at least two semiconductors, the core having a homogeneous composition and being characterized by a band-gap energy which is non-linear with respect to the molar ratio of the two semiconductors.

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Alternatively, the core may be non-homogeneous in nature, in which case the concentration of the first semiconductor increases gradually, starting from the center of the core, up to the surface of the core, and the concentration of the second semiconductor decreases gradually from the center of the core to the surface thereof.

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It is equally true of both cores that at least one of the semiconductors is a group II-group VI semiconductor or a group III-group V semiconductor (the group definition corresponds to the groups of the Periodic Table of the Elements). The alloy may be selected for example from the group of the following alloys: CdSeTe, CdSSe, CdSTe, ZnSeTe, ZnCdTe, CdHgS, CdHgTe, InGaAs, InGaP, GaAlAs, InGaN. These cores may additionally have a coating of inorganic material such as, for example, semiconductors (e.g. ZnS). This additional layer is known to the skilled worker as "capping" or "shell".

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Group II-group VI and group III-group V semiconductors are generally known and include for example  $\text{CdS}_{1-x}\text{Se}_x$ ,  $\text{CdS}_{1-x}\text{Te}_x$ ,  $\text{CdSe}_{1-x}\text{Te}_x$ ,  $\text{ZnSe}_{1-x}\text{Te}_x$ ,  $\text{Zn}_{1-x}\text{Cd}_x\text{Te}$ ,  $\text{Cd}_{1-x}\text{Hg}_x\text{S}$ ,  $\text{Cd}_{1-x}\text{Hg}_x\text{Te}$ ,  $\text{In}_{1-x}\text{Ga}_x\text{As}$ ,  $\text{Ga}_{1-x}\text{Al}_x\text{As}$  and  $\text{In}_{1-x}\text{Ga}_x\text{P}$ . The

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semiconductors preferably used are  $\text{CdSe}_{1-x}\text{Te}_x$ ,  $\text{CdS}_{1-x}\text{Te}_x$ ,  $\text{ZnSe}_{1-x}\text{Te}_x$ ,  $\text{Zn}_{1-x}\text{Cd}_x\text{Te}$ ,  $\text{Cd}_{1-x}\text{Hg}_x\text{S}$ ,  $\text{Cd}_{1-x}\text{Hg}_x\text{Te}$ ,  $\text{In}_{1-x}\text{Ga}_x\text{As}$ ,  $\text{In}_{1-x}\text{Ga}_x\text{P}$ , where  $x$  is a fraction from 0 to 1.

- 5 The molar ratio of the semiconductors can assume any molar ratio. However, in the case where the alloy comprises  $\text{CdSSe}$ , a preferred alloy has the molecular formulation  $\text{CdS}_{1-x}\text{Se}_x$ . In the case where the alloy comprises  $\text{CdSTe}$ , a preferred alloy has the molecular  
10 formulation  $\text{CdS}_{1-x}\text{Te}_x$ . In the case where the alloy comprises  $\text{ZnSeTe}$ , a preferred alloy has the molecular formulation  $\text{ZnSe}_{1-x}\text{Te}_x$ . In the case where the alloy comprises  $\text{ZnCdTe}$ , a preferred alloy has the molecular formulation solely composed of  $\text{CdTe}$ . In these  
15 statements,  $x$  is in each case a fraction between 0 and 1.

- These preferred inorganic cores of the nanoparticles of the invention can be prepared with the following steps:  
20 (i) preparation of a first solution under conditions which enable nanocrystals to form, (ii) preparation of a second solution which comprises a precursor of the semiconductors with a molar ratio under a condition which does not enable nanocrystals to form, (iii)  
25 addition of the second solution to the first solution enabling nanoparticles to form, and (iv) altering the conditions which terminate/stop the growth of the nanocrystals and the formation thereof. The method for preparing the cores is described in WO 2005/001889, to  
30 which reference is made concerning the disclosure of the preparation of this preferred embodiment of the inorganic core of the nanoparticles of the invention.

- In an alternative embodiment, the inorganic core may  
35 consist essentially of a noble metal cluster which preferably comprises 2 and 27 noble metal atoms. In a

preferred embodiment, the noble metal is selected from a group consisting of gold, silver, copper, platinum, palladium, osmium, iridium, ruthenium and rhodium. The cluster may have varying charges.

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These cores have the advantage that, owing to their strong absorption and emission, they can easily be detected as single so-called nanodots with a weak mercury lamp excitation. The nanoparticles of the invention with these cores are advantageously to be used as fluorescent single-molecule label and mass label.

In the context of the present invention, the term "noble metal" refers to an element group selected from a group consisting of gold, silver and copper and the platinum group metals (PGM) platinum, palladium, osmium, iridium, ruthenium and rhodium. In preferred embodiments of the present invention, the noble metals are selected from the group consisting of gold, silver and copper. In a particularly preferred embodiment, the noble metal is silver or gold.

The term "cluster" refers to a combination of 2-27 atoms of a metal. Clusters are known inter alia from the areas of chemical catalysis, of ceramics, of semiconductor technology and of material sciences. The skilled worker is therefore familiar with the preparation thereof. WO2004/003558 describes inter alia the preparation of noble metal clusters and additionally contains extensive further references to the literature concerning them. There is disclosure in particular of the preparation of noble metal nanoclusters associated with organic molecules. The term association is in this connection to be understood as every type of linkage, irrespective of the chemical

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or physical nature of the linkage (e.g. covalent, noncovalent, electrostatic or van-der-Waals linkage). Reference is made to WO2004/003558 concerning the preparation of the nanoclusters as core of the  
5 nanoparticles of the invention.

The nanoparticles of the invention have a passivating layer which increases the fluorescence intensity and improves the chemical and physical stability of the  
10 inorganic core. The nanoparticles thus emit light preferably with a quantum yield of more than 10%, preferably of more than 50%.

The nanoparticles of the invention preferably exhibit a  
15 storage stability in an aqueous environment at 4°C of at least 12 months and are preferably stable over a pH range from pH 5 to pH 10, i.e. they show deviations of less than 50% in relation to their specific spectral characteristics such as quantum yield, position of the  
20 emission maximum, half-width of the emission spectrum. Preferred particles show deviations of less than 10% in relation to these specific spectral characteristics.

They also show under biological conditions or in vivo  
25 essentially a constancy/stability of the properties of the core (including the passivating layer surrounding it) for a period of at least three days. Preferred particles show such a constancy/stability for a period of from 7 to 14 days or up to several weeks, where  
30 constancy means in the context of the invention a deviation/alteration of some or all of the abovementioned properties by 50%. Particularly preferred particles show a deviation/alteration of less than 10%.

