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(54) Title: CYCLODEXTRIN NANOSPONGES AS A CARRIER FOR BIOCATALYSTS, AND IN THE DELIVERY AND RELEASE OF ENZYMES, PROTEINS, VACCINES AND ANTIBODIES

(57) Abstract: Disclosed are crystalline or amorphous crosslinked cyclodextrin nanosponges as carriers for enzymes, antibodies, proteins, vaccines and macromolecules in general.

CYCLODEXTRIN NANOSPONGES AS A CARRIER FOR BIOCATALYSTS, AND IN THE DELIVERY AND RELEASE OF ENZYMES, PROTEINS, VACCINES AND ANTIBODIES

The present invention relates to the use of cyclodextrin nanosponges as carriers for enzymes, proteins, vaccines or antibodies, a process for the preparation of the enzymes carried by said nanosponges, and catalysts obtainable by said process.

Prior art

Proteins perform numerous functions, such as oxygen transport, maintenance of the structure of some tissues, reception and transduction of signals in the cells, immune response, and catalysis in metabolic processes. This latter function is performed by enzymes, which are divided into six classes on the basis of the generic reaction they catalyse.

Many industrial processes involving chemical transformation present operational disadvantages. Non-specific reactions lead to low yields, and the frequent need to operate at high temperatures and pressures requires the consumption of large amounts of energy, and very large amounts of cooling water in the "downstream" process. The reactors must often be made of materials resistant to drastic temperature, pressure, acidity or alkalinity reaction conditions, leading to an increase in plant manufacturing costs.

All these drawbacks can be eliminated or considerably reduced by using enzymes as biocatalysts. Enzymes operate under mild reaction conditions, have a high reaction speed, and are highly specific. In view of their efficiency, only small amounts of enzyme are needed to transform large volumes of reagents. They have a beneficial effect on the environment because they reduce energy consumption and reduce the production of pollutants.

Developments in genetic engineering have increased the stability,

economy and specificity of enzymes, and the number of their industrial applications is continually increasing.

Examples of industrially useful enzymes include alpha amylase, trypsin, cellulase and pectinase for fruit juice clarification processes, ligninase to break down lignin, lipase in the detergent industry and biodiesel production, etc.

Enzymes, like all catalysts, can be used in homogeneous phase (homogeneous catalysis) or heterogeneous phase (heterogeneous catalysis).

In the first case, the catalyst is in the same phase (liquid) as the reagents and the reaction products. Homogeneous catalysis is characterised by high selectivity, high activity, and constant reproducibility. The main disadvantages of this technique are the short life of the catalyst, its possible poisoning, and the difficulty of separating the reaction products from the catalyst and recycling the catalyst, which is always problematic.

In heterogeneous catalysis, the catalyst is in a different phase from the one that contains the reagents and reaction products. In this case, separation of the reaction products is easy, and work can be performed in continuous flow. However, lower selectivity and activity of the enzyme used is often observed.

The catalytic performance of the heterogenised enzyme depends on the substrate used and the immobilisation techniques. As catalytic activity depends mainly on the correct orientation of the active site, the enzyme must be immobilised in such a way as to minimise modifications to the catalytic site. The surface on which the enzyme is carried is responsible for retention of the enzyme structure, and the reaction micro-environment created can modify the fields of use of the enzyme, for example by extending the operational pH range.

Three immobilisation techniques are known:

1) interactions with carriers (physical adsorption, ion bond and

covalent bond);

2) crosslinking;

3) trapping.

Physical adsorption is usually the simplest method of immobilising enzymes. The enzyme is bonded to the carrier via hydrogen, saline and Van der Waals bonds. However, as these interactions are weak, desorption of the enzyme is often observed due to a change of temperature, pH, ionic strength, or even the substrate used.

It is also well known that proteins, peptides, enzymes and derivatives thereof can be used in the biomedical and therapeutic field. Proteolytic enzymes can be used to treat cancer or type I mucopolysaccharidosis, while DNA and oligonucleotides are used in gene therapy, for example. The administration of these molecules presents various problems and limitations. Most protein drugs are poorly absorbed through the biological membranes due to a combination of factors such as large molecular size, hydrophilic nature, ionicity, high surface charge, chemical and enzymatic instability, and low permeability through the mucous membranes. Following intravenous administration, protein molecules present rapid blood clearance, a significant ability to bond to the plasma proteins, and considerable sensitivity towards proteolytic enzymes. Oral administration presents the drawback of low bioavailability. Various approaches exist for therapeutic use, such as increasing the dose or using absorption promoters, which can cause toxicity problems, or using alternative administration routes. A number of systems for carrying enzymes and proteins have been developed, such as nano- and micro-particles, liposomes and hydrogels. Carriage in a particulate system can protect proteins from breakdown, modify their pharmacokinetics and improve their stability *in vivo*.

Cyclodextrins (CDs) are non-reducing cyclic oligosaccharides

consisting of 6-8 glucose molecules bonded with a 1,4-α-glucoside bond, which have a characteristic cone-frustum structure. The arrangement of the functional groups of the glucose molecules is such that the surface of the molecule is polar, while the inner cavity is relatively lipophilic.

The lipophilic cavity enables the CDs to form inclusion complexes which are also stable in solution with organic molecules of suitable polarity and dimensions.

CDs have therefore been studied, and present numerous applications in various fields (pharmaceuticals, analysis, catalysts, cosmetics, etc.) in which the characteristics of the inclusion compounds are exploited.

However, native or chemically modified cyclodextrins are generally used in these applications, never insoluble crosslinked polymers.

Nanosponges are crosslinked polymers of cyclodextrins with various bonds which have proved very useful in various applications ranging from environmental decontamination to controlled drug delivery and release.

Description of the invention

It has now been found that cyclodextrin nanosponges are a particularly suitable carrier to adsorb proteins, enzymes, antibodies and macromolecules in general. In particular when enzymes are used, it is possible to maintain their activity and efficiency, prolong their operation and extend the pH and temperature range of activity as well as allowing the conduct of continuous-flow processes. Moreover, proteins and other macromolecules can be carried by adsorbing or encapsulating them in cyclodextrin nanosponges. The interaction between proteins and nanosponges allows the formulation of slow, controlled-release systems.

The nanosponges usable according to the invention are described, for example, in WO 2006/002814, and prepared by performing a solvent-free reactions between cyclodextrins and organic carbonates, with or without

ultrasound irradiation. It has surprisingly been found that structurally amorphous nanosponges and nanosponges with a significant degree of crystallinity can be obtained (Figure 1a and 1b). Other types of nanosponges, obtainable by reacting a cyclodextrin with a multifunctional polyisocyanate crosslinker, as described in WO 98/22197, can also be used.

A new class of nanosponges containing polyamidoamine bonds have also been used as carriers; they can be obtained, for example, by reacting cyclodextrins with bis-acrylamides, the synthesis of which is described in detail in example 1. Synthesis is preferably conducted in the presence of ultrasound.

The immobilisation of enzymes on said nanosponges involves placing an aqueous solution of enzyme in contact with the crosslinked nanosponges for a time ranging between 10 and 720 minutes at temperatures of between 4 and 40°C. The aqueous solution is suitably buffered at a pH of between 5 and 9.

The invention relates both to the immobilisation process and to the enzymes immobilised on cyclodextrin crosslinked nanosponges.

Examples of enzymes which can be advantageously immobilised according to the invention include oxidoreductase, transferase, hydrolase, lyase, isomerase and ligase, and in particular amylase, trypsin, lipase, catalase, cellulase, ligninase, pectinase, protease and other enzymes of industrial interest.

