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(54) Title: NANOFIBERS CONTAINING PHOTOCURABLE ESTER DERIVATIVE OF HYALURONIC ACID OR ITS SALT, PHOTOCURED NANOFIBERS, METHOD OF SYNTHESIS THEREOF, PREPARATION CONTAINING PHOTOCURED NANOFIBERS AND USE THEREOF

(57) Abstract: The invention relates to nanofibers containing photocurable ester derivatives of hyaluronic acid or its salt, the method of preparation thereof, and the photocured preparation made from the nanofibers by light irradiation and the use thereof



Nanofibers containing photocurable ester derivative of hyaluronic acid or its salt, photocured nanofibers, method of synthesis thereof, preparation containing photocured nanofibers and use thereof

5 **Field of the invention**

The present invention relates to nanofibers containing a photocurable ester derivative of hyaluronic acid or its salt, photocured nanofibers and the methods of their synthesis and use of the preparation containing photocured nanofibers.

Background of the invention

10 Hyaluronic acid (HA or Hyaluronan, Figure A) is a linear glycosaminoglycan consisting of alternating D-glucuronic acid and N-acetyl-D-glucosamine units. HA is non-immunogenic and therefore it has a great potential in medicine. HA characterized by high molecular weight, has been found to be particularly useful in a variety of clinical fields, including wound healing, ophthalmic surgery and orthopaedic surgery due to its visco-elastic
15 properties. HA is also potentially useful in a variety of non-medical fields including cosmetic applications.

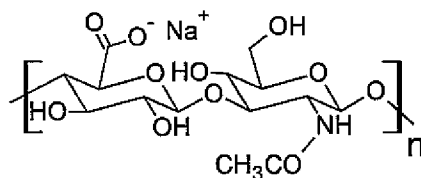


Figure A. Hyaluronic acid (Hyaluronan or HA)

HA is a water-soluble polymer and produces highly viscous solutions. HA has been
20 chemically cross-linked by means of different functional groups such as, epoxy compounds, divinylsulfone and the like, to prolong the half-life of the biopolymer *in vivo* as well as for preparing materials for medical use in the form of films or hydrogels. The main problem to be solved for medical and cosmetic applications of materials made thereof is the completely elimination of unreacted chemicals and/or catalysts after reaction. The elimination of these
25 components is challenging due to macromolecular conformation of HA, however, it is very important since when it is administered or implanted into living bodies, said residual chemicals may produce adverse effects, so that they are not recommended for a practical use in biomedical field.

On previously reported art, such as US patent No. 7951936 that describes a photo-cross-linked reactive hyaluronic acid wherein the carboxylic functional groups of HA was chemically modified. The functionalities use for photo-crosslinking may include cinnamic acid. These aminoalkyl ester derivatives were bonded to the polysaccharide by an amide bond
5 between the amino residue of the aminoalkyl group and the carboxyl group of HA. Such modification of carboxyl alter biocompatibility of the obtained materials because the carboxyl group of HA is known to be the recognition site of hyaluronidase and HA receptors.

US patent application No. 2012289478 (A1) describes ester derivatives of hyaluronic acid with hydroxy-cinnamic acid used as protective agents in dermatology. The synthesis of
10 such esters involves the use of formamide, which is very toxic and the activation of the carboxyl group of hydroxy-cinnamic acid of derivatives thereof is done using carbonyl diimidazol (CDI). The obtained highly viscous solutions or hydrogels cannot be used for electrospinning because CDI may cause self-cross-linking of HA.

Acylation of HA has been reported in several patent documents. For example, it is
15 catalyzed by chloroformates in WO 2010/105582. However, the application of that reagent for the functionalization of polysaccharides is still difficult due to the number of side reactions arising from their high reactivity such as described in a research article (Wondraczek, et al. Synthesis. *Carbohydrate Polymers* **2011**, 83, (3), 1112-1118). Furthermore a conversion of hyaluronic acid to its acid form decreases the recoveries of the polymer and increases
20 considerably the polydispersity. There is also claimed the necessity of anhydrous DMSO, which is very expensive in industrial scale. Additionally, one of the most important disadvantage is the high amount of 4-dimethyl-aminopyridine (or DMAP) used for the reaction feed. The problem of using this reagent is derived from its acute lethal effects (classified as T⁺). However, the reagent is very commonly used for acylation as acyl-transfer
25 agent. The use of DMAP for acylation of hyaluronic acid was recently published (Šmejkalová et al. *Carbohydrate Polymers*, 87(2), 2012, 1460).

Examples of the O-acylation in US Patent No. 7,041,310 included reactions employing one or more acid catalysts, for example, mineral acids such as hydrochloric acid or sulfuric acid, organic acids such as aromatic sulfonic acid and Lewis acids such as boron fluoride
30 etherate or the like, and a reaction with an organic acid employing one or more dehydrating agents, for example, N,N'-dicyclohexylcarbodiimide, 2-chloro-1-methyl pyridinium iodide and N,N'-carbonyl diimidazole or the like. However, the composition involves a complex of

hyaluronic acid or a salt thereof and a quaternary ammonium salt, which is in general consider as toxic.

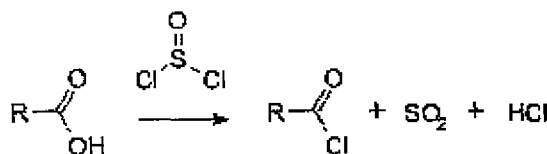
Similar reaction conditions were reported on Patent document WO2005092929A1 and related to hyaluronic acid butyric esters in which the hydroxyl groups of hyaluronic acid are partially esterified with butyric residues, characterized by a degree of substitution being equal or below 0.1. These esters with low degree of substitution are obtained by means of a process carried out in the homogeneous phase under anhydrous conditions, wherein hyaluronic acid is used in the form of a quaternary nitrogen salt. However, these conditions are difficult to be followed in high scale. US Patent No. 2005/0119219A1 relates to hyaluronic acid ester derivatives and hydrogels prepared by photo-curing. The main disadvantage of the synthesis is that is starting from the tetrabutylammonium salt of hyaluronic acid in anhydrous conditions, which is an expensive process and considerably degrades the polysaccharide.

US Patent No. 8247546B2 describes derivatives of acid polysaccharides, wherein the process of preparation comprises the reaction in homogeneous phase using a protic polar solvent such as formamide and an anhydride of an alkylcarboxylic acid, in the presence of a basic catalyst. There are several disadvantages of the synthetic pathway such as the use of formamide, a carcinogenic solvent that seriously limits the upscale of the reaction as well as the use of the modified polymer in medical devices. A second limiting is the use of high temperatures during the reaction (95°C), which degrades the polysaccharide. Similar conditions were used in US Patent No. 2012/0289478 A1. Synthesis of esters of hyaluronic acid and cinnamic, caffeic or ferulic acids was described and the use of the materials for cross-linking. However, this synthesis involves again the use of formamide for dissolution of the reaction mixture. US Patent No. 5,462,976 stated clearly that the synthesis of photo-curable derivatives of glycosaminoglycans requires high temperature and longer reaction time so that HA is degraded and produced unknown reaction sub-products.

In similar art, Japanese Patent number JP-19940307050 provides a cinnamic-photoreactive derivative having a spacer introduced between the reactive cinnamic group and the polymeric backbone. The disadvantage of its preparation is the long synthetic pathway as well as the use of very toxic reagents such as trifluoroacetic acid that impair an effective scale up.

Several activators of the carboxylic group have been reported in the literature such as the used ones for formation of peptide bond. **Scheme 1** shows the activation of the carboxylic group with thionyl chloride to form the acid chloride, published recently in *Molecules* **2012**,

17, 8661-8673. However, not all of such activators can be considered to be used for the modification of hyaluronic acid in aqueous solution since they may react violently and/or explosively and release dangerous gases upon contact with water and other reagents on the reaction feed.



Scheme 1. Activation of carboxylic group by thionyl chloride

On the other hand, electrospinning is a highly versatile method that produces fibers on the micro- or nano-scale. The technique of electrospinning of liquids and/or solutions is well known and described in previous art, such as U.S. Patents No. 4,043,331 and U.S. 5,522,879. The process of electrospinning involves the introduction of a liquid into an electric field, so that the fibers are produced. These fibers are generally drawn to a conductor at an attractive electrical potential for collection. During the conversion of the liquid into fibers, the fibers harden and/or dry. This hardening and/or drying may be caused by cooling of the liquid, i.e., where the liquid is normally a solid at room temperature; by evaporation of a solvent, or by a curing mechanism. As an example, US Patent Application No. 2011/0020917-A1 describes the electrospinning of poly-ε-caprolactone for the immobilization of biologically active compounds. There are some research papers related to electrospinning of polymers such as poly (vinyl alcohol) (PVA), but the authors make clear that the derivatives of PVA are not reactive enough for cross-linking, thereafter, the process requires heat treatment to favor the reaction (Zeng et al, Macromolecular Rapid communications, 2005, 26, 157). However, pre-heating is not favorable for sensitive polymers such as HA that considerably degrades with heating.

US Patent number US20110111012 describes the formation of wound dressings that use genipin, diethyl squarate or oxalic acid as cross-linking agents. However the incorporated cross-linking agents can be difficult to eliminate from the dressings. Another disadvantage of the process is high temperatures (150 °C) during drying, which cannot obviously be applied to HA.

US Patent Application No. 2010/002155 describes the external application of wound dressings made from nanofibers. However, this kind of material cannot be used as well for

internal use due to the presence of silver particles, which are not absorbable and biodegraded in the body.

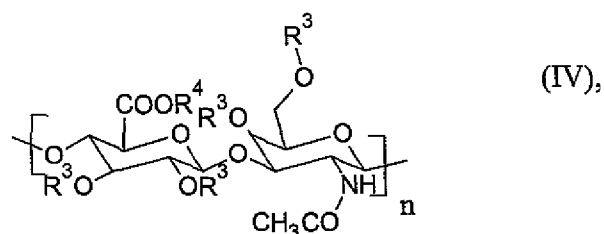
There are several research articles describing the electrospinning of native hyaluronic acid. The articles mainly deal with blends consisting of HA acid/gelatin (HA/GE) obtained in *N, N*-dimethylformamide and water-mixed solvents (Li et al. *Macromolecular Rapid Communications* 2006;27(2):114-120). Unfortunately, the use of DMF is not preferred for materials designed for biomedical applications due to its high toxicity. US Patent No. US20080071001 relates to the process of preparation of a polysaccharide sponge, wherein the cross-linking formation involves the cryo-processing of the material by cooling the solution to -80 °C.

Generally as mentioned above there are several disadvantages and problems concerning the process of preparation of material made from HA derivative applicable in biomedical field comprising the presence of toxic residues in the material that have to be removed in the complicated and expensive way, additionally using reaction conditions that are not suitable for HA, such as high temperature and anhydrous conditions.

SUMMARY OF THE INVENTION

The problems mentioned above are solved in the present invention concerning nanofibers based on photocurable ester derivative of hyaluronic acid or its salt and method of synthetic thereof. The photocurable ester derivatives of hyaluronic acid or its salt are suitable for forming of nanofibers by electrospinning and the photocured preparation from such nanofibers that is suitable for internal and external medical use. The main advantages of such method are performing the method using not extremely toxic components under the mild reaction conditions, especially using mixture of water and water-miscible polar or non-polar solvent. Furthermore, the nanofibers can be easily photo-cured (stabilized) by light irradiation - mediated cross-linking in the absence of initiators or activators.

The subject-matter of the inventions concerns nanofibers comprising a photocurable ester derivative of hyaluronic acid or its salt of the general formula IV



wherein n is integer in the range of 1 to 5000 dimers.

R^3 are independently H or COCHCHR^1 wherein R^1 is 5- or 6-membered aryl or 5- or 6-membered heteroaryl having at least one or more identical or different heteroatoms selected from the group comprising N, O, S, preferably phenyl, furyl, furfuryl, thienyl, thiofenyl, pyridyl or imidazolyl;

provided that at least one R^3 of the derivative is COCHCHR^1 ;

R^4 is H^+ or pharmaceutically acceptable salt, preferably selected from a group comprising any of ions of alkali metals or ions of alkaline-earth metals, more preferably Na^+ , K^+ , Mg^{2+} or Li^+ ;

and at least one carrying polymer.

The HA or its salt is mainly modified at least at one position of OH groups of D-glucuronic acid or N-acetyl-glucosamine unit of HA, preferably at position C6 of the N-acetyl-glucosamine of HA and randomly to the positions C2, C3 of glucuronic acid or C4 of N-acetyl-glucosamine.

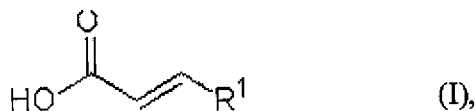
Amount of photoreactive groups ($-\text{COCHCHR}^1$) in the photocurable ester derivative of hyaluronic acid or its salt of the invention is from 0.1 to 20 % per 100 dimer of hyaluronic acid or its salt, preferably 5 to 10 %, more preferably 5%. Such amount of photoreactive groups corresponds the degree of substitution (DS) that was determined by integration of the signals on $^1\text{HNMR}$ (heteroaromatic moieties corresponding to for example furan, thiophene, pyridine, imidazole and the like) related to the methyl group corresponding to the N-acetyl group.

The carrying polymer is selected from a group comprising polyvinyl alcohol (PVA), polyacrylic acid (PAA), polyethylene oxide (PEO), polyvinyl pyrrolidone (PVP).

An amount of the carrying polymer in the nanofibers of the present invention is in the range of 50% (w/w) to 99% (w/w), preferably in the range of 70 % (w/w) to 90% (w/w), more preferably 80% (w/w).

The nanofibers according to the invention can comprise biologically compatible polymer relatively easily spinnable and biologically compatible polymer preferably carboxymethyl cellulose (CMC) gelatin, chitosan, polycaprolactone (PCL), polylactic acid (PLA), polyamide (PA), polyurethane (PUR), poly-(lactide-co-glycolic) acid; and their mixture or their copolymers.

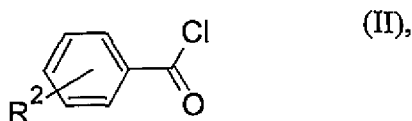
Another aspect of the present invention is a method of synthesis of nanofibers of the present invention containing the photocurable ester derivative of hyaluronic acid or its salt of the general formula IV as defined above comprising an activation reaction of α,β -unsaturated substituted acrylic acid of the general formula I



wherein

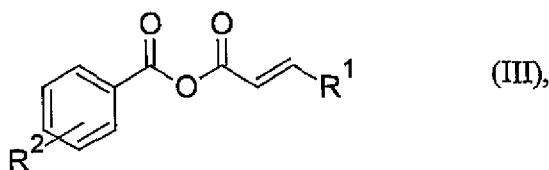
R^1 is 5- or 6-membered aryl or 5- or 6-membered heteroaryl having at least one or more identical or different heteroatoms selected from the group comprising N, O, S;

with substituted or non-substituted benzoyl chloride or its derivatives of the general formula II



wherein R^2 is one or more substituents selected from a group comprising H, $-\text{NO}_2$, $-\text{COOH}$,

halides, C_1 - C_6 alkylkoxy, preferably halides, methoxy or ethoxy, more preferably Cl; in the presence of an organic base that means aliphatic amine preferably a tertiary amine having a linear or branched, saturated or unsaturated, substituted or non-substituted C_3 - C_{30} alkyl group, preferably tertiary amine, more preferably triethylamine (TEA), diisopropylethylamine (DIPEA), N,N-Diisopropylethylamine (DIEA); a mixture of water and water-miscible polar or non-polar solvent to form a reactive anhydride of the general formula III



wherein

R^1 and R^2 are as defined above, (see also Scheme 2 below, Step A, an activation step), that reacts with hyaluronic acid or a salt thereof in the presence of an organic base, that means aliphatic amine preferably a tertiary amine having a linear or branched, saturated

or unsaturated, substituted or non-substituted C₃-C₃₀ alkyl group, preferably tertiary amine, more preferably triethylamine or diisopropylethylamine; a mixture of water and water-miscible polar or non-polar solvent, (see also Scheme 2 below, Step B, a modification step), to form the photocurable ester derivative of hyaluronic acid or its salt of the general formula

5 IV as defined above that is dissolved in the carrying polymer of molecular weight from 5×10^4 g/mol to 1×10^6 g/mol, preferably 4×10^5 g/mol in water to form a spinning solution that is spun by electrospinning.

