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(54) **Title**: METHOD OF PREPARATION OF HIGHLY SUBSTITUTED HYALURONIC ACID AMIDES

(57) **Abstract**: The invention relates to the method of preparation of highly substituted hyaluronic acid amides with a controllable degree of substitution, which are prepared in a polar aprotic environment from an acidic form of hyaluronic acid in the presence of ethylchloroformiate, by means of a primary amine.

Method of preparation of highly substituted hyaluronic acid amides

Field of the invention

The technical solution relates to the method of preparation of highly substituted amide derivatives of hyaluronic acid, which can be used in medicine, pharmacy and for preparation of hydrogels in tissue engineering.

Technical background and prior art

Hyaluronic acid is a linear heteropolysaccharide, composed of repetitive disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine. This non-branched polysaccharide, when isolated from natural source, may have molecular weight within the range from 50 000 to 5 000 000 Da, depending on the isolation method and teh source material.

Hyaluronic acid is the main component of intercellular substance and due to its visco-elastic properties is essential part of rheological, physiological and biological functions in organism. Due to its high lubrication capacity, ability of high sorption and water retention it is often applied in eye surgery. Hyaluronic acid eliminates free oxygen radicals and affects the proliferation and differentiation of cells. Furthermore, it prevents collagen deposition and in that way promotes healing of wounds and prevents formation of scars.

For wide clinical application of native hyaluronic acid, the complications consist especially in a very quick degradation thereof in solutions and in the relatively poor mechanical properties thereof. Chemical modification of hyaluronan allows to improve these properties and at the same time to increase its resistance to degradation.

Therefore, within the last years the attention was aimed at the preparation of synthetic hyaluronan derivatives which are soluble in aqueous solutions and after certain modifications can be applied as biodegradable and biocompatible materials in medicine, surgery and tissue engineering. One of the suitable modifications is cross-linking where hydrogels are formed from modified hyaluronic acid.

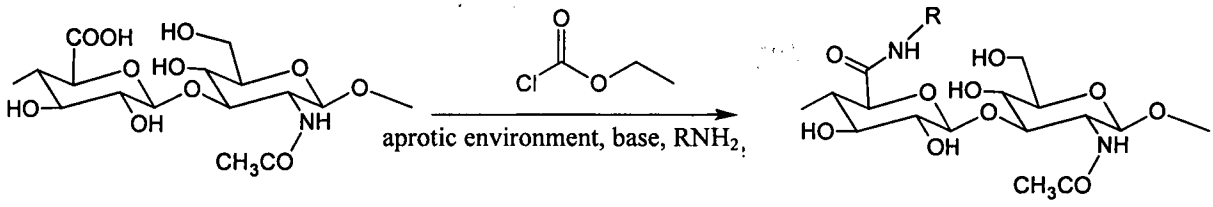
Hydrogels are hydrophilic polymer networks which can be used for controlled distribution of medicaments, bioactive and other substances in organisms, in the form of scaffolds or cutaneous filling substances. An important condition for the preparation of hydrogels is the stability of the derivatives which are used as input precursors for the cross-linking reaction.

A number of modified hyaluronan derivatives have been tested for the formation of hydrogels. Amide derivatives were one of them. These derivatives deserved a great attention due to their relatively high stability to degradation and hydrolysis in physiological conditions in an environment without any enzymatic activity.

In the present time, the preparation of hyaluronan amide derivatives for subsequent gelation is based on using various reaction conditions which, however, leads to only relatively low degrees of amidation (max. 30%) (WO/2000/001733 (A1)). A great disadvantage of some nowaday methods is mainly the fact that hyaluronan amidation itself is preceded by hyaluronan deacetylation (WO/2000/001733 (A1)). The hyaluronan amide derivative is then cross-linked until the hydrogel is formed. The cross-linking of azides and alkines, which are bonded to the polysaccharide by amidic bond, are most commonly catalyzed by copper (I) cations which are toxic. The lower substitution degree of amide derivatives entering the reaction, the higher amount of catalyst must be used (WO/2008/031525A1).

