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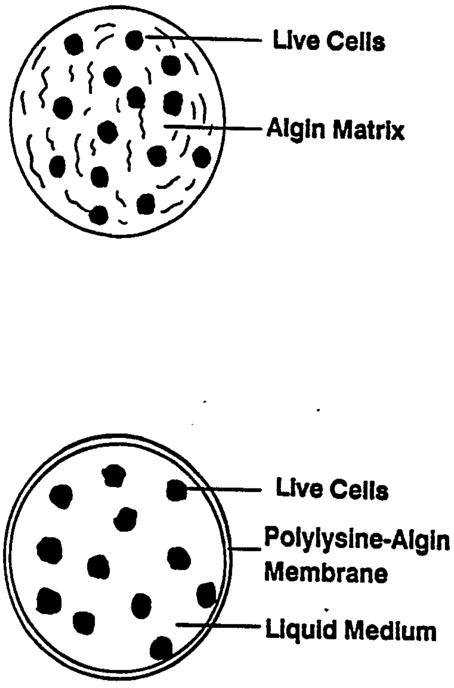
**(54) Title:** COMPOSITION AND METHOD OF PROMOTING HARD TISSUE HEALING

**(57) Abstract**

Osteoprogenitor cells encapsulated in alginate and alternatively, additionally encapsulated in polylysine and/or agarose promote regeneration of bone at the site of implantation. The present invention provides a composition comprising osteoprogenitor cells embedded or encapsulated in alginate and the use of said microcapsules for the facilitation of bone regeneration.



TWO TYPES OF ARTIFICIAL CELLS

* See back of page

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# COMPOSITION AND METHOD OF PROMOTING HARD TISSUE HEALING

## Field of the Invention

The present invention relates generally to the field of hard tissue healing and, more particularly, to the field of biodegradable implantable microcapsules to stimulate the natural process of hard tissue regeneration and bone wound healing.

## Background of the Invention

Defects in bone or osseous structures will initiate the process of bone healing. Healing often involves the replacement of injured tissue by connective tissue and leaves a scar. Bone, under optimal conditions, heals by regeneration in which injured tissues are replaced by their own kind and leave no scar. The success of regeneration following injury depends, among other things, on the type of injury, the adequacy of treatment and the systemic health of the patient. Osseous repair involves at least six physiological stages: impact, induction, inflammation, soft callus formation, hard callus formation, remodeling and regeneration. Heppenstall, Fracture Treatment and Healing, W.B. Saunders, Philadelphia, 1980, page 35.

With inadequate treatment, severe injury and/or metabolic bone disease, fracture healing is significantly retarded. For example, in the case of a metabolic bone disease such as osteoporosis, 40% of patients with decreased bone mass due to osteoporosis showed a markedly impaired fracture repair rate. Only 33% of women in whom significant osteoporosis was present were able to achieve a solid union following femoral neck fractures. In comparison, in 90% of women with physiologically normal bone mass a successful union was achieved. Lane et al., Osteoporosis, Orthopedics clinics North America 15: 711 (1984); Arnold, J. Bone Joint Surg. 66A: 847 (1984); Scileppe et al., Surg. Form 32: 543 (1981).

It is estimated that there are 200,000 hip fractures in osteoporetic women in the United States annually with a 40% mortality rate due to complications of repair of these fractures. As a result, there is a significant need to facilitate fracture repair in these types of patients. In addition, fractures in young accident and trauma victims result in loss of numerous productive days from the work place. For example, it takes an average of six weeks to complete repair even simple bone injuries in healthy individuals.

Bone fractures and bone wound healing following trauma or surgery account for considerable morbidity and mortality. For example, femoral neck fractures in patients under forty may be associated with avascular necrosis in as many as 40% of cases complicated by non-union. Kyle et al., Young Femoral Neck Fractures, Presented at the 52st Annual Meeting of American Acadeym of Orthopedic Surgery, Atlanta, Ga. (1984). Many other examples could be cited of the need for more expeditious methods to facilitate and/or accelerated fracture or hard tissue defect repair.

In addition, recent technological advances have made the replacement of joints and defective or diseased hard tissues common surgical procedures.

Since the feasibility of the preparation of artificial cells was first demonstrated in 1957 by Chang (Chang, T.M.S. (1964) Science:146, 524), numerous approaches to their production and use have been evaluated. Artificial cell membranes have been reported using a variety of synthetic and biological materials to give the desired membrane properties. A large variety of materials can be enclosed (microencapsulated) in artificial cells. This includes single and multienzyme systems, cell extracts, and combined enzyme-adsorbent systems (Chang, U.S. Patent No. 4,642,120). Biological cells have been encapsulated to prevent them from being adversely affected by external factors and immunological rejection (Chang, Biomedical Applications of Immobilized Enzymes and Proteins (Plenum: New York, 1977) Vols. 1 and 2; Mosbach et al. (1966) Acta Chem. Scan. 20: 2807; Lim et al. (1980) Science 210:908). More recently the microencapsulation of living biological cells that can be maintained in culture has been disclosed (Lim et al. (1980) Science 210: 908; U.S. Patent No. 4,391,909).

U.S. Patent No. 4,663,286 (Tsang et al.) discloses a process for encapsulating material is described for forming a capsule utilizing an alginate polymer with a polyvalent cation.

U.S. Patent No. 4,642,120 (Nevo et al.) discloses the repair of cartilage and bones by employment of a composition provided in gel form. The gel comprises certain types ot cells. These may be committed embryonal chondrocytes or any type of mesenchymally-derived cells which may differentiate into chondrocytes, generally as a consequence of the influence of chondrogenic inducing factors, in combination with fibrinogen, antiprotease, thrombin, and other factors. According to U.S. Patent No. 4,642,120, the cells should be of the same species as that to which the composition is transplanted. Incorporation of extracellular matrix (ECM) of chondrocytes, other hormones and/or growth factors such as SM (Somatomedin or IGF-I), FGF (fibroblast growth factor), CGF (cartilage growth factor), BDGF (bone derived growth factor) or a combination of any of these in the gel is also disclosed.

