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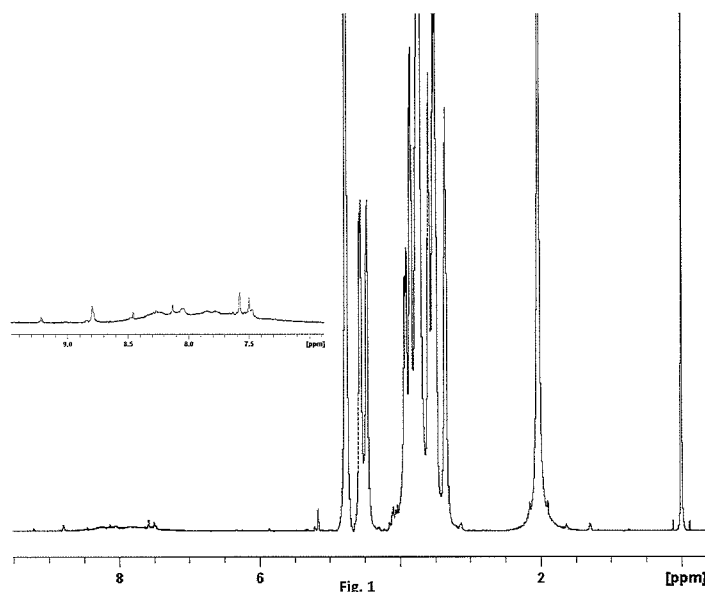
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(54) Title: FLUORESCENT CYPATE CONJUGATE OF HYALURONIC ACID OR SALT THEREOF, HYDROPHOBIZED CONJUGATE, METHODS OF PERPARATION AND USE THEREOF



(57) Abstract: The present invention relates to the fluorescent conjugate of hyaluronic acid containing cypate or salts thereof, the hydrophobized conjugate, methods of the preparation and use thereof in medicinal applications for in vivo imaging and the treatment of neoplasms.



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**FLUORESCENT CYPATE CONJUGATE OF HYALURONIC ACID OR SALT THEREOF,
HYDROPHOBIZED CONJUGATE, METHODS OF PERPARATION AND USE THEREOF**

- 5 Fluorescent conjugate of hyaluronic acid or salt thereof, hydrophobized conjugate, methods of preparation and use thereof.

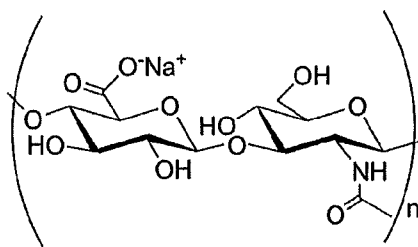
Field of the Invention

The present invention relates to a fluorescent ester conjugate of hyaluronic acid or a salt thereof containing *in vivo* diagnosable heptamethine indocyanine dye (Cypate) of the formula
 10 III or 1-[3-(2-carboxyethyl)-1,1-dimethyl-5,9b-dihydrobenzo[e]indol-3-ium-2-yl(chloride)]-octa-1,3,5,7-tetraenyl]-1,1-dimethyl-2H-benzo[e]indol-3-yl]propanoic acid. Further it is described a hydrophobized conjugate, methods of preparation thereof and an use for *in vivo* imaging applications and a treatment of neoplasms.

Background of the invention

15 *Hyaluronic acid*

Hyaluronic acid or a salt thereof (HA) is a linear polysaccharide belonging to the group of important glucosamino glycans. In the terms of the structure, it is a biopolymer formed with repeated disaccharide units (β -(1 $\rightarrow$ 4) glycosidic bond), consisting of D-glucuronic acid and *N*-acetyl-D-glucosamine mutually linked with a β -(1 $\rightarrow$ 3) glycosidic bond (see formula 1 below).



Formula 1. Hyaluronic acid

Hyaluronic acid occurs in the physiological environment in the form of sodium salt as a very hydrophilic and highly hydrated biopolymer (Schanté C. E.; *et al.*, *Carbohydr. Polym.* **2011**, 85 (3), 469–489). HA plays an important role in the structure and organization of
 25 the extracellular matrix and also forms suitable environment for cells, their proliferation, differentiation and mobility (D'Este M.; *et al.*, *Carbohydr. Polym.* **2014**, 108, 239–246; Schanté, C. E.; *et al.*, *F. Carbohydr. Polym.* **2011**, 85 (3), 469–489). HA is further contained in vertebrates in all body organs and the extracellular matrix of soft connective tissues (Eenschooten, C.; *et al.*, *Carbohydr. Polym.* **2010**, 79 (3), 597–605).

5 This, in the water soluble polysaccharide is relatively easily subjected to the chemical modification and it appears as a biomolecule very suitable for a conjugation with other compounds, including fluorescent agents. An advantage of HA is also the existence of several cellular receptors thereof, which can be used for specific aiming of derivatives, for example, into tumor tissues (Garg H. G.; *et al.*, *Chemistry and biology of hyaluronan*, 1st ed.; Elsevier:
10 Netherlands, **2004**).

In vivo diagnostics

Recently, one of the suitable methods for noninvasive diagnostics, beside nuclear imaging techniques, the roentgen radiography and the computer tomography, is the optical imaging through fluorescence. Fluorescence appears as a promising method for *in vivo* diagnostics, especially
15 thanks to its relatively high sensitivity, specificity and image gaining in the real time (Ye, Y.; *et al.*, *Bioconjugate Chem.* **2008**, *19* (1), 225–234). Among advantages of the fluorescent imaging are also relatively low cost, feasibility, non-invasiveness and safety in comparison to the ionizing radiation. This technic is very perspective for the detection, diagnostics and prevention in the tumor therapy, but also as a complement method for gaining complex information in clinic
20 applications in the imaging using the positron emission tomography (PET), SPECT („single-photonemission computed tomography“) or MRI („magnetic resonance imaging“), (Ye, Y.; *et al.*, *Theranostics* **2011**, *1*, 102–106).

In the application of the non-invasive diagnostic methods is the penetration of irradiation through tissues highly dependent on absorption properties and the refractive index (Frangioni, J.
25 V. *Curr. Opin. Chem. Biol.* **2003**, *7* (5), 626–634). The absorption and emission wavelength in the area 650 - 900 nm, that is radiation in the near infrared area (NIR), are considered as optimal. In comparison with shorter wavelengths of visible spectra, the radiation of these wavelengths penetrates deeper and at the same time no absorption by the endogenous fluorophores and rise of undesired autofluorescence occurs (Kobayashi, H.; *et al.*, *Chem. Rev.* **2010**, *110*, 2620–2640,
30 Luo, S. *et al.*; *Biomaterials* **2011**, *32* (29), 7127–7138).

NIR fluorescent agents

Recently, cyanine fluorescent dyes, derivatives of squarine, phtalocyanines, porphyrines and also some agents derived from boron-dipyrromethen (BODIPY) (Luo, S.; *et al.*, *Biomaterials* **2011**, *32* (29), 7127–7138) belong among important exogenous contrast agents.

5 Among very frequent agents used for imaging optical methods belong cyanine dyes. Structurally, they relate most to two heterocyclic structures, where one of these heterocycles carries positively charged nitrogen atom and they are further linked through the polymethine bridge, shown in the general formula of cyanine fluorescent agent, where



10 X and Y are heterocyclic nitrogen structures and $n = 1 - 3$. If $n = 1$, dyes count among tricyanine, $n = 2$ pentacyanine and $n = 3$ heptacyanine compounds.

Generally, the length of the polymethine bridge determines fluorescent properties of the given derivative and with every accruing n occurs the rise of the absorption and emission wavelengths of the compounds by about 100 nm. The typical wavelengths for the trimethine
15 dyes are about 500 nm, for pentamethine about 600 nm and heptamethine derivatives eventually rich wavelengths of the near infrared area. Bathochromic and hypsochromic shift of wavelengths is further partially influenced by the type of the heterocyclic structure that also determines absorption and radiance of the fluorescent dye (Hermanson, G. T. *Bioconjugate Techniques*, 2. ed.; Elsevier: United States of America, 2008).

20 *Conjugates of NIR fluorescent agents with various molecules*

In the past, it was possible to use NIR fluorescent agents alone only to nonspecific imaging, e.g. blood circuit and its purification (Frangioni, J. V. *Curr. Opin. Chem. Biol.* **2003**, 7 (5), 626–634). Polymethine cyanine dyes tend to an aggregate in the aqueous environment, which results in the loss of their fluorescence and it is therefore disadvantageous in *in vivo* diagnostics
25 (US6641798 B2). The said fluorescent agents are further characterized by very the short halftime of the degradation in the circuit system (150-180 s), that limits accumulation of the dye in the examined target, for example, in the tumor tissue and by that decreases also *in vivo* contrast (Hill T. K. *et. al.*, *Bioconjug Chem.* **2015**,; 26(2): 294–303). Simple and general approach how to increase the accumulation of the contrast agents in target places and tissues, or ensure the better
30 solubility in the aqueous environment, is their conjugation with the suitable ligand from the group of peptides, proteins, saccharides etc. (Frangioni, J. V. *Curr. Opin. Chem. Biol.* **2003**, 7 (5), 626–634). The conjugation of the contrast agents to the carrier polymer or the nanoparticle is mostly performed through a linker. The linker should be used to ensure fluorescent (to prevent quenching) and/or degradable conditions of system (Frangioni, J. V. *Curr. Opin. Chem. Biol.* **2003**, 7 (5), 626–634). The disadvantage of this solution is that the
35 reaction has more steps and it is very difficult, and derivatization of the entering agents is often

5 used, which is disadvantageous in the term of the purification of the products and/or the isolation of the reaction intermediates is necessary, which is again disadvantageous. In the case of nanoparticle systems, the linker length has to be chosen so, that no fluorescence quenching of the contrast agent occurs and the contrast agent is *in vivo* detectable (i.e. the linker must not be too short) (US20110104070).

10 Patent document US6641798 posts the demands to the general structure of the low-molecular conjugates of the bioactive molecules (for example peptides, proteins, antibodies, saccharides) with the cyanine fluorescent agents, prepared to increase the detection and the therapy of the tumor. A serious disadvantage of used derivatives in *in vivo* application is the short stability of fluorescence (c. 45 min) at the imaging.

15 In literature there were also described conjugates of NIR fluorescent agent Cypate with the several different ligands (amino saccharides, peptides, polysaccharides) in term of the contrast agents for *in vivo* diagnostics. The preparation of NIR fluorescent agent Cypate itself is described in document WO2002032285, the improved synthesis was further published in the publication Ye, Y.; *et al.*, *Bioconjugate Chem.* **2005**, *16*, 51–61. For *in vivo* studies prepared
20 Yunpeng Ye.; *et al.* (Ye, Y.; *et al.*, *J. Am. Chem. Soc.* **2004**, *126*, 7740–7741), polyvalent probes, where one or more monosaccharide units of D-(+)-glucosamine were bound on the carboxyl group of through the amide bond. Given NIR fluorescent agent formed the core of the dendrimeric formation and at the same time it served as a chromophore for nanoparticles, the biodistribution thereof was investigated using non-invasive optical methods *in vivo* and then *ex*
25 *vivo*. One disadvantage of this solution, where no polymer was used for the conjugation, is insolubility of the system in the aqueous solutions in the absence of organic solvent.

Another type of known nanoparticle for tumor imaging is a dually targeted polymer micelle on the basis of succinyl chitosan with a covalently bond Cypate using the amide bond, further with methionine and folic acid (Chen, H.; *et al.*, *Polym. Chem.* **2014**, *5*, 4734–4746). A
30 disadvantage of this solution is using of chitosan as a carrier polysaccharide, as chitosan is a body-foreign agent. Another disadvantage is very limited solubility of the native chitosan in the physiological conditions, the nature chitosan must be therefore always modified, because otherwise it would not be possible to use chitosan in the intravenous application. Furthermore, the modified chitosan is usually limitedly soluble in the physiological conditions. The
35 modification can cause changes in the biodegradability and biocompatibility of chitosan depending on the desired application (Balan, V.; *et al.*, *European Polymer Journal* **2014**, *53*, 171–188; Dumitriu, S. *Polymeric Biomaterials*, 2nd ed.; Marcel Dekker, Inc.: United States of

5 America, 2002). Chitosan conjugated with Pluronic F68 and with the cyanine dye (Cy5.5) was used in tracking the accumulation in the tumor mouse model (W. II Choi.; *et al.*, *Nanomedicine: Nanotechnology, Biology and Medicine* **2015**, *11*, 359–368). Without targeting of the carrier system with herceptin was the tumor tracking imaging *in vivo* possible, however, after 12 hours the NIR fluorescence intensity did not continue to increase and it can be therefore supposed that
10 this system does not enable the long term tracking of the tumor disease *in vivo* without the necessity of further carrier addition. The similar disadvantage of the short imaging time (maximum of 1-2 days after the application) is known also in the other polymer and non-polymer conjugates with NIR dyes (D. Kokuryo; *et al.*, *Journal of Controlled Release* **2013**, *178*, 125, Tan X.; *et al.*, *Biomaterials* **2012**, *33*, 2230–2239).

15 In literature, Cypate was further described as a part of multifunctional imaging probe, when on one carboxyl group of the said fluorescent agent was bound a chelating component of cation metals through the amide bond with the purpose of forming the optical-nuclear imaging system. Cypate was further conjugated to the cyclic RGD peptide with the purpose of targeting specific cells (Ye, Y.; *et al.*, *Bioconjugate Chem.* **2008**, *19*, 225–234). The disadvantage of this system is
20 that its fluorescent conditions change in the presence of metal cations and in some cases (presence of Fe^{3+} or Cu^{2+}) can lead up to the complete quenching of fluorescence (Ye, Y.; *et al.*, *Bioconjugate Chem.* **2008**, *19*, 225–234).

Conjugates of NIR fluorescent agents with hyaluronic acid.

In publications Choi K. (Choi, K. Y.; *et al.*, *J. Mater. Chem.* **2009**, *19* (24), 4102–4107 and
25 Choi, K. Y.; *et al.*, *Biomaterials* **2010**, *31* (1), 106–114), there was described a hydrophobized derivative of hyaluronic acid with 5 β -cholic acid. However it was found that that the action of 5 β -cholic acid leads to supporting of carcinogenesis of the intestine tumors in their early stages. (Baijal, K. P.; *et al.*, *Canadian Journal of Physiology and Pharmacology*, **1998**, *76*(12), 1095–1102). Further, NIR fluorescent agent Cy5.5 was conjugated on the carboxyl group of
30 hyaluronic acid, using ethyl-3(3-dimethylaminopropyl)-carbodiimide/hydroxy-benzotriazol. (EDC/HOBt) and linker dihydrazide of adipic acid. A disadvantage is not only multistep synthesis, but also used activator EDC/HOBt, that can cause undesirable intramolecular netting of hyaluronan and with that corresponding decrease of solubility of the final product (Huerta-Angeles, G.; *et al.*, *Carbohydr. Polym.* **2014**, *111* (13), 883–891). The derivative of hyaluronic
35 acid conjugated with Cy5.5 through the amide bond was further used for the surface treatment of superparamagnetic nanoparticles iron oxides (SPION) with the purpose of forming multimodal probes for combination of *in vivo* MRI/optical detection of activity of hyaluronidase (Lee, D.; *et*

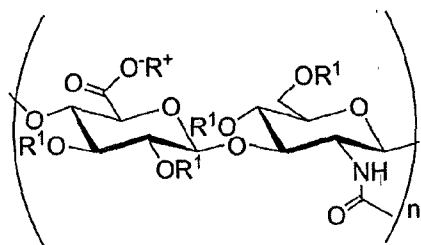
5 *al.*, *Macromol. Res.* **2011**, *19* (8), 861–867). With respect to *in vivo* diagnostics through the optical method, the fluorescent agent Cy5.5 from the group of pentamethine derivatives is not the ideal choice. In contrast to heptamethine derivatives, they do not reach such high absorption and emission wavelengths, that are for *in vivo* non-invasive diagnostics recommended, that is in using wavelengths typical for pentamethine derivatives can create the undesirable
10 autofluorescence of endogenous fluorophores.

In the case of heptamethine derivatives of cyanine is known the conjugate of hyaluronic acid with commercially available NIR fluorescent IR-783, modified four-step synthesis resulting in IR-783-S-Ph-COOH, where this derivative was consequently bound through the amide bond to hyaluronic acid. The derivative was prepared to study deeper understanding of pharmacokinetic
15 of HA *in vivo*, its degradation and role in the physiological and pathological conditions (Wang, W.; Cameron, A. G.; Shi, K. *Molecules* **2012**, *17*, 1520–1534). Unfortunately, the very complicated synthesis is not transformable into the industrial scale either in terms of economic or in terms of very low yield (Wang, W.; Cameron, A. G.; Shi, K. *Molecules* **2012**, *17*, 1520–1534).

20 Among other representatives of this type of fluorescent agents belongs indocyanine green (ICG). HA conjugated with indocyanine green and polyethylene glycol (PEG) makes aggregated particles in the aqueous environment, with the possibility of *in vivo* dual imaging of the tumor using optical methods and the photoacoustic detection (Miki, K.; *et al.*, *Biomacromolecules* **2015**, *16*, 219–227). The large disadvantage of using indocyanine green *in vivo* is hepatobiliary toxicity and fast removal of this dye by liver (G. R. Cherrick, et al. *J. Clinical Investigation*, 1960, *39*, 592–600). The disadvantage of derivatives of HA with NIR fluorescent agents, when it comes to their conjugation onto the carboxyl group of hyaluronic acid and that in modification of the carboxyl function of HA, results in the neutralization of the natural negative charge of biopolymer, further can influence the solubility of HA in the
25 physiological environment and not lastly also disturbs the recognition of HA by cell receptors
30 (Mero, A.; *et al.*, *Carbohydr. Polym.* **2010**, *79* (3), 597–605).

Summary of the Invention

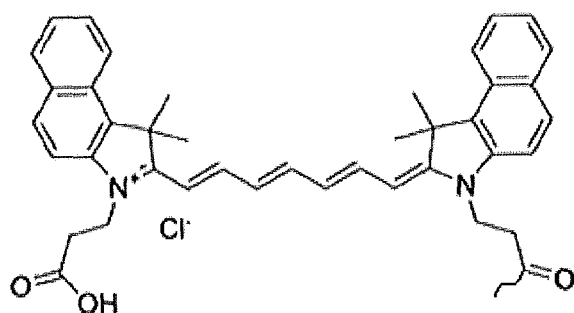
The said disadvantages of the prior art are overcome by a fluorescent conjugate of hyaluronic acid or a salt thereof of the general formula I



(I),

wherein R^+ is H^+ or a physiologically acceptable salt selected from the group containing Na^+ , K^+ , Mg^{2+} or Ca^{2+} ,

R^1 is $-H$ or the cypate residue of the formula II, where $\sim$ is a place of covalent bond of a cypate residue of the formula II



(II),

one R^1 being the cypate residue of the formula II in at least one repeated unit providing that if there is R^1 the cypate residue of the formula II in the unit, then the other R^1 in the unit are H ,

and where n is an integer in the range of 2 to 625.