The amount of enzyme immobilised on the nanosponges depends mainly on the incubation time of the polymer solution containing the nanosponges, as well as their nature and temperature. Broadly speaking, the immobilisation yields can be regulated within various limits, such as 1-50 mg per gram of nanosponges.

The enzyme activity is maintained for several days, and is more

resistant to temperature and pH conditions, thus allowing more efficient enzymatic reactions to be performed.

The invention is described in greater detail in the Examples below.

Example 1 - Synthesis of polyamidoamine nanosponges (Figure 2)

0.66 ml of distilled water, 339 mg of 2,2'-bis(acrylamido) acetic acid (BAC) and 84.7 mg of $LiOH.H_2O$ were introduced into a 10 ml flask, supplied with nitrogen flow, under stirring, in a nitrogen atmosphere,. When a clear solution had been obtained, 573 mg of β-cyclodextrin (βCD) and a further 63.5 mg of $LiOH.H_2O$ were added. The reaction was maintained under nitrogen flow for 10 minutes, after which the nitrogen inlet was closed, and the reaction was left to continue under inert atmosphere, in the dark, at ambient temperature, for 94 hours. When the reaction had finished, the crude product presented as a crosslinked polymer; it was crushed and swollen with an excess of water for 10 minutes under stirring, and then centrifuged to eliminate traces of base from the resin. The resin was swollen again in an excess of water and then acidified with 1M HCl to pH 2.5. The acidified resin was purified by washing three times in distilled water under vigorous stirring, followed by centrifugation. The traces of water were extracted with ethanol and by leaving the product under vacuum for two days. The end product took the form of an odourless fine white powder. The degrees of swelling of the resin in three buffer solutions at pH 2, 4.5 and 7 were evaluated, and are shown in Table 1. The resin was characterised by FT-IR spectrophotometry in KBr pastilles, and the main functional groups were identified as follows: 707 cm^{-1} (d_w secondary amide NH), 947 cm^{-1} (d C-O-H out of plane), 1030 cm^{-1} (n C-N aliphatic), 1087-1301 cm^{-1} (n C-O-C), 1520 cm^{-1} (d secondary amide NH, n C-N), 1650 cm^{-1} (n secondary amide C=O, n C=C) of d, 2921 cm^{-1} (n asymmetrical CH2), 3385 cm^{-1} (n OH).

The same reaction can be conducted with other stoichiometric ratios

and other types of crosslinker such as methylenebisacrylamide, methylpiperazine or mixtures thereof, and using other natural cyclodextrins (alpha and gamma) or derivatives thereof (e.g. monotosyl cyclodextrin, monoazidocyclodextrin, etc.)

Synthesis takes place more rapidly if the operations are ultrasound-assisted. Nanosponges with magnetic properties can also be obtained if synthesis is performed in the presence of magnetic particles, such as magnetite.

Example 2 - Loading of enzymes onto nanosponges

The best immobilisation yield was obtained from the crosslinked carrier described in WO 2006/002814, with a 1:2 molar ratio between β-cyclodextrins and the crosslinking agent.

To establish the best immobilisation conditions, 0.3 mg of 1,2-dioxygenase enzyme was immobilised on 1.0 g of both types of crosslinked carrier (1:2 and 1:4) at two different temperatures (4°C and 22°C). The carrier was pre-washed with the same solution in which immobilisation took place (50 mM Hepes, pH 8.0).

The quantity of immobilised enzyme was evaluated by calculating the difference between the total amount loaded onto the nanosponges and the amount not bonded to the resin which was present in the washing solutions. The quantity of enzyme was calculated by measuring the activity on catechol and calculating the quantity of enzyme in mg from the specific activity (activity/mg of protein).

The experiment was conducted by incubating the enzyme and the nanosponges for different times and calculating the immobilisation yield.

Figure 3 shows the immobilisation yield obtained after incubation of the enzyme and crosslinked carrier 1:2 at different times at 22°C.

When the enzyme loading conditions had been established, the enzyme

could be immobilised by absorption to a loading capacity of 28.9 mg of protein per gram of carrier.

Example 3 - Production of muconic acid

The enzyme 1,2-dioxygenase catalyses the reaction that transforms catechol into muconic acid. This reaction was exploited to evaluate the activity of the immobilised enzyme. The reaction was monitored for 60 seconds by means of the increase in absorption at 260 nm due to the formation of the reaction product, namely muconic acid. The reaction mixture contained 20 µl of wet resin as described in WO 2006/002814 with the immobilised enzyme, and 1 mM of catechol in 50 mM Hepes buffer, pH 8.0. The activity tests were conducted at 30°C in a 1 cm optical path cuvette. The absorption spectra of catechol and muconic acid are shown in Figure 4.

The formation of the reaction product was monitored by separation in HPLC, as shown in Figure 5. Reverse-phase separation with LiChrospher 100 RP-18 5 µm 250x4 (Merck) column was used. The mobile phase consisted of water and acetonitrile in the ratio of 60:40.

The two standard solutions of catechol and muconic acid were injected for checking purposes; the resulting retention time was approx. 2.7 minutes for muconic acid and approx. 6.7 minutes for catechol. The solution output by the bioreactor was then injected. The presence of a peak at the same retention time confirms the presence of the reaction product. The UV spectrum of the product was compared with that of standard muconic acid, and revealed the presence of the characteristic absorption peak at 260 nm.

The chromatogram (Figure 5) shows that catechol is almost totally converted to cis-cis muconic acid. Table 2 shows the kinetic constants of the reaction catalysed.

Example 4 - Enzyme activity over time

A column (10 x 2.5 mm) was packed with 1 g of the hyper-crosslinked

carrier described in WO 2006/002814, on which 0.3 mg of catechol 1,2-dioxygenase were immobilised.

A 1 mM catechol solution was injected into the bioreactor at regular intervals (once a day), and the column was washed with 50 mM Hepes buffer, pH 8.0, after 1 hour. The presence of the reaction product in the output of the bioreactor was monitored spectrophotometrically and by HPLC analysis. The reaction product was quantitated with a molar extinction coefficient at 260 nm of 17600 M^{-1} cm^{-1}.

Greater stability over time of the protein immobilised on the column was obtained with tests of immobilisation by adsorption. The enzyme was able to reconvert the catechol into cis-cis muconic acid for approx. 72 hours, as will be seen from the graph in Figure 6.

Example 5 - Influence of temperature on enzyme activity

To investigate the temperature stability of the immobilised enzyme on the hyper-crosslinked carrier disclosed in WO 2006/002814 and the stability of the enzyme in solution, activity tests were performed by incubating the enzyme (20 µl of wet resin with the immobilised enzyme) for 3 minutes in 50 mM Hepes buffer, pH 8.0, in a temperature range of 10°C to 70°C. After this incubation, the residual activity of the enzyme was verified by adding the substrate and monitoring the formation of the product.

As shown in Figure 7, the immobilised enzyme shows its greatest activity around 50°C, and still maintains 70% of its activity at 60°C.

Conversely, the optimum temperature range for the enzyme in solution is between 30°C and 40°C. Almost total loss of activity of the enzyme in solution was observed after incubation at 60°C. To monitor the stability of the enzyme catechol 1,2 dioxygenase at two different temperatures, 40°C and 60°C, the activity of the immobilised protein and that of the protein in solution were measured at different incubation times at the two selected

temperatures (Figures 8a and 8b).

The immobilised enzyme and the enzyme in solution were incubated in 50 mM Hepes buffer, pH 8.0, at the 2 different temperatures. At regular intervals, 20 µl of wet resin with the immobilised enzyme was taken up, and the residual activity was measured by adding the substrate and monitoring the formation of the product.