U.S. Patent No. 4,472,840 (Jefferies) discloses a method of inducing osseous formation by implanting bone graft material. Both demineralized bone particles (DBP) and bone inductive protein have demonstrated the capacity to induce the formation of osseous tissue in animal and human experiments. Reconstituted collagen conjugate is known to be highly biocompatible and can be fabricated in a variety of configurations, especially as a sponge. This material can be used as a grafting implant in plastic and reconstructive surgery, periodontal bone grafting, and in endodontic procedures. Structural durability is enhanced by crosslinking with glutaraldehyde which is also used to sterilize and disinfect the collagen conjugate prior to implantation.

U.S. Patent No. 4,132,746 (Urry et al.) discloses a crosslinked insoluble polypentapeptide elastomer capable of calcification by withdrawing calcium ions from a serum medium, thus making it useful as a calcifiable matrix for the formation of an artificial bone structure. The calcifiable material can be treated to make it useful in artificial vascular wall formation.

U.S. Patent No. 4,609,551 (Caplan et al.) discloses a material for stimulating growth of cartilage and bony tissue at anatomical sites. The material consists of a composition with a fibrin or allograft matrix containing soluble bone protein and fibroblast cells.

Summary of the Invention

In order to facilitate the healing of bone and other hard tissue fractures and defects and facilitate structural implant fixation, a microcapsule has been developed. Specifically, it has been discovered that osteoprogenitor cells can be embedded in or encapsulated by biocompatible materials and nonetheless retain their viability and biological function. The biocompatible encapsulating materials useful in practicing this invention can have different rates of biodegradeablity. The biocompatible material may be readily biodegradeable, slowly biodegradeable or relatively resistant to degradation in biological fluids. A readily biodegradeable material is one that is degraded 50% or more within hours to several days by contact with biological fluids. A slowly biodegradeable material will degrade at least 50% when in contact with biological fluids for more than several days up to a week or several weeks. A material resistant to biodegradation is one which retains its integrity for at least several weeks in the presence of biological fluids.

Materials which are readily biodegradeable (bioerodable) include naturally-occurring polymers such as alginates, polylysine, cellulose polymers, e.g., methylcellulose, collagen, gellen gum, casein, chitosan, and the like. Materials which are slowly degradeable include some polyesters, and polyanhydrides. Biocompatible materials which are relatively resistant to biodegradation include titanium oxide, hydroxyapitite, biocompatible metal compositions, biocompatible ceramic compositions, and the like. The microcapsules of the present invention can comprise one biodegradeable material or a combination of two or more biodegradeable materials.

In the latter case the microcapsule may contain biocompatible materials of varying rates of biodegradeablity.

A microcapsule comprising one or more biodegradeable materials can itself be coated or further encapsulated by a less readily degradeable substance in order to further delay complete release of the encapsulated material. By carefully choosing the materials used as the initial encapsulating material and the subsequent coating or encapsulating material, one of skill in the art may control the rate of release of one or several encapsulated materials, including the encapsulated osteoprogenitor cells. For instance, in one embodiment, alginate alone can be used as the sole encapsulating material. In a second embodiment, biodegradability is retarded by coating thus-prepared alginate microcapsules with a polyanionic polymer such as polylysine.

In yet another embodiment a core material relatively resistant to biodegradation, such as a ceramic material to which another material, e.g., one of the above mentioned growth factors, has been bound and from which this other material is slowly released, e.g., from the surface of the core material, may be encapsulated within a more readily biodegradeable material which itself contains the same or other treating materials, e.g., the same or another growth factor, antiviral agent, hormone, in order to sustain release of one or more of the encapsulated materials. For instance, a microcapsule comprising woven titanium mesh mixed with collagen may be also be embedded within the algin microcapsule containing osteoprogenitor cells. Prosthetic devices formed of the present invention will facilitate fixation of orthopedic devices or dental implants by enhancing the bone regeneration at the site of prosthetic implantation. In another embodiment, fixation of orthopedic implants at the surgical site can be facilitated by implantation of the composition of the present invention comprising ceramic hydroxyapitite adsorbed with bone derived growth factor or any other material which stimulates the differentiation or growth of osteoprogenitor or cartilage progenitor cells with the progenitor cells in the formable materials useful for practicing the present invention. This embodiment allows a stable and solid support for replacement and/or reconstruction of defective hard tissues while additionally providing the neccessary progenitor cells to repair and/or replace the defective hard tissue structures.

As indicated above, microcapsules prepared in accordance with this invention can additionally contain materials which aid in bone healing or in the prevention or treatment of complications of trauma. Such additional materials can include, but are not limited to, extracellular matrix of chondrocytes (ECM), hormones, growth factors such as somatomedins, fibroblast growth factor, bone morphogenic protein, platelet derived growth factor, bone inductive growth factor, osteoinductive growth factor, cartilage derived growth factor, prostaglandins, macrophage derived growth factors, bone derived growth factor, skeletal derived growth factor, epidermal growth factor, transforming growth factor ß, growth factor, cytokines, and the like, or a combination of any of these. Such materials may alternatively be termed herein hard tissue promoting factors. Other agents which aid in treatment or prevention of the complications of trauma may additionally be included. Examples of such other agents are, without limitation, antiviral agents, antibacterial agents and the like. The above agents and factors may be used alone or in combination in practicing the present invention. Such materials can be prepared by any method known to those skilled in the art, including purification from naturally occurring sources and recombinant technology.

The microcapsules of this invention, coated or uncoated, e.g., with polylysine, can be further surrounded by a material that can be formed into a hydrogel wafer, such as agar, gelatin, gellan gum or the like, in order to facilitate handling and transfer or implantation of encapsulated material(s) into the site of treatment.

The present invention provides compositions and a method to facilitate the healing or regeneration of bone, for instance, at fracture sites. This method comprises implantation or injection of any of the compositions of the present invention into a site or a device in an individual at which bone fixation, reconstruction, regeneration or healing is desired. The osteoprogenitor cells then proliferate and cause the deposition of new bone material at the implantation or injection site.

