Université de Montréal

Biocompatibilité des Microcapsules d'Alginate: Purification d'Alginate, Réaction Immunitaire de l'Hôte et Protection du Receveur

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Cette thèse intitulée :

Biocompatibilité des Microcapsules d'Alginate: Purification d'Alginate, Réaction Immunitaire de l'Hôte et Protection du Receveur.

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Résumé

L'immuno-isolation des îlots de Langerhans est proposée comme moyen d'effectuer des transplantations sans prise d'immunosuppresseurs par le patient. Cette immuno-isolation, par l'entremise d'une microcapsule composée d'alginate et de poly-L-lysine (microcapsule APA), protège le greffon d'une éventuelle attaque du système immunitaire du receveur grâce à sa membrane semi-perméable. Cette membrane empêche le système immunitaire du receveur de pénétrer la microcapsule tout en laissant diffuser librement les nutriments, le glucose et l'insuline. Avant l'application de cette technique chez l'humain, quelques défis doivent encore être relevés, dont la biocompatibilité de ce système. La biocompatibilité fait ici référence à la biocompatibilité du biomatériau utilisé pour la fabrication des microcapsules, l'alginate, mais aussi la biocompatibilité des microcapsules reliée à leur stabilité. En effet, il a été remarqué que, lors d'implantation *in vivo* de microcapsules fabriquées avec de l'alginate non purifiée, ceci induisait un phénomène nommé Réaction de l'Hôte contre la Microcapsule (RHM). De plus, il est connu que la stabilité des microcapsules APA peut influencer leur biocompatibilité puisqu'une microcapsule endommagée ou brisée pourrait laisser s'échapper les cellules du greffon chez le receveur. Nous croyons qu'une compréhension des processus d'initiation de la RHM en fonction de l'efficacité des procédés de purification d'alginate (et donc des quantités de contaminants présents dans l'alginate) ainsi que l'augmentation de la stabilité des microcapsules APA pourront améliorer la biocompatibilité de ce dispositif, ce que tente de démontrer les résultats présentés dans cette thèse. En effet, les résultats obtenus suggèrent que les protéines qui contaminent l'alginate jouent un rôle clé dans l'initiation de la RHM et qu'en diminuant ces quantités de protéines par l'amélioration des procédés de purification d'alginate, on améliore la biocompatibilité de l'alginate. Afin d'augmenter la stabilité des microcapsules APA, nous décrivons une nouvelle technique de fabrication des microcapsules qui implique la présence de liaisons covalentes. Ces nouvelles microcapsules APA réticulées sont très résistantes, n'affectent pas de façon négative la survie des cellules

encapsulées et confinent les cellules du greffon à l'intérieur des microcapsules. Cette dernière caractéristique nous permet donc d'augmenter la biocompatibilité des microcapsules APA en protégeant le receveur contre les cellules du greffon.

Mots-clés : Diabète de type I, transplantation d'îlots, immuno-isolation, microencapsulation, purification alginate, réaction à corps étranger, réticulation covalente, thérapies cellulaires.

Abstract

Islet of Langerhans inmmunoisolation is proposed as a way to avoid the use of immunosuppressive drugs after transplantation. Microcapsules, the immuno-isolating device, are composed of alginate and poly-L-lysine and the protection of the graft is granted by a semi-permeable membrane. This membrane allows small molecules to freely diffuse within the microcapsule, such as nutrients, glucose and insulin while protecting the graft against the host immune system. Biocompatibility is one of the challenges that must be addressed before the successful clinical application of this device. Microcapsules biocompatibility is related, first, to the biocompatibility of alginate, the polymer used to made microcapsules and second, to the *in vivo* stability of these microcapsules. In facts, it is well know that the use of an unpurified alginate containing many foreign contaminants to make microcapsules induce the host reaction against microcapsule (HRM). Moreover, damaged or broken microcapsules can allow the dissemination of cells from the encapsulated graft, activating the host immune system. We believe that a better understanding of the initiation processes of the HRM in terms of alginate purification efficacy to remove contamination as well as an improve microcapsule stability will increase microcapsules biocompatibility. Results reported in this thesis suggest that foreign proteins found in alginate are playing a key role in the initiation of HRM and that the reduction of these foreign proteins, by the improvement of alginate purification processes, improves microcapsules biocompatibility. In order to increase microcapsules stability, we also described and characterized an innovative type of microcapsules which involve covalent bonds. These covalently cross-linked microcapsules were found to by highly resistant and stable. The novel fabrication process of these microcapsules was not harmful for the encapsulated cell survival and was also found to confine the graft inside the microcapsules. This characteristic enables us to increase microcapsules biocompatibility by the protection of the host from the encapsulated cells.

Keywords : Type I diabetes, islet transplantation, immuno-isolation, microencapsulation, alginate purification, foreign-body reaction, covalent cross-linking, cell therapies.

Table des matières

Résumé ---------- *iii*

Abstract ---------- *v*

Introduction ---------- *1*

Pancréas et homéostasie du glucose ---------- 1

Diabète type I et auto-immunité---------- 3

Traitements du diabète ---------- 5

Insuline ---------- 5

Transplantation du pancréas total ---------- 6

Transplantation d'îlots ---------- 7

Rejet des greffes ---------- 8

Concept d'immuno-isolation----------11

Sites de transplantation----------13

Microencapsulation cellulaire ----------16

Polymères utilisés en microencapsulation----------16

Hydrogel d'alginate ----------17

Problématique ----------25

Partie I : Purification d'alginate et réaction immunitaire de l'hôte ----------27

Evaluation of Alginate Purification Methods: Effect on Polyphenol, Endotoxin and Protein Contamination----------37

CONTRIBUTIONS RESPECTIVES DES AUTEURS----------38

ABSTRACT ----------39

INTRODUCTION----------40

MATERIALS AND METHODS ----------42

RESULTS----------46

DISCUSSION ----------48

ACKNOWLEDGMENT ----------53

FIGURES, TABLES AND LEGENDS ---------- 54
REFERENCES ---------- 57

Impact of Residual Contamination on the Biofunctional Properties of Purified Alginates Used for Cell Encapsulation ---------- 63

