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

(19) World Intellectual Property Organization
International Bureau

PCT

(43) International Publication Date
8 July 2010 (08.07.2010)

(10) International Publication Number
WO 2010/077156 A1

(51) International Patent Classification:
A61K 47/36 (2006.01) *A61K 9/16* (2006.01)
A61K 47/38 (2006.01) *A61K 31/727* (2006.01)
A61K 9/70 (2006.01)

(21) International Application Number:
PCT/PL2009/000110

(22) International Filing Date:
22 December 2009 (22.12.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
P-386977 31 December 2008 (31.12.2008) PL

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(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, SM, 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))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2010/077156 A1

(54) **Title**: COMPOSITION FOR PROLONGED RELEASE OF HEPARIN AND USE OF THE ALGINATE-HYDROXYPROPYLCELLULOSE GEL FOR PROLONGED RELEASE OF HEPARIN

(57) **Abstract**: The composition for the prolonged release of heparin, wherein the gel obtained from the alkali metal alginate and hydroxypropylcellulose at the weight ratio from 2:1 to 5:1 is used subjected to the gelling process using calcium salt, containing immobilised heparin in the amount from 0.01 to 10% w/w. The invention also relates to the use of the gel obtained from sodium alginate and hydroxypropylcellulose for the prolonged release of heparin.

Composition for prolonged release of heparin and use of the alginate-hydroxypropylcellulose gel for prolonged release of heparin

The subject of the invention is the composition for prolonged release of heparin and use of alginate- hydroxypropylcellulose gel for prolonged release of heparin.

Heparin is an anticoagulant of natural origin. The physiological action of this compound is based on preventing the formation of blood clots and the growth of the already existing ones. It is one of the drugs existing the longest on the market, which is still in the widespread clinical use, mostly in the cases of acute myocardial infarctions and cardiosurgery. Short biological half-life of heparin (around one hour) constitutes the serious disadvantage. This problem can be partially solved by application of low molecular weight heparin (LMWH), which can be administered once daily. This solution, however, is not free from its shortcomings, one of them is the difficulty with fast and effective removal of LMWH heparin, which in some cases is absolutely necessary.

Heparin has also found numerous applications in tissue engineering of the blood vessels, one of the most promising techniques in the vessel surgery based on obtaining the living autologous tissue. In this technique heparin is used to prevent thrombosis upon implantation of the biodegradable tissue scaffolds. One of the ways to apply heparin is binding it to the scaffold surface, which, however, limits its activity considerably [G.B. Oliveira, L.B. Carvalho Jr., M.P.C. Silva, Properties of carbodiimide treated heparin, Biomaterials 24 (2003) 4777–4783; C.D. Ebert and S.W. Kim, Immobilized heparin: spacer arm effects on biological interactions. Thromb. Res. 26 (1982) 43-57]. Another approach, presented also in our research, is drug encapsulation inside the matrix that assures the continuous, slow release of the drug. The long-lasting constant blood level of the anticoagulant is also essential in other cases when clot prevention becomes necessary. For example thrombosis-preventing drugs are used in surgery also when the tissue being reconstructed is obtained *in vitro* and subsequently introduced into the injured place in the cardiac muscle tissue.

Stimuli-responsive polymers are widely used in the systems for controlled- release and delivery of the biologically active substances. Application of the thermo-sensitive polymers in the cell culturing was also reported. Using the conventional methods for detachment of the grown cells from the dish wall where the culture is growing may damage the cells to some

extent. Enzymatic methods destroy certain connections between cells and do not allow for the growth and subsequent detachment of the thin cell layers. This problem can be solved by application of the thermo-sensitive surfaces. Kim and coworkers [H. von Recuma, T. Okano S. Wan Kim, Growth factor release from thermally reversible tissue culture substrates, J. Control. Release 55 (1998) 121–130] covalently bound poly(N-isopropylacrylamide) (PNIPAM), the thermo-sensitive polymer, to the walls of the dishes in which cell cultures were grown. The aim was to enable the cells detachment upon the change from the physiological to the room temperature. Recently Hatakeyama and his coworkers [H. Hatakeyama, A. Kikuchi, M. Yamato, T. Okano, Bio-functionalized thermo-responsive interfaces facilitating cell adhesion and proliferation, Biomaterials 27 (2006) 5069–5078] obtained bioactive, thermo-sensitive surfaces for the cell cultures by immobilizing of the RGDS peptide responsible for the cell adhesion and the growth factor (insulin) on the copolymer matrix containing PNIPAM mers.

