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CELL CULTIVATION IN CHITOSAN ALGINATE HYDROGEL BEADS

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OTHER PUBLICATIONS
Loredo et al., Tissue Eng., 1996, 2, pp. 115-126.*

* cited by examiner

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ABSTRACT
The present invention relates to a method of producing hydrogel matrix comprising cartilage-forming cells wherein alginate, chitosan and cartilage-forming cells are mixed and subsequently polymerized into beads.

8 Claims, 6 Drawing Sheets
Figure 3

A

IL-6 production

- Alginate beads
- Chitosan 20kDa/alginate beads
- Chitosan 40kDa/alginate beads

pg/mg LDH

Days of culture

B

IL-6 production (0-9 days)

HCT116/Col-1

Alg + Ca
Alg + Ca/Sr
Alg + Sr
Alg/Chi + Ca
Alg/Chi + Ca/Sr
Alg/Chi + Sr
Figure 4
Figure 5
Figure 6

MMP-3 production

- Alginate beads
- Chitosan 20kDa/alginate beads
- Chitosan 40 kDa/alginate beads

Days of culture

ng/µg LDH
nition is 1/2). FIG. 3B shows IL-6 production in chondrocytes after 9 days of cultivation in alginate (left) or chitosan/alginate (right) beads using Ca++/Sr++ or a mixture of Ca++ and Sr++.

FIG. 4 shows the expression of alkaline phosphatase (AP) in alginate beads (closed bars) and in 0.6% chitosan/1.2% alginate beads (open bars) [chitosan 40 kD] in different media (ITS: medium comprising Insulin, transferrin and selenious acid; UG: Ulsrce G; FBS fetal bovine serum). AP concentrations are presented as Units alkaline phosphatase/pg ng lactate dehydrogenase). Panel A shows values after 21 days of cultivation, Panel B shows values after 28 days of cultivation.

FIG. 5 shows the expression of aggrecan (represented as nanogram aggrecan/pg ng lactate dehydrogenase). FIG. 5A shows the expression after 13 days of cultivation in alginate beads (left) chitosan (20 kD)/alginate beads (middle) and chitosan (40 kD)/alginate beads (chitosan/alginate mition is 1/2).

FIG. 5B shows aggrecan production in alginate beads after 9 days of cultivation in alginate (left) or chitosan/alginate (right) beads using Ca++, Sr++, or a mixture of Ca++ and Sr++. CM: Cell-associated Matrix; FRM: Further-Removed matrix*.

FIG. 6 shows the expression of MMP-3 (represented as nanogram MMP-3/pg lactate dehydrogenase) at different time points. Left bar: cells cultivated in calcium alginate; middle bar: cells cultivated in chitosan (20 kD)/calcium alginate beads; right bar: cells cultivated in chitosan (40 kD)/calcium alginate beads (chitosan/alginate ratio is 1/2).

FIG. 7 shows the implantation of a thermosensitive chitosan hydrogel comprising 0.6% chitosan/1.2% alginate beads (A. bisporus chitosan (40 kD)).

FIG. 8 shows the histological evaluation of the implant 15 days after implantation. A: Subchondral Bone; B: Cell-colonized hydrogel; C: cell colonized Chitosan/alginate beads; D: Cell embedded in chitosan lacunae.

DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention relates to a method of producing a hydrogel matrix comprising cartilage-forming cells. In this method cells are included in the gel constituents upon formation of the hydrogel matrix. This method comprises the following steps: providing a solution of alginate, providing a solution with chitosan of Mw lower than 60 kD, mixing the solution of alginate and the solution of chitosan with cartilage-forming cells, wherein the mixed solution comprises between 0.5 and 0.7 (w/v) of chitosan and between 1 and 1.4% alginate, introducing droplets of the mixed solution into a solution with Ca++ or Sr++ ions, isolating gelled beads from the solution with cations. The hydrogel which is obtained by this method results in a homogeneous matrix of calcium alginate and chitosan, wherein the cell are equally distributed throughout the matrix. The matrix as obtained in the present invention differs from prior art matrices which have a core of one component, coated with a layer of another component.