CONTRIBUTIONS RESPECTIVES DES AUTEURS ---------- 64
ABSTRACT ---------- 65
INTRODUCTION ---------- 66
MATERIALS AND METHODS ---------- 68
RESULTS ---------- 73
DISCUSSION ---------- 79
CONCLUSIONS ---------- 82
ACKNOWLEDGEMENTS ---------- 83
FIGURES, TABLES AND LEGENDS ---------- 84
REFERENCES ---------- 90

In Vivo Behaviour of Microcapsules Using Alginate of Varying Composition ---------- 95

CONTRIBUTIONS RESPECTIVES DES AUTEURS ---------- 96
ABSTRACT ---------- 97
INTRODUCTION ---------- 97
MATERIAL AND METHODS ---------- 99
RESULTS ---------- 105
DISCUSSION ---------- 108
FIGURE, TABLES AND LEGENDS ---------- 112
REFERENCES ---------- 118

Role of Protein Contaminants in the Immunogenicity of Alginates. ---------- 121

CONTRIBUTIONS RESPECTIVES DES AUTEURS ---------- 122
ABSTRACT ---------- 123
INTRODUCTION ---------- 124
MATERIALS AND METHODS ---------- 126
RESULTS ---------- 130
DISCUSSION ---------- 134
CONCLUSION ---------- 137
ACKNOWLEDGEMENTS ---------- 138

FIGURES, TABLES AND LEGENDS ---- 138
REFERENCES ---- 143

Discussion générale (Partie I) ---- 148

Partie II : Protection du receveur ---- 159

Microencapsulation of Living Cells in Semi-Permeable Membranes with Covalently Cross-Linked Layers. ---- 162

CONTRIBUTIONS RESPECTIVES DES AUTEURS ---- 163
ABSTRACT ---- 164
INTRODUCTION ---- 164
METHODS ---- 167
RESULTS ---- 172
DISCUSSION ---- 175
ACKNOWLEDGEMENTS ---- 178
REFERENCES ---- 179
FIGURES AND LEGENDS ---- 185

The Effect of Covalent Cross-links Between the Membrane Components of Microcapsules on the Dissemination of Encapsulted Malignant Cells ---- 191

CONTRIBUTIONS RESPECTIVES DES AUTEURS ---- 192
ABSTRACT ---- 193
INTRODUCTION ---- 193
MATERIALS AND METHODS ---- 196
RESULTS ---- 200
DISCUSSION ---- 203
ACKNOWLEDGEMENTS ---- 207
FIGURES AND LEGENDS ---- 208
REFERENCES ---- 213

Discussion générale (Partie II) ---- 216

Conclusion ---- 231

Bibliographie ---- I

Annexe I ---- XV

Attestation des co-auteurs et des éditeurs -- **XV**

Annexe II -- ***XXXVII***

Curriculum Vitae du candidat --**XXXVII**

Liste des tableaux

Tableau 1 : Influence des paramètres intrinsèques de l'alginate et de la PLL sur les propriétés des microcapsules APA [38]. 218

Liste des figures

Figure 1 : Représentation schématique d'un îlot de Langerhans. 3
Figure 2 : Estimation de la prévalence du diabète en 2025.. 4
Figure 3 : Les dix pays ayant l'incidence la plus important de diabète de type I chez les enfants âgés entre 0 et 14 ans. 4
Figure 4 : Représentation schématique de la présentation directe et indirecte des antigènes lors du rejet de la greffe. 10
Figure 5 : Concept d'immuno-isolation où la membrane semi-perméable permet la libre diffusion de petites molécules comme le glucose et l'insuline mais empêche le passage de molécules de plus grand poids moléculaire tels que les cellules immunes et les anticorps. 13
Figure 6 : Composition chimique de l'alginate. 19
Figure 7 : Illustration du modèle «egg-box». 21
Figure 8 : Schéma de la fabrication des billes d'alginate et des microcapsules complètes. 23
Figure 9 : Cinétique du développement de la réaction de l'hôte contre la microcapsule (RHM). 32
Figure 10 : Technique de l'angle de contact où l'angle formé sur une surface nous renseigne sur sa mouillabilité 35
Figure 11 : Étapes du développement d'un produit de combinaison pour approbation par la FDA. 155
Figure 12 : Modèles de réticulation covalente des microcapsules 220