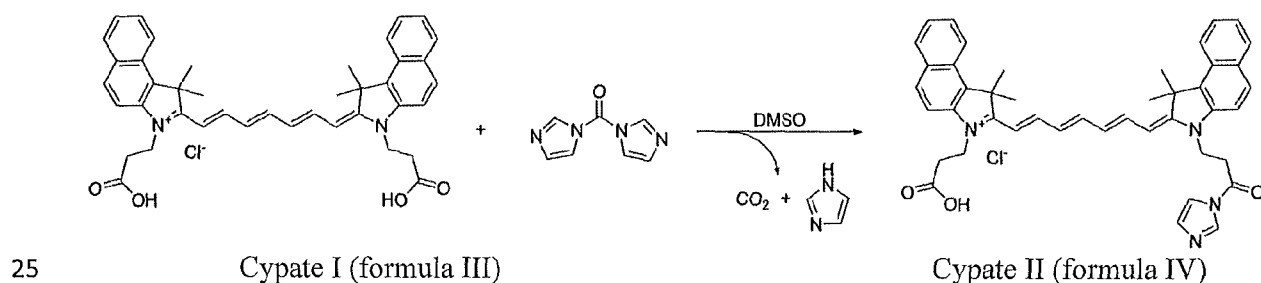
According to a preferred embodiment of the invention the cypate residue of the formula II is substituted at the position 6 of glucosamine part of the fluorescent conjugate of hyaluronic acid or the salt thereof of the general formula I.

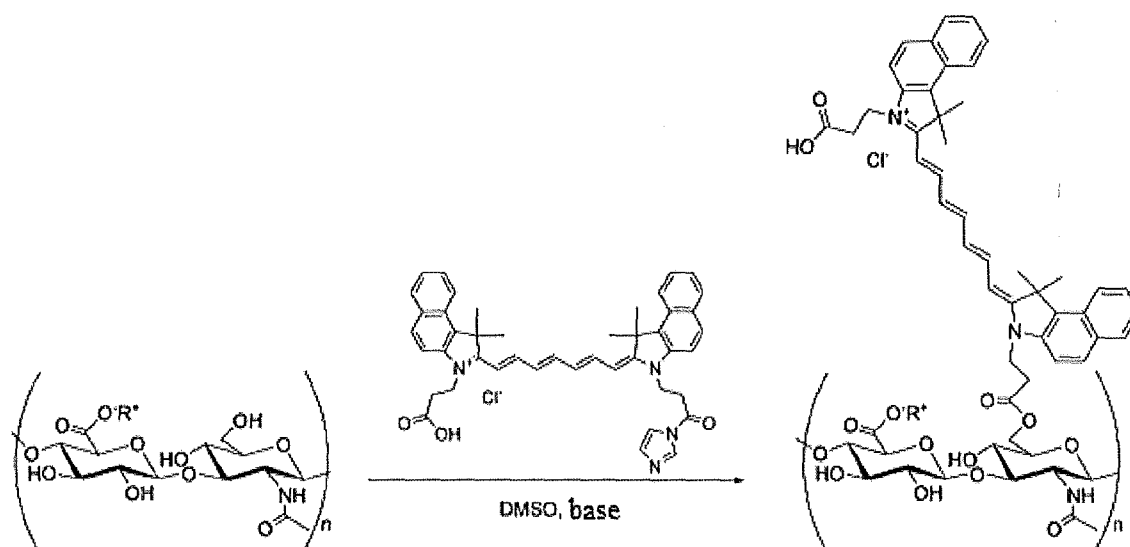
Cypate I is bound to a hydroxyl group of hyaluronic acid (see Scheme 1 and 2, below) after the activation, which is advantageous especially in terms of maintaining the biological properties of hyaluronic acid and also of its solubility in the physiological environment. The solubility of the conjugate of the present invention is 1 to 3 mg per 100 μl of physiological solution. The fluorescent conjugate of the invention is excited and absorbs the light in the area from 570 nm to 790 nm and emits the light in the area from 680 to 850 nm, preferably at 850 nm.

5 The degree of substitution of the cypate residue of the formula II bound in the conjugate of hyaluronic acid or the salt thereof of the general formula I is from 0.1 to 2%, preferably 1.0%. The low degree of substitution of the cypate residue in the conjugate of the present invention is preferred, since it enables to the image distribution of the conjugate *in vivo*, without the structure of HA to be significantly modified.

10 The preparation of the conjugate itself lies in the synthesis of the fluorescent agent: Cypate, further in the activation of the carboxyl group of the fluorescent agent and following esterification of hyaluronic acid.

The activation of the carboxyl group of Cypate I of the formula III is performed using *N,N'*-carbonyl diimidazole in the aprotic polar solvent (see Scheme 1, below) preferably selected from the group containing dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), formamide, or acetonitrile, more preferably DMSO. This activation is very effective even under the mild reaction conditions, wherein the reaction of the carboxyl group with *N,N'*-carbonyl diimidazole (CDI) results in the reactive intermediate mono-imidazolide (Cypate II of formula IV), where the reaction is driven by the release of carbon dioxide and imidazole (see Scheme 1, below). The activation reaction runs for 10 minutes to 24 hours, preferably 0.5 to 2 hours. The reaction temperature can be in the range from 20 °C to 60 °C, preferably in the range from 22 °C to 25 °C.





5

Scheme 2: The reaction of the activated Cypate with hyaluronic acid.

For the preparation of the conjugate, it is preferably used an acid form of hyaluronic acid or other organic salt for example tetrabutyl ammonium (TBA) (**Scheme 2**), that is used for the solubilization of hyaluronic acid in organic solvent (DMSO). Suitable molecule mass of the acid form or the other organic salt of hyaluronic acid for the given reaction is in the range from 5,000 – 250,000 g/mol, preferably (10,000 – 32,000 g/mol). Different molecule mass of HA of HA salt is not an obstacle for the reaction. The esterification of hydroxyl groups of HA in the aprotic polar solvent is further carried out by the addition of the fluorescent agent with the activated carboxyl group through CDI (N,N'-carbonyl diimidazole). The reaction runs at the presence of an organic base generated in situ in a form of imidazole or a added organic base selected for example from the group containing DABCO (1,4-diazabicyclo[2.2.2]octan), N,N,N',N'-tetramethyl-1,6-hexanediamine, N-methyl morfoline, imidazole, triethylamine (TEA) or N,N'-diisopropylethylamine (DIPEA), preferably imidazole generated in situ and the polar aprotic solvent, as is defined above. The reaction of the conjugate forming is performed at the temperature from 40 °C to 80 °C, preferably 40 °C to 60 °C, more preferably 60 °C for 12 to 48 hours, preferably 24 hours. A closer study revealed that the degree of substitution of HA with the fluorescent agent is dependent on an equivalent amount of Cypate I and also it is positively influenced by the presence of an equivalent of the organic base, the preferred combination is 0.5 molar equivalent of Cypate I : 1 molar equivalent of HA : 0.5 molar equivalent of N,N'-carbonyl diimidazole : 0.5 to 3.5 molar equivalents of the organic base, more preferably 1 molar equivalent of the organic base. Thus, it applies that the molar ratio of Cypate I : hyaluronic acid or a salt thereof : N,N'-carbonyl diimidazole : organic base is 0.5 : 1 : 0.5 : 0.5 to 3.5 in the

25

- 5 reaction mixture, preferably the molar ratio is 0.5 : 1 : 0.5 : 1. In the case of the organic base being generated *in situ* the molar ratio of cypate : hyaluronic acid or a salt thereof : N,N'-carbonyl diimidazole is 0.1 : 1 : 0.15 to 0.7 : 1 : 0.8, preferably 0.5 : 1 : 0.5.

The conjugate of hyaluronan with the heptamethine indocyanine fluorescent agent (or Cypate) of the general formula I, can be preferably further modified the forming of the hydrophobized fluorescent conjugate of the general formula I of the invention, where R^+ , R^1 and n are, as is defined above, applying at the same time, that at least in one repeated unit at least one R^1 is $-C(=O)R^2$, wherein R^2 is C_xH_y substituent, where x is an integer in the range of 5 to 17 and y is an integer in the range of 11 to 35, wherein it is a linear or branched, saturated or unsaturated C_6 - C_{18} aliphatic chain.

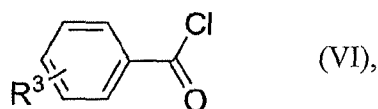
- 15 The degree of substitution of $-C(=O)R^2$ in the conjugate of hyaluronic acid or the salt thereof of the general formula I is from 1 to 70 %, preferably 5 to 12%. The hydrophobized fluorescent conjugate of the invention is excited and it absorbs the light in the range of wavelengths 570 nm to 790 nm and emits the light in the area of 680 to 850 nm, preferably at 850 nm.

- 20 The $C_6 - C_{18}$ acyl chain is the chain of fat acids that is linked through the ester bond to at least one hydroxyl group of HA. It binds preferably to the primary hydroxyl group (see Scheme 3), that is generally suitable for the esterification. The fat acid can have the short (SCFA), middle (MCFA), or long (LCFA) aliphatic chain and it can be essential or nonessential. The activation of the fat acid of the general formula V

- 25 R^2COOH (V),

wherein R^2 is defined above,

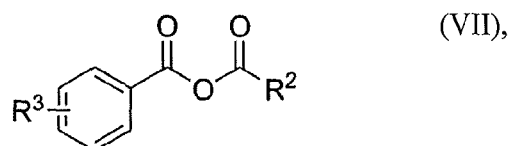
is performed for example through the substituted or unsubstituted benzoyl chloride of the general formula VI



- 30 wherein R^3 is one or more substituents selected from the group containing H, $-NO_2$, $-COOH$, halogenides, C_1 - C_6 alkyl alkoxy, preferably H;

5 in the presence of the organic base selected from the group containing e.g. DABCO (1,4-diazabicyclo[2.2.2]octan), N,N,N',N'-tetramethyl-1,6-hexanediamine, N-methyl morpholin, triethylamine (TEA) or N,N'-diisopropylethylamine (DIPEA), preferably TEA. The example of the activation is shown in Scheme 3A. The reaction environment is made with the polar solvent selected from the group containing isopropyl alcohol (IPA), tetrahydrofuran (THF), preferably

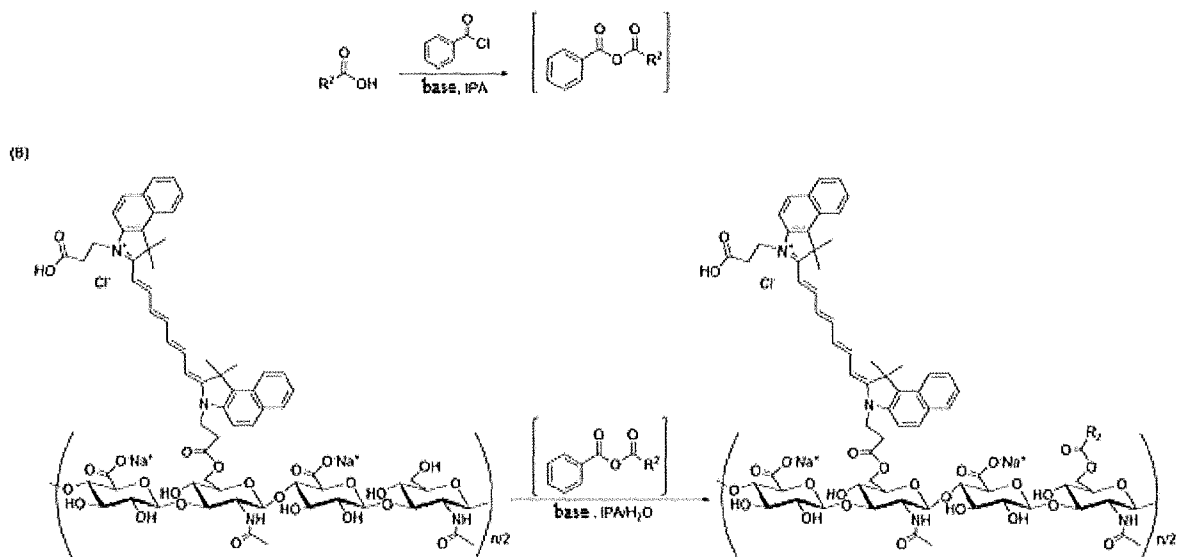
10 isopropyl alcohol, to form the reactive anhydride of the general formula VII



wherein

R^2 and R^3 are, as is defined above, that esterifies the fluorescent conjugate of hyaluronic acid or a salt thereof of the general formula I of the present invention (for example shown in

15 Scheme 3B).



20 **Scheme 3.** (A) The activation of the carboxyl group of the fat acid. (B) The hydrophobization of the fluorescent conjugate of hyaluronan with the saturated or unsaturated fat acid. Definitions of n and R^2 are stated above.

5 The esterification is performed with the activated carboxyl group of the fat acid in a mixture of water and the polar organic solvent miscible with water e.g. isopropyl alcohol (IPA), dimethyl sulfoxide (DMSO) or tetrahydrofuran (THF), preferably isopropyl alcohol. The esterification is performed in the mixture of water and the polar solvent miscible with water, wherein the water amount is in the range from 50 to 80 % v/v, preferably 50% v/v.

10 The reaction is also performed in the presence of the organic base preferably of amine selected from the group containing e.g. DABCO (1,4-diazabicyclo[2.2.2]octan), N,N,N',N'-tetramethyl-1,6-hexanediamine, N-methylmorpholine, imidazole, triethylamine (TEA) or N,N'-diisopropyl ethylamine (DIPEA), more preferably triethylamine. In the method of preparation of the hydrophobized conjugate of the present invention, the activation of the fat acid of the
15 general formula V is performed for 0.5 to 24 hours, at the temperature in the range 0 °C to 60 °C, preferably 0.5 hours at the temperature 0 °C to 25 °C and the esterification of the fluorescent conjugate of hyaluronic acid or the salt thereof is performed for 0.5 to 2 hours, preferably 2 hours, at the room temperature, i.e. at the temperature in the range of 22 °C to 25 °C.

According to a preferred embodiment of the method of preparation of the hydrophobized
20 conjugate of the present invention the amount of organic base corresponds to 2 to 6 molar equivalents, preferably to 4 molar equivalents per dimer of hyaluronic acid or the salt thereof. The amount of the substituted or unsubstituted benzoyl chloride corresponds to 0.2 to 2.0 molar equivalents, preferably 0.6 molar equivalents per dimer of hyaluronic acid or the salt thereof. The amount of the fat acid corresponds to 0.2 to 2.0 molar equivalents, preferably 0.6 molar
25 equivalents per the dimer of hyaluronic acid or the salt thereof. The closer study found out, that the degree of substitution of the hydrophobized conjugate of hyaluronic acid or the salt thereof of the invention with the fat acid depends on the equivalent amount of the activated fat acid and also it is positively influenced by the presence of the organic base.

The hydrophobized conjugate of hyaluronan with the heptamethine indocyanine
30 fluorescent agent (Cypate) of the general formula I of the present invention can be preferably used for the encapsulation (noncovalent bond) of nonpolar agents, preferably drugs or nanoparticles with hydrophobic surface. The hydrophobized conjugate is able to aggregate and form systems similar to polymer micelles with their behavior. Thus a composition is formed on the basis of the aggregated hydrophobized fluorescent conjugate of the present invention that
35 contains aggregates of the hydrophobized fluorescent conjugates and at least one or more nonpolar agents, preferably drugs, more preferably cytostatics, most preferably doxorubicin or paclitaxel, and/or nanoparticles, preferably superparamagnetic nanoparticles (i.e. spions). Spions

5 are preferably on the basis of iron oxides (Fe_2O_3 , Fe_3O_4), where the amount of iron in the composition is 0.3 to 3 wt.% preferably 1 to 1.5 wt.%. The size of superparamagnetic nanoparticles is 4 to 6 nm, preferably 5 nm. In the preferred embodiment, the composition contains the aggregated hydrophobized fluorescent conjugate of the present invention, wherein $\text{R}^1 - \text{C}(=\text{O})\text{C}_{17}\text{H}_{33}$ and nanoparticles, that are preferably superparamagnetic nanoparticles on the
10 basis of iron oxides (Fe_2O_3 , Fe_3O_4). Such composition can further preferably contain cytostatic, preferably doxorubicin or paclitaxel.

According to another embodiment of the present invention, the composition contains 2 to 15 wt.% of nonpolar agents in respect to the mass content of the hydrophobized fluorescent conjugate of hyaluronic acid or the salt thereof of present invention, preferably 2 to 6 wt.%.

15 The compositions of the present invention can be used in medicinal applications for *in vivo* imaging of tumors or for treating tumors.

In comparison with so far the described processes of preparation of the conjugates of hyaluronic acid with the cyanine fluorescent agents, the stated method of preparation of the conjugate of present invention brings about several advantages. In contrast to the technics
20 described in the publications, it relates to the direct synthesis without using any linker or previous modification of hyaluronic acid, or fluorescent agent. During the activation occurs the release of imidazole and CO_2 that are not toxic, and it can be easily removed from the reaction mixture. The release of imidazole can be further used in the preparation of the conjugate as in situ generated organic base and it need not be thus added as other organic base necessary
25 for the reaction proceeding. The esterification of hyaluronic acid proceeds in the organic solvent e.g. in DMSO. The activator of cypate is CDI. CDI can be used for the conjugation of Cypate to HA without the necessity of isolation of the intermediate. The advantage in this case is that the selective modification of the primary hydroxyl of HA occurs even in the case of non-protected secondary hydroxyl groups of HA and no undesired side reaction like oxidation of HA in DMSO
30 occurs (reaction Pfitzner–Moffatt).

Although the structure of Cypate contains two functional carboxyl groups, surprisingly no netting of the fluorescent conjugate of HA occurs (see Fig. 3-5), which would lead to decrease of the final product solubility.

Similar ester derivatives of HA are not easy to obtain using other processes, or activators as
35 are benzoyl chloride (BC) and 2,4,6-trichlorobenzoyl chloride (TBC) (WO2014082609). Neither ethyl chloroformate can be used that can activate carboxyl group (WO 2012034544), as it leads

- 5 to chromophore (cypate) degradation and loss of fluorescent properties. As another activator of carboxyl groups of hyaluronan in organic solvent is often used dicyclohexyl carbodiimide (DCC), its large disadvantage is, however, that it is indicated as strong allergen (Derm_Beruf_Umwelt, 1986; 34(4):110-1.) and it is highly toxic (Macrom. Rapid Commun. 2004, 25, 916-920).
- 10 The conjugate of hyaluronic acid with Cypate of general formula I and its hydrophobized conjugate of the present invention can be preferably excited in the area 570 nm to 790 nm and they emit at 680 to 850 nm, and thus suitable for the use in the medicinal applications for *in vivo* imaging of the conjugate distribution of the invention, preferably for *in vivo* imaging of organs selected from the group containing for example liver, skin; or imaging of tumors after the
- 15 intravenous, intraperitoneal or subcutaneous administration.