Summary of the invention

The low degree of hyaluronan amide derivatives can be increased by an application of specific reaction conditions. The main subject matter of this invention is a method of preparation of hyaluronic acid amides, where highly substituted products are obtained by selective amidation of hyaluronic acid carboxylic group. The principle of the reaction consists in the activation of hyaluronan carboxylic group by ethylchloroformiate in the presence of a base, wherein the resulting intermediate - activated hyaluronan - consequently reacts with a primary amine R-NH_2 and hyaluronic acid amide is formed, according to Scheme 1:



Scheme 1 Hyaluronic acid amidation

where R is an alkyl linear or branched chain C_1 – C_{30}, optionally containing aromatic or heteroaromatic groups. The preparation is preferably carried out in a polar aprotic environment, particularly in dimethylsulphoxide. The hyaluronic acid is preferably in an acidic form and has a weight average molecular weight within the range 10 to 500 kDa, particularly within the range 350 to 500 kDa.

In a preferred embodiment of the invention, the reaction is carried out at 25°C for 8 to 24 hours, preferably for 12 hours.

The degree of substitution of hyaluronic acid in the method of preparation according to the invention is preferably controlled by the molar quantity of the added amine R-NH_2, wherein the primary amine is preferably added to the reaction mixture at time 0.1 to 2 hours after the addition of ethylchloroformiate and in the molar quantity of 3 to 5 equivalents with respect to the molar amount of hyaluronan dimer. The primary amine R-NH_2 can be for example $CH{\equiv}C\text{-}CH_2\text{-}NH_2$, $NH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}N_3$ or $NH_2(CH_2)_5\text{-}CH_3$.

In a preferred embodiment of the invention, the molar amount of the added ethylchloroformiate is in a molar ratio of 3 to 5 equivalents with respect to the molar amount of hyaluronan dimer.

The base used in the method of the invention can be trialkylamine, such as triethylamine, preferably in the molar amount in a molar ratio of 3 to 5 equivalents with respect to the molar amount of hyaluronan dimer.

In comparison with the known analogues, the advantage is the selectable high degree of substitution which leads to a significant change of physical and chemical properties. Blocking of the hyaluronan carboxylic group in the cross-linked products causes a lower swelling capacity and consequently better mechanical stability of the prepared cross-linked products.

Hyaluronan amide derivatives prepared by this method show a controllable degree of substitution within the range from 10 to 99%. The substitution degree is controlled by the molar ratio of the primary amine, ethylchloroformiate and the base.

Amide derivatives are highly stable to degradation and hydrolysis and therefore, they can be used for the preparation of stable hydrogels. In case of a high substitution degree only a very small amount of catalyst is needed for the cross-linking reaction.

Examples

Example 1

Amidation of hyaluronic acid by propargylamine

0.5 g of hyaluronic acid (acidic form) having the molecular weight of 100 kDa and polydispersion of 1.9 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (5 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (3 eq) was added and the reaction mixture was stirred for 1 hour. Then 0.125 g (1.36 mmol) of propargylamine hydrochloride was added and the reaction mixture was stirred for further 24 hours at room temperature, the mixture was diluted to the total volume of 200 mL and is infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% $NaHCO_3$ solution (total volume of 10 liters) 3 x 24 hours and then against demineralized water 3 x 24 hours. The dialyzed derivative was evaporated until dry and is dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.3957 g

Mw of the derivative after the reaction: 80 kDa.

Degree of substitution = 44%.

^{1}H NMR (assigned by means of HSQC) (500 MHz, NaOD; δ ppm):

2.01 (s, 3H, 8a), 3.36 (bs, 1H, 2b), 3.46 (bs, 1H, 5a), 3.54 (bs, 1H, 4a), 3.70 (bs, 2H, 3a and 3b), 3.83 (bs, 1H, 4b), 3.90 (bs, 1H, 5b), 3.91 (d, 1H, 1α) 4.10 (d, 1H, 1β), 4.50 (bs, 2H, 1a and 1b)