Liste des sigles et abréviations

APA : Alginate-Poly-L-lysine-Alginate

AFU : Arbritary Fluorescence Units

ANB-NOS : N-5-Azido-2-nitrobenzoyloxysuccinimide

ATR-FTIR : Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy

Ba : Baryum

$BaCl_2$: Chlorure de Baryum

Ca : Calcium

$CaCl_2$: Chlorure de Calcium

CAM : Complexe d'attaque membranaire

Cd : Cadmium

Cl : Chlore

CMH : Complexe majeur d'histocompatibilité

Co : Cobalt

CO_2 : Dioxide de carbone

CPA : Cellule présentatrice d'antigène

cps : centipoise

CSE : Cellule souche embryonnaire

Cu : Cuivre

DCCT : Diabetes Control and Complications Trial

DMSO : Diméthyl sulfoxide

DNA : Deoxyribonucleotide acid

EDTA : Acide éthylène diamine tetra acétique

EU : Endotoxin unit

eV : Électronvolt

FDA : Food and Drug Administration

FITC : Fluorescéine Isothiocyanate

G : Acide guluronnique

HBSS : Hank's balance salt solution

HCl : Acide Chloridrique

HEPES : Acide sulfonique N-2-hydroxyméthylpipérazine-N'-2-éthane

IgG : Immunoglobuline G

IgM : Immunoglobuline M

IL : Interleukine

kDa : Kilodalton

$KHCO_3$: Potassium bicarbonate

kJ : Kilojoule

Ksec : Coefficient de partition

LAL : Limulus amebocyte lysate

LPS : Lipopolysaccharide

M : Acide Mannuronique

Mn : Manganèse

Mw : Molecular weight

MWCO : Molecular weight cut-off

N : Azote

Na : Sodium

NaCl : Chlorure de sodium

NaOH : Hydroxyl de sodium

NO : Oxide nitrique

NH_4Cl : Chlorure d'ammonium

P : Phosphore

Pb : Plomb

PBS : Phosphate buffered solution

PC : Phosphorylcholine

PE: Phycoerythrine

PEG : Polyéthylène glycol

PERV : Porcin endogeneous retovirus

PLL : Poly-L-lysine

PLO : Poly-L-ornithine

PVA : Polyvinyl alcool

RCT: Récepteur des cellules T

RHM: Réaction de l'hôte contre la microcapsule

$R\eta$: Rayon de viscosité

RT-PCR: Reverse transcriptase polymerase chain reaction

S: Soufre

SEC: Size exclusion chromatography

SEM: Standard error of the mean

Sr: Strontium

Sulfo-SANPAH: Sulphosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexane

TGF: Tissue growth factor

TNF: Tumor necrosis factor

UPLVG: Ultra purified low viscosity high G

UV: Ultraviolet

XPS: X-ray photoelectron spectroscopy

Zn: Zinc

I have frequently been questioned, especially by women, of how I could reconcile family life with a scientific career. Well, it has not been easy! *MARIE CURIE*

Remerciements

Je voudrais d'abord remercier mes directeurs de recherche, le Dr. Jean-Pierre Hallé à l'Hôpital Maisonneuve-Rosemont et L'Hocine Yahia à l'École Polytechnique. Jean-Pierre Hallé, avec qui j'ai partagé 10 ans de ma vie académique, ayant complété ma maîtrise ainsi que mon doctorat dans son laboratoire, et pour qui j'ai le plus grand des respects. Grâce à vous et à votre passion pour la recherche fondamentale, j'ai pu développer des qualités telles que la pensée critique, l'autonomie ainsi que la persévérance. L'Hocine, que je me de dois remercier aujourd'hui d'avoir cru en moi et de m'avoir convaincue de poursuivre mes études au niveau du doctorat.

Je profite de cette tribune pour rendre hommage à quatre personnes spéciales qui ont eu un impact majeur sur ma carrière académique :

Susan K. Tam, ma co-équipière de tous les instants dans ce projet. J'étais la biologie et toi l'ingénierie! Sans toi, ce projet un peu fou n'aurait pas pris une telle ampleur. Tu es une femme extraordinaire qui allie à la fois persévérance, rigueur et passion, autant comme scientifique que dans ta vie personnelle. Une ingénieure hors du commun qui possède également des cordes vocales en or! Et oui! Madame chante le Jazz! Je garderai toujours des souvenirs impérissables de nos aventures lors des congrès, promenades à vélo à Groningen, le Redlight d'Amsterdam, la bière Tibétaine, le magasinage à Vienne, le resto Népalais, le Pancake Boat…

Nathalie Henley fut mon premier contact avec la vraie vie de laboratoire. Elle m'a tout de suite prise sous son aile et m'a enseignée les rudiments de la Science. Oui, je sais, avec le temps on prend des mauvais plis, mais, ma base est impeccable! Nathalie, tu es aussi une oreille très attentive. Je ne peux plus compter le nombre de fois où je me suis retrouvé dans ton laboratoire de cytométrie, soit pour faire sortir mes frustrations ou encore pour partager les bonnes nouvelles avec toi. Tu es une amie fidèle avec qui j'ai énormément de complicité.

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Avant-propos

- L'originalité et la diversité des résultats présentés dans cette thèse découlent d'une étroite collaboration entre l'équipe du Dr Jean-Pierre Hallé de l'Hôpital Maisonneuve-Rosemont et l'équipe du Dr. L'Hocine Yahia de l'École Polytechnique et plus particulièrement entre moi, Julie Dusseault, étudiante au doctorat en Sciences Biomédicales et Susan K. Tam, étudiante à la maîtrise puis au doctorat en Génie Biomédical. Ces deux étudiantes ont constamment coopéré, échangé et également intégré différentes notions n'appartenant pas, a priori, à leur domaine d'études respectif. Se situant donc à la frontière entre la biologie et l'ingénierie, ce projet a généré un nombre important d'articles sur lesquels les deux étudiantes sont considérées comme co-premières auteures. Certains de ces articles feront donc partie des deux thèses de doctorat mais présenteront un point de vue différent, l'une se concentrant sur l'aspect biologique et l'autre sur l'aspect ingénierie. Il est à noter que ces deux étudiantes ont également d'autres publications originales, où elles sont premier auteure, ce qui distinguera davantage leur travail respectif.

- Mes travaux de doctorat se sont échelonnés de septembre 2002 à janvier 2009 (environ 6 ans ½). Durant cette période, j'ai dû interrompre mes études à 2 reprises pour des congés de maternité pour une période totale de 18 mois.

- Lors de ma maîtrise, j'ai travaillé activement à la mise au point de la technique de fabrication des microcapsules réticulées. À la fin de ces études, j'ai déposé un mémoire de maîtrise traditionnel (sans article publié) dans lequel je présente les conditions idéales pour obtenir des microcapsules réticulées ainsi que quelques résultats compatatifs entre les microcapsules standards et réticulées. J'estime qu'environ 50% des résultats publiés dans le premier article de la partie II dans la présente thèse, intitulé «Microencapsulation of living cells in semi-permeable membranes with covalently cross-linked layers» proviennent de mes études de maîtrise.