From the US 4 792 452 patent description is known of the pharmaceutical composition for the controlled release of the active substances of basic character, which contains alginate in the amount between 15 and 45% and hydrocolloidal gelling medium in the amount between 3 and 35%. As a gelling agent this composition may contain hydroxypropylcellulose. According to US 4 792 452 patent this composition formulation is designed for the release of the low molecular weight active substances of basic character and is used in pharmaceutical preparations in the form of tablets. In the patent description mentioned above there is no information about any compositions containing biologically active polymeric substances of non-basic character.

The invention provides the composition for the prolonged release of heparin, which is constituted by the alkali metal salt of alginate and hydroxypropylcellulose in the weight ratio from 2:1 to 5:1, subjected to the gelling process using the calcium salt, containing immobilized heparin in the amount between 0.01% and 10% w/w (from 10-4 mg to 0.1 mg heparin/1 mg of the sample). Preferably, the composition has the form of the microspheres, film or the film containing microspheres. Preferably, the alkali metal alginate is the sodium alginate.

The invention also relates to the use of the gel obtained from sodium alginate and hydroxypropylcellulose for the prolonged release of heparin.

The general description of the microsphere synthesis:

Microspheres are obtained via emulsification method. In the first stage in the reaction flask is placed so-called continuous phase, which is a liquid that is immiscible with water (e.g. cyclohexane, mineral oil). This liquid should, upon addition of the aqueous phase, form an emulsion in the presence of the suitable emulsifier (e.g. surfactant or a mixture of surfactants of the proper composition). To obtain the emulsion it is necessary to continuously and intensely stir the reaction mixture. The aqueous phase consists of 2 to 3% w/w aqueous solution of the alkali metal alginate containing dissolved heparin. To the mixture prepared as described above the aqueous solution of calcium chloride, which crosslinks the alginate polymer dissolved in the suspended droplets of water containing heparin. As a result the heparin-containing microspheres are formed. They are next incubated in the reaction mixture for ca. 30 minutes and then filtered off and washed.

Hydroxypropylcellulose shows lower critical solution temperature (LCST) at 43°C, whereas the LCST values obtained for the composition prepared according to the invention are considerably lower than the LCST of the native hydroxypropylcellulose. An increase in the alginate content increases the LCST value for the gel because alginate, as more hydrophilic, slows down the conformational changes in the sample. The dependence of the gel LCST on its composition allows adjusting the properties of the system to the various biomedical applications.

Composition according to the invention shows prolonged release of heparin for as long as over a dozen days. The release rate may be controlled by both the amounts of the components of the composition and temperature, because the system shows thermo-sensitivity in the range of physiological temperatures, whereas native hydroxypropylcellulose shows LCST above this range of temperatures. Heparin release from the composition is slower in elevated temperatures. Owing to this fact heparin, when introduced to the organism in the form of microspheres prepared from the composition according to the invention, is released longer than in the case of other known compositions. Slow release of heparin in physiological temperature constitutes an essential advantage of the system. Besides, the heparin release profile is very advantageous in many applications. After the initial burst release of the drug, it is then released continuously for several days keeping the high level of the active substance. Finally, its release rate drops down, but is still maintained for another week or two to assure the maximal therapeutic effect. Fast initial release after which the long-term process of the continuous slow release is observed allows reaching the optimal level of the delivered drug within a short time and then maintaining this level for a period of time necessary to ensure its

effective action. The optimal effect was obtained for the microspheres of the composition Alg/HPC equal to 4/1 w/w. In this case prolonged release of heparin was achieved for at least 16 days in the temperature of 37°C.

The composition according to the invention may find potential applications both in the oral delivery of heparin and in the preparation of the scaffolds for the tissue engineering of the blood vessels. In this latter application heparin-containing polymeric microspheres are introduced to the film obtained from the Alg/HPC mixture.

Figure 1 reports optical microscope images for the Alg/HPC microbeads of the various compositions and radius histograms of the microbeads of spherical shape.

Figure 2 reports a fluorescence microscope image of the 4:1 Alg/HPC microbeads, B-2E/C filter (excitation wavelengths: 465-495 nm; emission wavelengths: 515-555 nm).

Figure 3 reports SEM images of Alg/HPC 4:1 microparticle; 30 000 X magnification – single particle of spherical shape and rough surface.

Figure 4 reports dependence of the turbidity of 3:1 Alg/HPC gel on temperature and linear fit for LCST determination.

Figure 5 reports spectrophotometric detection of heparin: a) absorption spectra of solutions containing 4×10^{-5} M of Azure A and various concentrations of heparin; b) calibration curve.