As shown in Figures 7, 8a and 8b, the immobilisation process makes the enzyme more heat stable. The enzyme in solution completely loses its activity after incubation of the enzyme for 90 minutes at 40°C, whereas the immobilised enzyme still retains 50% of its initial activity after 90 minutes. Complete loss of the enzyme activity of the immobilised protein only takes place after 3 hours. The activity loss profile of the immobilised enzyme at 40°C shows a two-phase behaviour which is probably due to the presence of two catalytic sites stabilised in different ways by the immobilisation process. As no cases of allosterism between the two monomers of the enzyme catechol 1,2-dioxygenase have so far been reported in the literature, this behaviour is probably due to the immobilisation process, which can create two non-equivalent iron centres. Figure 8b shows the enzyme activity profile after incubation at 60°C. Complete loss of activity takes place after 15 minutes for the enzyme in solution, whereas at the same time, the immobilised enzyme still retains 70% of its initial activity.

Example 6 - Influence of pH on enzyme activity

To monitor the behaviour of the enzyme catechol 1,2- dioxygenase at different pH values, the activity of the immobilised protein and that of the protein in solution was measured in the pH range 5.5-10. The enzyme (20 µl of wet resin described in example 1 with the immobilised enzyme) was incubated for 3 minutes in the different buffer solutions at a given pH value at 30°C, and the residual activity was then evaluated.

The buffers used were: Mes (for pH 5.5 to 7), Hepes (for pH 7 to 8.5), and Ches (for pH 8.5 to 10). The concentration of the buffer was selected in such a way as to maintain a constant ionic strength.

As shown in Figure 9, the activity profiles are different, which demonstrates that the immobilisation process influences the catalytic site of the enzyme. The optimum pH for the enzyme activity of the immobilised protein is 9.5, while the peak activity for the enzyme in solution is observed at pH 8.5. Moreover, as indicated by the first part of the curve, the immobilised enzyme already has an activity of 40% at pH 6.5, whereas the protein in solution only has 8% of its peak activity.

Example 7 - Activity of HRP

To confirm the ability of the carrier to retain other enzymes by adsorption, commercially available horseradish peroxidase (Sigma Aldrich) was tested.

0.3 mg of enzyme was incubated with the nanosponges disclosed in WO 2006/002814 for 62 hours at 4°C in a potassium phosphate buffer solution at pH 7.4.

The concentration of the protein was estimated by absorption at 402 nm using a molar extinction coefficient of 93500 M-1 cm^{-1} (Keilin and Hartree, 1951), and the immobilisation yield was calculated as the difference between the quantity of protein incubated and the quantity of protein not bonded to the carrier.

The enzyme activity was measured on the substrate 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), following the formation of the cation radical ABTS.+ in the presence of H_2O_2 and peroxidase at 414 nm (Figure 10). The reaction mixture consisted of 20 µl of wet resin with the immobilised enzyme, 1 mM ABTS and 15 µM H_2O_2 in 50 mM phosphate buffer, pH 7.4.

Table 1. Loading of HRP onto different NSs

Carrier	Mg of protein immobilised/1 g of carrier	Turnover (μmoles produced/min/μmole of HRP)
Solution	-	245084.7
NS carbonate 1:2	0.083	200000
NS sol-gel	0.581	N/A
NS urethane	0.149	29530.2

Table 2. Kinetic constants of 1,2-dioxygenase

	Free catechol 1,2-dioxygenase	**Immobilised catechol 1,2-dioxygenase**
K_M (μM)	2.04 ± 0.3	16.62 ± 4.78
K_{Cat} (sec^{-1})	22 ± 1.2	27.31 ± 2.66
K_{cat}/K_M	10.78	1.64
peak activity pH	8.5	9.5
peak activity temperature (°C)	30-35	50
Activation energy (KJ/mole)	45.02	27.35

Example 8 - Immobilisation of lysozyme

5 ml of a solution of 200 ppm of lysozyme was incubated with increasing, known quantities of the nanosponge described in Example 1 at ambient temperature for 24 hours. At the end of said period, the suspension was centrifuged at 3000 rpm and the supernatant analysed with a UV-Vis spectrophotometer at 281 nm. As the initial absorbance was known, the quantity of lysozyme adsorbed by the nanosponges was calculated by subtraction from the absorbance observed. The results are reported in Figure 12, which shows that NSs have a very high affinity for lysozyme and bond over 80% of the protein, a percentage which tends to increase in proportion to the concentration of the protein in solution.

Example 9 - Delivery and release of albumin

50 mg of albumin is incubated with 50 mg of nanosponge overnight, under stirring, in 5 ml of water. The mixture is then freeze-dried. A solid product containing 50% albumin is obtained. A dissolution test was used to evaluate the release kinetics of albumin from the nanosponges. 25 mg of the nanosponges described in Example 1, containing 12.5 mg of albumin, are suspended in 25 ml of phosphate buffer at pH 7.4. The sample is left under stirring for 24 hours at 37°C. 0.5 ml of the sample is taken up at pre-set times, and replaced with 0.5 ml of buffer. The sample removed is centrifuged, and the supernatant analysed under the spectrophotometer at the wavelength of 278 nm. The results are shown in Figure 11.

CLAIMS

1. Crystalline or amorphous crosslinked cyclodextrin nanosponges as carriers for enzymes, antibodies, proteins, vaccines and macromolecules in general.

2. Nanosponges as claimed in claim 1, as a carrier for enzymes.

3. Nanosponges as claimed in claim 1 or 2, wherein the cyclodextrins are crosslinked with polyamidoamine in the presence or absence of ultrasound.

4. Process for the immobilisation of enzymes which involves contacting an aqueous solution of enzyme with the crosslinked nanosponges for a time ranging between 10 and 720 minutes at temperatures of between 4 and 40°C.

5. Process as claimed in claim 4, wherein the aqueous solution is buffered at a pH between 5 and 9.

6. Enzymes carried by crosslinked cyclodextrin nanosponges.

7. Enzymes as claimed in claim 6, selected from oxidoreductase, transferase, hydrolase, lyase, isomerase and ligase.

8. Enzymes as claimed in claim 7, selected from amylase, trypsin, lipase, catalase, cellulase, pectinase, ligninase and protease.