Introduction

Pancréas et homéostasie du glucose

Le pancréas est une glande amphicrine, c'est-à-dire qu'elle possède à la fois une partie exocrine, composée de cellules acineuses responsables de la sécrétion des sucs pancréatiques utiles à la digestion des aliments, et une partie endocrine, formée par les îlots de Langerhans qui produisent les hormones pancréatiques. Les îlots ne représentent que 1% du pancréas et sont principalement composés de 5 types de cellules endocrines (voir Figure 1, page 3). Les cellules β sont les plus abondantes, entre 70 à 80%, et sont responsables de la sécrétion d'insuline en fonction des taux de glucose sanguins. Le mécanisme est le suivant : lors d'un repas, le taux de glucose sanguin circulant augmente et ce glucose est capté par les transporteurs de glucose GLUT2 qui sont présents à la surface des cellules β. Ces transporteurs de glucose font pénétrer le glucose à l'intérieur des cellules β où il sera dégradé en pyruvate par une série d'enzymes spécialisées (glucokinase ou GK, phosphoglucose isomérase, phosphofructokinase ou PFK et glycérol-3-phosphate déhydrogénase ou GPDH). Ce pyruvate est ensuite acheminé aux mitochondries où il sera convertit en ATP. L'augmentation du niveau d'ATP dans la cellule induit l'activation des canaux potassiques ATP-dépendants qui font sortir le potassium de la cellule, ayant pour effet l'ouverture des canaux calciques qui eux font entrer le calcium. Cette augmentation de calcium intracellulaire induit la relâche de l'insuline qui était préalablement emmagasinée dans des granules de sécrétion. L'insuline a trois principales fonctions qui ont toutes pour but de diminuer la glycémie:

1. La liaison de l'insuline à son récepteur membranaire (IR) à la surface des cellules musculaires induit la translocation du transporteur de glucose GLUT4 à la surface cellulaire afin de faciliter l'entrée du glucose dans le muscle. Ce glucose est ensuite transformé en énergie par la glycolyse.
2. Tout comme dans le muscle, la liaison de l'insuline à son récepteur à la surface des adipocytes induit la translocation de GLUT4 qui favorise la diffusion du glucose

sauf que dans les cellules adipeuses, le glucose est convertit en glycérol-3-phosphate, puis en triglycérides, forme sous laquelle il sera emmagasiné.

3. Dans le foie, l'entrée du glucose n'est pas dépendante de la signalisation de l'insuline. En fait, les hépatocytes expriment GLUT2 à leur surface, tout comme les cellules β. Donc, lorsque la quantité de glucose sanguin augmente, il entre immédiatement dans les cellules hépatiques. Cependant, l'insuline relâchée par les cellules β, en se liant à son récepteur à la surface des hépatocytes stimule la synthèse du glycogène en activant les enzymes qui jouent un rôle dans la glycogénogénèse, telles que la glucokinase, la phosphofructokinase et la glycogène synthase.

Les cellules α, qui représentent environ 15-20% des cellules de l'îlot, produisent le glucagon qui a les effets contraires de l'insuline. Les cellules δ (3-5%) sécrètent la somatostatine, qui inhibe les sécrétions de l'îlot de façon paracrine, et les cellules PP (1%) qui fabriquent le polypeptide pancréatique ayant une fonction encore inconnue. Décrites plus récemment, les cellules ε, quant à elles, sécrètent la ghréline, une hormone connue pour stimuler l'appétit.

L'organisation tri-dimensionnelle des cellules à l'intérieur des îlots varie beaucoup en fonction des espèces [1]. Chez les rongeurs, les cellules β se retrouvent principalement concentrées au centre de l'îlot et entourées des quatres autres types cellulaires alors que chez l'humain, l'îlot ne présente pas cette organisation particulière, les différents types cellulaires étant distribués plus aléatoirement.

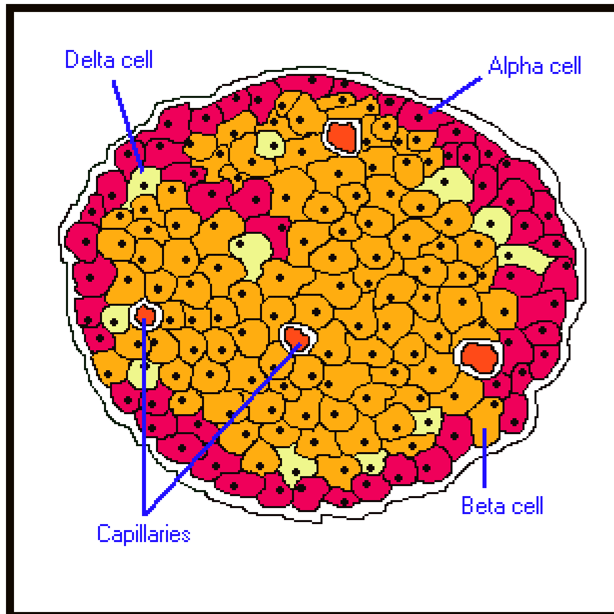
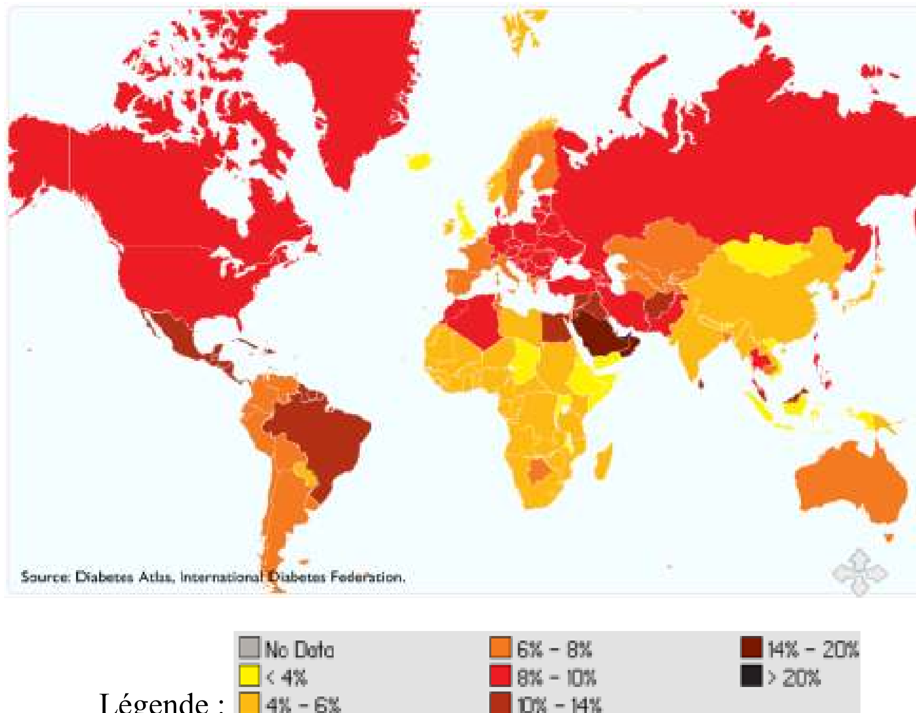