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The passivating layer comprises at least one compound

- 11 -

able to coordinate metal atoms or metal ions, e.g. zinc, mercury or cadmium ions. This compound may be a Lewis base or a cyclically or linearly unsaturated compound with resonant electrons. As cyclically  
5 unsaturated compound, it may also be a heterocycle or a heteroaromatic compound. The unsaturated or conjugated group is, in a preferred embodiment, located in a terminal position in relation to the structure of the molecule. The passivating layer may further include a  
10 crosslinker, or the cyclically or linearly unsaturated compound may also function as crosslinker.

The compounds coordinating metal atoms or metal ions may bind functionally through chelation, coordination  
15 or electron donor properties of Lewis bases to fluorescent inorganic cores and correspondingly include conjugated portions/groups. These molecules may moreover comprise portions which confer solubility or wettability in aqueous solutions on the cores coated  
20 with them.

These molecules or compounds may include a homogeneous or heterogeneous (heterocyclic) ring system having one, two or more linked (or else fused) rings. Examples of  
25 preferred heteroaromatic systems are thiazoles, thiazole derivatives, oxazoles, oxazole derivatives, pyrroles, pyrrole derivatives including doped or undoped polypyrrole oligomers, thiophenes, thiophene derivatives including doped and undoped polythiophenes,  
30 furans, furan derivatives, pyridine and pyridine derivatives, pyrimidine and its derivatives, pyrazines, pyrazine derivatives, triazine and triazine derivatives, triazoles, triazole derivatives, phthalocyanines and phthalocyanine derivatives, porphyrin and  
35 porphyrin derivatives. These compounds may include unsaturated (olefinic) hydrocarbons or their amines,

phosphorus derivatives or oxygen derivatives which may also include acetylene, propyne and allene, but are not limited thereto. It is preferred for the molecule to have an adequate p or pi electron density in order to  
5 take part in adduct formation or resonance on the surface of the semiconductor core.

The heteroaromatic compound is preferably an imidazole component. It is further preferred for an alkyl-  
10 phosphine compound to be added as crosslinker.

The term "imidazole component" means in the context of this description a heterocyclic or heteroaromatic molecule which comprises at least one imidazole group  
15 (including imidazole derivatives), and which is available for the linkage of the inorganic core or of the passivating layer to a metal such as cadmium, zinc, gallium or a metal cation or a substrate which comprises such a cation. In this connection, at least  
20 one imidazole group should preferably be in a terminal position in relation to the structure of the molecule. The imidazole component binds in its functional form via the ring which comprises delocalized molecular orbitals to the fluorescent nanocrystal. The nitrogens  
25 of the imidazole ring ordinarily serve as coordinating ligands in order to bind a metal ion such as cadmium or zinc in a functional manner.

In one embodiment, the imidazole component comprises  
30 reactive functional groups such as one or two amino acid(s) e.g. histidine, carnosine, anserine, baleine, homocarnosine, histidylphenylalanine, cyclo-histidyl-phenylalanine, 5-amino-4-imidazolecarboxamide, histidylleucine, 2-mercaptoimidazole, Boc-histidine,  
35 hydrazide, histinol, 1-methylhistidine, 3-methyl-histidine, imidazolysine, imidazole-containing

ornithine (e.g. 5-methylimidazole), imidazole-containing alanine (e.g. (beta)-(2-imidazolyl)-L(alpha)alanine), carzine, histamine. These histidine-based molecules or imidazole-containing amino acids can be synthesized by generally known methods.

The term "alkylphosphine" means in the context of the invention a molecule which includes at least one phosphine group (including derivatives thereof) for binding or chelating a nonmetal such as Se, S or other nonmetals, or substrates which comprise such atoms, and which provides at least one functional group (e.g. hydroxyl-, amino-, thiol-, carboxyl-, carboxamide-etc.) for reaction with adjacent molecules.

Preferably at least one phosphine group ought to be located in a terminal position in relation to the structure of the molecule. The phosphine portions serve as coordinating ligands in order to link a nonmetal or ion such as Se or S in functional form to a fluorescent core or a compound from the shielding layer.

In a preferred embodiment, the alkylphosphine-containing compound includes one, two or more phosphine groups which are coupled together (e.g. in polymeric form) and which may also include hydroxymethylphosphine compounds or the like, but are not limited thereto. Alkylphosphine-containing compounds can be synthesized by generally known methods. As is further known, alkylphosphine-containing compounds may additionally include one or more additional functional groups (e.g. hydroxyl-, amino-, thiol-, carboxyl-, carboxamide-etc.). Examples of derivatives are hydroxymethylphosphine derivatives, amides or esters, as long as the derivatization is compatible with the functions of the alkylphosphine as coating which are described herein.

Particularly preferred for coating the fluorescent inorganic cores of the nanoparticles of the invention are tris(hydroxymethyl)phosphine and  $\beta$ -[tris(hydroxymethyl)phosphino]propanoic acid. It is generally known that crosslinked alkylphosphine-containing compounds have the additional possibility of binding functionally to metal atom and/or ions such as Zn or Cd. In this regard, functionalized isocyanates or alkyl cyanoacrylates may be further useful crosslinkers for ligands and adduct formation with florescent cores.

The passivating effect of the passivating layer which is present according to the invention is based on the shielding of surface cadmium or zinc atoms or the like by the complexation with the heteroaromatic compound or heterocycle (preferably with the imidazole component) and the shielding of the counter atoms (Se or S or the like) via the complexation with the alkylphosphine-containing compounds.

The passivating layer of the nanoparticles of the invention is disclosed in US 2004/0247861 A1. This published specification describes the preparation of inorganic cores sheathed with the passivating layer, for example of quantum dots. Reference is therefore made to US 2004/0247861 for the purposes of disclosing the preparation of the passivating layer employed according to the invention and of the inorganic cores sheathed therewith.

The molecules of the passivating layer may further include or carry chemical groups in order to bind and crosslink target molecules and cells (specific ligands). In the presence of corresponding passivating reagents such as  $\text{ZnSO}_4$  and  $\text{Na}_2\text{S}$  these molecules or

compounds can form a passivating layer with the molecules on the fluorescent core ("capping" or "shell").

5 These reagents may also bind functionally to atoms or ions on the surface of the fluorescent nanocrystals so that this additional passivating layer can also be formed directly on the surface of the core.

10 The nanoparticles of the invention may, in an advantageous embodiment, additionally include modifiers which may consist of organic and/or inorganic portions. They serve to improve the compatibility, efficacy and/or solubility of the nanoparticles in a liquid or a  
15 suspending medium, in particular in the physiological environment. This surface modification is advantageous in particular in order to achieve minimal nonspecific adsorption and an increased compatibility in biological systems, especially in the human body.