Figure 6 reports release profiles for the microbeads of various Alg/HPC compositions at 25°C.

Figure 7 reports release profiles for 4:1 Alg/HPC microbeads at various temperatures: 25°C, 37°C and 50°C.

Figure 8 reports release profiles for heparin entrapped in the 4:1 Alginate/HPC microbeads, in the alginate/HPC film and in the film-microsphere matrix.

The subject of the invention was presented closer in the examples. The optical microscope imaging shown in the examples was performed using Nikon Eclipse TE2000 Inverted Research Microscope System. All the images were obtained in the air using Plan Achromat 50x objective. Small amount of sample studied (ca 2 mg) was dispersed in water using ultrasonic bath and spread over the glass slide immediately before the measurement. The size of the microspheres was measured for the central, fixed area of the image, always as a mean value of the diameter for the 250 microbeads . Histograms were made on the basis of these measurements to show the size distribution profiles of the microspheres. Nikon Eclipse TE2000, fitted with fluorescence and Hoffman Modulation Contrast microscope techniques

was used to obtain fluorescence images. They were measured using BV-2E/C filter (excitation wavelengths: 465-495 nm; emission wavelengths: 515-555 nm). Field-emission scanning electron microscopy (FE-SEM, Hitachi S-4700) equipped with energy dispersive X-ray spectroscope (EDS) was used to study the morphology and microstructure of microbeads (magnifications from 2500 X to 100 000 X).

Example 1.

a) Obtaining the Alginate/HPC microspheres

Aqueous solution of sodium alginate (2% w/v) was mixed with an appropriate amount of hydroxypropylcellulose in order to obtain viscous, homogenous and transparent solution. To the 5 ml of this solution heparin was added and the mixture was further stirred using magnetic stirrer for another 24h. Exact amounts of the polymer for each composition of the feed mixture are given in Table 1.

Stabilizing agent (TWEEN 85) was dissolved in cyclohexane; to the 100 ml round-bottom flask 45 ml of cyclohexane was introduced and then 0.702 g of TWEEN 85 was added. Afterwards the mixture was stirred for 10 minutes at the speed of 600 rpm to assure the complete dissolution of the stabilizing agent. 5 ml of the Alginate/HPC/Heparin mixture was added dropwise to the cyclohexane phase and mixed with the same speed for another 10 minutes. As s result the milk-white emulsion was obtained. Subsequently 10 ml of 0.2 m solution of calcium chloride was added to the emulsion to achieve physical crosslinking of the microspheres formed. After that the reaction mixture was stirred for another 30 minutes to allow the microspheres to harden. Next, they were filtered off and washed with water and isopropanol.

b) Formation of the heparin-containing film

5 ml of 2% w/v aqueous solution of sodium alginate was mixed with 25 mg of hydroxypropylcellulose (HPC) to obtain viscous, homogenous, transparent solution. Next 1 mg of heparin was added and mixed for another 24 hours. The solution was then poured to the small Petri dish (3 cm of diameter) to coat its bottom with the flat, even layer. Film was obtained by immersing the layer in the 0.2 M calcium chloride solution to initiate the crosslinking of the alginate and subsequently washed with distilled water.

c) Obtaining the film containing heparin-loaded microspheres

200 mg of the microspheres of Alg/HPC composition 4:1 and containing heparin was mixed with 800 mg of the aqueous solution containing alginate and HPC in the ratio 4 to 1 and then the mixture was poured to the small Petri dish (3 cm of diameter) to coat its bottom with the

flat, even layer. Film was obtained by immersing the layer in the 0.2 M calcium chloride solution to initiate the crosslinking of the alginate and subsequently washed with distilled water.

For fluorescence measurements 4:1 (w/w) Alg/HPC microbeads were prepared according to the above procedure (see Table 1 for details), but 33% w/w of alginate was replaced with dansyl alginate.

Table 1. Compositions of the emulsion for various Alg/HPC ratios (per 5 ml of solution).

Alginate/HPC ratio (w/w)	**2:1**	**3:1**	**4:1**	**5:1**
Alginate [mg]	100.38	100.46	100.01	100.17
HPC [mg]	50.19	33.18	25.01	20.52
Heparin [mg]	50.12	50.28	50.23	50.10

The shape, size, and size distribution of microbeads was analyzed using optical microscope (see Figure 1). When the content of HPC in the mixture exceeds 30% w/v, only unsymmetrical objects of various, irregular shapes are produced in small amount. That may be explained considering the fact that due to the high content of HPC in the blend the effective physical crosslinking is difficult to achieve. Part of the material is washed out from the beads and the collapse of the structure occurs. Therefore, those microparticles were not used in further studies. Average diameters of the obtained particles were estimated to ca 3.0 ± 1.6 μm in all samples and their sizes decreased slightly with the decreasing content of HPC in the composition.