Figure 1 : Représentation schématique d'un îlot de Langerhans.

Diabète type I et auto-immunité

Selon le «Diabetes Atlas» publié par la Fédération Internationale du Diabète, le diabète, quel qu'en soit la forme, affecte présentement 246 millions de personnes mondialement et il est prévu que ce nombre augmentera possiblement jusqu'à 380 millions en 2025 selon les projections (Figure 2, page 4). De ce nombre, environ 10 à 15% développent un diabète de type I, aussi appelé diabète insulino-dépendant, qui atteint principalement la population juvénile. L'incidence du diabète de type I est également augmenté d'environ 3% par année pour la population mondiale. Cependant, cette incidence n'est pas équivalente pour tous les pays. En effet, comme l'illustre bien la Figure 3 (page 4), certains pays ont une plus forte prévalence que les autres, le Canada arrivant au 5ième rang des pays ayant une plus grande prévalence.



Légende :

Figure 2 : Estimation de la prévalence du diabète en 2025.

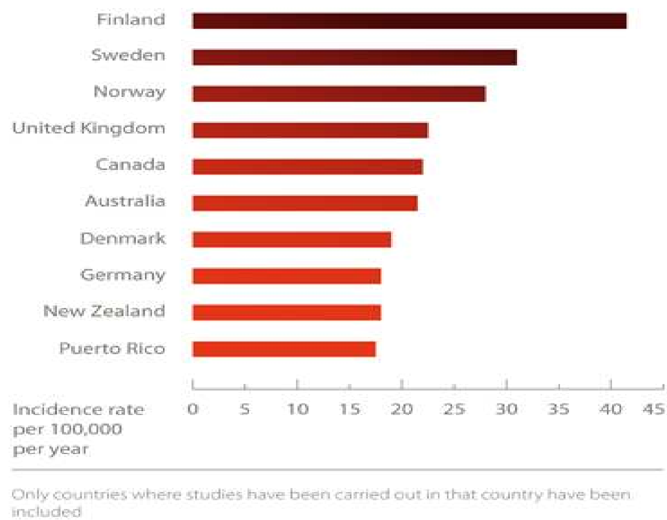


Figure 3 : Les dix pays ayant l'incidence la plus important de diabète de type I chez les enfants âgés entre 0 et 14 ans.

Le diabète de type I est une maladie dite auto-immune puisque, suite à un dérèglement du système immunitaire du patient, les cellules β qui sont responsables de la sécrétion d'insuline sont détruites par les cellules immunitaires. Les lymphocytes T naïfs circulants, entrent dans les ganglions lymphatiques, s'activent suite à un contact avec des cellules présentatrices d'antigènes qui sont chargées et présentent des antigènes provenant spécifiquement des cellules β. Ces lymphocytes T autoréactifs, qui sont normalement éliminés chez des sujets sains, retournent dans la circulation lymphatique et migrent dans le tissu pancréatique où ils sont alors réactivés et où ils déclenchent une cascade immunitaire spécifiquement contre les cellules β.

Les causes de ce dérèglement du système immunitaire ne sont pas encore totalement élucidées mais il semblerait que des facteurs environnementaux comme l'infection virale, l'exposition à des agents toxiques tels le gluten ou les nitrates et finalement l'aseptisation de l'environnement ainsi que des facteurs de prédispositions génétiques seraient à l'origine de ce dérèglement.

Traitements du diabète

Insuline

Afin de contrôler la glycémie, le traitement recommandé aux patients diabétiques est la multi-injection quotidienne d'insuline exogène. Même si l'insulinothérapie a permis d'améliorer grandement la qualité de vie des patients, ce traitement possède néanmoins ses limites et ne fait que retarder le développement des nombreuses complications reliées au diabète tels la néphropathie, la rétinopathie, la neuropathie et les maladies cardiovasculaires. En effet, l'administration d'insuline même plusieurs fois par jours ne permet pas un contrôle optimal et constant de la glycémie car cette glycémie fluctue à chaque minute de la journée en fonction, entre autre, des repas et des activités physiques. Selon l'étude clinique internationale et multicentrique «*Diabetes Control and*

Complications Trial (DCCT)», portant sur plus de 1440 patients atteints du diabète de type I, un meilleur contrôle de la glycémie permet une meilleure prévention du développement des complications ci-haut mentionnées ainsi qu'une diminution de l'hémoglobine glycosylée et de la glycémie moyenne [2]. Suite à ces observations, il parait clair que le développement d'un traitement plus efficace du diabète passe par la régulation «minute par minute» de la glycémie. Pour arriver à ce résultat, deux alternatives font présentement l'objet de recherches : 1) l'utilisation d'une pompe à insuline en circuit fermé avec un capteur constant de la glycémie, ou encore 2) le remplacement des cellules détruites par la transplantation de pancréas ou d'îlots de Langerhans.