The present invention also provides compositions and a method to facilitate the regeneration and healing of cartilagenous tissues.

It is, therefore, an object of the present invention to provide a composition to augment and/or facilitate the regeneration or healing of bone tissue at fracture sites.

A further object of the present invention is to provide a wafer delivery system for encapsulated osteoprogenitor cell-containing compositions of the present invention.

Yet another objective of the present invention is to provide viable encapsulated osteoprogenitor cells for implantation and timed-release at bone fracture sites to augment and facilitate healing of the fracture.

A still further object of the present invention is to provide a method of stimulating the healing of bone fractures.

These and other objects, as well as the nature, scope, advantages and utilization of this invention, will become readily apparent to those skilled in the art from the following description, the drawings and the appended claims.

Brief Description of the Drawings

Figure 1 shows a schematic of the two capsule types.

Figure 2 shows a schematic of the type of apparatus used to form one type of microcapsule.

Detailed Description of the Invention

In order to accomplish the above objects and objectives, the present invention provides, in one embodiment, osteoprogenitor cells embedded or encapsulated in an alginate matrix.

In one embodiment of the present invention, osteoprogenitor cells have been encapsulated, viability maintained within artificial membranes, and the cells when implanted in an animal model, subsequently proliferate and maintain their capacity to induce osteogenesis.

The osteoprogenitor cells useful in carrying out the present invention can be any cells capable of inducing the formation of regenerated bone (or cartilage)(or the deposition of calcium?). Preferably, these cells are autologous bone progenitor cells harvested from the individual in need of such treatment. These cells may be harvested from the site of the injury or from a distant site for transplantation to the injury site. The primary cells may be used directly or may be expanded by passage in cell culture. In another embodiment, the osteoprogenitor cells may be harvested from another individual of the same specie as the individual to be treated. However, the cells may also be selected from the group consisting of cell lines derived from any mesenchymally cells which will differentiate to form osseous orr cartilagenous tissue. In selecting the osteoprogenitor cells for use in the present invention it is only important to try to minimize as much as possible the rejection of the implanted cells for the period necessary to induce the regeneration of new bone.

The preferred composition of the present invention comprises osteoprogenitor cells embedded or encapsulated in a biodegradeable material. The cells may either be embedded in a matrix material by being dispersed within the matrix material itself or by surrounding the cells with a biodegradeable material. In either case, in order the decrease the rate of release of the osteoprogenitor cells from the microcapsule, the cells may be further encapsulated in a nonbiodegradeable material or a material which has a prolonged integrity in the host such as polylysine. Preferably, the matrix material is an alginate, such as sodium alginate. The matrix material may also be selected from the group consisting of gellan gum, chitosan, or agarose.

The method of encapsulating the osteoprogenitor cells comprises embedding or encapsulating the cells in a biodegradeable material by any of the techniques known to those of skill in the art. Preferably the osteoprogenitor cells are encapsulated by a modification of the method disclosed in U.S. Patent No. 4,391,909, incorporated herein by reference. Briefly, osteoprogenitor cells were gently dispersed in a solution of sterile sodium alginate and pumped through a needle into a collection bath of 1.3% calcium chloride containing Tween 20. The alginate embedded cells, also termed herein microcapsules, were harvested, washed with saline and either used directly for implantation or injection into the treatment site or further encapsulated to prolong the integrity in the host.

In a preferred embodiment, the microcapsules were formed into wafers to facilitate implantation. These wafers were preferably composed of agar, such a wafer is described in Example 4. The wafer can be of any material that is biocompatible and can be formed into a hydrogel having characteristics similar to agar such that the handling and placement of the microcapsules at the treatment site is facilitated.

The method of treating bone fractures of the present invention comprises implantation of the osteoprogenitor microcapsules of the present invention into a fracture site of an individual and allowing sufficient time for the formation of new bone at the treatment site. The osteoprogenitor microcapsules may be implanted by surgical procedures known to those of skill in the art or may be injected into the fracture site utilizing a suitable pharmaceutical carrier. The choice of such carriers will be obvious to those in the art.

The term "individual" is meant to include any animal, preferably a mammal, and most preferably a human, cat, dog, or horse.

Artificial cell preparation was carried out in a sterile environment. All equipment, materials, solutions, etc. were either sterilized by the appropriate means or purchased as sterile before use in the process.

Having now generally described the invention, a more complete understanding can be obtained by reference to the following specific examples. These examples are provided for purposes of illustration only and are not intended to be limiting unless otherwise specified.

## Example 1

## PROCEDURE FOR ENCAPSULATION OF OSTEOPROGENITOR CELLS

### A. Preparation of osteoprogenitor cells.

Cells were isolated from canine trabecular bone specimens. The specimens represented material obtained by biopsy of the iliac crest of four research grade mongrel dogs numbered as follows: 4452, 4386, 4593, and 4467. The biopsy specimens from each dog were processed individually in order to permit autologous implantation of the cellular material at a later date, thereby circumventing any possible rejection response and eliminating the need for immune suppression of the host dogs.

The biopsy material was washed multiple times in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (1000 units/ml), streptomycin (1000 ug/ml), and amphotericin-B (0.25 ug/ml) to remove adherent tissue and debris. The bony trabeculae were then cut into small pieces (1-2 mm2) followed by a second series of washings to remove hematogenous elements. The resulting clean pieces of bone were placed in a 100 mm cell culture dish in the absence of media and incubated at 37°C in an atmosphere of $O_2/CO_2$ (95/5 v/v). After 20 minutes, 10 ml DMEM containing 10% newborn calf serum (NCS) was carefully added to the dish without disturbing the bone fragments. The dishes were returned to the incubator and left undisturbed for 5 days. Subsequently, the media was changed every three days to fresh DMEM, 10% NCS. After 23 days of culture, the cells which had migrated from the bone fragments onto the surface of the culture dish were removed with trypsin/EDTA (0.125%/1 mM).