The matrix as obtained in the present invention has the advantage that the porosity of the matrix can be more accurately defined compared to matrices which are first lyophilised to obtain a certain degree of porosity. The matrix as obtained in the present invention has the advantage to be composed with a low molecular weight chi-
CELL CULTIVATION IN CHITOSAN ALGINATE HYDROGEL BEADS

This is a national stage of PCT/EP11/052013 filed Feb. 11, 2011 and published in English, which claims the priority of European application 10154712.3 filed Feb. 25, 2010, hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to scaffolds for the cultivation of cartilage-forming cells, more particular chondrocytes. The present invention further relates to cellulosed beads for implantation. The present invention further relates to methods and tools for cartilage repair.

BACKGROUND OF THE INVENTION

Isolated and expanded cartilage-forming cells have been used for implantation since several decades. The in vitro expansion to obtain a sufficient amount of cells however may lead to cells which upon implantation result in cells death, scar tissue or hyperdifferenitated cells. This is especially the case for cartilage repair where chondrocytes culture, upon implantation often produces fibrous or bone-like material instead of the envisaged hyaline cartilage.

Attempts have been made to cultivate chondrocyte cells in a three dimensional matrix wherein the extracellular matrix of cartilage is mimicked. A variety of gels has been used wherein cell is cultured. For example, the prior art discloses beads with a core of alginate and a coating of chitosan (Babister et al. (2008) Biomaterials 29, 58-65).

Methods to prepare hydrogel beads can be divided in three groups. A first type relates to porous matrices which are populated by cells. Herein the matrices have a large pore size to allow the migration of cells into the matrix. Ll et al. (2008) J. Biomed. Mater. Res. A. 86, 552-559, describe a porous scaffold containing 2.4% alginate/2.4% chitosan which is subsequently seeded with cells.

As the cells migrate from the outside into the interior of the matrix, these matrices often have many cells at the outer cells and a low amount of cells at the interior.

In a second type of methods cells are mixed with a constituent of the matrix whereafter the matrix is being formed. Bernstein et al. (2006) Biotechnol. Prog. 22, 1146-1152; describe methods wherein beads with 2.5% alginate and 1.4% chitosan are formed. The cartilage therein is described as being of low quality.

In a third method, as illustrated in WO2007/135114, cells are encapsulated in a gelling solution obtained from an aqueous solution of a mixture of a polysaccharide such as alginate and an oligosaccharide derivative of chitosan highly branched. The aqueous solution is gelled with gelling agents with the aim to encapsulate the cells.

In each type of methods the choice of hydrogel has a strong impact on the phenotype of the cells, and subsequently on the quality of the implant. Particularly in the hydrogel of WO2007/135114, oligosaccharide derivative of chitosan has a degree of derivatization of at least 40% and the polysaccharide mixture or alginate mixture has an ionic strength which is not optimal for keeping cells alive. Further this 3D matrix fails to keep chondrocyte phenotype stable as shown by an increase of cell proliferation and a decrease of aggrecan synthesis.

There remains a need for further improvements in the selection of matrix material and in the methods to produce cellulosed implants.

SUMMARY OF THE INVENTION

One aspect of the present invention relates to methods of producing a hydrogel matrix comprising cartilage-forming cells comprising the steps of:

1. providing a solution of alginate,
2. providing a solution with chitosan having Mw lower than 60 kDa,
3. mixing the solution of alginate and the solution of chitosan with cartilage-forming cells, wherein the mixed solution comprises between 0.5 and 0.7% (w/v) of chitosan and between 1 and 1.4% alginate (w/v),
4. introducing droplets of the mixed solution into a solution with Ca²⁺ or Sr²⁺ cations and
5. isolating gelled beads from the solution with cations.

In certain embodiments of methods of the present invention the droplets of the mixed solution are introduced in a solution Sr²⁺ ions.

In particular embodiments of this method the mixed solution comprises 0.6% chitosan and or comprises 1.2% alginate.

In particular embodiments of methods of the present invention the ratio between alginate and chitosan in the mixed solution is between 1.4 and 2.8, or between 1.75 and 2.25 or is about 2.