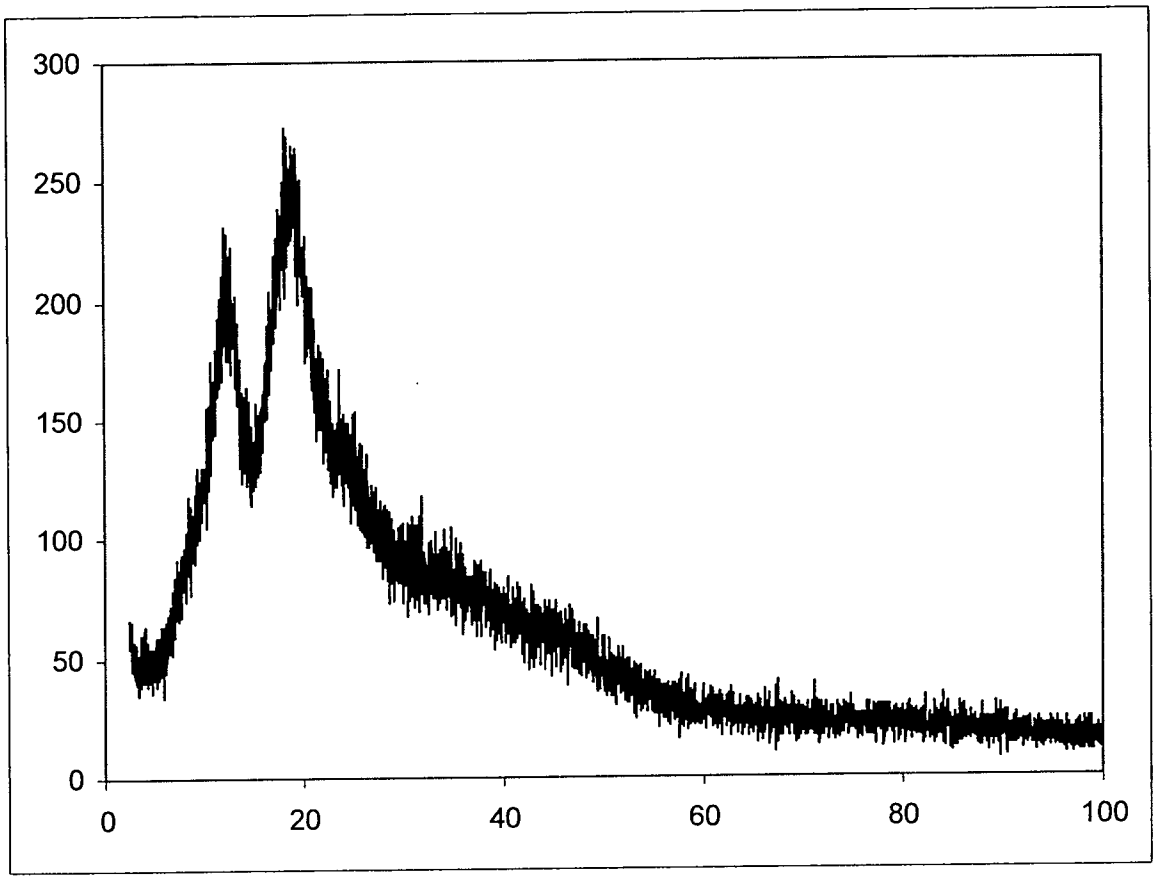

Figure 1a: XRD of amorphous nanosponge carbonate

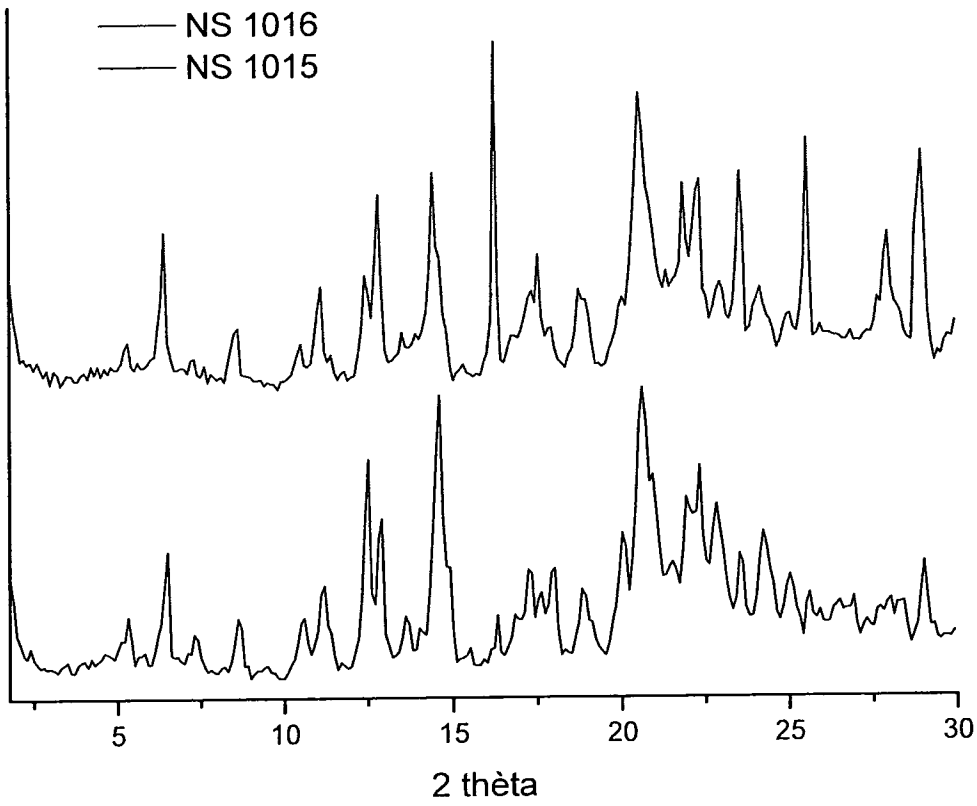

Figure 1b: XRD of crystalline nanosponge carbonate

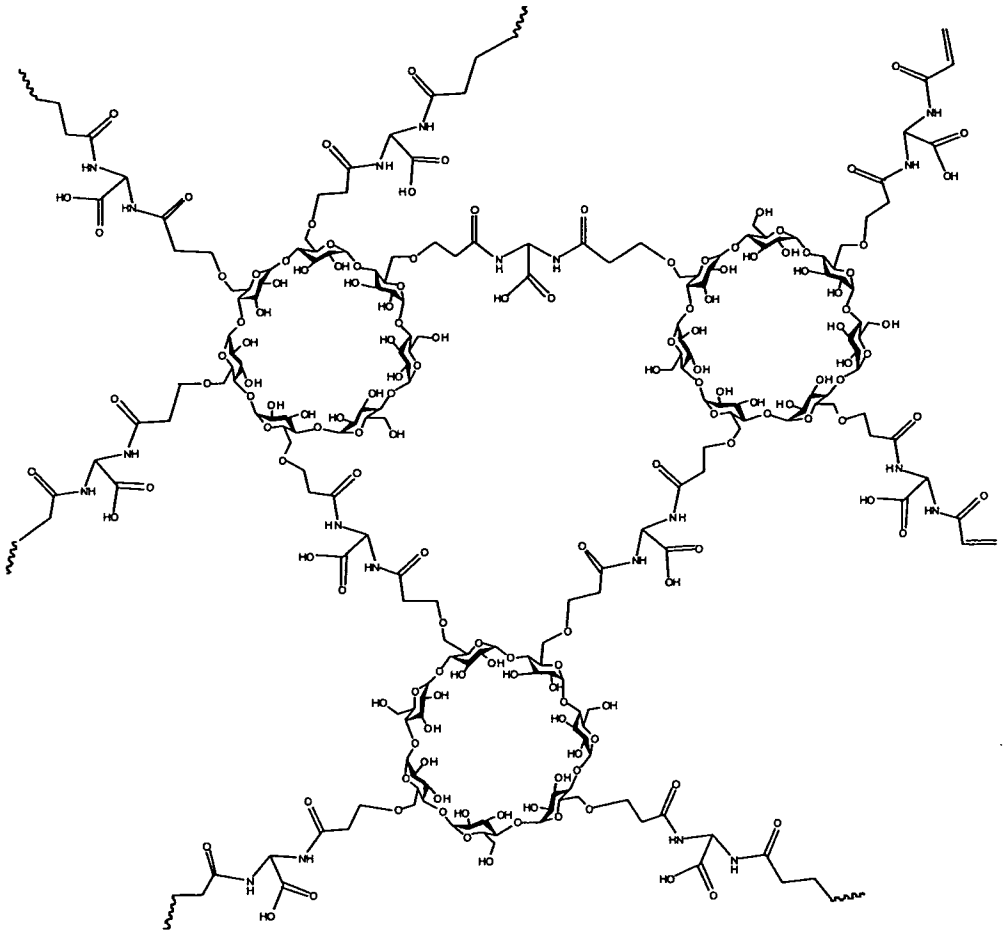

Figure 2: Structure of polyamidoamine nanosponges

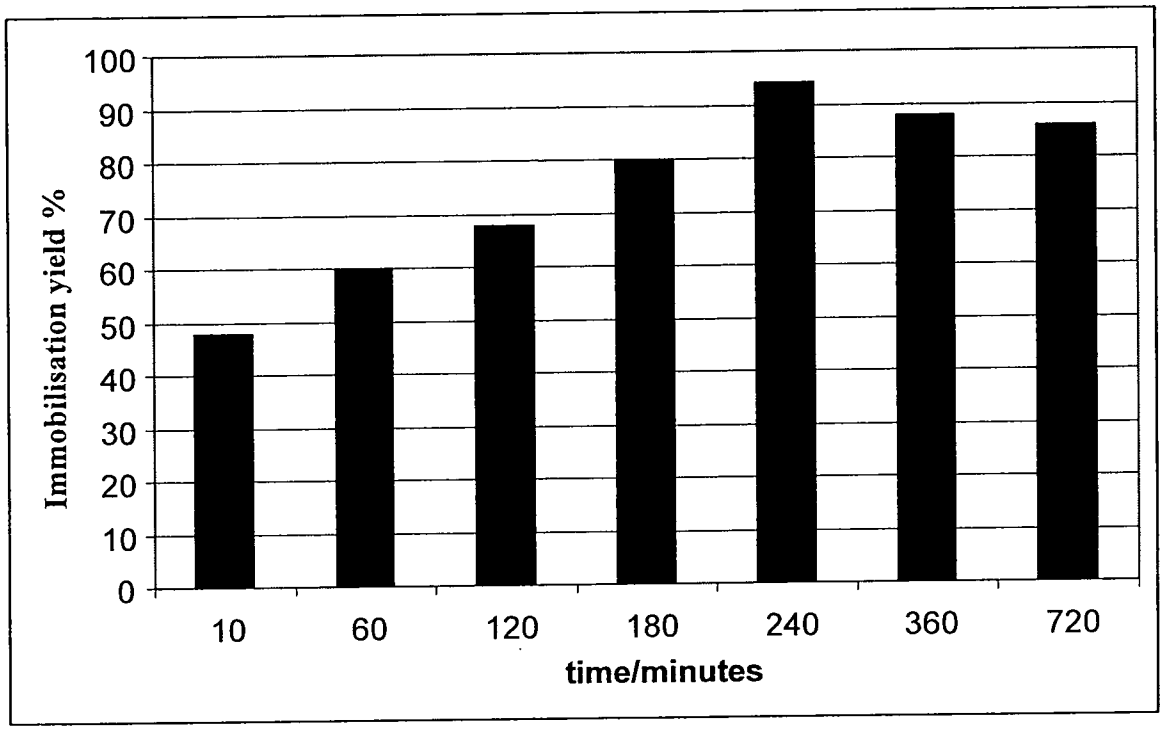

