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(54) Title: BIOCOMPATIBLE ENCAPSULATION SYSTEM

(57) Abstract: The invention relates to an encapsulation system comprising alginate biocapsules for the immunoisolation of living cells of therapeutics. Specifically, although by no means exclusively, the encapsulation system is for use in allo- and xeno- transplatation. The invention is also directed to methods of making and using the encapsulation system.

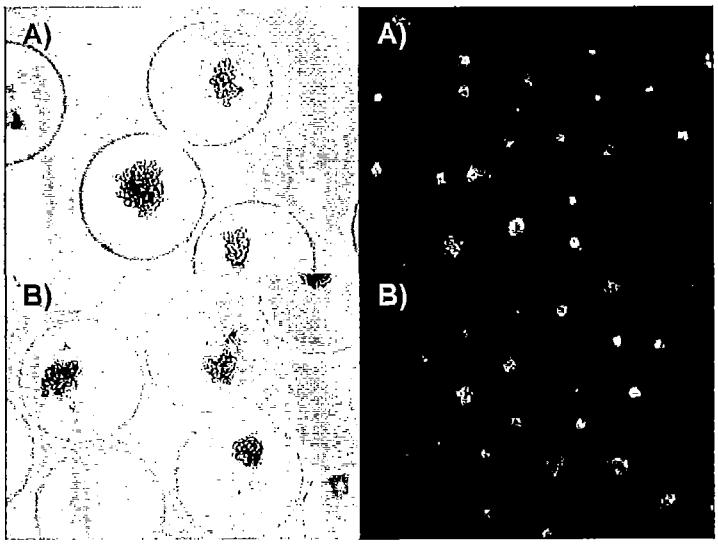

Figure 1

WO 2014/171842 A1

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BIOCOMPATIBLE ENCAPSULATION SYSTEM

FIELD OF THE INVENTION

The invention relates to an encapsulation system comprising alginate biocapsules for the immunoisolation of living cells or therapeutics. Specifically, although by no means exclusively, the encapsulation system is for use in allo- and xeno- transplantation. The invention is also directed to methods of making and using the encapsulation system.

BACKGROUND OF THE INVENTION

Clinical therapies involving cell transplantation are increasingly successful. One iteration of cell transplantation takes advantage of developments in material science, cell biology, and drug delivery to develop micro- and macro-encapsulated cell therapy platforms. These include 2-D and 3-D tissue engineered conformations composed of nonerodible thermoplastic polymers, bioerodible materials, and hybrid combinations. These constructs allow for the controlled delivery of therapeutic molecules for the treatment of acute and chronic diseases, but their widespread use is precluded by the need for frequent administration for erodible materials, and retrieval and chronic biocompatibility issues for nondegradable materials. In the case of biodegradable materials, the success of encapsulated cell therapy will depend to a large degree on an understanding of the stability of the material once transplanted and ultimately how that stability impacts the ability of the graft to support cell survival, protein secretion and diffusion, immunoisolation, biocompatibility, physical placement and fixation, degradation, and the efficacy and pharmacodynamics of the secreted product. One of the most common materials used for such biocapsules for cell therapy is alginate, a bioerodible carbohydrate.

Alginate has long been studied as a biomaterial in a wide range of physiologic and therapeutic applications. Its potential as a biocompatible implant material was first explored in 1964 in the surgical role of artificially expanding plasma volume (1). More than a decade later, the matrix capability of alginate for cell support was realized in vitro in a series of experiments that demonstrated microbial cell survival for 23 days (2). Over the last twenty years, there has been considerable progress in alginate cell microencapsulation for the treatment of diabetes (3-10), chronic pain (11), hemophilia (12; 13), central nervous system (CNS) disorders (14-24), and others. Despite success in numerous animal models and in limited clinical allotransplantation, existing encapsulation systems have been hampered by variable degradation kinetics impacting diffusion, immunoisolation, stability, biocompatibility, and ultimately leading to loss of graft survival and rejection. The general understanding of the biocompatibility and

stability of alginate-polycation capsules *in vivo* is limited, and this in turn limits their use. Accordingly, there is a need to better understand and control certain aspects of alginate degradation and alginate biocompatibility *in vivo*.

It is an object of the present invention to go some way towards improving the stability and biocompatibility of alginate biocapsules, for example to produce more stable or more biocompatible microcapsules for *in vivo* applications, and/or to provide the public with a useful choice.

Other objects of the invention may become apparent from the following description which is given by way of example only.

SUMMARY OF THE INVENTION

The invention is directed to a biocompatible composition comprising alginate and one or more neutralized polycations for producing microcapsules. Such microcapsules may be produced by standard methods following the teachings of the present specification. The composition of the present invention is advantageous over known compositions as it can be used to produce microcapsules that are more biocompatible than known microcapsules and thus may allow for prolonged protection from the host immune system when otherwise immunogenic agents, such as allogenic or xenogenic cells, are encapsulated. This is demonstrated herein, whereby a decreased rate of degradation *in vivo* and increased function was observed for microcapsules composed of the composition of the present invention. The microcapsules exhibit enhanced biocompatibility and may be administered to sites which, previously, were inaccessible or undesirable, for example hyperinflammatory sites, as set out below.

In a first aspect, the invention provides a composition comprising alginate and one or more polycations, such as poly-L-ornithine, wherein the polycation comprises one or more amine groups covalently bound to a neutralizing agent or blocking group.

In a second aspect, the invention provides biocompatible microcapsules comprising a core layer of alginate cross-linked with a cationic cross-linking agent, and an outer layer of one or more polycations forming a semi-permeable membrane, wherein at least one of the one or more polycations comprises one or more amine groups covalently bound to a blocking group.

In a third aspect, the present invention comprises a method for preparing biocompatible microcapsules comprising the steps:

a. dissolving alginate in isotonic saline to a concentration of between about 0.5% w/v to 2.5% w/v;
b. spraying the dissolved alginate solution of step a) through an air- or frequency-based droplet generator into a stirring solution of an excess of a cross-linking agent to form gelled microcapsules;
c. coating the gelled microcapsules of step b) with one or more polycations comprising one or more amine groups;
d. contacting the microcapsules with a solution comprising one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine;
e. collecting the one or more microcapsules.

In a fourth aspect, the present invention comprises a method of preparing microencapsulated cells comprising the steps:

a. incubating living cells in a solution of alginate dissolved in isotonic saline to a concentration of between about 0.5% w/v and 2.5% w/v;
b. spraying the cell-containing alginate solution of step c) through an air- or frequency-based droplet generator into a stirring solution of an excess of a cross-linking agent to form one or more gelled cell-containing microcapsules;
c. coating the one or more gelled cell-containing microcapsules of step b) with one or more polycations comprising one or more amines;
d. contacting the microcapsules with a solution comprising one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine; and
e. collecting the one or more cell-containing microcapsules to provide the microencapsulated cells.

In a fifth aspect, the present invention comprises a method for coating a non-degradable cell delivery construct comprising the steps:

a. immerising the non-degradable cell delivery construct in a solution of alginate dissolved in isotonic saline to a concentration of between 1.0% w/v to 2.0% w/v;
b. incubating the construct of step a) in a solution containing an excess of a cross-linking agent to form a gelled coating;
c. further coating the gelled construct of step b) with one or more polycations comprising one or more amines;

d. contacting the gelled construct with a solution comprising one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine to produce an immunoisolatory membrane coated non-degradable cell delivery construct; and
e. recovering the immunoisolatory membrane coated non-degradable cell delivery construct.

In a sixth aspect, the invention provides a method for encapsulating one or more small molecules, proteins or DNA therapeutics comprising the steps

a. incubating the one or more small molecules, proteins or DNA therapeutics in a solution of alginate dissolved in isotonic saline to a concentration of between about 0.5% w/v and 2.5% w/v;
b. spraying the cell-containing alginate solution of step c) through an air- or frequency-based droplet generator into a stirring solution of an excess of a cross-linking agent to form one or more microcapsules containing the small molecules, proteins or DNA therapeutics;
c. coating the one or more gelled microcapsules of step b) with one or more polycations comprising one or more amines;
d. contacting the microcapsules with a solution comprising one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine; and
e. collecting the one or more microcapsules to provide the microencapsulated small molecules, proteins or DNA therapeutics.

In a further aspect, the invention provides a use of alginate and one or more polycations comprising one or more amines covalently bound to a blocking group in the manufacture of a microcapsule preparation for use in the methods of the invention, including use in allo- or xeno- transplantation applications and use in the treatment or prevention of one or more diseases or conditions as herein described.

In a further aspect, the invention provides a method of treating or preventing a disease or condition in a subject comprising transplanting an effective amount of a cell-containing microcapsule of any one of the preceding claims into said subject, when one or more of the cells present in the microcapsule secrete a therapeutic that is effective at treating or preventing said disease or condition.

In still a further aspect, the invention provides a use of biocompatible microcapsules of the invention in the manufacture of a medicament for use in the methods of the

invention, including use in allo- or xeno- transplantation applications and use in the treatment or prevention of one or more diseases or conditions as herein described.

A method of treating or preventing a disease or condition in a subject comprising transplanting an effective amount of a therapeutic-containing microcapsules as claimed in any one of the preceding claims in the said subject, when said therapeutic is effective at treating or preventing said disease or condition.

Accordingly, the microcapsule preparations of the invention may be administered to a subject.

The following embodiments are applicable to any of the aspects described herein.

In certain embodiments, the one or more amine groups of the one or more polycations are primary amines.

In certain embodiments of the preparative methods above, step c) comprises contacting the one or more microcapsules, constructs or cells with more than one polycation, or with more than one solution of one or more polycations. In various embodiments step c) is repeated with more than one polycation or more than one solution of polycation(s), or both. In certain embodiments, the repeated contacting of step c) is performed under the same or different conditions, including differing duration, temperature, or pressure.

In various embodiments, the one or more polycations are provided as a solution or in more than one solution. In one example, the one or more polycations are provided as a solution comprising a first concentration of one or more polycations. In another example, the one or more polycations are provided as more than one solution of one or more polycations, wherein the more than one solution of one or more polycations differ in the identity, concentration, amount, or composition of the one or more polycations present. For example, the one or more polycations are provided as a solution comprising a first concentration of one or more polycations, and a second solution comprising a different concentration of the one or more polycations.

In certain embodiments, the polycations are the same, or are different.

In certain embodiments, the microcapsules further comprise living cells, for example within the core layer or within the microcapsule lumen. In certain embodiments, the cells comprise naturally occurring cells, and in other embodiments are genetically engineered cells. In certain embodiments, the cells are in the form of single cells or cell clusters. Particularly contemplated exemplary cells are selected from the group comprising β islet cells, hepatocytes, neuronal cells such as choroid plexus cells, pituitary cells, chromaffin

cells, chondrocytes, and any other cell type capable of secreting factors that would be useful in the treatment of a disease or condition.

In certain embodiments, the polycation, for example poly-L- ornithine, is present in the composition in a relatively purified form, for example whereby the range of molecular weight species is limited and the polydispersity index (ie average MW ÷ median MW) is low, for example, less than 1.5, for example less than 1.2, for example less than 1.1.

In certain embodiments, the polycation, for example poly-L- ornithine, is present in the composition in a relatively purified form, for example whereby the range of molecular weight species is limited and the polydispersity index (ie average MW ÷ median MW) is low, for example, less than 1.5, less than 1.2, or less than 1.1.

In one embodiment the composition or microcapsule additionally comprises one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine.

In one embodiment, the one or more amine groups are primary amines.

In one embodiment the composition or microcapsule is the polycation poly-L-ornithine.

In a further embodiment the polycation is poly-L-ornithine having an average molecular weight of between about 10 – 100 KDa.

In one embodiment the average molecular weight of the poly-L-ornithine is between about 15 and 30 KDa.

In another embodiment the average molecular weight of the poly-L-ornithine is between 20 and 25 KDa.