These conjugates are able to penetrate after intravenous or intraperitoneal administration into tumor tissues (i.e. neoplasms), preferably into palpable tumors and/or into very small (non-palpable) tumors, and thus are suitable for the imaging to the diagnose disease, especially tumor disease. In comparison with the pentamethine compounds is heptamethine cyanine conjugate of

20 hyaluronan advantageous in respect to fluorescent properties – especially of larger depth of the radiation penetration and further limitation of undesired autofluorescence. Diagnostics of tumor disease is preferably applicable for the tumor tissues selectively uptaking low-molecular hyaluronan (e.g. tissues with higher expression of CD44). The solubility of the hydrophobized conjugate of the present invention is 1 to 3 mg per 100 µl of the saline solution.

- 25 The hydrophobized conjugate of the present invention is in the term of the fluorescent properties very stable and after the intravenous administration it can be preferably imaged at least for 15 days without the necessity of the repeated administration of the conjugate. The conjugate of present invention is very easily concentrated even in small (palpably non-detectable) tumors. Another advantage of the hydrophobized conjugate of the present invention
- 30 is the possibility of the noncovalent binding of the anticancer agent (cytostatic) and thus their usage for a construction of theranostics, i.e. carrier with diagnostic and therapeutic function at the same time. The main advantage of the hydrophobized conjugate of the present invention as a theranostic is the use of its ability to accumulate in tumor tissues (especially in breast tumors), long term imaging and application to treatment of tumor itself.

5 *Terms definition:*

Cypate includes the structure of the general formula III (cypate I) or 1-[3-(2-carboxyethyl)-1,1-dimethyl-5,9b-dihydrobenzo[e]indol-3-ium-2-yl(chloride)]-octa-1,3,5,7-tetraenyl]-1,1-dimethyl-2H-benzo[e]indol-3-yl]propanoic acid, heptamethine indocyanine dye.

10 SS = degree of substitution = percentage amount of modified disaccharide units of hyaluronan per 100 disaccharide units of hyaluronan (100 % states, that per 100 of disaccharide units of hyaluronan was detected 100 modifying units)

The term "room temperature" refers to the range of temperatures in the room of 22 °C to 25 °C.

15 The equivalent (eqv.) refers to the dimer of hyaluronic acid, it refers to the molar equivalent if not stated otherwise.

The binding capacity is the amount of bound agent expressed in the percent by weight, if not stated otherwise.

20 The term "nonpolar agent" refers to a compound with symmetric distribution of charges. It refers to an agent that is soluble in organic solvents, especially in alcohols and insoluble in water.

Description of figures in drawings.

Fig. 1: ¹H NMR (D₂O) conjugate HA-Cypate.

Fig. 2: DOSY NMR spectrum (D₂O) conjugate HA-Cypate.

25 Fig. 3: Chromatogram record of SEC-MALLS (HA-Cypate 14,000g/mol) conjugate HA-Cypate (Example 3).

Fig. 4: Chromatogram record of SEC-MALLS (HA-Cypate 14,000g/mol) conjugate HA-Cypate (Example 7).

Fig. 5: Chromatogram record of SEC-MALLS (HA-Cypate 58,000g/mol) conjugate HA-Cypate (Example 8).

30 Fig. 6: Chromatogram record of SEC-MALLS (HA-Cypate 72,000g/mol) conjugate HA-Cypate (Example 9).

5 Fig. 7: The emission spectrum of the fluorescence of conjugate HA-Cypate in the aqueous solution at the excitation 650, 660, 665 and 670 nm.

Fig. 8: The emission fluorescence of the conjugate in the aqueous solution at the excitation with laser $\lambda = 632.8$ nm without (left panel) and in a combination with the filter transmitting wavelengths over $\lambda = 635$ nm (right panel).

10 Fig. 9: *In vivo* fluorescent imaging: HA-Cypate applied subcutaneously. The place of application is indicated with letter S. Figures show the detection of emission using the different excitation and emission filters.

Fig. 10: *In vivo* fluorescent imaging in time after the intraperitoneal application of HA-Cypate.

15 Fig. 11: *In vivo* fluorescent imaging in time after the intravenous application of HA-Cypate-C18:1.

Fig. 12: *In vivo* fluorescent imaging in time after the intravenous application of HA-Cypate-C18:1 (mouse with tumor, tumor cells indicated with chemiluminescent luciferase).

20 Fig. 13: The evaluation of *in vivo* luminescence of tumor after the administration of (i) HA-Cypate-C18:1 (=HA cyp), (ii) HA-Cypate-C18:1+doxorubicin (=HA cyp dox), (iii) HA-Cypate-C18:1+doxorubicin+spion (=HA cyp dox+spions).

Fig. 14: The comparison of spleen and liver weight after the administration of (i) HA-Cypate-C18:1 (=HA cyp), (ii) HA-Cypate-C18:1+doxorubicin (=HA cyp dox), (iii) HA-Cypate-C18:1+doxorubicin+spion (=HA cyp dox+spions).

25 Fig. 15: *In vivo* fluorescent imaging in time after the intraperitoneal application of HA-Cypate-C18:1 (mouse with tumor, tumor cells indicated with luminescent luciferase).

Fig. 16: The mass spectrum of dimer HA-Cypate obtained from the enzymatic degradation of the conjugate HA-Cypate with the hyaluronan lyase.

Examples of the Embodiments of the Present Invention

Description of instrumentation

30 NMR spectra were recorded on BRUKER AVANCE 500 at frequency 500.13 MHz (^1H). For the processing of experimental data was used the software by Bruker TOPSPIN 1.2 or software SpinWorks 3.1. For the interpretation of the spectra from NMR analyses were used abbreviations: s (singlet), d (doublet), t (triplet), m (multiplet). For UV/Vis spectra measurement

5 in the wavelength range 190 – 800 nm was used UV/Vis spectrophotometer Varian Cary 100. Fluorescent spectra were recorded on the apparatus PTI Quantamaster 400. ESI-MS analyses of Cypate were performed on the mass spectrophotometer with ionic trap amaZon X (BrukerDaltonics) equipped with the electrospray ionizing source and the quadrupole mass analyzer. Measurements were performed in the positive and negative mode. The structure of
10 conjugate of HA-cypate was confirmed using LC-MS analysis of the conjugate after its enzymatic cleaving with hyaluronan lyase. The mixture of oligosaccharides was separated on column Kinetex 1.7 μ m F5 100A (Phenomenex) using gradient 0.1% HCOOH in H₂O and acetonitrile. The detection was performed on Synapt G2-Si in negative resolution mode with ionization with electrospray. Analysis of samples and molecule mass of initial hyaluronan was
15 determined using the method SEC-MALLS (HPLC Alliance) with UV/VIS 2489 and the refractometry detector RID 2414 and the detector of light scattering mini DAWN TREOS. Data were processed using the software Astra Version 5.3.4.20 (Wyatt Technology EuropeGmbH). All *in vivo* imaging analyses were carried out on the apparatus IVIS Luminia XR Series III) on a laboratory mice of the strain Balb/c.

20 ***Example 1. Synthesis of 3-(2-carboxyethyl)-1,2,2-trimethyl-1H-benzo[e]indolium-bromide***

2.0 g (9.6 mmol) of 1,1,2-trimethyl-1H-benzindol and 2.2 g (14.3 mmol) 3-bromopropanoic acid were dissolved in 10 ml 1,2-dichlorobenzen and under the constant stirring warmed at 115 °C for 16 hours. The crude reaction mixture was cooled down to the room temperature and the resulting precipitate was washed with 1,2-dichloromethane (10 x 50 ml). The final product
25 was separated using the filtration, dried under vacuum on the rotary evaporator (RE) and it was obtained in the form of light gray crystal powder (yield 2.2 g (64 %)).

¹H NMR (DMSO-*d*₆, 500 MHz): δ 8.38 (d, J = 8.35, 1H_{arom}), 8.29 (d, J = 9.00, 1H_{arom}), 8.23 (d, J = 8.35, 1H_{arom}), 8.18 (d, J = 9.00, 1H_{arom}), 7.80 – 7.71 (m, 2H_{arom}), 4.79 (t, J = 6.95, 2H, -CH₂-), 3.05 (t, J = 6.95, 2H, -CH₂-), 2.98 (s, 3H, -CH₃), 1.76 (s, 6H, -CH₃) ppm

30 ESI-MS: ⁺MS [M]⁺ = 282

Example 2. Preparation of Cypate

0.8 g (2.8 mmol) glutaconic dialdehyde dianiline hydrochloride was dissolved in 8 ml 1,2-dichloromethane and tempered to 0 – 5 °C. 521 μ l (5.5 mmol) of the anhydride of acetic acid, 481 μ l (2.8 mmol) and DIPEA in the small volume of 1,2-dichlormethan (0.5 ml) was added
35 dropwise into the solution and the reaction mixture was left while cooling and stirring to react

5 for 3 hours. In the meantime 2 g of (5.5 mmol) 3-(2-carboxyethyl)-1,2,2-trimethyl-1*H*-benzo[*e*]indolium-bromide prepared in Example 1 and 0.9 g (11.0 mmol) of sodium acetate in the mixture of solvents acetonitrile/water 95/5 of volume 15 ml were brought to reflux and the first reaction mixture was added dropwise. The reaction proceeded for 18 hours under stirring at reflux in dark. The crude reaction mixture was cooled down to the room temperature, washed
10 with 400 ml ethyl-acetate and 400 ml 1M HCl, the product was filtrated and dried under the low pressure. The resulting product was obtained in the form of dark green crystal powder yielding 1.62 g (88 %).

¹H NMR (DMSO-*d*₆, 500 MHz): δ 8.25 (d, *J* = 8.75, 2H), 8.07 – 7.97 (m, 6H), 7.82 (t, *J* = 12.65, 1H), 7.73 (d, *J* = 8.75, 2H), 7.69 – 7.62 (m, 2H), 7.54 – 7.48 (m, 2H), 6.60 (t, *J* = 12.65,
15 2H), 6.47 (d, *J* = 13.75, 2H), 4.43 (bt, 4H -CH₂-), 2.77 (t, *J* = 6.9, 4H -CH₂-), 1.92 (s, 12H -CH₃) ppm

ESI-MS: ⁺MS [M]⁺ = 625; ⁻MS [M-2H]⁻ = 623 m/z

UV/Vis: λ_{abs.max} = 782 nm (MeOH)

Example 3: Preparation of conjugate HA-Cypate

20 87 mg (0.13 mmol, 0.5 eqv.) Cypate from Example 2 was dissolved in 2 ml DMSO, 22 mg (0.13 mmol, 0.5 eqv.) *N,N'*-carbonyl diimidazole was added and under the constant stirring was let activate 2 hours at the room temperature. In the meantime 100 mg (0.27 mmol, 1 eqv.) acid form of hyaluronic acid M_w 14,000 g/mol was dissolved in DMSO at 60 °C. Then 15 μl (0.13 mmol, 0.5 eqv.) *N*-methylmorpholine and the first reaction mixture without the previous isolation
25 was added into the solution. The reaction proceeded under the constant stirring in dark at 60 °C 24 hours. The reaction was stopped by adding ten-fold of 100% isopropyl alcohol (AIPA) in respect to the initial volume of the reaction mixture and the saturated solution of NaCl, when precipitation of the desired product occurred. The crude product was purified with 5 x 100 ml AIPA, dissolved in 50 ml demineralized water and transferred into dialysis tube. The protonized
30 form of HA was neutralized 1st day in 0.5% solution of NaCl and 0.5% NaHCO₃, further dialyzed 2nd and 3rd day in the demineralized water. The tube content was frozen and lyophilized. The resulting product in the form of hyaluronan was obtained as green lyophilizate of mass 89 mg (87 %).

¹H NMR (D₂O) (Fig. 1): cypate: δ 8.80 (s, 2H), 8.50– 8.47 (m, 2H), 8.46 – 8.43 (m, 2H),
35 8.29 – 8.19 (m, 2H), 8.16 – 8.10 (m, 2H), 8.08 – 7.89 (m, 2H), 7.88 – 7.81 (m, 2H), 7.79 – 7.73

- 5 (m, 2H), 7.58 (s, 2H), 7.51 – 7.46 (m, 2H), 5.16, - 5.14 (m, 4H), 3.14 – 3.09 (m, 4H), 2.02 (12H, CH₃, overlaid with signals of HA), HA: δ 4.62 – 4.39 (m, 2H anomer), 4.01 – 3.28 (m, 10H skeletal), 2.02 (s, 3H, -CH₃) ppm

DOSY (D₂O): (Fig. 2)

SS = 1.5 % (determined from ¹H NMR)

- 10 SEC-MALLS-LS-UV/Vis-RI: (Fig. 3)

Fluorimeter: $\lambda_{em,max}$ = 695 nm (at $\lambda_{exc.}$ = 665 nm; H₂O); (Fig. 7)

Fluorescence of the conjugate excited with the laser wavelength λ = 632.8 nm (Fig. 8)

ESI-MS: [M-H]⁻ = 984 m/z (Fig. 16, dimer detected after the enzymatic degradation of the conjugate with lyase)

15 **Example 4: Preparation of conjugate HA-Cypate**

- 87 mg (0.13 mmol, 0.5 eqv.) Cypate from Example 2 was dissolved in 2 ml DMSO, 22 mg (0.13 mmol, 0.5 eqv.) *N,N'*-carbonyl diimidazole was added and under the constant stirring let to activate 30 min. at the room temperature. 100 mg (0.27 mmol, 1 eqv.) of the acid form of hyaluronic acid of M_w 14,000 g/mol in DMSO at 60 °C was added. The reaction proceeded
- 20 under the constant stirring in dark at 60 °C for 24 hours. The reaction was stopped by addition of ten-fold volume of 100% isopropyl alcohol (AIPA) in respect to the initial volume of the reaction mixture and the saturated solution of NaCl, then the precipitation of the desired product occurred. The crude product was purified with 5 x 100 ml AIPA, dissolved in 50 ml demineralized water and transferred into the dialysis tube. The protonized form of HA was
- 25 neutralized 1st day in 0.5% solution NaCl and 0.5% NaHCO₃, further dialyzed 2nd and 3rd day in demineralized water. The tube content was frozen and lyophilized. The resulting product in the form of hyaluronan was obtained as green lyophilizate of mass 89 mg (87 %).

- ¹H NMR (D₂O) (Fig. 1): cypate: δ 8.80 (s, 2H), 8.50– 8.47 (m, 2H), 8.46 – 8.43 (m, 2H), 8.29 – 8.19 (m, 2H), 8.16 – 8.10 (m, 2H), 8.08 – 7.89 (m, 2H), 7.88 – 7.81 (m, 2H), 7.79 – 7.73
- 30 (m, 2H), 7.58 (s, 2H), 7.51 – 7.46 (m, 2H), 5.16, - 5.14 (m, 4H), 3.14 – 3.09 (m, 4H), 2.02 (12H, CH₃, overlaid with signals of HA), HA: δ 4.62 – 4.39 (m, 2H anomer), 4.01 – 3.28 (m, 10H skeletal), 2.02 (s, 3H, -CH₃) ppm

SS = 1 % (determined from ¹H NMR)

5 Fluorimeter: $\lambda_{em,max} = 695$ nm (at $\lambda_{exc.} = 665$ nm; H₂O)

Example 5: Preparation of conjugate HA-Cypate

17 mg (0.03 mmol, 0.1 eqv.) Cypate from Example 2 was dissolved in 1 ml DMSO, 6 mg (0.04 mmol, 0.15 eqv.) *N,N'*-carbonyl diimidazole was added and under the constant stirring was let to activate 30 min. at the room temperature. 100 mg (0.27 mmol, 1 eqv.) of the acid form of
 10 hyaluronic acid of M_w 14,000 g/mol in DMSO was dissolved at 60 °C. The reaction proceeded under the constant stirring in dark at 60 °C 24 hours. The reaction was stopped with the addition of ten-fold volume of 100% isopropyl alcohol (AIPA) in respect to the initial reaction mixture volume and the saturated solution of NaCl, then the precipitation of the desired product occurred. The crude product was purified with 5 x 100 ml AIPA, dissolved in 50 ml demineralized water
 15 and transferred into the dialysis tube. The protonized form of HA was neutralized 1st day in 0.5% solution of NaCl and 0.5% NaHCO₃, further dialyzed 2nd and 3rd day in the demineralized water. The tube content was frozen and lyophilized. The resulting product in the form of hyaluronan was obtained as green lyophilizate of mass 92 mg (90 %).

¹H NMR (D₂O): cypate: δ 8.80 (s, 2H), 8.50–8.47 (m, 2H), 8.46–8.43 (m, 2H), 8.29–8.19 (m, 2H), 8.16–8.10 (m, 2H), 8.08–7.89 (m, 2H), 7.88–7.81 (m, 2H), 7.79–7.73 (m, 2H), 7.58 (s, 2H), 7.51–7.46 (m, 2H), 5.16, - 5.14 (m, 4H), 3.14–3.09 (m, 4H), 2.02 (12H, CH₃, overlaid with signals of HA), HA: δ 4.62–4.39 (m, 2H anomer), 4.01–3.28 (m, 10H skeletal), 2.02 (s, 3H, -CH₃) ppm

SS = 0.7 % (determined from ¹H NMR)

25 Fluorimeter: $\lambda_{em,max} = 695$ nm (at $\lambda_{exc.} = 665$ nm; H₂O)

Example 6: Preparation of conjugate HA-Cypate

122 mg (0.19 mmol, 0.7 eqv.) Cypate from Example 2 was dissolved in 1 ml DMSO, 34 mg (0.21 mmol, 0.8 eqv.) *N,N'*-carbonyl diimidazole was added and under the constant stirring was let to activate 30 min. at the room temperature. 100 mg (0.27 mmol, 1 eqv.) of the acid form of
 30 hyaluronic acid of M_w 14,000 g/mol in DMSO was dissolved at 60 °C. The reaction proceeded under the constant stirring in dark at 60 °C 24 hours. The reaction was stopped with the addition of ten-fold volume of 100% isopropyl alcohol (AIPA) in respect to the initial reaction mixture volume and the saturated solution of NaCl, then the precipitation of the desired product occurred. The crude product was purified with 5 x 100 ml AIPA, dissolved in 50 ml demineralized water
 35 and transferred into the dialysis tube. The protonized form of HA was neutralized 1st day

5 in 0.5% solution of NaCl and 0.5% NaHCO₃, further dialyzed 2nd and 3rd day in the demineralized water. The tube content was frozen and then lyophilized. The resulting product was obtain in the form of hyaluronan as green lyofilizate of mass 85 mg (88 %).

