(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 January 2010 (14.01.2010)

PCT

(10) International Publication Number
WO 2010/003797 A1

(51) International Patent Classification:
A61K 31/728 (2006.01) *A61L 12/14* (2006.01)
A61P 27/02 (2006.01) *C11D 3/00* (2006.01)
A61P 27/04 (2006.01)

(21) International Application Number:
PCT/EP2009/057585

(22) International Filing Date:
18 June 2009 (18.06.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
08160011.6 9 July 2008 (09.07.2008) EP

(71) Applicant *(for all designated States except US)*: **NOVOZYMES BIOPHARMA DK A/S** [DK/DK]; Krogshoejvej 36, DK-2880 Bagsvaerd (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SCHWACH-ABDELLAOUI, Khadija** [FR/DK]; Martensens Allé 8, DK-1828 Frederiksberg C (DK). **FURRER, Pascal** [CH/CH]; 2 rue du Grand-Bureau, CH-1227 Les Acacias (CH). **GURNY, Robert** [CH/CH]; 7 rue Jean-Calvin, CH-1204 Geneve (CH).

(74) Agent: **NOVOZYMES A/S**; Patents, Krogshoejvej 36, DK-2880 Bagsvaerd (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2010/003797 A1

(54) **Title**: HYALURONIC ACID FOR CORNEAL WOUND HEALING

(57) **Abstract**: The invention relates to methods of using a composition comprising at least one hyaluronic acid fraction, or salt thereof, with an average molecular weight in the range of 20 - 1,200 kDa; preferably in the range of 25 - 1,000 kDa; or 30 - 800 kDa; or 35 - 600 kDa; or most preferably in the range of 40 - 400 kDa, for the manufacture of a medicament for the treatment of a corneal wound.

TITLE: HYALURONIC ACID FOR CORNEAL WOUND HEALING

FIELD OF THE INVENTION

The present invention relates to the manufacture of a medicament for the treatment of a corneal wound using a composition comprising one or more hyaluronic acid (or salt thereof) fraction with a certain average molecular weight and to the treatment of a corneal wound with such a medicament or composition.

BACKGROUND OF THE INVENTION

Hyaluronic acid (HA) is a natural and linear carbohydrate polymer belonging to the class of non-sulfated glycosaminoglycans. It is composed of beta-1,3-*N*-acetyl glucosamine and beta-1,4-glucuronic acid repeating disaccharide units with a molecular weight (MW) up to 6 MDa. HA is present in hyaline cartilage, synovial joint fluid, and skin tissue, both dermis and epidermis. HA may be extracted from natural tissues including the connective tissue of vertebrates, from the human umbilical cord and from cocks' combs. However, it is preferred today to prepare it by microbiological methods to minimize the potential risk of transferring infectious agents, and to increase product uniformity, quality and availability (U.S. Patent No. 6,951,743; WO 03/0175902).

Numerous roles of HA in the body have been identified. It plays an important role in biological organisms, as a mechanical support for cells of many tissues, such as skin, tendons, muscles and cartilage. HA is involved in key biological processes, such as the moistening of tissues, and lubrication. It is also suspected of having a role in numerous physiological functions, such as adhesion, development, cell motility, cancer, angiogenesis, and wound healing. Due to the unique physical and biological properties of HA (including viscoelasticity, biocompatibility, and biodegradability), HA is employed in a wide range of current and developing applications within cosmetics, ophthalmology, rheumatology, drug and gene delivery, wound healing and tissue engineering.

SUMMARY OF THE INVENTION

The experiments showed herein demonstrate improved healing over 48 hours of rabbit corneal wounds when different hyaluronic acid fractions of 3 average molecular weights, 51 kDa, 320 kDa and 774 kDa were applied to the wound. In particular the two fractions having the lowest average molecular weight were surprisingly effective.

Accordingly, in a first aspect the invention relates to the use of a composition comprising at least one hyaluronic acid fraction, or salt thereof, with an average molecular weight in the range of 20 – 1,200 kDa; preferably in the range of 25 – 1,000 kDa; or 30 – 800 kDa; or 35 - 600 kDa; or most preferably in the range of 40 – 400 kDa, for the manufacture of

a medicament for the treatment of a corneal wound.

In a second aspect, the invention relates to a method of treating a corneal wound with a composition comprising at least one hyaluronic acid fraction, or salt thereof, with an average molecular weight in the range of 20 – 1,200 kDa; preferably in the range of 25 – 1,000 kDa; or 30 – 800 kDa; or 35 - 600 kDa; or most preferably in the range of 40 – 400 kDa.

A third aspect of the invention relates to a contact lens multipurpose solution or a liquid preparation for a contact lens comprising at least one hyaluronic acid fraction, or salt thereof, with an average molecular weight in the range of 20 – 1,200 kDa; preferably in the range of 25 – 1,000 kDa; or 30 – 800 kDa; or 35 - 600 kDa; or most preferably in the range of 40 – 400 kDa.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the rabbit corneal wound healing of a circular mechanical wound of 6 mm diameter (n=3, mean + SD) expressed as percentage of fluorescent corneal surface (measured by confocal microscopy). The effects on wound healing of hyaluronic acid of four different molecular weights (51 kDa, 320 kDa, 774 kDa, and 1500 kDa) applied three times a day for 4 days in the form of a hydrogel are compared with a saline solution ('vehicle') and the absence of treatment (without treatment).