20 One possibility is modification of the surface with the polyethylene glycol (PEG) which is already approved for certain medical applications, especially in low molecular weight forms, in order to maintain a small  
25 overall size of the nanoparticle. This can increase both the biocompatibility of the nanoparticles and the blood circulation time thereof and the efficiency of uptake into cells. By combining a low molecular weight PEG layer with further substances such as vitamins such  
30 as, for example, folic acid it is possible to achieve a smaller uptake of the nanoparticles in macrophages, because the protein adsorption to the nanoparticles is reduced thereby, thus impeding recognition of the nanoparticles by the immune system.

35 Coating with monosaccharides, di- or trisaccharides up

to low molecular weight polysaccharides composed of one or different monosaccharides represents a further possibility for advantageous surface modification through use of modifiers. One possible embodiment is  
5 modification with, for example, polyglucose, in which it is possible to employ dextran which is approved medically as blood substitute. It shows good biocompatibility/tolerability. A further embodiment is the use of stereoisomeric forms (D-/L-) of the  
10 saccharides in order to counteract possible degradation.

A further embodiment is the use of biologically compatible hydrophilic vitamins as modifiers such as,  
15 for example, thiamine, riboflavin, niacin, pyridoxine, cobalamin, pantothenic acid, ascorbic acid and folic acid. Thus, for example, folic acid may lead to a preferred binding of nanoparticles to cancer cells. This vitamin shows only low immunogenicity and thus  
20 high biocompatibility. Binding to the membrane-associated folic acid receptor facilitates internalization of the nanoparticles.

The surface modification with lipophilic vitamins such  
25 as retinol, cholecalciferol, tocopherol and phylloquinone is likewise possible. Thus, for example, vitamin E may lead to an increased cellular uptake of nanoparticles.

30 Fatty acids such as, for example, 1-octadecenes or 18-methyleicosanoid acid and derivatives thereof may increase the solubility and stability of the colloids and have a terminal functional carboxyl group which can be utilized for subsequent binding of specific ligands.  
35 It is therefore worthwhile also to include fatty acids as modifiers.

A further embodiment of the surface modification is a coating with polyalcohols such as, for example, diethylene glycol (DEG) which are able particularly well to reduce nonspecific protein adsorption. The same applies to polytetrafluoroethylene (PTFE, Teflon), especially in its low molecular weight forms, on the basis of which it is possible to achieve reduced protein adsorption. Polytetrafluoroethylene is frequently employed in cardiosurgical applications.

A surface modification can likewise be undertaken with one or more naturally occurring amino acids, which include both the proteinogenic and nonproteinogenic amino acids, and synthetic amino acids. It is possible in this connection to use both stereoisomers (D and L forms). Di-, tri-, tetra- up to small polypeptides composed of the abovementioned amino acids scarcely stimulate the immune system and are thus likewise suitable for a thin compatibility layer. Possibilities in this connection are artificial amino acid sequences and sequences from biological proteins. Peptide derivatives of natural proteins such as, for example, of phytochelatin can likewise be used for surface modification. Surface modification with Tat peptide and Tat peptide-containing peptides is a further possibility for making nanoparticles available for use in biomedical applications. The Tat peptide is an efficient molecule for bringing, for example, gold nanoparticles through the cell membrane into the nucleus.

A further embodiment of the possible modifiers is the formation of a phosphorylcholine coating. Phosphorylcholine reduces possible nonspecific protein adsorption, such as, for example, on contact lenses. A

phosphorylcholine modification can, because of the non-thrombogenic properties, be employed well in biological systems and is distinguished by high long-term stability.

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Since polylactate is biocompatible, this substance is employed in diverse medical applications. Low molecular weight forms of polylactate in particular are a further possibility for surface modification of the nanoparticles of the invention. It is possible in this connection to employ both stereoisomers (D/L form) in order to reduce possible biodegradation.

Besides the surface modifications mentioned it is possible to link nonspecific proteins in a proteolytically cleavable manner to the nanoparticles. This may result in an increase in the biocompatibility/tolerability. Elimination of the large protein can take place at the target site to release the small nanoparticles in the tissue. It is likewise possible for the elimination to take place after an appropriate residence time. Suitable and preferred for this purpose are widely used proteins such as, for example, transferrin lactoferrin, ceruloplasmin, elastin and albumin as well as other proteins which reduce nonspecific adsorption. Thus, for example, a surface coating composed of combinations of polypeptides with elastin can prevent unwanted thrombus formation and thus increase the biocompatibility of the nanoparticles.

The main serum protein albumin is able to reduce nonspecific interactions with plasma membranes. The correspondingly modified nanoparticle moreover retains the ability of forming specific interactions with target cells through simultaneous binding of a specific

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ligand to the particle surface. Coating with serum albumin may lead to a substantially longer blood circulation time through the prevention of rapid uptake by macrophages after intravenous administration than is  
5 the case with uncoated nanoparticles.

Besides the nonspecific coatings outlined above, the nanoparticles of the invention carry a selective labeling with target cell-specific ligands, for example  
10 they are conjugated to proteins, antibodies, peptides or, particularly preferably, to small, high-affinity protein domains, antibody fragments or other organic molecules which bind, for example, to tumor cell-specific structures or other targets.

15 The combination of reduced hydrodynamic diameter, which leads to the higher rate of diffusion and perfusion mentioned, together with the previously described properties and improvements and with the high  
20 fluorescence intensity in particular in the visible red region of light makes the nanoparticles of the invention a simple diagnostic aid with diverse possible uses for selective and accurate discrimination of tissue types in vivo. These possibilities in  
25 combination with tissue-specific biomarkers serve in particular for differentiating abnormal, (pre)carcinogenic from normal tissue, assisting visual assessment for more precise tumor resection during a surgical intervention. The nanoparticles of the  
30 invention which can be employed in this connection thus serve as contrast agents.

According to the present invention, the nanoparticles can be employed either as in vitro or in vivo  
35 diagnostic aid, theranostic agent and/or therapeutic agent. They can for this purpose be administered

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locally (e.g. intratumorally, intramuscularly or into surgically accessible tissues/organs) or else systemically (e.g. intravenously). Local/topical administration can be envisaged as liquid, spray  
5 solution, gel, foam, cream, active patch. This may be preferred in particular for the treatment/diagnosis of hollow organs. Oral intake is also possible, e.g. as liquid or in the form of tablets or capsules. Inhalation is equally possible (e.g. spray). Anal  
10 administration by suppository is envisaged. In one variant, the nanoparticles can be implanted in depot form. The term "diagnostic aid" is used in the context of the present invention as synonym for "contrast agent", i.e. it serves for the discriminating  
15 visualization of morphological or functional structures in biological systems, especially in living people, to assist a medical intervention.

The nanoparticles can be employed as diagnostic aid  
20 especially in surgical interventions. They can likewise be used in minimally invasive methods (e.g. endoscopy, laparoscopy). Combination with imaging methods such as PET, MRI, CT etc. is worthwhile.

