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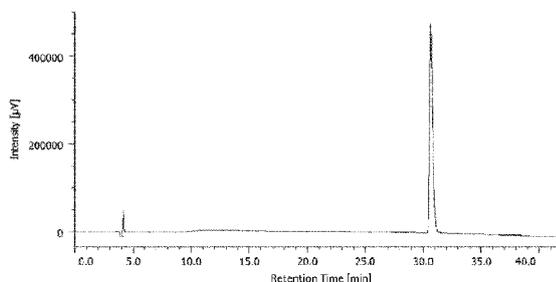
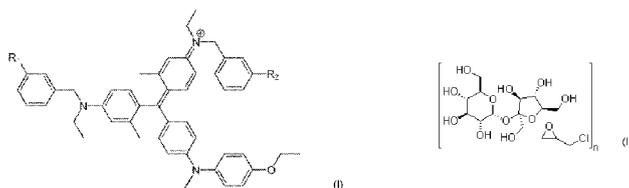


FIGURE 4

(57) Abstract: A dye molecule having the structure (I): where R₁ is an SO₃⁻ group bound to a hydrogen H atom or to another atom or to an ammonium NH₄ group or to a lysine salt or to an arginine salt or to a different monovalent cation, and R₂ is an SO₃⁻ group, in particular for making dye preparations for use in methods for the treatment of the bodies of humans or animals, for example for dyeing the internal limiting membrane ILM and/or epiretinal membranes EPRM in a surgical method which involves subsequent removal respectively of the ILM and/or of the EPRM; as well as a dye preparation having a density higher than 1.01 g/cm³ and comprising at least one dye and at least one agent for increasing its density which is a polymer with the empirical formula (C₁₂H₂₂O₁₁·C₃H₅ClO)_n, and structure (II).

WO 2016/189475 A1

DESCRIPTION

DYE MOLECULE AND DYE PREPARATIONS, IN PARTICULAR FOR USE IN SURGICAL METHODS OF OPHTHALMIC SURGERY AND FOR DYEING PROTEINS

Technical field

This invention relates to a new dye molecule, in particular a molecule usable for making preparations for dyeing the internal limiting membrane (hereinafter referred to as ILM) of an eye, as well as at least several types of epiretinal membranes (hereinafter referred to as ERPM), with the aim of creating a colour difference between the membranes dyed in that way (ILM and ERPM) and the underlying tissue during vitreoretinal surgeries involving the removal of such membranes. Furthermore, the molecule of this invention is intended to be used for dyeing protein chains. This invention also relates to a dye preparation that comprises said new molecule, in particular for medical use, and even more particularly for the medical uses indicated above. The invention also relates to the use of a preparation comprising said dye molecule, both in the medical sector, in particular for the specific medical uses indicated above, and in the non-medical sector for dyeing proteins. Not least, this invention also relates to dye preparations comprising a new thickening agent, both as such and for use in methods for the treatment of the bodies of humans or animals, in particular in the methods indicated above. Finally, it relates to the use of dye preparations comprising a new thickening agent both of the medical and non-medical type.

Background art

As is fully described in the prior documents EP 1819366 A1 or US 2011/190728 A1, in many pathologies of the eye, vitrectomy surgery is often the best solution. However, during the surgery, it is important to minimise the risk of causing damage to the retina. One of the generally

recognised precautionary measures consists of removing, during the surgery, the ILM, and if necessary any epiretinal membrane that has formed on it, in order to prevent intravitreal tensions from affecting the macula. According to the surgical technique generally used now, removal
5 of the ILM and any epiretinal membranes is performed by mechanically detaching them using a suitable gripper, with a mechanical action called peeling. However, consequently, it has been realised how, for the surgeon, it is essential to be able to distinguish as much as possible between the membrane to be removed and the underlying retina.

10 For this reason, over the years, similarly to what occurs for other ophthalmic surgeries, it was suggested that the membranes to be removed should be selectively dyed, so that they can be visually distinguished from undyed underlying structures. Obviously, in order to be able to be used for that purpose, a dye must meet many requirements. In
15 particular, on one hand it must be biocompatible and not cytotoxic or harmful to cells. On the other hand, it must preferably be soluble in water, it must be able to dye the membranes as selectively as possible and it must be easily flushed out of the eye at the end of the surgery, but not too easily during the surgery.

20 Many dyes have been proposed for this purpose, but until now none of them has proved completely satisfactory. The initial dyes used included Indocyanine Green (hereinafter referred to as ICG – see for example “Indocyanine green-assisted peeling of the retinal internal limiting
25 membrane”. Burk SE et al. Ophthalmology. 2000;107:2010-2014) and Trypan Blue (hereinafter referred to as TB). However, the various studies carried out have highlighted several problems with such dyes. In particular, whilst for ICG many doubts were raised about its safety for use in human eyes, TB proved able to satisfactorily dye epiretinal membranes but not the ILM.

30 Other dyes were subsequently put forward, such as Brilliant Blue G (hereinafter referred to as BBG), Brilliant Blue R (hereinafter referred to as

BBR), Patent Blue V or Methylene Blue.

In particular, in EP 1819366 A1 it was proposed that BBG, a salt of BBG or a hydrate of BBG should be used.

BBG is also indicated as the preferred dye in US 2011/190728 A1, a more
5 generic document that describes the use of at least one dye selected from triphenylmethane dyes and/or azo dyes and/or cyanine dyes and/or natural dyes such as anthocyanins and anthocyanidines.

In that document the dye preparation based on the selected dye is also made with a density of between 1.01 g/cm³ and 1.5 g/cm³, preferably
10 between 1.01 g/cm³ and 1.3 g/cm³, in order to guarantee greater contact during the surgery between the dyed solution and the ILM and to overcome problems of excessive flushing away of the dye which may occur with dyes having a lower density, due to the continuous irrigation of the eye during the surgery.

15 According to that document, the agent used to increase the density is selected from heavy water D₂O, disaccharides or polysaccharides, or neutral polymers such as polyethers, polyvinyl alcohol, polyesters, polyacrylic acid copolymers and polyvinyl pyrrolidone.

However, of all of the various dyes indicated above, the only one that was
20 subsequently marketed for dyeing the ILM, is BBG (which, it is no coincidence, is indicated as the preferred dye in the above-mentioned patent documents).

However, as already indicated, even that dye did not prove to be free of disadvantages.

25 In particular, BBG is a dye that is difficult to synthesize with high levels of purity, at least at a reasonable cost in commercial terms.

In fact, BBG is obtained by modification of BBR and the yields of the synthesis reaction are limited, therefore, the commercially available product very often has a level of purity that is even well below the declared
30 90% (tests conducted by the Applicant even showed levels of purity of 80%) with high contamination by the starting BBR. It is easy to imagine

that, without a preliminary purity test, it can be difficult to make BBG-based dye preparations that have a precisely controlled dye content.

At the same time, attempting to further purify BBG is not advantageous in terms of the cost-benefit ratio.

- 5 In addition to these disadvantages, it should be noticed that BBG is not an intrinsically biocompatible substance, but is cytotoxic. The possibility of using it or not in preparations intended for medical use or in any case for dyeing living material, depends exclusively on its concentration. Based on the scientific literature now available, in particular, BBG is considered not
10 to be cytotoxic only when its concentration is not higher than 0.3 g/l (equal to 0.03% w/v - see tests carried out in accordance with DIN EN ISO 10993 shown on the sheet of the commercial product Brilliant Peel® from the company Geuder AG). However, according to many surgeons, at those concentrations the dyeing effect that can be achieved may not always be
15 satisfactory, for example in the case of the eyes of people who are very short-sighted.