In particular embodiments of methods of the present invention the chitosan has a Mw of between 35 and 45 kDa and is of animal or preferably of vegetable origin.

In other particular embodiments of methods of the present invention the method further comprises the step of cultivating the beads comprising cartilage-forming cells in a growth medium. Such a cultivation can be performed for up to 7, 14, 21 or even 28 days.

In other particular embodiments of methods of the present invention the growth medium comprises serum.

In other particular embodiments of methods of the present invention the formation of beads is performed by passing droplets through a needle to obtain a particle with a diameter between about 0.2 and 5 mm.

In other particular embodiments of methods of the present invention, the method further comprises the step of mixing the cultivated beads in a thermosensitive hydrogel. Herein the ratio between beads and hydrogel is for example between 5/1 and 1/1, or between 4/1 and 2/1.

Another aspect of the present invention refers to a spherical hydrogel bead comprising a homogeneous mixture of chitosan and alginate and further comprising cartilage-forming cells, wherein said bead is obtainable by the methods as described above.

Another aspect of the present invention refers to a spherical hydrogel bead with a diameter between 0.01 and 5 mm, comprising a homogeneous mixture of chitosan and alginate and further comprising cartilage-forming cells within the matrix, characterised in that the bead comprises between 1 and 1.4% alginate and between 0.5 and 0.7% chitosan, for example the bead comprises 1.2% alginate, or for example the bead comprises 0.6% chitosan.

In particular embodiments of beads of the present invention the ratio of alginate/chitosan is between 1.4 and 2.8, or between 1.75 and 2.25, or between 1.8 and 2.2.

In other particular embodiments of beads of the present invention the chitosan has a Mw between 35 and 45 kDa.

In other particular embodiments of beads of the present invention the cartilage-forming cells have a concentration of between 50000 or 60000 to 100000 or 150000 cells per bead.

In other particular embodiments of beads of the present invention the cartilage-forming cells, after 21 days of culti-
The matrix as obtained in the present invention, wherein cells are included into the matrix upon gellation, has the advantage that the cells are homogeneously distributed over the matrix beads. Porous beads which are seeded afterwards with cells typically will create a gradient of cells whereby the interior core of the matrix contains less beads than the exterior shell of the beads. This often results in cell populations wherein the cells at the core have different properties than those at the outside.

As indicated in the examples section, the alginate and chitosan which is used to prepare the hydrogels is dissolved in strong alkaline or acidic buffers which have a sterilising effect. This is an additional advantage of the invention.

In the methods in accordance with the present invention the alginate and chitosan solutions can be mixed to obtain beads with different concentrations. Particular embodiments of the present invention relate to beads wherein the composition, prior the gelification by calcium or strontium ions comprises 0.4, 0.45, 0.5, 0.55, 0.60, 0.65, 0.70, 0.75 or 0.80% (w/v) chitosan, and independently thereof comprises 0.9, 0.95, 0.1, 0.105, 0.11, 0.115, 0.12, 0.125, 0.13, 0.135, 0.14, 0.145 or 1.5% (w/v) alginate. In particular embodiments the concentration of chitosan ranges from 0.5 to 0.7%, or from 0.55 to 0.65%. In other particular embodiments the concentration of alginate ranges from 1. to 1.4% or from 1.25 to 1.55%. A particular embodiment of hydrogel comprises about 0.6% chitosan and about 1.2% alginate.

Further embodiments of methods and compositions of the present invention relate to gel bed composition and beads obtained thereof wherein the ratio between alginate and chitosan in the mixed solution is between 1.4 and 2.8, more particularly between 1.5 and 2.7, more particularly between 1.6 and 2.5, or between 1.75 and 2.25. Particular values of this ratio are about 1.9, 1.95, 2.9, 2.05 and 1.

In the methods of the present invention the average size of the beads can be adapted and empirically determined by adjusting the diameter of the needle which is used to form the droplets which are introduced into the calcium or strontium solution. Envisaged herein are beads with a diameter between 0.01 and 5 mm. These dimensions provide a compromise between the ease of manipulation and the diffusion of nutrients into the beads.