Transplantation du pancréas total

Lorsque les patients diabétiques prenant de l'insuline ont un diabète labile, mal contrôlé et développent plusieurs complications causées par ce diabète (néphropathie, rétinopathie…), la transplantation de pancréas peut être envisagée. La transplantation de pancréas total est une technique bien établie puisqu'il n'existe pas moins de 15 000 cas cliniques à travers le monde [3, 4] et dans la plupart des cas, elle est combinée à une transplantation rénale qui a déjà justifié l'immunosuppression [4, 5]. La transplantation de pancréas total offre l'avantage majeur d'un meilleur contrôle de la glycémie, ce qui n'est pas le cas pour les injections quotidiennes d'insuline. Le pancréas transplanté agit comme un pancréas normal en sécrétant les quantités d'insuline nécessaires au bon fonctionnement du métabolisme du glucose et donc à la régulation de la glycémie. Comme souligné par l'étude clinique du DCCT, un meilleur contrôle glycémique diminue significativement le développement des complications reliées au diabète [2]. Cette intervention comporte cependant deux principaux risques et inconvénients. Tout d'abord, le type de chirurgie associé à la transplantation du pancréas total est lourd et peut ainsi entraîner plusieurs complications postopératoires [6-8]. De plus, afin de prévenir le rejet du transplant ainsi

que la récidive de la maladie auto-immune, les patients doivent impérativement prendre une médication immunosuppressive puissante et ce de façon chronique. Ces immunosuppresseurs minent la qualité de vie des patients en affaiblissant de façon générale leur système immunitaire sans oublier les multiples effets secondaires. Finalement, comme la quantité de donneurs de pancréas est faible, ce ne sont pas tous les patients présentant un diabète labile qui peuvent avoir accès à une telle chirurgie, de plus, les bénéfices envisagés par cette transplantation doivent surpasser les inconvénients de l'insulinothérapie.

Transplantation d'îlots

Une autre alternative à la transplantation de pancréas total est la greffe d'îlots de Langerhans. Le concept est simple; on digère le tissu pancréatique afin de ne récupérer que les îlots qui sont ensuite greffés dans le foie des patients de façon non-invasive. Cette technique offre un contrôle glycémique constant comme lors de la transplantation de pancréas, mais sans l'inconvénient de la lourde chirurgie et de la longue récupération postopératoire. Malheureusement, les résultats obtenus lors d'allogreffe d'îlots sont décevants permettant seulement une normalisation de la glycémie dans seulement 12,4% des cas entre 1990 et 1998 (sur un total de 267 greffes) et uniquement 8,2% après un an [9, 10]. C'est en l'an 2000, lors de la publication très médiatisée du protocole d'Edmonton dans le New England Journal of Medicine que la greffe des îlots a regagné en popularité [9]. Dans ce protocole, l'élaboration d'un nouveau cocktail d'immunosuppresseurs sans utilisation de corticostéroïdes, connus pour avoir des propriétés diabétogéniques, a permis l'atteinte d'un taux de succès de 100%, c'est-à-dire que tous les patients transplantés sous ce protocole sont devenus insulino-indépendants [9]. Suite à cette publication, plusieurs centres d'isolement et de transplantation d'îlots ont vu le jour partout à travers le monde [11, 12]. Malgré ce succès, lors d'une publication subséquente concernant le suivi de tous les patients greffés, l'on constate que la durée

moyenne de l'insulino-indépendance est d'environ 15 mois et que seulement 10% des patients n'ont pas eu à recommencer à s'injecter de l'insuline 5 ans suite à la greffe [13]. Par contre, la qualité de vie des patients greffés ainsi que le contrôle général de leur diabète sont restés supérieurs à ce qu'ils étaient avant le greffe d'îlots. La greffe d'îlots de Langerhans partage tout de même quelques inconvénients avec la transplantation de pancréas total. En effet, les patients ayant subis une greffe d'îlots doivent également prendre des immunosuppresseurs afin d'éviter le rejet de la greffe et de la récidive de la maladie auto-immune. De plus, comme le nombre de donneurs de pancréas cadavériques est faible, l'accessibilité à une greffe d'îlots est réservée aux patients présentant des conditions particulières et un mauvais contrôle de leur glycémie malgré les injections quotidiennes d'insuline. En fait, seulement 1% des receveurs potentiels atteint du diabète de type I pourraient bénéficier d'une greffe d'îlots.

Rejet des greffes

Dans certaines maladies où la sécrétion hormonale est compromise, la transplantation cellulaire, dans le but de remplacer les cellules défectueuses, peut être une approche intéressante. Les maladies endocriniennes comme le diabète [9], l'hypoparathyroïdie [14], et l'insuffisance hypophysaire en sont de bons exemples. Ces désordres endocriniens peuvent parfois être causés pas des maladies auto-immunes comme dans le cas du diabète de type I [15], de la maladie Hashimoto [16] ainsi que certaines formes d'hypoparathyroïdie [17] et d'hypogonadisme [18]. Dans ces cas particuliers, si l'on envisage effectuer une transplantation cellulaire, le greffon doit faire face à deux phénomènes immunitaires pouvant causer sa destruction, soit le rejet de la greffe [19, 20] et la récidive de la maladie auto-immune [21, 22].