DOSY NMR:

log D (2.03 ppm CH_3-CO-NH-Polymer) ~-10.5 m^2/s

log D (2.7 ppm $-CH_2NH$-R) ~-10.5 m^2/s

log D (3.0 ppm $-CH_2NH$-R) ~-10.5) m^2/s

Example 2

Amidation of hyaluronic acid by propargylamine

0.5 g of hyaluronic acid (acidic form) having the molecular weight of 248 kDa and polydispersion of 1.6 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (5 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (3 eq) was added and the reaction mixture was stirred for 1 hour. Then 0.125 g (1.36 mmol) of propargylamine hydrochloride was added and the reaction mixture was stirred for 24 hours at room temperature, the mixture was diluted to the total volume of 200 mL and infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% $NaHCO_3$ solution (total volume of 10 liters) 3 x 24 hours and then against demineralized water 3 x 24 hours. The dialyzed derivative was evaporated until dry and dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.3957 g

Mw of the derivative after the reaction: 120 kDa.

Degree of substitution = 98%.

^1H NMR (assigned by means of HSQC) (500 MHz, NaOD, δ ppm): see Example 1

DOSY NMR: see Example 1

Example 3

Amidation of hyaluronic acid by propargylamine

0.5 g of hyaluronic acid (acidic form) having the molecular weight of 366 kDa and polydispersion of 1.6 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (3 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (3 eq) was added and the reaction mixture was stirred for 1 hour. Then 0.125 g (1.36 mmol) of propargylamine hydrochloride and the reaction

mixture was stirred for further 12 hours at room temperature, the mixture was diluted to the total volume of 200 mL and infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% $NaHCO_3$ solution (total volume of 10 liters) 3 x 24 hours and then against demineralized water 3 x 24 hours. The dialyzed derivative was evaporated until dry and dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.3957 g

Mw of the derivative after the reaction: 177.4 kDa.

Degree of substitution = 50%.

^{1}H NMR (assigned by means of HSQC) (500 MHz, NaOD, δ ppm): see Example 1

DOSY NMR: see Example 1

Example 4

Amidation of hyaluronic acid I

0.5 g of hyaluronic acid (acidic form) having the molecular weight of 393 kDa and polydispersion of 1.6 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (5 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (3 eq) was added and the reaction mixture was stirred for 1 hour. Then 0.111 g (1.32 mmol) of propargylamine hydrochloride was added and the reaction mixture was stirred for further 12 hours at room temperature, the mixture was diluted to the total volume of 200 mL and infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% $NaHCO_3$ solution (total volume of 10 liters) 3 x 24 hours and then against demineralized water 3 x 24 hours. The dialyzed derivative was evaporated until dry and dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.4452 g

Mw of the derivative after the reaction: 240 kDa.

Degree of substitution = 60%.

^{1}H NMR (assigned by means of HSQC) (500 MHz, NaOD, δ ppm): see Example 1

DOSY NMR: see Example 1

Example 5

Amidation of hyaluronic acid by propargylamine

0.5 g of hyaluronic acid (acidic form) having the molecular weight of 485 kDa and polydispersion of 1.6 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (5 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (3 eq) was added and the reaction mixture was stirred for 1 hour. Then 0.111 g (1.32 mmol) of propargylamine hydrochloride was added and the reaction mixture was stirred for further 12 hours at room temperature, the mixture was diluted to the total volume of 200 mL and infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% $NaHCO_3$ solution (total volume of 10 liters) 3 x 24 hours and then against demineralized water 3 x 24 hours. Dialyzed derivative was evaporated until dry and dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.4452 g

Mw of the derivative after the reaction: 133.4 kDa

Degree of substitution = 88%.

^1H NMR (assigned by means of HSQC) (500 MHz, NaOD; δ ppm): see Example 1

DOSY NMR: see Example 1

Example 6

Amidation of hyaluronic acid by 3-azidopropyl amine

1.0 g of hyaluronic acid (acidic form) having the molecular weight of 42 kDa and polydispersion of 1.6 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (3 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (3 eq) was added and the reaction mixture was stirred for 1 hour. Then 0.228 g (2.65 mmol) of 3-azidopropyl amine was added and the reaction mixture was stirred for further 12 hours at room temperature, the mixture was diluted to the total volume of 200 mL and infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% $NaHCO_3$ solution (total volume of 10 liters) 3 x 24 hours and

then against demineralized water 3 x 24 hours. The dialyzed derivative was evaporated until dry and dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.8001 g

Mw of the derivative after the reaction: 20 kDa

Degree of substitution = 75%.