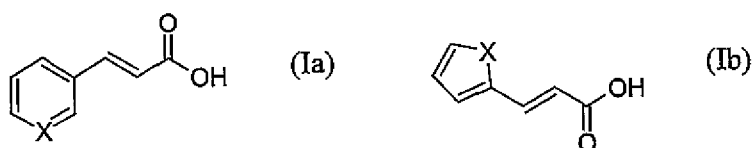
The spinning solutions can be formed with PEO of different molecular weights depending of the specific application.

10 The concentration of the photocurable ester derivative of hyaluronic acid or its salt in the spinning solution is in the range of 0.5 to 12% (w/w), preferably 5 to 7.5% (w/w), more preferably 5%(w/w).

The substituents R² of benzoyl chloride or its derivatives of the general formula II as defined above can be located in positions *ortho*-, *meta*- or *para*- to the acyl chloride-group, preferably in *ortho*- or *para*- positions. The use of benzoyl chloride and its derivatives as the

15 activators is not generally used for chemical modification of HA because it is believed that catalyzes transesterification reactions and it may react with common organic solvents used for the chemical modification of HA and respective isolation and purification, such as ethanol, methanol and higher alkyl-alcohols.

20 According to one preferred embodiment of the invention and also Scheme 2 below (see Part A), the activation step is carried out by mixing of 5-membered or 6-membered heteroaromatic acrylic acid of general formulae Ia or Ib below



wherein X is selected from O, S, N, preferably O or N, preferably 3-(2-furyl)acrylic acid, with

25 benzoyl chloride as the activating agent.

The activation step may be performed for 5 to 120 minutes at any temperature between 0 °C to 60°C. However, if the temperature is higher than 60°C, the reaction may generate sub-products, while if the reaction is cooled down the reaction will become slow but the reaction is still proceeding. Therefore, it is preferable to carry out the reaction at a temperature

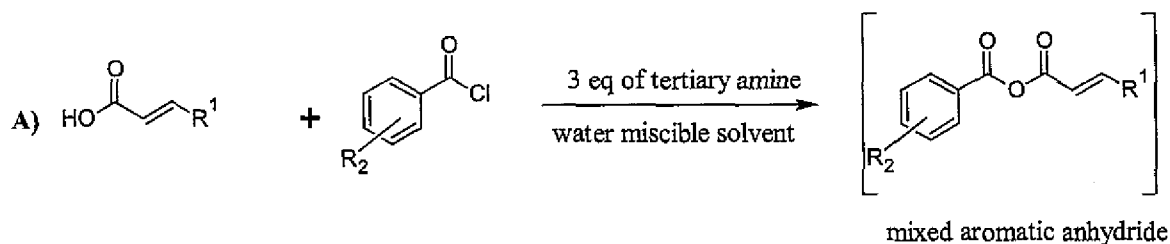
30 of 25 °C to 60 °C, more preferably for 30 minutes at 25°C.

The activation of the benzoyl chloride with any one of derivative of the Figures Ia or Ib is carried out almost instantly. However, the reactive intermediate of the structure III can

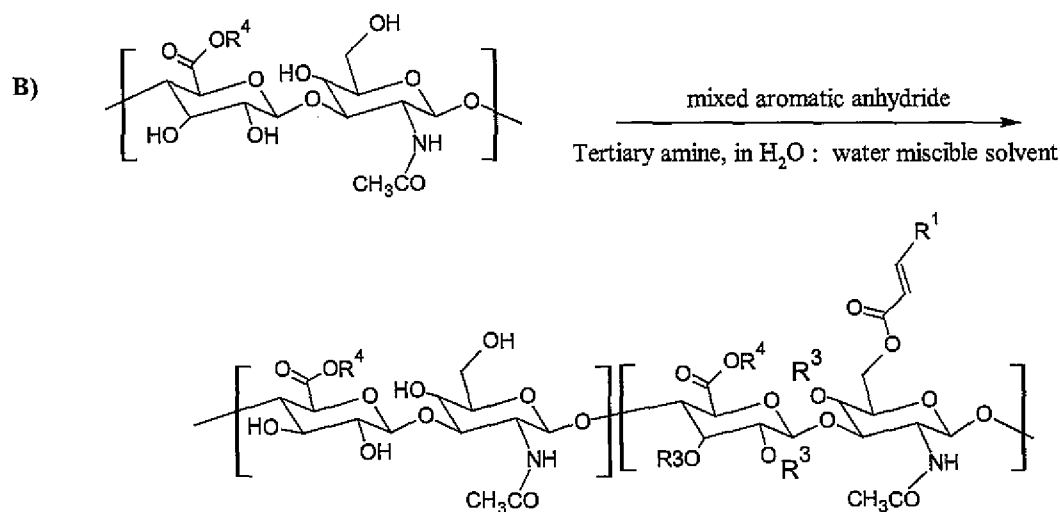
be prepared by reacting the intermediate Ia or Ib with II under vigorous stirring for two to five hours, in the presence of a tertiary amine. The reaction time can be changed due to stirring factors, whether the reaction is carried out in higher scale.

Moreover, the reaction depicted in the Part B is carried out without the presence of 4-dimethylaminopyridine (DMAP), an organocatalysts commonly used in acylation of alcohols. This catalyst is very toxic and not acceptable for materials intended for medical use. This is a great advantage of this methodology compared to the previously reported art. Additionally, it was found that using of the combination benzoyl chloride or its derivatives of the general formula II and DMAP produces higher degree of substitution (>30%) and self-crosslinking of the HA derivatives. This is undesirable since it precludes the use of the derivatives of hyaluronan for electrospinning (see Examples 14 and 16)

A) An activation of α,β -unsaturated substituted acrylic acid and a formation of the reactive anhydride – the activation step.



B) A formation of the photocurable ester of HA or its salt – the modification step



R^1 = 5 or 6 membered aryl or heteroaryl

R^3 = $-H$, or $-COCHCHR^1$

R^4 = H^+ or ions of alkaline metals or ions of alkaline-earth metals

Scheme 2

Moreover the hyaluronic acid or its salt, used in the modification step according to the method of the invention has a molecular weight (weight average of molecular weight) from 5×10^3 to $1,6 \times 10^6$ g/mol, preferably from $1,5 \times 10^4$ to $2,5 \times 10^5$ g/mol, more preferably $8,5 \times 10^4$ to $1,2 \times 10^5$ g/mol. However the method of the invention is not limited to these scopes of HA or its salt. The mean molecular weight and number average of molecular weights of the polymers were determined by SEC-MALLS as well as the polydispersity of the derivatives.

The reaction of the part B (see Scheme 2 above, the modification step) is carried out for 1 to 48 hours, preferably 1 to 3 hours; at temperatures in the range of 0 °C to 80 °C, preferably from 20 °C to 60 °C, more preferably at 25°C.

Amount of reactive anhydride of the invention mixed with HA or its salt corresponds to 0.01 to 5 equivalents, preferably 0.5 to 2 equivalents, more preferably 0,5 equivalents per dimer of hyaluronic acid or its salt to produce the photocurable ester derivative of HA or its salt of the invention having covalently bonded the photocurable group ($-\text{COCHCHR}^1$). The photocurable ester derivatives hyaluronic acid or its salt of the invention have not considerably changed the viscosity and physico-chemical characteristics of HA and they are characterized by low polydispersity. Moreover, the molecular weight of the derivatives has shown a slightly increased after the chemical modification. These derivatives were fully characterized by ^1H and HSQC-NMR, IR and UV spectroscopies.

The steps of the activation and the modification (see Scheme 2 above) are carried out as described above in the mixture of water and a water miscible polar or non-polar solvent miscible with water. The solvent is selected from a group comprising isopropanol, acetone, ethyl acetate, dimethyl sulfoxide, acetonitrile, dimethylformamide, tetrahydrofurane, but is preferably the use of isopropanol. The amount of water in the mixture water and water miscible polar solvent is from 10 %v/v to 99 %v/v, preferably 50 %v/v.

Additionally, this methodology of the above described method of the invention allows an easily upscale to allow the preparation of nanofibers of the invention comprising higher amounts of the photocurable ester HA-derivatives.

The nanofibers in accordance to the present invention can be prepared for example by electro-spinning in the device for preparation of nanofibers (for example 4SPIN®, CONTIPRO-group s.r.o.) or by another electrospinning method known in the art, for example uniaxial electrospinning, coaxial electrospinning, or multiaxial electrospinning. The characterization of the fiber mesh was effected by scanning electronic microscopy (SEM) as described in the known art and shown in Figures 3, 4, 5.

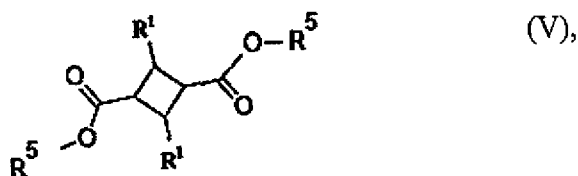
Photocurable ester derivatives of the invention possess different degree of substitution (DS). All of them were tested for the electrospinning (Table 1, Examples 22, 23 and 24). According to experimental data the ester HA-derivatives of the invention having lower degree of substitution (5-20%) are easily electrospun and also they have shown a slightly increase of viscosity due to the modification (see Figure 6). The method of chemical modification of HA
 5 as described in the modification step (see Scheme 2) is able to give the required viscosity for the electrospinning, which is very important for obtaining a large recovery (a large amount of product obtained from the process).

The nanofibers of the present invention as described above are preferably gel-forming and
 10 the average diameter thereof is between 99 to 150 nm.

The obtained fibers are able to retain their structural integrity on absorption of exudate. Alternatively the biologically compatible polymers contained in the nanofibers of the present invention increase the mechanical stability of the nanofibers as itself as well as of the final preparation.

15 The gel-forming fibers have preferably an absorbency in the range of at least 2 to 25 grams of 0.9% saline solution per gram of nanofiber (as measured by the free swell method), preferably at least 10 g/g. Such low water absorption capacity (about 20%) means that the materials made thereof are mechanically robust.

Additionally according to another embodiment nanofibers of the invention nanofibers
 20 as defined above can be photocured by the light irradiating within the range of UV-Vis wavelengths whereby cycloaddition of at least two ester groups of at least two molecules of hyaluronic acid or its salt of the formula IV occurs, forming the compound having cyclobutane ring of the general formula V



25 wherein

R^1 is as defined above and

R^5 is a backbone chain of hyaluronic acid or its salt.

The light irradiating is done in the range of UV-Vis wavelengths, preferably in the range from 280 nm to 750 nm, more preferably at 302 nm. The irradiation according to the invention is preferably carried out at room temperature, in an exposure time between 2 minutes and 60 minutes, and more preferably between 3 to 10 minutes. After cross-linking the fibers mat is interconnected and forms a 3D network (See Figure 5).

Figure 7 shows UV-Vis spectrum of sodium 2-furyl acrylic hyaluronate nanofibers irradiated for different periods of time at 302 nm to induce cross-linking. Different aliquots were taken from the soluble part of the nanofibers after swelling in water. As it is observed the intensity of the absorption maxima decreased. With increase of UV irradiation time a significant decrease was observed. After exposure to UV-light for 60 minutes almost no absorbance was observed. These materials after electrospinning perfectly react in the solid state without the presence of initiators neither activators and are photo-cured. The photo-curing can be as well effected by a free radical polymerization process.

The photocured nanofibers of the invention comprising the compound having cyclobutane ring of the formula V, as defined above are preferably a part of the preparation which is in the form selected from a group comprising layer, film, mat, wound dressing or tissue scaffold used in cosmetics, medicine or regenerative medicine for example in wound care device on patches for external or internal use.

The biocompatibility of the photocurable ester derivatives of the present invention was determined by 3T3-NIH fibroblast cells cytotoxicity study (Figure 8 and 10), included as examples in this document. The biocompatibility of the products was evaluated as well as their photo-cytotoxicity (Figures 9 and 11). Thus, some examples have demonstrated that the nanofiber layer produced according to the present invention can be used to cover different wounds because the films made thereof have been in contact with fibroblasts (fibrous tissue cells) without apparent cytotoxicity. Some other cells or a combination thereof can be cultivated on the nanofiber layer. The nanofibers described in the present invention can be used for the formation of layer, film, mat, or coating that is a part of a wound dressing, or tissue scaffold form but not limited to this group.

The preparations of the present invention such as the films are not soluble in water anymore after crosslinking and can be applied to a specific point as gel or bandage made thereof. The preparation of the invention can be used in cosmetics, medicine or regenerative medicine, preferably in wound care device, or in patches for the external or internal use, as it is expected for chemical modified derivatives of HA.

Advantages of the method of synthesis the nanofibers comprising photocurable ester derivate of the invention can be found in simplicity, low cost price and also that it can be carried out at mild reaction conditions using non-toxic reagents. The derivatives of the present invention were classified as biocompatible and non-cytotoxic, thus, they are suitable for *in vivo* use. They can be easily electrospun in highly efficiency. The method of photo-curing is fast and efficient and is characterized by been effected without the use of an external catalyst or initiator.

The definitions of the terms

The term "halides" means F, Cl, Br or I.

10 The term "mixed aromatic anhydride" means substituted benzoic arylpropen-2-enoic anhydride or benzoic heteroarylpropen-2-enoic anhydride, or an organic component

characterized by the formula
$$\text{R}_x\text{C}(=\text{O})\text{O}\text{C}(=\text{O})\text{R}_y$$
, wherein $\text{R}_x \neq \text{R}_y$, in other words a non symmetrical acid anhydride.

15 The term "organic base" is an aliphatic amine preferably a tertiary amine having a linear or branched, saturated or unsaturated, substituted or non-substituted $\text{C}_3\text{-C}_{30}$ alkyl group.

The term "HA dimer" means repeating disaccharide units of hyaluronic acid. That means glucuronic acid and N-acetyl glucosamine.

20 The term "pharmaceutically acceptable salt" as used herein, means those salts of ester derivatives of HA of the invention that are safe and effective for topical *in vivo* use and that possess a desired biological activity. Pharmaceutically acceptable salts include salts of acidic or basic groups present in of ester derivatives of HA of the invention, preferably ions of alkali metals or ions of alkaline-earth metals, more preferably Na^+ , K^+ , Mg^+ or Li^+ .

25 The term "degree of substitution of the photocurable ester HA-derivative" is the (average) number of COCHCHR^1 attached per 100 dimers of HA or it salt. For example 5 % means that 5 of each 100 dimers of HA can be substituted with a group COCHCHR^1 .

The term "photo-curing" means photo-polymerization or solid state cross-linking. The term mostly refers to a process characterized by free radical or cationic photo-initiated polymerization, which can produce a cross-linking or interpenetrating polymeric network.

30 The term "gel-forming nanofiber" means that the fibers are hygroscopic which upon the uptake of wound exudate become moist, slippery or gelatinous and thus reduce the tendency for the surrounding fibers to adhere to the wound.

The term "film" means a complex structure composed of nanofibers characterized by a high surface area and a high porosity and is prepared by electrospinning or the like technique.

These fibers are characterized by a diameter ranging from 50 nm to 1000 nm or greater. The nanofibers, which can be cross-linked or not, comprising the ester derivatives of hyaluronic acid or its salt described in this invention, can be used for coating or wrapping.

The term "carying-polymer" is referred to a polymer which is biocompatible and electrospinnable. In other words, it is a natural or synthetic polymer which is intended for pharmaceutical applications and it is not causing any undesirable or systemic toxicity.