DETAILED DESCRIPTION OF THE INVENTION

"Hyaluronic acid" is defined herein as an unsulphated glycosaminoglycan composed of repeating disaccharide units of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA) linked together by alternating beta-1,4 and beta-1,3 glycosidic bonds. Hyaluronic acid is also known as hyaluronan, hyaluronate, or HA. The terms hyaluronan, hyaluronic acid and HA are used interchangeably herein.

The level of hyaluronic acid may be determined according to the modified carbazole method (Bitter and Muir, 1962, *Anal Biochem.* 4: 330-334). The method of choice to determine the absolute molecular weight and polydispersity of hyaluronic acid is to use Size Exclusion Chromatography combined with refractive index coupled to multi angle laser light scattering detection (SEC-MALLS-RI). For separation of hyaluronan into different molecular weight fractions, a hydrophilic column with the appropriate pore size is required (*Standard guide for characterization and testing of hyaluronan as starting materials intended for use in biomedical and tissue engineered medical product applications, ASTM International, F 2347-03*). Accordingly, the chromatography system consisted of a Waters Alliance HPLC (Waters 2695, Milford, MA, USA) equipped with Wyatt's Multi Angle Laser Light Scattering (Dawn EOS, Santa Barbara, CA, USA) and Wyatt's Optilab rex Refractive Index (RI) detector

(Optilab rEX, Santa Barbara, CA, USA). Three TSK columns (4000, 5000, and 6000 PWXL) connected in series were eluted with a buffer of 50 mM NaH_2PO_4 and 150 mM NaCl at a flow rate of 0.5 mL/min at 30°C. Dn/dc and A2 values used were 0.153 and 2.3×10^{-3}, respectively. All data were calculated in Astra software v. 5.1.3.0. (Wyatt, 1993, *Anal. Chim. Acta* 272: 1-40).

The first aspect of the invention relates to the use of a composition comprising at least one hyaluronic acid fraction, or salt thereof, with an average molecular weight in the range of 20 – 1,200 kDa; preferably in the range of 25 – 1,000 kDa; or 30 – 800 kDa; or 35 - 600 kDa; or most preferably in the range of 40 – 400 kDa, for the manufacture of a medicament for the treatment of a corneal wound.

In a preferred embodiments the the composition comprises at least two hyaluronic acid fractions having different average molecular weights that differ by at least 250 kDa; preferably by at least 200 kDa; more preferably by at least 150 kDa; still more preferably by at least 100 kDa; and most preferably by at least 50 kDa.

In another preferred embodiment, the composition further comprises at least one additional hyaluronic acid fraction, or salt thereof, with a average molecular weight in the range of 600 – 10,000 kDa; preferably in the range of 650 – 5,000 kDa; more preferably in the range of 700 – 2,500 kDa.

In a preferred embodiment the medicament provides a corneal wound surface area below 22% or a reduction in the corneal wound surface area of at least 32% compared to a control treatment when measured at 48 hours in the rabbit corneal wound healing model defined herein.

It is preferred that the composition comprises at least 0.01% (w/w) of the at least one hyaluronic acid fraction, or salt thereof; preferably at least 0.1% (w/w) of the at least one hyaluronic acid fraction, or salt thereof.

Likewise, it is preferable that the medicament also comprises at least one pharmaceutically active compound, preferably an antibiotic compound, a bacteriostatic compound or an anaesthetic compound.

The second aspect of the invention relates to a method of treating a corneal wound with a composition comprising at least one hyaluronic acid fraction, or salt thereof, with an average molecular weight in the range of 20 – 1,200 kDa; preferably in the range of 25 – 1,000 kDa; or 30 – 800 kDa; or 35 - 600 kDa; or most preferably in the range of 40 – 400 kDa.

It is preferred that the composition comprises at least two hyaluronic acid fractions having different average molecular weights that differ by at least 250 kDa; preferably by at least 200 kDa; more preferably by at least 150 kDa; still more preferably by at least 100 kDa; and most preferably by at least 50 kDa.

Preferably the composition further comprises at least one additional hyaluronic acid fraction, or salt thereof, with a average molecular weight in the range of 600 – 10,000 kDa; preferably in the range of 650 – 5,000 kDa; more preferably in the range of 700 – 2,500 kDa.

A preferred embodiment relates to the method of the second aspect, which provides a corneal wound surface area below 22% or a reduction in the corneal wound surface area of at least 32% compared to a control treatment when measured at 48 hours in the rabbit corneal wound healing model defined herein.

It is also preferred that the composition comprises at least 0.01% (w/w) of the at least one hyaluronic acid fraction, or salt thereof; or preferably at least 0.1% (w/w) of the at least one hyaluronic acid fraction, or salt thereof.

In another preferred embodiment the composition comprises at least one pharmaceutically active compound, preferably an antibiotic compound, a bacteriostatic compound or an anaesthetic compound.

The third aspect of the invention relates to a contact lens multipurpose solution or a liquid preparation for a contact lens comprising at least one hyaluronic acid fraction, or salt thereof, with an average molecular weight in the range of 20 – 1,200 kDa; preferably in the range of 25 – 1,000 kDa; or 30 – 800 kDa; or 35 - 600 kDa; or most preferably in the range of 40 – 400 kDa.