Figure 3: Immobilisation yield of catechol 1,2-dioxygenase at 22°C at different times

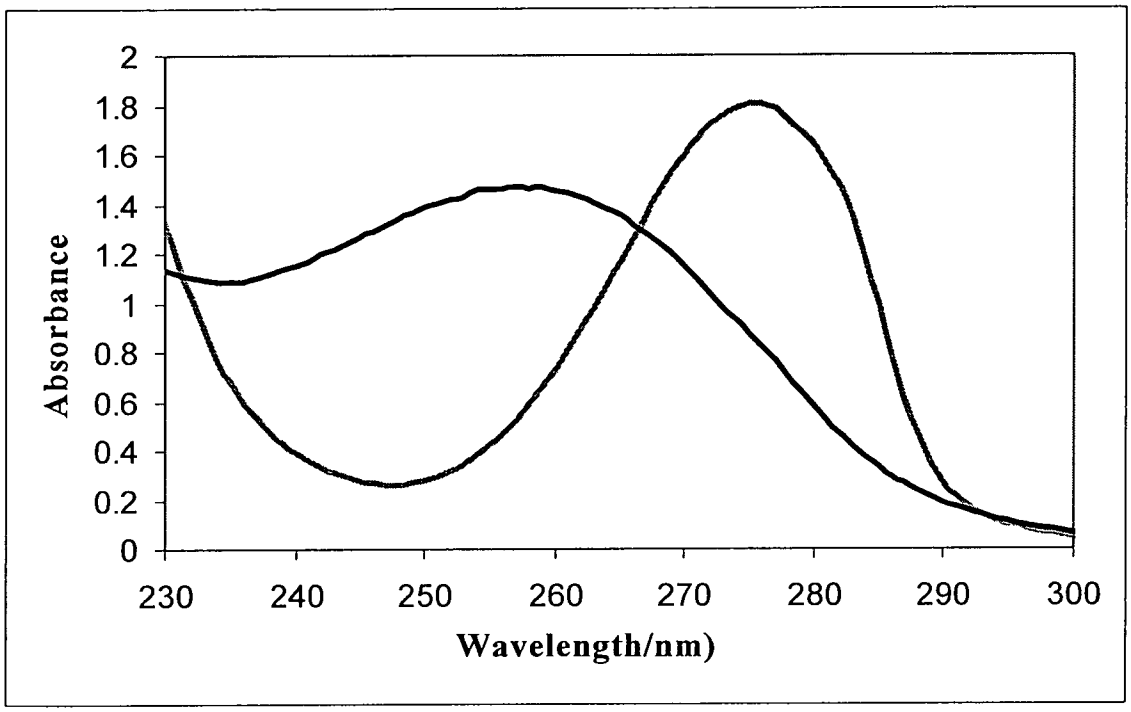

Figure 4: Absorption spectra of catechol (substrate) and muconic acid (product)

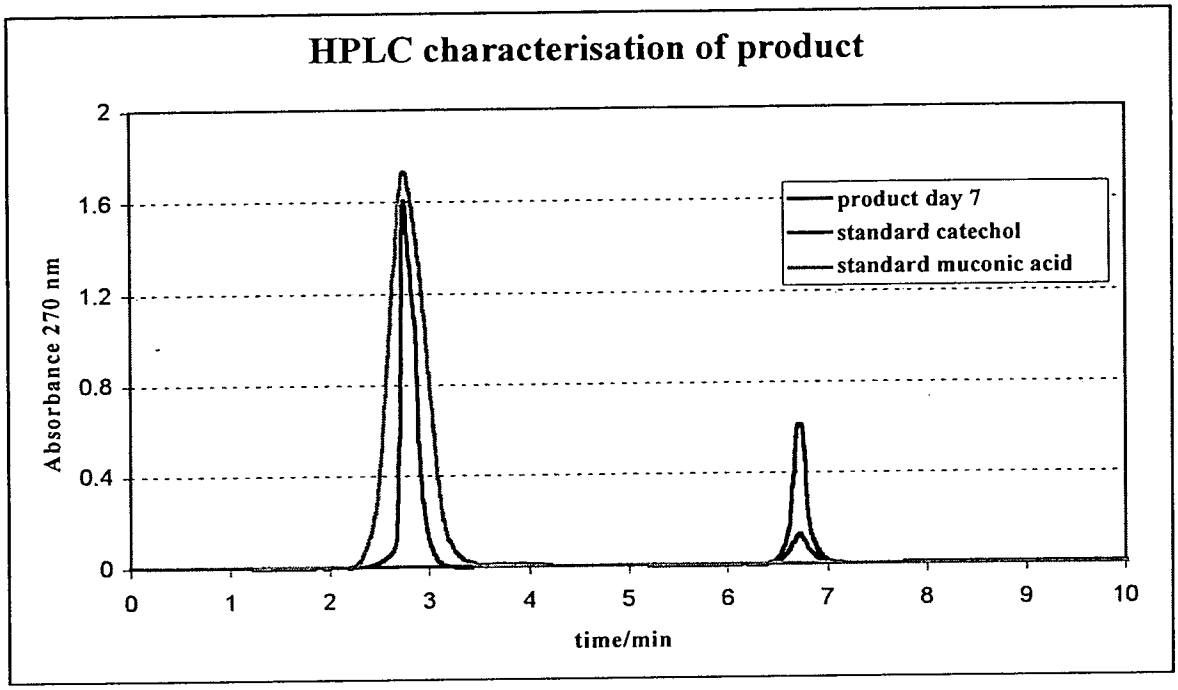

Figure 5: HPLC chromatogram of product output from column after 7 days' immobilisation, recorded at 270 nm