In a further embodiment the average molecular weight of the poly-L-ornithine is between 5 and 15 KDa.

In one embodiment the polycation contains less than 20% of a molecular weight species of 10 KDa or less.

In a further embodiment the polycation contains less than 20% of a molecular weight species of 100 KDa or more.

In one embodiment more than about 60% of the amine groups of the one or more polycations are covalently bound to a blocking group.

In another embodiment more than about 90% of the amine groups of the one or more polycations are covalently bound to a blocking group.

In one embodiment more than about 99% of the amine groups of the one or more polycations are covalently bound to a blocking group.

In one embodiment the blocking group acylates one or more of the amines to form a non-reversible acetamide modification.

In another embodiment blocking group is provided by or the composition or microcapsule comprises an N-hydroxysulfosuccinimide ester.

In one embodiment the N-hydroxysulfosuccinimide ester is Sulfo-N-hydroxysulfosuccinimide acetate.

In various embodiments the blocking group is or is provided by or the composition or microcapsule comprises a polyethylene glycol.

In various embodiments the polyethylene glycol is selected from the group comprising bis(succinimidyl) ethylene glycols, succinimidyl (N-methyl) ethyleneglycol-esters, mono-functional linear polyethylene glycols, NHS active PEG esters, NHS carbonate PEGs, and branched methyl PEG NHS esters.

In one embodiment the bis(succinimidyl) ethylene glycol has the formula BS(PEG)n, where n = 3 – 60.

In one embodiment the bis(succinimidyl) ethylene glycol has the formula BS(PEG)n, where n = 6 – 30.

In one embodiment the bis(succinimidyl) ethylene glycol is BS(PEG)9.

In one embodiment the succinimidyl (N-methyl) ethyleneglycol-esters has the formula MS(PEG)n, where n = 3 – 60.

In one embodiment the succinimidyl (N-methyl) ethyleneglycol-esters has the formula MS(PEG)n, where n = 6 – 30.

In one embodiment the succinimidyl (N-methyl) ethyleneglycol-esters is MS(PEG)12.

In one embodiment the ratio of alginate to polycation is from about 5:1 to about 50:1.

In one embodiment the alginate has an average molecule weight of greater than about 400 KDa.

In one embodiment the alginate has an average molecular weight of greater than about 600KDa.

In one embodiment, the microcapsule comprises living cells within the core layer.

In one embodiment, the microcapsule comprises living cells within the microcapsule lumen.

In one embodiment, the cells encapsulated by the microcapsule are selected from naturally occurring and genetically altered cells.

In one embodiment the cells are present as single cells and/or cell clusters selected from the group consisting of β islet cells, hepatocytes, neuronal cells and any other cell type capable of secreting factors useful in the treatment of a disease or condition.

In one embodiment the neuronal cells are selected from the group comprising choroid plexus cells, pituitary cells, chromafin cells and chondrocytes.

In one embodiment the cross-linking agent is selected from salts of the group consisting of Ag+, Al3+, Ba2+, Ca2+, Cd2+, Cu2+, Fe2+, Fe3+, H+, K+, Li+, Mg2+, Mn2+, Na+, NH4+, Ni2+, Pb2+, Sn2+ and Zn2+.

In one embodiment the cross-linking agent is calcium chloride.

In one embodiment the outer layer of the microcapsule is between about 10 and about 80 microns in thickness.

In one embodiment the core layer of the microcapsule is depolymerised by a chelation agent to form a hollow lumen.

In one embodiment the chelation agent is selected from sodium citrate and EDTA.

In one embodiment the ratio of the core layer to the outer layer of the microcapsule is about 5:1 to about 50:1 by weight.

In one embodiment the ratio of the core layer to the outer layer of the microcapsule is about 10:1 to about 20:1 by weight.

In one embodiment the diameter of between 50 and 2000 microns.

In one embodiment the composition comprises less than about 1% calcium chloride and/or sodium chloride.

In one embodiment step b) of the method comprises stirring in about 15mM to about 120mM calcium chloride for between about 5 to about 30 minutes.

In one embodiment step c) comprises coating the capsules with poly-L-ornithine at a concentration of between about 0.02% to about 0.10% (w/v) for between about 1 to about 45 minutes.

In one embodiment step c) comprises coating the capsules with poly-L-ornithine at a concentration of about 0.05% (w/v) for about 10 minutes.

In one embodiment the blocking agent of step d) is selected from the group comprising blocking agents that acylate one or more of the amines to form a non-reversible acetamide modification, and polyethylene glycols.

In one embodiment step d) comprises contacting the one or more microcapsules with about 1mM to about 100mM of a blocking agent selected from the group comprising blocking agents that acylate one or more of the amines to form a non-reversible acetamide modification, and polyethylene glycols.

In one embodiment step d) comprises contacting the one or more microcapsules with about 1mM to about 100mM Sulfo-N-hydroxysulfosuccinimide acetate.

In one embodiment step e) comprises contacting the one or more microcapsules with about 0.5mM to about 100mM BS(PEG)9 or MS(PEG)12.

In one embodiment the disease or condition is Type I diabetes mellitus.

In one embodiment the one or more of the cells are islet β cells.

In one embodiment the cells comprise hepatocytes and said disease or condition is a disease or disorder of the liver.

In one embodiment the cells comprise neuronal cells selected from the group consisting of choroids plexus cells, pituitary cells, chromafin cells, chondrocytes and any other neuronal cell capable of secreting neuronal factors, and the disease or condition is a neurological disease or condition.

In one embodiment the polycation is selected from the group consisting of chitosan, chitosan glutamate, chitosan glycol, modified dextran, poly-L-lysine, poly-L-ornithine, salmine sulfate, protamine sulfate, polyacrylimide, polyacrylimide-co-methacryloxyethyltrimethylammonium bromide, polyallylamine, polyamide, polyamine, polybrene, Polybutylacrylate-co-Methacryloxyethyl Trimethylammonium Bromide

(80/20), Poly-3-chloro-2-hydroxypropylmethacryl-oxyethyl dimethylammonium Chloride, Polydiallyldimethylammonium, Polydiallyldimethylammonium Chloride, Polydiallyldimethylammonium Chloride-co-Acrylamide, Polydiallyldimethylammonium Chloride-co-N-Isopropyl Acrylamide, Polydimethylamine-co-epichlorohydrin, Polydimethylaminoethylacrylate-co- Acrylamide, Polydimethylaminoethylmethacrylate, Polydimethylaminoethyl Methacrylate, Polyethyleneimine, Polyethyleneimine-Epichlorohydrin Modified, Polyethyleneimine, Poly-2-hydroxy-3-methacryloxypropyl Trimethylammonium Chloride, Poly-2-hydroxy-3-methacryloxyethyl, Trimethylammonium Chloride, Polyhdroxyproplymethacryloxy Ethyldimethyl Ammonium Chloride, Polyimadazoline (Quaternary), Poly-2-methacryloxyethyltrimethylammonium Bromide, Polymethacryloxyethyltrimethylammonium Bromide/Chloride, Polymethyldiethylaminoethylmethacrylate-co-Acrylamide, Poly-l-methyl-2-vinylpyridinium Bromide, Poly-l-methyl-4-vinylpyridinium Bromide, Polymethylene-co-Guanidine Hydrochloride, Polyvinylamine, Poly-N-vinylpyrrolidone-co-Dimethylaminoelhyl-Methacrylate, Poly-4-vinylbenzyltrimethylammonium Chloride, Poly-4-vinylbenzyltrimethylammonium Chloride, PEI-g-Chitosan, cationic gelatin, cationic cellulose, collagen, cationic cyclodextrin, poly(aminoamines), poly(amino-co-ester), PEG HCl derivatives including 0-2(-aminoethyl)-o-2(succunylamino) ethyl) PEG HCl, o-(2-aminoethyl)-O-(2-carboxyethyl) PEG HCl, and modified PEG including O-(2-aminoethyl)PEG.

It is intended that reference to a range of numbers disclosed herein (for example, 1 to 10) also incorporates reference to all rational numbers within that range (for example, 1, 1.1, 2, 3, 3.9, 4, 5, 6, 6.5, 7, 8, 9 and 10) and also any range of rational numbers within that range (for example, 2 to 8, 1.5 to 5.5 and 3.1 to 4.7) and, therefore, all sub-ranges of all ranges expressly disclosed herein are hereby expressly disclosed. These are only examples of what is specifically intended and all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application in a similar manner.

In this specification where reference has been made to patent specifications, other external documents, or other sources of information, this is generally for the purpose of providing a context for discussing the features of the invention. Unless specifically stated otherwise, reference to such external documents is not to be construed as an admission that such documents, or such sources of information, in any jurisdiction, are prior art, or form part of the common general knowledge in the art.

This invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively,

and any or all combinations of any two or more of said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

DESCRIPTION OF THE DRAWINGS

The invention will now be described with reference to the figures of the accompanying drawings in which:

Figure 1 depicts light microscopy and fluorescent microscopy images of encapsulated porcine pancreatic islet cells after storage at 37°C for 1 month in RPMI-CPM media. A) APA capsule, B) Sulfo-NHS acetate treated APA capsule.

Figure 2 shows the QCM-D analysis of alginate/poly-L-ornithine layer build-up and the covalent attachment of sulfo-NHS acetate.

Figure 3 depicts confocal microscopy images of AP capsules covalently modified with FITC-PEG-NHS.

Figure 4 shows the QCM-D analysis demonstrating layer-by-layer deposition of alginate and poly-O-ornithine layers and subsequent covalent attachment of MS-PEG_{12}.

Figure 5 depicts fibrotic APA capsules post *in vivo* retrieval from CD1 mice.

Figure 6 depicts light microscopy images of free floating capsules taken post retrieval from the peritoneum of CD1 mice. a) APA capsules and b) $BS(peg)_9$ modified capsules and c) capsules treated with 10 mM sulfo-NHS acetate.

Figure 7 depicts histological sections of fat tissue retrieved from the peritoneum of mice implanted with modified APA capsules. A) AP capsules, B) AP capsules Treated with 5 mM BS-PEG, C) capsules treated with 10 mM sulfo-NHS acetate D) AP capsules treated with 5mM MS-PEG.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to an encapsulation system for living cells and therapeutics which has improved biocompatibility when the encapsulated cells and therapeutics are implanted into a subject. This improved biocompatibility enables the encapsulated cells and therapeutics to remain within a living body and to retain viability and function for longer periods than is currently the case, in turn leading to improved therapeutic delivery and thus treatment efficacy.

As used herein the term "and/or" means "and" or "or", or both.

As used herein "(s)" following a noun means the plural and/or singular forms of the noun.

The term "comprising" as used in this specification means "consisting at least in part of". When interpreting statements in this specification which include that term, the features, prefaced by that term in each statement or claim, all need to be present but other features can also be present. Related terms such as "comprise" and "comprised" are to be interpreted in the same manner.

Alginate

Alginate is a polysaccharide composed of glucuronic (G) and mannuronic (M) acid linked by (1,4)-α- and -β-glycoside bonds. The ratio of these monomers contributes to certain physical characteristics of the polysaccharide. It has been reported that once cationically crosslinked, alginates high in G, due to a more networked structure resulting from α(1-4) bonds, are more brittle with a higher elastic modulus, while those that are high in M, with more linear β(1-4) linkages, exhibit decreased 3-D crosslinking and greater elasticity and form stable microcapsules when tested in vivo.

Thus, in certain embodiments the present invention provides a composition comprising an alginate having more than 50% mannuronic acid residues, and a polycation, such as poly-L-ornithine, comprising one or more amine groups covalently bound to a blocking group. In one embodiment, the alginate and the polycation are in a ratio of approximately 5:1 to 50:1 by weight, for example about 10:1 to 40:1 by weight or about 10:1 to about 20:1 by weight. In certain examples, the composition of the present invention additionally includes a source of chloride ions, for example calcium chloride or sodium chloride, or both.