The experiments of the present invention indicate that the physical properties of the chitosan may contribute to the phenotype of the cartilage-forming chondrocytes. Chitosan has been isolated from different animal sources such as crustaceans (praw shells) or squid. Alternatively the chitosan is of vegetal, more of particularly fungal origin, such as Mucor racematus, Mucor racemosus and Cunninghamella elegans, Gorgonella butleri, Aspergillus niger, Rhizopus oryzae, Lentinus edodes, Pleurotus sajou-caju, Zoysoccharomyces rouxii, Candida albicans or Agaricus bisporus.

Chitosan further exists in different types of molecular weight. Herein, the chain length of chitosan may contribute to the three dimensional structure of the hydrogels. Typical chitosans, for use in the present invention has an average molecular weight lower than 60 kD, particularly between 15 and 50 kD, more particularly between 35 and 45 kD.

Beads which are produced in accordance with the present invention comprise cartilage-forming cells and are cultured to obtain the desired amount of cells for further purposes, such as implantation. The cultivation period can depend from certain factors such as cell type and initial concentration in the bead.

The beads of the present invention are prepared together with chondrocytes, chondrocyte precursors, cartilage-forming mesenchymal stem cells or cartilage-forming stem cells or with mixtures of chondrocytes and cartilage-forming precursor or stem cells.

It has been found that freshly isolated chondrocytes can be cultivated in the beads of the present invention for 7, 14, 21, or 28 days or even longer while maintaining their property to generate hyaline cartilage upon implantation.

The appropriate time of cultivation for the other above mentioned cell types is determined experimentally upon analysis of markers for chondrocyte stability which are described in the examples section of the present invention.

The chitosan/alginate beads of the present condition allow the cultivation of cells in conditions which are described as detrimental for the cultivation of chondrocytes, more particularly allow the growth of chondrocytes in a medium comprising 5, 10, 15% (volume serum/volume growth medium) serum of foetal or adult animals and overcomes the use of specialised synthetic media for chondrocyte cultivation.

The method of manufacturing beads as described above results in the formation of spherical hydrogel beads which comprise a homogeneous mixture of chitosan and alginate and further comprise cartilage-forming cells which are also homogenously distributed in the matrix of a bead.

Compared to other types of cellularised beads, the bead of the present invention can be prepared in a more reproducible ways than beads which are freeze-dried to provide cavities for the entry and cultivation of cells.

The superior properties of the cells which are cultivated in beads which are prepared in accordance with the methods of the present invention are demonstrated by the limited expression of cell death markers such as lactate dehydrogenase (LDH), the limited expression of inflammation markers, such as interleukins, the limited expression of markers which are typical for bone forming cells, such as alkaline phosphatase and the enhanced expression of cartilage markers such as aggrecan (FIG. 5). The expression of markers which are relevant for assessing the phenotypic stability of the chondrocytes have been experimentally determined by cultivating cells in chitosan/alginate beads of the present invention and in alginate beads, in the presence of serum. It has been further observed that the type of cation that has been used in gelification has an effect on the expression of at least aggrecan and interleukin-6. Herein, the use of strontium instead of calcium decreases as well the expression of IL-6 a marker for inflammation as of aggrecan, a marker for ECM production.

In view of the surprising properties of cartilage-forming cells, grown in beads as prepared in the present invention, one aspect of the invention relates to the use of cartilage-forming cells comprising in a bead as prepared in the present invention for the treatment of cartilage defects, more particularly osteoarthritids. Depending on the type of cartilage defect, the beads can be implanted shortly after formation of the beads, such that the cells will mainly grow after implantation. Alternatively the beads are implanted after in vitro cultivation for several days or weeks.

In particular embodiments, beads with chondrocytes are formulated as a biphasic implant material formed by beads and gel. This implant includes a polymeric matrix ("hydrogel"), and the spherical three-dimensional beads comprising chitosan and alginate and cells.