These cells were placed in a T-75 culture flask and designated first passage cells. The cells were passaged two more times to yield third passage cells which, when confluent, were encapsulated as described below (Runs 1-30A through 1-31B). Examination of aliquots of the encapsulated cells suspended in DMEM containing the vital dye trypan blue, indicated that the cells had retained their viability during the encapsulation procedure. The encapsulated cells were maintained in DMEM containing 10% NCS at 37°C in an atmosphere of $0_2/C0_2$ (95/5 v/v) for 24-48 hours prior to preparation for implantation into nonunion sites prepared in the radii of dogs. Viability experiments revealed that the encapsulated cells could be maintained in this manner for three days without a decrease in cell number. In fact, the cell number increased by 70-90% during this time period.

Cells for implantation in nonunion fracture sites in dogs were harvested and grown in culture as described above. Osteoprogenitor cells were incubated in an incubator (37°C) until ready for use in the encapsulation process.

B. Encapsulation of Cells

Cells were encapsulated by a modification of the method described in U.S. Patent No. 4,391,909 (Lim). Two types of encapsulated cells were prepared. In one, cells were encapsulated (or embedded) in an algin matrix. In the second, the process was carried further and the alginate embedded cells were further encapsulated using poly-L-lysine/algin as the capsule membrane. A schematic of the two capsule types is shown in Figure 1.

The encapsulated cells were prepared as follows and used for implantation into animals to demonstrate the effect on fracture healing. Cells from several flasks were combined, placed in a 15-ml sterile culture tube and rinsed 3 times with sterile 0.9% saline solution. After decanting the saline solution from culture tube, 10 ml of sterile sodium alginate solution (about 1%) was added. The alginate used for most of the cell encapsulation was sterile Macrocarrier* solution obtained from Bellco Glass, Inc. The cells were gently dispersed and the cell/alginate solution was transferred to a sterile syringe. The syringe was placed in a sterile pump device and connected to the encapsulation device with sterile tubing.

A sterile collection bath (containing a 1.3% calcium chloride solution with 0.25 ml of 10% Tween 20) was placed under the encapsulation device. The cells were encapsulated in the alginate and collected in the collection bath. After the alginate encapsulated cells remained in the collection bath for 3-5 minutes, they were passed through fine wire screen baskets. The alginate embedded (encapsulated) cells were then rinsed 2 times with 0.9% saline solution and used in this form as the alginate matrix cell preparation.

In some situations when it is desirable to provide polylysine encapsulated osteoprogenitor cells, the alginate embedded (encapsulated) cells were rinsed once with a polylysine solution, preferably about 0.2%. The poly-L-lysine used in the encapsulation was obtained from Sigma Chemical Company and had a molecular weight of approximately 38,000. The cells were then incubated in the polylysine solution for 5-7 minutes, rinsed 2 times with 0.9% saline solution, and finally, rinsed once with an approximately 1.5% sodium citrate solution by incubating the encapsulated cells in the sodium citrate solution for 5-7 minutes. The cells were then rinsed 2 times with 0.9% saline solution and 3 times with DME (Dulbecco's Modified Eagle's Medium) for 2-3 minutes.

The cells suspended in approximately 40 ml DME were transferred to a sterile T-75 flask and incubated at 37°C until implantation.

The results of the encapsulation procedures are shown on Table 1. In the initial runs (1-1A through 1-9D) only placebo capsules were prepared in order to adjust process parameters to produce the desired type of capsule. Matrix materials evaluated during this period included alginates, casein, chondroitin sulfate, and collagen. In the preferred embodiment, spheres were formed using sodium alginate collected in a calcium chloride ($CaCl_2$) bath as shown in Run 1-7A in Table 1. In forming the capsule, air regulation was used to control the droplet size. A schematic of the apparatus used is shown in Figure 2.

Runs following 1-12A were carried out with encapsulating live cells unless otherwise stated. The encapsulation of osteoprogenitor cells are designated as 1-30A through 1-31B in Table 1.

Histopathologic analysis was performed on encapsulated cells maintained in vitro as well as on tissues removed at necropsy from animals implanted with microencapsulated osteoprogenitor cells for in vivo evaluation. This was accomplished in three phases as described in Examples 2, 3, and 4, below.

## Example 2

## In Vitro Cell Analysis

Following encapsulation of the cells, in vitro studies were conducted to determine osteoprogenitor cell viability and define their morphology within artificial cell membranes. Histologic sections were prepared and stained with hematoxylin and eosin using encapsulated cells in the following combinations:

K-1 Alginate + U2OS cells (an osteosarcoma cell line)
K-2 Polylysine + U2OS cells
K-3 Alginate + normal dog cells (animal #4452)
K-4 Polylysine + normal dog cells (animal #4452)
K-5 Alginate + FL cell tumor (a transformed human tumor cell line capable of bone formation)

U2OS cells encapsulated in alginate appeared as small nests or colonies numbering approximately 2-15 cells, each with an average of approximately 10 cells per group. The cells had basophilic staining nuclei which were round and regular with prominent nucleoli noted at random. Cell cytoplasm was moderately eosinophilic and cell boundaries were relatively distinct. The algin matrix was amorphous and slightly basophilic but obviously degrading as a consequence of the histologic processing procedure necessary to produce the sections.

U2OS cells encapsulated in polylysine also appeared as clusters with morphology not significantly different from that described above, however, the artificial polylysine membranes were histologically distinct as slightly basophilic undulating cuticular surfaces enclosing cell nests. The undulation was interpreted as an artifact of dehydration, again necessary for processing.

Normal dog cells when encapsulated in alginate, appeared as isolated groups, usually of 2-3 cells. Morphologically the cells had the characteristics of osteoblasts with eccentrically located round nuclei and relatively conspicuous eosinophilic cytoplasm. In some cells there was evidence of a perinuclear eosinophilic condensation typical of osteoblasts. Again, the alginate membranes appeared to be degrading as a result of the histological preparation.