¹H NMR (D₂O): cypate: δ 8.80 (s, 2H), 8.50–8.47 (m, 2H), 8.46–8.43 (m, 2H), 8.29–8.19 (m, 2H), 8.16–8.10 (m, 2H), 8.08–7.89 (m, 2H), 7.88–7.81 (m, 2H), 7.79–7.73 (m, 2H),
 10 7.58 (s, 2H), 7.51–7.46 (m, 2H), 5.16, - 5.14 (m, 4H), 3.14–3.09 (m, 4H), 2.02 (12H, CH₃, overlaid with signals of HA), HA: δ 4.62–4.39 (m, 2H anomer), 4.01–3.28 (m, 10H skeletal), 2.02 (s, 3H, -CH₃) ppm

SS = 1.3 % (determined from ¹H NMR)

Fluorimeter: λ_{em,max} = 695 nm (at λ_{exc.} = 665 nm; H₂O)

15 **Example 7: Preparation of conjugate HA-Cypate**

87 mg (0.13 mmol, 0.5 eqv.) Cypate from Example 2 was dissolved in 2 ml DMSO, 22 mg (0.13 mmol, 0.5 eqv.) CDI was added and under the constant stirring was let to activate 2 h at the room temperature. 100 mg (0.27 mmol, 1 eqv.) the acid form of hyaluronic acid of Mw (14,000 g/mol) was dissolved in DMSO at 60 °C. Then was 138 μl (0.79 mmol, 3 eqv.) DIPEA and the
 20 first reaction mixture added to this solution. The reaction proceeded under the constant stirring in dark at 60 °C 24 hours.

The reaction was stopped by the addition of ten-fold volume of AIPA in respect to the initial volume of the reaction mixture and the saturated solution of NaCl, then occurred the precipitation of the desired product. The product was purified with 5 x 100 ml AIPA, dissolved
 25 in 50 ml demineralized water and transferred in the dialysis tube. The protonized form of HA was neutralized 1st day in 0.5% solution of NaCl and 0.5% NaHCO₃, further dialyzed 2nd and 3rd day in demineralized water. The tube content was frozen and lyophilized. The resulting product in the form of hyaluronan was obtained as lyofilizate of mass 86 mg (84 %).

¹H NMR (D₂O): cypate: δ 8.81 (s, 2H), 8.50–8.47 (m, 2H), 8.46–8.43 (m, 2H), 8.29–8.19 (m, 2H), 8.16–8.10 (m, 2H), 8.08–7.89 (m, 2H), 7.88–7.81 (m, 2H), 7.79–7.73 (m, 2H),
 30 7.58 (s, 2H), 7.51–7.46 (m, 2H), 5.16, - 5.14 (m, 4H), 3.14–3.09 (m, 4H), 2.03 (12H, CH₃, overlaid with signals of HA), HA: δ 4.62–4.38 (m, 2H anomer), 4.01–3.26 (m, 10H skeletal), 2.03 (s, 3H, -CH₃) ppm

SS = 1.5 % (determined from ¹H NMR)

5 SEC-MALLS-LS-UV/Vis-RI: (Figure 4)

Fluorimeter: $\lambda_{em,max} = 695$ nm (at $\lambda_{exc,max} = 665$ nm; H₂O)

Example 8: Preparation of conjugate HA-Cypate

87 mg (0.13 mmol, 0.5 eqv.) Cypate from Example 2 was dissolved in 2 ml DMSO, 22 mg (0.13 mmol, 0.5 eqv.) CDI was added and under the constant stirring let activate for 2h at the
10 room temperature. 100 mg (0.27 mmol, 1 eqv.) of the acid form of hyaluronic acid of Mw (5.8×10^4 g/mol) in DMSO was dissolved at 60 °C. Into this solution was then added 15 μ l (0.13 mmol, 0.5 eqv.) *N*-methyl morfolin and the first reaction mixture without the previous purification. The reaction proceeded under the constant stirring in dark at 60 °C 24 hours.

The reaction was quenched by the addition of ten-fold of AIPA in respect to the initial
15 volume of the reaction mixture and saturated solution of NaCl, then the precipitation of the desired product occurred. The crude product was purified with 5 x 100 ml AIPA, dissolved in 50 ml demineralized water and transferred into the dialysis tube. The protonized form of HA was neutralized 1st day in 0.5 % solution NaCl and 0.5% NaHCO₃, further dialyzed 2nd and 3rd day in demineralized water. The tube content was frozen and lyophilized. The resulting product in the
20 form of hyaluronan was obtained as green lyophilizate of mass 95 mg (93 %).

¹H NMR (D₂O): cypate: δ 9.2 (m, 2H), 8.82–8.77 (m, 2H), 8.46–8.43 (m, 2H), 8.29–8.19 (m, 2H), 8.16–8.10 (m, 2H), 8.08–7.89 (m, 2H), 7.89–7.81 (m, 2H), 7.79–7.73 (m, 2H), 7.58 (s, 2H), 7.51–7.46 (m, 2H), 5.16, - 5.14 (m, 4H), 3.14–3.09 (m, 4H), 2.03 (12H, CH₃, overlaid with signals of HA), HA: δ 4.64–4.38 (m, 2H anomer), 4.04–3.21 (m, 10H skeletal),
25 2.03 (s, 3H, -CH₃) ppm

[SS = 1.0 % (determined from ¹H NMR)

SEC-MALLS-LS-UV/Vis-RI: (Fig.5)

Fluorimeter: $\lambda_{em,max} = 695$ nm (at $\lambda_{exc.} = 665$ nm; H₂O))

Example 9: Preparation of conjugate HA-Cypate

30 87 mg (0.13 mmol, 0.5 eqv.) Cypate from Example 2 was dissolved in 2 ml DMSO, 22 mg (0.13 mmol, 0.5 eqv.) *N,N'*-carbonyl diimidazole was added and with the constant stirring let activate for 2h at the room temperature. 100 mg (0.27 mmol, 1 eqv.) acid form of hyaluronic acid of Mw (7.2×10^4 g/mol) in DMSO was dissolved at 60 °C. 15 μ l (0.13 mmol, 0.5 eqv.) *N*-

5 methyl morfoline and the first reaction mixture was then added into this solution. The reaction proceeded with the constant stirring in dark at 60 °C 24 hours.

The reaction was stopped by addition of ten-fold volume of AIPA in respect to the initial volume of the reaction mixture and the saturated solution of NaCl, then occurred the precipitation of the desired product. The crude product was purified with 5 x 100 ml 100%
 10 AIPA, dissolved in 50 ml demineralized water and transferred into the dialysis tube. The protonized form of HA was neutralized 1st day in 0.5% solution NaCl and 0.5% NaHCO₃, further dialyzed 2nd and 3rd day in demineralized water. The tube content was frozen and lyophilized. The resulting product in the form of hyaluronan was obtained as lyophilizate of mass 96 mg (94 %).

¹H NMR (D₂O): cypate: δ 9.2 (m, 2H), 8.83– 8.5 (m, 2H), 8.45 – 8.43 (m, 2H), 8.30 – 8.18
 15 (m, 2H), 8.16 – 7.99 (m, 2H), 7.99 – 7.89 (m, 2H), 7.89 – 7.81 (m, 2H), 7.79 – 7.73 (m, 2H), 7.58 (s, 2H), 7.51 – 7.46 (m, 2H), 5.16, - 5.14 (m, 4H), 3.14 – 3.09 (m, 4H), 2.03 (12H, CH₃, overlaid with signals of HA), HA: δ 4.64 – 4.38 (m, 2H anomer), 4.04 – 3.21 (m, 10H skeletal), 2.03 (s, 3H, -CH₃) ppm

SS = 1.0 % (determined from ¹H NMR)

20 SEC-MALLS-LS-UV/Vis-RI: (Fig. 6)

Fluorimeter: λ_{em,max} = 695 nm (at λ_{exc.} = 665 nm; H₂O))

Example 10: Preparation of conjugate HA-Cypate

87 mg (0.3 mmol, 0.5 eqv.) Cypate from Example 2 was dissolved in 2 ml DMSO, 22 mg (0.13 mmol, 0.5 eqv.) *N,N'*-carbonyl diimidazole was added and under the constant stirring let
 25 activate 2h at the room temperature. 100 mg (0.27 mmol, 1 eqv.) of the acid form of hyaluronic acid of Mw (2.5 x 10⁵ g/mol) was dissolved in DMSO at 40 °C. *N*-methyl morfolin and first reaction mixture was then added 15 µl (0.13 mmol, 0.5 eqv.) into this solution. The reaction proceeded under the constant stirring in dark at 40 °C 24 hours

The reaction was stopped by the addition of ten-fold volume of AIPA in respect to the initial
 30 volume of the reaction mixture and the saturated solution of NaCl, then the precipitation of desired product occurred. The crude product was purified with 5 x 100 ml AIPA, dissolved in 50 ml demineralized water and transferred into the dialysis tube. The protonized form of HA was neutralized 1st day in 0.5% solution NaCl and 0.5% NaHCO₃, further dialyzed 2nd and 3rd day in

- 5 demineralized water. The tube content was frozen and lyophilized. The resulting product in the form of hyaluronan was obtained as lyophilizate of mass 93 mg (92 %).

^1H NMR (D_2O): cypate: δ 9.2 (m, 2H), 8.83– 8.5 (m, 2H), 8.45 – 8.43 (m, 2H), 8.30 – 8.17 (m, 2H), 8.16 – 7.99 (m, 2H), 7.99 – 7.89 (m, 2H), 7.89 – 7.81 (m, 2H), 7.79 – 7.73 (m, 2H), 7.58 (s, 2H), 7.51 – 7.42 (m, 2H), 5.16, - 5.14 (m, 4H), 3.14 – 3.09 (m, 4H), 2.03 (12H, CH_3 , overlaid with signals of HA), HA: δ 4.62 – 4.38 (m, 2H anomer), 4.01 – 3.26 (m, 10H skeletal), 2.03 (s, 3H, $-\text{CH}_3$) ppm

SS = 0.5 % (determined from ^1H NMR)

Fluorimeter: $\lambda_{\text{em,max}} = 695 \text{ nm}$ (at $\lambda_{\text{exc.}} = 665 \text{ nm}$; H_2O)

Example 11: Esterification of HA-Cypate with hexanoic acid

- 15 300 mg (0.73 mmol, 1 eqv.) of the conjugate HA-Cypate prepared according to Example 3 was dissolved in 15 ml demi water and 13 ml AIPA, 382 μl (2.20 mmol, 3 eqv.) DIPEA and 4.5 mg (0.04 mmol, 0.05 eqv) DMAP were then added. 165 μl (1.32 mmol, 1.8 eqv.) hexanoic acid was dissolved in 2 ml AIPA, 255 μl (1.46 mmol, 2 eqv.) DIPEA 153 μl (1.32 mmol, 1.8 eqv.) benzoyl chloride was added and let react under the constant stirring for 30 minutes at 0 °C. After
20 the period of time was everything quantitatively transferred to the first reaction mixture, then the esterification proceeded for 2 hours at the room temperature.

The reaction was quenched by addition of the high excess of AIPA and the saturated solution of NaCl, whereas the precipitation of product occurred. The crude product was washed with 4 x 200 ml AIPA, further dissolved in demineralized water and transferred into the dialysis
25 tube. Dialysis proceeded for 24 hours against 0.5% NaHCO_3 , 0.5% NaCl and further 8 hours in demineralized water. The product was obtained in the form of green lyophilizate yielding 230 mg (73%).

^1H NMR additional signals compared with example 3 (D_2O , 500 MHz): δ 2.4 (m, 2H, αCH_2), 1.6 (m, 2H, βCH_2), 1.31 (m, 4H, γCH_2), 0.8 (m, 3H, $-\text{CH}_2-\text{CH}_3$) ppm

- 30 SS (acylation) = 70 % (determined from ^1H NMR)

Fluorimeter: $\lambda_{\text{em,max}} = 700 \text{ nm}$ (at $\lambda_{\text{exc.}} = 665 \text{ nm}$; H_2O)

Example 12: Esterification of HA-Cypate with palmitic acid

5 300 mg (0.73 mmol, 1 eqv.) of the conjugate of HA-Cypate prepared according to Example 3 was dissolved in 15 ml demi water, and then was added 306 μ l (2.20 mmol, 3 eqv.) TEA and 4.5 mg (0.04 mmol, 0.05 eqv.) DMAP were added. In the meantime 94 mg (0.37 mmol, 0.5 eqv.) of hexadecanoic acid dissolved in 3 ml THF, 153 μ l (1.10 mmol, 1.5 eqv.) TEA, 43 μ l (0.37 mmol, 0.5 eqv.) benzoyl chloride was added and let react under the constant stirring for 30 minutes at
10 the room temperature. After the period of time was everything quantitatively transferred to the first reaction mixture, the esterification then proceeded for 2 hours at the room temperature.

The reaction was quenched by addition of the high excess of AIPA and saturated solution of NaCl, whereas the precipitation of the product occurred. The crude product was washed with 4 x 200 ml AIPA, further dissolved in demineralized water and transferred into the the dialysis tube.
15 Dialysis proceeded for 24 hours against 0.5% NaHCO₃ and 0.5% NaCl and further for 48 hours in demineralized water. The product was obtained in the form of green lyofilizate yielding 189 mg (61%).

¹H NMR additional signals to example 3 (D₂O, 500 MHz): δ 2.76 – 2.66 (m, 2H, α CH₂), 1.64 – 1.48 (m, 2H, β CH₂), 1.31 – 1.08 (m, 24H, CH₂), 0.98 – 0.78 (m, 3H, -CH₂-CH₃) ppm

20 SS acylation = 7 % (determined from ¹H NMR)

Fluorimeter: $\lambda_{em,max}$ = 710 nm (at $\lambda_{exc.}$ = 685 nm; H₂O)

Example 13: Esterification of HA-Cypate with palmitic acid

200 mg (0.49 mmol, 1 eqv.) of the conjugate of HA-Cypate prepared according to Example 3 was dissolved in 10 ml demi water, and then 136 μ l (0.98 mmol, 2 eqv.) TEA and 3 mg (0.02
25 mmol, 0.04 eqv.) DMAP were added. 38 mg (0.16 mmol, 0.3 eqv.) of palmitic acid dissolved in 3 ml THF, 68 μ l (0.48 mmol, 1 eqv.) TEA, 17 μ l (0.16 mmol, 0.3 ekv.) benzoyl chloride was added and let react with the constant stirring for 30 minutes at the room temperature. After the period of time was everything quantitatively transferred to the first reaction mixture, the esterification then proceeded for 2 hours at the room temperature.

30 The eaction was quenched by the addition of high excess of AIPA and saturated solution of NaCl, whereas the precipitation of product occurred. The crude product was washed with 4 x 200 ml AIPA, further dissolved in demineralized water and transferred into the dialysis tube. Dialysis proceeded for 24 hours against 0.5% NaHCO₃ and 0.5% NaCl and further for 48 hours in demineralized water. The product was obtained in the form of green lyofilizate yielding 76 mg
35 (37 %).

5 ^1H NMR (D_2O , 500 MHz) additional signals to Example 3: δ 2.76 – 2.66 (m, 2H, αCH_2), 1.64 – 1.48 (m, 2H, βCH_2), 1.31 – 1.08 (m, 24H, CH_2), 0.98 – 0.78 (m, 3H, $-\text{CH}_2-\underline{\text{CH}_3}$) ppm

SS (acylation) = 5 % (determined from ^1H NMR)

Fluorimeter: $\lambda_{\text{em,max}} = 710$ nm (at $\lambda_{\text{exc.}} = 685$ nm; H_2O)

Example 14: Esterification of HA-Cypate with palmitic acid

10 200 mg (0.49 mmol, 1 eqv.) of the conjugate HA-Cypate prepared according to Example 3 was dissolved in 10 ml demi water, and then 255 μl (1.47 mmol, 3 eqv.) DIPEA and 3 mg (0.02 mmol, 0.04 eqv.) DMAP were added. 38 mg (0.15 mmol, 0.3 eqv.) palmitic acid was dissolved in 3 ml THF, 85 μl (0.4-9 mmol, 1 eqv.) DIPEA, 17 μl (0.15 mmol, 0.3 eqv.) benzoyl chloride was added and let react under the constant stirring for 30 minutes at the room temperature. After
15 the period of time was everything quantitatively transferred to the first reaction mixture, the esterification then proceeded for 2 hours at the room temperature.

The reaction was quenched by the addition of high excess of AIPA and the saturated solution of NaCl, whereas the precipitation of the product occurred. The crude product was washed with 4 x 200 ml AIPA, further dissolved in demineralized water and transferred into the
20 dialysis tube. Dialysis proceeded for 24 h against 0.5% NaHCO_3 and 0.5% NaCl and further for 48 hours in demineralized water. The product was obtained in the form of green lyophilizate yielding 93 mg (45 %).

^1H NMR (D_2O , 500 MHz) additional signals to Example 3: δ 2.76 – 2.66 (m, 2H, αCH_2), 1.64 – 1.48 (m, 2H, βCH_2), 1.31 – 1.08 (m, 24H, CH_2), 0.98 – 0.78 (m, 3H, $-\text{CH}_2-\underline{\text{CH}_3}$) ppm

25 SS (acylation) = 5 % (determined from ^1H NMR)

Fluorimeter: $\lambda_{\text{em,max}} = 710$ nm (at $\lambda_{\text{exc.}} = 685$ nm; H_2O)

Example 15: Esterification of HA-Cypate with oleic acid

300 mg HA-Cypate (0.73 mmol, 1 eqv.) from Example 3 was dissolved in 15 ml demi water and 13 ml AIPA, 306 μl (2.20 mmol, 3 eqv.) TEA and 4.5 mg (0.04 mmol, 0.05 eqv.) DMAP
30 were then added. 186 μl (0.59 mmol, 0.8 eqv.) cis-octadec-9-enoid acid dissolved in 2 ml AIPA, 306 μl (2.20 mmol, 3 eqv.) TEA, 68 μl (0.59 mmol, 0.8 eqv.) benzoyl chloride was added and everything was let react under the constant stirring for 30 minutes at the room temperature.

- 5 After the period of time was everything quantitatively transferred to the first reaction mixture, esterification then proceeded for 2 hours at the room temperature.

The reaction was quenched by the addition of the high excess of AIPA and saturated solution of NaCl, whereas the precipitation of product occurred. The crude product was washed 4 x 200 ml AIPA, further dissolved in demineralized water and transferred into the dialysis tube.

- 10 Dialysis proceeded for 24 hours against 0.5% NaHCO₃ and 0.5% NaCl and further 48 hours in demineralized water. The product was obtained in the form of green lyophilizate yielding 210 mg (66 %).