In certain examples, the composition comprises alginate at a concentration of about 99% to about 90%, for example about 95%. In certain examples the composition comprises poly-L-ornithine at a concentration of about 1% to about 10%, for example about 5%. In certain examples, the composition additionally includes calcium chloride at a concentration of less than about 1% and sodium chloride at a concentration of less than about 1%.

In one embodiment, the average molecular weight of the alginate is greater than about 400 KDa, for example greater than about 600 KDa.

For application in the present invention, the alginate source is purified, ideally containing less than 1 endotoxin unit/ml of 1.8% (w/v) alginate. Examples of commercially available alginates suitable for use in the present invention include Keltone LVCR and Pronova SLM20. However, any other alginate with suitable high mannuronic acid content (or suitable M:G ratios) can be used as a raw material for use in the present invention.

In one embodiment, the alginate has a pH of 7.0 ± 0.4 when dissolved in saline at a concentration of 1.8% (w/v).

Polycations

The nature of the polycation, for example, its molecular weight, the amount or distribution of amines present in the polycation, the presence of solely primary amines versus the presence of secondary amines, is also important to the biocompatibility and stability of microcapsules of the invention. The applicants have established for the first time that microcapsules comprising one or more polycations wherein one or more of the amine groups present in the polycations, for example one or more primary amines present in the polycations, are blocked by a blocking group have enhanced biocompatibility and stability, and enhanced retention of immunoisolatory function.

Methods and agents suitable for covalently binding a blocking group to the one or more amines of the one or more polycations will be evident to a person skilled in the art on reading the instant specification. Examples of such methods are presented herein.

In certain embodiments, greater than about 60% of the amines of the one or more polycations are covalently bound to a blocking group. For example, greater than about 70%, greater than about 80%, greater than about 90%, greater than about 95%, or substantially all of the amines of the one or more polycations are covalently bound to a blocking group.

In certain embodiments, the variability of molecular weight of polycation species is minimized, such that polycations having a polydispersity index of less than about 1.5, for example less than about 1.2 or less than 1.1, can result in superior microcapsules which are highly stable and can remain *in vivo* for long periods of time, and certainly for more than one month.

Polycatonic agents comprising a high polydispersity index and therefore including a wide range of MW species have been reported to result in inferior microcapsules. This has

been proposed to be caused by the larger MW molecules being unable to diffuse into the alginate coat, resulting in a weak coating, while the smaller MW molecules on the other hand, can diffuse too rapidly into the alginate coating and can penetrate into the core, disrupting the function of or displacing therapeutic agents present within the core or microcapsule lumen, such as living cells. Thus, in particular embodiments of the invention, one or more polycations with a limited range of MW species are specifically contemplated.

For example, when the polycation is poly-L-ornithine, or poly-L-lysine, exemplary average MW for the polycation is from between 10 to 40 KDa, for example between 15 to 30 KDa, including around 20-25 KDa. Certainly polycation preparations having other ranges of MW are contemplated in the present invention, but such polycation preparations will desirably have low polydispersity.

In certain embodiments, the polycation, for example the poly-L-lysine or poly-L-ornithine, will contain less than about 20% of molecules having a MW of 10 KDa or less, for example less than about 10% of molecules having a MW of 10 KDa or less.

The invention further provides biocompatible microcapsules prepared using the composition of the invention, and comprising a core layer of alginate cross-linked with a cationic cross-linking agent, and an outer layer of one or more polycations comprising one or more amines covalently bound to a blocking group.

In certain embodiments, the alginate comprises from more than about 50% mannuronic acid residues, for example from about 60% to about 70% mannuronic acid residues.

In certain embodiments, the cationic cross-linking agent is selected from salts of the group consisting of Ag^{+}, Al^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, H^{+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, NH^{4+}, Ni^{2+}, Pb^{2+}, Sn^{2+} and Zn^{2+}. In certain examples, the cationic cross-linking agent is calcium chloride. The cross-linking agent is generally in excess, for example from 15mM to 120mM calcium chloride, such as 110mM calcium chloride.

In certain embodiments, the polycationic agent is selected from the group consisting of chitosan, chitosan glutamate, chitosan glycol, modified dextran, poly-L-lysine, poly-L-ornithine, salmine sulfate, protamine sulfate, polyacrylimide, polyacrylimide-co-methacryloxyethyltrimethylammonium bromide, polyallylamine, polyamide, polyamine, polybrene, Polybutylacrylate-co-Methacryloxyethyl Trimethylammonium Bromide (80/20), Poly-3-chloro-2-hydroxypropylmethacryl-oxyethyl dimethylammonium Chloride, Polydiallyldimethylammonium, Polydiallyldimethylammonium Chloride,

Polydiallyldimethylammonium Chloride-co-Acrylamide, Polydiallyldimethylammonium Chloride-co-N-Isopropyl Acrylamide, Polydimethylamine-co-epichlorohydrin, Polydimethylaminoethylacrylate-co- Acrylamide, Polydimethylaminoethylmethacrylate, Polydimethylaminoethyl Methacrylate, Polyethyleneimine, Polyethyleneimine-Epichlorohydrin Modified, Polyethyleneimine, Poly-2-hydroxy-3-methacryloxypropyl Trimethylammonium Chloride, Poly-2-hydroxy-3-methacryloxyethyl, Trimethylammonium Chloride, Polyhdroxyproplymethacryloxy Ethyldimethyl Ammonium Chloride, Polyimadazoline (Quaternary), Poly-2-methacryloxyethyltrimethylammonium Bromide, Polymethacryloxyethyltrimethylammonium Bromide/Chloride, Polymethyldiethylaminoethylmethacrylate-co-Acrylamide, Poly-l-methyl-2-vinylpyridinium Bromide, Poly-l-methyl-4-vinylpyridinium Bromide, Polymethylene-co-Guanidine Hydrochloride, Polyvinylamine, Poly-N-vinylpyrrolidone-co-Dimethylaminoelhyl-Methacrylate, Poly-4-vinylbenzyltrimethylammonium Chloride, Poly-4-vinylbenzyltrimethylammonium Chloride, PEI-g-Chitosan, cationic gelatin, cationic cellulose, collagen, cationic cyclodextrin, poly(aminoamines), poly(amino-co-ester), PEG HCl derivatives including 0-2(-aminoethyl)-o-2(succunylamino) ethyl) PEG HCl, o-(2-aminoethyl)-O-(2-carboxyethyl) PEG HCl, and modified PEG including O-(2-aminoethyl)PEG.

In particularly contemplated examples, the polycationic agent is poly-L-ornithine, for example poly-L-ornithine at a concentration of between 0.02% and 0.2%w/v, including for example a concentration of about 0.05% to about 0.1%w/v.

In certain embodiments, poly-L-ornithine is advantageously purified to remove the higher and/or lower MW species to give a polydispersity index of less than 1.2, for example less than 1.1. Specifically, in exemplary embodiments the average MW for the poly-L-ornithine polycationic agent is from between 10 to 40 KDa, for example between 15 and 30 KDa, including around 20 to 25 KDa. Such ranges can be achieved by removing any molecules having a molecular weight below 10 KDa and above 40 KDa, for example by dialysis or other known methods. In specifically contemplated examples, the poly-L-ornithine used in the present invention comprises less than about 20% of molecules having a MW of 10 KDa or less, for example less than 10% of molecules having a MW of 10 KDa or less.

In various embodiments, the outer layer, which is formed of polycations around the core layer, comprise a semi-permeable membrane of between about 10 and about 80 μm in thickness.

In certain embodiments the alginate of the core layer is solid, while in other embodiments the core is depolymerised by a chelation agent to form a lumen or hollow core. Examples of suitable chelation agents are sodium citrate and EDTA.

Previously it has been thought that chelation of the alginate (degelling) core solubilises the internal structural support of the capsule, thereby adversely affecting the durability of the microcapsule. This problem has been avoided in the prior art by not carrying out the chelation step so that the core is solid (see US 6,365,385, for example). However, the use of the compositons and methods of the present invention increases the durability of the microcapsules even when the core is hollowed to form a lumen, for example by chelation. Nevertheless, in certain embodiments the microcapsules of the present invention have a solid core for further enhanced stability and durability.

In certain embodiments, the ratio of the core layer (of alginate) to the outer layer (of polycation) is 5:1 to about 50:1 by weight, for example about 10:1 to about 20:1 by weight.

It will be appreciated that using the compositions and methods of the present invention, the surface of the microcapsule when formed has an ionically neutral surface.

In certain embodiments, the microcapsules further comprise living cells within the core layer. In certain embodiments, the cells comprise naturally occurring or genetically engineered cells, for example in the form of single cells and/or cell clusters selected from the group consisting of β islet cells, hepatocytes, neuronal cells such as choroid plexus cells, pituitary cells, chromafin cells, chondrocytes and any other cell type capable of secreting factors that would be useful in the treatment of a disease or condition.

For example, the cells are islet cells capable of secretory insulin useful for the treatment of diabetes.

In certain embodiments, the cells comprise hepatocyte or non-hepatocyte cells capable of secreting liver secretory factors useful in the treatment of liver diseases or disorders.

In certain embodiments, the cells comprise neuronal cells, such as choroids plexus, pituitary cells, chromoffin cells, chondrocytes and any other cell capable of secreting neuronal factors useful in the treatment of neuronal diseases such as Parkinson's disease, Alzheimer's disease, epilepsy, Huntington's disease, stroke, motor neurone disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, aging, vascular disease, Menkes Kinky Hair Syndrome, Wilson's disease, trauma or injury to the nervous system.

In certain embodiments, the microcapsules of the present invention are between 50 and 2000 microns in diameter. In certain embodiments the microcapsules are between about 100 and 1000 microns in diameter, for example between about 500 and 700 microns in diameter. Examples of such microcapsules are presented herein.

It is expected that the microcapsules of the present invention will be able to remain functional in vivo in a subject for a significant period of time and certainly in light of the experimental support presented herein in the Examples, for periods greater than one month.

The functional duration of the microcapsules can be controlled by one or more of the following methods:

by varying the polydispersity of the alginate range used in the core layer of the microcapsule;

by varying the total protein content of the core alginate layer;

by inducing calcification of the alginate layer;

by varying the degree of covalent bonding of blocking group to the one or more amines (particularly primary amines) of the one or more polycations,

by varying the range and distribution of molecular weight of the one or more polycations;

by varying the uniformity of the polycation concentration, creating a gradient across the intermediate layer of the microcapsule.

The present invention further provides a method for preparing the biocompatible microcapsules of the invention comprising the steps:

a. incubating living cells in a solution of alginate dissolved in isotonic saline to a concentration of between about 0.5% w/v and 2.5% w/v;
b. spraying the cell-containing alginate solution of step c) through an air- or frequency-based droplet generator into a stirring solution of an excess of a cross-linking agent to form one or more gelled cell-containing microcapsules;
c. coating the one or more gelled cell-containing microcapsules of step b) with one or more polycations comprising one or more amines;
d. contacting the microcapsules with a solution comprising one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine; and

e. collecting the one or more cell-containing microcapsules to provide the microencapsulated cells.

The present invention further provides a method of preparing microencapsulated cells comprising the steps:

a. incubating living cells in a solution of alginate dissolved in isotonic saline to a concentration of between about 0.5% w/v and 2.5% w/v;
b. spraying the cell-containing alginate solution of step c) through an air- or frequency-based droplet generator into a stirring solution of an excess of a cross-linking agent to form one or more gelled cell-containing microcapsules;
c. coating the one or more gelled cell-containing microcapsules of step b) with one or more polycations comprising one or more amines;
d. contacting the microcapsules with a solution comprising one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine; and
e. collecting the one or more cell-containing microcapsules to provide the microencapsulated cells.