As already indicated, a second field of application of this invention is the generic dyeing of proteins and protein chains.

In fact, even in this sector, one of the most used dyes today is BBG.

- 20 However, the Applicant has realised that although it can dye proteins, BBG actually has a relatively low affinity with the self-same proteins. Tests performed on the protein commonly accepted as the reference protein (egg albumin), by means of circular dichroism (hereinafter CD) analysis, in fact highlighted for BBG-250 in a phosphate buffer (1.22 mg/ml in D₂O) an
25 association constant K_a between BBG and albumin of approximately 38000 M⁻¹. It should be noticed that, to carry out the tests, the commercial BBG with a purity of 80% was preliminarily purified and the CD spectra were measured in the 800-400 nm region in the presence of increasing quantities of albumin. Finally, in the known way, the value of the
30 association constant was determined using a non-linear regression of the intensity of the dichroic signal expressed in ΔA ($A_L - A_R$, where A_L and A_R

respectively indicate the absorbency of the preparation as regards the two circularly polarized light waves used for the circular dichroism study, the left wave and the right wave) depending on the concentration of the egg white albumin, used as a reference. However, on this point what is indicated in the subsequent Affinity Tests section should also be considered.

Disclosure of the invention

In this context the technical purpose which forms the basis of this invention is to provide a new dye molecule which overcomes the above-mentioned disadvantages.

In particular, the technical purpose of this invention is to provide a dye molecule that can be used in preparations for dyeing the ILM, in particular better than is currently possible using BBG.

Another technical purpose of this invention is to provide a new dye molecule that has a much greater affinity with proteins and protein chains than BBG.

Yet another technical purpose of this invention is to provide a heavy dye preparation that has better dyeing capability than the prior art ones.

Not least, the technical purpose of this invention is to provide a dye preparation that can be used in methods for treatment of the bodies of humans or animals.

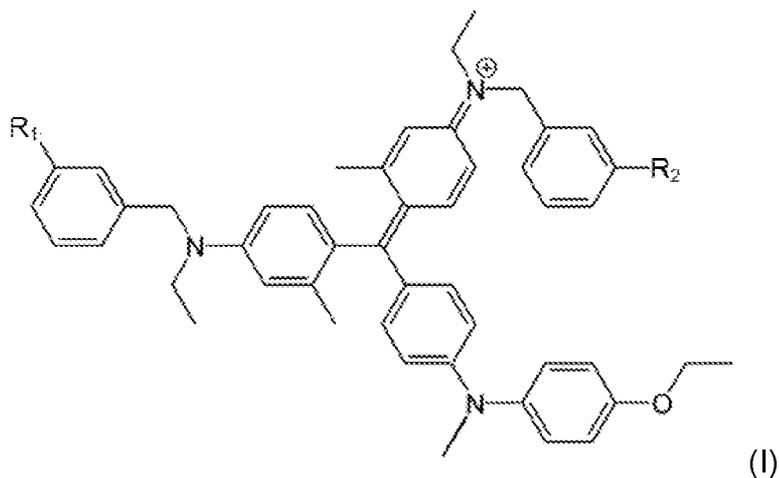
The technical purpose specified and the aims indicated are substantially achieved by a dye molecule and by a dye preparation which comprises it, as described in the appended claims.

Further features and the advantages of this invention are more apparent in the detailed description with reference to several preferred, non-limiting embodiments of this invention illustrated below.

Detailed description of preferred embodiments of the invention

Regarding the dye molecule, in the context of this invention, a family of it

was provided that is represented by the following structure (I):



where R₂ is constituted of an SO₃⁻ group (which forms an inner salt with the nearby positively charged nitrogen N atom) and R₁ is constituted of an SO₃⁻ group bound with an ionic bond to a hydrogen H atom or to another atom (for example, sodium Na) or to an ammonium NH₄ group or to a lysine salt or to an arginine salt or to a different monovalent cation.

As can be seen, the molecule disclosed according to this invention differs from a molecule of BBG (it should be noticed that in the context of this invention, that name is also intended to indicate any product identified by synonyms, such as Coomassie® brilliant blue, Acid Blue 90, C. I. 42655 and Brilliant Blue G 250, all uniquely identified by CAS number: 6104-58-1) due to the presence of a methyl group rather than a hydrogen atom bound to the disubstituted amino nitrogen atom.

Synthesis of the new molecule disclosed may be achieved by means of a methylation reaction to the disubstituted amino nitrogen of a starting product constituted of Brilliant Blue G250. In particular, the reaction is particularly advantageous starting with commercial BBG which, as said, usually has a level of purity less than or equal to 90% and is contaminated with BBR. In fact, at the end of the methylation reaction and of the subsequent purification, the contamination with BBR has been eliminated. Generally speaking, the synthesis method involves making the starting

product react in one or more successive steps with sodium hydroxide and methyl iodide, if necessary varying their proportions to one another each time.

EXAMPLE

- 5 The following is a description of a preferred production example. Obviously, the quantities indicated could be varied, preserving the proportions between the various substances, it being understood that the process described leads to the production of approximately 1.6 g of molecule disclosed, and the production of significantly larger quantities
- 10 could require some modifications to the process for its industrial optimisation.

Materials and Methods

Reagents

Reagents	Manufacturer	Quantity for a batch of approx. 1.6 grams
Brilliant Blue G –pure (BBG)	(Sigma Aldrich, B0770)	2g
RP grade methanol	(Sigma Aldrich)	50 ml
Sodium hydroxide	(Sigma Aldrich)	0.20 g
Methyl iodide	(Sigma Aldrich)	2.20 ml
HPLC grade acetonitrile	(Sigma Aldrich)	4 l
Deionised water		10 l
UV grade trifluoroacetic acid	(Sigma Aldrich)	40 ml
HPLC grade methanol	(Sigma Aldrich)	4 l

- 15 As regards the starting BBG, in the tests carried out it was a product with 91% purity (Analysis certificate B0770, Sigma Aldrich, batch SLBJ8621V). Confirming that, Figure 1 shows its mass spectrum which indicates at least two significant peaks.

20 *Materials*

Materials	Quantity for a batch of approx. 1.6 grams

250 ml glass flask	1
5 ml pipettes	2
500 ml, 1 l freeze-drying flasks	5
16*160 mm glass test tubes washed in a laboratory washing machine	300
200 ul vials for HPLC autosampler	600

Instruments

Instruments	Manufacturer
Medium Pressure Liquid Chromatography	
REVELERIS C18 WP 40 g column	Grace
Freeze Dryer	Freeze Dryer Modulyo Edwards
HPLC	Shimadzu LC10
Magnetic stirrer	Ika
Rotary evaporator	Buchi

Description of reaction

5 0.12 g of sodium hydroxide and 0.92 ml of methyl iodide are added to 1.7 g of BBG dissolved in methanol-water 1:1 v/v. The reaction is allowed to run for around two days at ambient temperature. The reaction mixture can then be checked using HPLC to highlight how the starting product is still present. The results of the HPLC analysis for the tests carried out are

10 shown in Figure 3 and highlight three peaks that correspond, from left to right, the first to the solvent used in the reaction, the second to the molecule of BBG and the third to the new molecule disclosed. At this point, another 0.92 ml of methyl iodide and 0.08 g of sodium hydroxide are added. After 7 days, another 0.36 ml of methyl iodide and 0.08 g of

15 sodium hydroxide are added and the reaction is allowed to finish for another two days.

The reaction mixture is then concentrated in small volumes and the unrefined mixture is purified by means of Medium Pressure Liquid Chromatography (MPLC) using a REVELERIS C18 WP 40 g column

20 (Grace) eluted with a mixture of water (A) and 90% v/v acetonitrile in water

(B) both containing 0.05% v/v of trifluoroacetic acid.