25 As already stated above, the use according to the invention in the form of local administration is particularly advantageous. The amount of Cd employed on local administration in this connection advantageously does not exceed one tenth of the total exposure which  
30 normally accumulate anyway during the course of life in the liver and kidney of a person of advanced age and usual lifestyle. The total exposure of these organs is about 18 mg (Saturag et al. 2000; "British Journal of Nutrition; 2000, (84), 791-802). Accordingly, it is  
35 advantageous on local administration for the amount of nanoparticles to be limited so that the amount of Cd

supplied at least does not substantially exceed the value of 2 mg. In a particularly preferred embodiment, the tumor visualization is possible even when an amount of contrast agent which does not exceed a total amount  
5 of 0.6 mg, particularly preferably 0.2 mg, of cadmium.

The particular advantage of this embodiment is that the use of the nanoparticles in medical application on a living person is thereby possible for the first time  
10 because otherwise - i.e. as systemic administration - this is precluded because of the toxicity associated therewith. This is because local administration reduces the dose of nanoparticles necessary for adequate visualization.

15 It has emerged that the Cd-containing contrast agent is advantageously employed according to the invention for visualizing a tumor in vivo in a dose corresponding to an amount of from 0.002 to 0.02 mg of Cd per  $\text{cm}^3$  of  
20 tumor tissue. Dosages of the contrast agent of from 0.002 to 0.015 mg of Cd/ $\text{cm}^3$  of tumor tissue are particularly advantageous, in particular between 0.002 and 0.010 mg of Cd/ $\text{cm}^3$ . It is possible with this advantageous dosage to visualize tumors with a volume  
25 of up to about 150  $\text{cm}^3$  in vivo without thereby exceeding the normally acceptable upper limit of exposure for humans.

The investigations may relate to all accessible  
30 tissues/organs of the patient, especially the skin, hollow organs (e.g. in the gastrointestinal, urogenital, respiratory tract) or else externally accessible regions of the sensory organs and also the cardiovascular system.

35 Use as in vitro diagnostic aid is also possible, e.g.

immunohistochemistry or FACS, and ELISA. A combination of in vivo and in vitro diagnosis (e.g. biopsy material) is particularly advantageous.

- 5 Where the nanoparticles are employed according to the invention for therapeutic purposes, only some of the ligands of the nanoparticle may carry effector molecules or active substances, i.e. represent effectors. An effector in this connection is a ligand
- 10 with a selected function. The nanoparticle advantageously carries both specific ligands for targeted localization of the nanoparticle in the body or in the tissue, and a ligand with effector molecule.
- 15 The effector may remain linked to the nanoparticle or be able to be eliminated, or detached or released. The effector may for example exert its function via an activation/inactivation of a receptor, a masking of (surface) structures, activation of the immune system
- 20 ("priming"), modulation of signaling pathways, activation or deactivation of an enzyme, gene therapy (e.g. by targeted delivery of plasmids or siRNA), targeted delivery of toxins/chemotherapeutics/cytostatics or a stimulating effect on, for example,
- 25 metabolism, hormone production inter alia. Protection of cells, e.g. insulin-producing B cells, is also possible.

## 1. Methods

- 30 a) Preparation of a BP619-neutravidin conjugate with linked biotinylated monoclonal antibody

### Chemicals and materials

- 35 Nanoparticles

BP619                      200 µg/ml

The BP619 or BP617 nanoparticle is a nanoparticle of the invention, i.e. it has a  $\text{CdS}_x\text{S}_e$  core ("alloy core") and a passivating layer as disclosed in US 2004/0247861 A1.

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#### Protein

Purified protein is present in phosphate/NaCl buffer (storage at  $-80^\circ\text{C}$ )

- 10 MES buffer substance (Sigma), NaCl, KCl,  $\text{Na}_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4$ , EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), S-NHS (N-hydroxysulfosuccinimide), dialysis chambers (Slide-A-Lyzer), Vivaspinn (MWCO 50 kDa, VivaScience)

15

#### Buffer

##### D-PBS (10×)

1370 mM NaCl (80 g/l)

27 mM KCl (2 g/l)

- 20 42 mM  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  (15.4 g/l) / 7.652 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$   
14 mM  $\text{KH}_2\text{PO}_4$  (2 g/l)

make up to 1000 ml, the pH should be about 7.5, autoclave, storage at RT.

- 25 For a 1×PBS solution, dilute 100 ml of 10× buffer with  $\text{ddH}_2\text{O}$  and, before making up completely to 1 l, adjust the desired pH (pH 7.4 (Ab) or 8.0 (QD)) with a few drops of 2 M NaOH.

- 30 MES buffer = activation buffer

Make up fresh (organic buffer, cannot be autoclaved)

Formula for 0.8 l (0.1 M MES, 0.25 M NaCl, pH 6.0):

15.616 g MES

11.688 g NaCl

- 35 ad 800 ml  $\text{ddH}_2\text{O}$ , adjust pH 6.0

Make up to a maximum of 0.7 l with  $\text{ddH}_2\text{O}$ , then adjust

the pH with 2 M or 5 M NaOH. Subsequently make up to 800 ml with ddH<sub>2</sub>O.

1 M glycine solution (can be stored for a lengthy  
5 period at 4°C if sterilized by filtration), frees  
aliquots

#### Procedure

10 Firstly, MES buffer (activation buffer) is prepared in  
a glass measuring cylinder. The dialysis chamber is  
hydrated in MES buffer for 1 to 2 minutes before use.  
100 µl of BP619 (20 µg) are made up to a final volume  
of 400 µl with MES buffer in a sterile Eppendorf vial  
and thoroughly mixed with a pipette. The BP619 are  
15 transferred into the dialysis chamber (3.5 kDa). In the  
first dialysis, the BP619 are dialyzed against 800 ml  
of MES activation buffer at room temperature with  
continuous mixing and protecting from light for one  
hour. After the first dialysis, the BP619 are removed  
20 from the dialysis chamber and transferred into an  
Eppendorf vial. For mixing EDC and S-NHS with the  
BP619, stock solutions of in each case 100 mM EDC and  
100 mM S-NHS are prepared immediately before use. After  
the first dialysis, 33 µl of 100 mM S-NHS and 13 µl of  
25 100 mM EDC are pipetted into the BP619 and shaken at  
room temperature and 350 rpm, protecting from light,  
for 15 minutes. After the incubation, the BP619 are  
dialyzed against PBS. For this purpose, the BP619 are  
transferred into the dialysis chamber (MWCO 3.5 kDa)  
30 and dialyzed against PBS with pH 8.0, protecting from  
light, for one hour. After the second dialysis, the  
BP619 are removed from the dialysis chamber and  
transferred into an Eppendorf vial. The activated BP619  
are mixed with 80 µg of neutravidin (8 µl at 10 mg/ml,  
35 20 µl final volume with D-PBS). This reaction mixture  
is then shaken at room temperature and at 350 rpm,

protecting from light, for 2 hours. After the conjugation, the conjugation mixture is stored at 4°C, protecting from light. The following day, 1 M glycine is pipetted in to a final glycine concentration of 10 mM in order to saturate any reactive groups still present.