The alginate solution of step a) comprises an alginate concentration of about 1.0% to 2.0% w/v.

The alginate solution of step d) comprises an alginate concentration of about 0.01 to 1.7% w/v.

The cells may be naturally occurring or genetically engineered cells which may be in the form of single cells and/or cell clusters selected from the group comprising of β islet cells, hepatocytes, neuronal cells such as choroid plexus cells, pituitary cells, chromaffin cells, chondrocytes and any other cell type capable of secreting factors that would be useful in the treatment of a disease or condition.

In certain embodiments, the cells are isolated from the same species as a recipient host, for use in allo-transplantation, while in other embodiments, the cells are from a different species, for use in xeno-transplantation.

In certain embodiments, the cells are contained within the core alginate layer, but can alternatively or additionally be contained within the outer layer.

In certain embodiments, the non-degradable cell delivery construct is selected from the group consisting of hollow-fiber membrane devices, flat sheets, porous scaffolds for cell ingrowth and other novel scaffolding constructs, as would be appreciated by a skilled worker.

In certain embodiments, the non-degradable cell delivery construct comprises living cells, for example naturally occurring or genetically engineered cells in the form of single cells and/or cell clusters, including cells or cell clusters selected from β islet cells, hepatocytes, neuronal cells such as choroids plexus cells, pituatary cells, chromaffin cells, chondrocytes and any other cell type capable of secreting factors that would be useful in the treatment of a disease or condition.

The invention further provides a method for encapsulating small molecule, protein or DNA therapeutics comprising the steps

a. incubating the one or more small molecules, proteins or DNA therapeutics in a solution of alginate dissolved in isotonic saline to a concentration of between about 0.5% w/v and 2.5% w/v;
b. spraying the cell-containing alginate solution of step c) through an air- or frequency-based droplet generator into a stirring solution of an excess of a cross-linking agent to form one or more microcapsules containing the small molecules, proteins or DNA therapeutics;
c. coating the one or more gelled microcapsules of step b) with one or more polycations comprising one or more amines;
d. contacting the microcapsules with a solution comprising one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine; and
e. collecting the one or more microcapsules to provide the microencapsulated small molecules, proteins or DNA therapeutics.

In certain embodiments, the small molecule, protein or DNA therapeutic is in the microcapsule lumen, or is contained within the core alginate layer. Alternatively, the small molecule, protein or DNA therapeutic is contained within the (polycationic) outer layer. In such embodiments, it will be appreciated that exposure at the surface of the microcapsule may be desirable in certain circumstances, bearing in mind the general desirability for complete encapsulation of immunogenic agents.

Examples of suitable protein therapeutics include erythropoietin, insulin, CNTF, BDNF, GDNF, GH, and others, as would be appreciated by a skilled worker.

The invention further provides a method of treating or preventing a disease or condition in an animal, including a human, comprising transplanting an effective amount of the cell-containing microcapsules of the invention into said animal, wherein said cells secrete a therapeutic that is effective at treating or preventing said disease or condition.

The invention further provides a method of treating or preventing a disease or condition in an animal, including a human, comprising transplanting an effective amount of the cell-containing immunoisolatory membrane coated non-degradable cell delivery construct of the invention into said animal, wherein said cells secrete a therapeutic that is effective at treating or preventing said disease or condition.

The invention further provides a method of treating or preventing a disease or condition in an animal, including a human, comprising transplanting an effective amount of the therapeutic-containing microcapsules of the invention into said animal, wherein said therapeutic is effective at treating or preventing said disease or condition.

In these methods of treatment, the microcapsules or coated delivery constructs of the invention may be administered in an amount that would deliver sufficient therapeutic so as to be effective against the disease. For example, in the treatment of diabetes, a single mL of microcapsules would contain approximately 10,000-60,000 β islet equivalents and approximately 1-10 mL microcapsules would be implanted per kg body weight into a subject to secrete the required amount of insulin to control blood glucose levels.

A skilled practitioner would be able to test the secretion rate of the particular therapeutic from the microcapsules in vitro and, for any particular patient need, be able to calculate how many microcapsules would be required to treat that particular patient effectively.

The microcapsules of the invention may be formulated for allo- or xeno- transplantation depending on the source of the living cells and/or therapeutics.

In certain embodiments, porcine cells, such as neonatal porcine cells, are specifically contemplated. In particular, neonatal porcine β islet cells are specifically contemplated for the treatment of Type I diabetes mellitus.

The microcapsules of the invention may be transplanted within the tissues of the body or within fluid-filled spaces of the body, which ever is the most appropriate in terms of accessibility and efficacy. For example, if the living cells within the microcapsules are β

islet cells, they may be transplanted in the peritoneal cavity. If the living cells with the microcapsules are choroid plexus cells and are for treating neurological disorders and any therapeutic agent secreted by the cells must be in contact with the cerebro spinal fluid surrounding the brain, such microcapsules may be implanted into or onto the brain.

Alternatively, the microcapsules may be formulated for oral or topical administration, particularly when they contain a therapeutic bioactive agent, such as an antibiotic.

The invention provides a use of an alginate containing between about 50 and about 95% mannuronic acid residues and a polycation in the manufacture of a microcapsule preparation for use in allo- or xeno- transplantation applications.

Such microcapsules may comprise living cells comprising naturally occurring or genetically or genetically engineered cells which may be in he form of single cells and/or cell clusters selected from the group comprising of β islet cells, hepatocytes, neuronal cells such as choroid plexus cells, pituitary cells, chromaffin cells, chondrocytes and any other cell type capable of secreting factors that would be useful in the treatment of a disease or condition.

Alternatively the microcapsules may comprise a therapeutic agent.

This invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, and any or all combinations of any two or more said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

The invention consists in the foregoing and also envisages constructions of which the following gives examples only.

EXAMPLES

The invention consists in the foregoing and also envisages constructions of which the following gives examples only. The following examples are included to demonstrate particular embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in

the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Introduction

This example explores the biocompatibility of encapsulation systems of the invention and their suitability for encapsulating pancreatic islet cells for implantation.

Methods

APA Capsule Preparation

Alginate (Alg) with a molecular weight of 140-150 kDa was purified as described in a previous publication (25). Porcine pancreatic islets were mixed with 1.8% (w/v) alginate prior to capsule formation and a homogeneous mixture of the two was pumped through a needle supplied with a coaxial airflow. Alginate beads were cross-linked in 200 ml of 109 mM $CaCl_2$ for 5 minutes resulting in the formation of encapsulated islets. The encapsulated islets were subsequently coated with 0.1% poly-L-ornithine (PLO) for 10 minutes, 0.05% PLO for 6 minutes, and 0.18% alginate for 6 minutes. After capsule preparation the alginate core was dissolved using 55 mM isotonic sodium citrate (2 minutes). The samples were washed with saline solution (0.9 M NaCl) between each step and all solutions were filter sterilized before filtering through a 0.2 µm PES filter. Empty capsules were formed using the same method in the absence of cells.

Preparation of AP-Sulfo-NHS-Acetate Capsules

Capsules were prepared as described above but after coating with 0.05% PLO the capsules were washed 3 times with ice-cold PBS to remove any amine-containing culture media and proteins from the cells. Sulfo-NHS acetate (5-10 mM) was prepared in ultra-pure water at room temperature; added to the capsules, and allowed to react for 1 hour at room temperature. After treatment the capsules were washed three times with PBS and then treated with 55 mM sodium citrate for 2 minutes. Following core dissolution, the capsules were once again washed three times with PBS, re-suspended in RPMI-CPN media and stored at 37°C in a 5% CO_2 environment.

Preparation of AP-BS$(PEG)_9$ and AP-MS$(PEG)_{12}$ Capsules

Capsules were prepared as described for APA capsules, but after the 0.05% PLO step, the capsules were washed three times with ice-cold PBS to remove any amine-containing

culture media and proteins from the cells. A 250 mM stock solution of $BS(PEG)_9$ was prepared in DMSO, diluted in concentration range 1 – 5 mM. This was added to the capsules and allowed to react for 30 minutes at room temperature or 4°C. After treatment the capsules were washed three times with PBS and then treated with 55 mM sodium citrate for 2 minutes. Following core dissolution, the capsules were once again washed three times with PBS, re-suspended in RPMI-CPN media and stored at 37°C under a 5% CO_2 environment.

Fluorescein-PEG-NHS

AP capsules were prepared as described above but the final layer of alginate was not applied. A stock solution (83.33 mM) of FITC-PEG-NHS was prepared in DMSO and diluted down to 0.1-0.5 mM in PBS. Capsules were suspended in this and the reaction was allowed to commence for 30 minutes under continuous stirring. The alginate core was dissolved by treatment with 55 mM isotonic sodium citrate and the capsules were washed three times in PBS after each step. Finally, the capsules were re-suspended in RPMI-CPN media and incubated in a 5% CO_2 environment at 37°C.

Microscopy

An Olympus IX51 microscope was used in both visible light and fluorescence modes to image encapsulated islets and empty capsules using 2 x, 4 x and 10 x magnification. Acridine orange (AO) was visualized in the green field (excitation 502 nm, emission 525 nm) and propidium iodide (PI) in the red field (excitation at 590 nm, emission at 620 nm).

Confocal Imaging

Confocal images were captured with a Leica TCS SP2 microscope. Encapsulated islets were washed three times in HBSS buffer. 100 µl of sample was applied to a glass slide fixed with a CoverWell™ imaging chamber gasket (Invitrogen) with dimensions of 20 mm diameter by 1 mm deep. Excess moisture was removed and the sample was submerged in 300 µl of 1% agarose. Once dry, each sample was coated with 100 µl of ProLong® Gold antifade reagent.

Islet Viability

Islet survival was monitored at timed intervals *in vitro* over the course of a month using AO and PI staining for viable cell counting. An aliquot of encapsulated islets was taken from each sample at the designated time points, and treated with AOPI. Islet viability

was assessed using fluorescence microscopy. These tests were all performed to GMP level.

Static Glucose Stimulation Test (SGS)

For assessment of *in vitro* viability, a static incubation assay was used to determine the secretory response of the islets to glucose after encapsulation at timed intervals over the course of a month. The cultured fractions were recovered from the T-flask, washed, and aliquots of 1000-2000 islet equivalents (IEQ) were incubated for 60 minutes in 5 ml of RPMI (minus glucose) medium supplemented with 2 mmol/liter l-glutamine, 0.5% BSA and either 2.8 mmol/liter dextrose, 19.6 mmol/liter dextrose or 19.6 mmol/liter dextrose plus 10 mmol/liter theophylline. Encapsulated cells and medium were then separated by centrifugation and assayed for their respective insulin contents. The insulin content of the medium was expressed as a percentage of the total content. Stimulation indices were calculated by dividing the amount of insulin release at 19.6 mmol/liter dextrose (+theophylline) by that released at 2.8 mmol/liter. All tests were performed to good manufacturing practice (GMP) level and passed strict Quality Control (QC) performance criteria prior to release, including statistical relevance.

Quartz Crystal Microbalance with Dissipation (QCM-D) Analysis of Film Growth

Layer-by-layer (LbL) film build-up for APA, AP-MS$(PEG)_{12}$ and AP-sulfo-NHS acetate films was monitored in situ by QCM-D, using the Q-sense Omega Auto unit (Q-sense, Sweden), which allowed for the simultaneous monitoring of the changes in frequencies (Δf) and energy dissipation (ΔD) after each deposition step. Briefly, the QCM-D method involves the following steps: the crystal is excited at its fundamental frequency (f = 5 MHz) as well as at the third, fifth, and seventh overtones (denoted by v = 3, 5 and 7, which correspond respectively to 15, 25 and 35 MHz). Changes in the resonance frequencies, Δf, are measured once the excitation is stopped at the four frequencies. The apparatus also allows for measurement of the dissipation (D) of the vibrational energy stored in the resonator. For control 2D APA layers, polyelectrolyte films were formed by injecting 200 µl of the polymer solutions into the measuring cell (10 minutes allowed for adsorption of each layer), followed by rinsing with 200 µl of 0.9% NaCl buffer (data not shown). AP-MS$(PEG)_{12}$ films were formed as described for the control but instead of deposition of the final alginate layer, 1mM MS$(PEG)_{12}$ was applied to the surface. Following this, the surface was washed with PBS. AP-Sulfo-NHS acetate 2D films were formed by successive deposition of 1.8% alginate, 0.1% PLO, 0.05% PLO and a final layer of 10mM sulfo-NHS acetate (Figure 7). The steps used to prepare the AP-Sulfo-NHS acetate and AP-MS-PEG_{12} 2D films are listed in Tables 1 and 2, respectively.