^{1}H NMR (assigned by means of HSQC) (500 MHz, NaOD, δ ppm):

1.8 (q, 3H, 2), 2.01 (s, 3H, 8a), 3.19 (bs, 1H, 1α), 3.30 (bs, 1H, 2b), 3.36 (bs, 2H, 3), 3.50 (bs, 3H, 1β, 6a, 5a), 3.57 (bs, 1H, 3b), 3.70 (bs, 4H, 6a, 3b, 4b, 3a), 3.89 (bs, 5H, 6a, 3b, 4b, 3a, 5b), 4.50 (bs, 2H, 1a and 1b)

DOSY NMR:

log D (2.03 ppm *CH*$_3$-CO-NH-Polymer) ~-10.5 m^2/s

log D (2.7 ppm –CH$_2$ NH-R) ~-10.5 m^2/s

log D (3.0 ppm –CH$_2$NH-R) ~-10.5) m^2/s

Example 7

Amidation of hyaluronic acid by 3-azidopropyl amine

1.0 g of hyaluronic acid (acidic form) having the molecular weight of 42 kDa and polydispersion of 1.6 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (3 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (4 eq) was added and the reaction mixture was stirred for 1 hour. Then 0.228 g (2.65 mmol) of 3-azidopropyl amine was added and the reaction mixture was stirred for further 12 hours at room temperature, the mixture was diluted to the total volume of 200 mL and infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% NaHCO$_3$ solution (total volume of 10 liters) 3 x 24 hours and then against demineralized water 3 x 24 hours. The dialyzed derivative was evaporated until dry and dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.8067 g

Mw of the derivative after the reaction: 20 kDa

Degree of substitution = 50%.

^{1}H NMR (assigned by means of HSQC) (500 MHz, NaOD, δ ppm): see Example 6

DOSY NMR: see Example 6

Example 8

Amidation of hyaluronic acid by 3-azidopropyl amine

1.0 g of hyaluronic acid (acidic form) having the molecular weight of 42 kDa and polydispersion of 1.6 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (3 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (5 eq) was added and the reaction mixture was stirred for 1 hour. Then 0.228 g (2.65 mmol) of 3-azidopropyl amine was added and the reaction mixture was stirred for further 12 hours at room temperature, the mixture was diluted to the total volume of 200 mL and infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% $NaHCO_3$ solution (total volume of 10 liters) 3 x 24 hours and then against demineralized water 3 x 24 hours. The dialyzed derivative was evaporated until dry and dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.8067 g

Mw of the derivative after the reaction: 20 kDa

Degree of substitution = 40%.

^{1}H NMR (assigned by means of HSQC) (500 MHz, NaOD, δ ppm): see Example 6

DOSY NMR: see Example 6

Example 9

Amidation of hyaluronic acid by 3-azidopropyl amine

1.0 g of hyaluronic acid (acidic form) having the molecular weight of 72 kDa and polydispersion of 1.7 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (3 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (5 eq) was added and the reaction mixture was stirred

for 1 hour. Then 0.228 g (2.65 mmol) of 3-azidopropyl amine was added and the reaction mixture was stirred for further 24 hours at room temperature, the mixture was diluted to the total volume of 200 mL and infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% $NaHCO_3$ solution (total volume of 10 liters) 3 x 24 hours and then against demineralized water 3 x 24 hours. The dialyzed derivative was evaporated until dry and dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.7826 g

Mw of the derivative after the reaction: 52 kDa

Degree of substitution = 54%.