The BP619 conjugates are concentrated using Vivaspin centrifuge tubes. The conjugates are centrifuged until the desired concentration is reached. This is followed by stoichiometric addition of biotinylated monoclonal antibody directed against the membrane-associated and tumor-associated glucose transporter 1 (GLUT1) antigen.

#### b) Conjugation of BP619 with EGF-His

##### Chemicals and materials

##### Nanoparticles

BP619 200 µg/ml

##### Protein

Purified protein is present in phosphate/NaCl buffer (storage at -80°C)

MES buffer substance (Sigma), NaCl, KCl, Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), S-NHS (N-hydroxysulfosuccinimide), dialysis chambers (Slide-A-Lyzer), Vivaspin (MWCO 50 kDa, VivaScience)

##### Buffer

D-PBS (10x)

1370 mM NaCl (80 g/l)

27 mM KCl (2 g/l)

42 mM Na<sub>2</sub>HPO<sub>4</sub>\*12H<sub>2</sub>O (15.4 g/l)/7.652 g/l Na<sub>2</sub>HPO<sub>4</sub>\*2H<sub>2</sub>O

- 26 -

14 mM  $\text{KH}_2\text{PO}_4$  (2 g/l)

make up to 1000 ml, the pH should be about 7.5,  
autoclave, storage at RT.

- 5 For a 1xPBS solution, dilute 100 ml of 10x buffer with  $\text{ddH}_2\text{O}$  and, before making up completely to 1 l, adjust the desired pH (pH 7.4 (Ab) or 8.0 (QD)) with a few drops of 2 M NaOH.
- 10 MES buffer = activation buffer  
Make up fresh (organic buffer, cannot be autoclaved)  
Formula for 0.8 l (0.1 M MES, 0.25 M NaCl, pH 6.0):  
15.616 g MES  
11.688 g NaCl
- 15 ad 800 ml  $\text{ddH}_2\text{O}$ , adjust pH 6.0  
Make up to a maximum of 0.7 l with  $\text{ddH}_2\text{O}$ , then adjust the pH with 2 M or 5 M NaOH. Subsequently make up to 800 ml with  $\text{ddH}_2\text{O}$ .
- 20 1 M glycine solution (can be stored for a lengthy period at 4°C if sterilized by filtration), freeze aliquots

#### Procedure

- 25 Firstly, MES buffer (activation buffer) is prepared in a glass measuring cylinder. The dialysis chamber (3.5 kDa) is hydrated in MES buffer for 1 to 2 minutes before use. 100  $\mu\text{l}$  of BP619 (20  $\mu\text{g}$ ) are made up to a final volume of 400  $\mu\text{l}$  with MES buffer in a sterile
- 30 Eppendorf vial and thoroughly mixed with a pipette. The BP619 are transferred into the dialysis chamber (3.5 kDa). In the first dialysis, the BP619 are dialyzed against 800 ml of MES activation buffer at room temperature with continuous mixing and protecting
- 35 from light for one hour. After the first dialysis, the BP619 are removed from the dialysis chamber and

transferred into an Eppendorf vial. For mixing EDC and S-NHS with the BP619, stock solutions of in each case 100 mM EDC and 100 mM S-NHS are prepared immediately before use. After the first dialysis, 33  $\mu$ l of 100 mM S-NHS and 13  $\mu$ l of 100 mM EDC are pipetted into the BP619 and shaken at room temperature and 350 rpm, protecting from light, for 15 minutes. After the incubation, the BP619 are dialyzed against PBS. For this purpose, the BP619 are transferred into the dialysis chamber (3.5 kDa) and dialyzed against PBS with pH 8.0, protecting from light, for one hour. After the second dialysis, the BP619 are removed from the dialysis chamber and transferred into an Eppendorf vial. The activated BP619 are conjugated with 4.92  $\mu$ g of EGF-His (diluted with PBS to a final volume of 20  $\mu$ l). For this purpose, the activated BP619 are pipetted into EGF-His and thoroughly mixed with the pipette. This reaction mixture is then shaken at room temperature and at 350 rpm, protecting from light, for 2 hours. After the conjugation, the conjugation mixture is stored at 4°C, protecting from light. The following day, 1 M glycine is pipetted in to a final glycine concentration of 10 mM in order to saturate any reactive groups still present.

25

The BP619-EGF-His conjugates are concentrated using Vivaspin centrifuge tubes (50 kDa MWCO). In this case, the membrane is prewashed once with 4 ml of ddH<sub>2</sub>O and then washed again with 4 ml of PBS. The BP619-EGF-His conjugates are diluted in 2 ml of PBS and loaded onto the membrane. The BP619-EGF-His conjugates are then washed again with 2 ml of PBS. The conjugates are centrifuged until the desired concentration is reached.

35 c) Animal experiment

## i) Procedure

For this purpose, human colon carcinoma cells of the HT29 cell line were injected subcutaneously into nude mice (without thymus and therefore immunosuppressed) and formed solid tumors after a growth time of about 2 to 3 weeks.

Each mouse is anesthetized with Hypnomidate in order to undertake the injection and is injected intratumorally with 25  $\mu$ l of a nanoparticle solution; the injection in this case takes place centrally at one site in the tumor.

Kinetics of the fluorescence of the material in the tumor are recorded from time 0 to 5 and 60 min.

After sacrifice, firstly the tumor with epidermis is removed and then the organs spleen, liver, kidneys are removed.

The tumor is removed with epidermis-dermis, frozen with one drop of OCT on aluminum foil (outside pointing upwards), packed in aluminum foil and shock-frozen in  $N_2$ . Then stored at  $-80^\circ\text{C}$  until transported back on dry ice, further storage at  $-80^\circ\text{C}$ .

The organs spleen, liver, kidneys are removed from all the mice, shock-frozen in  $N_2$  and stored at  $-80^\circ\text{C}$  until transported back.

## ii) Photographic documentation

Materials/equipment used

Nikon Coolpix P2 camera

24W cold light source (Eltrotec LB24)

Optical filters:

- Shortpass filter 50% cutoff wavelength 550 nm

(Melles Geriot 03SWP408 or 03SWP608)

- "Green" color filter 550 nm (Melles Geriot 03FCG087/OG550)
- "Orange" color filter 570 nm (Melles Geriot 03FCG089/OG570)
- "Red" color filter 590 nm (Melles Geriot 03FCG098/OG590)

black clay board as background.