***In vivo* studies**

After one week in culture media, empty capsules were transplanted into the peritoneal cavity of 5-9 week old CD1 mice. Before implantation the characteristics and size of the capsules were determined. The abdomen was treated with EMLA cream (topical local analgesic) before implantation of capsules. Each animal was implanted with approximately 500 capsules (approximately 700 IEQ in 500 capsules for islet containing capsules) in 1.0 mL of saline into the peritoneal cavity using a 20G catheter. 3 males and 3 female mice were used for each condition (n=6). All animals were monitored daily and the capsules recovered through peritoneal lavage after euthanasia by CO_2 at 14-16 days post-implantation. After retrieval, the capsules were assessed by microscopy, counted and the degree of fibrosis estimated. Free floating and attached capsules were then mounted in agar blocks, paraffin embedded, subsequently sectioned and stained using hematoxylin and eosin stains. All procedures adhere to the guidelines of the New Zealand National Ethics Council and protocol submissions were formally reviewed and approved by the LCT Animal Ethics Committee.

Table 1: Conditions used to prepare AP-Sulfo-NHS acetate films.

Step #	Pos	Name	Duration	Flow	Volume
1	R1 A1	0.9% NaCl buffer	5 min	20 µl/min	100 µl
2	R1 A2	1.8% alginate	10 min	20 µl/min	200 µl
3	R1 A1	0.9% NaCl buffer	10 min	20 µl/min	200 µl
4	R1 A3	0.1% PLO	10 min	20 µl/min	200 µl
5	R1 A1	0.9% NaCl buffer	10 min	20 µl/min	200 µl
6	R1 A4	0.05% PLO	10 min	20 µl/min	200 µl
7	R1 A1	0.9% NaCl buffer	10 min	20 µl/min	200 µl
8	R1 B4	miliQ	5 min	20 µl/min	100 µl
9	R1 B2	Sulfo-NHS acetate	20 min	20 µl/min	400 µl
10	R1 B4	miliQ	5 min	20 µl/min	100 µl
11	R1 B3	PBS	5 min	20 µl/min	100 µl
12	R1 B1	BSA (100 µg/ml)	10 min	20 µl/min	200 µl
13	R1 B3	PBS	3 hrs	20 µl/min	3.6 ml
14	R1 A1	0.9% NaCl buffer	10 min	20 µl/min	200 µl

Table 2: Conditions used to prepare AP-MS-PEG$_{12}$ acetate films.

Step #	Pos	Name	Duration	Flow	Volume
1	R1 A1	0.9% NaCl buffer	5 min	20 µl/min	100 µl
2	R1 A2	1.8% alginate	10 min	20 µl/min	200 µl
3	R1 A1	0.9% NaCl buffer	10 min	20 µl/min	200 µl
4	R1 A3	0.1% PLO	10 min	20 µl/min	200 µl
5	R1 A1	0.9% NaCl buffer	10 min	20 µl/min	200 µl
6	R1 A4	0.05% PLO	10 min	20 µl/min	200 µl
7	R1 A1	0.9% NaCl buffer	10 min	20 µl/min	200 µl
8	R1 B3	PBS	5 min	20 µl/min	100 µl
9	R1 A6	1mM MS-PEG$_{12}$	10 min	20 µl/min	200 µl
10	R1 B3	PBS	5 min	20 µl/min	100 µl
11	R1 B1	BSA (100 µg/ml)	5 min	20 µl/min	100 µl
12	R1 A1	0.9% NaCl buffer	10 min	20 µl/min	200 µl
13	R1 B1	BSA (100 µg/ml)	10 min	20 µl/min	200 µl
14	R1 B3	PBS	3 hrs	20 µl/min	3.6 ml
15	R1 A1	0.9% NaCl buffer	10 min	20 µl/min	200 µl

Results

Integrity of capsules and viability of encapsulated islets

AP capsules modified with Sulfo-NHS acetate, BS(PEG)$_9$, or MS(PEG)$_{12}$ were analysed using light microscopy to determine their size, uniformity and integrity compared with APA capsules. The viability of the encapsulated porcine pancreatic islets was also determined.

QCM-D was used to monitor the deposition of alginate and poly-L-ornithine (PLO) layers on a QCM sensor as a means of demonstrating layer-by-layer (LbL) formation of this membrane (2D). Additionally, fluorescence microscopy was used to observe covalent attachment of the blocking agent to PLO.

The use of sulfo-NHS acetate to block the amine groups on AP capsules had no significant effect on the size, uniformity or integrity of the AP-Sulfo-NHS-acetate capsules compared with unmodified APA capsules as shown in Table 3. The size, uniformity and integrity of the cells was unaffected by treatment with 5 mM or 10 mM Sulfo-NHS-acetate (Table 4). The viability of the encapsulated porcine pancreatic islet cells was not affected by modification with Sulfo-NHS-acetate at a concentration of 5 mM or 10 mM (Tables 3 and 4). Analysis of capsules using light and fluorescence microscopy confirmed the viability of AP capsules modified with Sulfo-NHS-acetate as shown in Figure 1. Table 4 shows that the proportion of capsules encapsulating islets is unchanged by modification with Sulfo-NHS-acetate.

Covalent attachment of sulfo-NHS acetate to PLO was observed using QCD-M as shown in Figure 2.

Table 3: Viability of encapsulated porcine pancreatic islets, and size, uniformity and integrity of AP capsules modified with 10 mM Sulfo-NHS acetate.

BR day	Capsule Description	Viability >85%	Capsule Size (um)	Uniformity >90%	Integrity >90%
9	APA control	99	743	100	98
	Alginate/PLO/PLO/Sulfo-NHS acetate (10mM)	97	648	100	99
22	APA control	100	637	100	98
	Alginate/PLO/PLO/Sulfo-NHS actetate (10mM)	100	646	100	99
35	APA control	100	647	100	96
	Alginate/PLO/PLO/Sulfo-NHS actetate (10mM)	100	650	100	98

BR day – days post islet isolation

Table 4: Viability of encapsulated porcine pancreatic islets, the percentage of capsules containing islets, and the size, uniformity and integrity of AP capsules modified with 5 mM or 10 mM Sulfo-NHS acetate.

BR day	Capsule Description	Viability >85%	Capsule Size μm	Uniformity >90%	Integrity >90%	With Islets >70%
13	APA control	99	638	100	96	86
	Alginate/PLO/PLO/Sulfo-NHS actetate (10mM)	100	646	100	91	78
	Alginate/PLO/PLO/Sulfo-NHS actetate (5mM)	100	629	100	98	86
35						
	APA control	100	641	100	95	84
	Alginate/PLO/PLO/Sulfo-NHS actetate (10mM)	96	649	100	93	87
	Alginate/PLO/PLO/Sulfo-NHS actetate (5mM)	97	631	100	96	89

The use of $BS(PEG)_9$ to block the amine groups on AP capsules had no significant effect on the size, uniformity or integrity of the AP-Sulfo-NHS-acetate capsules compared with unmodified APA capsules as shown in Table 5. Incubation of BS(PEG) at lower temperatures preserved islet viability. The proportion of capsules encapsulating islets is unchanged by modification with BS(PEG) as shown in Table 5.

Table 5. Viability of encapsulated porcine pancreatic islets after two and four weeks, the percentage of capsules containing islets, and the size, uniformity and integrity of AP capsules modified with BS(PEG)$_9$.

BR day	Capsule Description	Viability >85%	Capsule Size μm)	Uniformity >90%	Integrity >90%	With Islets >70%
13	APA Control	99	638	100	96	86
	Alginate/PLO/PLO/BS(PEG)9 1mM @ RT)	85	635	100	94	84
	Alginate/PLO/PLO/BS(PEG)9 1mM @ 4ºC)	88	637	100	90	84
35	APA Control	100	641	100	95	84
	Alginate/PLO/PLO/BS(PEG)9 1mM @ RT)	97	637	100	92	82
	Alginate/PLO/PLO/BS(PEG)9 1mM @ 4ºC)	100	637	100	93	81

Results show data for capsules prepared at room temperature and at 4°C.

AP capsules blocked with FITC-PEG-NHS were used to confirm covalent attachment of PEG-NHS to the PLO layer by fluorescence microscopy as shown in Figure 3. QCM-D analysis also demonstrated MS-PEG$_{12}$ binding to layers of alginate and poly-L-ornithine as shown in Figure 4.

***In vitro* insulin release studies**

Insulin release from porcine pancreatic islets encapsulated in AP capsules modified by treatment with Sulfo-NHS-acetate or BS(PEG)$_9$ were measured *in vitro* over the course of a month using the static glucose stimulation test described above.

The results presented in Table 6 show that amine blocking by Sulfo-NHS-acetate did not affect insulin release by encapsulated islet cells, as indicated by the maximum insulin released over the course of the experiment (days 9, 22 and 35). Insulin was not affected by modification with Sulfo-NHS-acetate at a concentration of 5 mM or 10 mM as shown in Table 7.

Table 6: Maximum insulin release data from APA capsules and AP capsules treated with 10 mM Sulfo-NHS Acetate monitored at batch record days 9, 22 and 35.

BR day	Capsule Description	L1 (A/C)	MIR ≥39	L3	ISI1 ≥ 3	ISI2 ≥ 3
9	APA control	1.8	179	8.8	102	20.4
	Alginate/PLO/PLO/Sulfo-NHS actetate (10 mM)	1.9	209	9.8	108	21.3
22	APA control	5.1	590	14.6	115	40
	Alginate/PLO/PLO/Sulfo-NHS actetate (10 mM)	5.5	678	16.2	123	42
35	APA control	15.4	1776	69.6	115	26
	Alginate/PLO/PLO/Sulfo-NHS actetate (10 mM)	39.9	2375	116.4	59	20

*Abbreviations: L1: low glucose incubation step 1; L3: low glucose incubation step 3; MIR - maximum insulin release, ISI- insulin stimulation index. Where ISI1 is the ratio of MIR to L1 and ISI2 is the ratio of MIR to L3 Insulin measured with ELISA expressed as µU/ml/100IEQ/h, where IEQ = islet equivalents.

Table 7: Maximum insulin release data from APA capsules and AP capsules treated with 5 or 10 mM Sulfo-NHS Acetate monitored on batch record days 13 and 35.

BR day	Capsule Description	L1	MIR ≥ 39	L3	ISI 1 ≥3	ISI 2 ≥3
13	APA control	2	120	2.8	59	43
	Alginate/PLO/PLO/Sulfo-NHS actetate (10mM)	1.4	88	1.6	65	55
	Alginate/PLO/PLO/Sulfo-NHS actetate (5mM)	1.2	100	2.3	81	44
35						
	APA control	13.4	806	31.9	60	25
	Alginate/PLO/PLO/Sulfo-NHS actetate (10mM)	22.3	892	32.1	40	28
	Alginate/PLO/PLO/Sulfo-NHS actetate (5mM)	20.5	902	38	44	24

*Abbreviations: L1: low glucose incubation step 1; L3: low glucose incubation step 3; MIR - maximum insulin release, ISI- insulin stimulation index. Where ISI1 is the ratio of MIR to L1 and ISI2 is the ratio of MIR to L3. Insulin measured with ELISA expressed as µU/ml/100IEQ/h, where IEQ = islet equivalents.