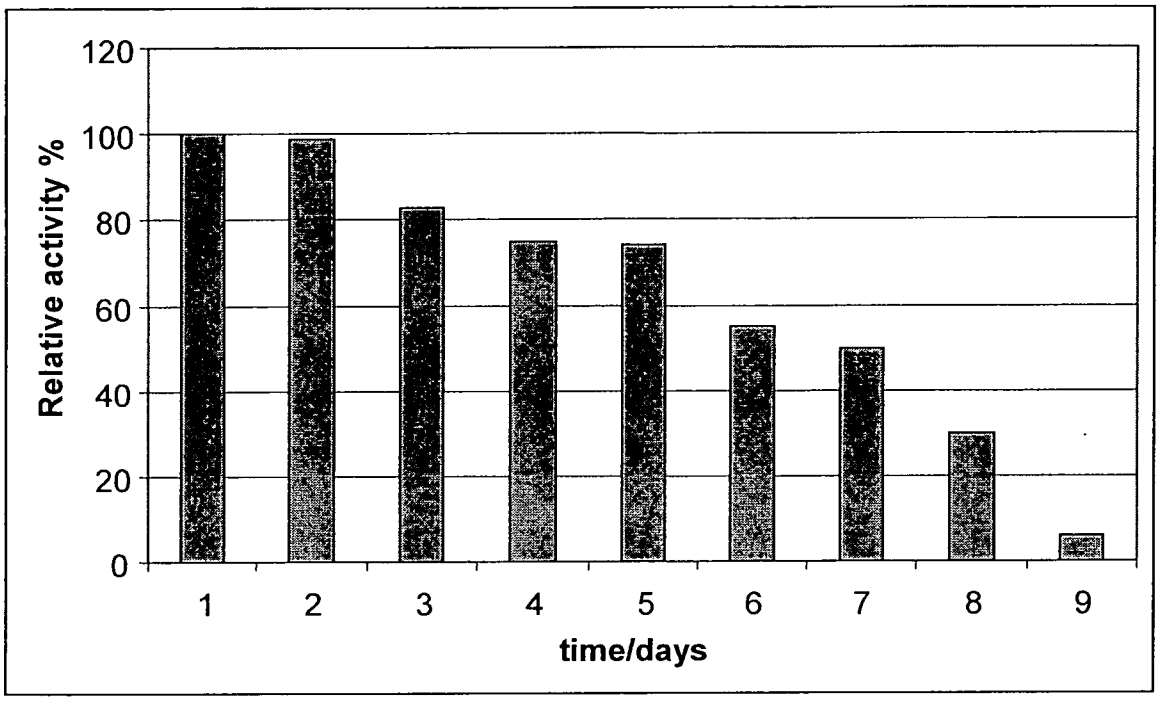

Figure 6: Ability of on-column immobilised enzyme (%) to convert catechol into cic-cis muconic acid, over time

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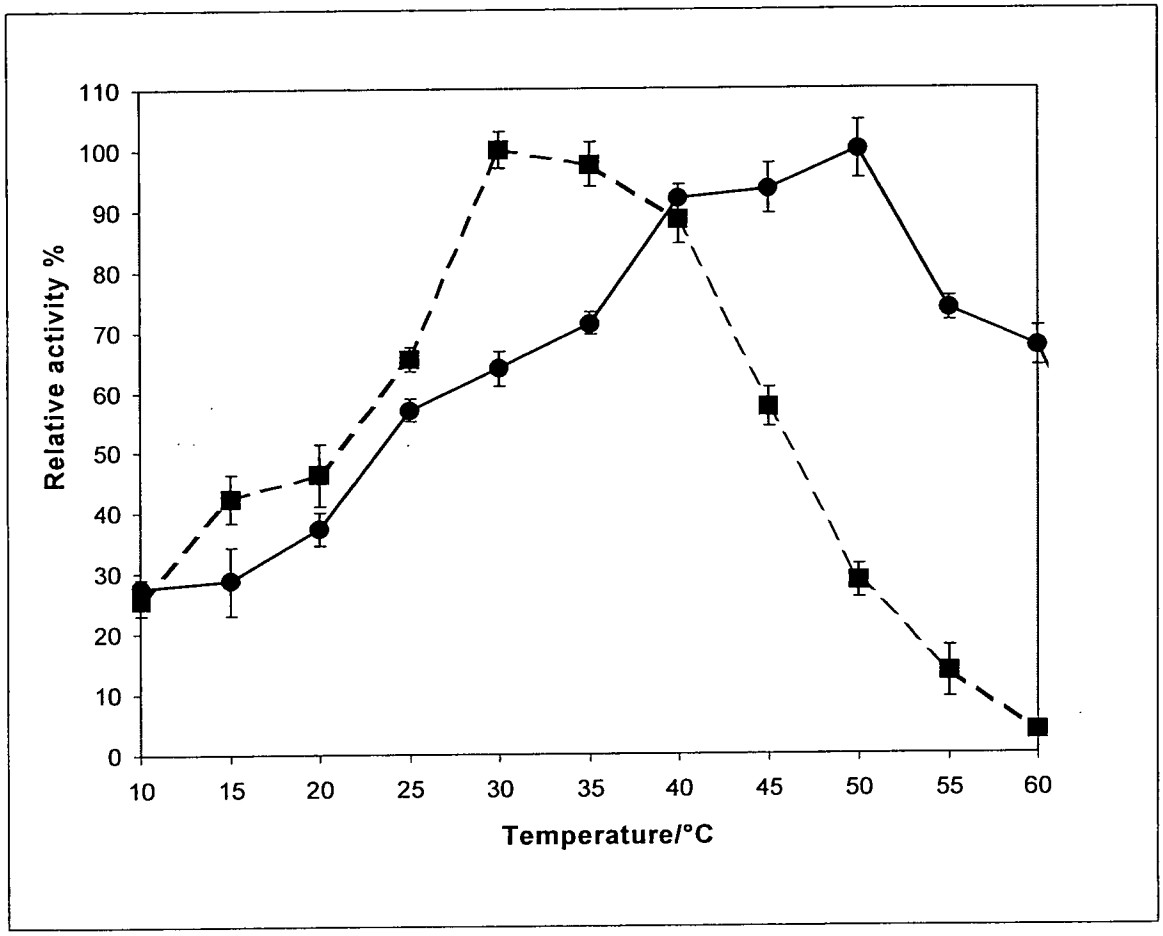

Figure 7: Comparison between percentage activity of enzyme in solution (dotted line) and immobilised enzyme at different temperatures