Brief description of the drawings

Figure 1. HSQC spectra of Sodium 2-Furyl acrylate Hyaluronate obtained by the reaction described in Example 1

Figure 2. ^1H spectra of sodium *trans*-3-(3-Pyridyl) acrylic Hyaluronate

Figure 3. SEM micrographs displaying the morphology of fibrous mats after electro-spinning, observed at two different amplifications (A) 2 μm and (B) 20 μm (C) after 10 minutes irradiation (D) after 20 minutes irradiation (302 nm), which corresponds to Example 22, Table 1 (Example 2)

Figure 4. SEM micrographs displaying the morphology of fibrous mats electro-spun which corresponds to material described in Example 22, Table 1 (Example 3), observed at A) 2 μm and (B) 20 μm .

Figure 5. SEM images of fibers electrospun from samples described in Example 24

Figure 6. SEM micrographs depict the morphology of fibrous mats electro-spun after cross-linking of the derivative (10 minutes) and swelling for 24 hours in PBS, as described in Example 27.

Figure 7. Dynamic viscosity of the samples before spinning, showing a progressive increase of viscosity as a function of the degree of substitution

Figure 8. UV-Vis spectrum of Sodium 2-Furyl acrylic Hyaluronate nanofibers irradiated for different periods at 302 nm.

Figure 9. Sodium 2-Furyl acrylic Hyaluronate and its influence on cell viability tested on cells 3T3-NIH, MTT, $n=6$

Figure 10. Sodium 2-Furyl acrylic Hyaluronate and phototoxicity assay tested on concentrations ($c=1-1000\mu\text{g/ml}$), 3T3-NIH cell line, MTT, T24, $n=5$.

Figure 11. Sodium Thiophenyl-acrylic ester of Hyaluronan and its influence on cell viability tested on cells 3T3-NIH, MTT, $n=6$.

Figure 12. Sodium Thiophenyl-acrylic ester of Hyaluronan and phototoxicity assay tested on concentrations ($c=1-1000\mu\text{g/ml}$), 3T3-NIH cell line, MTT, T24, $n=5$.

Examples

Example 1. Activation of 2-Furyl acrylic acid by benzoyl chloride and reaction with Hyaluronan

5 Sodium Hyaluronate (1.0 g, 2.5 mmol) with a mean molecular weight of 1.5×10^4 g/mol was dissolved in 20 ml of distilled water. Isopropanol (IPA, 10 ml) was slowly added to that solution to avoid a possible precipitation of the polysaccharide. Subsequently, 0.7 ml of triethylamine corresponding to 2 equivalents to HA dimer was added to the reaction mixture. In a second flask, 0.17 g (1.25 mmol) of 2-furyl-acrylic acid was dissolved in isopropanol (10
10 ml). After that 0.7 ml of triethylamine (TEA) (corresponding to 2 equivalents to HA dimer) were added to the reaction, followed by the addition of 0.15 ml of benzoyl chloride (corresponding to 0.5 equivalents of HA dimer). The activation was carried out for 30 minutes at room temperature (25°C). After that, the solution was added to the first solution (containing HA). The reaction is carried out for 3 hours at room temperature. The reaction was finished by
15 the addition of water, and a solution of sodium chloride. After that the product was precipitated with an excess of isopropanol (250 ml). The product was washed one time by using 50 ml of isopropanol (100%) after that 4 times washed using 50 ml of mixtures of isopropanol and water (85% v/v). Finally, the precipitate was washed three times more with 100% isopropanol and dried in an oven at 40°C. Yield of the reaction 95%, Mw of the
20 derivative (SEC-MALLS) = 1.21×10^4 g/mol and polydispersity = 1.19.

Chemical description of the derivative

Degree of substitution was calculated to be 2.5% according to the ^1H NMR.

^1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H),
25 7.62(d,1H), 7.95(d, 1H)

2D HSQC (Figure 2), δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D_2O): 6.40-7.95 ppm: -11 to -12 $\log(\text{m}^2/\text{s})$, 1.90-4.70 ppm: -11 -12
30 $\log(\text{m}^2/\text{s})$

UV/vis (0.05 %; H_2O) λ_{max} = 309 nm.

Example 2. Activation of 2-Furyl acrylic acid by benzoyl chloride and reaction with Hyaluronan

Sodium Hyaluronate (1.0 g, 2.5 mmol) with a mean molecular weight of 4.0×10^4 g/mol was dissolved in 20 ml of distilled water. Isopropanol (10 ml) was slowly added to that solution to avoid a possible precipitation of the polysaccharide. Subsequently, 0.7 ml of triethylamine corresponding to 2 equivalents to HA dimer was added to the reaction mixture. In a second flask, 0.17 g (1.25 mmol) of 2-furyl-acrylic acid was dissolved in isopropanol (10 ml). After that 0.7 ml of TEA (corresponding to 2 equivalents to HA dimer) were added to the reaction, followed by the addition of 0.15 ml of benzoyl chloride (corresponding to 0.5 equivalents of HA dimer). The activation was carried out for 30 minutes at room temperature (25°C). After that, the solution was added to the first solution (containing HA). The reaction is carried out for 3 hours at room temperature. The reaction was finished by the addition of water, and a solution of sodium chloride. After that the product was precipitated with an excess of isopropanol (250 ml). The product was washed one time by using 50 ml of isopropanol (100%) after that 4 times using 50 ml of mixtures of isopropanol and water (85% v/v). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. The yield of the reaction 98.8%. %, Mw of the derivative (SEC-MALLS) = 4.2×10^4 g/mol and polydispersity = 1.5.

Chemical description of the derivative

Degree of substitution was calculated to be 3.5 mol % according to the ^1H NMR.

^1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)

2D HSQC (Figure 2), δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47 132.2 (signal 3), 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D_2O): 6.40-7.95 ppm: -11 to -12 $\log(\text{m}^2/\text{s})$, 1.90 4.70 ppm: -11 -12 $\log(\text{m}^2/\text{s})$

UV/vis (0.05 %; H_2O) $\lambda_{\text{max}} = 309$ nm.

Example 3. Activation of 2-Furyl acrylic acid by benzoyl chloride and reaction with Hyaluronan

Sodium Hyaluronate (1.0 g, 2.5 mmol) with a mean molecular weight of 8.0×10^4 g/mol was dissolved in 20 ml of distilled water. Isopropanol (10 ml) was slowly added to that solution to avoid a possible precipitation of the polysaccharide. Subsequently, 0.7 ml of triethylamine corresponding to 2 equivalents to HA dimer was added to the reaction mixture. In a second flask, 0.17 g (1.25 mmol) of 2-furyl-acrylic acid was dissolved in isopropanol (10 ml). After that 0.7 ml of TEA (corresponding to 2 equivalents to HA dimer) were added to the reaction, followed by the addition of 0.15 ml of benzoyl chloride (corresponding to 0.5 equivalents of HA dimer). The activation was carried out for 30 minutes at room temperature (25°C). After that, the solution was added to the first solution (containing HA). The reaction is carried out for 3 hours at room temperature. The reaction was finished by the addition of water, and a solution of sodium chloride. After that the product was precipitated with an excess of isopropanol (250 ml). The product was washed one time by using 50 ml of isopropanol (100%) after that 4 times using 50 ml of mixtures of isopropanol and water (85% v/v). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. Yield of the reaction 92.8%, Mw of the derivative (SEC-MALLS) = 8.5×10^4 g/mol and polydispersity = 1.5.

Chemical description of the derivative

Degree of substitution was calculated to be 5.3 % according to the ^1H NMR.

^1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)

2D HSQC (Figure 2), δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62 129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D_2O): 6.40-7.95 ppm: -11 to -12 log (m^2/s), 1.90-4.70 ppm: -11 -12 log (m^2/s)

UV/vis (0.05 %; H_2O) λ_{max} = 309 nm.

Example 4. Activation of 2-Furyl acrylic acid by benzoyl chloride and reaction with Hyaluronan

Sodium Hyaluronate (30.0 g, 75 mmol) with a mean molecular weight of 8.3×10^4 g/mol was dissolved in 500 ml of distilled water. Isopropanol (250 ml) was slowly added to that solution to avoid a possible precipitation of the polysaccharide. Subsequently, 21 ml of triethylamine

corresponding to 2 equivalents to HA dimer was added to the reaction mixture. In a second flask, 10.37 g (75 mmol) of 2-furyl-acrylic acid was dissolved in isopropanol (50 ml). After that 21 ml of TEA (corresponding to 2 equivalents to HA dimer) were added to the reaction, followed by the addition of 9 ml of benzoyl chloride (corresponding to 0.5 equivalents of HA dimer). The activation was carried out for 30 minutes at room temperature (25°C). After that, the solution was added to the first solution (containing HA). The reaction is carried out for 3 hours at room temperature. The reaction was finished by the addition of water, and a solution of sodium chloride. After that the product was precipitated with an excess of isopropanol (2500 ml). The product was washed one time by using 300 ml of isopropanol (100%) after that 4 times using 200 ml of mixtures of isopropanol and water (85% v/v). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. Yield of the reaction 92.8%, Mw of the derivative (SEC-MALLS) = 8.9×10^4 g/mol and polydispersity= 1.5

15 Chemical description of the derivative

Degree of substitution was calculated to be 10 % according to the ^1H NMR.

^1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)

2D HSQC (Figure 2), δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D_2O): 6.40-7.95 ppm: -11 to -12 log (m²/s), 1.90-4.70 ppm: -11 -12 log (m²/s)

UV/vis (0.05 %; H_2O) λ_{max} = 309 nm.

25 Example 5. Activation of 2-Furyl acrylic acid by benzoyl chloride and reaction with Hyaluronan

Sodium Hyaluronate (1.0 g, 2.5 mmol) with a mean molecular weight of 2.7×10^5 g/mol was dissolved in 20 ml of distilled water. Isopropanol (10 ml) was slowly added to that solution to avoid a possible precipitation of the polysaccharide. Subsequently, 0.7 ml of triethylamine corresponding to 2 equivalents to HA dimer was added to the reaction mixture. In a second flask, 0.346 g (2.5 mmol) of 2-furyl-acrylic acid was dissolved in isopropanol (10 ml). After that 0.7 ml of TEA (corresponding to 2 equivalents to HA dimer) were added to the reaction,

followed by the addition of 0.30 ml of benzoyl chloride (corresponding to 0.5 equivalents of HA dimer). The activation was carried out for 30 minutes at room temperature (25°C). After that, the solution was added to the first solution (containing HA). The reaction is carried out for 3 hours at room temperature. The reaction was finished by the addition of water, and a solution of sodium chloride. After that the product was precipitated with an excess of isopropanol (250 ml). The product was washed one time by using 50 ml of isopropanol (100%) after that 4 times using 50 ml of mixtures of isopropanol and water (85% v/v). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. Yield of the reaction 92.5%, Mw of the derivative (SEC-MALLS) = 2.9×10^5 g/mol and polydispersity = 1.65

Chemical description of the derivative

Degree of substitution was calculated to be 17 % according to the ^1H NMR.

^1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)

2D HSQC (Figure 2), δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D_2O): 6.40-7.95 ppm: -11 to -12 log (m^2/s), 1.90-4.70 ppm: 11 -12 log (m^2/s)

UV/vis (0.05 %; H_2O) λ_{max} = 309 nm.

Example 6. Activation of 2-Furyl acrylic acid by benzoyl chloride and reaction with Hyaluronan

Sodium Hyaluronate (1.0 g, 2.5 mmol) with a mean molecular weight of 5.0×10^5 g/mol was dissolved in 17 ml of distilled water. Isopropanol (10 ml) was slowly added to that solution to avoid a possible precipitation of the polysaccharide. Subsequently, 1.0 ml of triethylamine corresponding to 3 equivalents to HA dimer was added to the reaction mixture. In a second flask, 0.173 g (1.25 mmol) of 2-furyl-acrylic acid was dissolved in isopropanol (10 ml). After that 1.0 ml of TEA (corresponding to 3 equivalents to HA dimer) were added to the reaction, followed by the addition of 0.15 ml of benzoyl chloride (corresponding to 0.5 equivalents of HA dimer). The activation was carried out for 30 minutes at room temperature (25°C). After that, the solution was added to the first solution (containing HA). The reaction is carried out

for 3 hours at room temperature. The reaction was finished by the addition of water, and a solution of sodium chloride. After that the product was precipitated with an excess of isopropanol (250 ml). The product was washed one time by using 50 ml of isopropanol (100%) after that 4 times using 50 ml of mixtures of isopropanol and water (85% v/v).
 5 Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. Yield of the reaction 95.8%, Mw of the derivative (SEC-MALLS) = 5.2×10^5 g/mol and polydispersity = 1.65

Chemical description of the derivative

- 10 Degree of substitution was calculated to be 14 % according to the ^1H NMR.
 ^1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)
 2D HSQC (Figure 2), δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).
 15 H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.
 DOSY NMR (500 MHz, D_2O): 6.40-7.95 ppm: -11 to -12 $\log(\text{m}^2/\text{s})$, 1.90-4.70 ppm: -11 -12 $\log(\text{m}^2/\text{s})$
 UV/vis (0.05 %; H_2O) $\lambda_{\text{max}} = 309$ nm.

20 Example 7. Activation of 2-Furyl acrylic acid by benzoyl chloride and reaction with Hyaluronan

- Sodium Hyaluronate (1.0 g, 2.5 mmol) with a mean molecular weight of 1.0×10^6 g/mol was dissolved in 20 ml of distilled water. Isopropanol (10 ml) was slowly added to that solution to
 25 avoid a possible precipitation of the polysaccharide. Subsequently, 0.7 ml of triethylamine corresponding to 2 equivalents to HA dimer was added to the reaction mixture. In a second flask, 0.17 g (1.25 mmol) of 2-furyl-acrylic acid was dissolved in isopropanol (10 ml). After that 0.7 ml of TEA (corresponding to 2 equivalents to HA dimer) were added to the reaction, followed by the addition of 0.15 ml of benzoyl chloride (corresponding to 0.5 equivalents of
 30 HA dimer). The activation was carried out for 30 minutes at room temperature (25°C). After that, the solution was added to the first solution (containing HA). The reaction is carried out for 3 hours at room temperature. The reaction was finished by the addition of water, and a solution of sodium chloride. After that the product was precipitated with an excess of isopropanol (250 ml). The product was washed one time by using 50 ml of isopropanol

(100%) after that 4 times using 50 ml of mixtures of isopropanol and water (85% v/v). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. Yield of the reaction 95.8%, Mw of the derivative (SEC-MALLS) = 1.0×10^6 g/mol and polydispersity = 1.8

5

Chemical description of the derivative

Degree of substitution was calculated to be 4.8 % according to the ^1H NMR.

^1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d, 1H), 7.95(d, 1H)

10 2D HSQC (Figure 2), δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D_2O): 6.40-7.95 ppm: -11 to -12 $\log(\text{m}^2/\text{s})$, 1.90-4.70 ppm: -11 -12 $\log(\text{m}^2/\text{s})$

15 UV/vis (0.05 %; H_2O) $\lambda_{\text{max}} = 309$ nm.

Example 8. Activation of 2-Furyl acrylic acid by benzoyl chloride and reaction with Hyaluronan

20 Sodium Hyaluronate (1.0 g, 2.5 mmol) with a mean molecular weight of 1.6×10^6 g/mol was dissolved in 20 ml of distilled water. Isopropanol (10 ml) was slowly added to that solution to avoid a possible precipitation of the polysaccharide. Subsequently, 0.7 ml of triethylamine corresponding to 2 equivalents to HA dimer was added to the reaction mixture. In a second flask, 0.17 g (1.25 mmol) of 2-furyl-acrylic acid was dissolved in isopropanol (10 ml). After
25 that 0.7 ml of TEA (corresponding to 2 equivalents to HA dimer) were added to the reaction, followed by the addition of 0.15 ml of benzoyl chloride (corresponding to 0.5 equivalents of HA dimer). The activation was carried out for 30 minutes at room temperature (25°C). After that, the solution was added to the first solution (containing HA). The reaction is carried out for 3 hours at room temperature. The reaction was finished by the addition of water, and a
30 solution of sodium chloride. After that the product was precipitated with an excess of isopropanol (250 ml). The product was washed one time by using 50 ml of isopropanol (100%) after that 4 times using 50 ml of mixtures of isopropanol and water (85% v/v). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at

40°C. Yield of the reaction 95.8%, Mw of the derivative (SEC-MALLS) = 1.7×10^6 g/mol and polydispersity = 1.7

Chemical description of the derivative

- 5 Degree of substitution was calculated to be 4.2 % according to the ^1H NMR.
1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)
2D HSQC (Figure 2), δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).
10 H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19 7.62ppm, 7.47-7.19 ppm.
DOSY NMR (500 MHz, D_2O): 6.40-7.95 ppm: -11 to -12 log (m^2/s), 1.90-4.70 ppm: 11 -12 log (m^2/s)
UV/vis (0.05 %; H_2O) λ_{max} = 309 nm.