In a preferred embodiment the solution or preparation of the third aspect comprises at least two hyaluronic acid fractions having different average molecular weights that differ by at least 250 kDa; preferably by at least 200 kDa; more preferably by at least 150 kDa; still more preferably by at least 100 kDa; and most preferably by at least 50 kDa.

Preferably, the solution or preparation of the third aspect further comprises at least one additional hyaluronic acid fraction, or salt thereof, with a average molecular weight in the range of 600 – 10,000 kDa; preferably in the range of 650 – 5,000 kDa; more preferably in the range of 700 – 2,500 kDa.

It is also preferred that the solution or preparation of the third aspect comprises at least 0.01% (w/w) of the at least one hyaluronic acid fraction, or salt thereof; or preferably at least 0.1% (w/w) of the at least one hyaluronic acid fraction, or salt thereof.

Finally, it is preferred that the solution or preparation of the third aspect also comprises at least one pharmaceutically active compound, preferably an antibiotic compound, a bacteriostatic compound or an anaesthetic compound.

HA sources

Rooster combs are a significant commercial source for hyaluronan. Microorganisms are an alternative source. U.S. Patent No. 4,801,539 discloses a fermentation method for preparing hyaluronic acid involving a strain of *Streptococcus zooepidemicus* with reported

yields of about 3.6 g of hyaluronic acid per liter. European Patent No. EP0694616 discloses fermentation processes using an improved strain of *Streptococcus zooepidemicus* with reported yields of about 3.5 g of hyaluronic acid per liter.

In a preferred embodiment the hyaluronic acid or salt thereof is of microbial origin, preferably from a strain of *Streptococcus*.

As disclosed in WO 03/054163 (Novozymes), which is incorporated herein in its entirety, hyaluronic acid or salts thereof may be recombinantly produced, e.g., in a Gram-positive *Bacillus* host.

Hyaluronan synthases have been described from vertebrates, bacterial pathogens, and algal viruses (DeAngelis, P. L., 1999, Cell. Mol. Life Sci. 56: 670-682). WO 99/23227 discloses a Group I hyaluronate synthase from *Streptococcus equisimilis*. WO 99/51265 and WO 00/27437 describe a Group II hyaluronate synthase from *Pasturella multocida*. Ferretti et al. disclose the hyaluronan synthase operon of *Streptococcus pyogenes*, which is composed of three genes, *hasA, hasB,* and *hasC*, that encode hyaluronate synthase, UDP glucose dehydrogenase, and UDP-glucose pyrophosphorylase, respectively (Proc. Natl. Acad. Sci. USA. 98, 4658-4663, 2001). WO 99/51265 describes a nucleic acid segment having a coding region for a *Streptococcus equisimilis* hyaluronan synthase.

Since the hyaluronan of a recombinant *Bacillus* cell is expressed directly to the culture medium, a simple process may be used to isolate the hyaluronan from the culture medium. First, the *Bacillus* cells and cellular debris are physically removed from the culture medium. The culture medium may be diluted first, if desired, to reduce the viscosity of the medium. Many methods are known to those skilled in the art for removing cells from culture medium, such as centrifugation or microfiltration. If desired, the remaining supernatant may then be filtered, such as by ultrafiltration, to concentrate and remove small molecule contaminants from the hyaluronan. Following removal of the cells and cellular debris, a simple precipitation of the hyaluronan from the medium is performed by known mechanisms. Salt, alcohol, or combinations of salt and alcohol may be used to precipitate the hyaluronan from the filtrate. Once reduced to a precipitate, the hyaluronan can be easily isolated from the solution by physical means. The hyaluronan may be dried or concentrated from the filtrate solution by using evaporative techniques known to the art, such as lyophilization or spraydrying.

Accordingly, in a preferred embodiment the hyaluronic acid or salt thereof is recombinantly produced, preferably by a Gram-positive bacterium or host cell, more preferably by a bacterium of the genus *Bacillus*.

In another preferred embodiment the hyaluronic acid or salt thereof is recombinantly produced, preferably by expressing a heterologous hyaluronic acid synthase gene(s) in a strain of *Bacillus*.

The host cell may be any *Bacillus* cell suitable for recombinant production of hyaluronic acid. The *Bacillus* host cell may be a wild-type *Bacillus* cell or a mutant thereof. *Bacillus* cells useful in the practice of the present invention include, but are not limited to, *Bacillus agaraderhens, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans, Bacillus clausii, Bacillus coagulans, Bacillus firmus, Bacillus lautus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus stearothermophilus, Bacillus subtilis,* and *Bacillus thuringiensis* cells. Mutant *Bacillus subtilis* cells particularly adapted for recombinant expression are described in WO 98/22598. Non-encapsulating *Bacillus* cells are particularly useful in the present invention.