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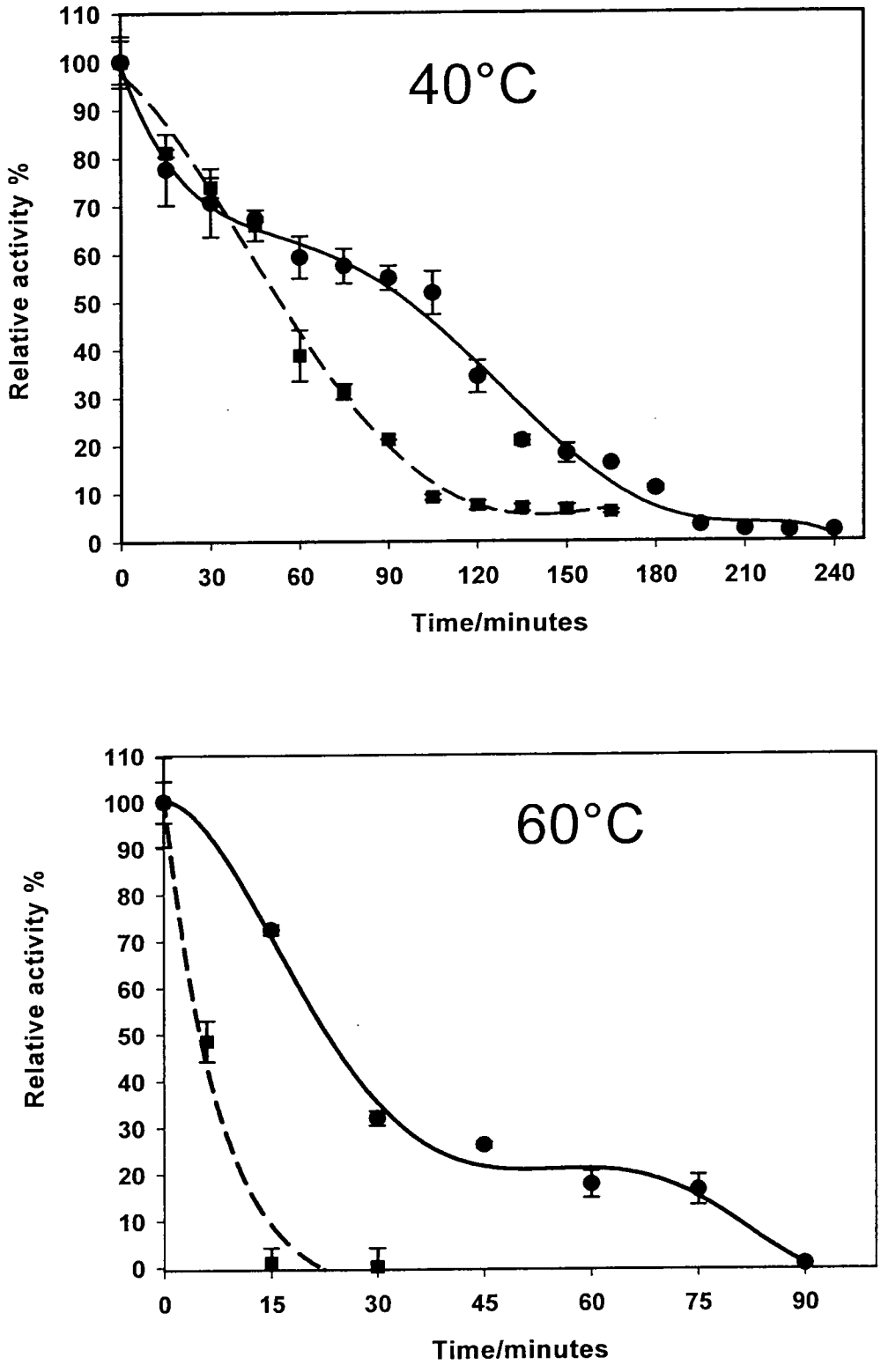

Figure 8: Loss of activity over time of catechol 1,2-dioxygenase in solution and immobilised at 40°C and 60°C (8a and 8b)

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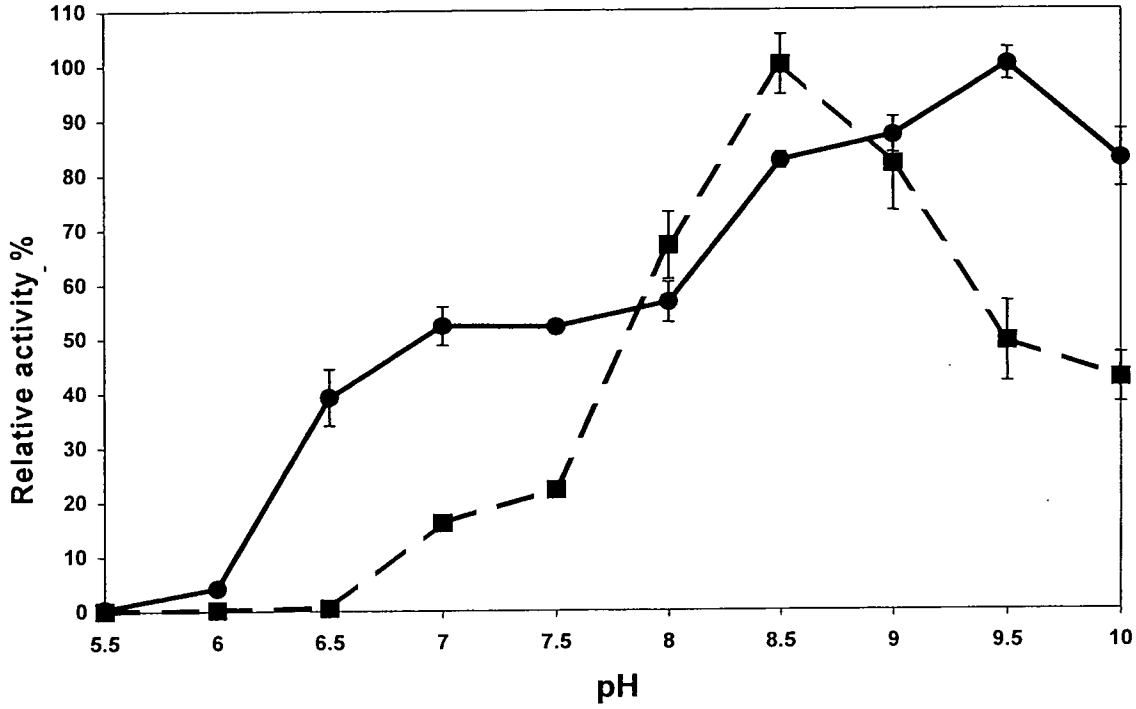

Figure 9: Comparison between percentage activity of enzyme in solution (dotted line) and enzymes immobilised at various pH values