15 **Example 9. Activation of 2-Furyl acrylic acid by benzoyl chloride and reaction with Hyaluronan**

- Sodium Hyaluronate (1.0 g, 2.5 mmol) with a mean molecular weight of 1.6×10^6 g/mol was dissolved in 20 ml of distilled water. Isopropanol (10 ml) was slowly added to that solution to
20 avoid a possible precipitation of the polysaccharide. Subsequently, 0.7 ml of triethylamine corresponding to 2 equivalents to HA dimer was added to the reaction mixture. In a second flask, 0.259 g (1.88 mmol) or 0.75 equivalents of 2-furyl-acrylic acid was dissolved in isopropanol (10 ml). After that 0.7 ml of TEA (corresponding to 2 equivalents to HA dimer) were added to the reaction, followed by the addition of 0.21 ml of benzoyl chloride
25 (corresponding to 0.75 equivalents of HA dimer). The activation was carried out for 30 minutes at room temperature (0°C). After that, the solution was added to the first solution (containing HA). The reaction is carried out for 3 hours at 60°C. The reaction was finished by the addition of water, and a solution of sodium chloride. After that the product was precipitated with an excess of isopropanol (250 ml). The product was washed one time by
30 using 50 ml of isopropanol (100%) after that 4 times using 50 ml of mixtures of isopropanol and water (85% v/v). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. Yield of the reaction 80%. The average molecular weight of the derivative was determined as 1.59×10^6 and polydispersity of 1.52.

Chemical description of the derivative

Degree of substitution was calculated to be 10 % according to the ^1H NMR.

^1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)

- 5 2D HSQC (Figure 2), δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62 129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D_2O): 6.40-7.95 ppm: -11 to -12 $\log(\text{m}^2/\text{s})$, 1.90 4.70 ppm: 11 -12 $\log(\text{m}^2/\text{s})$

- 10 UV/vis (0.05 %; H_2O) $\lambda_{\text{max}} = 309 \text{ nm}$.

Example 10. Activation of 2-Furyl acrylic acid by benzoyl chloride and reaction with Hyaluronan

- 15 Sodium Hyaluronate (1.0 g, 2.5 mmol) with a mean molecular weight of $1.8 \times 10^6 \text{ g/mol}$ was dissolved in 10 ml of distilled water. Isopropanol (10 ml) was slowly added to that solution to avoid a possible precipitation of the polysaccharide. Subsequently, 0.7 ml of triethylamine corresponding to 2 equivalents to HA dimer was added to the reaction mixture. In a second flask, 0.34 g (2.5 mmol) of 2-furyl-acrylic acid was dissolved in isopropanol (10 ml). After
20 that 0.7 ml of TEA (corresponding to 2 equivalents to HA dimer) were added to the reaction, followed by the addition of 0.29 ml of benzoyl chloride (corresponding to 1.0 equivalents of HA dimer). The activation was carried out for 30 minutes at room temperature (25°C). After that, the solution was filtered and added to the first solution (containing HA). The reaction is carried out for 2 hours at 40°C . The reaction was finished by the addition of water, and a
25 solution of sodium chloride. After that the product was precipitated with an excess of isopropanol (250 ml). The product was washed one time by IPA, after that with a solution IPA: water (85% v/v, 4 x 50 ml). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C . Yield of the reaction 99%, Mw of the derivative (SEC-MALLS) was determined as 1.82×10^6 and polydispersity= 1.61

30

Chemical description of the derivative

Degree of substitution (corresponding to 12% of photoreactive groups)

¹H NMR (500 MHz, D₂O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)

2D HSQC, δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).

5 **H-H COSY**: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D₂O): 6.40-7.95 ppm: -11 to -12 log (m²/s), 1.90-4.70 ppm: -11 -12 log (m²/s)

UV/vis (0.05 %; H₂O) λ_{max} = 309 nm.

10 **Example 11. Activation of 3-(2-Furyl) acrylic acid by 2, 4, 6-trichlorobenzoyl chloride and reaction with Hyaluronan**

Sodium hyaluronate (30 g, 75 mmol) with a mean molecular weight of 1x10⁶ g/mol was dissolved in 1000 ml of distilled water. THF (1000 ml) were slowly added to that solution of
 15 avoid a possible precipitation of the polysaccharide. Subsequently, 21 ml of triethylamine (TEA) was added to the reaction mixture (corresponding to 2 equivalents to HA dimer). In a second flask, 5.18 (37.4 mmol) g of 2-furfuryl-acrylic acid was dissolved in THF (300 ml). After that 21 ml of TEA (corresponding to 2 equivalents to HA dimer) were added to the reaction, followed by the addition of 0.4 ml of 2, 4, 6-trichlorobenzoyl chloride
 20 (corresponding to 0.5 equivalents to HA dimer). The activation was carried out for 30 minutes. After that, the filtrated solution (soluble part) was added to the first solution (containing HA). The reaction is carried out for 3 hours at room temperature. The reaction was finished by the addition of water, and a solution of sodium chloride. The product was washed one time by IPA, after that with a solution IPA: water (85% v/v, 4 x 500 ml). Finally,
 25 the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. For all the reactions, "yield of the reaction" is reported. Yield of the reaction: 98% (average of three repetitions).

Chemical description of the derivative

30 Degree of substitution (corresponding to 3% of photosensitive groups)

¹H NMR (500 MHz, D₂O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)

2D HSQC, δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62 ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D₂O): 6.40-7.95 ppm: -11 to -12 logs (m²/s), 1.90-4.70 ppm: -11 -12 log (m²/s).

UV/vis (0.05 %; H₂O) λ_{max} = 309 nm.

Example 12. Activation of 3-(2-Furyl) acrylic acid by 2, 4, 6-trichlorobenzoyl chloride and reaction with Hyaluronan

Sodium hyaluronate (30 g, 75 mmol) with a mean molecular weight of 8.5×10^4 g/mol was dissolved in 600 ml of distilled water. THF (300 ml) were slowly added to that solution of avoid a possible precipitation of the polysaccharide. Subsequently, 21 ml of triethylamine (TEA) was added to the reaction mixture (corresponding to 2 equivalents to HA dimer). In a second vessel, 10.36 g (75 mmol) of 2-furfuryl-acrylic acid were dissolved in THF (300 ml). After that 21 ml of TEA (corresponding to 2 equivalents to HA dimer) were added to the reaction, followed by the addition of 12 ml of 2, 4, 6-trichlorobenzoyl chloride (corresponding to 1 equivalent to HA dimer). The activation was carried out for 30 minutes. After that, the filtrated solution (soluble part) was added to the first solution (containing HA). The reaction is carried out for 3 hours at room temperature. The reaction was finished by the addition of water, and a solution of sodium chloride. The product was washed one time by IPA, after that with a solution IPA: water (85% v/v, 4 x 500 ml). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. For all the reactions, "yield of the reaction" is reported. That means the percentage gain/loss of the product compared to the amount of weight HA-Na in the reaction. A yield of the reaction was 91% (average of three reactions). Determination of Molecular weight by SEC-MALLS is 9.0×10^4 g/mol and polydispersity= 1.52

Chemical description of the derivative

Degree of substitution (corresponding to 6.2% of photosensitive groups)

¹H NMR (500 MHz, D₂O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)

2D HSQC, δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D₂O): 6.40-7.95 ppm: -11 to -12 log (m²/s), 1.90-4.70 ppm: -11 -12

5 log (m²/s)

UV/vis (0.05 %; H₂O) λ_{max} = 309 nm.

Example 13. Activation of 3-(2-Furyl) acrylic acid by benzoyl chloride and reaction with Hyaluronan

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Sodium hyaluronate (30 g, 75 mmol) with a mean molecular weight of 8.5×10^4 g/mol was dissolved in 600 ml of distilled water. THF (300 ml) were slowly added to that solution of avoid a possible precipitation of the polysaccharide. Subsequently, 31ml of triethylamine (TEA) was added to the reaction mixture (corresponding to 3 equivalents to HA dimer). In a second flask, 20.73 g of 2-furfuryl-acrylic acid corresponding to 2 equivalents of HA dimer were dissolved in THF (300 ml). After that 31 ml of TEA (corresponding to 3 equivalents to HA dimer) were added to the reaction, followed by the addition of 0.2 ml of benzoyl chloride (corresponding to 2 equivalents to HA dimer). The activation was carried out for 30 minutes at 0°C. After that, the filtrated solution (soluble part) was added to the first solution (containing HA). The reaction is carried out for 3 hours at 0°C. The reaction was finished by the addition of water, and a solution of sodium chloride. After that the product was precipitated with an excess of isopropanol (1000 ml). The product was washed one time by IPA, after that with a solution IPA: water (85% v/v, 4 x 500 ml). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. Determination of

15

20

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Molecular weight by SEC-MALLS is 9.0×10^4 g/mol and polydispersity= 1.52.

Chemical description of the derivative

Degree of substitution (corresponding to $18 \pm 2\%$ of photosensitive groups)

¹H NMR (500 MHz, D₂O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H),

30 7.62(d,1H), 7.95(d, 1H)

2D HSQC, δ ppm: 6.4-114.9 (cross peak, signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D₂O): 6.40-7.95 ppm: -11 to -12 log (m²/s), 1.90-4.70 ppm: -11 -12 log (m²/s)

UV/vis (0.05 %; H₂O) λ_{\max} = 309 nm.

5 Example 14. Activation of 3-(2-Furyl) acrylic acid by 2, 4, 6-trichlorobenzoyl chloride and addition to Hyaluronan, wherein reaction is catalyzed by DMAP

Sodium hyaluronate (30 g, 75 mmol) with a mean molecular weight of 8.5×10^4 g/mol was dissolved in 600 ml of distilled water. THF (300 ml) were slowly added to that solution of
10 avoid a possible precipitation of the polysaccharide. Subsequently, 31 ml of triethylamine (TEA) was added to the reaction mixture (corresponding to 3 equivalents to HA dimer), followed by 0,458 g of 4-Dimethyl aminopyridine (DMAP), corresponding to 0.05 equivalents of HA dimer. In a second flask, 31 g of 2-furfuryl-acrylic acid corresponding to 3 equivalents of HA dimer were dissolved in THF (300 ml). After that 31 ml of TEA
15 (corresponding to 3 equivalents to HA dimer) were added to the reaction, followed by the addition of 35.1 ml of 2, 4, 6 trichlorobenzoyl chloride (corresponding to 3 equivalents to HA dimer). The activation was carried out for 30 minutes. After that, the filtrated solution (soluble part) was added to the first solution (containing HA). The reaction is carried out for 3 hours at room temperature. The reaction was finished by the addition of water, and a solution of
20 sodium chloride. After that the product was precipitated with an excess of isopropanol (1000 ml). The product was washed one time by IPA, after that with a solution IPA: water (85% v/v, 4 x 250 ml). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. For all the reactions, we have reported "yield of the reaction". That means the percentage gain/loss of the product compared to the amount of weight HA-Na in the
25 reaction. Yield of the reaction: 99%. Due to the high viscosity of the derivative, the molecular weight cannot be determined by SEC-MALLS methodology due to possible cross-linking.

Chemical description of the derivative

30 Degree of substitution (corresponding to 30-40% of photosensitive groups)

¹H NMR (500 MHz, D₂O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)

2D HSQC, δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3), 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D₂O): 6.40-7.95 ppm: -11 to -12 log (m²/s), 1.90-4.70 ppm: -11 -12

5 log (m²/s)

UV/vis (0.05 %; H₂O) λ_{\max} = 309 nm.

Example 15. Activation of 2-thienyl acrylic acid by 2, 4, 6-trichlorobenzoyl chloride and reaction with Hyaluronan

10 Sodium Hyaluronate (5.0 g, 12.5 mmol) with a mean molecular weight of 1.01×10^5 g/mol was dissolved in 100 ml of distilled water. THF (50 ml) were slowly added to that solution of avoid a possible precipitation of the polysaccharide. Subsequently, 3.5 ml of triethylamine corresponding to 2 equivalents to HA dimer was added to the reaction mixture. In a second flask, 1.0 g of 2-thienyl-acrylic acid was dissolved in THF (50 ml). After that 2.5 ml of TEA
15 (corresponding to 3 equivalents to HA dimer) were added to the reaction, followed by the addition of 0.95 ml of benzoyl chloride (corresponding to 0.5 equivalents of HA dimer). The activation was carried out for 30 minutes at 60°C. After that, the solution was filtered and added to the solution containing HA. The reaction mixture was stirred out for 3 hours at 60°C. The reaction was finished by the addition of water, and a solution of sodium chloride. After
20 that the product was precipitated with an excess of isopropanol (250 ml). The product was washed one time by IPA, after that with a solution IPA: water (85% v/v, 4 x 50 ml). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. Yield of the reaction 92.18%, Mw of the derivative (SEC-MALLS) = 1.08×10^5 and polydispersity= 1.482

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Chemical description of the derivative

Degree of substitution (corresponding to 5% of photosensitive groups)

¹H NMR (500 MHz, D₂O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H),

7.62(d,1H), 7.95(d, 1H)

30 **2D HSQC**, δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3),

7.62-129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D₂O): 6.40-7.95 ppm: -11 až -12 log (m²/s), 1.90-4.70 ppm: -11 -12 log (m²/s)

UV/vis (0.05 %; H₂O) λ_{\max} = 314 nm.

5 Example 16. Activation of 3-(2-Thienyl) acrylic acid by 2, 4, 6-trichlorobenzoyl chloride and addition to Hyaluronan, wherein reaction is catalyzed by DMAP

Sodium Hyaluronate (5.0 g, 12.5 mmol) with a mean molecular weight of 1.01×10^5 g/mol was dissolved in 100 ml of distilled water. THF (50 ml) were slowly added to that solution of
 10 avoid a possible precipitation of the polysaccharide. Subsequently, 5.2 ml of triethylamine corresponding to 3 equivalents to HA dimer was added to the reaction mixture, followed by 0.076 g of 4-dimethyl aminopyridine (DMAP). In a second flask, 6.36 g of 2-thienyl-acrylic acid was dissolved in THF (50 ml). After that 5.2 ml of TEA (corresponding to 3 equivalents to HA dimer) were added to the reaction, followed by the addition of 5.8 ml of 2, 4, 6
 15 trichlorobenzoyl chloride (corresponding to 3 equivalents of HA dimer). The activation was carried out for 30 minutes at room temperature (25°C). After that, the solution was filtered and added to the first solution (containing HA). The reaction is carried out for 3 hours at room temperature. The reaction was finished by the addition of water, and a solution of sodium chloride. The product was washed one time by IPA, after that with a solution IPA: water (85%
 20 v/v, 4 x 50 ml). Finally, the precipitate was washed three times more with 100% IPA and dried in an oven at 40°C. Yield of the reaction 95%. Due to the high viscosity of the derivative, the molecular weight cannot be determined by SEC-MALLS methodology

Chemical description of the derivative

25 Degree of substitution (corresponding to 30% of photosensitive groups)

¹H NMR (500 MHz, D₂O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)

2D HSQC, δ ppm: 6.4-114.9 (signal 1), 7.18-128.6 (signal 4), 7.47-132.2 (signal 3),
 30 7.62-129.7 (signal 5), 7.95-139.1 (signal 2).

H-H COSY: cross-peak signal 6.40-7.95 ppm, 7.19-7.62ppm, 7.47-7.19 ppm.

DOSY NMR (500 MHz, D₂O): 6.40-7.95 ppm: -11 až -12 log (m²/s), 1.90-4.70 ppm: -11 -12 log (m²/s)

UV/vis (0.05 %; H₂O) λ_{max} = 314 nm.