Comme les cellules greffées sont dans la plupart de cas allogéniques, c'est – à dire de provenance humaine mais non autologue, elles sont reconnues par le système immunitaire du receveur comme étant un corps étranger et induisent un phénomène de rejet [19, 20]. On le décrit en trois différentes phases : le rejet hyperaigu, aigu et chronique [19]. Le rejet hyperaigu se déploie seulement lorsque le receveur possède déjà, dans son répertoire immunologique, des anticorps préformés spécifiques au greffon [23]. Cette réaction immune est très forte et se développe de façon très rapide allant de quelques minutes à quelques heures seulement. Le rejet d'une xénogreffe discordante dans lequel des immunoglobulines (IgM) préformés contre les épitopes α-galactosyl des cellules endothéliales du greffon, un épitope inexistant sur les cellules humaines, est un bon exemple de rejet de greffe hyperaigu [24, 25]. Le rejet aigu quant à lui, est le rejet de greffe le plus communément rencontré lors de transplantations et il survient dans le premier mois suivant la greffe. Cette réaction immunitaire est spécifique à la reconnaissance du greffon comme étant du non-soi [19, 26]. Finalement, le rejet chronique, qui prend place graduellement, allant de quelques mois à quelques années, est un phénomène encore mal caractérisé. Celui-ci semblerait lié à une dégradation à long terme du greffon initiant une réaction immune graduelle et induisant le début de la fibrose autour du transplant [27, 28].

Le rejet aigu des greffes est le phénomène le plus important dans les cas d'allogreffe (donneur de la même espèce que le receveur) et de xénogreffes (donneur d'une espèce différente du receveur). Cette réaction est médiée par la reconnaissance des antigènes du donneur comme étant du non-soi de la part des lymphocytes T du receveur (soi) [19]. Il y a deux modes de reconnaissance du greffon dans le rejet aigu, soit la reconnaissance directe ou indirecte de l'antigène (Figure 4A, page 10). La reconnaissance directe survient lorsque le récepteur des cellules T (RCT) du receveur réagit avec le complexe majeur d'histocompatibilité (CMH) non-soi sur la cellule présentatrice d'antigène (CPA) du donneur [29]. Les CPAs les plus connus étant les macrophages et les cellules dendritiques. Ce concept est appelé alloréactivité. En fait, lorsque l'on compare

deux individus, de 1 à 10% des cellules T du patient A réagiront avec les CPAs du patient B. Il est connu que les tissus ou les cellules greffées sont infiltrés par des CPAS résidents. Ces CPAs peuvent migrer dans les ganglions lymphatiques et initier une réponse immunitaire allogénique des cellules T. Par l'entremise de contact cellule-cellule dans les ganglions, les CPAs du receveur peuvent acquérir le CMH allogénique du donneur et ainsi devenir compétentes à induire une réponse allogénique, menant au rejet de la greffe. La reconnaissance indirecte de l'antigène quant à elle se fait par l'entremise de complexes CMH/antigène inclus dans les vésicules nécrotiques ou apoptotiques [29]. Ces vésicules qui sont relâchées de la surface membranaire des cellules en nécrose ou apoptose, sont prises en charge et processées pas les CPAs du receveur et ensuite présentés au RCTs du receveur. La présentation directe des antigènes est la voie principale empruntée par le système immunitaire du receveur dans les cas d'allotransplantations alors que la voie indirecte est prédominante pour les xénotransplantations, puisque dans la xénotransplantation, les CMHs du donneur et du receveur sont trop différents pour permettre une réaction croisée. Les antigènes du donneur doivent donc être pris en charge par les CPAs du receveur et présentés aux CMHs de classe II du receveur.

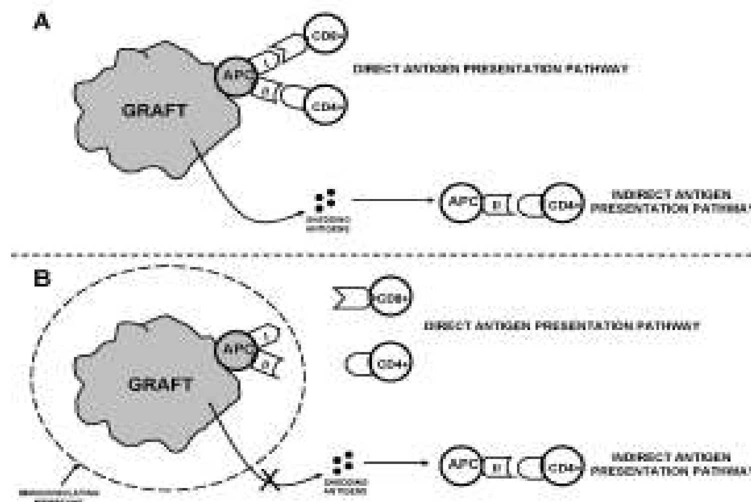


Figure 4 : Représentation schématique de la présentation directe et indirecte des antigènes lors du rejet de la greffe. A. Greffon non encapsulé. B. Greffon encapsulé.

L'activation des lymphocytes T CD4+ via l'interaction RCT-CMH ainsi que les signaux des molécules de co-stimulations induisent une réponse immunitaire spécifique aux cellules β. L'action des lymphocytes T CD4+ se fait de trois façons [22]. Tout d'abord, ces lymphocytes peuvent agir comme cellules auxiliaires en activant les lymphocytes T CD8+ et en les aidants à acquérir des fonctions cytotoxiques. De plus, les lymphocytes T CD4+ peuvent stimuler les lymphocytes B à produire des anticorps spécifiques et finalement, elles peuvent également jouer un rôle effecteur en interagissant directement avec le greffon. En plus des lymphocytes T CD8+ qui sont reconnus pour leur activité cytotoxique, une sous-classe de lymphocytes CD4+ peut également jouer ce rôle cytotoxique. Cette cytotoxicitée est médiée par la relâche de molécules cytotoxiques comme la perforine, le granzyme B ou encore par l'activation du système CD95-CD95L (Fas-FasL).

Concept d'immuno-isolation

L'immuno-isolation de cellules à l'aide de microcapsules à base d'hydrogel est une stratégie proposée afin d'isoler et de protéger celles-ci du système immunitaire du receveur et d'empêcher la prise d'immunosuppreseurs (Figure 5, page 13). À la lumière des progrès faits dans la compréhension des mécanismes de rejet de greffes, il est maintenant connu que, dans le cas d'une allogreffe où la voie de présentation directe de l'antigène est dominante, la seule prévention des interactions cellulaires entre le système immunitaire du receveur et le greffon par une membrane semi-perméable est suffisante pour empêcher l'activation du système immunitaire (Figure 4B, page 10). En effet, plusieurs expériences utilisant l'encapsulation cellulaire l'ont démontré [30, 31]. Entre autres lors de l'utilisation de microcapsules ayant une perméabilité pouvant permettre le passage de grandes molécules telles les anticorps, aucun phénomène de rejet ne fut observé [32].