After purification, the fractions containing the desired product are put together, concentrated in small volumes and freeze-dried. The solid dissolved in sodium hydroxide 0.01 is then purified using the same column
5 with a mixture of water (A) - methanol (B). MPLC conditions: flow rate 20 ml/min, detection 254 nm, gradient 40% (B) for 5 min, 40-60% (B) in 40 min, 60-95% (B) in 2 min, 95% (B) 2 min, 95-40% (B) 2 min.

Figure 4 shows the result of the HPLC check on the purified and freeze-dried end product obtained in the tests carried out, and shows the
10 presence of a single peak corresponding to the molecule disclosed (in the form of sodium salt). Confirming that, Figure 2 shows the ESI-MS mass spectrum of the purified end product.

Also in the tests carried out, the purity of the end product was approximately 96.6% as calculated starting with the HPLC spectra, whilst
15 the yield of the reaction was around 60% unrefined product (100 g of BBG-250 therefore yielded approximately 60 g of the molecule disclosed, in the form of sodium salt).

As indicated, this invention also relates to a dye preparation comprising at least one first dye whose molecule is the new dye molecule disclosed, or a
20 pharmaceutically acceptable salt of it or a hydrate of it.

Preferably, the dye preparation is an aqueous solution and/or has a phosphate buffered matrix. Moreover, it is liquid at least in the temperature range between 0°C and 50°C.

Moreover, at least for the uses in the context of methods for treatment of
25 the bodies of humans or animals, the preparation is also advantageously sterile and biocompatible.

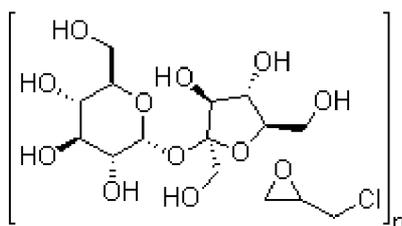
Advantageously, the first dye is present in a quantity, by weight relative to the total volume of the preparation (w/v), of between 0.0001% and 0.5%, preferably between 0.015% and 0.05%.

30 Moreover, in some preferred embodiments the dye preparation has a density of between 1.01 g/cm³ and 1.5 g/cm³ thanks to the additional

presence of at least one agent for increasing its density.

The at least one agent for increasing the density may be selected according to requirements. For example, it may be selected in the group consisting of: heavy water D_2O , monosaccharides, disaccharides, polysaccharides, and neutral polymers; amongst the neutral polymers in particular it may be selected from polyethers, polyvinyl alcohol, polyesters, polyacrylic acid copolymers, mannitol and polyvinyl pyrrolidone.

However, in a particularly preferred embodiment the agent for increasing the density is a polymer with the empirical formula $(C_{12}H_{22}O_{11} \cdot C_3H_5ClO)_n$, and structure



That agent is commercially known as Ficoll® or Polysucrose (CAS number: 26873-85-8), and is advantageously present in a quantity, by weight relative to the total volume of the preparation (w/v), of between 0.001% and 20%, preferably between 0.1% and 10%. Hereinafter, when reference is made to Ficoll, the intention is to indicate that agent.

In a particularly preferred formulation the preparation comprises 0.05% (w/v) of the molecule disclosed and 4% (w/v) of Ficoll in a phosphate buffer.

Depending on the applications for which it is intended, the dye preparation may also comprise other substances, and in particular it may also comprise at least one second dye that is different to the first dye. In a preferred embodiment in which the dye preparation is intended to be used in the context of methods for treatment of the bodies of humans or animals, the second dye is advantageously trypan blue.

As regards the other properties of the dye preparation, such as dynamic viscosity, pH, osmolality, etc., in each case the expert in the field will be able to adapt them to the use of the dye preparation. For example, in the case of use of the dye preparation in the bodies of humans or animals, if
5 necessary they can be adjusted in such a way that they are as close as possible to the physiological conditions.

As regards the possible uses of the dye preparation, one of those for which this invention is specifically intended is use in methods for treatment of the bodies of humans or animals.

10 Advantageously, in particular, the dye preparation is intended for use in a surgical method of vitreoretinal surgery.

Even more advantageously, the dye preparation is usable for dyeing the internal limiting membrane ILM and/or epiretinal membranes in a surgical method which involves subsequent removal of the ILM and/or of the
15 EPRM. In fact, it has been seen that the dye molecule disclosed is able to selectively dye at least the ILM in such a way that during the step of detaching the ILM (peeling) and the EPRM, a clearly visible difference is created between the dyed ILM and the undyed underlying structures. To guarantee that all possible EPRM are also dyed, the dye preparation may
20 also comprise a second dye, able to selectively dye them (such as trypan blue).

A further preferred use of the dye preparation disclosed is in a method in which it dyes proteins, for example to make a tissue to which the proteins belong more visible.

25 EXPERIMENTAL DATA

The following are some experimental data obtained following tests and analyses carried out by the Applicant.

Cytotoxicity analysis

The Applicant carried out various tests which highlighted how dye
30 preparations according to this invention are not cytotoxic.

The following, by way of example, are the results obtained by the Eurofins

Biolab S.r.l. laboratories in Vimodrone (Milan province), Italy, for three different batches of a dye preparation comprising 0.05% (w/v) of a molecule made in accordance with this invention (with the group R₁ constituted of an SO₃⁻ group bound to a sodium cation), 4% (w/v) Ficoll in a phosphate buffer, which show how none of the three samples were cytotoxic. It should be noticed how the concentration used is much higher than that (0.03 % w/v) at which BBG is declared to be cytotoxic.

batch	sterilisation	% reduction in viability		degree of cellular degeneration	
		value measured	% cytotoxicity limit	value measured	degree cytotoxicity limit
1	autoclave 121.1°C x20'	1.6	>30	0	>2
2	autoclave 121.1°C x20'	-0.42	>30	0	>2
3	autoclave 121.1°C x20'	-2.77	>30	0	>2

10 Affinity tests

These are tests carried out by the Applicant to compare the molecule disclosed and commercial BBG-250, in terms of affinity with proteins and protein chains. As in the previous case, the molecule produced according to this invention used in the tests is a molecule obtained with the method described in the Example, and in which the group R₁ is constituted of an SO₃⁻ group bound to a sodium cation.

In contrast, as regards the commercial BBG-250, given that the purity of said compound was approximately 91% (compared with the declared 80%), before carrying out the tests it was purified to approximately 98%.

In particular, tests were carried out on four different dye preparations, water-based in a phosphate buffer: for each molecule one comprising Ficoll (at 4% w/v) and one comprising heavy water D₂O (at 13% v/v). In all of the dye preparations tested, the phosphate buffer comprised:

- 5 - Sodium phosphate monobasic monohydrate NaH₂PO₄ · H₂O: 0.266 mg/ml;
- Sodium phosphate dibasic anhydrous Na₂HPO₄: 1.51 mg/ml;
- Sodium Chloride NaCl: 8.2 mg/ml.

Affinity was determined by means of CD (circular dichroism) spectroscopy.

- 10 The CD spectra were measured in the 800-400 nm region in the presence of increasing quantities of egg white albumin which, as is known, for analyses of this type is commonly considered to be representative of proteins in general.

- It should be noticed that in the conditions used (400-800 nm) the albumin
15 does not absorb and therefore does not give rise to any dichroic signal, whilst the dyes have a high level of absorption, but do not show any dichroic signal. In contrast, the interaction of the albumin with the dye molecule (BBG-250 or disclosed herein) induces a chirality in the dye that can be measured by means of CD. Consequently, titrating the dye with the
20 albumin it is possible to determine its apparent binding constant or association constant, by means of a non-linear regression of the titration curve.