Example 17. Activation of 3-(2-Furanyl) acrylic acid by 4-methoxybenzoyl chloride and reaction with Hyaluronan

- 5 1.0 g of Hyaluronic acid sodium salt (2.5 mmol, 8.5×10^4 g/mol) was dissolved in 20 ml of distilled water. Tetrahydrofuran (10 ml) was slowly added to that solution followed by 0.7 ml of triethylamine (5 mmol). In a second flask, 0.17 g of Furyl acrylic acid (1.25 mmol) was solubilized in 10 ml of tetrahydrofuran, after 5 minutes of mixing 0.7 ml of triethylamine (TEA) were added, followed by 0.17 ml of p-methoxy benzoyl chloride (1.25 mmol). The
- 10 activation was allowed to occur for 30 minutes. After that, the second solution was added to the first one (containing Hyaluronic acid). The mixture was allowed to react for 2 hours at room temperature. After that, 200 ml of absolute isopropanol were added, followed by 1 ml of super saturated solution of sodium chloride. The product was washed out 3 times by 100% isopropanol, and 4 times with a solution of isopropanol: water (85:15) and 2 times once more
- 15 with 100% isopropanol. The reaction product was dried in an oven overnight and characterized. Determination of Molecular weight by SEC-MALLS is 9.5×10^4 g/mol and polydispersity= 1.52

Chemical description of the derivative

- 20 Degree of substitution (corresponding to 1% of photosensitive groups)
- ¹H NMR (500 MHz, D₂O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H)
- UV/vis (0.05 %; H₂O) λ_{max} = 309 nm.

- 25 **Example 18. Activation of 3-(2-Furanyl) acrylic acid by 4-nitrobenzoyl chloride and reaction with Hyaluronan**

- 1.0 g of Hyaluronic acid sodium salt (2.5 mmol, 5×10^3 g/mol) was dissolved in 20 ml of distilled water. To that solution 10 ml of tetrahydrofuran were slowly added followed by 0.7
- 30 ml of triethylamine (5 mmol). In a second flask, 0.17 g of Furyl acrylic acid (1.25 mmol) was solubilized in 10 ml of tetrahydrofuran, after 5 minutes of mixing 0.7 ml of triethylamine (TEA) were added, followed by 0.23 g of p-nitro-benzoyl chloride (1.25 mmol). The

activation was allowed to occur for 30 minutes. After that, the second solution was added to the first one (containing Hyaluronic acid). The mixture was allowed to react for 2 hours at room temperature. After that, 200 ml of absolute isopropanol were added, followed by 1 ml of super saturated solution of sodium chloride. The product was washed out 3 times by absolute isopropanol, and 4 times with a solution of isopropanol: water (85:15) and 2 times with absolute isopropanol. The reaction product was dried in an oven overnight and characterized. Determination of Molecular weight by SEC-MALLS is 5.5×10^3 g/mol and polydispersity= 1.52

10 **Chemical description of the derivative**

Degree of substitution (corresponding to 1% of photosensitive groups)

^1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.40 (d, 1H), 7.18 (t, 1H), 7.47 (s, 1H), 7.62(d,1H), 7.95(d, 1H).

UV/vis (0.05 %; H_2O) λ_{max} = 309 nm.

15 **Example 19 Activation of *Trans*-3-(3-Pyridyl) acrylic acid and reaction with Hyaluronan**

1.0 g of Hyaluronic acid sodium salt (2.5 mmol, 8.5×10^4 g/mol) was dissolved in 20 ml of distilled water. Isopropanol (10 ml) was slowly added to that solution, followed by 0.7 ml of triethylamine (5 mmol). In a second flask, 0.19 g of *trans*-3-(3-Pyridyl) acrylic acid (1.25 mmol) was solubilized in 10 ml of isopropanol, after 5 minutes of mixing 0.8 ml of Diisopropylethylamine (DIPEA) were added, followed by 0.15ml of benzoyl chloride (1.25 mmol). The activation was allowed occur for 30 minutes. After that, the second solution was added to the first one (containing Hyaluronic acid). The mixture was allowed to react for 2 hours at room temperature. After that, 200 ml of absolute isopropanol were added, followed by 1 ml of super saturated solution of sodium chloride. The product was washed out 3 times by absolute isopropanol, and 4 times with a solution of isopropanol: water (85:15) and 2 times with absolute isopropanol. The reaction product was dried in an oven overnight and characterized. Determination of Molecular weight by SEC-MALLS is 9.0×10^4 g/mol and polydispersity= 1.6

30 Degree of substitution was determined as 9% (corresponding to the amount of photosensitive groups). ^1H NMR spectra is depicted in Figure 2.

Chemical description of the derivative

^1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.74 (d, 1H, $J=15.9$), 7.53 (m, 1H), 7.70 (d, 1H, $J=17.5$), 8.09 (m, 1H), 8.55 (m, 1H), 8.76 (m, 1H).

UV/vis (0.05 %; H_2O) $\lambda_{\text{max}} = 263$ nm.

5 Example 20. Activation of 3-(4-imidazolyl) acrylic acid and reaction with Hyaluronan

1.0 g of Hyaluronic acid sodium salt (2.5 mmol, 8.5×10^4 g/mol) was dissolved in 20 ml of distilled water. Isopropanol (10 ml) was slowly added to that solution, followed by 0.7 ml of triethylamine (5 mmol). In a second flask, 0.173 g of 3-(4-imidazolyl) acrylic acid (1.25 mmol) was solubilized in 10 ml of isopropanol, after 5 minutes of mixing 0.7 ml of triethylamine (TEA) were added, followed by 0.15 ml of benzoyl chloride (1.25 mmol). The activation was allowed to occur for 30 minutes. After that, the second solution was added to the first one (containing Hyaluronic acid). The mixture was allowed to react for 2 hours at room temperature. After that, 200 ml of absolute isopropanol were added, followed by 1 ml of super saturated solution of sodium chloride. The product was washed out 3 times by absolute isopropanol, and 4 times with a solution of isopropanol: water (85:15) and 2 times with absolute isopropanol. The reaction product was dried in an oven overnight and characterized.

Chemical description of the derivative

20 Degree of substitution (corresponding to 5% of photosensitive groups)

^1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.40 (m, 1H), 7.30 (m, 1H), 7.38 m, 1H), 7.52 (2H), 7.71 (m, 1H), 8.0 (1H).

UV/vis (0.05 %; H_2O) $\lambda_{\text{max}} = 286$ nm.

25 Example 21. Activation of phenyl acrylic acid by benzoyl chloride and reaction with Hyaluronic acid

1.0 g of Hyaluronic acid sodium salt (2.5 mmol, 5×10^3 g/mol) was dissolved in 10 ml of distilled water. Isopropanol (5 ml) was slowly added to that solution, followed by 0.7 ml of triethylamine (5 mmol). In a second flask, 0.37 g of phenyl acrylic acid (2.5 mmol) was solubilized in 5 ml of isopropanol, after 5 minutes of mixing 0.7 ml of triethylamine (5 mmol) were added, followed by 0.29 ml of benzoyl chloride (2.5 mmol). The activation was carried out for 30 minutes. After that, the second solution was added to the first one (containing

Hyaluronic acid). The mixture was allowed to react for 2 hours at room temperature. After that, 200 ml of absolute isopropanol were added, followed by 1 ml of super saturated solution of sodium chloride. The product was washed out 3 times by absolute isopropanol, and 4 times with a solution of isopropanol: water (85:15) and 2 times with absolute isopropanol. The reaction product was dried in an oven overnight and characterized.

Chemical description of the derivative

Degree of substitution (corresponding to 9% of photosensitive groups)

^1H NMR (500 MHz, D_2O), δ ppm, for linker: 6.64 (d, $J=16.4$, 1H), 7.50 (bs, 3H), 7.70 (m, 2H), 7.52 (2H), 7.82 (d, $J=16.5$, 1H).

UV/vis (0.05 %; H_2O) $\lambda_{\text{max}} = 281$ nm.

Example 22. Obtention of nanofibers comprising native HA and ester derivatives of HA

A solution is prepared by dissolving the derivatives described in Table 1. Thus, the solutions having a concentration of the particular ester derivative of HA of previously described examples and polyethylene oxide was 5% (w/v) in water, keeping a ratio of 80:20 (HA/PEO of 4×10^5 g/mol) were prepared and used. These nanofiber layers were formed from those solutions. The electrospinning was performed using a wide multi-nozzle using the device 4spin® (CONTIPRO, Biotech s.r.o.). However, the production of the nanofibers is not limited to that device. The nanofibers were produced by using a collecting electrode at a distance of 20 cm and varying electric voltage from 60-80kV. And a surface tension of the solution ($58.1 \text{ mN} \cdot \text{m}^{-1}$). The conductivity and viscosity of the produced solutions are resumed in Table 1. Nanofibers of different diameter size averages from 99 to 150 nm can be obtained (see Table 1). The microstructure of the nanofibers was characterized by SEM (As a selected example, the microstructure of the derivative with degree of substitution 5% corresponding to preceding examples are shown on Figures 3 and 4, respectively). The table contains as well a reference sample of nanofibers obtained in based of Native Hyaluronic acid (on Entry 1) and the ester derivatives prepared in previously described examples which formed (Examples 2,3,4,12,13) or non-formed nanofibers (described in Examples 14, 16). The last two examples are used the toxic catalyst DMAP that induces self-cross-linking of the derivatives before electrospinning. The nanofibers were at least prepared three independent times.

Table 1. Processing parameters of HA and Sodium 3-(2-Furanyl) acryloyl Hyaluronate as described in proceeding Examples.

Examples	DS _{Photo} (%)	Viscosity	Electrical conductivity $\mu\text{S cm}^{-1}$	Average fiber diameter (nm)
HA+ PEO (400,000)	0	3.54	6.2-7.3	109.1-121.68
2	4.2 \pm 2	1.25	4.1	133.1
3	5.3 \pm 2	1.40	4.9	120.7 \pm 10
12	6.2 \pm 2	1.54	4.5	127.4
4	10 \pm 2	1.79	4.9	126.5 \pm 3.5
13	18 \pm 2	1.85	4.4	141.5 \pm 15.9
14,16	30-40	3.5	4.2	no

no= non obtained

Example 23. Obtention of nanofibers comprising the ester derivative of HA prepared in the Example 3 and different composition of polyethylene oxide

- 5 Spinning solution was prepared by dissolving the derivatives of Example 3, characterized by a molecular weight of 8.6×10^4 g/mol and a polyethylene oxide of molecular weight of 6×10^5 g/mol in water. The solution was prepared by stirring for 8 hours. The used concentration was 9.7% (w/w) and the weight ratio of HA/PEO was (74/26). The spinning was carried out by electrospinning from a wide multi-nozzle in the device 4Spin® from
- 10 Contipro Biotech s.r.o, wherein the collecting electrode had an electrical voltage between (60 to 80) kV, the solution had shown a surface tension of solution of 55.1 mN.m⁻¹ and a conductivity of 7.35 mS.cm⁻¹, and viscosity of 6.72 Pa s. The average diameter of electrostatically spun nanofibers detected by scanning electron microscopy and image analysis was about 261 nm.

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Example 24. Obtention of nanofibers comprising the ester derivative of HA in the Example 3 and polyvinyl alcohol

- A solution is prepared by dissolving the derivatives described in Example 3. Thus, a concentration of 5% (w/w in water and polyvinyl alcohol (PVA) of molecular weight 4×10^5 g/mol keeping a ratio of 80:20 (HA/PVA). These nanofiber layers were formed from those
- 20 solutions. The electrospinning was performed using a wide multi-nozzle using the device 4spin® (CONTIPRO, Biotech s.r.o.). However, the production of the nanofibers is not limited

to that device. The nanofibers were produced by using a collecting electrode at a distance of 20 cm and varying electric voltage from 60-80kV. And a surface tension of the solution (58.1 mN. m⁻¹) and a conductivity of 7.35 mS.cm⁻¹, and viscosity of 6.5 Pa s. The microstructure of electrostatically spun nanofibers is shown in Figure 5

5

Example 25. Obtention of nanofibers comprising the ester derivative of HA in the Example 3 and polyvinyl pyrrolidone

A solution is prepared by dissolving the derivatives described in Example 3 in a mixture 1:1 of water and ethanol. Thus, a concentration of 5% (w/w in water and polyvinylpyrrolidone (PVP) of molecular weight 4×10^5 g/mol keeping a ratio of 80:20 (HA/PVP). These nanofiber layers were formed from those solutions. The electrospinning was performed using a wide multi-nozzle using the device 4spin® (CONTIPRO, Biotech s.r.o.). However, the production of the nanofibers is not limited to that device. The nanofibers were produced by using a collecting electrode at a distance of 20 cm and varying electric voltage from 60-80kV.

15

Example 26. Obtention of nanofibers comprising ester derivatives of HA and PEO

A solution is prepared by dissolving the derivatives described in Example 15 or with the derivative described in Example 20. Thus, the solutions having a concentration of this particular ester derivative of HA of previously described examples and polyethylene oxide was 5% (w/v) in water, keeping a ratio of 80:20 (HA/PEO of 4×10^5 g/mol) were prepared and used. These nanofiber layers were formed from those solutions. The electrospinning was performed using a wide multi-nozzle using the device 4spin® (CONTIPRO, Biotech s.r.o.). However, the production of the nanofibers is not limited to that device. The nanofibers were produced by using a collecting electrode at a distance of 20 cm and varying electric voltage from 60-80kV.

25

Example	DS _{photo} (%)	Viscosity Pa.s	Electrical conductivity $\mu\text{S cm}^{-1}$	Average fiber diameter
15	5±2	1.29	4.8	99.58 ± 10.9
20	5±2	1.35	5.2	100.22 ± 4.4

Example 27. UV-induced cross-linking of nanofibers

Different specimens of nanofiber mats were prepared as described in Example 22 and exposed to UV irradiation inside an oven designed by Eppendorf Czech & Slovakia s.r.o

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under the designation CL-1000 (302 nm). This device provides uniform UV radiation in the UV/Vis region of the spectrum is usually from 280 to 315 nm with a constant power (Approximately 6.75 mW.cm^{-2}). The maximum value of the relative radiation energy is declared at a wavelength of 302 nm. UV radiation is emitted by five parallel gas-discharge tubes, each of which has an output of 8 watts located below the upper wall. The radiation dose was $E = 24300 \text{ } \mu\text{J.cm}^{-2}$ and at a constant power (6.75 mW.cm^{-2}). The nanofibers are exposed for 5, 10, 20, 30 and 60 minutes for 10 or 60 minutes. The microstructure of the nanofibers after swelling in PBS corresponding to example 3 on the table, 1 is shown in Figure 6.

Example 28. Water content calculation of the nanofibrous material obtained thereof

Samples of circular shape with a mean diameter of 5.0 cm were cut out with a special tool. The area of the samples was determined as 0.0078 square meters. To be more precise, the initial amount of derivative was weighted to have in all the cases circa 0.2 g or 0.5g per sample respectively. Thereafter, the samples were immersed in demineralized water or saline solution with a concentration of 0.9 % (w/v) of sodium chloride dissolved in water. The samples were immersed in a definite volume of 30 ml for 0.5 g sample and 12 ml in the case of 0.2 g of sample for 5 minutes. After soaking the material, the sample is filtered through a mesh (pore size of 90-160 μm). The material was then weighted, so that the amount of absorbed water or saline solution was determined (w_s). Water absorption is determined by the formula $Q_w = (w_d - w_s / w_d) \times 100$; Where w_d and w_s are the weight of the dried sample and swollen, respectively.