In the fluorescence microscopy studies (see Figure 2) fluorescently labeled microbeads were used. They were prepared form the formulation in which 30% of sodium alginate was replaced with dansyl alginate. As expected, alginate is almost homogeneously distributed in the beads, although in some particles the higher density of the alginate was observed close to the bead surface.

Scanning electron microscopy (SEM) was used to study further the morphology and microstructure of the microbeads (see Figure 3). The most regular beads were obtained for 4:1

Alg/HPC composition. SEM images show mostly regular spherical shaped beads, although some of them show slight deformations.

The LCST for the Alg/HPC compositions studied has been determined at the same concentrations of polymers and calcium chloride as those used to prepare microspheres. LCST measurements were performed using a Hewlett-Packard 8452A diode array spectrophotometer equipped with a Hewlett-Packard 89090A Peltier temperature-control accessory allowing for the precise digital temperature control (± 0.1°C). Alg/HPC solutions of the same composition and concentration as those used to obtain microspheres were placed in a 1-cm cuvette and appropriate amount of calcium chloride was added (same as used for microbeads crosslinking). Temperature was measured with a Hewlett–Packard 89102A temperature sensor immersed in the solution. LCST values were determined from changes in the sample transmittance at $\lambda = 400$ nm (see Figure 4).

Importantly, the LCST values for all samples were found to be in the range of physiological temperatures (36-38°C), i.e. 5-7°C lower than those of the native HPC (see Table 2).

Table 2. LCST values for various Alg/HPC gels.

Microbeads composition	*LCST[°C]*
Alginate/HPC 2:1	36.0
Alginate/HPC 5:2	36.7
Alginate/HPC 3:1	36.9
Alginate/HPC 4:1	37.1
Alginate/HPC 5:1	37.5

Example 2

Determination of the encapsulation efficiency

To determine the efficiency of heparin encapsulation ca. 3 mg samples of the microbeads were placed in a small glass bottle and 1 ml of 5 mM EDTA solution was added. EDTA is a strong chelator of divalent cations and was used to remove the Ca^{2+} crosslinks in the alginate gels in order to solubilize the alginate. The sample was stirred with the magnetic stirrer for 24 hours to ensure complete dissolution of the microbeads and then the amount of heparin was determined using the same method as in the release studies. The calculations were done based on the calibration curve obtained for the standard solutions prepared in 5 mM EDTA solution. Each experiment was done in triplicate.

Heparin encapsulation efficiency (see Table 3 for details) was comparable for all compositions of Alginate/HPC studied (between 57 and 64%), with a small tendency to decrease with the increase in HPC content in the sample.

Table 3. Heparin encapsulation efficiency for various microbeads compositions.

Microbeads type	*Encapsulation efficiency [%]*
Alginate/HPC 3:1	57.0 ± 3.0
Alginate/HPC 4:1	61.3 ± 3.1
Alginate/HPC 5:1	63.3 ± 6.2

Example 3

Heparin release studies

a) Heparin release studies in solution

The weighed amount (ca 3 mg) of the microbeads was placed in a 7 ml centrifuge tube, 1 ml of distilled water was added, and the sample was placed on the magnetic stirrer with temperature control (±1°C) in the oil bath and stirred continuously at the fixed temperature. After defined time intervals the sample was centrifuged at 6000 rpm for 4 minutes, and then the solution from above the microbeads was decanted. The new portion of distilled water was added to the microbeads and the system was placed again in the oil bath. To the collected

solution 100 μl of 0.2 M calcium chloride solution was added to remove any trace of dissolved alginate, which could interfere with heparin detection. The alginate gel formed with calcium ions was removed from the solution by subsequent centrifugation.

b) Studies on heparin release from the films

1 ml aliquots of distilled water were introduced to each small round plastic well where the film was previously formed and then, after the precisely determined periods of time, the solution from above the film sample was exchanged with a new portion of distilled water (1 ml) and the extracted solution was analysed according to the procedure described below.

The method of the heparin detection

The spectrophotometric method of heparin detection was based on colour changes of the solution occurring during interactions of heparin anion with cationic Azure A dye, which aggregates on the surface of the heparin macromolecular anion (Němcová et al. 1999). To determine the amount of heparin in the collected solution 0.8 ml of the solution was mixed with 0.8 ml of Azure A (8×10^{-5} M) aqueous solution and the absorption was measured at 512 nm. The concentration of heparin was calculated using the calibration curve for heparin. In a procedure of calibration curve preparation the 100 μl of 0.2 M calcium chloride solution was also added to account for the fact that increased salt concentration caused a decrease in the sensitivity of the Azure A- heparin assay.