This allows to formulate an injectable gel which upon implantation, ensures an optimal spatial distribution of cells (chondrocytes or others) in the host tissue or organ. The injectable gel which is typically used herein is a thermo-sensitive gel. These gels remain liquid at ambient temperature.
(in a device used for introduction in to the patient), but become a solid gel upon introduction into the body at about 37°C. This in situ gelification maintains the beads (and the cells therein) in their spatial distribution. In vitro tests have demonstrated that beads containing cells were homogeneously distributed in such a hydrogel when heated to 37°C. Examples of thermosensitive hydrogels include poly(N-isopropylacrylamide (PNIPAAm). A particular type hereof is chitosan.

Without being bound by theory it is believed that the chitosan network within the microbeads confers particular mechanical properties at the beads such that they are less compressible and more resistant to pressure than the beads made of alginate only. The matrices of the present invention provide inter-connected chitosan trabeculae within an alginate gel, resulting in an environment which is favourable for cellular culture providing an aqueous medium with a neutral pH.

Such trabeculae are obtained by insoluble chitosan which forms coacervates which create a basket-like structure network or trabeculae when mixed with alginate. The trabeculae are of varying thickness and length which provides to the beads particular biological and mechanical properties such as phenotype stabilisation, deformability, elasticity and compressive modulus.

EXAMPLES

Example 1

Isolation of Chondrocytes

Fragments of cartilage are collected by biopsy and then submitted to three successive enzymatic treatments (hyaluronidase 0.5 mg/ml during 30 min at 37°C; pronase 1 mg/ml during 1 h at 37°C; collagenase 0.5 mg/ml during 1 night at 37°C. In the presence of 1% Ultroser G) to remove the extracellular matrix. (Ultroser G is a serum substitute of Pall Corporation). After passage on a cells strainer, the chondrocytes are rinsed and centrifuged. The pellet is collected and suspended in the chitosan/alginate solution.

Example 2

Preparation of Alginate/Chitosan Beads

Beads are prepared from a homogeneous mixture of chitosan (0.6% final) and alginate (1.2% final). The two solutions are prepared separately before being mixed. The solutions of alginate and chitosan are prepared in the following way: an alginate solution 2.4% (W/v) in 0.16 M NaOH and a solution of chitosan 1.333% (W/v) in 1.666 M HAc are prepared. To 10 volumes of the alginate solution, 1 volume of a 1 M Hepes solution is added. After homogenisation, we 9 volumes of chitosan solution are progressively added, while mixing regularly and vigorously.

Hereafter a cellular pellet of chondrocytes was administered and cautiously dispersed in the chitosan/alginate solution at a concentration of 6x10³ cells/ml. The chitosan/alginate solution with the cells was slowly passed through a 25 gauge needle in a dropwise fashion into a 102 mM CaCl2 solution (Sigma-Aldrich, Bornem, Belgium). After instantaneous gelation, the beads were allowed to gelify further for 10 min in this CaCl2 solution. In the gelled beads, the chondrocytes are distributed in a homogeneous way. At microscopic scale, the chitosan (stained in red by the eosin) forms a basket-like structure, composed of trabeculae or fibres of varying thickness and length (see FIGS. 1 and 2). The interstices herein are filled by alginate (hematoxylin stained in violet) which contains the chondrocytes.

Example 3

Cultivation of Chondrocytes in Alginate/Chitosan Beads

The gelled beads comprising chondrocytes, were washed with a saline solution. Beads were cultured in a 24-well plates by providing 10 beads in one ml of culture medium supplemented with 1% ITS+ (ICN Biomedicals, Asse-Relegem, Belgium), 10 mM HEPES, penicillin (100 U/ml) and streptomycin (100 U/ml), 200 μg/ml glutamine (Biowhittaker Europe, Verviers, Belgium), 50 μg/ml ascorbic acid (Sigma-Aldrich, Bornem, Belgium), 2 mM proline (Gibco, Merelbeke, Belgium)). ITS+ is a pre-mixed cell growth system containing in one ml: 0.625 mg insulin, 0.625 mg transferrin, 0.625 μg selenious acid, 0.125 g bovine serum albumin and 0.535 mg linoleic acid).

Example 4

Characterisation of Chondrocytes

Chondrocytes in FIG. 2B were stained with hematoxylin/eosin.