#### 10 Camera settings

The photographs to document the fluorescence are taken with a commercially available digital compact camera (Nikon Coolpix P2). The settings made on the camera are summarized below.

15

| Setting               | Value                     |
|-----------------------|---------------------------|
| White balance (WB)    | "Direct sunlight" (fixed) |
| Exposure metering     | "Spot metering"           |
| Continuous shooting   | "Single image"            |
| Best shot selector    | "Off"                     |
| Bracketing            | "Bracketing"              |
| Flash compensation    | "0"                       |
| Contrast              | "Normal"                  |
| Sharpening            | "Off"                     |
| Color saturation      | "+/-0"                    |
| ISO sensitivity       | 64                        |
| Image quality         | "fine"                    |
| Image size            | "2592x1944"               |
| Compression           | "Medium"                  |
| Autofocus             | "single autofocus"        |
| Fixed aperture        | "On"                      |
| Noise reduction       | "Off"                     |
| Exposure compensation | Variable (std. -2)        |

The respective image settings can be taken from the

EXIF information in the image file (e.g. with Photoshop or PixVue).

### Procedure

- 5 The camera is mounted with the filter holder on a tripod and adjusted with the aid of the 3D head so that the distance between lens and mouse/surface is about 15-20 cm. The angle should be as steep as possible from above, as far as permitted by the positioning of the  
10 tripod. The photographic filter is mounted so that the distance from the lens is as small as possible.

- The illumination and excitation of the substance takes place by a cold light source whose spectrum is  
15 controlled by a short pass filter (see above). Because of the heat produced by the light source and to improve manipulability, a flexible light guide with a lens is used to focus the light cone. The filter holder with the short pass filter is mounted on this light guide.  
20 The light guide is then fixed in a laboratory stand so that the distance to the surface is about 15 cm. The light cone should moreover be adjusted so that its diameter is about 8 cm. The angle of illumination from above should moreover be as steep as possible to reduce  
25 shadows.

- The mouse and the laboratory stand for the illumination are positioned on black clay board to improve the contrast. The mouse is then positioned in the middle of  
30 the light cone. Care should be taken in this connection that the tumor produces minimal shadow and is well lighted.

- The autofocus display should be noted when the  
35 photograph is taken.

The photographs should be taken in the normal case with the maximum wide angle. With relatively large zoom the camera may become unfocused and, in addition, the aperture setting changes.

5

If the photographic filter is changed between photographs, if possible nothing else should be changed in the experimental setup in order to ensure comparability and facilitate later processing.

10

#### d) Cell-binding assay

##### Materials and equipment

Fluorescence microscope: Leica DMIL, Zeiss LSM510META

15 Signal enhancer, ProLong Gold Antifading Reagent

##### Buffer

D-PBS (pH 7.4) (10×)

1370 mM NaCl (80 g/l)

20 27 mM KCl (2 g/l)

42 mM  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  (15.4 g/l) / 7.652 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

14 mM  $\text{KH}_2\text{PO}_4$  (2 g/l)

Adjust pH 7.4 and make up to 1000 ml

autoclave, storage at RT

25

Triton X-100 solution

0.1% (v/v) in D-PBS

Storage at 4°C

30 BSA solution

3% (w/v) in D-PBS

Make up fresh or from -20°C stock

4% PFA solution

35 5.71 ml of formaldehyde (35%)

5 ml of 10× D-PBS

- 32 -

adjust/check pH 7.4

ad 50 ml with ddH<sub>2</sub>O, storage at 4°C

0.1 M glycine solution

5 0.375 g of glycine

ad 50 ml D-PBS, adjust/check pH 7.4, sterilize by filtration, storage at 4°C

Mowiol/DABCO

10 Mixing of 2.4 g of Mowiol in 6 g of glycerol (extra pure) is followed by addition of 6 ml of ddH<sub>2</sub>O and then stirring at RT for several h. 12 ml of 0.2 M tris (pH 8.5) are added thereto, and the mixture is heated at 50°C with stirring for 10 min. After the Mowiol has  
15 dissolved (takes longer than 10 min), the mixture is centrifuged at 5000 × g for 15 min and finally 20 mg/ml DABCO are added.

Storage: in aliquots at -20°C, can be used for only a few weeks at 4°C, slowly hardens

20

#### Antibodies

First antibody: Anti-EGFR mAb (mouse) Dianova Ab-5  
1:100

Second antibody: goat anti-mouse with Alexa488

25

#### Procedure

2 days beforehand, HT29 cells are seeded on circular coverslips. For the seeding,  $5 \times 10^4$  cells are transferred so that, after growth at 37°C for 48 hours,  
30 a 50-70% confluent monolayer is present at the start of the immunostaining.

The BP619-EGF-His conjugates are preincubated in 50 µl of McCoy medium containing 3% BSA at room temperature  
35 for 30 minutes.

- 33 -

When the HT29 cells reach confluence, the medium is aspirated off and the cells are washed at least once with D-PBS.

- 5    30    to    50  $\mu$ l    of    the    preincubated    BP619-EGF-His  
conjugates are pipetted onto the washed cells and then  
incubated in an incubator at 37°C/7.5% CO<sub>2</sub> for one  
hour.
- 10    After the cells have been stained with the BP619-EGF-  
His conjugates, the cells are washed once with PBS and  
then fixed with 300  $\mu$ l of 4% PFA solution at room  
temperature for 15 to 20 minutes. After the fixation,  
the cells are washed once with D-PBS and quenched with  
15    0.1 M glycine at room temperature for 5 minutes. After  
the quenching, the cells are washed once with D-PBS and  
then permeabilized with 0.1% Triton X-100-PBS at room  
temperature for 10 minutes. The cells are blocked by  
subsequently incubating the cells with 3% BSA at room  
20    temperature for 30 minutes.

- After blocking of the stained cells, the cells can be  
counterstained or the BP619-EGF-His conjugate staining  
can be analyzed directly under a microscope.
- 25    Counterstaining is necessary to analyze the  
colocalization.

- The counterstaining is done with the first antibody,  
anti-EGFR, a monoclonal antibody from Dianova. The
- 30    anti-EGFR antibody is diluted 1:100 in 30  $\mu$ l of 1% BSA-  
PBS and then pipetted onto the cells. Anti-EGFR  
incubates on the cells at room temperature for 60  
minutes. After the incubation, the cells are washed  
with D-PBS for 3  $\times$  5 minutes. The second antibody with  
35    fluorochrome for the counterstaining is goat anti-mouse  
with Alexa488. For this purpose, the second antibody is

diluted 1:200 in 30  $\mu$ l of 1% BSA and then pipetted onto the cells. The goat anti-mouse Alexa488 second antibody is incubated on the cells at room temperature for 60 minutes. After the incubation, the cells are washed  
5 with D-PBS for 3  $\times$  5 minutes. The cells are embedded in Mowiol/DABCO and analyzed under a microscope.