Figure 5a shows the absorption spectra of the standard solutions containing Azure A (4×10^{-5} M) and various heparin concentrations and Figure 5b shows the calibration curve.

The release profiles for heparin-loaded microbeads of various compositions have a similar shape; however, they differ in the amounts of the heparin released in the same time intervals (Figure 6). These differences may be explained considering differences in encapsulation efficiencies and in microbead morphology. The heparin release is a three-stage process, indeed. Initially, in the first 4 hours fast release is observed which is followed by the slower one occurring during the next 5 days. Then the third stage begins, characterized by even slower, linear release. After as long as 16 days, slow heparin release is still observed.

In the next step the temperature effect on the heparin release profiles from the 4:1 Alg/HPC thermosensitive microbeads was studied. All the profiles have shown similar shape, with the initial fast stage, followed by the slow release. It was observed, however, that the efficiency of release is lower at elevated temperatures than at room temperature (Figure 7).

To study the applicability of the obtained microspheres as a support for the growing cells the films containing 20% w/w of the microspheres were prepared from the

Alginate/HPC 4:1 mixture. The microsphere gel was of the same composition as the film mixture and contained additionally ca. 1.8% w/w of heparin. Release profiles for this system were measured in room temperature (25°C), and next compared with the heparin release profiles obtained for the same microspheres studied in water and for the same amount of heparin released from the film containing no microspheres. The amount of heparin encapsulated in the film matrix and in the film-microsphere matrix was the same.

As can be seen in the Figure 8 the initial burst effect was considerably suppressed for heparin entrapped in the film, but after longer release times the amount of the heparin released is approaching that released from the microspheres in water. The amount of heparin released from the film-microsphere matrix was 1000-times lower taking into account the amount of heparin in microspheres such film has a potential to maintain the stable level of heparin for at least 2 weeks or longer. That could suggest its potential application as a support for the cell cultures and its usefulness as an active component in the preparation of the scaffolds for tissue engineering.

Claims

1. The composition for the prolonged release of heparin, wherein the gel obtained from the alkali metal alginate and hydroxypropylcellulose at the weight ratio from 2:1 to 5:1 is used subjected to the gelling process using calcium salt, containing immobilised heparin in the amount from 0.01 to 10% w/w.
2. Composition according to claim 1, wherein the alkali metal alginate is the sodium alginate.
3. Composition according to claim 1, wherein the gel has a form of microspheres, film or microspheres-containing film.
4. Use of the gel obtained from sodium alginate and hydroxypropylcellulose for the prolonged release of heparin.