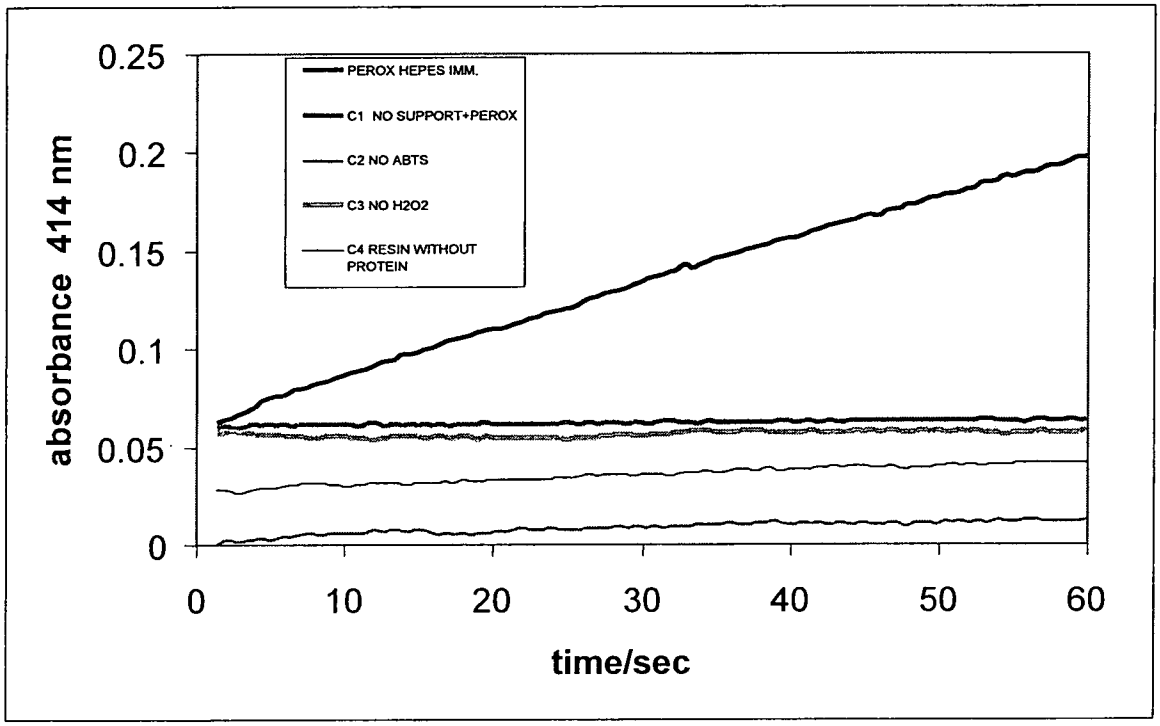

Figure 10: Activity of immobilised peroxidase on crosslinked cyclodextrins

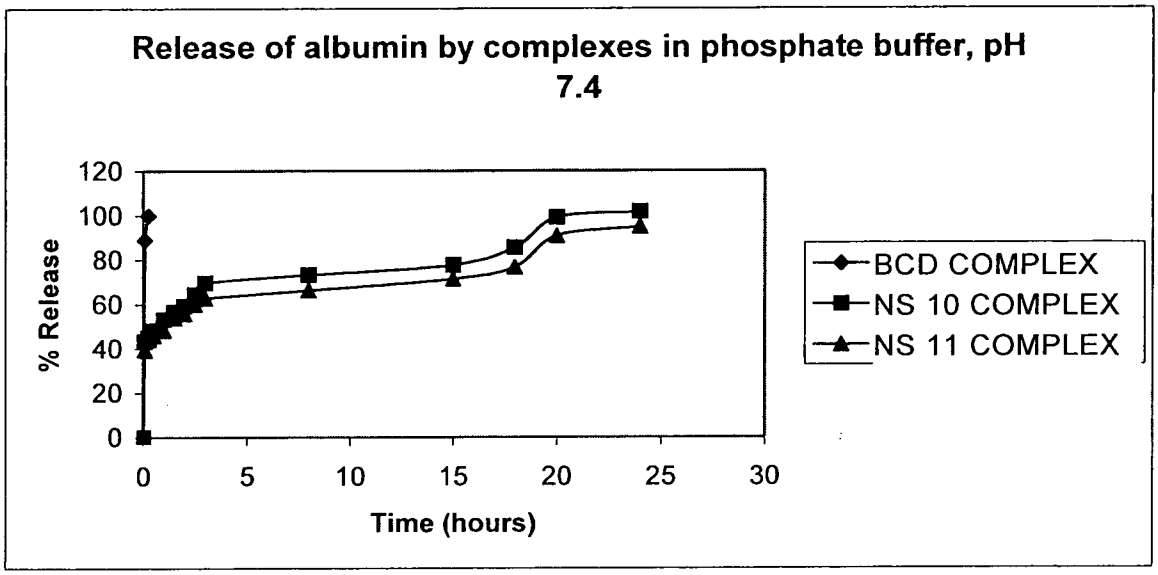

Figure 11: Release of albumin by carbonated nanosponges

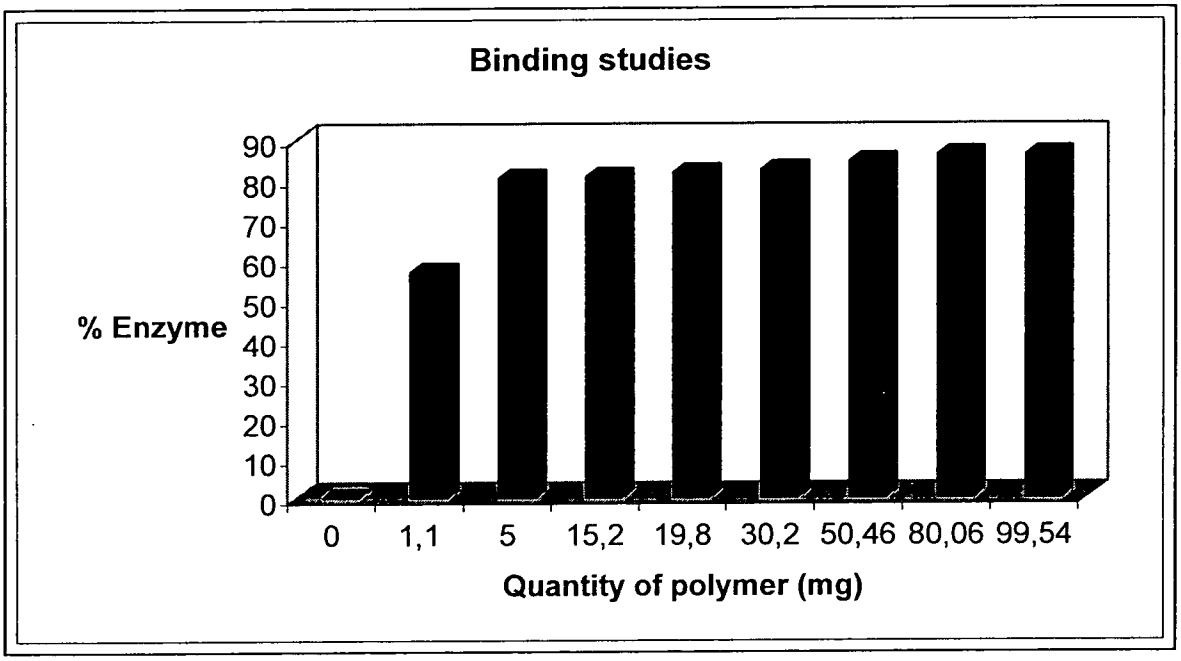

Figure 12: Quantity of lysozyme adsorbed on NS carbonate

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/004098

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N11/10 C08B37/00

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)
C12N C08B

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, 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 5 718 905 A (SKIBA MOHAMED [FR] ET AL) 17 February 1998 (1998-02-17) column 3 - column 4; claim 6	1,2,4,5
X	TSUTSUMI T, HIRAYAMA F, UEKAMA K, ARIMA H.: "Potential use of polyamidoamine dendrimer/alpha-cyclodextrin conjugate (generation 3, G3) as a novel carrier for short hairpin RNA-expressing plasmid DNA." JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 97, no. 8, 11 October 2007 (2007-10-11), - 11 October 2007 (2007-10-11) pages 3022-3034, XP002547969 the whole document	1-3

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

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 2009	13/10/2009

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	Stoyanov, Borislav

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/004098

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 2006/002814 A (SEA MARCONI TECHNOLOGIES DI W [IT]; TROTTA FRANCESCO [IT]; CAVALLI ROB) 12 January 2006 (2006-01-12) page 1 - page 12	1,2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2009/004098

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		US 2008213384 A1	04-09-2008