Aggrecan and IL-6 were quantified with ELISA (Biosource Europe) Alkaline Phosphatase was quantified with a spectrophotometric assay. Briefly, Fifty μl of cell extract were incubated with 100 μl of P-nitrophenylphosphate (KEM-ENTIC, Kobenhavn, Denmark). In the presence of AP, P-NPP is transformed to p-nitrophenol and inorganic phosphate. P-nitrophenol absorbance is measured at 405 nm. A standard preparation of p-nitrophenol was used for calibration. Results were expressed in nmoles of p-nitrophenol released per min and per μg of DNA. One unit AP is defined as one n mole of p-nitrophenol liberated per min. Lactate dehydrogenase was quantified by testing its enzymatic activity in the culture supernatant. 100 μl of the supernatant or dilutions of standard solution (LDH from rabbit muscle) were mixed with 50 μl of Tris buffer (10 mM Tris-HCl [pH 8.5], 0.1% BSA) containing 800 mM lactate. 100 μl of colorimetric regent (1.6 mg/ml iodonitrotetrazolium chloride (Sigma-Aldrich, Bornem, Belgium), 4 mg/ml nicotinamide adenine dinucleotide (Roche Diagnostics, Brussels, Belgium), 0.4 mg/ml phenazine methosulfate (Sigma-Aldrich, Bornem, Belgium) was added and the absorbance at 492 nm was read after 10 min of incubation at room temperature.

These data show that chondrocytes which are cultivated in the matrix of the present invention show a number of superior properties:

- cells cultivated in chitosan/alginate show less apoptosis or necrosis compared to alginate beads as indicated by the measurement of lactate dehydrogenase.
- cells cultivated in chitosan/alginate show less signs of inflammation.

Chondrocytes cultivated in chitosan/alginate beads do not produce or produce very little amounts of IL-6, IL-8 and NO.

FIG. 3 shows a dramatic reduction of IL-6 production during the culture of the chondrocytes in mixed beads (alginate/ chitosan) compared to alginate beads.

IL-6 is a very active cytokine in the inflammatory reaction and the associated tissue proteolysis. The stimulating effects
of IL-1β on the production of mediators of inflammation or of proteolytic enzymes are less important in alginate/chitosan beads than in alginate beads.

Chondrocytes cultivated in chitosan/alginate show less signs of catabolic events as indicated by lower production of a matrix metalloproteinase MMP-3, which is involved in the cartilaginous matrix degradation compared to chondrocytes cultivated in alginate beads. The growth of chondrocytes in the chitosan/alginate beads has a beneficial and stabilising effect on the chondrocyte phenotype.

Example 5

Formulation of Beads in a Thermosensitive Hydrogel

Culture medium is removed by per aspiration, and the beads are mixed with a vegetable (Agaricus bisporus) chitosan hydrogel (Kitozyme, Allleur, Belgium). This step is performed below 27°C to avoid hydrogel gelification. A ratio of beads/hydrogel of 3/1 (v/v) has been used.

Example 6

Effect of Cations on Chondrocyte Phenotype

FIGS. 3B and 5B show the effect of the choice of cation used in the hydrogel formulation. Whereas the choice of calcium or strontium has no effect on the expression of the markers interleukin-6 and aggrecan in beads which consist entirely of alginate, a significant effect is observed when chitosan/alginate are formed with either calcium or strontium ions.

Example 7

Implantation in an Animal Model

A gel as described under example 5, without chondrocytes, has been implanted in a cartilage defect in a rabbit joint (FIG. 7). After 15 days of implantation, the implant was evaluated (FIG. 8). The lesion remains filled with the implant. Further it is observed that the implant is colonised with cells originating from the underlying bone marrow. Cells were encountered in the settled thermosensitive chitosan hydrogel (B) as well as in the chitosan alginate beads (C) (chitosan trabeculae are indicated by D). This test confirms that the bioplastic implant can be easily handled and grafted. The biodegradable nature of the implant ensures a progressively resorption after implantation.