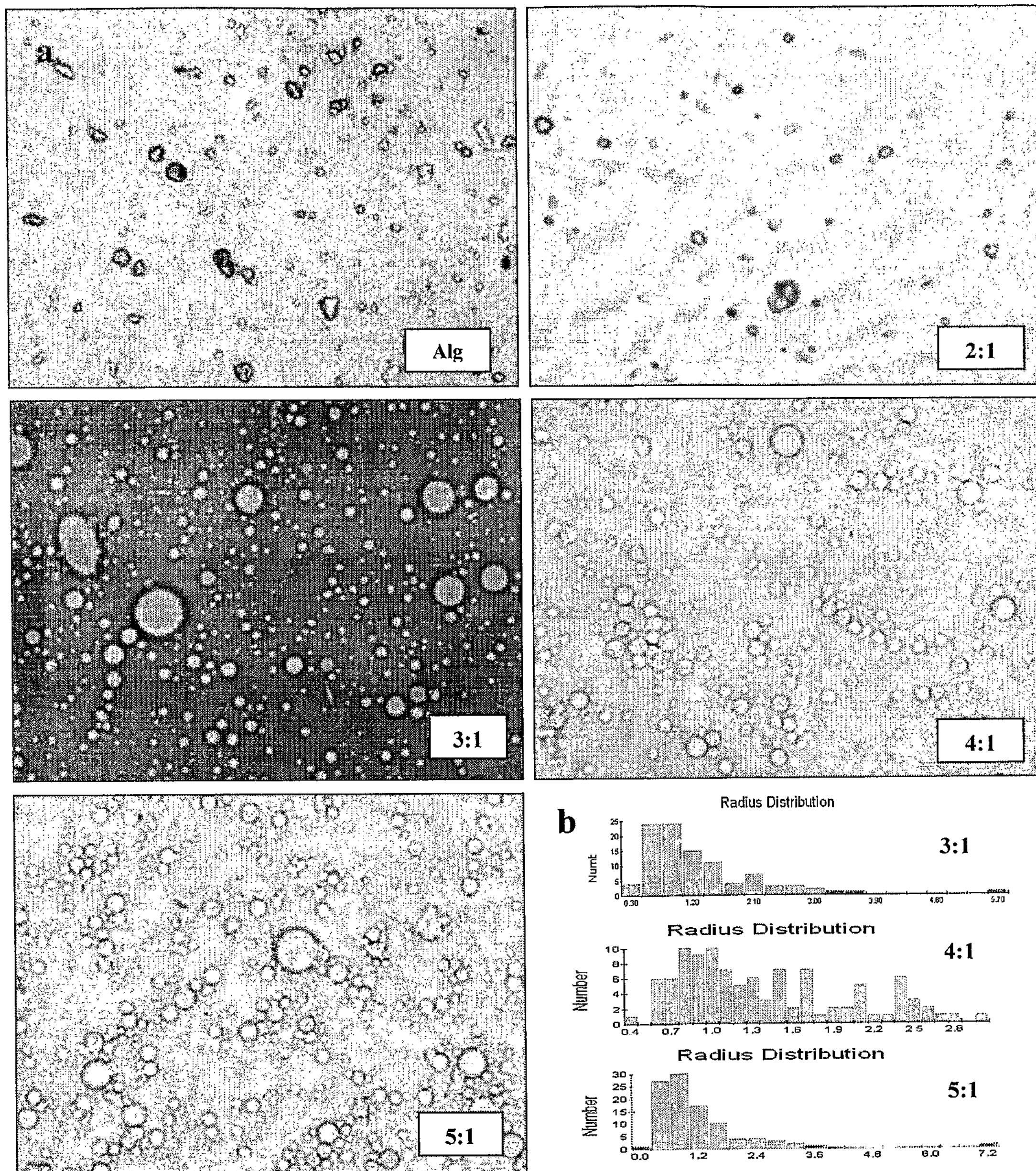

Figure 1.

Figure 2.

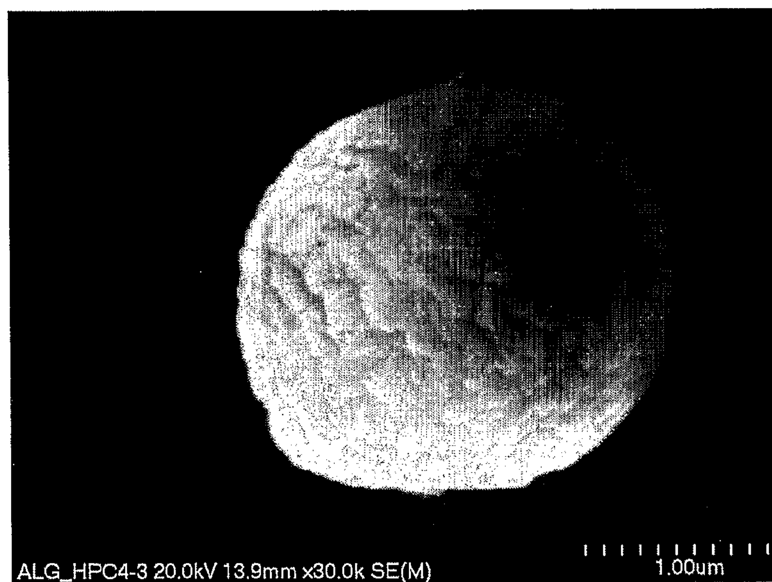

Figure 3.

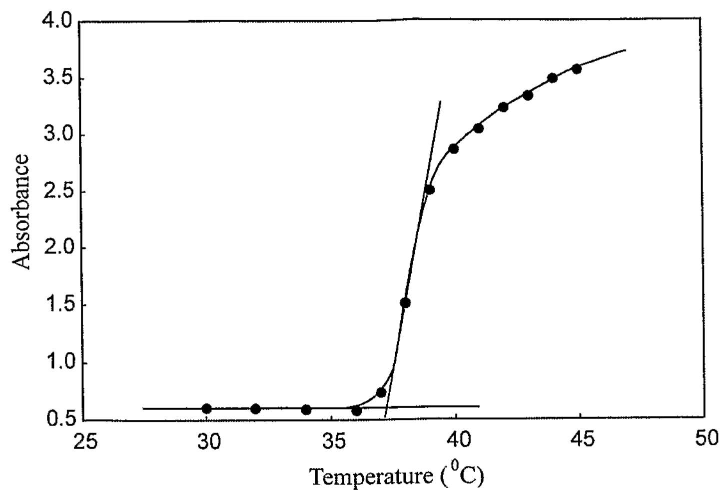

Figure 4.