Insulin release data as shown in Table 8 showed that treating capsules with BS(PEG) reduced insulin release, which may be attributed to diffusion of the molecule through the capsule wall and coating of the islets, therefore a higher molecular weight PEG chain may be required.

Table 8: Maximum insulin release data for islets encapsulated within APA capsules and BS(PEG)$_9$ modified AP capsules monitored on batch record days 13 and 35.

BR Day	Capsule Description	L1	MIR ≥39	L3	ISI 1 ≥3	ISI 2 ≥3
13	APA Control	2	120	2.8	59	43
	Alginate/PLO/PLO/BS(PEG)9 1mM @ RT)	1.4	20	0.8	14	25
	Alginate/PLO/PLO/BS(PEG)9 1mM @ 4ºC)	1.2	56	1.5	48	37
35	APA Control	13.4	806	31.9	60	25
	Alginate/PLO/PLO/BS(PEG)9 1mM @ RT)	4	131	6.7	33	20
	Alginate/PLO/PLO/BS(PEG)9 1mM @ 4ºC)	7.4	426	20.4	58	21

*Abbreviations: L1: low glucose incubation step 1; L3: low glucose incubation step 3; MIR - maximum insulin release, ISI- insulin stimulation index. Where ISI1 is the ratio of MIR to L1 and ISI2 is the ratio of MIR to L3 Insulin measured with ELISA expressed as µU/ml/100IEQ/h, where IEQ = islet equivalents.

***In vivo* studies**

Unmodified APA capsules or AP capsules (lacking a second alginate coat), and AP capsules modified with 5 mM sulfo-NHS-acetate, or 5 mM BS(PEG)$_9$ or 5 mM MS(PEG)$_{12}$ were implanted into the peritoneum of 5-9 week old CD1 mice and recovered by peritoneal lavage 2 weeks later as described above.

Analysis of free floating APA capsules showed a high percentage of fibrotic reaction and therefore significant immune rejection of the capsules as shown in Figure 5.

Imaging of free floating AP capsules modified with sulfo-NHS-acetate or BS(PEG)$_9$ showed reduced fibrotic reaction compared with unmodified AP and APA capsules, suggesting a reduced immune rejection to the capsules as shown in Figure 6.

Histological analysis showed reduced fibrotic growth around free floating and fat bound AP capsules modified with sulfo-NHS-acetate or BS(PEG)$_9$ in fat tissue compared with the unmodified capsules as shown in Figure 7. AP capsules modified with 5mM BS-PEG9 showed a large reduction in fibrotic response and ~60 % of free floating capsules were clean (Table 9 and Figure 6). AP capsules modified with 5mM MS(PEG)12 also showed a reduction in fibrotic response and ~50% of the free floating capsules were clean (Table 9).

Without an outer coat of alginate, the AP capsules bound significantly to the fatty tissue and liver, and subsequently less capsules were found free floating as shown in Figure 7 and Table 9. This is no doubt due to the extreme charge on the PLO layer which tends to bind to most surfaces. In the presence of an outer layer of alginate ~80% of the capsules were free floating, but initiated a very strong immune response.

Table 9: *In vivo* post-mortem data for capsules retrieved from the peritoneal cavity of CD1 mice two weeks post implantation.

			FF Capsules	
Conditions	**%FF Capsules**	**% Attached**	**0-10%**	**>10%**
Alg/PLO/Alg	86.691	18.309	29.698	70.302
Alg/PLO	48.783	53.232	27.929	65.660
Alg/PLO/Sulfo-NHS-acetate (5mM)	51.295	48.705	17.195	78.236
Alg/PLO/Sulfo-NHS-acetate (10mM)	69.239	30.761	4.901	66.630
Alg/PLO/BS-PEG (5mM)	71.486	28.514	60.784	39.290
Alg/PLO/MS-PEG (5mM)	49.036	50.964	46.254	29.403

The % of free floating (ff) and attached capsules are shown for each group and the degree of fibrotic reaction on free floating capsules post-mortem is shown in the final two columns.

This study demonstrates that modification of AP capsules with Sulfo-NHS-acetate, $BS(PEG)_9$ or $MS(PEG)_{12}$ to block the amine groups present on PLO improved the biocompatibility of the capsules without impairing insulin release by pancreatic cells encapsulated by the capsules.

Discussion

The microcapsules of the invention are superior with regard to biocompatibility, viability and longevity when compared to prior art microcapsules tested, particularly in terms of minimization of fibrotic response, minimization of cell adhesion, and maintenance of insulin secretion *in vivo*.

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INDUSTRIAL APPLICATION

The compositions and methods of the present invention are useful in the formation of immunoisolatory microcapsules for use in delivering therapeutic agents, such as living cells capable of secreting therapeutics, or to deliver therapeutics per se, for the treatment of diseases or disorders.

It is not the intention to limit the scope of the invention to the abovementioned examples only. As would be appreciated by a skilled person in the art, many variations are possible without departing from the scope of the invention as outlined in the accompanying claims.

CLAIMS

1. A composition comprising alginate and one or more polycations, wherein at least one of the one or more polycations comprises one or more amine groups covalently bound to a blocking group.
2. The composition of claim 1 or claim 2 additionally comprising one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine.
3. A biocompatible microcapsule comprising a core layer of alginate cross-linked with a cationic cross-linking agent, and an outer layer of one or more polycations forming a semi-permeable membrane, wherein at least one of the one or more polycations comprises one or more amine groups covalently bound to a blocking group.
4. The composition or microcapsule as claimed in any one of the preceding claims wherein the one or more amine groups are primary amines.
5. The composition or microcapsule as claimed in any one of the preceding claims wherein the polycation is poly-L-ornithine.
6. The composition or microcapsule as claimed in any one of the preceding claims, where in the polycation is poly-L-ornithine having an average molecular weight of between about 10 – 100 KDa.
7. The composition or microcapsule as claimed in any one of the preceding claims, wherein the average molecular weight of the poly-L-ornithine is between about 15 and 30 KDa.
8. The composition or microcapsule as claimed in any one of the preceding claims, wherein the average molecular weight of the poly-L-ornithine is between 20 and 25 KDa
9. The composition or microcapsule as claimed in any one of the preceding claims, wherein the polycation contains less than 20% of a molecular weight species of 10 KDa or less.
10. The composition or microcapsule as claimed in any one of the preceding claims, wherein the polycation contains less than 20% of a molecular weight species of 100 KDa or more.
11. The composition or microcapsule as claimed in any one of the preceding claims, wherein more than about 60% of the amine groups of the one or more polycations are covalently bound to a blocking group.
12. The composition or microcapsule as claimed in any one of the preceding claims, wherein more than about 90% of the amine groups of the one or more polycations are covalently bound to a blocking group.

13. The composition or microcapsule as claimed in any one of the preceding claims, wherein more than about 99% of the amine groups of the one or more polycations are covalently bound to a blocking group.
14. The composition or microcapsule as claimed in any one of the preceding claims, wherein the blocking group acylates one or more of the amines to form a non-reversible acetamide modification.
15. The composition or microcapsule as claimed in claim 14, wherein the blocking group is provided by or the composition or microcapsule comprises an N-hydroxysulfosuccinimide ester.
16. The composition or microcapsule as claimed in claim 15, wherein the N-hydroxysulfosuccinimide ester is Sulfo-N-hydroxysulfosuccinimide acetate.
17. The composition or microcapsule as claimed in any one of the preceding claims, wherein the blocking group is or is provided by or the composition or microcapsule comprises a polyethylene glycol.
18. The composition or microcapsule as claimed in claim 17, wherein the polyethylene glycol is selected from the group comprising bis(succinimidyl) ethylene glycols, succinimidyl (N-methyl) ethyleneglycol-esters, mono-functional linear polyethylene glycols, NHS active PEG esters, NHS carbonate PEGs, and branched methyl PEG NHS esters.
19. The composition or microcapsule as claimed in claim 18, wherein the bis(succinimidyl) ethylene glycol has the formula BS(PEG)n, where n = 3 – 60.
20. The composition or microcapsule as claimed in claim 18, wherein the bis(succinimidyl) ethylene glycol has the formula BS(PEG)n, where n = 6 – 30.
21. The composition or microcapsule as claimed in claim 18, wherein the bis(succinimidyl) ethylene glycol is $BS(PEG)_9$.
22. The composition or microcapsule as claimed in claim 18, wherein the succinimidyl (N-methyl) ethyleneglycol-esters has the formula MS(PEG)n, where n = 3 – 60.
23. The composition or microcapsule as claimed in claim 18, wherein the succinimidyl (N-methyl) ethyleneglycol-esters has the formula MS(PEG)n, where n = 6 – 30.
24. The composition or microcapsule as claimed in claim 18, wherein the succinimidyl (N-methyl) ethyleneglycol-esters is $MS(PEG)_{12}$.
25. The composition or microcapsule as claimed in any one of the preceding claims, wherein the ratio of alginate to polycation is from about 5:1 to about 50:1.
26. The composition or microcapsule as claimed in any one of the preceding claims, wherein the alginate has an average molecule weight of greater than about 400 KDa.
27. The composition or microcapsule as claimed in any one of the preceding claims, wherein the alginate has an average molecular weight of greater than about 600KDa.

28. The microcapsule as claimed in any one of the preceding claims, comprising living cells within the core layer.
29. The microcapsule as claimed in any one of the preceding claims, comprising living cells within the microcapsule lumen.
30. The microcapsule as claimed in any one of the preceding claims, wherein the cells are selected from naturally occurring and genetically altered cells.
31. The microcapsule as claimed in any one of the preceding claims, wherein the cells are present as single cells and/or cell clusters selected from the group consisting of β islet cells, hepatocytes, neuronal cells and any other cell type capable of secreting factors useful in the treatment of a disease or condition.
32. The microcapsule as claimed in any one of the preceding claims, wherein the neuronal cells are selected from the group comprising choroid plexus cells, pituitary cells, chromafin cells and chondrocytes.
33. The microcapsule as claimed in any one of the preceding claims, wherein the cross-linking agent is selected from salts of the group consisting of Ag^{+}, Al^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, H^{+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, NH_4^{+}, Ni^{2+}, Pb^{2+}, Sn^{2+} and Zn^{2+}.
34. The microcapsule as claimed in any one of the preceding claims, wherein the cross-linking agent is calcium chloride.
35. The microcapsule as claimed in any one of the preceding claims, wherein the outer layer is between about 10 and about 80 microns in thickness.
36. The microcapsule as claimed in any one of the preceding claims, wherein the core layer is depolymerised by a chelation agent to form a hollow lumen.
37. The microcapsule as claimed in any one of the preceding claims, wherein the chelation agent is selected from sodium citrate and EDTA.
38. The microcapsule as claimed in any one of the preceding claims, wherein the ratio of the core layer to the outer layer is about 5:1 to about 50:1 by weight.
39. The microcapsule as claimed in any one of the preceding claims, wherein the ratio of the core layer to the outer layer is about 10:1 to about 20:1 by weight.
40. The microcapsule as claimed in any one of the preceding claims, having a diameter of between 50 and 2000 microns.
41. The composition as claimed in any one of the preceding claims, further comprising less than about 1% calcium chloride and/or sodium chloride.
42. A method of preparing one or more biocompatible microcapsules comprising the steps:
    a. dissolving alginate in isotonic saline to a concentration of between about 0.5% w/v to 2.5% w/v;

b. spraying the dissolved alginate solution of step a) through an air- or frequency-based droplet generator into a stirring solution of an excess of a cross-linking agent to form gelled microcapsules;
c. coating the gelled microcapsules of step b) with one or more polycations comprising one or more amine groups;
d. contacting the microcapsules with a solution comprising one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine;
e. collecting the one or more microcapsules.