#### e) Preparation and staining of cryo sections

10 The tumors are stored at -80°C and transported in a Styropor box with cooling units (cooled to -80°C) for sectioning. The sectioning takes place with a cry-microtome. The resulting sections are 10  $\mu$ m thick.

#### 15 Materials and equipment

Fluorescence microscope: Leica DMIL, Zeiss LSM510META, troughs for washing, humidity chamber, grease pen for marking the tissue region, 4% strength paraformaldehyde solution (see cell-binding assay), PBS (see cell-  
20 binding assay), 3% strength BSA solution (see cell-binding assay), Triton X-100 solution (see cell-binding assay), 0.1 M glycine solution (see cell-binding assay)

Appropriate primary and secondary antibodies, where  
25 appropriate further reagents for counterstaining

#### Procedure

The cryo sections are thawed at RT and dried (about 10-20 min). After the tissue region has been marked with a  
30 grease pen, the tissue is fixed with 4% strength paraformaldehyde solution in a humidity chamber for 20 min. After washing with D-PBS subsequently quenching with 0.1 M glycine solution for 5 min.

35 Washing in PBS and permeabilization with 0.1% Triton X-100 are followed by blocking with 3% strength BSA

solution at RT for 1 h.

An anti-EGFR-A488 direct conjugate is used for detecting EGFR in the plasma membrane. This conjugate  
5 is diluted 1:100 in 1% BSA/PBS, and the sections are incubated therewith in a humidity chamber at RT for 1 h. Washing with D-PBS takes place in a trough for at least 15 min with at least one buffer change.

10 Mowiol/DABCO is employed for the embedding, and the sections (unstained and stained) are analyzed under a microscope.

#### **f) Microscopic analysis**

15 The microscopic analysis of the specimens takes place with a Zeiss LSM510. The following filters are employed for this purpose:

for NP fluorescence analysis:

20 FSet 15= FilterSet 15 488015-0000

Excitation: BP546

Beamsplitter: FT580

Emission: LP 590

25 for Alexa488 fluorescence analysis:

FSet 46= FilterSet 46 1196-681

Excitation: BP500/20

Beamsplitter: FT515

Emission: BP535/30

30

A confocal laser analysis was also carried out with some of the specimens (see instructions for operating the microscope).

35 **2. Exemplary embodiments**

Exemplary embodiment 1:

In vivo experiment: Animal experiment with HT29 xenograft tumors in nude mice with intratumoral injection of neutravidin-antibody complexes according to the invention

5 A specific tumor targeting of antibody conjugates according to the invention was shown in an in vivo experiment on mice with xenograft tumors. For this purpose, human colon carcinoma cells of the HT29 cell  
10 line were injected subcutaneously into nude mice (without thymus and therefore immunosuppressed) and formed solid tumors after a growth period of 3 weeks.

For a selective tumor marking, an antibody complex  
15 according to the invention was prepared, or a neutravidin conjugate according to the invention with a biotinylated monoclonal antibody linked thereto. This monoclonal antibody is directed against the membrane-associated tumor-associated glucose transporter 1  
20 antigen (GLUT1) which is expressed on many types of human colorectal carcinomas.

After intratumoral injection of the complexes, the tumors were immediately identifiable visually by red  
25 fluorescence with UV excitation. After up to 48 h after injection it was possible to detect the complexes of the invention in the prepared cryo sections of the tumors.

30 Fig. 1: Red signal (conjugate of neutravidin and biotinylated antibody against GLUT1 membrane protein). Specific binding to HT-29 cells but not to murine cells is evident (homogeneous marking of the complete tumor not yet achieved).

35

Fig. 2: Red signal (conjugate of neutravidin and

biotinylated antibody against GLUT1 membrane protein). Specific binding to HT-29 cells in the direct vicinity of intratumoral ducts but not to murine cells is evident. (Homogeneous marking of the complete tumor not yet achieved).

Exemplary embodiment 2:

i) Comparison of the intensity of Biopixels 618 (material according to the invention) with Crystalplex alloy nanoparticles 630 (NC 630) by spectral analysis. The NC630 nanoparticles are nanoparticles having a  $\text{CdS}_x\text{Se}_{1-x}/\text{ZnS}$  core and functionalized with COOH groups.

The following values are evident from fig. 3:

|    |                         |                       |
|----|-------------------------|-----------------------|
| 15 | BP618 concentration     | 1.67 $\mu\text{g/ml}$ |
|    | NC630 concentration     | 6.70 $\mu\text{g/ml}$ |
|    | BP618 excitation 360 nm | 55 000 cps            |
|    | NC630 excitation 360 nm | 22 000 cps            |
| 20 | BP618 excitation 488 nm | 25 000 cps            |
|    | NC630 excitation 488 nm | 10 000 cps            |

When the higher concentration, by a factor of 4, of the NC630 nanoparticles is taken into account, the emission intensity of the BP618 material of the invention is a factor of 10 higher.

This difference in intensity is very essential for the use according to the invention of the contrast agent for direct visualization, e.g. on medical application in surgery. Whereas with the material of the invention it is possible for the treating clinician to observe the fluorescence directly and merely with the aid of fluorescence filters, the NC630 nanoparticles would need additional electronic amplification to be made

visible.

ii) Characterization of the BioPixels 619 by gel filtration

5 Fig. 4 reveals that the elution volume is 16.2 ml. This value correlates with a Stokes diameter of 10.8 nm.

iii) Comparison of mouse tumor after injection of EGF-coupled BioPixels 619 NC630 nanoparticles

10

Conjugation of BP619 with EGF-His was carried out by the method described under 1b). In this case, 1.4  $\mu$ M protein were employed, and 40  $\mu$ g of nanoparticles (= duplicate) were conjugated. After purification/concentration with Vivaspin centrifuge units, a total of about 14.3  $\mu$ g of nanoparticles were injected into the tumor.

15

Photographic documentation took place as described under 1c). The tumors into which the NC630 material was injected showed no fluorescence (fig. 5a, see marking), whereas on use of EGF-coupled BioPixels 619 of the invention a fluorescence is distinctly evident (fig. 5b, see marking).

20

25

iv) Microscopy of the tissue marking in the tumor with EGF-coupled BioPixels 619 of the invention

30

The removed tumors were sectioned using a cryotome (section thickness 10  $\mu$ m) and treated for microscopic analysis as described above (1e). The analysis took place with a Zeiss microscope (LSM510) using the FSet15 for detecting the BioPixel fluorescence (see 1f). The marking of the tumor (see fig. 6; white areas on a dark background) is not homogeneous; some areas are marked less strongly (see fig. 6a), where others show stronger

35

marking (see fig. 6b).