^1H NMR (assigned by means of HSQC) (500 MHz, NaOD, δ ppm): see Example 6

DOSY NMR: see Example 6

Example 10

Amidation of hyaluronic acid by 3-azidopropyl amine

1.0 g of hyaluronic acid (acidic form) having the molecular weight of 70 kDa and polydispersion of 1.7 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (3 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (5 eq) was added and the reaction mixture was stirred for 1 hour. Then 0.228 g (2.65 mmol) of 3-azidopropyl amine was added and the reaction mixture was stirred for further 24 hours at room temperature, the mixture was diluted to the total volume of 200 mL and infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% $NaHCO_3$ solution (total volume of 10 liters) 3 x 24 hours and then against demineralized water 3 x 24 hours. The dialyzed derivative was evaporated until dry and dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.7826 g

Mw of the derivative after the reaction: 35 kDa

Degree of substitution = 60%.

^1H NMR (assigned by means of HSQC) (500 MHz, NaOD, δ ppm): see Example 6

DOSY NMR: see Example 6

Example 11

Amidation of hyaluronic acid by 3-azidopropyl amine

1.0 g of hyaluronic acid (acidic form) having the molecular weight of 393 kDa and polydispersion of 1.7 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (3 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (3 eq) was added and the reaction mixture was stirred for 1 hour. Then 0.228 g (2.65 mmol) of 3-azidopropyl amine was added and the reaction mixture was stirred for further 8 hours at room temperature, the mixture was diluted to the total volume of 200 mL and infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% $NaHCO_3$ solution (total volume of 10 liters) 3 x 24 hours and then against demineralized water 3 x 24 hours. The dialyzed derivative was evaporated until dry and dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.7826 g

Mw of the derivative after the reaction: 165 kDa

Degree of substitution = 85%.

^{1}H NMR (assigned by means of HSQC) (500 MHz, NaOD, δ ppm): see Example 6

DOSY NMR: see Example 6

Example 12

Amidation of hyaluronic acid by 3-azidopropyl amine

1.0 g of hyaluronic acid (acidic form) having the molecular weight of 485 kDa and polydispersion of 1.7 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (3 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (3 eq) was added and the reaction mixture was stirred for 1 hour. Then 0.228 g (2.65 mmol) of 3-azidopropyl amine was added and the reaction

mixture was stirred for further 10 hours at room temperature, the mixture was diluted to the total volume of 200 mL and infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% $NaHCO_3$ solution (total volume of 10 liters) 3 x 24 hours and then against demineralized water 3 x 24 hours. The dialyzed derivative was evaporated until dry and dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.7826 g

Mw of the derivative after the reaction: 177 kDa

Degree of substitution = 98%.

^{1}H NMR (assigned by means of HSQC) (500 MHz, NaOD, δ ppm): see Example 6

DOSY NMR: see Example 6

Example 13

Amidation of hyaluronic acid by hexylamine

1.0 g of hyaluronic acid (acidic form) having the molecular weight of 485 kDa and polydispersion of 1.7 was dissolved in 50 mL of DMSO at 60°C. After the dissolution, the hyaluronic acid solution was allowed to cool to the room temperature. After cooling, 0.922 mL of triethylamine (5 eq) was added and the reaction mixture was stirred for 10 minutes. Then 0.378 mL of ethylchloroformiate (3 eq) was added and the reaction mixture was stirred for 1 hour. Then 0.253 g (2.5 mmol) of hexylamine was added and the reaction mixture was stirred for further 12 hours at room temperature, the mixture was diluted to the total volume of 200 mL and infused into dialyzing tubes (cut-off 12-14 kDa) and dialyzed against 0.1% NaCl and 0.1% $NaHCO_3$ solution (total volume of 10 liters) 3 x 24 hours and then against demineralized water 3 x 24 hours. The dialyzed derivative was evaporated until dry and dried by means of acetone. The structure of the derivative was confirmed by NMR analysis.

Reaction yield: 0.7826 g

Mw of derivative after reaction: 200 kDa

Degree of substitution = 88%.