- The CD spectroscopy data relative to a dye preparation comprising the molecule disclosed and Ficoll are shown in Figure 5 where the number 1
25 indicates the dichroic spectrum of the dye preparation in the absence of protein, whilst the other lines relate to the same preparation in the presence of increasing quantities of albumin (according to the arrow).

- As already indicated, using a non-linear regression of the intensity of the dichroic signal expressed in ΔA ($A_L - A_R$) depending on the concentration of
30 egg white albumin, the values of the association constant K_a are determined. Figure 6 shows the trend of the regression concerning the dye

preparation comprising the molecule disclosed and Ficoll. It should be noticed that in the calculation of the association constant K_a the different concentrations of the two dyes was taken into account.

The results obtained are as follows:

- 5 - Dye preparation with BBG-250 in Ficoll 4% in a phosphate buffer (sterilised): K_a approximately 60,000 M^{-1} ;
- Dye preparation with the molecule disclosed in Ficoll 4% in a phosphate buffer (sterilised): K_a approximately 300,000 M^{-1} ;
- Dye preparation with BBG-250 in a phosphate buffer (1.22 mg/ml in
10 D_2O) (sterilised): K_a approximately 38,000 M^{-1} ;
- Dye preparation with the molecule disclosed in a phosphate buffer (0.58 mg/ml in D_2O) (sterilised): K_a approximately 180,000 M^{-1} .

Therefore, as can be seen, irrespective of the agent for increasing the density used, the molecule disclosed surprisingly showed an affinity for the
15 albumin equal to 4.7-5 times that of commercial BBG-250.

Moreover, it was possible to establish that, for both of the dye molecules, the presence of Ficoll promoted (by 1.66 – 1.58 times) the association of the dye with the albumin compared with used in heavy water. Although the reason for this latter result is not currently known for sure, the theory of the
20 Applicant is that the Ficoll, in addition to the role of agent for increasing the density, may also fulfil the role of “crowding agent” (hereinafter referred to as CA) and promote the interaction of the dyes with the egg albumin by means of “excluded volume” and spatial factors.

In fact, recently, Ficoll, together with PEG and dextran, has been studied
25 and used as a CA, that is to say, as a filling/crowding agent, to mimic the conditions of the intracellular environment which is characterised by an extremely crowded environment, with a limited quantity of free water and an almost total absence of space. Many studies on CA (see, for example, “What Macromolecular Crowding Can Do to a Protein”, Irina M.
30 Kuznetsova 1,2, Konstantin K. Turoverov 1,2 and Vladimir N. Uversky 1,3,4,5) have in fact shown how “macromolecular crowding” created by the

CA could affect the structure of the proteins, the folding, the form, shape, stability, the bond with the small molecules, enzymatic activity, protein-protein interactions, protein-nucleic acid interactions, and pathological aggregation.

- 5 The main mechanism of CA is to act for “excluded volume”, which corresponds to spatial occupation and, therefore, to movement of the other molecules into the remaining free spaces. Locally this may result in an increase in concentration or in steric molecular changes of solutes. Therefore, this could also occur in the case in question.
- 10 In light of the experimental results just discussed concerning the surprising advantages than can be obtained using Ficoll as the agent for increasing the density, to conclude, this invention also relates to a dye preparation for use in a surgical method of ophthalmic surgery having a density higher than 1.01 g/cm^3 and comprising Ficoll as an agent for increasing its
- 15 density. That preparation may be used for all of the uses indicated above with reference to the new dye molecule disclosed.
- Advantageously, this dye preparation may comprise at least one dye selected from the group consisting of: triphenylmethane dyes, azo dyes, cyanine dyes, natural dyes and/or mixtures of them, but preferably it will
- 20 comprise BBG or a structural analogue of it.
- Amongst the latter, those preferred are the pharmaceutically acceptable salts of BBG, BBG hydrates and BBG methylates.
- From an operating viewpoint, in the case of use of a dyed preparation which uses Ficoll to obtain a density higher than 1.01 g/cm^3 , for dyeing the
- 25 ILM, it should be remembered that in these surgeries following removal of the vitreous by means of the vitrectomy, the dye preparation is injected into the posterior chamber. Thanks to its high density it is deposited on the ILM with which it remains in contact only for several seconds before being removed by means of the usual abundant washing of the posterior
- 30 chamber with BSS. However, despite just several seconds of contact, the dye preparation dyes the ILM.

It should be noticed, however, that in these conditions of use (therefore even in the case of other ophthalmic surgeries performed under similar conditions), any Ficoll used as a thickener is not able to act as a CA, since the dye preparation is removed immediately after dyeing the membrane
5 and is diluted in the posterior chamber with BSS.

Therefore, limited to the use for intraoperative dyeing in ophthalmic surgery methods, using Ficoll gives “only” performance similar to that obtainable, the density of the dye preparation being equal, with normal agents for increasing the density, such as heavy water.

10 This invention brings important advantages.

Regarding the new dye molecule provided, first the great advantage was achieved of that molecule proving able to dye better than commercial BBG not just the ILM (circumstance declared by all of the surgeons involved in the experimental tests), but also the proteins and protein chains (see the
15 results of the tests shown above), the consequence being that it is possible to use less dye to achieve the same results.

Moreover, it is not cytotoxic at concentrations significantly higher than those currently permitted for commercial BBG.

In contrast, as regards the use of Ficoll as an agent for increasing the
20 density, as an alternative to those used in the sector until now, it was possible to establish that its use allows in some applications results similar to those obtainable until now (as in the case of ophthalmic surgery), whilst in other applications it allows the use of less dye to achieve the same results.

25 Finally, it should be noticed that this invention is relatively easy to produce and that even the cost linked to implementing the invention is not very high.

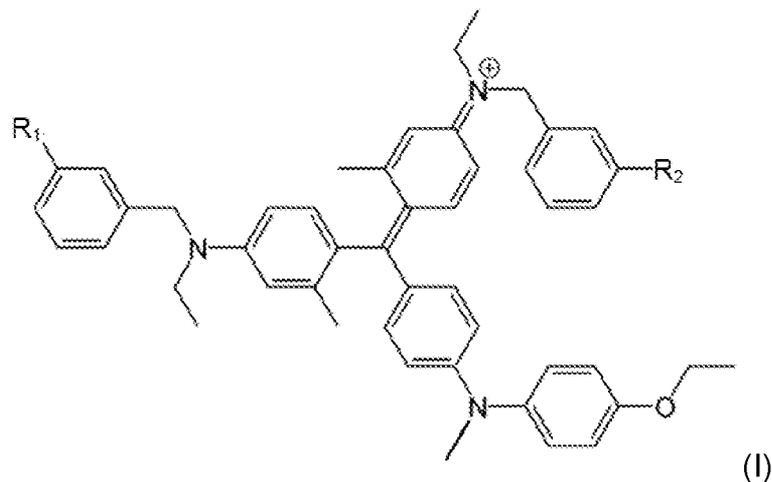
The invention described above may be modified and adapted in several ways without thereby departing from the scope of the inventive concept.

30 All details of the invention may be substituted with other technically equivalent elements and the materials used, as well as the shapes and

dimensions of the various components, may vary according to requirements.