- ¹H NMR (D₂O, 500 MHz) additional signals to Example 3: δ 5.41 – 5.34 (m, 2H, CH=CH), 2.48 – 2.40 (m, 2H, -CH₂-CO-), 1.68 – 1.54 (m, 2H, -CH₂-CH₂-CO-), 1.40 – 1.23 (m, 24 H, (-CH₂)₁₂), 0.91 – 0.68 (m, 3H, -CH₂-CH₃) ppm
- 15

SS (acylation) = 10 % (determined from ¹H NMR)

Fluorimeter: λ_{em,max} = 710 nm (at λ_{exc.} = 685 nm; H₂O)

Example 16: Esterification of HA-Cypate with oleic acid

- 300 mg (0.73 mmol, 1 eqv.) of the conjugate of HA-Cypate prepared according to Example 3 was dissolved in 15 ml demi water and 13 ml AIPA, then 306 μl (2.20 mmol, 3 eqv.) TEA and 4.5 mg (0.04 mmol, 0.05 eqv.) DMAP were added. 116 μl (0.36 mmol, 0.5 eqv.) of cis-octadec-9-enoic acid dissolved in 2 ml AIPA, 153 μl (1.08 mmol, 1.5 eqv.) TEA, 43 μl (0.36 mmol, 0.5 eqv.) benzoyl chloride was added and let react under the constant stirring for 30 minutes at the room temperature. After the period of time everything was quantitatively transferred to the first reaction mixture, the esterification then proceeded for 2 hours at the room temperature.
- 25

- The reaction was quenched with the addition of the high excess of AIPA and saturated solution of NaCl, whereas the precipitation of the product occurred. The crude product was washed with 4 x 200 ml AIPA, further dissolved in demineralized water and transferred into the dialysis tube. Dialysis proceeded for 24 hours against 0.5% NaHCO₃ and 0.5% NaCl and further for 48 hours in demineralized water. The product was obtained in the form of green lyophilizate yielding 205 mg (73 %).
- 30

¹H NMR (D₂O, 500 MHz) additional signals to Example 3: 5.41 – 5.34 (m, 2H, CH=CH), 2.48 – 2.40 (m, 2H, -CH₂-CO-), 1.68 – 1.54 (m, 2H, -CH₂-CH₂-CO-), 1.40 – 1.23 (m, 24 H, (-CH₂)₁₂), 0.91 – 0.68 (m, 3H, -CH₂-CH₃) ppm

5 SS (acylation) = 8 % (determined from ^1H NMR)

Fluorimeter: $\lambda_{\text{em,max}} = 710 \text{ nm}$ (at $\lambda_{\text{exc.}} = 685 \text{ nm}$; H_2O)

Example 17: Esterification of HA-Cypate with oleic acid

300 mg (0.73 mmol, 1 eqv.) of the conjugate of HA-Cypate prepared according to Example 3 was dissolved in 15 ml demi water and 13 ml AIPA, 382 μl (2.20 mmol, 3 eqv.) DIPEA and 4.5 mg (0.04 mmol, 0.05 eqv.) DMAP were added. 139 μl (0.45 mmol, 0.6 eqv.) of cis-octadec-9-enoic acid dissolved in 2 ml AIPA, 229 μl (1.32 mmol, 1.8 eqv.) DIPEA, 51 μl (0.44 mmol, 0.6 eqv.) benzoyl chloride was added and let react under the constant stirring 30 minutes at 0 °C. After the given period was everything quantitatively transferred to the first reaction mixture, and the esterification proceeded for 2 hours at the room temperature.

15 The reaction was quenched by the addition of high excess of AIPA and saturated solution of NaCl, whereas the precipitation of the product occurred. The crude product was washed with 4 x 200 ml AIPA, further dissolved in demineralized water and transferred into the dialysis tube. Dialysis proceeded for 24 hours against 0,5% NaHCO_3 and 0,5% NaCl and further for 48 hrs in demineralized water. The product was obtained in the form of green lyofilizate yielding 204 mg (65 %).

^1H NMR (D_2O , 500 MHz) additional signals to Example 3: δ 5.41 – 5.34 (m, 2H, $\text{CH}=\text{CH}$), 2.48 – 2.40 (m, 2H, $-\text{CH}_2-\text{CO}-$), 1.68 – 1.54 (m, 2H, $-\text{CH}_2-\text{CH}_2-\text{CO}-$), 1.40 – 1.23 (m, 24 H, $(-\text{CH}_2)_{12}$), 0.91 – 0.68 (m, 3H, $-\text{CH}_2-\text{CH}_3$) ppm

SS (acylation) = 12 % (determined from ^1H NMR)

25 Fluorimeter: $\lambda_{\text{em,max}} = 710 \text{ nm}$ (at $\lambda_{\text{exc.}} = 685 \text{ nm}$; H_2O)

Example 18: Esterification of HA-Cypate with oleic acid

300 mg (0.73 mmol, 1 eqv.) of the conjugate HA-Cypate prepared according to Example 3 was dissolved in 15 ml demi water and 11 ml AIPA, then was 306 μl (2.20 mmol, 3 eqv.) TEA and 4.5 mg (0.04 mmol, 0.05 eqv.) DMAP added. 231 μl (0.73 mmol, 1 eqv.) of cis-octadec-9-enoic acid dissolved in 2 ml AIPA, 204 μl (1.46 mmol, 2 eqv.) TEA, 85 μl (0.73 mmol, 1 eqv.) benzoyl chloride were added and let react with the constant stirring for 30 minutes at the room

5 temperature. After the given period of time everything was quantitatively transferred into the first reaction mixture, and the esterification proceeded for 2 hours at the room temperature.

The reaction was quenched by the addition of the high excess of AIPA and saturated solution of NaCl, whereas the precipitation of the product occurred. The crude product was washed with 4 x 200 ml AIPA, further dissolved in the demineralized water and transferred into dialysis tube.

10 Dialysis proceeded for 24 hours against 0.5% NaHCO₃ and 0.5% NaCl and further for 48 hours in demineralized water. The product was obtained in the form of green lyophilizate yielding 226 mg (70 %).

¹H NMR (D₂O, 500 MHz) additional signals to Example 3: δ 5.41 – 5.34 (m, 2H, CH=CH), 2.48 – 2.40 (m, 2H, -CH₂-CO-), 1.68 – 1.54 (m, 2H, -CH₂-CH₂-CO-), 1.40 – 1.23 (m, 24 H, (-CH₂)₁₂), 0.91 – 0.68 (m, 3H, -CH₂-CH₃) ppm

SS (acylation) = 12 % (determined from ¹H NMR)

Fluorimeter: λ_{em,max.} = 710 nm (at λ_{exc.} = 685 nm; H₂O)

Example 19: Esterification of HA-Cypate with oleic acid

300 mg (0.73 mmol, 1 eqv.) of the conjugate HA-Cypate prepared according to Example 3
20 was dissolved in 15 ml demi water and 11 ml AIPA, then 375 μl (1.76 mmol, 2.4 eqv.) N,N,N',N'-tetramethyl-1,6-hexanediamine and 4.5 mg (0.04 mmol, 0.05 eqv.) DMAP was added. 139 μl (0.44 mmol, 0.6 eqv.) of cis-octadec-9-enoic acid dissolved in 2 ml AIPA, 375 μl (1.76 mmol, 2.4 eqv.) N,N,N',N'-tetramethyl-1,6-hexanediamine, 51 μl (0.44 mmol, 0.6 eqv.) benzoyl chloride were added and let react under the constant stirring for 30 minutes at the room
25 temperature. After the given period everything was quantitatively transferred into the first reaction mixture, and the esterification proceeded for 2 hours at the room temperature.

The reaction was quenched by the addition of the high excess of AIPA and saturated solution of NaCl, whereas the precipitation of the product occurred. The crude product was washed with 4 x 200 ml AIPA, further dissolved in demineralized water and transferred into the dialysis tube.
30 Dialysis proceeded for 24 hours against 0.5% NaHCO₃ and 0.5% NaCl and further for 48 hrs in demineralized water. The product was obtained in the form of green lyophilizate yielding 217 mg (70 %).

- 5 ^1H NMR (D_2O , 500 MHz) additional signals to Example 3: δ 5.41 – 5.34 (m, 2H, $\text{CH}=\text{CH}$), 2.48 – 2.40 (m, 2H, $-\text{CH}_2-\text{CO}-$), 1.68 – 1.54 (m, 2H, $-\text{CH}_2-\text{CH}_2,-\text{CO}-$), 1.40 – 1.23 (m, 24 H, $(-\text{CH}_2)_{12}$), 0.91 – 0.68 (m, 3H, $-\text{CH}_2-\text{CH}_3$) ppm

SS (acylation) = 6 % (determined from ^1H NMR)

Fluorimeter: $\lambda_{\text{em,max}} = 710$ nm (at $\lambda_{\text{exc.}} = 685$ nm; H_2O)

10 **Example 20: Esterification of HA-Cypate with oleic acid**

- 300 mg (0.73 mmol, 1 eqv.) of the conjugate HA-Cypate prepared according to Example 3 was dissolved in 15 ml demi water and 11 ml AIPA, and then 241 μl (2.20 mmol, 3 eqv.) N-methyl morpholine and 4.5 mg (0.04 mmol, 0.05 eqv.) DMAP were added. 139 μl (0.44 mmol, 0.6 eqv.) cis-octadec-9-enoic acid dissolved in 2 ml AIPA, 193 μl (1.76 mmol, 2.4 eqv.) N-methyl
 15 morpholine, 51 μl (0.44 mmol, 0.6 eqv.) benzoyl chloride were added and let react under the constant stirring for 30 minutes at the room temperature. After the given period was everything quantitatively transferred into the first reaction mixture, and the esterification proceeded for 2 hours at the room temperature.

- The reaction was quenched by the addition of the high excess of AIPA and saturated solution of
 20 NaCl, whereas the precipitation of the product occurred. The crude product was washed with 4 x 200 ml AIPA, further dissolved in demineralized water and transferred into the dialysis tube. Dialysis proceeded for 24 hours against 0.5% NaHCO_3 and 0.5% NaCl and further for 48 hrs in demineralized water. Product was obtained in the form of green lyophilizate yielding 264 mg (82 %).

- 25 ^1H NMR (D_2O , 500 MHz) additional signals to Example 3: δ 5.41 – 5.34 (m, 2H, $\text{CH}=\text{CH}$), 2.48 – 2.40 (m, 2H, $-\text{CH}_2-\text{CO}-$), 1.68 – 1.54 (m, 2H, $-\text{CH}_2-\text{CH}_2,-\text{CO}-$), 1.40 – 1.23 (m, 24 H, $(-\text{CH}_2)_{12}$), 0.91 – 0.68 (m, 3H, $-\text{CH}_2-\text{CH}_3$) ppm

SS (acylation) = 12 % (determined from ^1H NMR)

Fluorimeter: $\lambda_{\text{em,max}} = 710$ nm (at $\lambda_{\text{exc.}} = 685$ nm; H_2O)

30 **Example 21: Esterification of HA-Cypate with oleic acid**

300 mg (0.73 mmol, 1 eqv.) of the conjugate HA-Cypate prepared according to Example 3 was dissolved in 15 ml demi water and 11 ml AIPA, and then 246 mg (2.20 mmol, 3 eqv.) DABCO and 4.5 mg (0.04 mmol, 0.05 eqv.) DMAP were added. 139 μl (0.44 mmol, 0.6 eqv.)

- 5 cis-octadec-9-enoic acid dissolved in 2 ml AIPA, 148 mg (1.32 mmol, 1.8 eqv.) DABCO, 51 μ l (0.44 mmol, 0.6 eqv.) benzoyl chloride were added and let react with the constant stirring for 30 minutes at the room temperature. After the given period everything was quantitatively transferred into the first reaction mixture, and the esterification proceeded for 2 hours at the room temperature.
- 10 The reaction was quenched by the addition of the high excess of AIPA and saturated solution of NaCl, whereas the precipitation of product occurred. The crude product was washed with 4 x 200 ml AIPA, further dissolved in demineralized water and transferred into the dialysis tube. Dialysis proceeded for 24 hours against 0.5% NaHCO₃ and 0.5% NaCl and further 48 hours in demineralized water. The product was obtained in the form of green lyophilizate yielding 183 mg
- 15 (60 %).

¹H NMR (D₂O, 500 MHz) additional signals to Example 3: δ 5.41 – 5.34 (m, 2H, CH=CH), 2.48 – 2.40 (m, 2H, -CH₂-CO-), 1.68 – 1.54 (m, 2H, -CH₂-CH₂-CO-), 1.40 – 1.23 (m, 24 H, (-CH₂)₁₂), 0.91 – 0.68 (m, 3H, -CH₂-CH₃) ppm

SS (acylation) = 3 % (determined from ¹H NMR)

- 20 Fluorimeter: $\lambda_{em,max}$ = 710 nm (at λ_{exc} = 685 nm; H₂O)

Example 22: Loading of hydrophobized HA-Cypate with nonpolar compound

- 150 mg of the acylated conjugate of HA-cypate prepared according to Example 16 was 2 hours dissolved in 15 ml demi water under the constant stirring on the magnetic stirrer. Then was gradually added 10 mg paclitaxel in 2 ml chloroform and the resulting mixture was evaporated
- 25 (RE) to dry and then hydrated with demi water (15 ml). Unbound paclitaxel was removed with the filtration through 1.0 μ m glass filter and the resulting product was lyophilized.

The amount of unbound paclitaxel (HPLC determination): 4.2% (wt.)

Example 23: Loading of hydrophobized HA-Cypate with nonpolar compound

- 150 mg of the acylated conjugate HA-cypate prepared according to Example 18 was 2 hours
- 30 dissolved in 15 ml demi water with the constant stirring on the magnetic stirrer. Then 15 mg doxorubicin was gradually added in 2 ml chloroform and the resulting mixture was first sonicated (pulse sonication cca 15 min, 200 W, amplitude 65%, cycle 0.5 s) until reaching of the homogenous mixture and then evaporated (RE) to dry and then hydrated with demi water (15

- 5 ml). Unbound doxorubicin was removed with filtration through 1.0 μm glass filter and the resulting product was lyophilized.

The amount of unbound doxorubicin (HPLC determination): 7.5% (wt.)

10 ***Example 24: Loading of hydrophobized HA-Cypate with nonpolar compound***

- 150 mg of the acylated conjugate HA-cypate prepared according to Example 18 was 2 hours dissolved in 15 ml demineralized water with the constant stirring on the magnetic stirrer. Then 2 mg of spions on the basis of iron (Fe_2O_3 , Fe_3O_4) (5 mm) and 20 mg doxorubicin was gradually added in 5 ml chloroform and the resulting mixture was first sonicated (pulse sonication, cca 15 min, 200 W, amplitude 65%, cycle 0.5s) until reaching homogenous mixture and then evaporated (RE) to dry and then hydrated with demi water (15 ml). Unbound doxorubicin was removed with filtration through 1.0 μm glass filter and the resulting product was lyophilized.

The amount of unbound doxorubicin (HPLC determination): 6.5% (wt.), unbound spions: 2% (wt.)

20 ***Example 25: In vivo experiment – fluorescence of conjugate HA-Cypate***

- For *in vivo* experiments was HA-Cypate, prepared according to Example 3, dissolved in the saline solution ($c = 3.8$ mg derivative in 100 μl solution) and then sterilized by filtration (0.22 μm). 50 μl (Fig. 9) and/or intraperitoneally 150 μl (Fig. 10) sterile solution of the given derivative was subcutaneously applied to the laboratory mouse of the type Balb/c in narcosis.
- 25 Fluorescence was detected using the combination of different excitation wavelengths and emission filters.

- Fig. 9 a 10 show the sufficient intensity of fluorescence of the derivative after the subcutaneous and intraperitoneal application for *in vivo* imaging. The imaging can be performed using the various excitation wavelengths (530-745 nm), and filters for the emission (ICG, Cy5.5). The filter DsRed cannot be used. From Fig. 10 the stability of the derivative as
- 30 fluorophore after intraperitoneal administration is further apparent.

Example 26: In vivo experiment – fluorescence of conjugate HA-Cypate-C18:1

5 HA-Cypate-C18:1 (SS =10 %) prepared according to Example 15 was dissolved in phosphate buffer (c = 3.6 mg of the conjugate in 100 µl solution and sterilized by filtration (0.22 µm). 100 µl of such prepared solution was applied intravenously to two model mice Balb/c (in narcosis) and observed its fluorescence *in vivo* for the period of 7 days (Fig. 11).

10 Fig. 11 show that HA-Cypate-C18:1 is distributed after i.v. administration in the healthy mouse especially into liver. Fluorescence of the conjugate is sufficient for *in vivo* imaging, fluorescence of the conjugate is further very stable, after one administration can be imaging performed for 2 weeks.

Example 27: In vivo experiment – fluorescence of conjugate HA-Cypate-C18:1

15 HA-Cypate-C18:1 (SS =10 %) prepared according to Example 15 was dissolved in the saline solution (c = 1.8 mg of the conjugate in 100 µl solution) and sterilized by the filtration (0.22 µm). 100 µl of such prepared solution was applied intravenously to 3 model mice Balb/c (in narcosis) with non-palpable breast tumor (orthotopic administration 4T1 luc cells, displayable through chemiluminescence –detection of luciferase activity after i.p. injection of luciferin) and
20 observable its fluorescence *in vivo* for period 72 hours (Fig. 12).

Fig. 12 shows that HA-Cypate-C18:1 is distributed after i.v. administration into liver and further after 24 hours into very small (non-palpable) tumor. In tumor, there is the accumulation of HA-Cypate-C18:1 growing with time and the presence of the conjugate in tumor is very significant even after 15 days after the administration of the conjugate. Fluorescence of the
25 conjugate *in vivo* is thus very stable and the conjugate can be used to imaging of very small tumors and further to tumor observation in time. In left panel of the figure is the control image of tumor with the luminescent imaging (the detection of luciferase activity).

Example 28: In vivo experiment – fluorescence of conjugate HA-Cypate-C18:1

To 9 model mice Balb/c (in narcosis) were orthotopically administered 4T1 luc cells, (cells
30 displayable with chemiluminescence –detection of luciferase activity after i.p. application of luciferin) and breast tumor was let grow for 14 days. 14th day were mice divided into 3 groups (1 group = 3 animals). To every group was 14th, 21th and 28th day intravenously applied 1 mg of selected conjugate in 100 µl phosphate buffer and then: to first group was applied HA-Cypate-C18:1 prepared in Example15, to second group HA-Cypate-C18:1 loaded with doxorubicin,
35 prepared in Example 23 and to third group HA-Cypate-C18:1 loaded with doxorubicin and

5 spions, prepared in Example 24. The radiation was evaluated for 35 days *in vivo* (Fig. 13), then mice were sacrificed and the tumor and spleen weight in individual groups was compared (Fig. 14).

Fig. 13 shows that the tumor grew most in the group, to which only HA-Cypate was administered. Slower grow was detected in contrast in groups, to which HA-Cypate-
10 C18:1+doxorubicin, and/or HA-Cypate-C18:1+doxorubicin+spion was applied.

Fig. 14 shows, that the first (not treated) group had the highest spleen and tumor weight. In given model, increased spleen indicates progression of disease (duPre et al., *Experimental and Molecular Pathology* 2007, 82, 12–24). The second (treated) and the third (treated) group had spleen significantly smaller and tumor smaller compared to the first group. The smallest tumor
15 size was found in the third group. The carrier system can thus not only image but also cure the tumor and fills the function of theranostatic.

Example 29: *In vivo* experiment – fluorescence of conjugate HA-Cypate-C18:1

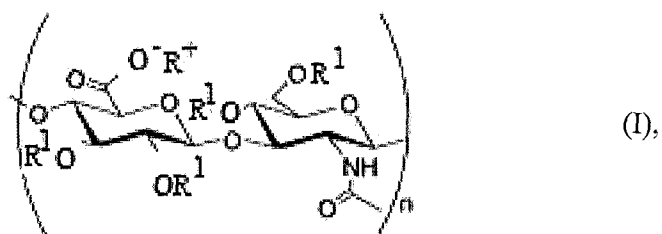
HA-Cypate-C18:1 (SS =10 %) prepared according to Example 15 was dissolved in the saline solution ($c = 0; 0.625; 1.25; 2.5$ mg of the conjugate in 100 μ l solution) and sterilized by
20 the filtration (0.22 μ m). 100 μ l of such prepared solutions was applied intraperitoneally to 4 model mice Balb/C (in narcosis) with the breast tumor (35th day after orthotopic administration of 4T1 luc cells, displayable with chemiluminescence detection of luciferase activity after i.p. injection of luciferin) and observed its fluorescence *in vivo* for 7 days (Fig. 15).