**c) Exemplary embodiment 3**

Marking of tumor cells with EGF coupled BioPixels 619  
5 of the invention with intracellular uptake of the  
biopixels.

In this case, a cell-binding assay was carried out with  
HT29 tumor cells by the method described (1d). Fig. 7a  
10 shows the BP619 fluorescence. In this case, some of the  
signals whose fluorescence is caused exclusively by  
BP619 have been marked by circles. The fluorescence of  
the antibody A488 was detected in fig. 7b. The first  
antibody employed here was an EGFR antibody and the  
15 second antibody was goat anti-mouse A488 (see 1d). Once  
again, some of the signals whose fluorescence is  
attributable exclusively to A488 have been circled.  
Finally, fig. 7c shows the picture copied together from  
7a and 7b. Many of the signals reveal a colocalization,  
20 i.e. they are to be found both on fig. 7a and fig. 7b.

### CLAIMS

1. The use of fluorescent nanoparticles comprising an inorganic core, a passivating layer comprising an imidazole component, and specific ligands having a hydrodynamic diameter of the inorganic core with the passivating layer of not more than 15 nm, preferably of not more than 10 nm, particularly preferably of not more than 5 nm, as in vivo contrast agent, characterized in that the nanoparticles show an emission of less than 700 nm, and the contrast agent is suitable for local application in humans.
2. The use as claimed in the preceding claim, characterized in that the nanoparticles are suitable to be administered or injected systemically, locally or topically.
3. The use as claimed in either of the preceding claims suitable for tissue marking in surgical, endoscopic or minimally invasive interventions.
4. The use as claimed in any of the preceding claims, characterized in that the nanoparticles additionally include at least one modifier selected from the group of the following compounds: polyethylene glycol, monosaccharides, disaccharides, trisaccharides, hydrophilic vitamins, lipophilic vitamins, fatty acids, polyalcohols, Teflon, amino acids, phosphorylcholine and polylactate.
5. The use as claimed in any of preceding claims 1 to 4, characterized in that the inorganic core comprises an alloy/semiconductor core, where the core has a homogeneous composition and is characterized by a band-gap energy which is non-linear in relation to the molar ratio of the two semiconductors.
6. The use as claimed in any of claims 1 to 4, characterized in that the inorganic core comprises an alloy of a first semiconductor and of a second semiconductor, where the concentration of the first semiconductor increases gradually from the center of the core to its surface, and the concentration of the second semiconductor decreases gradually from the center of the core to its surface.
7. The use as claimed in either of claims 5 and 6, characterized in that at least one of the semiconductors is a group II-group VI semiconductor or a group III-group V semiconductor.

8. The use as claimed in any of claims 5 to 7, characterized in that the core comprises an alloy selected from the group of the following alloys: CdSeTe, CdSSe, CdSTe, ZnSeTe, ZnCdTe, CdHgS, CdHgTe, InGaAs, GaAlAs, InGaN, InGaP, CdSe or CdTe.
9. The use as claimed in any of claims 1 to 8, characterized in that the imidazole component comprises one or more compounds selected from the following group: histidine, carnosine, anserine, baleine, homocarnosine, histidyl-phenylalanine, cyclo-histidylphenylalanine, 5-amino-4-imidazolecarboxamide, histidylleucine, 2-mercaptoimidazole, boc-histidine, hydrazide, histinol, 1-methylhistidine, 3-methylhistidine, imidazolysine, imidazole-containing ornithine (e.g. 5-methylimidazole), imidazole-containing alanine (e.g. (beta)-(2-imidazolyl)-L(alpha)alanine), carzine and histamine, each of which may in turn be substituted by reactive groups (for example amino, thiol, carboxyl or carboxamide).
10. The use as claimed in any of claims 1 to 9, characterized in that the passivating layer comprises a crosslinker for crosslinking the imidazole component.
11. The use as claimed in claim 10, characterized in that the crosslinking component comprises an alkylphosphine.
12. An in vivo contrast agent comprising nanoparticles comprising an inorganic core, a passivating layer comprising an imidazole component and specific ligands having a hydrodynamic diameter of the inorganic core with the passivating layer of not more than 15 nm, preferably of not more than 10 nm, particularly preferably of not more than 5 nm, characterized in that they show an emission of from 600 to 700 nm, preferably from 620 to 650 nm.
13. The in vivo contrast agent comprising nanoparticles as claimed in claim 12, characterized in that they additionally include at least one modifier selected from the group of the following compounds: polyethylene glycol, monosaccharides, disaccharides, trisaccharides, hydrophilic vitamins, lipophilic vitamins, fatty acids, polyalcohols, Teflon, amino acids, phosphorylcholine and polylactate.
14. The in vivo contrast agent comprising nanoparticles as claimed in claim 12 or 13, characterized in that the inorganic core comprises an alloy/semiconductor

core, where the core has a homogeneous composition and is characterized by a band-gap energy which is non-linear in relation to the molar ratio of the two semiconductors.

15. The in vivo contrast agent comprising nanoparticles as claimed in either of claims 13 or 14, characterized in that the inorganic core comprises an alloy of a first semiconductor and of a second semiconductor, where the concentration of the first semiconductor increases gradually from the center of the core to its surface, and the concentration of the second semiconductor decreases gradually from the center of the core to its surface.
16. The in vivo contrast agent comprising nanoparticles as claimed in either of claims 14 or 15, characterized in that at least one of the semiconductors is a group II-group VI semiconductor or a group III-group V semiconductor.
17. The in vivo contrast agent comprising nanoparticles as claimed in claim 16, characterized in that the core comprises an alloy selected from the group of the following alloys: CdSeTe, CdSSe, CdSTe, ZnSeTe, ZnCdTe, CdHgS, CdHgTe, InGaAs, GaAlAs, InGaN, InGaP, CdSe and CdTe.
18. The in vivo contrast agent comprising nanoparticles as claimed in any of claims 13 to 17, characterized in that the imidazole component comprises one or more compounds selected from the following group: histidine, carnosine, anserine, baleine, homocarnosine, histidylphenylalanine, cyclohistidyl-phenylalanine, 5-amino-4-imidazolecarboxamide, histidylleucine, 2-mercapto-imidazole, boc-histidine, hydrazide, histinol, 1-methylhistidine, 3-methyl-histidine, imidazolysine, imidazole-containing ornithine (e.g. 5-methyl-imidazole), imidazole-containing alanine (e.g. (beta)-(2-imidazolyl)-L(alpha)alanine), carzine and histamine, each of which may in turn be substituted by reactive groups (for example amino, thiol, carboxyl or carboxamide).
19. The in vivo contrast agent as claimed in claim 13 to 18, characterized in that the crosslinking component comprises an alkylphosphine.