CLAIMS

1. A dye molecule having the structure:

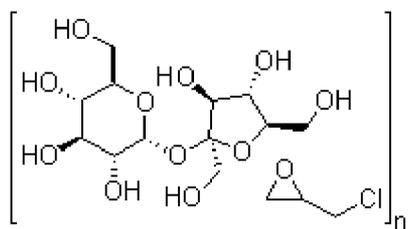


where:

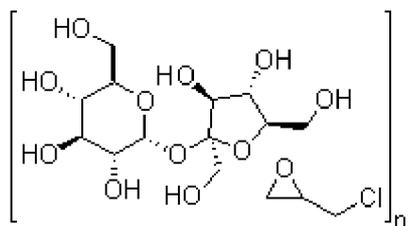
- 5 R_1 is constituted of an SO_3^- group bound with an ionic bond to a hydrogen H atom or to another atom or to an ammonium NH_4 group or to a lysine salt or to an arginine salt or to a different monovalent cation; and
- R_2 is constituted of an SO_3^- group.
2. A dye preparation comprising at least one first dye with the molecule according to claim 1, or a pharmaceutically acceptable salt of it or a hydrate of it.
3. The dye preparation according to claim 2, characterised in that the first dye is present in a quantity, by weight relative to the total volume of the preparation (w/v), of between 0.0001% and 0.5%.
- 15 4. The dye preparation according to claim 2, also comprising a second dye which is different to the first dye.
5. The dye preparation according to claim 4, wherein the second dye is trypan blue.
6. The dye preparation according to any one of claims 2 to 5, characterised in that it has a density of between 1.01 g/cm^3 and 1.5 g/cm^3 and in that it also comprises at least one agent for increasing the density.
- 20 7. The dye preparation according to claim 6, characterised in that the at least one agent for increasing the density is selected in the group

consisting of: heavy water D_2O , monosaccharides, disaccharides, polysaccharides, neutral polymers such as polyethers, polyvinyl alcohol, polyesters, polyacrylic acid copolymers, mannitol and polyvinyl pyrrolidone.

- 5 8. The dye preparation according to claim 6, characterised in that the agent for increasing its density is a polymer with the empirical formula $(C_{12}H_{22}O_{11} \cdot C_3H_5ClO)_n$, and the structure



9. A dye preparation having a density greater than 1.01 g/cm^3 and
 10 comprising at least one dye and at least one agent for increasing its density that is a polymer with the empirical formula $(C_{12}H_{22}O_{11} \cdot C_3H_5ClO)_x$, and the structure



10. The dye preparation according to claim 9, comprising at least one dye
 15 selected from the group consisting of triphenylmethane dyes, azo dyes, cyanine dyes, natural dyes and/or mixtures of them.

11. The dye preparation according to claim 10, wherein the dye is Brilliant Blue G (BBG) or a structural analogue of it.

12. The dye preparation according to claim 10, wherein the dye is selected
 20 from BBG, a pharmaceutically acceptable salt of BBG, a hydrate of BBG or a methylate of BBG.

13. The dye preparation according any one of claims 8 to 12, wherein the agent for increasing its density is present in a quantity, by weight relative to the total volume of the preparation (w/v), of between 0.001% and 20%.

14. The dye preparation according to any one of claims 2 to 13, characterised in that it also comprises a phosphate buffered matrix.
15. The dye preparation according to any one of claims 2 to 14, characterised in that it is also liquid at least in the temperature range
5 between 0°C and 50°C.
16. The dye preparation according to any one of claims 2 to 15 for use in methods for the treatment of the bodies of humans or animals.
17. The dye preparation according to claim 16, for use in a surgical method for vitreoretinal surgery.
- 10 18. The dye preparation according to claim 16 or 17, for use in dyeing the internal limiting membrane ILM and/or epiretinal membranes EPRM in a surgical method which involves subsequent removal respectively of the ILM and/or of the EPRM.
- 15 19. The dye preparation according to claim 18, when it is dependent on claim 4, for use also in dyeing any epiretinal membranes, wherein the first dye in use dyes at least the ILM and the second dye dyes the epiretinal membranes EPRM.
- 20 20. The dye preparation according to claim 16 for use in a method in which the dye preparation dyes proteins to make a tissue to which the proteins belong more visible.
21. Use of a dye preparation according to any one of claims 2 to 14 for dyeing proteins.
22. A method for synthesising the dye molecule according to claim 1, characterised in that it uses a methylation reaction of a starting product
25 constituted of Brilliant Blue G250.
23. The method according to the preceding claim, wherein the methylation reaction comprises making the starting product react in one or more steps with sodium hydroxide and methyl iodide.
24. Use of a dye preparation according to any one of claims 2 to 15 in a
30 method for the treatment of the bodies of humans or animals.
25. Use of a dye preparation according to any one of claims 2 to 15 in a

surgical method of vitreoretinal surgery.

26. Use of a dye preparation according to any one of claims 2 to 15 for dyeing the internal limiting membrane ILM and/or epiretinal membranes EPRM in a surgical method which involves subsequent removal
5 respectively of the ILM and/or of the EPRM.

27. Use of a dye preparation according to claim 4 or any one of claims 5 to 8 when they are dependent on claim 4, for dyeing the internal limiting membrane ILM and epiretinal membranes EPRM, in a surgical method which involves subsequent removal respectively of the ILM and of the
10 EPRM, wherein the first dye in use dyes at least the ILM and the second dye dyes the epiretinal membranes EPRM.

28. Use of a dye preparation according to any one of claims 2 to 15 for dyeing proteins for making a tissue to which the proteins belong more visible.

15 29. Use of a dye preparation according to any one of claims 2 to 15 for production of a preparation for use in a surgical method of vitreoretinal surgery.

30. Use of a dye preparation according to any one of claims 2 to 15 for production of a preparation for dyeing the internal limiting membrane ILM
20 and/or epiretinal membranes EPRM in a surgical method which involves subsequent removal respectively of the ILM and/or of the EPRM.

31. Use of a dye preparation according to claim 4 or any one of claims 5 to 8 when they are dependent on claim 4, for production of a preparation for dyeing the internal limiting membrane ILM and epiretinal membranes
25 EPRM, in a surgical method which involves subsequent removal respectively of the ILM and of the EPRM, wherein the first dye in use dyes at least the ILM and the second dye dyes the epiretinal membranes EPRM.

32. Use of a dye preparation according to any one of claims 2 to 15 for
30 production of a preparation for dyeing proteins for making a tissue to which the proteins belong more visible.