Swelling media	Initial weight (g)	cross-linking time (m)	Water absorption Q_w
H ₂ O	0.2	10	20.0
H ₂ O	0.5	10	20.6
0.9% (w/v) NaCl	0.2	10	10.2
0.9%(w/v) NaCl	0.2	60	17.1

Example 29. Cell viability assay of Sodium Furan-2-acrylate ester of Hyaluronan

The biocompatibility of the esters of hyaluronic acid prepared as described in previously described examples (1-9) were examined using on 3T3-NIH cell lines. Thus, 0.1 (w/v) % solution of the derivative was dissolved in complete cell culture media thorough mixing (overnight). After that, the solution was filtered thorough sterile filtration device (0.22 μm), forming final concentrations of the derivative such as 1, 0.5 and 0.1 mg/ml. 3000 cells lines (3T3-NIH) were seeded into wells of 96-well test plates. The cells were cultured for 24 hours before treated with the tested solutions. Cell viability was measured 0, 24, 48, 72 hours after treatment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 20 μL of MTT stock solution (5 mg/ml) was added to 200 μL of cell culture medium in each well. The plates were incubated at 37°C in a cell culture incubator for 2.5 hours. MTT solution was removed and added 220 μL of lysis solution. Cells were lysed for 30 minutes at room temperature on an orbital shaker and the optical density was measured by Microplate reader VERSAmax at 570 nm. The experiment design was completed with a set of control cells cultured in common media without treatment and blank samples. MTT test was utilized to obtain basic information about cell metabolism and proliferation. Thus, these derivatives were found to be non-cytotoxic up to concentration of 1000 $\mu\text{g/ml}$.

Example 30. Evaluation of photo-cytotoxicity for the derivative Sodium Furan-2-acrylate ester of Hyaluronan

10,000 cells (3T3-NIH) were seeded into wells of 96-well test plates. The cells were cultured for 24 hours before treatment with the tested solutions in full medium. After that, the cells were rinsed with PBS and incubated with for one hour with the derivative previously dissolved in PBS (1-1000 $\mu\text{g/ml}$). The cells with derivative were irradiated with a dose of 0.1 J/cm² UVA/UVB with lamp (Oriol Instruments). Power of the lamp was determined using a photometer PMA 2100 (Solar light Co.). 10 minutes after exposure the supernatant was sucked out and full media was added. Cell viability was measured 24 hours after irradiation and using the MTT method. The experiment design was completed with a set of control cells cultured in common media without treatment and UV irradiation and also with positive and negative control. Phototoxicity assay can be utilized to identify a possible phototoxic effect of the derivative Sodium Furanyl-acrylic ester of Hyaluronan. The phototoxicity assay was repeated minimally four times and the mean (percentage) relative to the control and standard error of the means (SEMs) are calculated. Optical density was measured and the percentage relative to the control was calculated as a function of time.

Example 31. Cell viability assay of Sodium thiophenyl-acrylic ester of Hyaluronan

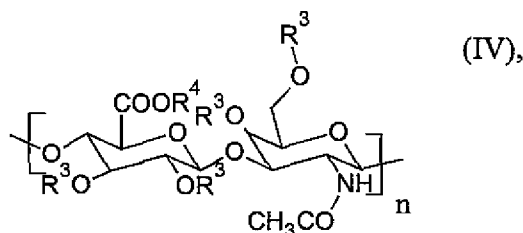
Sodium Thiophenyl-acrylic ester of Hyaluronan prepared as described in above examples and its biocompatibility was examined using on 3T3-NIH cell lines. Thus, a 0.1 (w/v) % solution of the derivative was dissolved in complete cell culture media thorough mixing (overnight). The method described in Example 7 was performed as well for this derivative. These derivatives were also found to be non-cytotoxic up to concentration of 1000 µg/mL.

Example 32. Evaluation of photo-cytotoxicity for the derivative sodium thiophenyl-acrylic ester of Hyaluronan

Sodium thiophenyl-acrylic ester of Hyaluronan prepared as described in above examples and its biocompatibility was examined using 3T3-NIH cell lines and photocytotoxicity test. The method described in Example 9 was performed as well for this derivative. This derivative was also found to be non-photocytotoxic. All derivatives have been tested as non-cytotoxic and non-photocytotoxic for 3T3-NIH cell lines. Thus, the viability of cells after their treatment has not significantly changed in whole monitored interval.

Claims

1. Nanofibers based on ester derivatives of hyaluronic acid and its salt **characterized in that** they comprise a photocurable ester derivative of hyaluronic acid or its salt of the general formula IV



wherein n is integer in the range of 1 to 5000 dimers,

R^3 are independently H or COCHCHR^1 wherein R^1 is 5- or 6-membered aryl or 5- or 6-membered heteroaryl having at least one or more identical or different heteroatoms selected from a group comprising N, O, S, provided that at least one R^3 of the derivative is COCHCHR^1 ;

R^4 is H^+ or pharmaceutically acceptable salt;

and at least one carrying polymer.

2. Nanofibers according to Claim 1 **characterized in that** R^1 is selected from a group comprising phenyl, furyl, furfuryl, thienyl, thiofenyl, pyridyl or imidazolyl;

R^4 is selected from a group comprising any of ions of alkali metals or ions of alkaline-earth metals, preferably Na^+ , K^+ , Mg^{2+} or Li^+ .

3. Nanofibers according to any one of Claims 1 to 2 **characterized in that** the amount of COCHCHR^1 in the photocurable ester HA-derivative is from 0.1 to 20 % with respect to 100 dimers of hyaluronic acid or its salt, preferably 5 to 10 %, more preferably 5%.

4. Nanofibers according to any one of Claims 1 to 3 **characterized in that** the carrying polymer is selected from a group comprising polyvinyl alcohol, polyacrylic acid, polyethylene oxide, polyvinyl pyrrolidone.

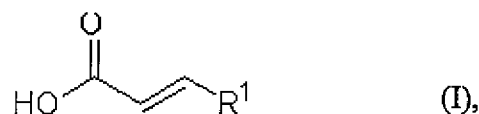
5. Nanofibers according to Claim 4 **characterized in that** the amount of the carrying polymer is in the range of 50% (w/w) to 99% (w/w), preferably in the range of 70 to 90% (w/w), more preferably 80% (w/w).

6. The nanofibers according to any one of Claims 1 to Claim 5 **characterized in that** it comprises additionally biologically compatible polymer preferably carboxymethyl cellulose, gelatin, chitosan, polycaprolactone, polylactic acid, polyamide, polyurethane, poly-(lactide-co-glycolic) acid; and their mixture or their copolymers.

7. The nanofibers according to any one of Claims 1 to Claim 6 **characterized in that** the average diameter thereof is between 1 to 500 nm, preferably 99 to 300 nm.

8. The nanofibers according to any one of Claims 1 to Claim 7 **characterized in that** they are in the form of gel.

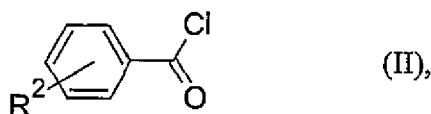
9. A method of synthesis of nanofibers according to any one of Claims 1 to 8 **characterized in that** α,β -unsaturated substituted acrylic acid of a general formula I



wherein

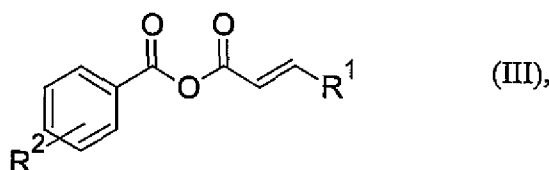
R^1 is 5- or 6-membered aryl or 5- or 6-membered heteroaryl having at least one or more identical or different heteroatoms selected from the group comprising N, O or S;

is activated with substituted or non-substituted benzoyl chloride or its derivatives of a general formula II



wherein R^2 is one or more substituents selected from a group comprising H, $-\text{NO}_2$, $-\text{COOH}$, halides, C_1 - C_6 alkylkoxy, preferably halides, methoxy or ethoxy, more preferably Cl;

in the presence of an organic base, preferably tertiary amine, more preferably triethylamine, diisopropylethylamine or N,N-diisopropylethylamine; a mixture of water and water-miscible polar or non-polar solvent to form a reactive anhydride of the general formula III

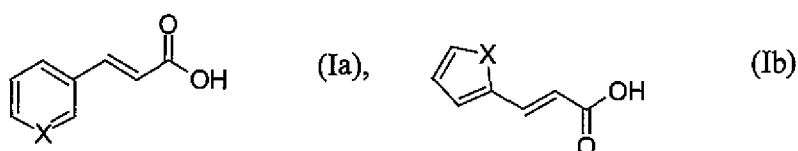


wherein

R^1 and R^2 are as defined above

that reacts with hyaluronic acid or a salt thereof in the presence of an organic base, preferably tertiary amine, more preferably triethylamine, diisopropylethylamine or N,N-diisopropylethylamine; a mixture of water and water-miscible polar or non-polar solvent, to form the photocurable ester derivative of hyaluronic acid or its salt of the general formula IV as defined in Claim 1 or Claim 2 that is dissolved in a carrying polymer of molecular weight from 5×10^4 g/mol to 1×10^6 g/mol, preferably 4×10^5 g/mol in water to form a spinning solution that is spun by electrospinning.

10. The method according to Claim 9 **characterized in that** the α,β -unsaturated substituted acrylic acid is 5-membered or 6-membered aromatic or heteroaromatic acrylic acid of general formulae Ia or Ib



wherein X is selected from C, O, S, N, preferably O or N and the substituted benzoyl chloride of the general formula II is trichlorobenzoyl chloride.

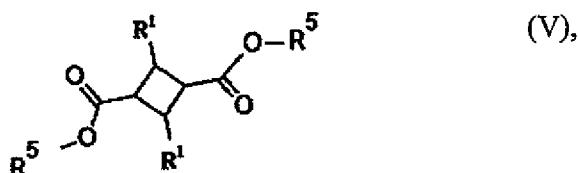
11. The method according to Claim 10 **characterized in that** α,β -unsaturated substituted acrylic acid is 3-(2-furyl)acrylic acid.

12. The method according to any one of Claims 9 to 11 **characterized in that** the carrying polymer is selected from a group comprising polyvinyl alcohol, polyacrylic acid, polyethylene oxide, polyvinyl pyrrolidone.

13. The method according to any one of Claim 8 to 11 **characterized in that** the activation of the α,β -unsaturated substituted acrylic acid of the general formula I is carried out for 5 to 120 minutes at a temperature in a range from 0 °C to 60 °C, preferably 25 °C to 60 °C, more preferably for 30 minutes at 25°C.
14. The method according to Claim 9 **characterized in that** the amount of reactive anhydride mixed with hyaluronic acid or its salt corresponds to 0.01 to 5 equivalents, preferably 0.5 to 2 equivalents, more preferably 0,5 equivalents per hyaluronic acid dimer or its salt.
15. The method according to any one of Claims 9 to 14 **characterized in that** hyaluronic acid or its salt has a molecular weight from 5×10^3 to 1.6×10^6 g/mol, preferably from 1.5×10^4 to 2.5×10^5 g/mol, more preferably 8.5×10^4 to 1.20×10^5 g/mol.
16. The method according to any one of Claims 9 to 15 **characterized in that** forming of the photocurable ester derivative of hyaluronic acid or its salt is carried out for 1 to 48 hours, preferably 1 to 3 hours.
17. The method according to any one of Claims 9 to 16 **characterized in that** forming of the photocurable ester derivative of hyaluronic acid or its salt is carried out at temperatures within the range of 0 °C to 80°C, preferably from 20 °C to 60 °C, more preferably at 25°C.
18. The method according to any one of Claims 9 to 17 **characterized in that** the water miscible polar or non-polar solvent is selected from a group comprising isopropanol, acetone, ethyl acetate, dimethyl sulfoxide, acetonitrile, dimethylformamide, tetrahydrofuran, preferably isopropanol, tetrahydrofuran, more preferably isopropanol.
19. The method according to any one of Claims 9 to 18 **characterized in that** the amount of water in the mixture water and water miscible polar solvent is from 10 % to 99 %v/v, preferably 50 %v/v.

20. The method according to any one of Claims 9 to 19 **characterized in that** the concentration of the photocurable ester derivative of hyaluronic acid or its salt in the spinning solution is in the range of 0.5 to 12% (w/v), preferably 5 to 7.5% (w/v), more preferably 5% (w/v).

21. A method of photo-curing of nanofibers defined in any one of Claims 1 to 9 **characterized in that** the nanofibers are photocured by light irradiating within the range of UV-Vis wavelengths whereby cycloaddition of at least two ester groups of at least two molecules of hyaluronic acid or its salt of the formula IV occurs, forming the compound having cyclobutane ring of the general formula V



wherein

R¹ is as defined in Claim 1 and

R⁵ is a backbone chain of hyaluronic acid or its salt.

22. The method according to Claim 21 **characterized in that** UV-light has the wavelength range from 280 nm to 750 nm, preferably 302 nm.

23. The method according to Claim 21 or Claim 22 **characterized in that** photocuring is carried out for 2 min to 60 min, preferably for 3 min to 10 min.

24. Photocured nanofibers based on hyaluronic acid derivatives **characterized in that** they comprise the compound having cyclobutane ring of the formula V, defined in Claim 21.

25. A preparation comprising photocured nanofibers **characterized in that** the photocured nanofibers are as defined in Claim 24 and that the preparation is in a form selected from a group comprising layer, film, mat, wound dressing or tissue scaffold.