In a preferred embodiment, the Bacillus host cell is a *Bacillus amyloliquefaciens, Bacillus clausii, Bacillus lentus, Bacillus licheniformis, Bacillus stearothermophilus* or *Bacillus subtilis* cell. In a more preferred embodiment, the *Bacillus* cell is a *Bacillus amyloliquefaciens* cell. In another more preferred embodiment, the *Bacillus* cell is a *Bacillus clausii* cell. In another more preferred embodiment, the *Bacillus* cell is a *Bacillus lentus* cell. In another more preferred embodiment, the *Bacillus* cell is a *Bacillus licheniformis* cell. In another more preferred embodiment, the *Bacillus* cell is a *Bacillus subtilis* cell. In a most preferred embodiment, the *Bacillus* host cell is *Bacillus subtilis* A164Δ5 (see U.S. Patent No. 5,891,701) or *Bacillus subtilis* 168Δ4.

Transformation of the Bacillus host cell with a nucleic acid construct of the present invention may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, Molecular General Genetics 168: 111-115), by using competent cells (see, e.g., Young and Spizizen, 1961, Journal of Bacteriology 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, Journal of Molecular Biology 56: 209-221), by electroporation (see, e.g., Shigekawa and Dower, 1988, Biotechniques 6: 742-751), or by conjugation (see, e.g., Koehler and Thorne, 1987, Journal of Bacteriology 169: 5271-5278).

In a preferred embodiment the salt of hyaluronic acid is an inorganic salt, preferably sodium hyaluronate, potassium hyaluronate, ammonium hyaluronate, calcium hyaluronate, magnesium hyaluronate, zinc hyaluronate, or cobalt hyaluronate.

EXAMPLES

Example 1: Production of low molecular weight HA by acid hydrolysis

Hyaluronic acid (0.5 g) was dissolved overnight at room temperature with vigorous agitation in 50 ml MilliQ™ water in 5 x sealable 250 ml bottles. The solutions were pre-warmed to 60^{O}C before 4 M HCl was added under vigorous stirring (800 rpm) for 1 minute to give acid concentrations of 0; 0.10; 0.50; 1.0 and 2.0 M. The total HA concentrations were adjusted to 10 mg/ml. The bottles were left at weak shaking at 60^{O}C in a water bath for a total of 52 hours. Samples of 5 ml were withdrawn at 1h10min, 2h17min, 4h10min, 5h05min,

23h, 48h15min and 52h10min from each solution and neutralized with equimolar amounts of NaOH (1M solution) before freezing (-20°C) and lyophilisation.

Example 2: Preparation and characterization of HA-based formulations

HA (molecular weight: 51kDa, or 320 kDa, or 774 kDa, or 1500 kDa) was dissolved in a neutral, isotonic and sterile phosphate buffer solution (PBS) at room temperature and under magnetic stirring to give a final concentration of 0.2% (w/v). The buffer was sterilized by filtration (Sartorius filters of nominal pore size 0.22 microns).

The formulations were freshly prepared for all *in vivo* experiments to avoid the addition of preservatives and were conditioned in radiation sterilized eye dropper glass bottles of 10 mL. The PBS buffer had a pH of 7.33 and an osmotic pressure of 282 mOsm/kg. These physico-chemical characteristics measured were compatible with physiological parameters of the tear film, thus allowing topical administration to the cornea.

Example 3: Evaluation of effect of hyaluronic acid on corneal wound healing

New Zealand white rabbit females weighing 3.3 - 5.0 kg (University Medical Center, Geneva, Switzerland) have been used in this study. Animals were individually housed in stainless steel cages and maintained in a 12 hour light/dark cycle at 19 ± 1°C. They were allowed water and food ad libitum. All animals were healthy and free of clinical observable ocular abnormalities. All experiments have been performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research (ARVO, 1984) and were approved by the local veterinary authority for animal experimentation.

Mechanical wounds in form of circular superficial epithelial abrasion were performed on rabbit corneas by means of a sterile Algerbrush burr with a 1 mm tip (Jannach, Italy). Animals were anesthetized with an intramuscular injection of a 1:1 mixture of ketamine hydrochloride (37.5 mg/kg body weight) and xylazine hydrochloride (10 mg/kg body weight). A drop of topical anaesthetic (oxybuprocain hydrochloride 0.4%, NOVESIN®, Ciba Vision, Switzerland) was instilled on the cornea prior to the surgical procedure.

Circular wounds of 6-mm diameter were generated. The wound size was controlled by applying onto the cornea a sterilized transparent stencil film (PARAFILM® M, American Can Company, Greenwich, USA) with a hole cut up by a punch of 6-mm diameter. Immediately after the creation of the mechanical wound, the rabbit eye was thoroughly rinsed with a sterile saline physiological solution (isotonic sodium chloride solution).

Twelve rabbits were included in the study, three for each formulation. Each rabbit received only one HA-formulation. The isotonic hydrogel HA-formulations based on 0.2% hyaluronic acid were applied to the right eye immediately after the surgical treatment at the

beginning of the experiment. Then the rabbits were treated with the different formulations three times per day at 9:00, 13:00, and 17:00 o'clock till the end of the experiment (that is 96 h after surgery).

The abraded corneal surface was revealed by instilling a sterile isotonic sodium fluorescein solution (0.5%, 25 microliter). After 2 minutes dyeing, the excess fluorescein was washed out during one minute with a sterile NaCl 0.9% solution. The corneas were then observed under confocal microscopy. The HA formulations were compared with a saline vehicle and with absence of treatment.