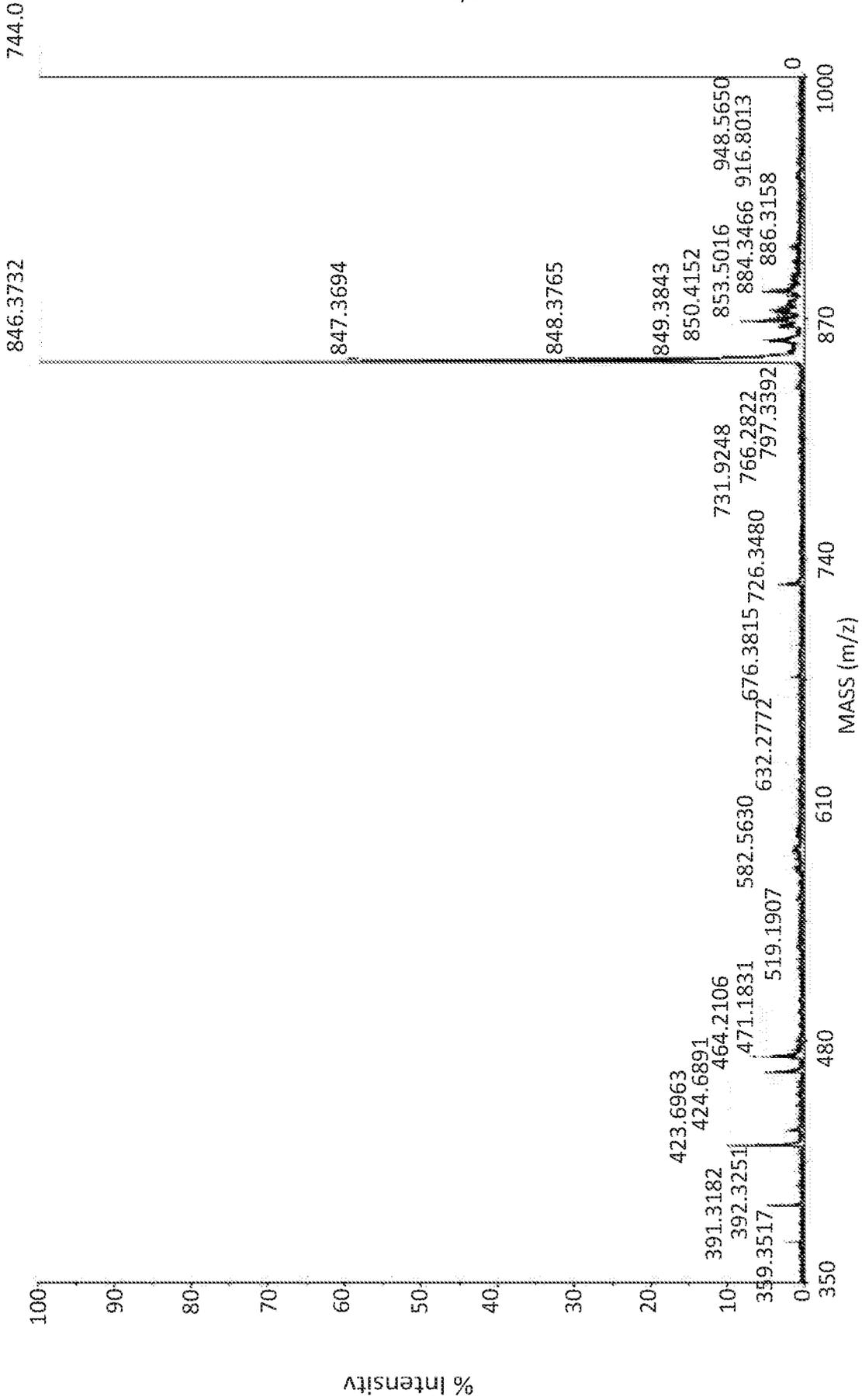


FIGURE 1

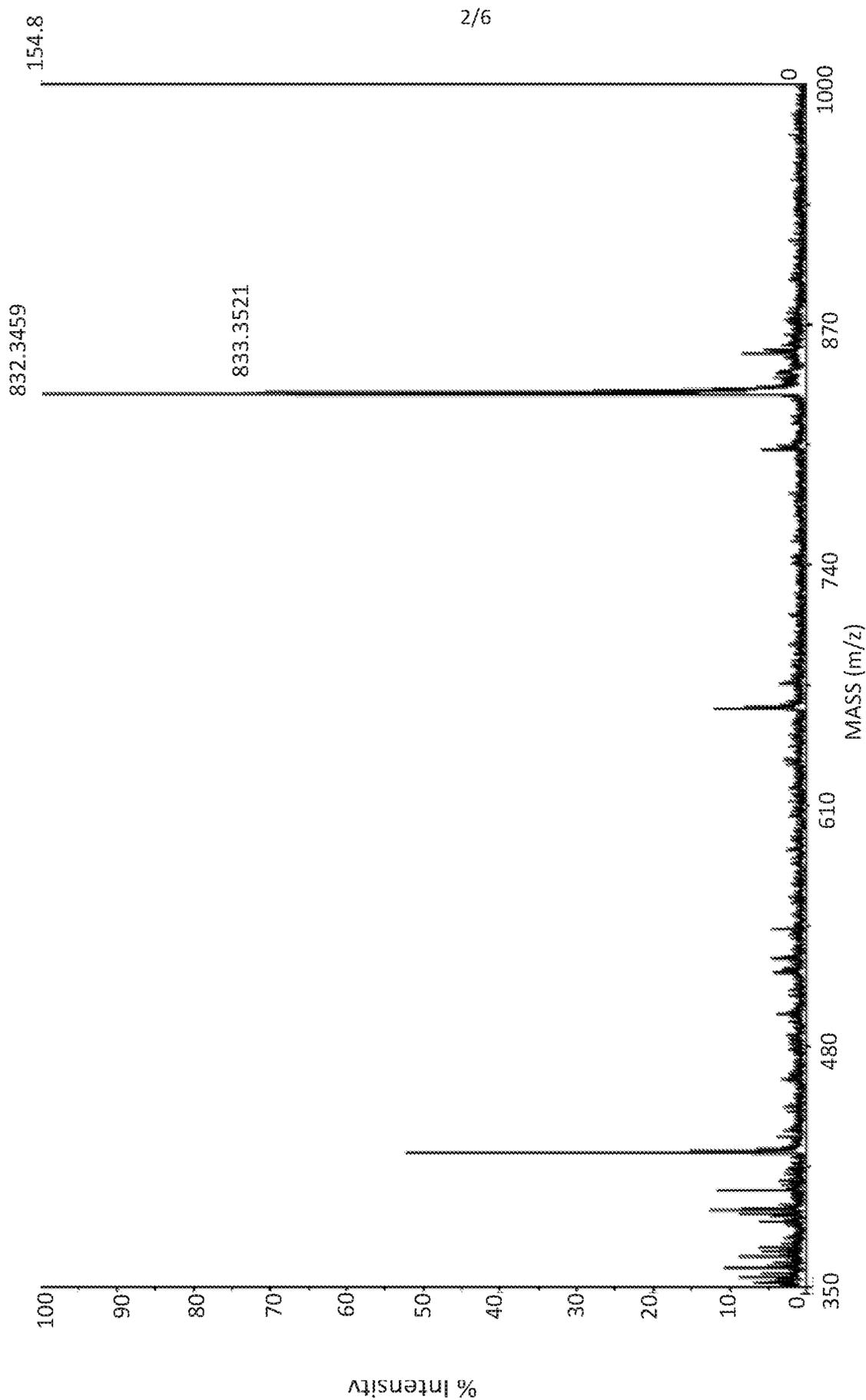


FIGURE 2

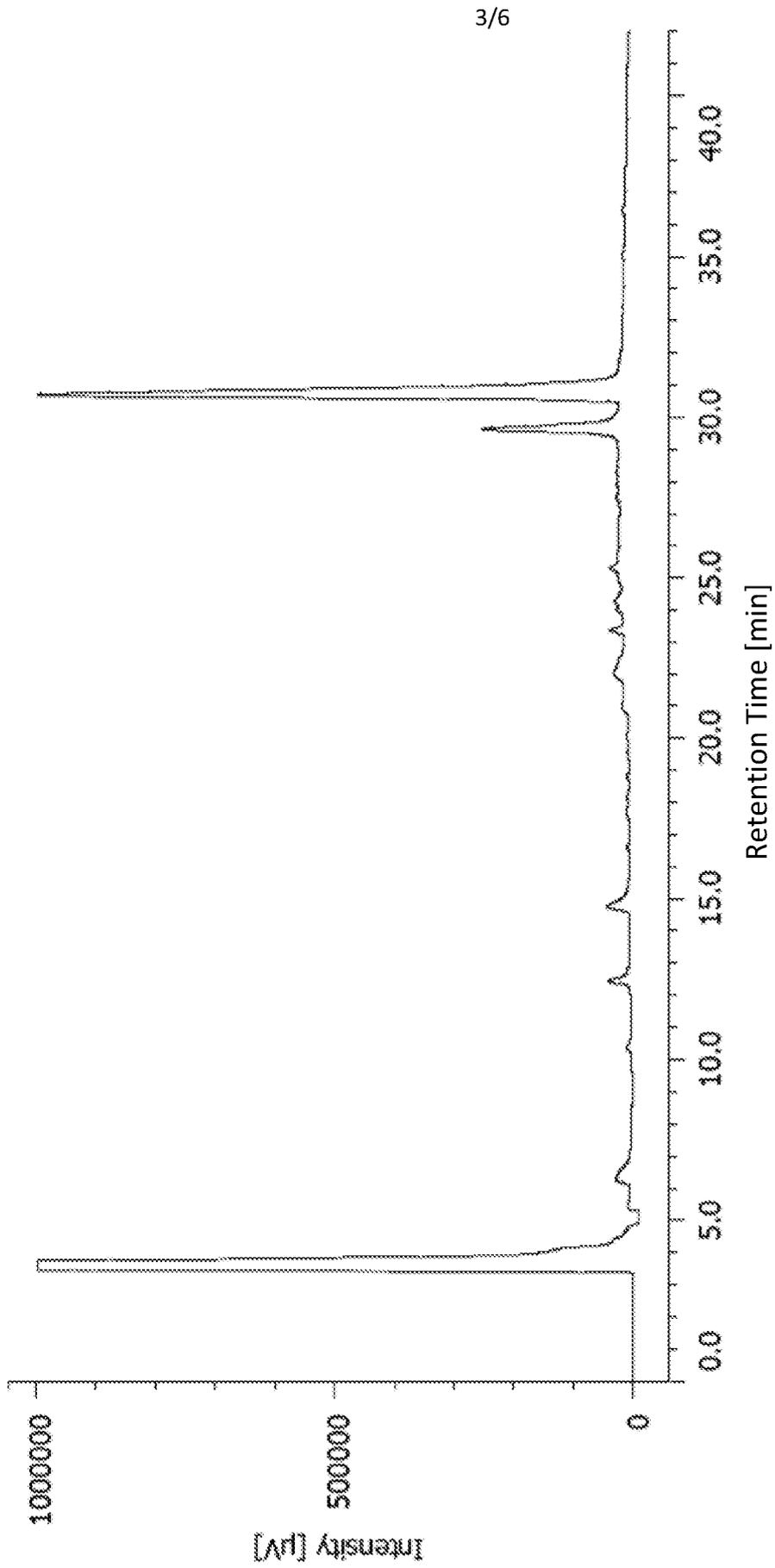


FIGURE 3

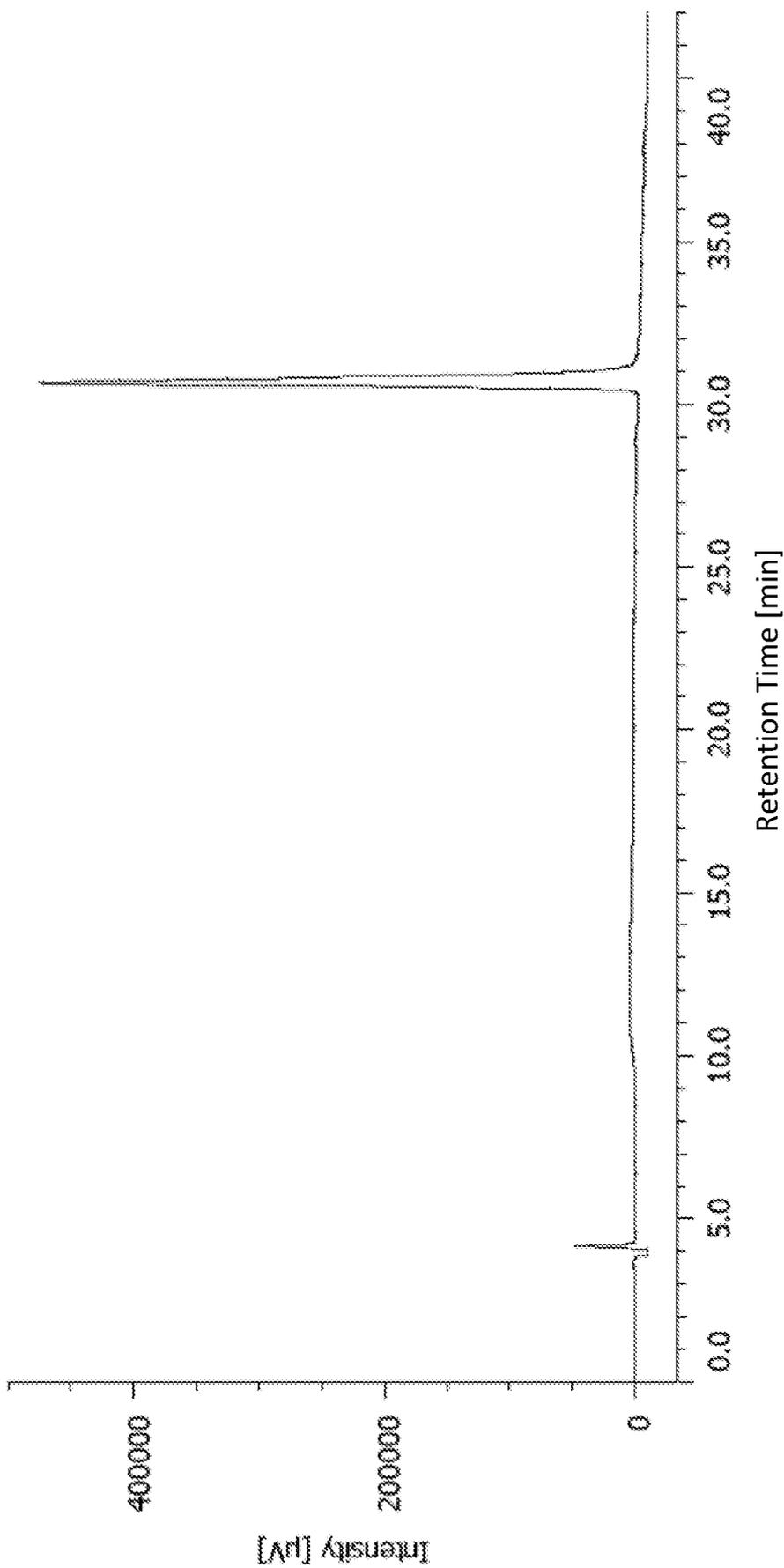


FIGURE 4

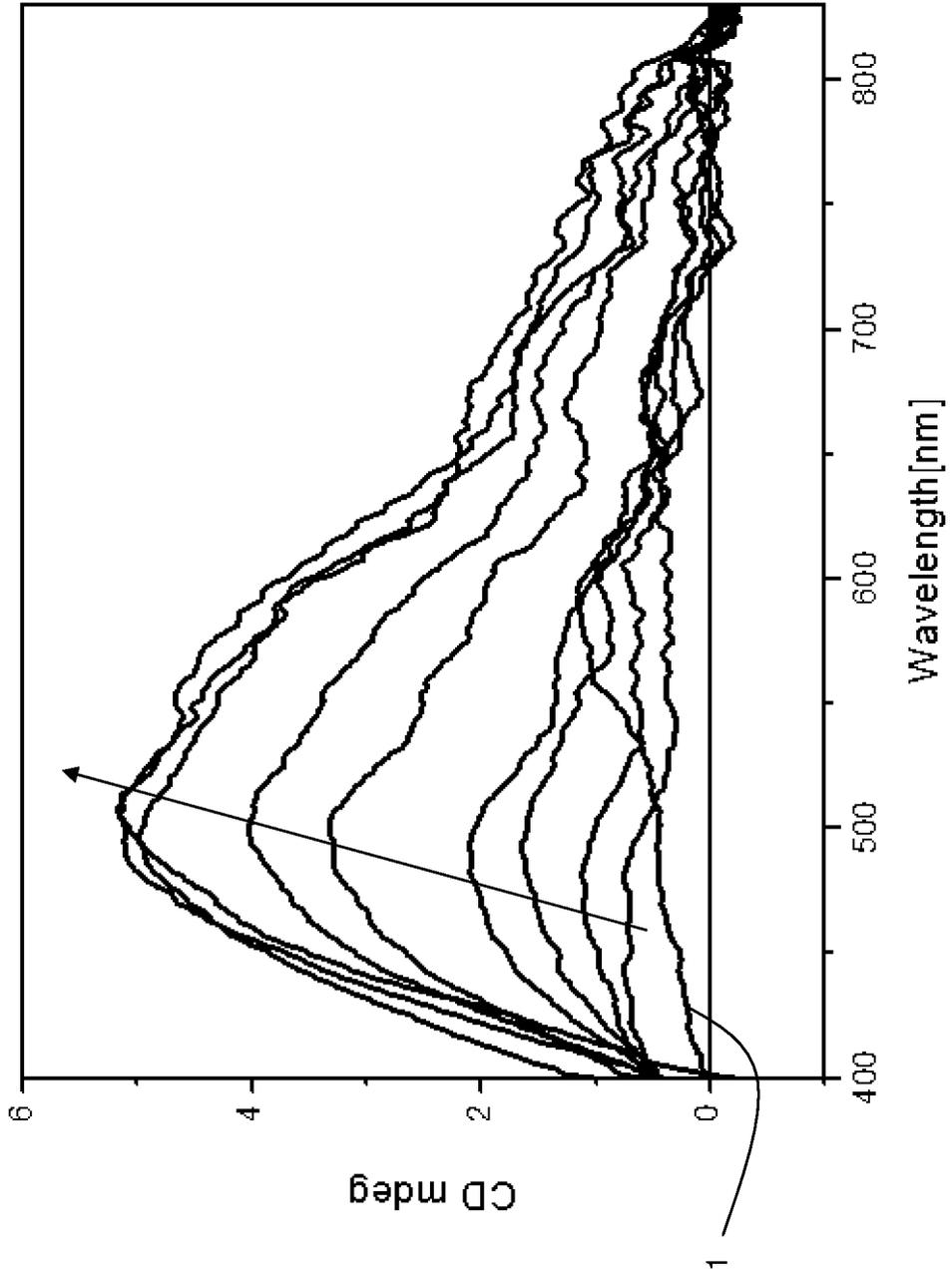


FIGURE 5

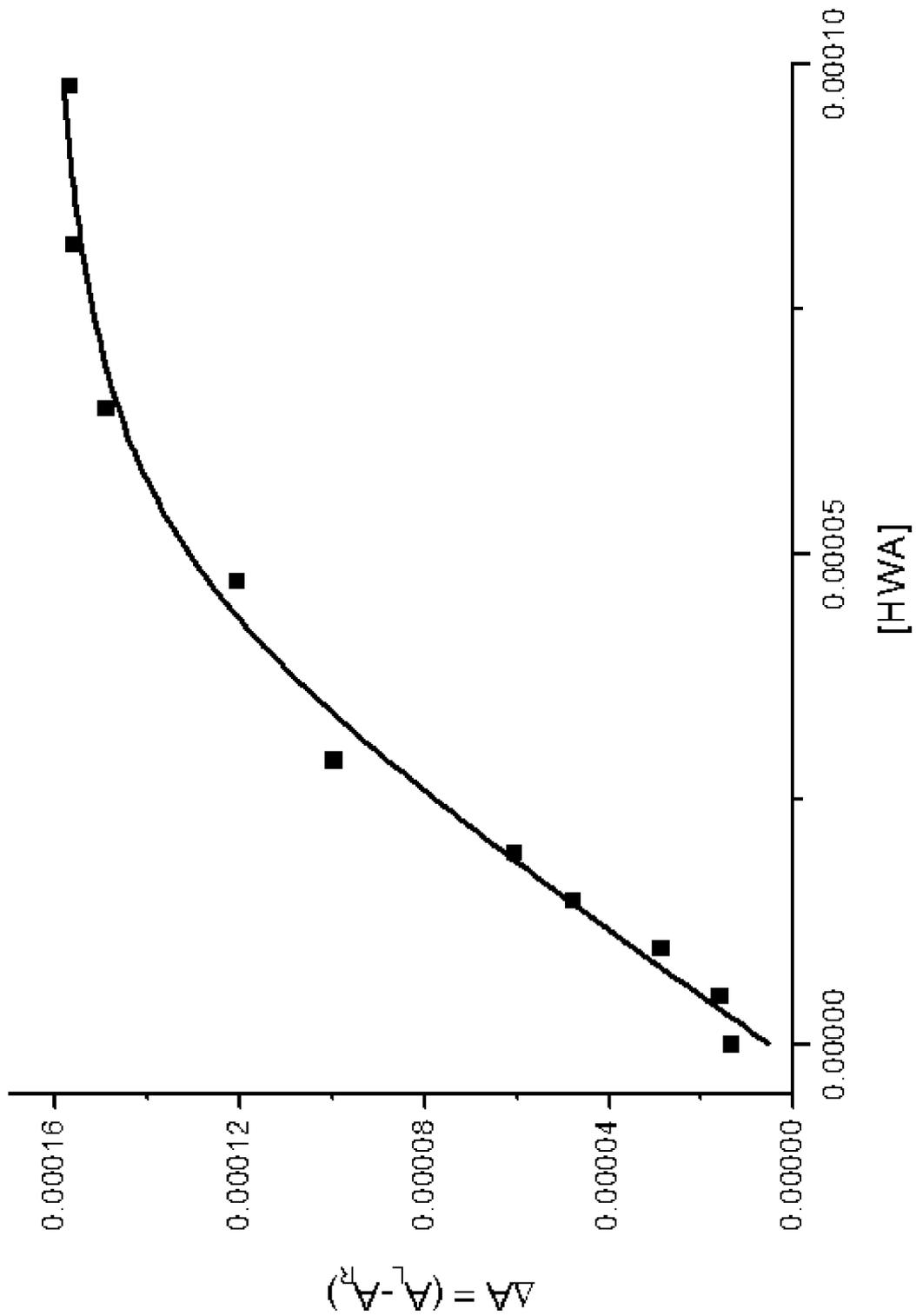


FIGURE 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2016/053062

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K49/00 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, CHEM ABS Data, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/062233 A1 (NAT UNIVERSITY CORP KYUSHU UNI [JP]; ENAIDA HIROSHI [JP]; HISATOMI TOS) 15 June 2006 (2006-06-15) cited in the application the whole document <p style="text-align: center;">-----</p>	1-32
<input type="checkbox"/> Further documents are listed in the continuation of Box C.		
<input checked="" type="checkbox"/> 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	"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	
"E" earlier application or patent but published on or after the international filing date	"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	
"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)	"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	
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	
"P" document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search <p style="text-align: center;">11 August 2016</p>	Date of mailing of the international search report <p style="text-align: center;">19/08/2016</p>	
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 <p style="text-align: center;">Diederens, Jeroen</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2016/053062

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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