Fig. 15 shows, that HA-Cypate-C18:1 is distributed after the i.p. administration into tumor
25 and liver. The tumor is without any problem displayable *in vivo* at minimum for 7 days after the administration of the conjugate in all concentrations used. In left panel of the figure is the control image of the tumor with the luminescent imaging (detection of luciferase). The right panel of the figure shows 4 mice, to which the solution of the derivative was administered – from left to right grows the concentration: (0; 0.625; 1.25; 2.5 mg of the conjugate administered in 100
30 μ l solution).

5

CLAIMS

1. A fluorescent conjugate of hyaluronic acid or a salt thereof of the general formula I,

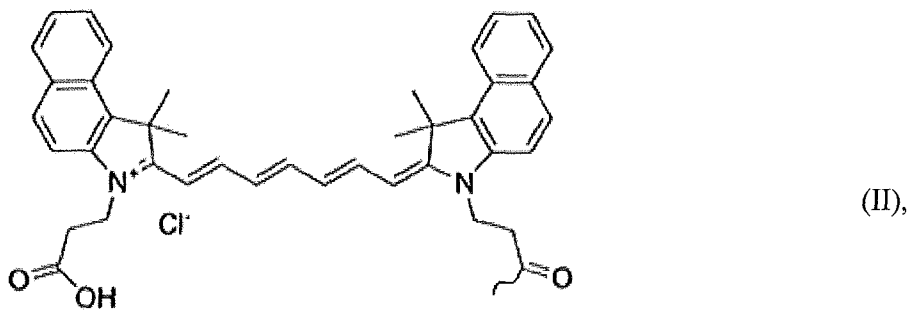


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wherein R^+ is H^+ or physiologically acceptable salts selected from the group containing Na^+ , K^+ , Mg^{2+} or Ca^{2+} ,

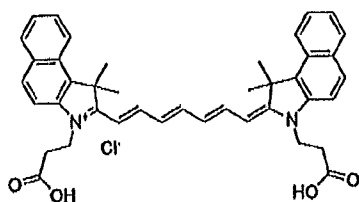
R^1 is $-H$ or a cypate residue of the formula II, where $\sim$ is in the place of covalent bond of cypate residue of the formula II

15



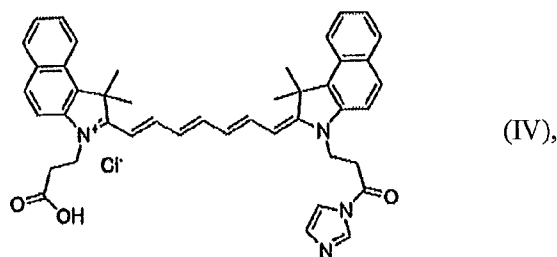
20

- one R¹ being cypate residue of formula II in at least one repeated unit providing that if there is R¹ cypate residue of formula II in the unit, then the other R¹ in the unit are H,
and wherein n is an integer in the range 2 to 625.
2. The fluorescent conjugate of Claim 1, where the residue of cypate of the formula II is in the position 6 of the glucosamine part of the fluorescent conjugate of the hyaluronic acid or the salt thereof of the general formula I.
 3. The fluorescent conjugate of Claim 1 or Claim 2, where the degree of substitution of the residue of cypate of the formula II in the conjugate of hyaluronic acid or the salt thereof of the general formula I is from 0.1 to 2%, preferably 1.0%.
 4. The fluorescent conjugate of any one of Claims 1 to 3 that it absorbs the light in the area of 570 nm to 790 nm and it emits the light in the area of 680 nm to 850 nm, preferably at 850 nm.
 5. The fluorescent conjugate of hyaluronic acid or the salt thereof of any one of Claims 1 to 3, wherein R⁺, R¹ and n are, as defined in Claim 1, applying at the same time that in at least one repeated unit at least one R¹ is -C(=O)R², where R² is C_xH_y substituent, where x is an integer in the range of 5 to 17 and y is an integer 11 to 35, whereas it is linear or branched, saturated or unsaturated C₆-C₁₈ aliphatic chain.
 6. The fluorescent conjugate of Claim 5, wherein the degree of substitution of -C(=O)R² in the conjugate of hyaluronic acid or the salt thereof of the general formula I is from 3 to 70 %, preferably 5 to 12%.
 7. The fluorescent conjugate of Claim 5 or Claim 6, that it absorbs the light in the range of wavelengths 570 nm to 790 nm and it emits the light at 680 to 850 nm, preferably 850 nm.
 8. A method of preparation of the conjugate of any one of Claims 1 to 4, **characterized in, that** cypate I of the formula III

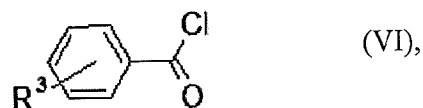


(III),

- 5 is activated with N,N'-carbonyl diimidazole (CDI) in the aprotic polar solvent selected from the group containing dimethyl sulfoxide, dimethyl formamide, formamide or acetonitrile, preferably dimethyl sulfoxide; resulting in a reactive intermediate mono-imidazolide of the formula IV



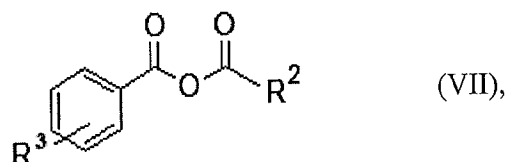
- 10 that reacts with hyaluronic acid or the salt thereof in the presence of the organic base, that is either generated *in situ* in the form of imidazole, or it is added to the reaction mixture, the added organic base being selected from the group containing 1,4-diazabicyclo[2.2.2]octan, N,N,N',N'-tetramethyl-1,6-hexandiamine, N-methyl morfolin, imidazole, triethylamine, or N,N'-diisopropyl ethylamine, preferably imidazole generated *in situ*; and polar aprotic solvent, as is defined above.
- 15
9. The method of Claim 8, **characterized in, that** the activation of cypate is performed at the temperature in the range 20 °C to 60 °C, preferably at 22 °C to 25 °C; for 10 minutes to 20 hours, preferably 0.5 to 2 hours
10. The method of Claim 8, **characterized in, that** the formation of the conjugate of hyaluronic acid or the salt thereof is performed at temperature 40 °C to 80 °C, preferably 40 °C to 20 60 °C, more preferably 60 °C; for 12 to 48 hours, preferably 24 hours.
11. The method of any one of Claims 8 to 10, **characterized in, that** the molar ratio of cypate I : hyaluronic acid or the salt thereof: N,N'-carbonyl diimidazole : the organic base is 0.5 : 1 : 0.5 : 0.5 to 3.5 in the reaction mixture, preferably the weight ratio is 0.5 : 1 : 0.5 : 1.
- 25 12. A method of a preparation of the fluorescent conjugate of any one of Claims 5 to 7, **characterized in, that** the activation of the fat acid of the general formula V is performed
- R²COOH (V),
- wherein R² is C_xH_y, whereas x is an integer in the range 5 to 17 and y is an integer 11 to 35 and C_xH_y is linear or the branched, saturated or unsaturated chain; using the substituted or 30 unsubstituted benzoyl chloride of the general formula VI



wherein R^3 is one or more substituents selected from the group containing H, $-\text{NO}_2$, $-\text{COOH}$, halogenides, $\text{C}_1\text{-C}_6$ alkyl alkoxy, preferably H;

in the presence of the organic base selected from the group containing 1,4-diazabicyclo[2.2.2]octan, N,N,N',N'-tetramethyl-1,6-hexandiamine, N-methyl morfolin, triethylamin or N,N'-diisopropyl ethylamine, preferably triethylamine; and the polar solvent selected from the group containing isopropyl alcohol, tetrahydrofuran, preferably isopropyl alcohol

to form the reactive anhydride of the general formula VII



wherein

R^2 and R^3 are, as is defined above,

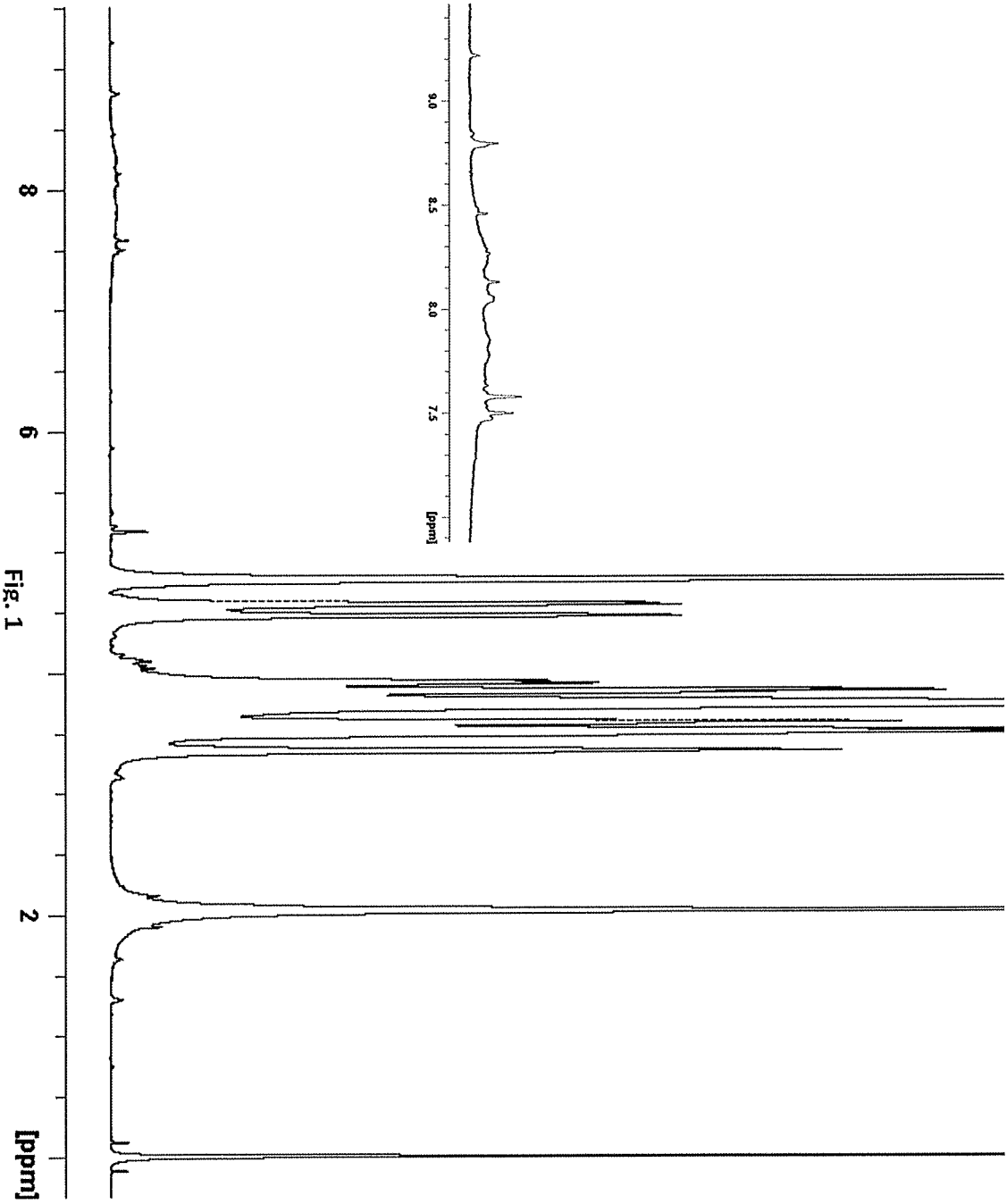
that it esterifies the fluorescent conjugate of hyaluronic acid or the salt thereof of the general formula (I), as defined in any one of Claims 1 to 3, in the presence of the organic base, preferably amine selected from the group containing (1,4-diazabi-cyclo[2.2.2]octan), N,N,N',N'-tetramethyl-1,6-hexandiamine, N-methyl morfolin, imidazole, triethylamin or N,N'-diisopropyl ethylamine, more preferably triethylamine; the mixture of water and the polar solvent miscible with water selected from the group containing isopropyl alcohol, dimethyl sulfoxide or tetrahydrofuran, preferably isopropyl alcohol.

13. The method of Claim 12, **characterized in, that** the activation of the fat acid of the general formula V is performed for 0.5 to 24 hours, at the temperature in the range $0\text{ }^{\circ}\text{C}$ to $60\text{ }^{\circ}\text{C}$, preferably 0.5 hours at the temperature from $0\text{ }^{\circ}\text{C}$ to $25\text{ }^{\circ}\text{C}$, and the esterification of the fluorescent conjugate of hyaluronic acid or the salt thereof is performed for 0.5 to 2 hours, preferably 2 hours, at the temperature in the range of $22\text{ }^{\circ}\text{C}$ to $25\text{ }^{\circ}\text{C}$.

14. The method of Claim 12 or Claim 13 **characterized in, that**

- 5 the amount of the organic base corresponds to 2 to 6 molar equivalents, preferably 4 molar equivalents per the dimer of hyaluronic acid or the salt thereof ;
- the amount of the substituted or unsubstituted benzoyl chloride corresponds to 0.2 to 2.0 molar equivalents, preferably 0.6 molar equivalents per the dimer of hyaluronic acid or the salt thereof;
- 10 the amount of the fat acid corresponds to 0.2 to 2.0 molar equivalents, preferably 0.6 molar equivalents of hyaluronic acid or the salt thereof.
15. The method of any one of claims 12 to 14 **characterized in, that** the amount of water in the mixture water and the polar solvent miscible with water is 50 to 80 % v/v, preferably 50% v/v.
- 15 16. The fluorescent conjugate of any one of Claims 1 to 7 for use in medicinal applications for *in vivo* imaging of the conjugate distribution, preferably for *in vivo* imaging of organs or neoplasms.
17. The fluorescent conjugate of claim 16 for use in the intravenous, intraperitoneal or subcutaneous application.
- 20 18. The fluorescent conjugate of claim 16 for use in administration for *in vivo* imaging of non-palpable and/or palpable tumors.
19. The fluorescent conjugate of claim 18 for use in the intravenous, intraperitoneal administration.
- 25 20. A composition on the basis of aggregated fluorescent conjugate of any one of Claims 5 to 7 **characterized in that** it contains an aggregate of fluorescent conjugates and at least one or more nonpolar agents, preferably drugs and/or nanoparticles.
21. The composition of Claim 20 **characterized in that** the drug is a cytostatic, preferably doxorubicin or paclitaxel.
- 30 22. The composition of Claim 20 **characterized in that** the fluorescent conjugate is the conjugate of any one of claims 5 to 7, where $R^1 -C(=O)C_{17}H_{33}$ and nanoparticles are superparamagnetic nanoparticles.

- 5 23. The composition of any one of Claims 20 to 22 **characterized in that** it contains 2 to 15 wt.%, preferably contains 2 to 6 wt.% of nonpolar compounds in respect to weight content of the fluorescent conjugate of hyaluronic acid or the salt thereof.
24. The composition of any one of Claims 20 to 23 for use in medicinal applications for *in vivo* imaging of neoplasms.
- 10 25. The composition of any one of Claims 20 to 23 for use in the treatment of neoplasms.