Microscopic observation was performed using a confocal laser scanning ophthalmoscope (CLSO Zeiss, Oberkochen, Germany) modified by addition of a set of lenses in order to view the cornea instead of the retina. An argon ion laser operating at 488 nm wavelength was used as the excitation light source. The fluorescence signal was detected by a photomultiplier. Images were obtained using Epiplan-Neofluar (2.5x/0.075 NA objective lens (Zeiss, Oberkochen, Germany). Optical sectioning was performed parallel to the corneal surface, at 16 equidistant different focal planes, the focus shifting (from 0 to 470 microns) covering the whole corneal thickness. The images were displayed on a digital video monitor. An image processing system (Analysis SIS, Münster, Germany) carried out the following operation step: addition of the 16 digitized images in one stack to produce a three-dimensional reconstruction, projection of this stack and calculation of the total surface of the fluorescence areas on the projection. No anesthesia of the animals was necessary during the microscopic observation.

The results were evaluated in three ways. Firstly, the influence of the different treatments on the percentages of fluorescent corneal surface was compared to the vehicle treatment and to the absence of treatment using the Student t test (unpaired sample, level of significance: $p>0.05$) after the Fisher-Snedecor analysis of variance (supposing a normal distribution of the value). Secondly, using curvefitting in a form of a cubic spline (Maple 10 software, Maplesoft, Ontario, Canada), the mathematical equation of each curve (percentage of fluorescent area as a function of time) was calculated (J.H. Ahlberg, E.N. Nilson et al. 1967; C. de Boor, 1978; G.D. Knott, 2000). Thirdly, using the same software, the Area Under the Curve (AUC) was determined for each curve (integration limits: 0 h and 96 h). The AUC is a parameter indicating the healing rate: the smaller the AUC, the faster the wound healing rate. For biexponential curves, the AUC was calculated taking the interval of integration from 0 h until the time corresponding to the curve minimum point where the first derivative of the curve is zero. The enabled to take into account the first healing phase without the epithelial reorganization one.

Example 4: Evolution of the wound healing and wound healing rate after mechanical

corneal injury

The evolution of the wound healing after mechanical corneal injury for different hyaluronic acids (51 kDa, 320 kDa, 774 kDa and 1500 kDa) is detailed in Table 1. The mean area under the curve (AUC) indicating the rate after instillation of the four types of hyaluronic acids is presented in Table 2.

The results were compared to a vehicle (saline solution) and to the absence of treatment ('without treatment') (Figure 1). The data showed a healing evolution quite similar for all tested ophthalmic formulations. This evolution consisted of a rapid decrease of the percentage of fluorescent surface in 48 h, followed by a slower decrease between 48h and 96h. Sometimes, the percentage of fluorescent surface even decreased slightly between 72h and 96h. This evolution indicated a two-phase corneal repair mechanism. In the first phase, the wound closed up more or less rapidly, and in the second phase, a rearrangement of the epithelial cells occurred, thus explaining the possible and transient slight increase in the percentage of fluorescent surface.

Moreover, due to important standard deviation, the statistical data confrontation of each HA formulation with the saline solution (vehicle) at different times (24h, 48,h, 72h, and 96h after surgery) did not discriminate the performance if the different formulations at 24h. Presumably, at this time, tissue inflammation was too important to be influenced by the instillation of a HA hydrogel.

At 48h, formulations based on 51 kDa HA, 320 kDa HA, and 774 kDa significantly stimulated corneal wound healing compared to saline control solution. Low MW HA (51 kDa and 320 kDa) promoted wound healing 48 h after surgery compared to the absence of treatment, suggesting that these HAs covered and protected the corneal wound adequately and accelerated the wound healing rate during the first phase of corneal repair where the wound was closed up by bordering cells. The healing process was enhanced by 50% when applying these two low molecular weight HA-based formulations compared to what it would be when applying no treatment. At 48h, HA can provide additional comfort.

Later, that is 72h and 96h after surgery, the administration of HA of different molecular weights did not seem to accelerate the wound healing rate. During the second phase of wound healing, a cell rearrangement occurred, explaining the slight decrease in fluorescence in a few cases.

No difference was observed between formulations based on 1500 kDa and the control formulations regardless the time of analysis. High molecular weight HA is too viscous and creates a barrier for cells.

Table 1: Effects of the different HA molecular weights (applied three times per day during 5 days in the form of an hydrogel) on the wound healing rate, compared to a saline solution

(vehicle) and the absence of treatment (without treatment). The percentage of fluorescent corneal surface is assessed by confocal microscopy. All values are means of triplicates (n=3). The data is shown graphically in figure 1.

Time (h)	Without treatment*		Vehicle*		HA (51 kDa)*	
	Mean corneal surface	Standard deviation	Mean corneal surface	Standard deviation	Mean corneal surface	Standard deviation
0	100.0 %		100.0 %		100.0 %	
24	83.8 %	22.1	77.0 %	1.7	73.8 %	11.2
48	25.2 %	2.9	25.9 %	2.8	17.5 %	2.4
72	11.4 %	0.9	20.8 %	7.8	11.6 %	5.5
96	8.0 %	6.5	7.5 %	1.5	12.4 %	7.0

Time (h)	HA (320 kDa)*		HA (774 kDa)*		HA (1500 kDa)*	
	Mean corneal surface	Standard deviation	Mean corneal surface	Standard deviation	Mean corneal surface	Standard deviation
0	100.0 %		100.0 %		100.0 %	
24	62.6 %	17.2	81.4 %	4.3	75.1 %	2.8
48	12.8 %	4.4	21.0 %	2.6	29.6 %	4.6
72	11.3 %	6.6	6.6 %	1.7	11.3 %	2.9
96	14.9 %	10.8	7.5 %	2.6	5.7 %	4.9

Table 2: Wound healing rate after mechanical corneal injury as measured by the area under the curve (AUC) in a graph plotting the decrease of wound surface over time 0 – 96 hours (n=3).