Normal osteoprogenitor dog cells encapsulated in polylysine showed similar morphology to those encapsulated in alginate alone. Again, polylysine membranes were distinct as described with the U2OS cells above.

Alginate embedded FL cells also showed isolated cells or groups of 2-4 cells with round, eccentrically located nuclei, occasional prominent nucleoli and eosinophilic cytoplasm, but differing from U2OS cell lines in that clusters were in general smaller and less numerous within the artificial membranes.

In summary, all artificial cell preparations contained viable cells with morphology varying as to the derivation of the particular cell type indicating that no deleterious effects resulted from the encapsulation process.

Alginate cell membranes degraded during histological processing and thus were not visible in subsequent sections produced from animal studies. Polylysine membranes were more distinct and durable and remained visible at least in early phases of the animal studies. The interpretation of the in vivo data shown in the Examples below was made in accordance with these observations .

These studies demonstrate that cells may be encapsulated, their viability maintained, and sections prepared for histologic analysis. Intact cells were noted within the confines of the artificial membranes and, as a consequence, these formulations rendered viable cells for implantation studies.

## Example 3

### In Vivo Studies of Encapsulated Cell Lines Implanted in Nude Mice

Cell viability following encapsulation was evaluated in vivo using FL cells, a transformed line of human amnion cells capable of tumor formation in the nude mouse. The rationale for these experiments follows. Cells encapsulated in alginate and implanted beneath the skin of the nude mouse formed tumors as rapidly as nonencapsulated cells injected subcutaneously, since the alginate is rapidly dissolved in vivo. Formation of tumors by cells encapsulated in polylysine was delayed, since polylysine is not readily dissolved in the host and cells first have to multiply within the capsules in sufficient mass to burst them.

Encapsulated FL cells (Runs 1-15B, 1-16B, 1-36A, and 1-36B) were maintained overnight at 37°C in an atmosphere of O2/CO2(95/5 v/v). The following morning 0.5 ml alginate or polylysine encapsulated cells were surgically implanted beneath the skin of 3-week old nude mice of the nu/nu strain (Harlan). The mice were sacrificed at 16 and 32 days after implantation for gross and histological evaluation of tumor formation.

FL cell lines encapsulated in alginate and implanted for a period of 16 days, demonstrated at necropsy, viable cells with histologic features remarkably similar to those described in the in vitro experiments with the exceptions that the cell clusters were now much larger, often forming confluent nests in excess of several hundred cells.

Alginate membranes, as expected, were not visible but the general outlines of the artificial cells were present in some areas, perhaps attributable to fibrocollagenous connective tissue proliferating in proximity with the artificial cell membranes. In other areas, FL cells had grown into confluent nests with the subcutaneous tissue and muscles, violating and disrupting the boundaries of the artificial cell membranes. In these areas of host tumor interface, conspicuous bone and osteoid production was noted.

FL cells encapsulated in polylysine and implanted for 16 days again showed large viable cell clusters with morphologic features as described with the exception that the cell membranes of polylysine remained intact. Most cell groups within the membranes had grown to confluency. No evidence of cell penetration into adjacent tissues, as was noted above, was apparent. No bone or osteoid production was visible.

FL cell lines encapsulated in alginate/polylysine harvested 32 days after implantation showed a large bulk of tumor (larger than 2.0 X 1.0 cm) with FL cell line morphology. It was composed of confluent nests and sheets of cells proliferating in no discernable pattern with a few groups similar to those described above still present. Most membrane material apparently had been resorbed and was inconspicuous. There was overt invasion of host tissue by the FL cell lines with conspicuous bone and osteoid production.

Thus, the above results have demonstrated the viability of encapsulated cells and further that this viability could be maintained throughout the implantation or injection procedure with the encapsulated cells subsequently proliferating within artificial membranes, rupturing the membranes and invading into host tissues.

Additionally, the above results demonstrate that cell lines induced bone production, evidence of the maintenance of cell capacity to exhibit their normal function following the encapsulation process. Alginate and polylysine microcapsules apparently degrade at different rates, since discernable differences between polylysine and alginate encapsulated cells were noted at 16 days with alginate tending to degrade earlier than polylysine.

In order to determine if artificially encapsulated cells would survive in vivo, 7 nude mice were injected with encapsulated cells formulated in varying matrices. If injection of encapsulated cells is delayed, viability is significantly suppressed. Vital cells encapsulated in polylysine and alginate membranes could be observed 24 days following injection. Surviving cells which had been injected alone or in a carrageenan matrix were not detectable at 24 days.

## Example 4

## In vivo Studies of Treatment of Fracture Non-unions Produced in Dogs.

Fracture nonunions were experimentally induced in 11 research grade dogs. The nonunions were performed by surgically removing a 3 mm disc of cortical and cancellous bone from the mid-radius. Dogs were then allowed to resume normal weight bearing activities, and after 12 weeks, stable fracture nonunions were produced. The dogs were then divided into groups consisting of controls receiving only matrix material with no osteoprogenitor cells and four animals receiving osteoprogenitor cells formatted in varying ways. Each dog received cells which had been harvested at the time of the initial surgery and maintained in tissue culture as described in Example 1 above.

In order to facilitate handling during the implantation procedures and to insure retention at the nonunion site, the encapsulated cells were prepared in a gel of low melt agarose (Sigma TYPE VII). A "doughnut" prepared with 3 ml of 4% agarose was formed in a 28 mm diameter culture dish with a 12 mm diameter post in the center. After the agarose had gelled, the centerpost was removed. A suspension was prepared from 3 ml encapsulated cells and 3ml 2% agarose. The hole in the center of the 4% agarose doughnut was filled with 1.5 ml of this suspension. After the central portion had gelled, the entire doughnut was transferred to a cell culture dish, covered with DMEM, and returned to the incubator. The doughnuts were implanted into the nonunion defects within 15-18 hours. The outer rim of the doughnut was substantial enough to permit gentle handling with forceps. The central core was rigid enough to hold the encapsulated cells at the implant site, while still allowing for diffusion of wound and tissue fluid to the cells. These discs were then implanted following excision of the fibrous nonunion material and the radii splinted with a 4-hole stainless steel splint. The dogs then resumed weight bearing activity for an additional 12 weeks at which time the animal was sacrificed and material taken for detailed histologic evaluation.