43. The method as claimed in 42 wherein the polycation is poly-L-ornithine.
44. The method as claimed in any one of the preceding claims, wherein step b) comprises stirring in about 15mM to about 120mM calcium chloride for between about 5 to about 30 minutes.
45. The method as claimed in any one of the preceding claims, wherein step c) comprises coating the capsules with poly-L-ornithine at a concentration of between about 0.02% to about 0.10% (w/v) for between about 1 to about 45 minutes.
46. The method as claimed in any one of the preceding claims, wherein the poly-L-ornithine has an average molecular weight of between about 10 and 40 KDa.
47. The method as claimed in any one of the preceding claims, wherein step c) comprises coating the capsules with poly-L-ornithine at a concentration of about 0.05% (w/v) for about 10 minutes.
48. The method as claimed in any one of the preceding claims, wherein the blocking agent of step d) is selected from the group comprising blocking agents that acylate one or more of the amines to form a non-reversible acetamide modification, and polyethylene glycols.
49. The method as claimed in any one of the preceding claims, wherein step d) comprises contacting the one or more microcapsules with about 1mM to about 100mM of a blocking agent selected from the group comprising blocking agents that acylate one or more of the amines to form a non-reversible acetamide modification, and polyethylene glycols.
50. The method as claimed in any one of the preceding claims, wherein step d) comprises contacting the one or more microcapsules with about 1mM to about 100mM Sulfo-N-hydroxysulfosuccinimide acetate.
51. The method as claimed in any one of the preceding claims, wherein step e) comprises contacting the one or more microcapsules with about 0.5mM to about 100mM $BS(PEG)_9$ or $MS(PEG)_{12}$.
52. A method of preparing microencapsulated cells comprising the steps:

a. incubating living cells in a solution of alginate dissolved in isotonic saline to a concentration of between about 0.5% w/v and 2.5% w/v;
b. spraying the cell-containing alginate solution of step c) through an air- or frequency-based droplet generator into a stirring solution of an excess of a cross-linking agent to form one or more gelled cell-containing microcapsules;
c. coating the one or more gelled cell-containing microcapsules of step b) with one or more polycations comprising one or more amines;
d. contacting the microcapsules with a solution comprising one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine; and
e. collecting the one or more cell-containing microcapsules to provide the microencapsulated cells.

53. A method for coating a non-degradable cell delivery construct comprising the steps:
a. immerising the non-degradable cell delivery construct in a solution of alginate dissolved in isotonic saline to a concentration of between 1.0% w/v to 2.0% w/v;
b. incubating the construct of step a) in a solution containing an excess of a cross-linking agent to form a gelled coating;
c. further coating the gelled construct of step b) with one or more polycations comprising one or more amines;
d. contacting the gelled construct with a solution comprising one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine to produce an immunoisolatory membrane coated non-degradable cell delivery construct; and
e. recovering the immunoisolatory membrane coated non-degradable cell delivery construct.

54. A method for encapsulating small molecule, protein or DNA therapeutic agents comprising the steps:
a. incubating the one or more small molecules, proteins or DNA therapeutics in a solution of alginate dissolved in isotonic saline to a concentration of between about 0.5% w/v and 2.5% w/v;
b. spraying the cell-containing alginate solution of step c) through an air- or frequency-based droplet generator into a stirring solution of an excess of a cross-linking agent to form one or more microcapsules containing the small molecules, proteins or DNA therapeutics;
c. coating the one or more gelled microcapsules of step b) with one or more polycations comprising one or more amines;

d. contacting the microcapsules with a solution comprising one or more blocking agents capable of providing a blocking group itself capable of covalently binding to an amine; and

e. collecting the one or more microcapsules to provide the microencapsulated small molecules, proteins or DNA therapeutics.

55. A biocompatible microcapsule prepared by the method of any one of claims any one of the preceding claims.

56. A cell-containing microcapsule prepared by the method of any one of claims any one of the preceding claims.

57. An immuniosolatory membrane coated non-degradable cell delivery construct prepared by the method of any one of the preceding claims.

58. A therapeutic agent-containing microcapsule prepared by the method of any one of the preceding claims.

59. A method of treating or preventing a disease or condition in a subject comprising transplanting an effective amount of a cell-containing microcapsule of any one of the preceding claims into said subject, when one or more of the cells present in the microcapsule secrete a therapeutic that is effective at treating or preventing said disease or condition.

60. The method according to claim 59 wherein the disease or condition is Type I diabetes mellitus.

61. The method according to claim 59 or 60 wherein one or more of the cells are islet β cells.

62. A method of treating or preventing a disease or condition in a subject comprising transplanting an effective amount of a therapeutic-containing microcapsules as claimed in any one of the preceding claims in the said subject, when said therapeutic is effective at treating or preventing said disease or condition.

63. The method as claimed in 62, wherein the cells comprise hepatocytes and said disease or condition is a disease or disorder of the liver.

64. The method as claimed in claims 62, wherein the cells comprise neuronal cells selected from the group consisting of choroids plexus cells, pituitary cells, chromafin cells, chondrocytes and any other neuronal cell capable of secreting neuronal factors, and the disease or condition is a neurological disease or condition.

65. The composition as claimed in claim 1, where the polycation is selected from the group consisting of chitosan, chitosan glutamate, chitosan glycol, modified dextran, poly-L-lysine, poly-L-ornithine, salmine sulfate, protamine sulfate, polyacrylimide, polyacrylimide-co-methacryloxyethyltrimethylammonium bromide, polyallylamine, polyamide, polyamine, polybrene, Polybutylacrylate-co-Methacryloxyethyl Trimethylammonium Bromide (80/20), Poly-3-chloro-2-

hydroxypropylmethacryl-oxyethyl dimethylammonium Chloride, Polydiallyldimethylammonium, Polydiallyldimethylammonium Chloride, Polydiallyldimethylammonium Chloride-co-Acrylamide, Polydiallyldimethylammonium Chloride-co-N-Isopropyl Acrylamide, Polydimethylamine-co-epichlorohydrin, Polydimethylaminoethylacrylate-co-Acrylamide, Polydimethylaminoethylmethacrylate, Polydimethylaminoethyl Methacrylate, Polyethyleneimine, Polyethyleneimine-Epichlorohydrin Modified, Polyethyleneimine, Poly-2-hydroxy-3-methacryloxypropyl Trimethylammonium Chloride, Poly-2-hydroxy-3-methacryloxyethyl, Trimethylammonium Chloride, Polyhdroxyproplymethacryloxy Ethyldimethyl Ammonium Chloride, Polyimadazoline (Quaternary), Poly-2-methacryloxyethyltrimethylammonium Bromide, Polymethacryloxyethyltrimethylammonium Bromide/Chloride, Polymethyldiethylaminoethylmethacrylate-co-Acrylamide, Poly-l-methyl-2-vinylpyridinium Bromide, Poly-l-methyl-4-vinylpyridinium Bromide, Polymethylene-co-Guanidine Hydrochloride, Polyvinylamine, Poly-N-vinylpyrrolidone-co-Dimethylaminoelhyl-Methacrylate, Poly-4-vinylbenzyltrimethylammonium Chloride, Poly-4-vinylbenzyltrimethylammonium Chloride, PEI-g-Chitosan, cationic gelatin, cationic cellulose, collagen, cationic cyclodextrin, poly(aminoamines), poly(amino-co-ester), PEG HCl derivatives including 0-2(-aminoethyl)-o-2(succunylamino) ethyl) PEG HCl, o-(2-aminoethyl)-O-(2-carboxyethyl) PEG HCl, and modified PEG including O-(2-aminoethyl)PEG.