Sample	Mean AUC	Standard deviation
HA 51 KDa	3375.8	343.8
HA 320 kDa	2920.9	498.9
HA 774 kDa	3713.5	37.9
HA 1500 kDa	3830.2	153.3
Vehicle	3621.8	241.7

CLAIMS

1. Use of a composition comprising at least one hyaluronic acid fraction, or salt thereof, with an average molecular weight in the range of 20 – 1,200 kDa; preferably in the range of 25 – 1,000 kDa; or 30 – 800 kDa; or 35 - 600 kDa; or most preferably in the range of 40 – 400 kDa, for the manufacture of a medicament for the treatment of a corneal wound.

2. The use according to claim 1, wherein the composition comprises at least two hyaluronic acid fractions having different average molecular weights that differ by at least 250 kDa; preferably by at least 200 kDa; more preferably by at least 150 kDa; still more preferably by at least 100 kDa; and most preferably by at least 50 kDa.

3. The use according to any of claims 1 - 2, wherein the composition further comprises at least one additional hyaluronic acid fraction, or salt thereof, with a average molecular weight in the range of 600 – 10,000 kDa; preferably in the range of 650 – 5,000 kDa; more preferably in the range of 700 – 2,500 kDa.

4. The use of any of claims 1 – 3, wherein the medicament provides a corneal wound surface area below 22% when measured at 48 hours in the rabbit corneal wound healing model defined herein.

5. The use of any of claims 1 – 4, wherein the medicament provides a reduction in the corneal wound surface area of at least 32% compared to a control treatment when measured at 48 hours in the rabbit corneal wound healing model defined herein.

6. The use of any of claims 1 – 5, wherein the composition comprises at least 0.01% (w/w) of the at least one hyaluronic acid fraction, or salt thereof; preferably at least 0.1% (w/w) of the at least one hyaluronic acid fraction, or salt thereof.

7. The use of any of claims 1 – 6, wherein the medicament also comprises at least one pharmaceutically active compound, preferably an antibiotic compound, a bacteriostatic compound or an anaesthetic compound.

8. A method of treating a corneal wound with a composition comprising at least one hyaluronic acid fraction, or salt thereof, with an average molecular weight in the range of 20 – 1,200 kDa; preferably in the range of 25 – 1,000 kDa; or 30 – 800 kDa; or 35 - 600 kDa; or most preferably in the range of 40 – 400 kDa.

9. The method of claim 8, wherein the composition comprises at least two hyaluronic acid fractions having different average molecular weights that differ by at least 250 kDa; preferably by at least 200 kDa; more preferably by at least 150 kDa; still more preferably by at least 100 kDa; and most preferably by at least 50 kDa.

10. The method of claim 8 or 9, wherein the composition further comprises at least one additional hyaluronic acid fraction, or salt thereof, with a average molecular weight in the range of 600 – 10,000 kDa; preferably in the range of 650 – 5,000 kDa; more preferably in the range of 700 – 2,500 kDa.

11. The method of any of claims 8 – 10, which provides a corneal wound surface area below 22% when measured at 48 hours in the rabbit corneal wound healing model defined herein.

12. The method of any of claims 8 – 11, which provides a reduction in the corneal wound surface area of at least 32% compared to a control treatment when measured at 48 hours in the rabbit corneal wound healing model defined herein.

13. The method of any of claims 8 – 12, wherein the composition comprises at least 0.01% (w/w) of the at least one hyaluronic acid fraction, or salt thereof; or preferably at least 0.1% (w/w) of the at least one hyaluronic acid fraction, or salt thereof.

14. The method of any of claims 8 – 13, wherein the composition also comprises at least one pharmaceutically active compound, preferably an antibiotic compound, a bacteriostatic compound or an anaesthetic compound.

15. A contact lens multipurpose solution or a liquid preparation for a contact lens comprising at least one hyaluronic acid fraction, or salt thereof, with an average molecular weight in the range of 20 – 1,200 kDa; preferably in the range of 25 – 1,000 kDa; or 30 – 800 kDa; or 35 - 600 kDa; or most preferably in the range of 40 – 400 kDa.

16. The solution or preparation of claim 15, which comprises at least two hyaluronic acid fractions having different average molecular weights that differ by at least 250 kDa; preferably by at least 200 kDa; more preferably by at least 150 kDa; still more preferably by at least 100 kDa; and most preferably by at least 50 kDa.