The two dogs receiving only polylysine matrix material showed a persistent nonunion defect occupying approximately 8.5-11% of the original nonunion defect volume on histomorphometric analysis. The trabecular bone volume in these areas was calculated at 6.5 and 24.75% respectively with 46.9 and 18.9% fibrous connective tissue intermixed as well as a small amount of fibrocartilage. In addition, a significant quantity of polylysine matrix, visible as irregularly shaped refractile material was noted throughout the defect. There was a modest multinucleate foreign body giant cell response to this material as well as minimal chronic inflammatory cell infiltration. The histologic features from the two control dog studies were identical to six control dogs from previous studies involving the encapsulation and implantation of bone inductive proteins in nonunion fractures.

When autologous osteoprogenitor cells were encapsulated in an artificial matrix of alginate and implanted in a dog nonunion, histologic examination showed a dramatic and complete healing of the fracture nonunion. This was apparent on histomorphometric analysis with 100% of the original defect being filled with new bone. The trabecular bone volume in this area was 55% with no interposed fibrous connective tissue. Relatively normal cancellous space was present instead. This was in dramatic contrast to the controls and other test animals receiving inductive proteins. Also apparent were isolated small cell clusters and groups of cells with round, elliptically located nuclei and relatively distinct cytoplasmic membranes with slight eosinophilia to the cytoplasm. These were identical to cell clusters noted in the in vitro and nude mouse in vivo experiments. These cells could be observed within the cancellous space and at times in intimate adaptation with an acellular eosinophilic homogeneous material consistent with osteoid.

When autologous osteoprogenitor cells were encapsulated in a polylysine matrix and implanted in a dog nonunion, histologic examination 3 weeks demonstrated evidence of degrading artificial cell membranes consistent with polylysine and a few artificial cell nests as described above in the in vitro and in vivo nude mouse studies, as well as the dog previously described. Throughout the nonunion site there was evidence of brisk osteoblastic activity with production of homogeneous, eosinophilic acellular osteoid as noted in the previous dog. The histologic features demonstrated healing at a significantly advanced stage compared with that anticipated for control animals from previous nonunion experiments. The two remaining dogs each received polylysine encapsulated cells or alginate encapsulated cells. Both dogs were carried to 13 weeks. The polylysine cells showed some evidence of osteoid production and remnants of artificial cells, but no significant fill of the nonunion defect. The same was true of the last dog receiving alginate encapsulated cells.

The results of the implantation of osteoprogenitor cells encapsulated in alginate (with or without poly-L-lysine) demonstrated that the method of the present invention causes complete healing of the fibrous nonunion, the healed fracture being composed of mature bone with lamellar characteristics and evidence that remodeling of the fracture site into a functional state had occurred. This Example conclusively demonstrates that osteoprogenitor cells may be encapsulated in artificial membranes, their viability maintained, and these cells subsequently implanted in living subjects (mice and dogs). The cells subsequently proliferate out of the artificial confines to produce osteoid and new bone which contributes to the healing process.

Although all dogs receiving encapsulated osteoprogenitor cells did not demonstrate the same amount of nonunion fracture healing, this result may relate to a number of complex interrelated factors. These include the kinetics of artificial cell membrane degradation, cell release from artificial membranes, proliferative capabilities of individual autologous cells, differences inherent in healing capacity of each animal, or combinations of these.

In addition, bone inductive factors may be necessary in the artificial membranes to completely signal encapsulated cell populations to begin proliferation within the unfavorable environment of a healing wound. Some of these variables may be overcome by inclusion of bone cell differentiation factors within the microcapsule at the time of encapsulation.

### Example 5

### Device Fixation Enhancement in Primates

In order to improve the fixation of titanium bone implants, in vivo studies of implantation of microencapsules containing osteoprogenitor cells and bone inductive factors were performed. Osteoprogenitor cells were encapsulated in either alginate or alginate coated with polylysine as described in Example 1. Additionally, microcapsules were prepared as in Example 1 however, bone inductive growth factor was also included in the microcapsules. The microcapsules were implanted within the internal aspects of a titanium bone implant in six sites in each of two baboons (animal no. 713 and 609) to determine whether bone growth into titanium prosthetic implants could be enhanced, facilitating of the fixation of the prothesis in the baboon tibia.

One of six titanium implant sites was used as a control and received no microcapsule material. Each of of the remaining five titanium implant sites in baboon tibia recieved the same microencapsulation composition. At one week intervals for six weeks, tissue within the internal aspects of one of the six titanium implant sites in each baboon was retrieved for histologic evaluation. The status of the encapsulated material and the quantity of bone in the internal aspects of the titanium implant was determined. Histologic analysis of tissue within the implant at the site of microcapsule implantation was carried out weekly for six weeks. The encapsulating materials were highly biocompatible and did not elicit a giant cell foreign body response. The amount of encapsulating material present in histologic sections decreased as the treatment period progressed, indicating that the implanted microcapsules were biodegraded at the site of the titanium prosthetic implant. Gross histological examination revealed bone regeneration in all titanium prosthetic implant sites which received the microcapsule composition of the present invention. Further analysis may reveal quantitative or qualitative differences in the regenerating hard tissue due to the presence of the bone inductive growth factor.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth below.

WHAT IS CLAIMED AS NEW AND IS DESIRED TO BE COVERED UNDER LETTERS PATENT IS:

# CLAIMS

1. A composition comprising osteoprogenitor cells encapsulated in a biocompatible microcapsule.

2. The composition of claim 1, wherein said microcapsule comprises a biodegradeable polymer.

3. The composition of claim 2, wherein said biodegradeable polymer is selected from the group consisting of alginate, polylysine, methylcellulose, collagen, gellen gum, casein and chitosan.