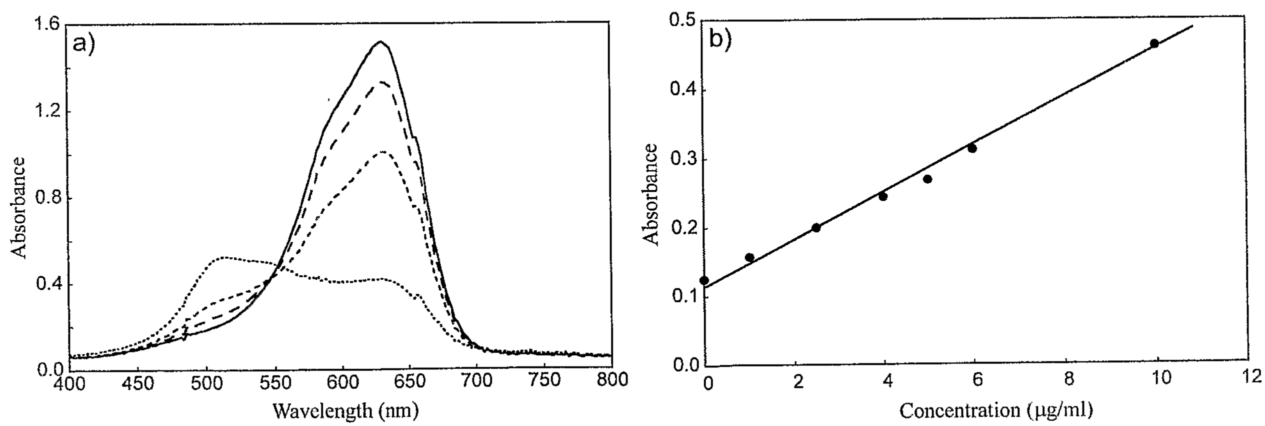

Figure 5.

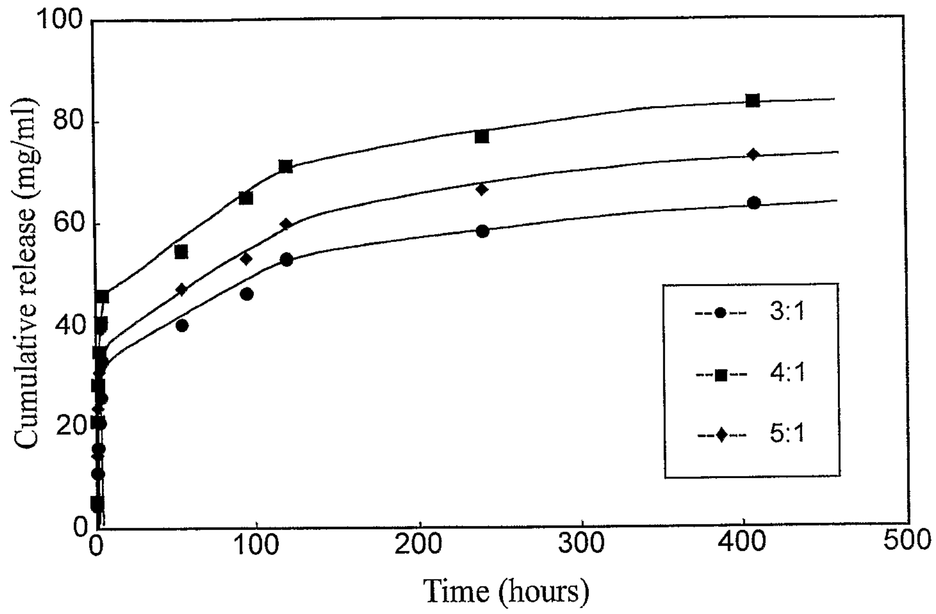

Figure 6.