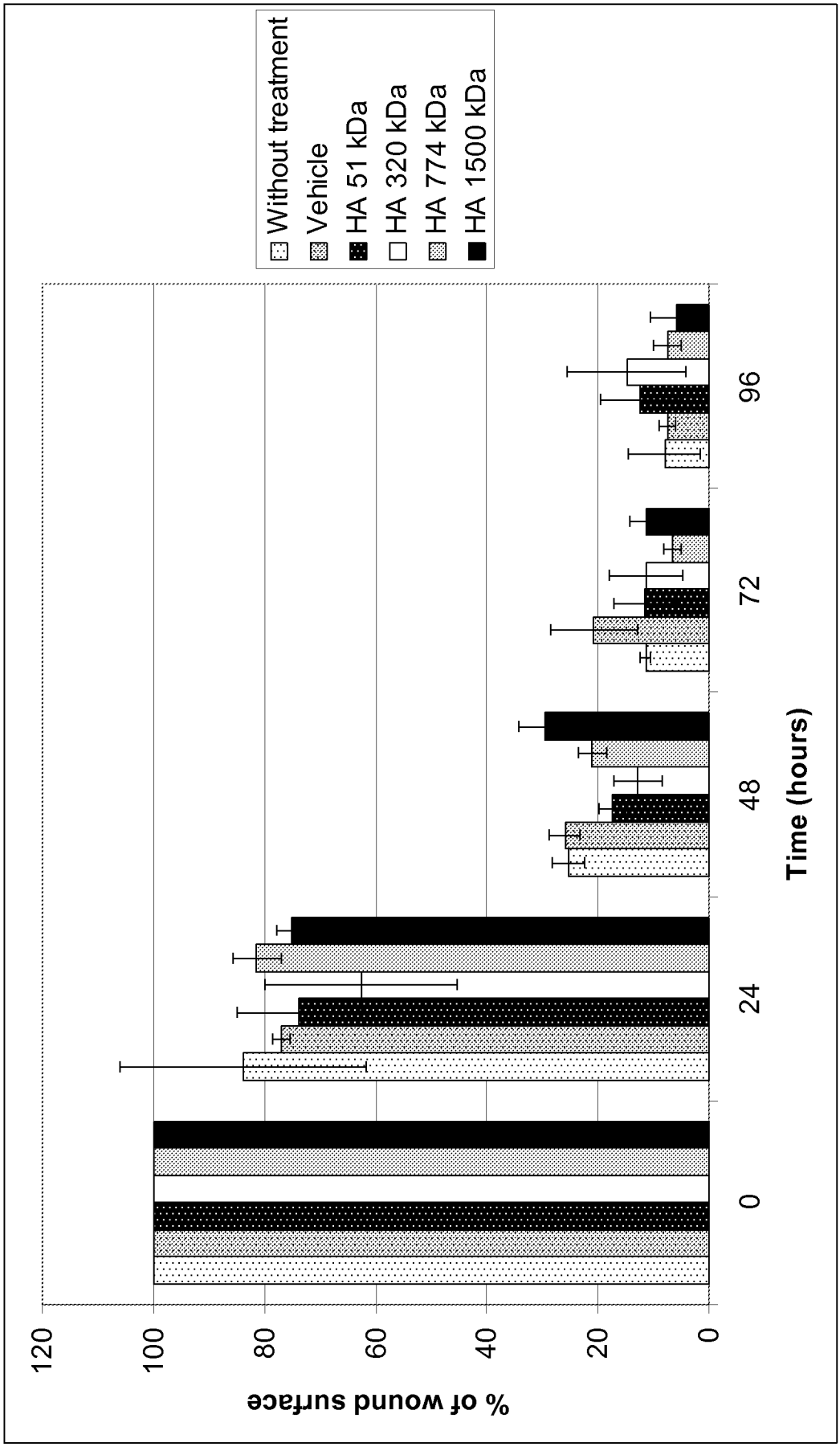
17. The solution or preparation of claim 15 or 16, which further comprises at least one

additional hyaluronic acid fraction, or salt thereof, with a average molecular weight in the range of 600 – 10,000 kDa; preferably in the range of 650 – 5,000 kDa; more preferably in the range of 700 – 2,500 kDa.

18. The solution or preparation of any of claims 15 - 17, which comprises at least 0.01% (w/w) of the at least one hyaluronic acid fraction, or salt thereof; or preferably at least 0.1% (w/w) of the at least one hyaluronic acid fraction, or salt thereof.

19. The solution or preparation of any of claims 15 - 18, which also comprises at least one pharmaceutically active compound, preferably an antibiotic compound, a bacteriostatic compound or an anaesthetic compound.

FIG. 1


Without treatment
Vehicle
HA 51 kDa
HA 320 kDa
HA 774 kDa
HA 1500 kDa
% of wound surface
120
100
80
60
40
20
0
0
24
48
72
96
Time (hours)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/057585

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/728 A61P27/02 A61P27/04 A61L12/14 C11D3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C11D

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 practical, search terms used)
EPO-Internal, EMBASE, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 166 331 A (DELLA VALLE FRANCESCO [IT] ET AL) 24 November 1992 (1992-11-24) column 1, lines 17-25 column 2, lines 46-57 column 3, lines 42-59 column 4, lines 19-62 columns 7-8; table 2 column 45, lines 20-25 claims 1-18	1-14
X	EP 0 555 898 A (FIDIA SPA [IT] FIDIA SPA [CA]) 18 August 1993 (1993-08-18) page 18, line 15 - page 29, line 19 claims 1-7	1-14

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 October 2009	23/10/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hillebrecht, Dieter