4. The composition of claim 3 wherein said polymer is alginate.

5. The composition of claim 3 wherein said polymer is polylysine.

6. The composition of claim 1 wherein said microcapsule comprises alginate and polylysine.

7. The composition of claim 1 wherein said microcapsule comprises alginate coated with polylysine.

8. The composition of claim 1 further comprising a material selected from the group consisting of extracellular matrix of chondrocytes (ECM), a hormone, a growth factor, an antiviral agent, and an antibacterial agent.

9. The composition of claim 8 wherein said growth factor is selected from the group consisting of somatomedin, fibroblast growth factor, epidermal growth factor and bone derived growth factor.

10. The composition of claim 1 contained in a hydrogel wafer.

11. The composition of claim 10 wherein said hydrogel wafer comprises a material selected from the group consisting of agar, gelatin, gellan gum and agarose.

12. A method for promoting bone regeneration, comprising administration of the composition of any one of claims 1 - 11, inclusive, to an individual in need of said treatment.

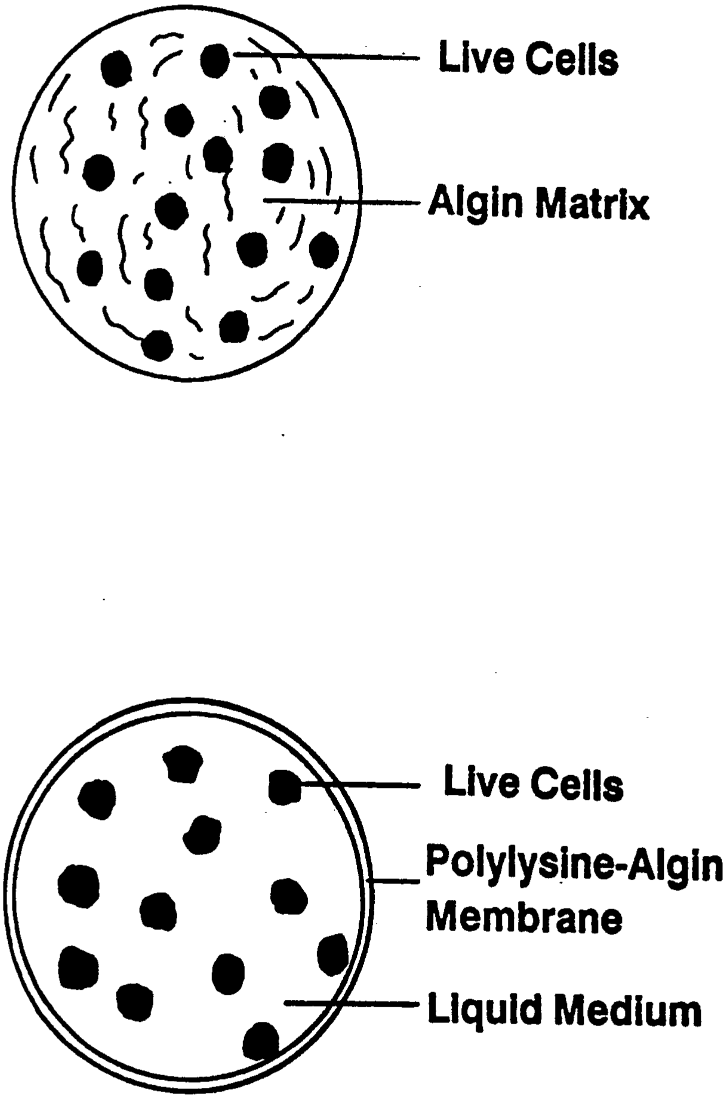
13. The method of claim 12 wherein said individual is a mammal.

14. The method of claim 13 wherein said mammal is selected from the group consisting of a human, a dog, a cat, and a horse.

15. The method of claim 14 where in said mammal is a human.

16. A composition comprising a material resistant to degradation in biological fluids, wherein said resistant material is selected from the group consisting of titanium oxide, hydroxyapitite, biocompatible metal compositions and biocompatible ceramic compositions; hard tissue promoting factors bound to the resistant material; and contained within a readily degradeable polymer.