26. The preparation defined in Claim 25 for use in cosmetics, medicine or regenerative medicine.

27. The preparation defined in Claim 26 in wound care device, or in patches for external or internal use.

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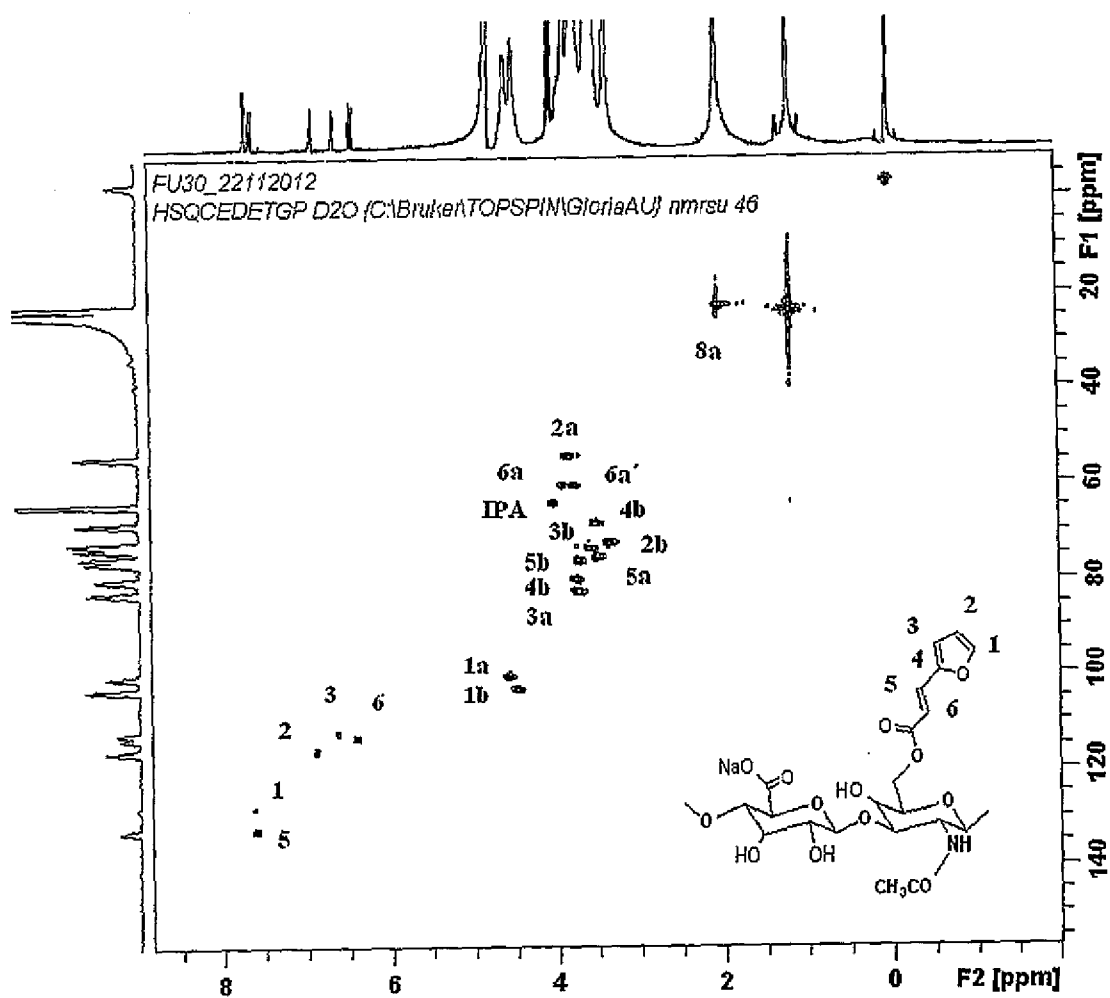
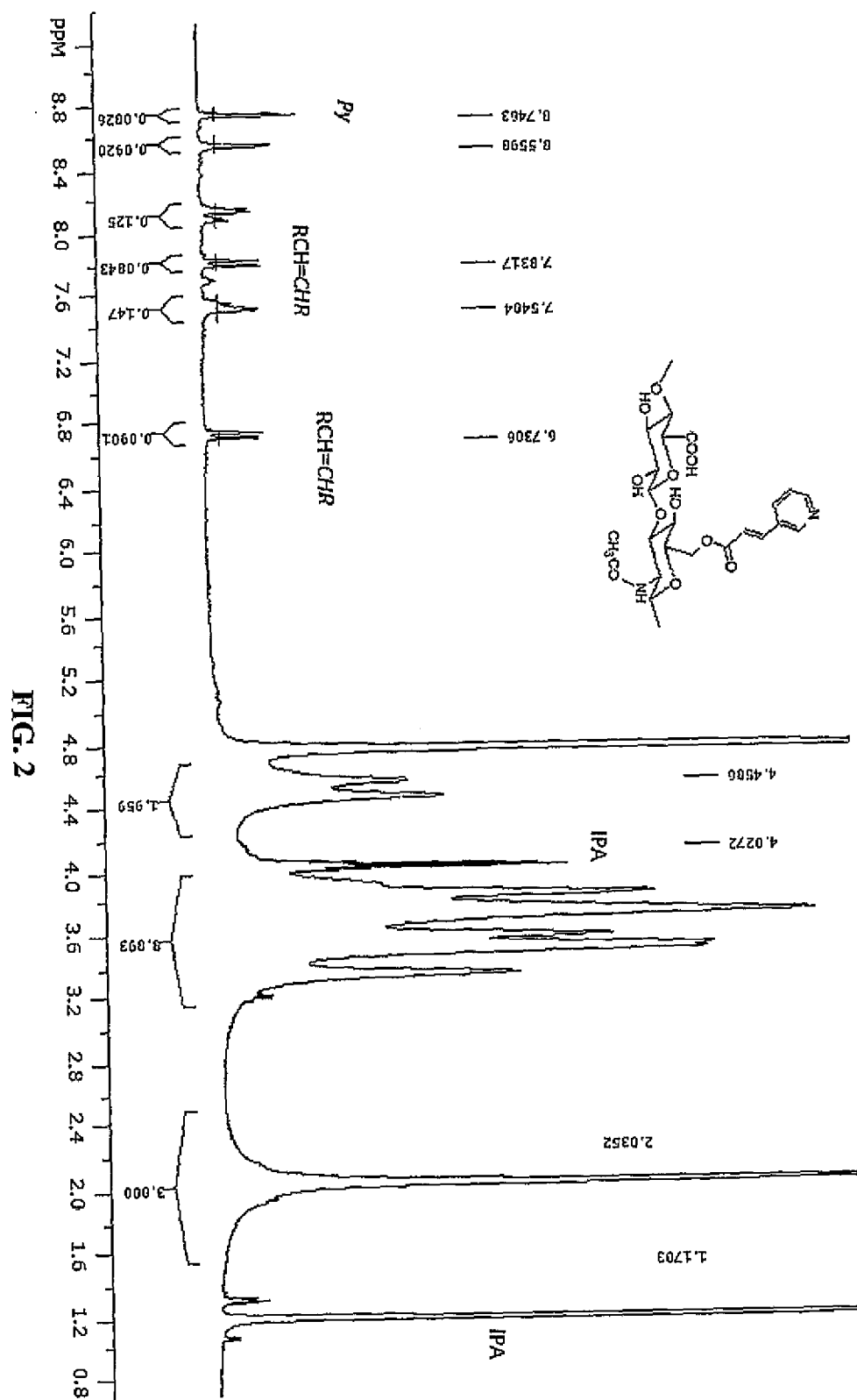


FIG.1



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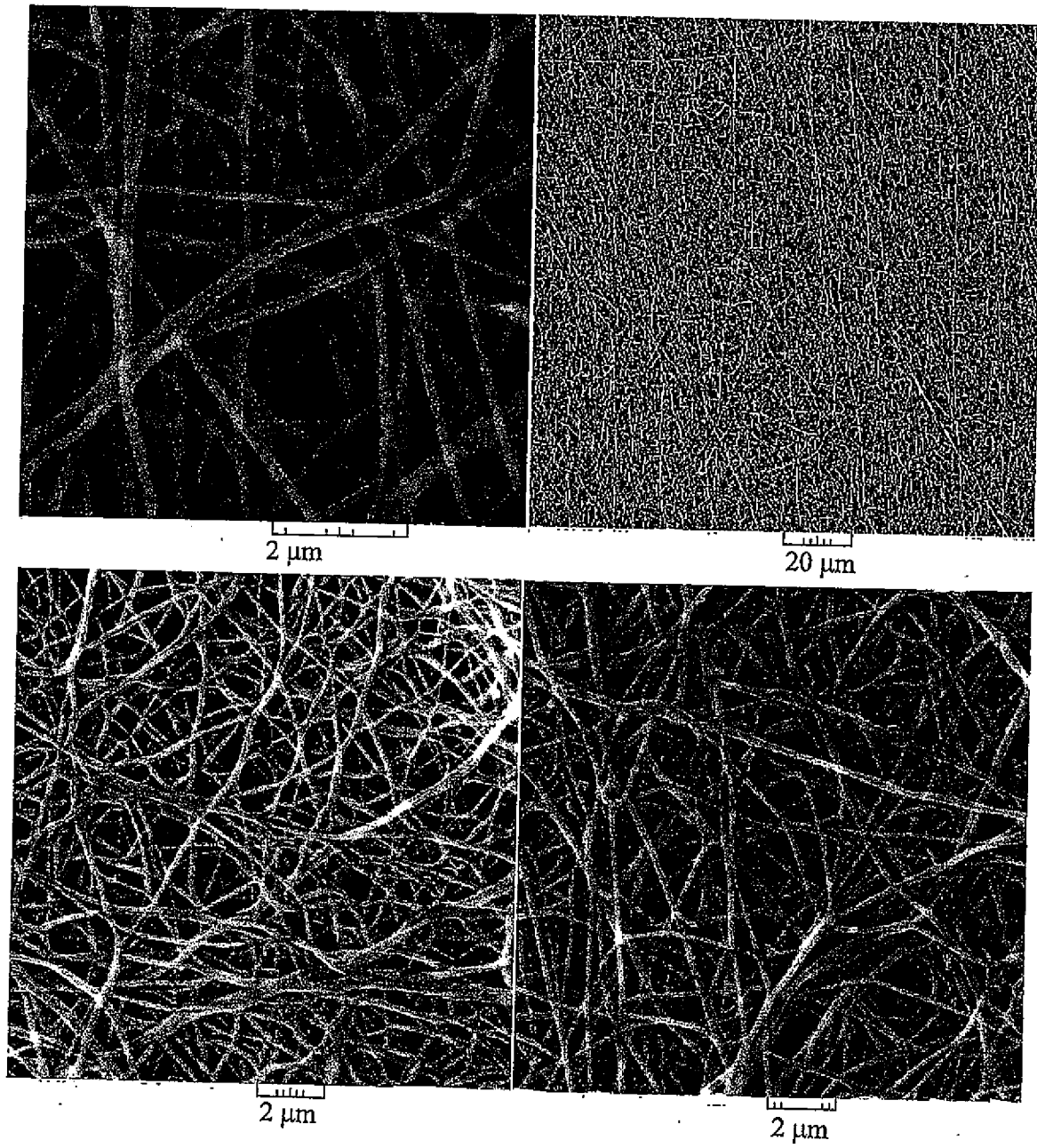


FIG. 3

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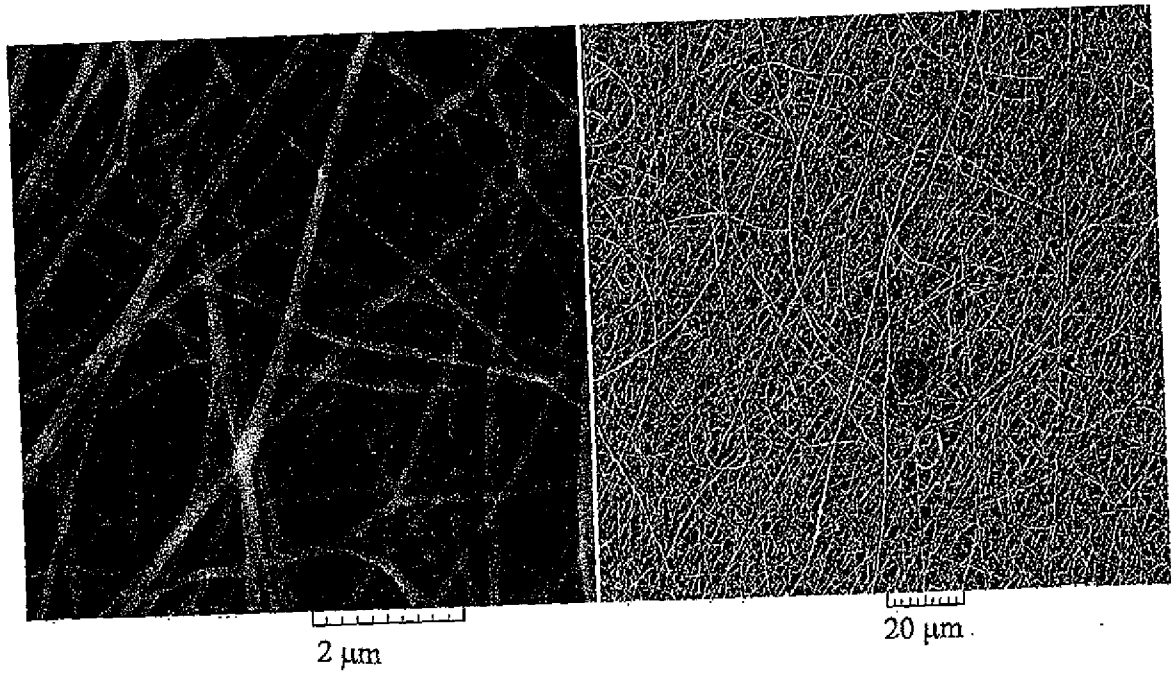


FIG. 4

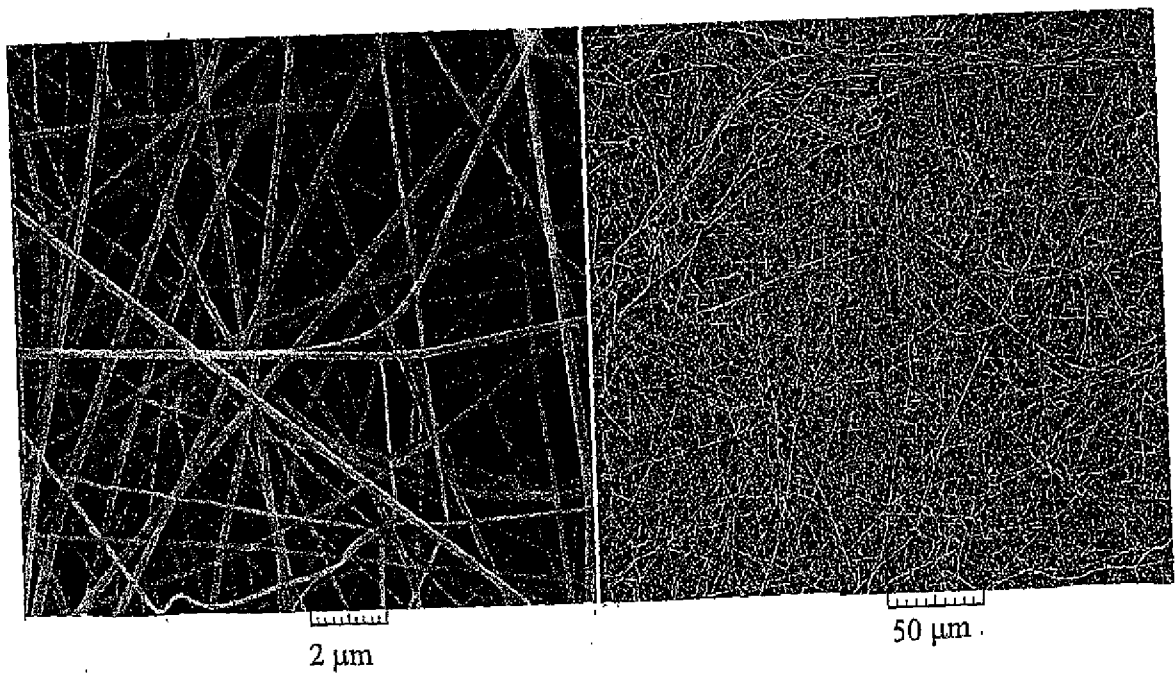


FIG.5

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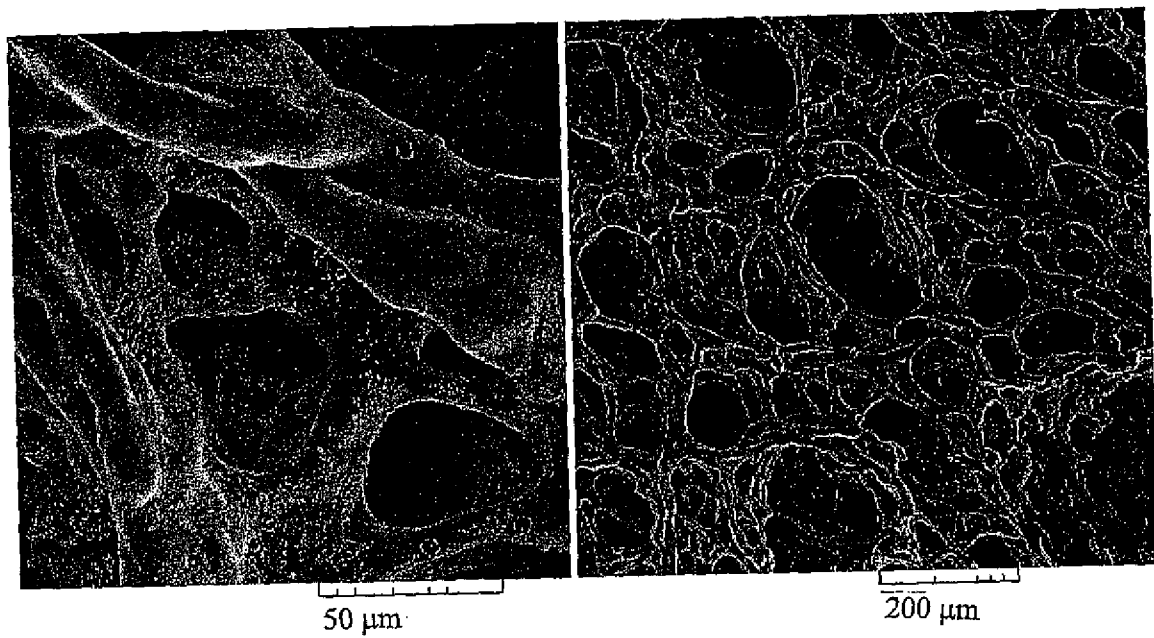