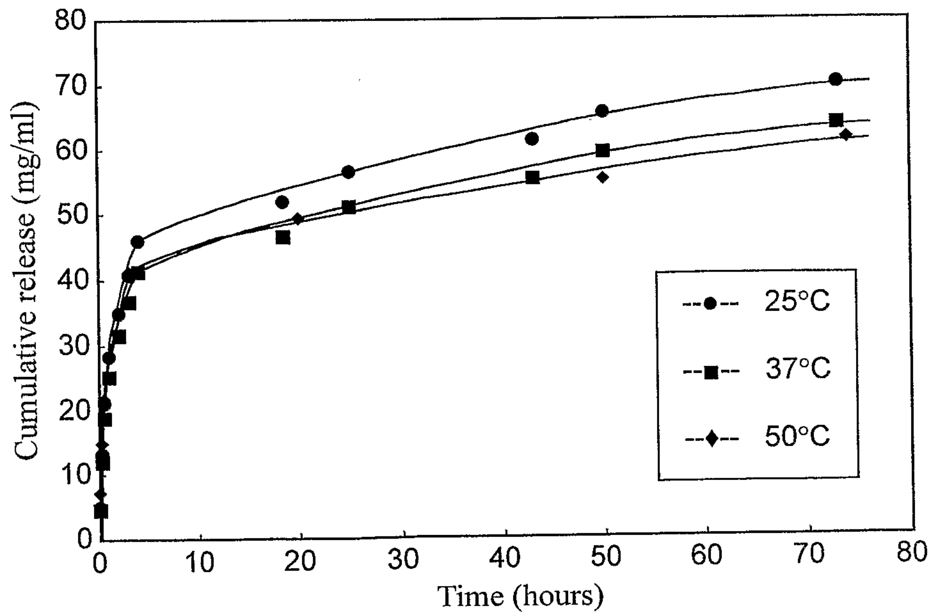

Figure 7.

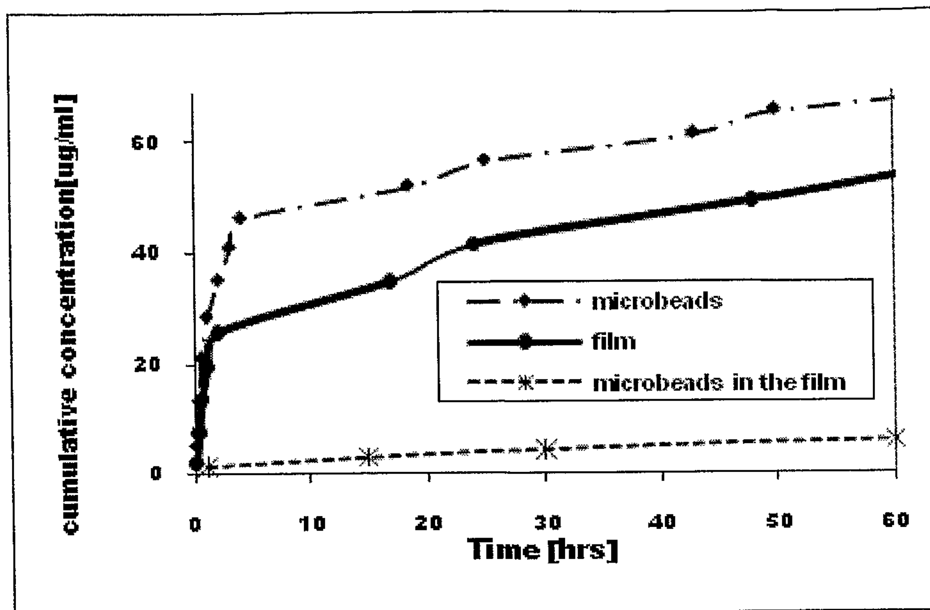

Figure 8.

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2009/000110

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/36 A61K47/38 A61K9/70 A61K9/16 A61K31/727
ADD.

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

B. FIELDS SEARCHED

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

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, 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	GB 2 207 353 A (SQUIBB & SONS INC SQUIBB & SONS INC [US]) 1 February 1989 (1989-02-01) claims 1-20	1-4
A	WO 2004/037290 A1 (HANMI PHARM IND CO LTD [KR]) 6 May 2004 (2004-05-06) claims 1-4	1-4
A	WO 97/12605 A1 (EURO CELTIQUE SA [LU]; KRISHNAMURTHY THINNAYAM NAGANA [CA]) 10 April 1997 (1997-04-10)	1-4
A	WO 96/26717 A1 (HALLMARK PHARM INC [US]) 6 September 1996 (1996-09-06) claims 1-3	1-4
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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7 May 2010	17/05/2010

Name and mailing address of the ISA/	Authorized officer
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	WO 2009/079537 A1 (NEW WORLD PHARMACEUTICALS LLC [US]; SEXTON FREDERICK A [US]; KRISHNAN) 25 June 2009 (2009-06-25) the whole document -----	1-4

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/PL2009/000110

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