17. The composition of claim 16 additionally comprising unnbound hard tissue promoting factors.



TWO TYPES OF ARTIFICIAL CELLS

Figure 1

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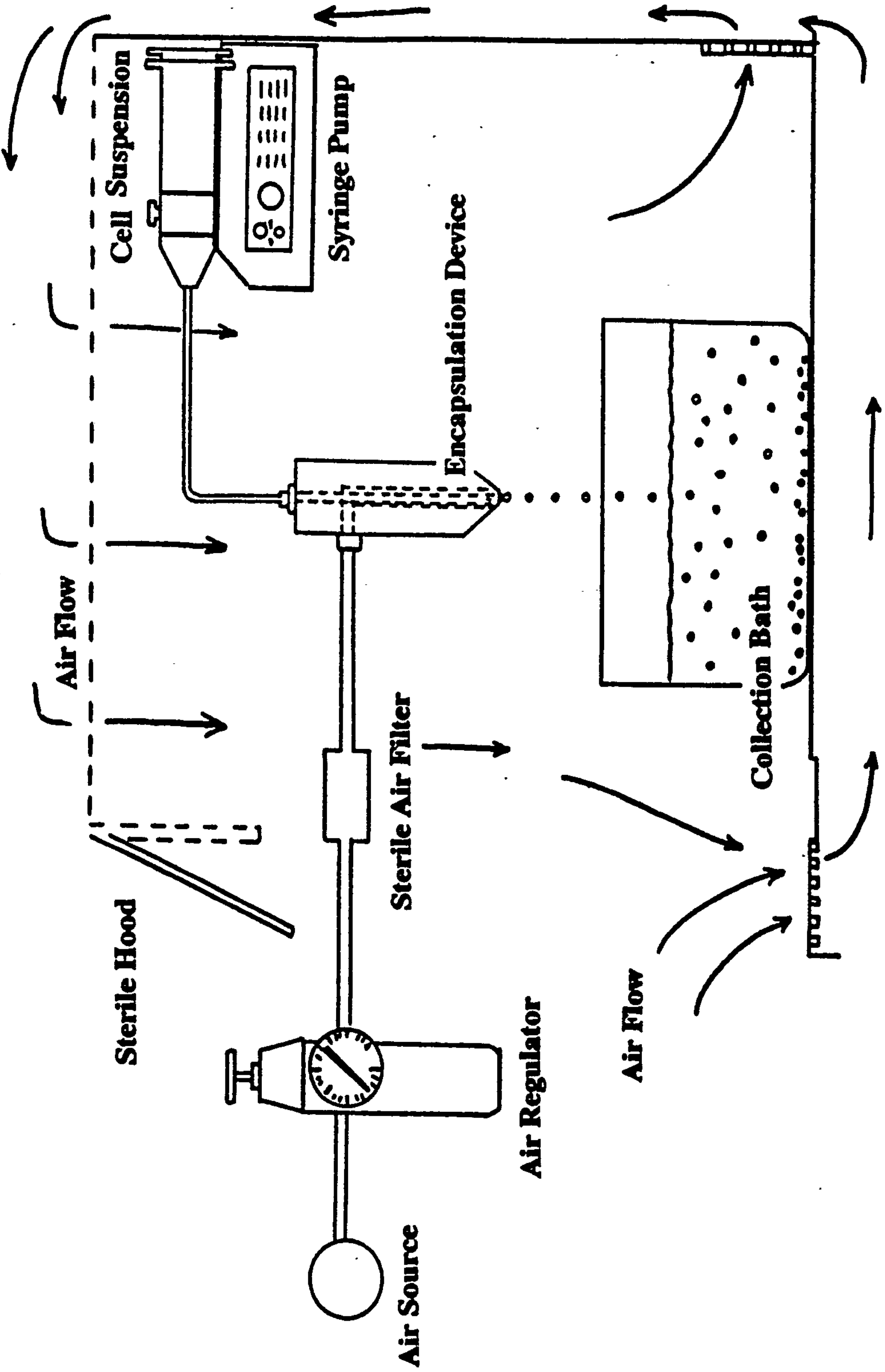

CELL ENCAPSULATION PROCESS

Figure 2

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/04381

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 9/14,9/16,9/52,9/64,37/24; C12N 11/04,11/10,11/12
U.S. CL. 424/484,485,488,491,; 435/178,182; 530/399

**II. FIELDS SEARCHED**

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 424/484, 485, 488, 491; 435/178, 179, 182<br>530/399 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

**III. DOCUMENTS CONSIDERED TO BE RELEVANT** [9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| A,P | US, A, 4,904,259 (ITAY) 27 FEBRUARY 1990<br>See Abstract. | 1,2, 12-15 |
| Y | US, A, 4,642,120 (NEVO) 10 FEBRUARY 1987<br>See Abstract and column 4, lines 35-36. | 1,2,8,9,<br>12-15 |
| Y | US, A, 4,609,551 (CAPLAN) 02 SEPTEMBER 1986<br>See Abstract, column 2, lines 1-2, 17-22, 54 and column 3, lines 34-48. | 1-3,8,12-15 |
| Y | US, A, 4,647,536 (MOSBACH) 03 MARCH 1987<br>See Abstract and column 1, lines 10-15. | 1, 2, 3 |
| Y | US, A, 4,663,286 (TSANG) 05 MAY 1987<br>See Abstract, column 1, lines 5-7 and column 3, lines 4-38. | 1-7 |
| Y | US, A, 4,391,909 (LIM) 05 JULY 1983<br>See Abstract, column 4, lines 9-16, column 9, and Example 1. | 1-7 |
| Y | US, A, 4,673,566 (GOOSEN) 16 JUNE 1987<br>See Abstract, column 8 and Example 1. | 1-7, 10, 11 |

* Special categories of cited documents: [10]

"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

"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

**IV. CERTIFICATION**

| | |
|---|---|
| Date of the Actual Completion of the International Search<br>02 OCTOBER 1990 | Date of Mailing of this International Search Report<br>09 JAN 1991 |
| International Searching Authority<br>ISA/US | Signature of Authorized Officer S. Kishore<br>Gollamudi S. Kishore |

International Application No. PCT/US90/04381

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| Y | US, A, 4,798,786 (TICE) 17 JANUARY 1989 See Abstract, column 10 and claim 3. | 1-3, 11-15 |
| Y | US, A, 4,620,327 (CAPLAN) 04 NOVEMBER 1986 See Abstract, column 2, lines 4-54 and column 3, lines 27-34. | 16 & 17 |
| Y | US, A, 4,610,692 (EITENMULLER) 09 SEPTEMBER 1986; See Abstract. | 16 & 17 |
| Y | US, A, 4,595,713 (ST. JOHN) 17 JUNE 1986 See Abstract, and column 6, lines 23-41. | 16 & 17 |
| Y | US, A, 4,888,366 (CHU) 19 DECEMBER 1989 See Abstract, column 2, lines 54-64 and column 7, lines 7-65. | 16 & 17 |

International Application No. PCT/US90/04381

**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**

**V.** ☐ **OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE** [1]

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

1.☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers , 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 [13], specifically:

3.☐ Claim numbers___________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

**VI.** ☒ **OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING** [2]

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

I. Claims 1-15: A composition comprising osteoprogenitor cells encapsulated in a microcapsule.

II. Claims 16-17: A composition containing no osteoprogenitor cells. (See attachment)

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

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ 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 claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Con't. from PCT/ISA/210 supplemental sheet (2).

The international application lacks unity of invention under PCT Rule 13 because of the following reason:

Inventions I and II are independent and distinct in that the composition in invention I contains osteoprogenitor cells where as the composition in invention II does not require the presence of these cells, but contain additional factors.