^{1}H NMR (assigned by means of HSQC) (500 MHz, NaOD, δ ppm):

0.8 (bs, 2H, 6), 1.2 (bs, 2H, 5), 1.45 (bs, 6H, 3, 4 and 5), 1.8 (q, 3H, 8), 2.01 (s, 3H, 8a), 3.19 (bs, 1H, 1α), 3.30 (bs, 1H, 2b), 3.36 (bs, 2H, 3), 3.50 (bs, 3H, 1β, 6a, 5a), 3.57 (bs,

1H, 3b), 3.70 (bs, 4H, 6a, 3b, 4b, 3a), 3.89 (bs, 5H, 6a, 3b, 4b, 3a, 5b), 4.50 (bs, 2H, 1a and 1b)

DOSY NMR:

log D (2.03 ppm *CH*$_3$-CO-NH-Polymer) ~-10.5 m^2/s

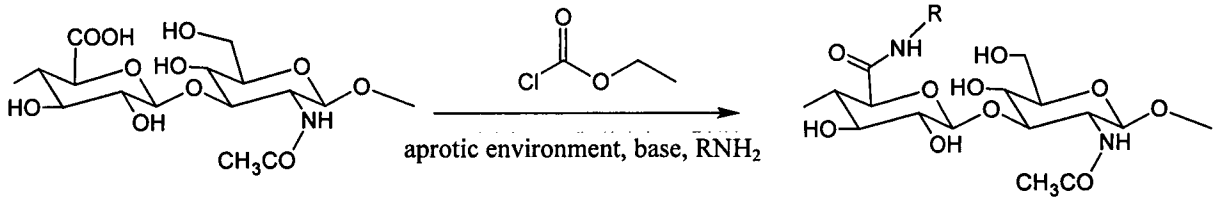
log D (2.7 ppm –CH_2 NH-R) ~-10.5 m^2/s

log D (3.0 ppm –CH_2NH-R) ~-10.5) m^2/s

The examples above only illustrate the invention and are neither meant to be limiting, nor to represent an exhaustive list of all possible embodiments of the invention.

CLAIMS

1. A method of preparation of amide derivatives of hyaluronic acid, **characterized by that** first the COOH group of the hyaluronic acid is activated by ethylchloroformiate in the presence of a base, forming a reactive intermediate which consequently reacts with a primary amine R-NH_2, forming an hyaluronic acid amide according to the Scheme 1:



Scheme 1

where R is an alkyl linear or branched chain $C_1 - C_{30}$, optionally containing aromatic or heteroaromatic groups.

2. The method of preparation according to claim 1, **characterized by that** it is carried out in a polar aprotic environment, particularly in dimethylsulphoxide.
3. The method of preparation according to any one of claims 1 and 2, **characterized by that** hyaluronic acid is in its acidic form and has a weight average molecular weight within the range of 10 to 500 kDa, particularly within the range of 350 to 500 kDa.
4. The method of preparation according to any one of claims 1 to 3, **characterized by that** the reaction is carried out at 25°C for 8 to 24 hours, preferably for 12 hours.
5. The method of preparation according to any one of claims 1 to 4, **characterized by that** the substitution degree of hyaluronic acid is controlled by the molar quantity of the added amine R-NH_2.
6. The method of preparation according to any one of claims 1 to 5, **characterized by that** the primary amine is added to the reaction mixture at time 0.1 to 2 hours after the addition of ethylchloroformiate.
7. The method of preparation according to any one of claims 1 to 6, **characterized by that** the primary amine is added to the reaction mixture in a molar quantity of 3 to 5 equivalents with respect to the molar amount of hyaluronan dimer.
8. The method of preparation according to any one of claims 1 to 7, **characterized by that** the primary amine R-NH_2 is $CH{\equiv}C\text{-}CH_2\text{-}NH_2$, $NH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}N_3$ or $NH_2(CH_2)_5\text{-}CH_3$.

9. The method of preparation according to any one of claims 1 to 8, **characterized by that** the molar amount of the added ethylchloroformiate is in a molar ratio of 3 to 5 equivalents with respect to the molar amount of hyaluronan dimer.
10. The method of preparation according to any one of claims 1 to 9, **characterized by that** the base is trialkylamine, particularly triethylamine.
11. The method of preparation according to any one of claims 1 to 10, **characterized by that** the molar amount of the added base is in a molar ratio of 3 to 5 equivalents with respect to the molar amount of hyaluronan dimer.