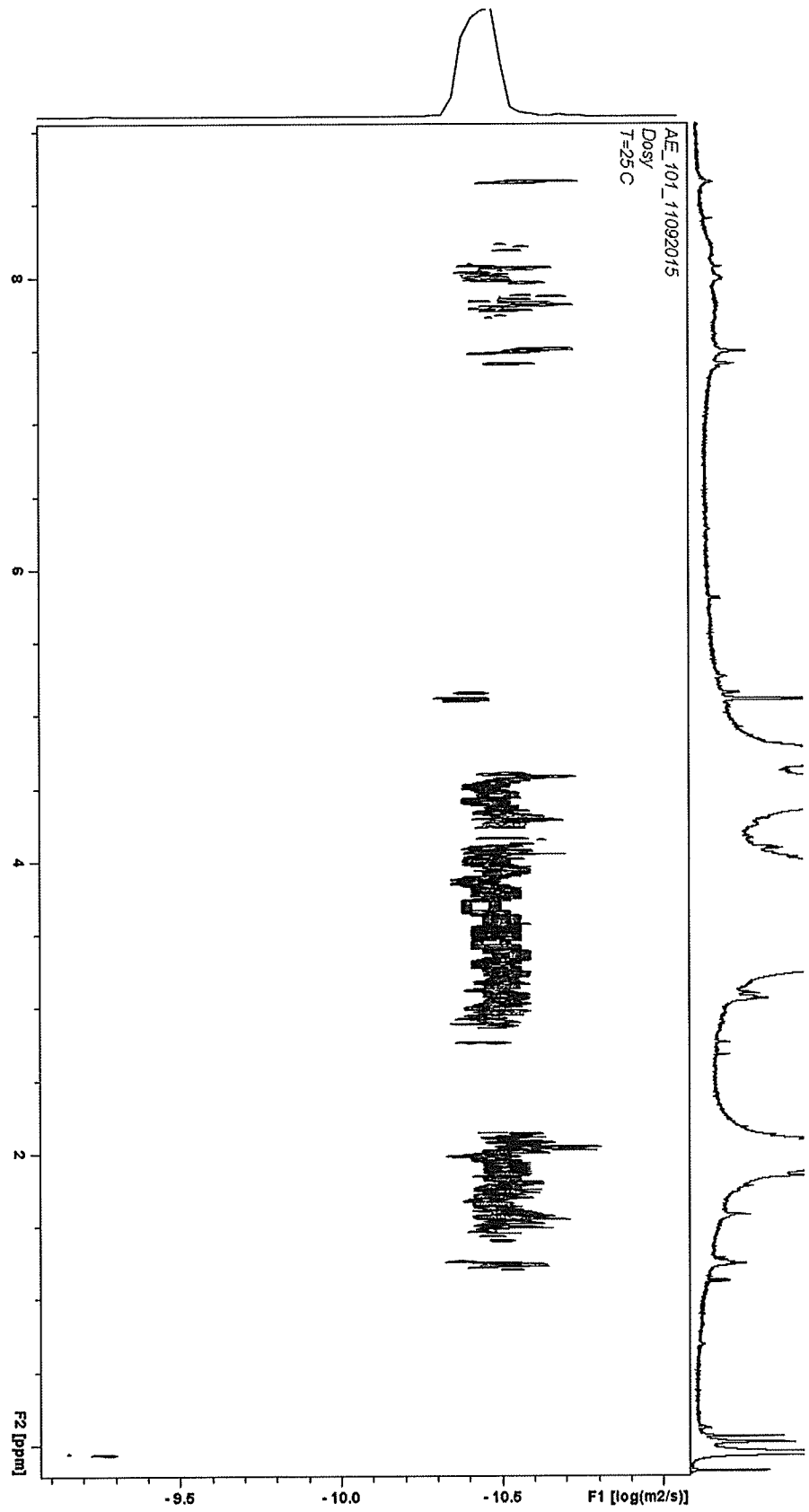


Fig. 2

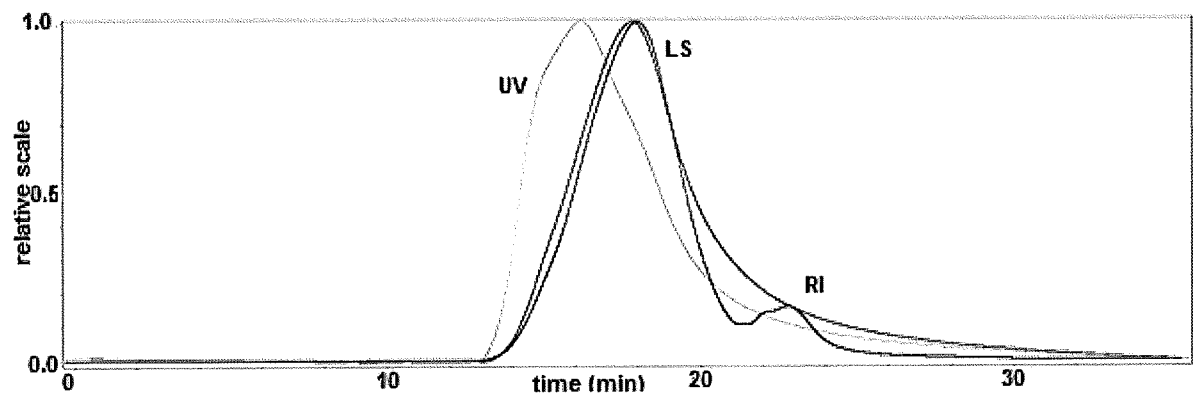


Fig. 3

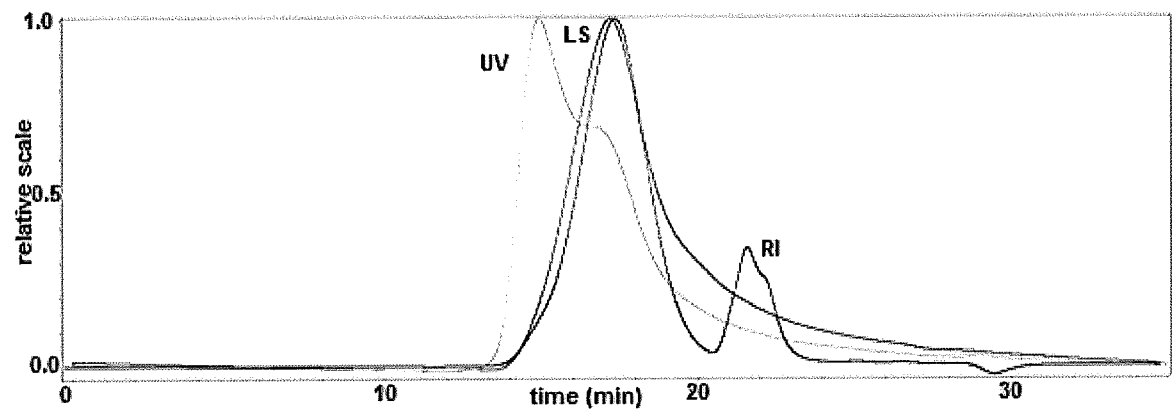


Fig. 4

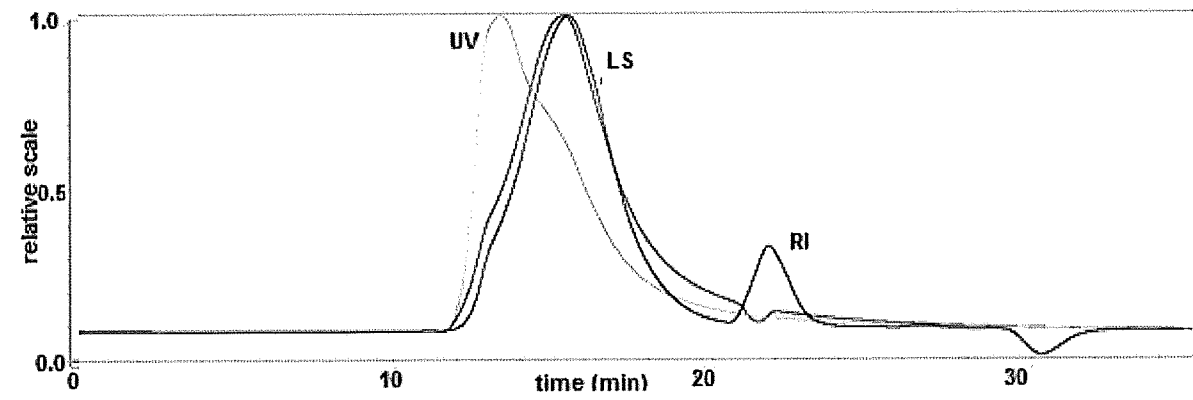


Fig. 5

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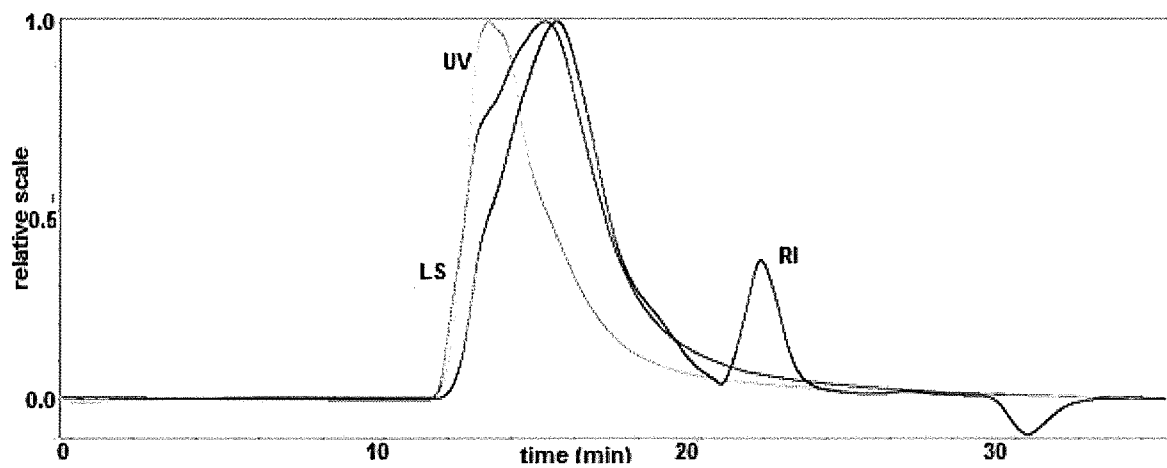


Fig. 6

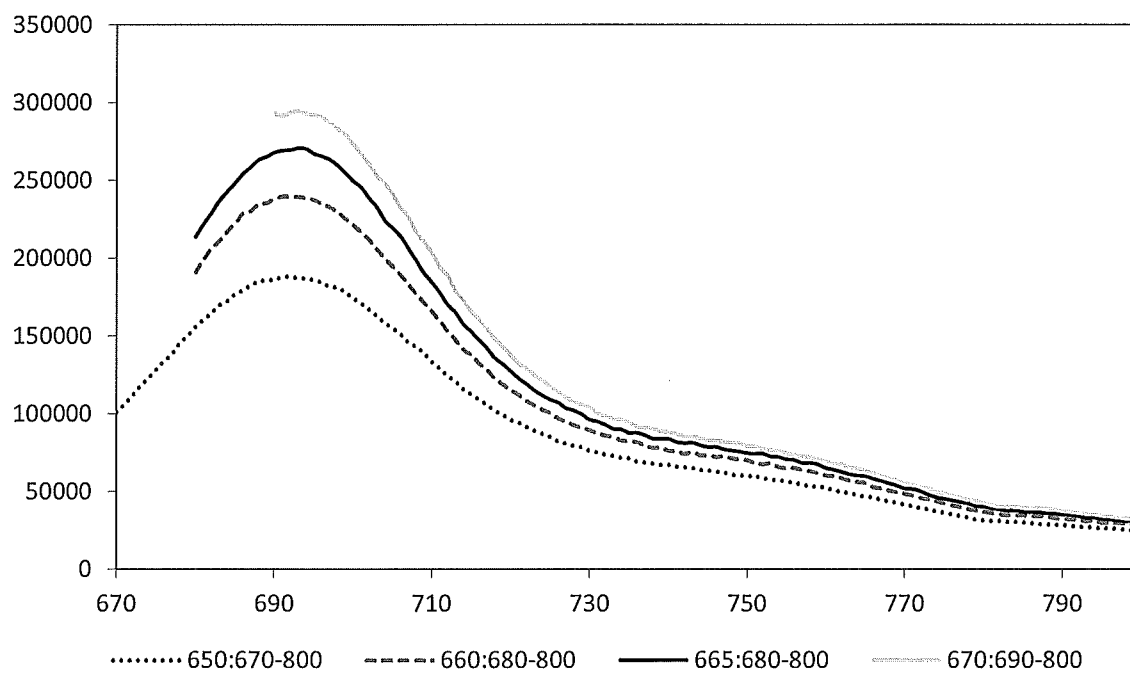


Fig. 7

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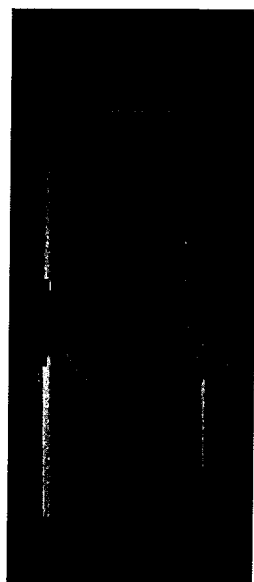


Fig. 8

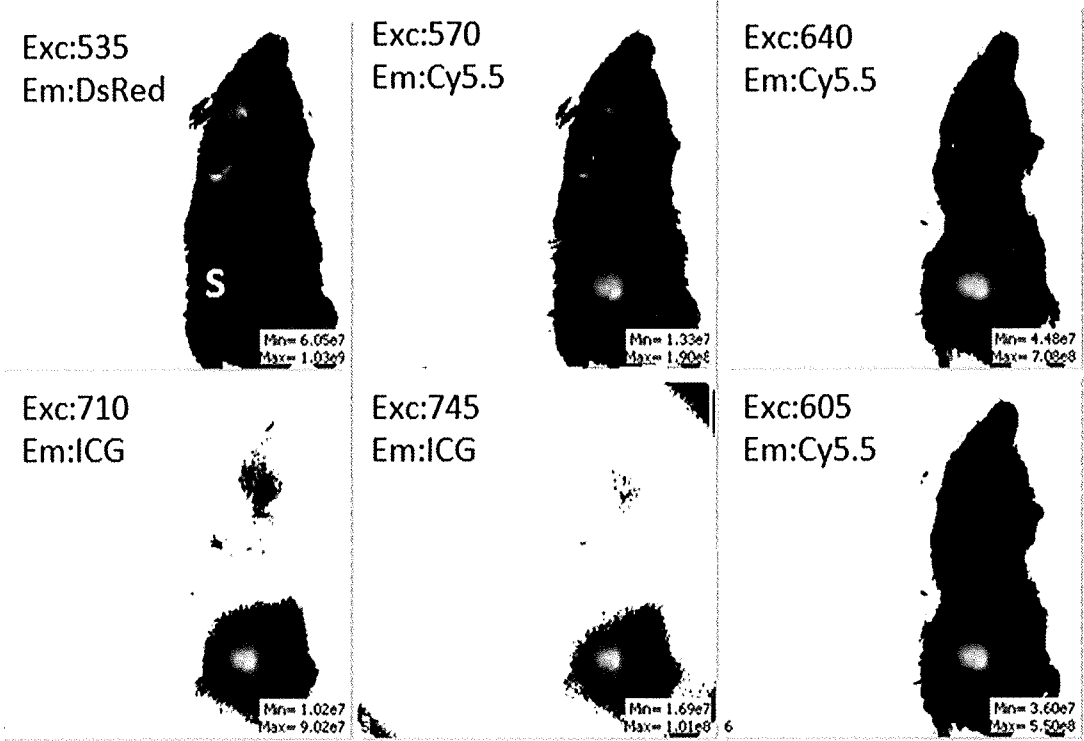


Fig. 9

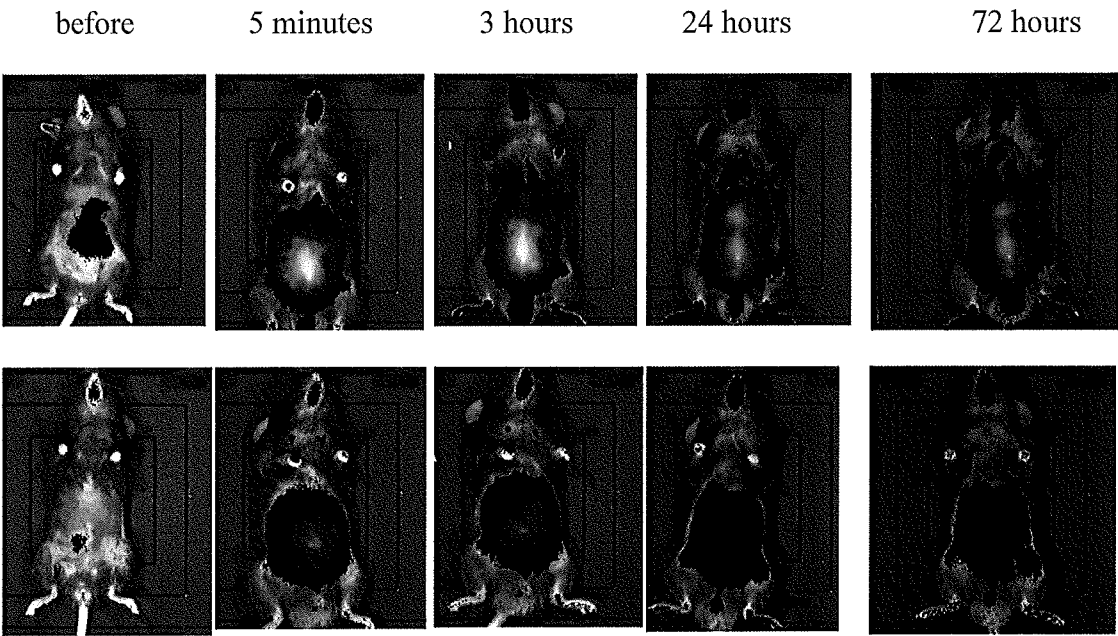


Fig. 10

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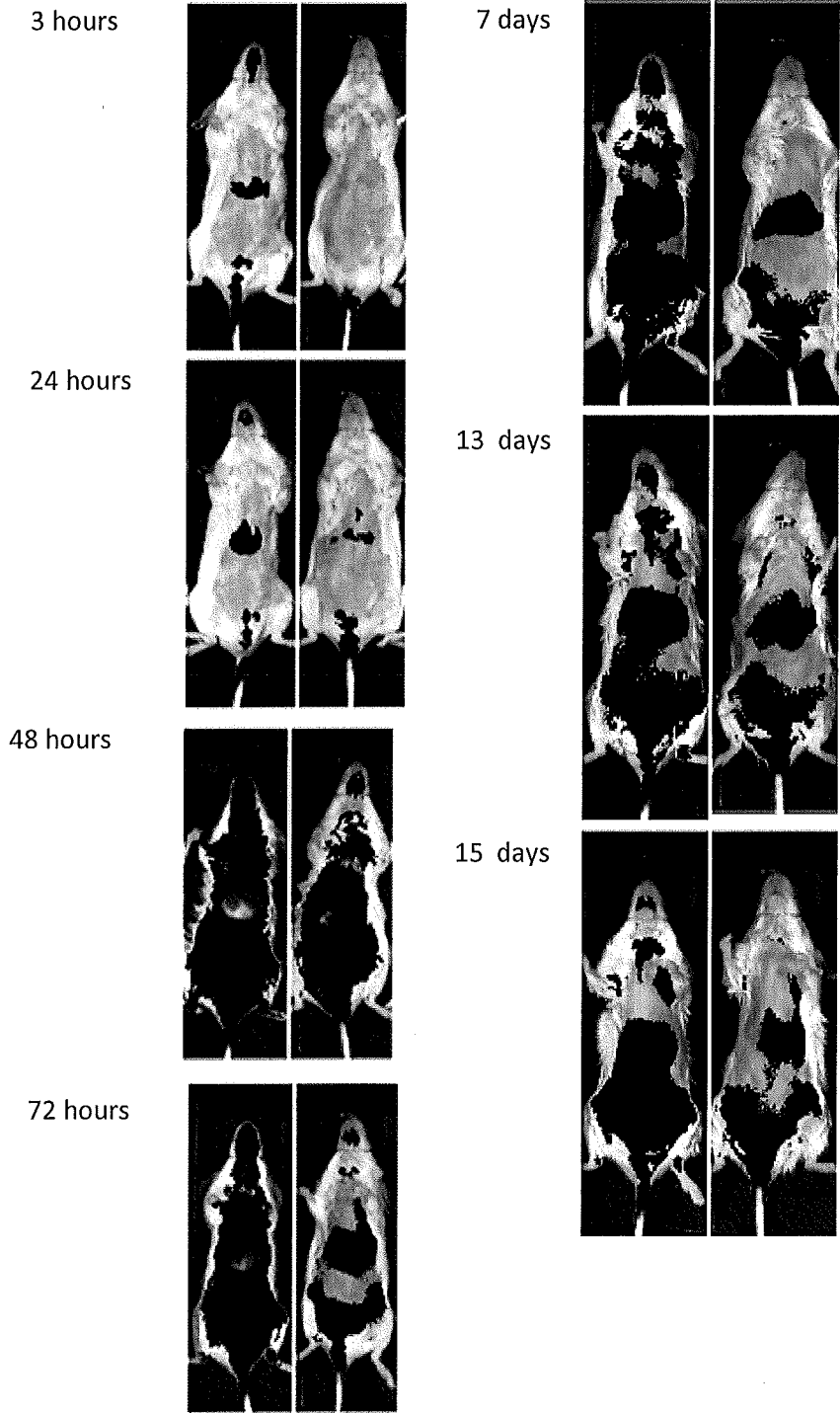