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NAKAMURA M ET AL: "Concentration and molecular weight dependency of rabbit corneal epithelial wound healing on hyaluronan" CURRENT EYE RESEARCH 1992 GB, vol. 11, no. 10, 1992, pages 981-986, XP008097255 ISSN: 0271-3683 abstract page 981, column 1, paragraph 2 page 984, column 2, paragraph 2 - last paragraph; figure 5	1,4-6,8, 11-13
X	NAKAMURA M ET AL: "Recent developments in the use of hyaluronan in wound healing" EXPERT OPINION ON INVESTIGATIONAL DRUGS 1995 GB, vol. 4, no. 3, 1995, pages 175-188, XP008097254 ISSN: 1354-3784 page 178, column 1, last paragraph - page 182, column 2, last paragraph	1,4-6,8, 11-13
A	CAMILLIERI G ET AL: "HYALURONAN-INDUCED STIMULATION OF CORNEAL WOUND HEALING IS A PURE PHARMACOLOGICAL EFFECT" JOURNAL OF OCULAR PHARMACOLOGY AND THERAPEUTICS, MARY ANN LIEBERT, INC., NEW YORK, NY, US, vol. 20, no. 6, 1 January 2004 (2004-01-01), pages 548-553, XP008063687 ISSN: 1080-7683 abstract page 548, last paragraph - page 549, paragraph 1 page 550, paragraph 2-4 page 551, paragraph 1 - page 552, paragraph 2	1-14
A	US 5 925 626 A (DELLA VALLE FRANCESCO [IT] ET AL) 20 July 1999 (1999-07-20) column 2, lines 46-62 claims 1-22	1-14
A	WO 93/15744 A (ALLERGAN INC [US]) 19 August 1993 (1993-08-19) the whole document	1-14
P,X	WO 2009/024677 A (AGRO IND RECH S ET DEV A R D [FR]; INST NAT SANTE RECH MED [FR]; BRESI) 26 February 2009 (2009-02-26) claims 1,9	1

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/057585

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/076506 A (BAUSCH & LOMB) 26 June 2008 (2008-06-26) page 11, last paragraph - page 12, paragraph 1; claims	15-19
X	WO 2004/062660 A (ADVANCED MEDICAL OPTICS INC) 29 July 2004 (2004-07-29) paragraphs [0002] - [0005], [0010], [0027]; claims; examples	15-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2009/057585

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-14 (entirely)

Use of a composition comprising at least one hyaluronic acid fraction or salt thereof with an average molecular weight in the range of 20-1,200 kDa as defined in claims 1-7 for the manufacture of a medicament for the treatment of a corneal wound or
a method of treating a corneal wound with said composition as defined in claims 8-14.

---

2. claims: 15-19 (entirely)

A contact lens multipurpose solution or a liquid preparation for a contact lens comprising at least one hyaluronic acid fraction or salt thereof with an average molecular weight in the range of 20-1,200 kDa as defined in claims 15-19.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2009/057585

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5166331 A	24-11-1992	NONE	
EP 0555898 A	18-08-1993	AR 245369 A1	31-01-1994
		AT 98495 T	15-01-1994
		AU 592077 B2	04-01-1990
		AU 5566286 A	16-10-1986
		BE 904547 A1	03-10-1986
		CA 1340825 C	23-11-1999
		CA 1341418 C	21-01-2003
		CH 672886 A5	15-01-1990
		CN 85102921 A	08-06-1988
		DE 3689384 D1	27-01-1994
		DE 3689384 T2	07-07-1994
		DK 149886 A	06-10-1986
		EP 0197718 A2	15-10-1986
		ES 8800055 A1	01-01-1988
		FI 861395 A	06-10-1986
		FR 2579895 A1	10-10-1986
		HU 40579 A2	28-01-1987
		HU 204202 B	30-12-1991
		IE 64440 B1	09-08-1995
		IN 165867 A1	03-02-1990
		IT 1229075 B	17-07-1991
		JP 2585216 B2	26-02-1997
		JP 61236732 A	22-10-1986
		JP 2677778 B2	17-11-1997
		JP 9169667 A	30-06-1997
		LU 86386 A1	02-09-1986
		NO 861331 A	06-10-1986
		NZ 215676 A	29-08-1989
		PH 29979 A	29-10-1996
		PT 82342 A	01-05-1986
		US 4736024 A	05-04-1988
		ZA 8602463 A	30-12-1987
US 5925626 A	20-07-1999	NONE	
WO 9315744 A	19-08-1993	AT 241366 T	15-06-2003
		AU 3658793 A	03-09-1993
		CA 2129238 A1	19-08-1993
		DE 69333005 D1	03-07-2003
		DE 69333005 T2	01-04-2004
		EP 0625904 A1	30-11-1994
		ES 2199940 T3	01-03-2004
		JP 7503958 T	27-04-1995
		JP 3751017 B2	01-03-2006
WO 2009024677 A	26-02-2009	FR 2919185 A1	30-01-2009
WO 2008076506 A	26-06-2008	US 2008141628 A1	19-06-2008
WO 2004062660 A	29-07-2004	AU 2004204734 A1	29-07-2004
		BR PI0406636 A	06-12-2005
		CA 2512320 A1	29-07-2004
		EP 1581211 A1	05-10-2005
		JP 2006516032 T	15-06-2006