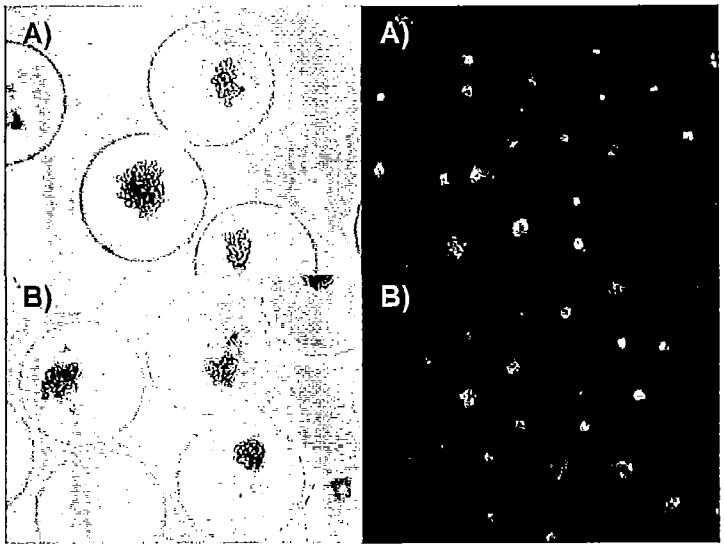


Figure 1

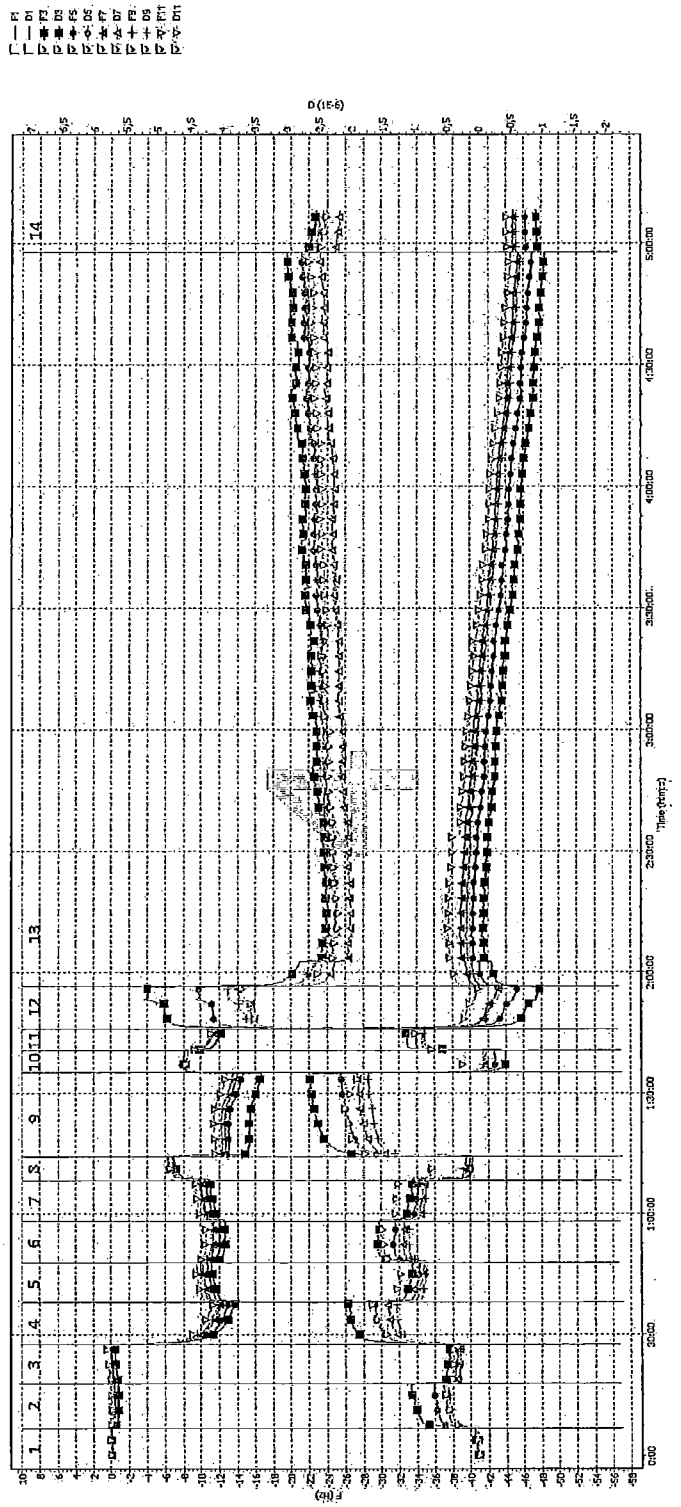


Figure 2

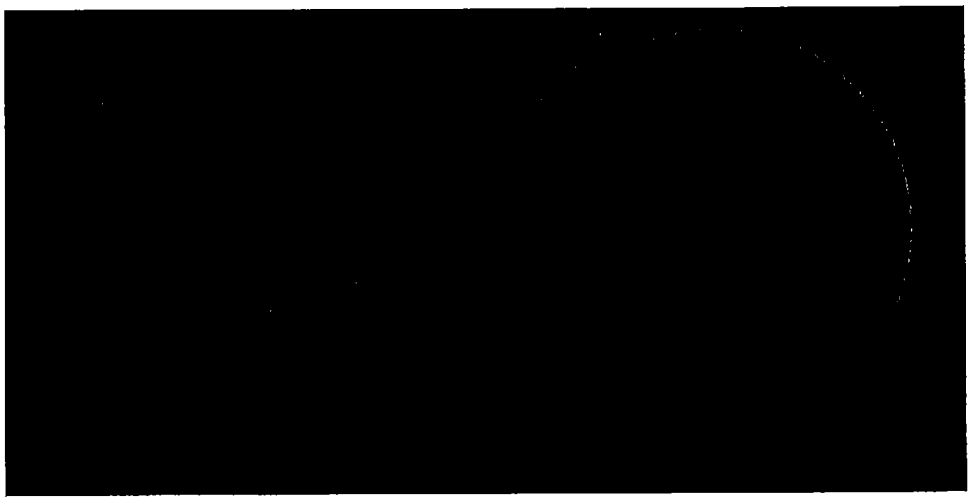
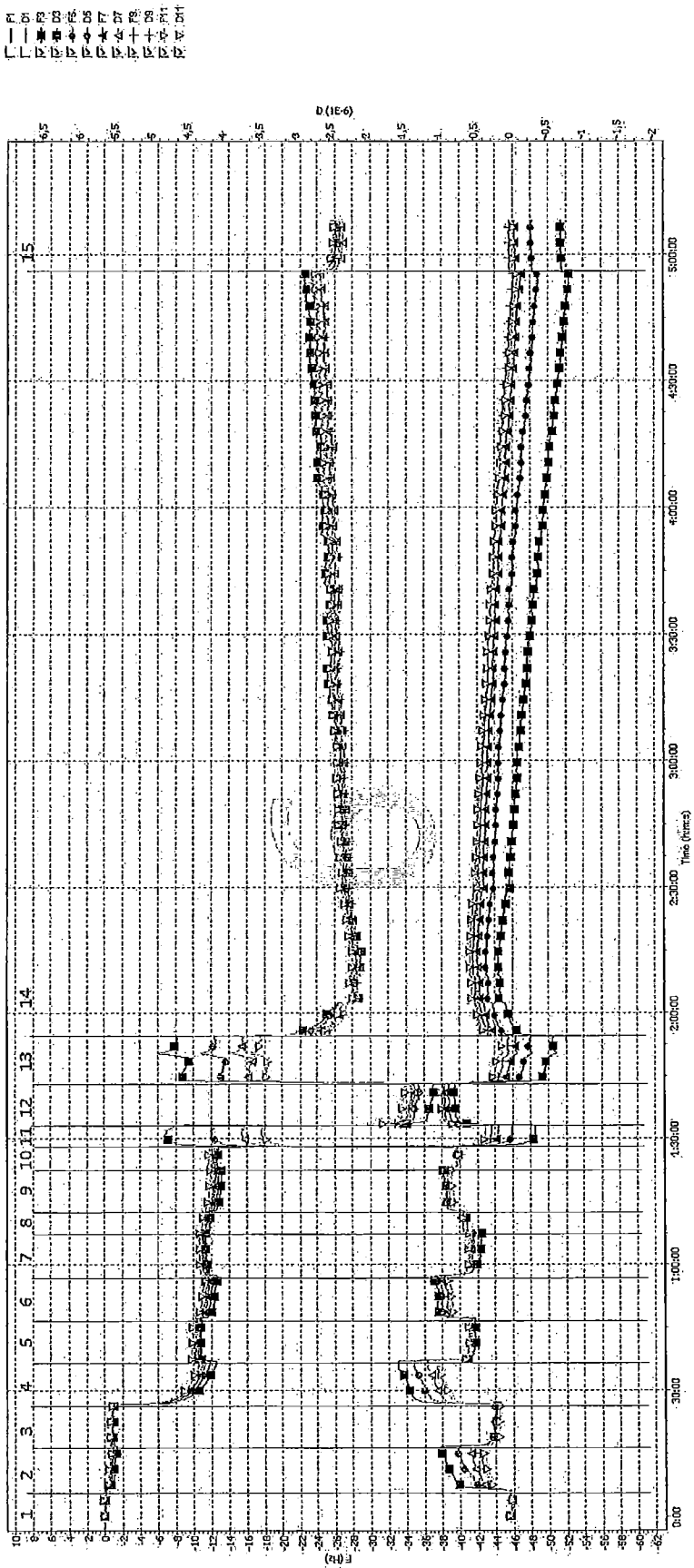


Figure 3


P1
D1
P3
D3
P5
D5
P7
D7
P9
D9
P11
D11
D (1E-6)
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Time (mins)
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30:00
1:00:00
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4:30:00
5:00:00

Figure 4

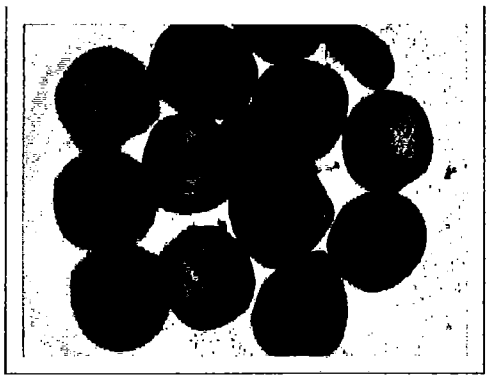

Figure 5

Figure 6

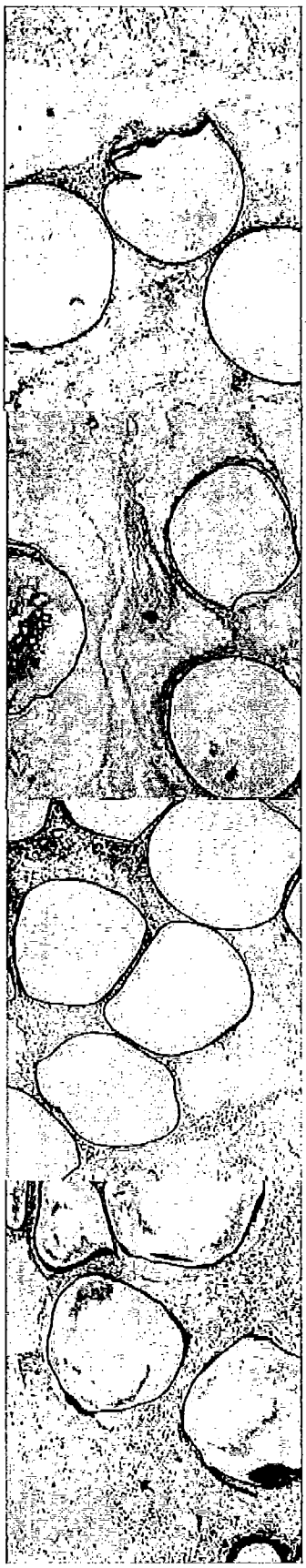

Figure 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2014/000068

A. CLASSIFICATION OF SUBJECT MATTER

A61K 47/38 (2006.01) ***A61K 47/40 (2006.01)*** ***A61K 47/48 (2006.01)*** ***A61K 47/32 (2006.01)*** ***A61K 9/50 (2006.01)*** ***B01J 13/02 (2006.01)***

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)

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 practicable, search terms used)

Databases Consulted: WPI, EPODOC, MEDLINE, CAPLUS, BIOSIS, ESPACENET

Search terms used: alginate, alginic acid, polycation, polyamine, polyamide, polyammonium, chitosan, dextran, poly-L-lysine, poly-L-ornithine, protamine, salmine, polyethylene, block, acylate, hydroxylsulfosuccinimide, MS-PEG, BS-PEG, pegylate, acetamide, protect, biocompatible, microcapsule, implant, encapsulate, construct, beta-islet, hepatocyte, neuron, protein, DNA, Living Cell Technologies, *and related terms.*

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 September 2014	09 September 2014
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Safiea Goolam AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832521

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	**PCT/NZ2014/000068**

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X	HAQUE T, et al. 'Investigation of a new microcapsule membrane combining alginate, chitosan, polyethylene glycol and poly-L-lysine for cell transplantation applications,' *The International Journal of Artificial Organs,* (2005) Vol 28 No 6, pp 631-637. Abstract; page 632 column 1, adjoining paragraphs at columns 1-2; adjoining paragraphs at pages 632-633; page 633 adjoining paragraphs at columns 1-2; page 635 column 1; page 636 column 2	1-65
X	ZHENG JN, et al. 'Chitosan-*g*-MPEG Alginate/Chitosan Hydrogel Microcapsules: A Quantitative Study of the Effect of Polymer Architecture on the Resistance to Protein Adsorption,' *Langmuir*, (2010) Vol 26 No 22, pp 17156-17164. Abstract; page 17158 coumn 1; page 17157 at item 2.4, page 17158 at item 2.5,	1-65
X	PAUL A, et al. 'BacMam Virus Transduced Cardiomyoblasts Can Be Used for Myocardial Transplantation Using AP-PEG-A Microcapsules: Molecular Cloning, Preparation and *In Vitro* Analysis, *Journal of Biomedicine and Biotechnology*, (2010), Article ID 858094, pp 1-12. Abstract; page 4 item 2.8; page 8 item 3.4; page 10 item 3.6; page 10 at conclusion	1-65
X	CHEN JP, et al.'Microencapsulation of Islets in PEG-Amine Modified Alginate-Poly(L-Lysine)-Alginate Microcapsules for Constructing Bioartificial Pancreas,' *Journal of Fermentation and Bioengineering*, (1998) Vol 86 No 2, pp 185-190. Abstract; page 186 column 1, adjoining paragraph at columns 1-2; page 188 last paragraph - page 189 column 1	1-65
X	CHANDY T, et al. 'Delivery of LMW Heparin via Surface Coated Chitosan/peg-Alginate Microspheres Prevents Thrombosis,' *Drug Delivery*, (2002) Vol 9, pp 87-96. Abstract; page 88 column 2; page 89 column 2; page 95 column 1	1-65
X	US 5,573,934 A (HUBBELL et al.) 12 November 1996 Column 1 lines 15-17; column 17 lines 14-64; column 10 lines 6-51; Example 10 at columns 21-22	1-65
A	WO 2007/046719 A2 (LIVING CELL PRODUCTS PTY LIMITED) 26 April 2007 Whole document. Particularly abstract, claims, examples.	1-65
P,X	CN 103301788 A (DALIAN CHEMICAL PHYSICS INST.) 18 September 2013 Abstract; [0006]; [0029]-[0032]; claim 6; [0014]-[0026]; claim 7; claim 10; claim 8	1-4, 14, 17, 26, 27, 28, 30, 31, 33, 37, 42, 48, 55, 56, 58 and 65

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/NZ2014/000068**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/NZ2014/000068**

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INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/NZ2014/000068**

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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/NZ2014/000068**

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CN 103301788 A	18 September 2013	None	

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.