Fig. 11

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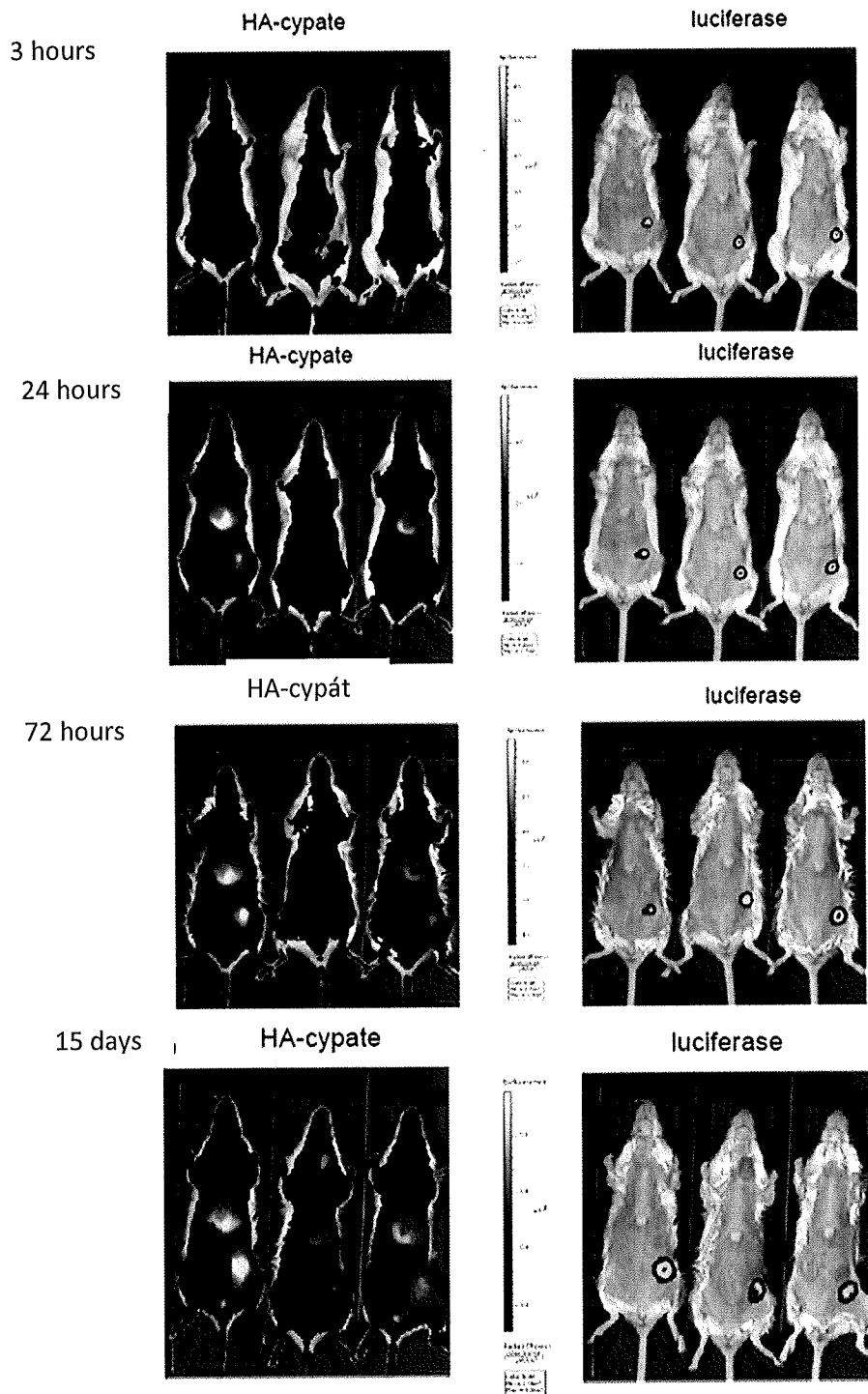


Fig. 12

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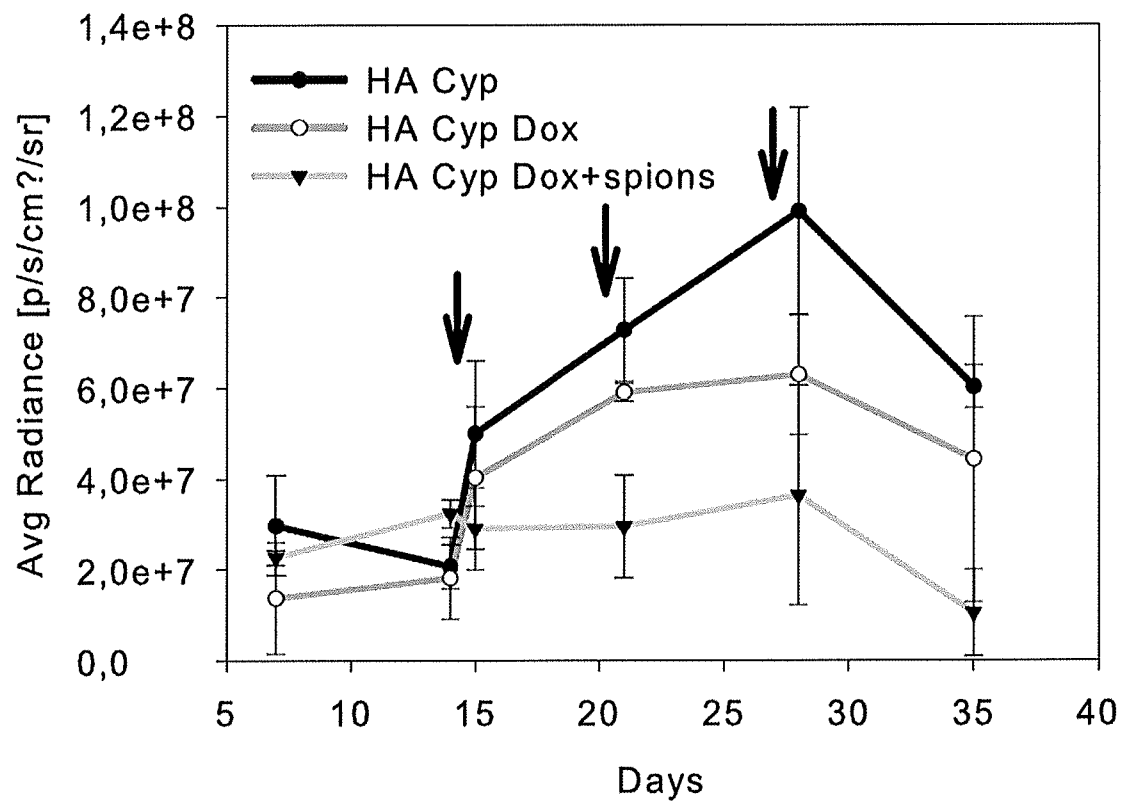


Fig.13

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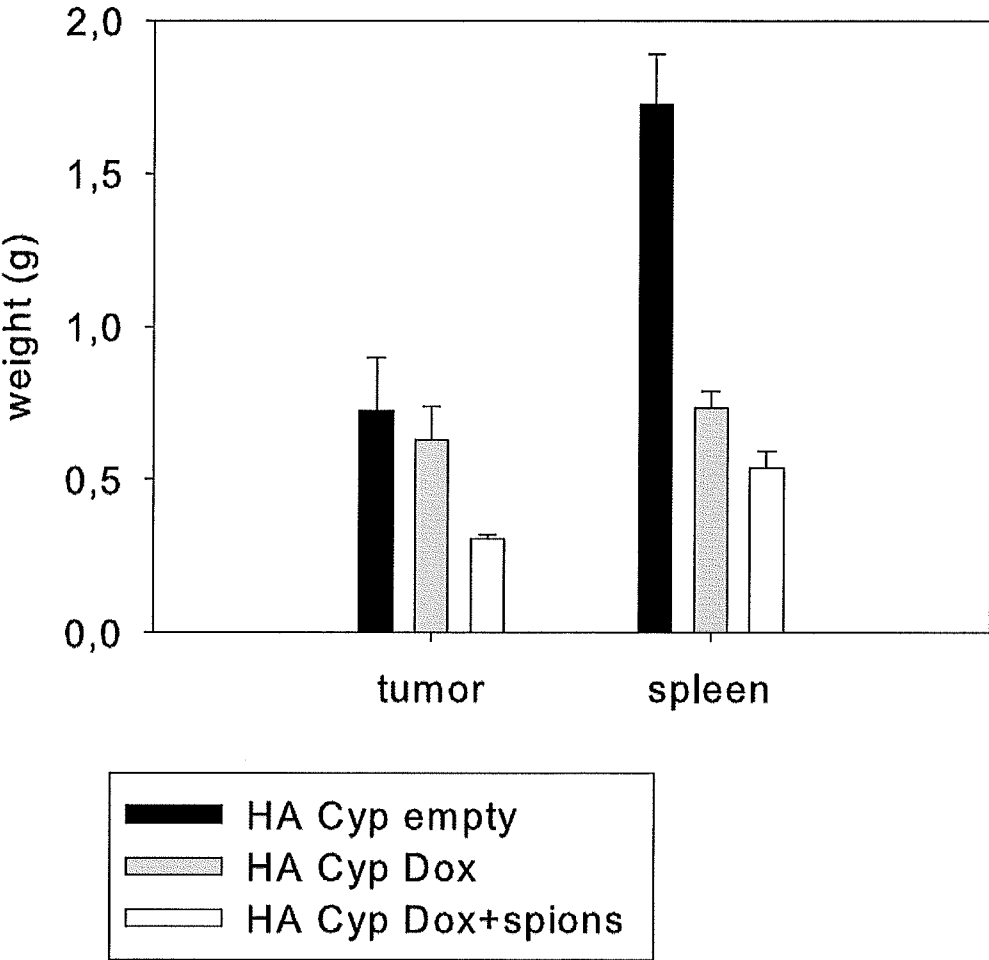


Fig.14

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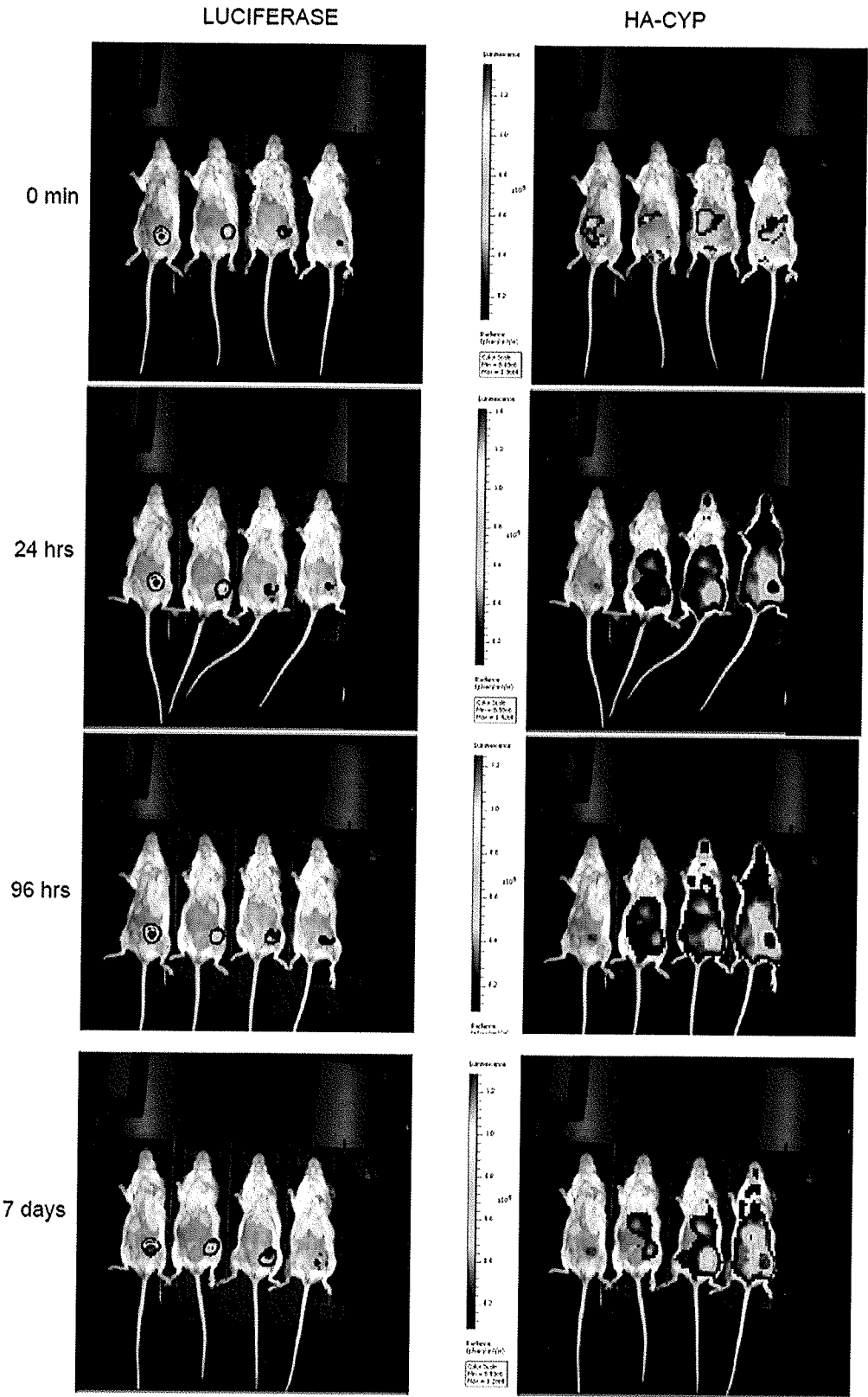


Fig.15

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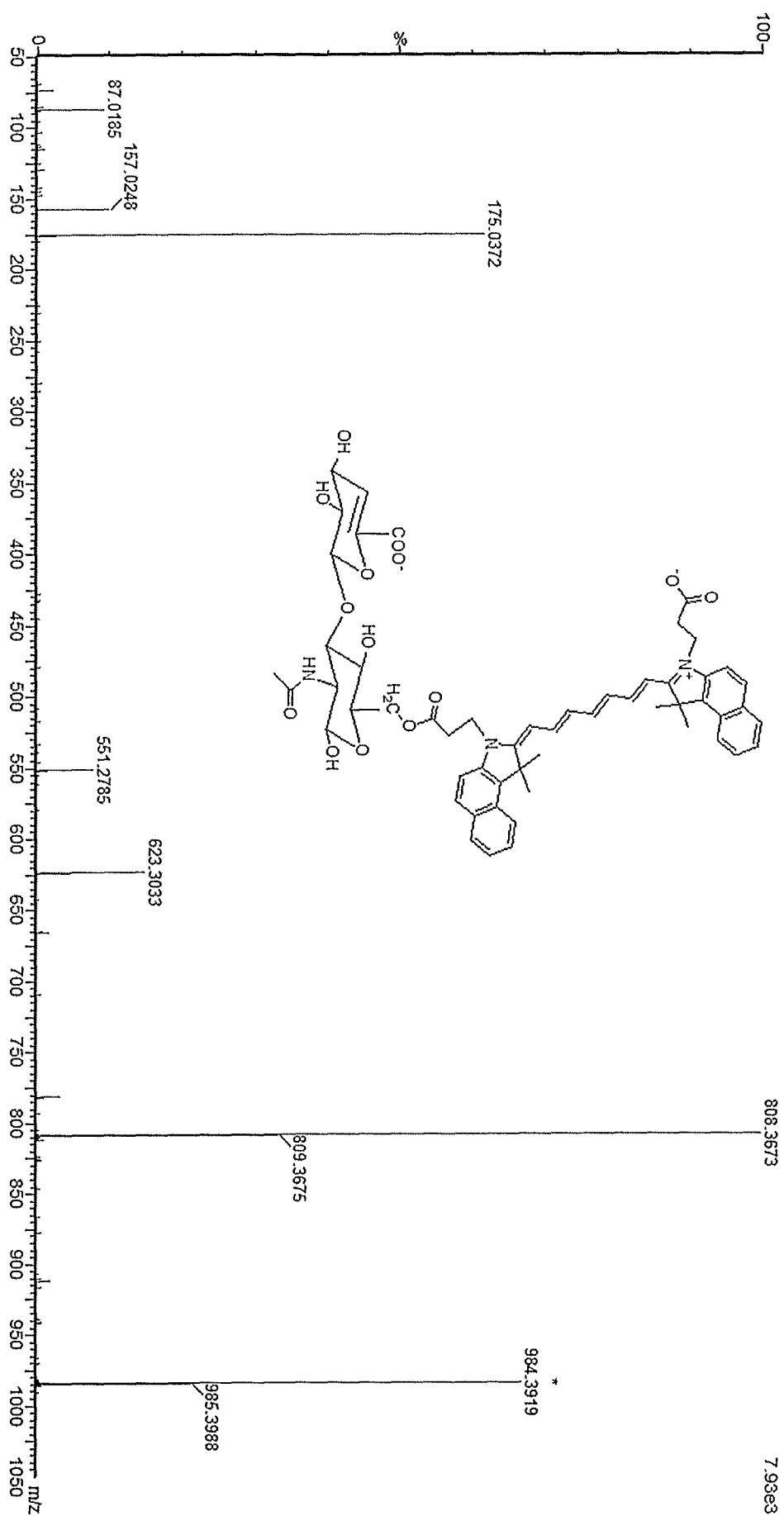


FIG. 16

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2016/050048

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K49/00 A61K47/54 A61K9/107 A61K49/18 A61P35/00 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		
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, BIOSIS, CHEM ABS Data, COMPENDEX, EMBASE, INSPEC, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KOJI MIKI ET AL: "Near-Infrared Dye-Conjugated Amphiphilic Hyaluronic Acid Derivatives as a Dual Contrast Agent for In Vivo Optical and Photoacoustic Tumor Imaging", BIOMACROMOLECULES, vol. 16, no. 1, 12 January 2015 (2015-01-12), pages 219-227, XP055366080, ISSN: 1525-7797, DOI: 10.1021/bm501438e cited in the application abstract figure 1 scheme 2 ----- -/--	1-25
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 21 April 2017		Date of mailing of the international search report 03/05/2017
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Villard, Anne-Laure

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2016/050048

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Week 201381 Thomson Scientific, London, GB; AN 2013-M47637 XP002769418, & KR 2013 0085294 A (UNIV IND & ACADEMIC COOP IN CHUNGNAM NAT) 29 July 2013 (2013-07-29) abstract -----	1-25
A	US 2009/028788 A1 (ACHILEFU SAMUEL [US]) 29 January 2009 (2009-01-29) figures 11C,11D -----	1-25
A	WO 2014/082609 A1 (CONTIPRO BIOTECH SRO [CZ]) 5 June 2014 (2014-06-05) cited in the application examples 18,27 -----	1-25
A	EENSCHOOTEN C ET AL: "Preparation and structural characterisation of novel and versatile amphiphilic octenyl succinic anhydride-modified hyaluronic acid derivatives", CARBOHYDRATE POLYMERS, APPLIED SCIENCE PUBLISHERS, LTD. BARKING, GB, vol. 79, no. 3, 11 February 2010 (2010-02-11), pages 597-605, XP026806649, ISSN: 0144-8617 [retrieved on 2009-09-19] cited in the application page 598, left-hand column, last paragraph - right-hand column, paragraph 1 -----	1-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/CZ2016/050048

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		KR 20150088848 A	03-08-2015
		RU 2015125076 A	13-01-2017
		US 2015320873 A1	12-11-2015
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