Example 8

Effect of Chitosan Molecular Weight in Beads Formation

Different molecular weights of native chitosan have been used in the process according to the invention. Different physical parameters such as pH and viscosity of the mixed solution (with chondrocytes) were measured. Osmolarity of the resulting hydrogel is measured according to a well-known technique in the art.

<table>
<thead>
<tr>
<th>Solution</th>
<th>pH</th>
<th>Viscosity (CPS)</th>
<th>Osmolarity (in Osm/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate 1.2%/chitosan 22 kDa 0.6%</td>
<td>7.8</td>
<td>109.7</td>
<td>324</td>
</tr>
<tr>
<td>Alginate 1.2%/chitosan 30 kDa 0.6%</td>
<td>8.3</td>
<td>230</td>
<td>305</td>
</tr>
<tr>
<td>Alginate 1.2%/chitosan 32 kDa 0.6%</td>
<td>7.8</td>
<td>192</td>
<td>308</td>
</tr>
<tr>
<td>Alginate 1.2%/chitosan 55 kDa 0.6%</td>
<td>8.0</td>
<td>279</td>
<td>290</td>
</tr>
<tr>
<td>Alginate 1.2%/chitosan 55 kDa 0.6%</td>
<td>8.2</td>
<td>336</td>
<td>302</td>
</tr>
<tr>
<td>Alginate 1.2%/chitosan 91 kDa 0.6%</td>
<td>Impossible to mix</td>
<td>Impossible to mix</td>
<td></td>
</tr>
</tbody>
</table>

We concluded that mixed beads can be made with native chitosan below 55 kDa. For example, at 91 kDa, beads cannot be made using the process described in our invention and at the ratio alginate 1.2% and chitosan 0.6%, the chitosan solution is too viscous. Therefore, the selection of chitosan molecular weight is an essential element in our invention.

Example 9

Effect of Chitosan Molecular Weight on Chondrocytes Behaviour

Molecular weight has an influence on the chondrocytes behaviour as illustrated in FIGS 3A, 3B, 3C, and 6. The effect of chitosan 40 kDa on MMP-3, IL-6 and aggrecan is significantly different from that obtained with 20 kDa. Chitosan 20 kDa is less efficient on IL-6 and MMP-3 synthesis than 40 kDa chitosan, but more effective on aggrecan synthesis than 40 kDa. This clearly demonstrates that the biological activity of native chitosan is directly dependent of the molecular weight.

The invention claimed is:

1. A method of producing homogenous polymerised beads comprising cartilage-forming cells, said method comprising the steps of:
   - providing the cartilage-forming cells,
   - providing a strong alkaline solution of alginate, providing an acidic solution with chitosan of Mw between 22 kDa to 55 kDa,
   - mixing the solution of alginate and the solution of chitosan, wherein said mixed solution comprises between 0.5% (w/v) to 0.7% (w/v) of chitosan and between 1% (w/v) and 1.4% (w/v) of alginate,
   - dispersing the cartilage-forming cells in the mixed solution of alginate and chitosan, introducing droplets of said mixed solution into a solution with Ca²⁺ or Sr²⁺ cations to form homogeneous polymerised beads wherein the cartilage-forming cells are homogeneously distributed within the homogeneous polymerised beads, wherein the homogenous gelified beads comprising interconnected chitosan trabeculae within an alginate gel, and
   - isolating the homogenous polymerised beads from said solution with cations.

2. The method according to claim 1, wherein said mixed solution comprises 0.6% (w/v) chitosan.
3. The method according to claim 1, wherein said mixed solution comprises 1.2% (w/v) alginate.
4. The method according to claim 1, wherein alginate and chitosan in said mixed solution are present in a ratio between 1.75:1 to 2.25:1.
5. The method according to claim 1, wherein said chitosan has a Mw from 35 kDa to 45 kDa.
6. The method according to claim 1, further comprising the step of cultivating said beads comprising cartilage-forming cells in a growth medium.

7. The method according to claim 6, wherein said growth medium comprises serum.

8. The method according to claim 1, wherein said droplets are passed through a needle to obtain a particle with a diameter between 0.01 and 5 mm.

* * * * *