FIG.6

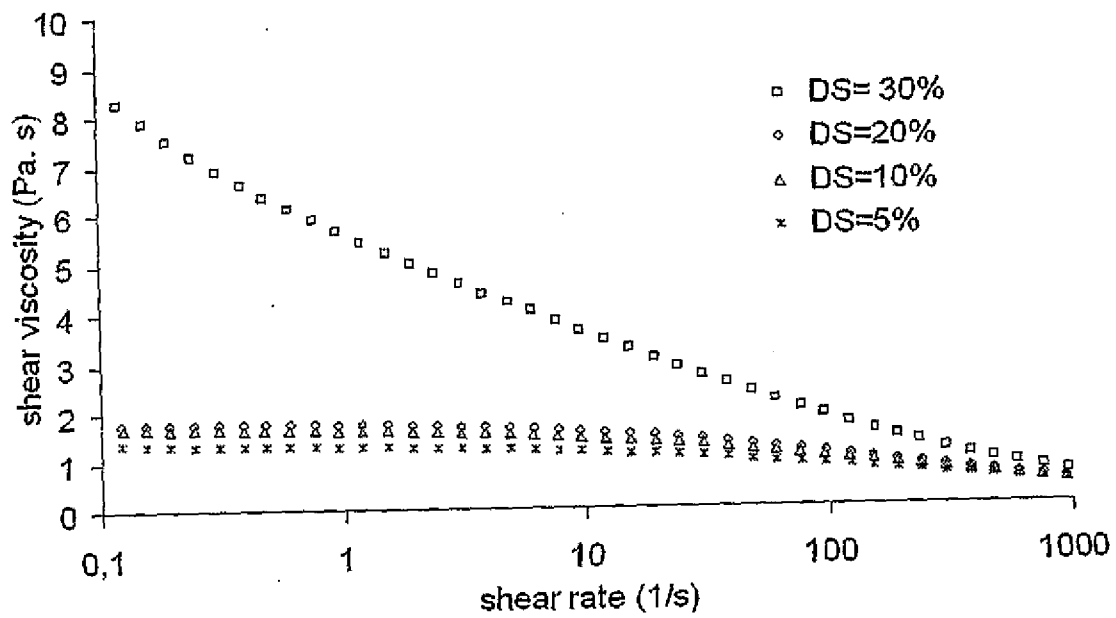


FIG.7

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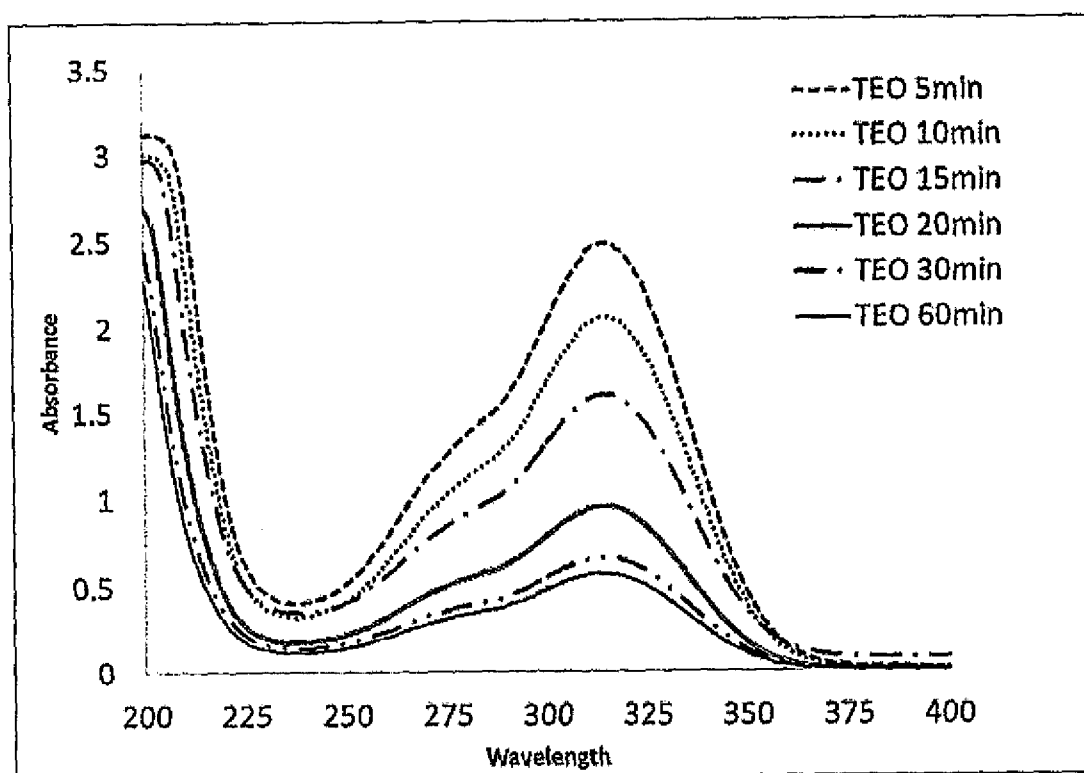


FIG. 8

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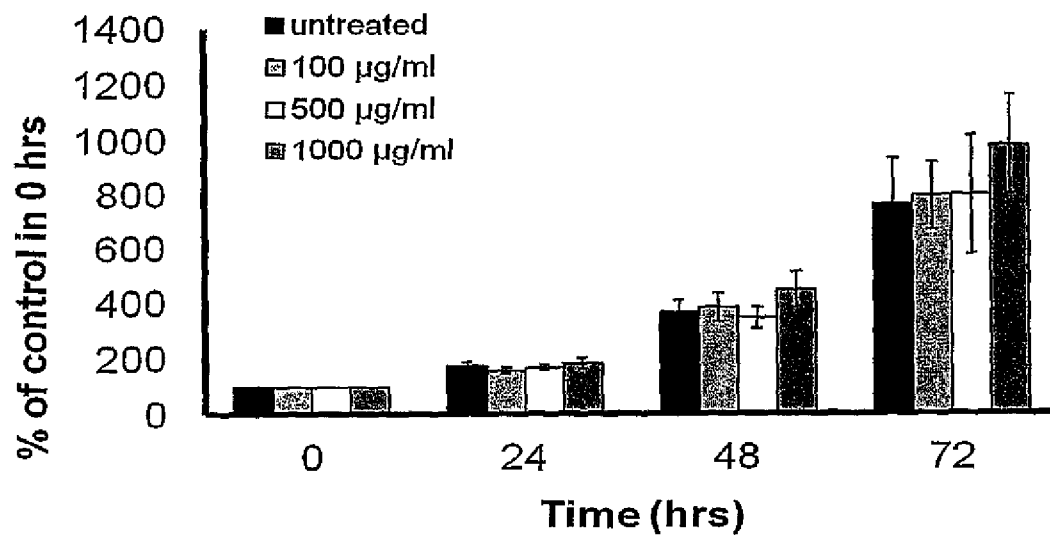


FIG.9

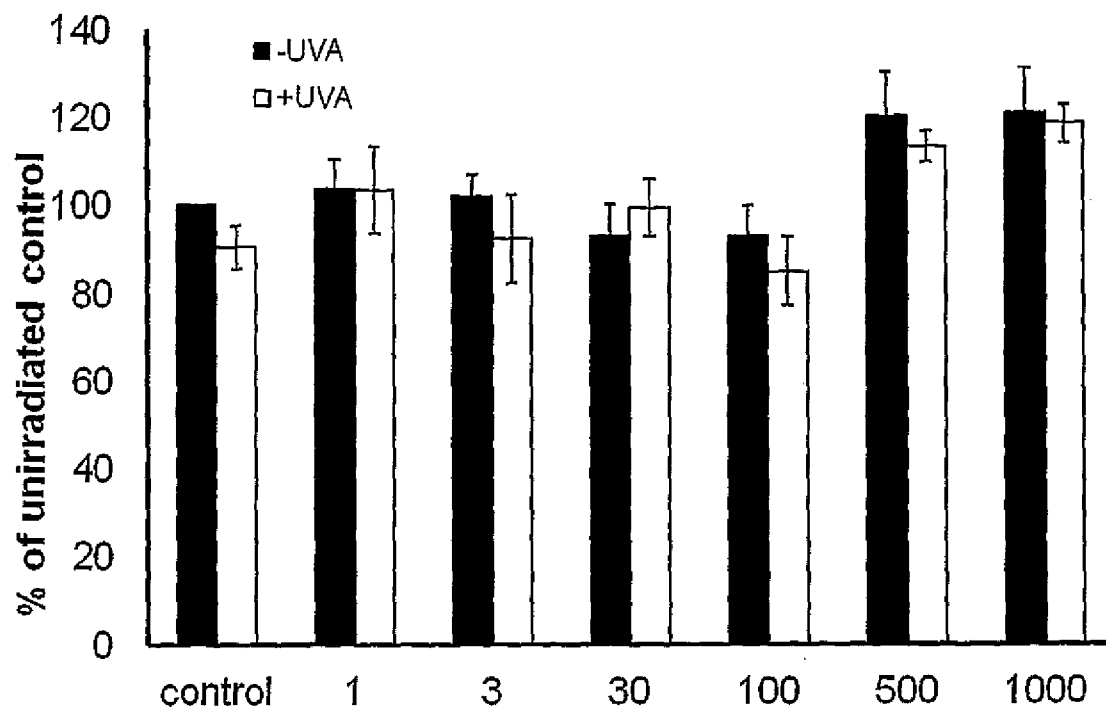


FIG.10

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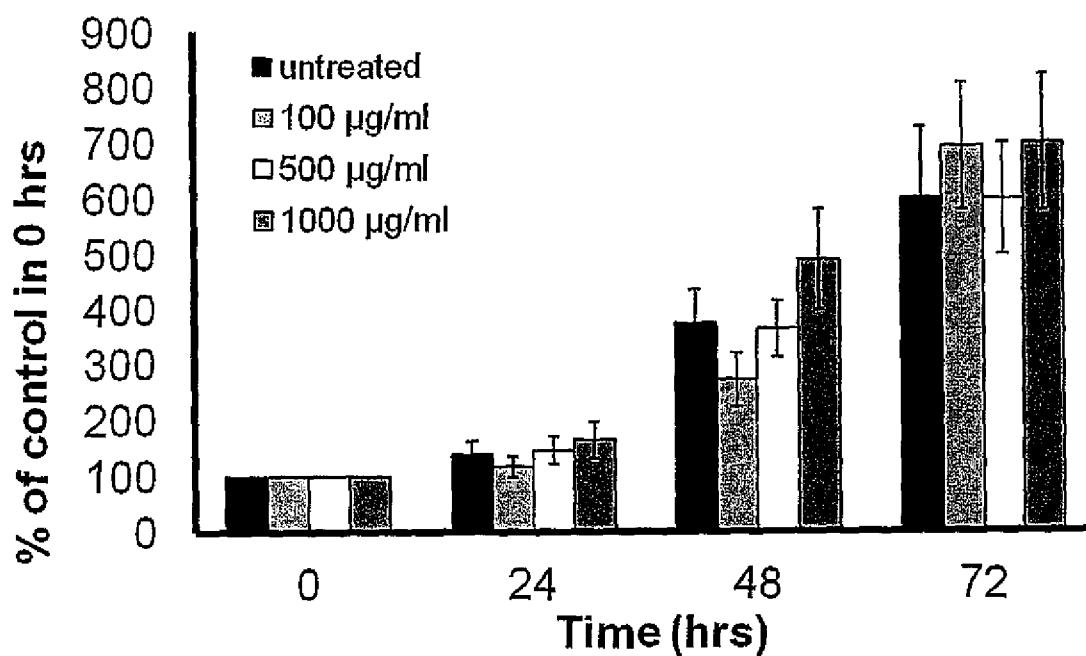


FIG. 11

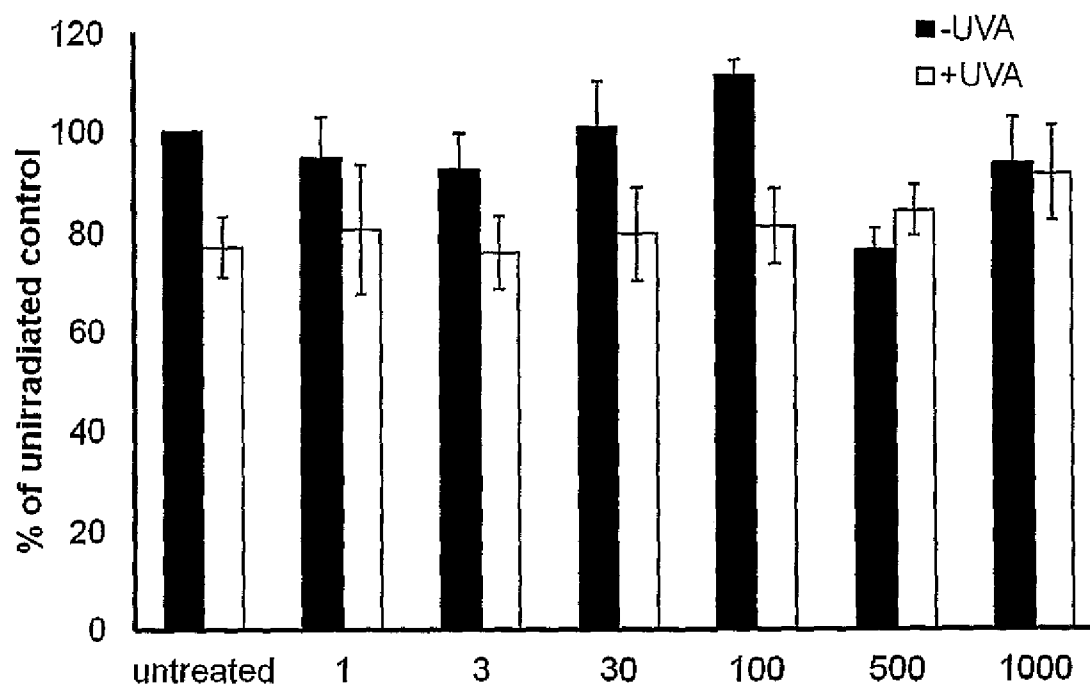


FIG. 12

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2014/000138

A. CLASSIFICATION OF SUBJECT MATTER
INV. C08B37/08 A61K8/00 C08L5/08
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)

C08B A61K C08L

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)

EP0-Internal, 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	WO 2013/159757 A1 (CONTIPRO BIOTECH SRO [CZ]) 31 October 2013 (2013-10-31) page 4, lines 11-22 page 5, lines 12-19 page 8, line 11 - page 9, line 5 compounds I - III -----	1-27
A	EP 1 369 441 A1 (SEIKAGAKU KOGYO CO LTD [JP]) 10 December 2003 (2003-12-10) paragraphs [0139] - [040,] -----	1-27
A	EP 2 522 337 A2 (ROTTAPHARM SPA [IT]) 14 November 2012 (2012-11-14) examples; paragraphs [0001] - [0004], [0012] ----- -/--	1-27

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search

13 April 2015

Date of mailing of the international search report

04/05/2015

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Zellner, Armin

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2014/000138

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	<p>WO 2014/082608 A1 (CONTIPRO BIOTECH SRO [CZ]) 5 June 2014 (2014-06-05) page 8, paragraph 3 - page 9, paragraph 1; figure 3</p> <p>-----</p>	1-27

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/CZ2014/000138

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013159757 A1	31-10-2013	EP 2841462 A1 KR 20150005937 A WO 2013159757 A1	04-03-2015 15-01-2015 31-10-2013
EP 1369441 A1	10-12-2003	CA 2435491 A1 EP 1369441 A1 JP 4135502 B2 US 2004076811 A1 US 2008071001 A1 US 2008071050 A1 US 2011098455 A1 WO 02060971 A1	08-08-2002 10-12-2003 20-08-2008 22-04-2004 20-03-2008 20-03-2008 28-04-2011 08-08-2002
EP 2522337 A2	14-11-2012	CA 2776222 A1 CN 102775514 A EP 2522337 A2 JP 2012241192 A RU 2012119458 A US 2012289478 A1	13-11-2012 14-11-2012 14-11-2012 10-12-2012 20-11-2013 15-11-2012
WO 2014082608 A1	05-06-2014	CZ 304267 B6 WO 2014082608 A1	05-02-2014 05-06-2014