Étant donné le manque de donneur de pancréas, on ne peut rejeter du revers de la main la possible utilisation des xénogreffes comme source alternative d'îlots. Comme mentionné précédemment, dans le cas de xénogreffes, la voie de la présentation indirecte de l'antigène est la principale voie activée lors du rejet. Pour que cette présentation ait lieu, les complexes CMH/antigène doivent traverser la membrane semi-perméable ce qui ne semble pas être le cas puisque ces complexes sont inclus dans des vésicules apoptotiques ou nécrotiques qui ne pourraient pas, théoriquement, diffuser hors de cette membrane. Quoique ce point soit encore controversé dans la littérature, il existe quelques évidences soutenant ces phénomènes dans lesquels des succès de xénogreffes d'îlots furent rapportés dans quelques modèles animaux [33-36]. La microencapsulation cellulaire pourrait donc permettre la transplantation d'allo et de xénogreffes en bloquant les deux voies de présentation d'antigènes.

L'immuno-isolation offre également un autre avantage non négligeable dans le cas du traitement du diabète de type I, une protection contre la récidive de la maladie auto-immune. Comme lors de l'évolution de cette maladie il y a développement d'anticorps et de cellules T auto-réactives, la transplantation d'îlots présentant le même type d'antigènes que les cellules d'origine du pancréas pourrait réactiver le processus auto-immun en détruisant le greffon [31]. La microencapsulation des îlots à l'intérieur de membranes semi-perméables qui empêchent les cellules T et les anticorps d'atteindre et de détruire le greffon est un moyen de les protéger contre la maladie auto-immune. Le succès de transplantations d'îlots microencapsulés dans des modèles animaux de diabète auto-immun ont d'ailleurs déjà été publiés [31, 37].

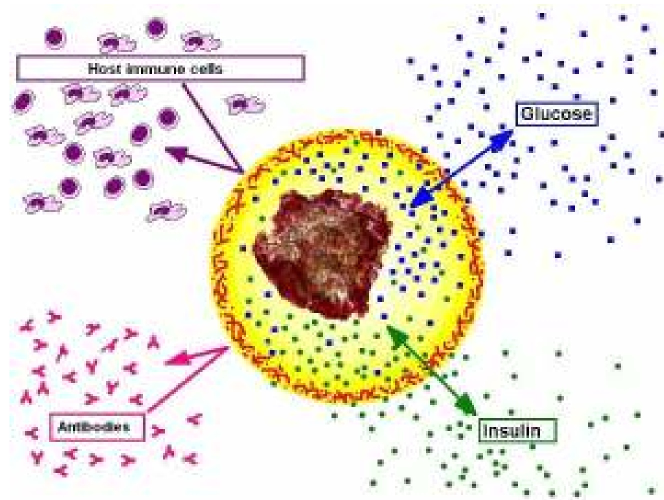


Figure 5 : Concept d'immuno-isolation où la membrane semi-perméable permet la libre diffusion de petites molécules comme le glucose et l'insuline mais empêche le passage de molécules de plus grand poids moléculaire tels que les cellules immunes et les anticorps [38].

Sites de transplantation

Que ce soit pour des îlots encapsulés ou non-encapsulés, le site de transplantation choisi pour effectuer la greffe est d'une importance primordiale puisqu'il peut influencer directement le succès de la transplantation. Pour des raisons historiques, le site de transplantation le plus utilisé pour la transplantation d'îlots microencapsulés est le péritoine. La transplantation de microcapsules dans le péritoine est extrêmement facile car celles-ci sont tout simplement injectées par l'entremise d'un cathéter et ne requiert aucune anesthésie chez l'animal. Le péritoine à également l'avantage de pouvoir accueillir un grand volume [39]. Ceci était une condition déterminant à l'époque où la taille des microcapsules variait entre 700 µm et 1,5 mm de diamètre et que le volume d'îlots encapsulés à transplanter était élevé. Avec le développement de nouvelles techniques d'encapsulation, comme l'utilisation d'un générateur d'ondes électrostatiques, la taille des

microcapsules a diminuée pour atteindre un diamètre moyen variant de 200 μm à 350 μm, amoindrissant ainsi le volume à transplanter de façon considérable (ex : le volume à transplanter pour des microcapsules de 800 μm vs 300 μm est de 264 ml vs 14 ml respectivement, pour 1 million de microcapsules). La diminution du volume à transplanter a donc ouvert la porte à d'autres sites potentiels pour la transplantation des îlots microcapsulés comme le foie [40-42] et la capsule rénale [43-45]. Le nombre élevé d'études ayant été conduites dans le péritoine ont également mis en évidences d'autres désavantages reliés à ce site. Le péritoine est un site peu vascularisé et où la tension d'oxygène est faible, ce qui implique que la diffusion des nutriments et du glucose vers l'îlot ou de l'insuline vers la circulation sanguine est retardée [46-48] et que les îlots encapsulés seront exposés à l'hypoxie [49, 50]. Finalement, la composition cellulaire du liquide péritonéal est également problématique puisque constituée majoritairement de macrophages, à plus de 85%, qui sont les cellules jouant un rôle déterminant lors de réactions à corps étranger [51].

Le foie est déjà largement utilisé lors de la transplantation d'îlots non encapsulés [9, 11, 52]. C'est un site dans lequel on peu aisément transplanter via l'utilisation de techniques de chirurgie minimalement invasives [53]. De plus, le transplant baigne directement dans le flot sanguin, lui permettant de réagir rapidement en fonction de la glycémie. Cependant, il a été démontré que ces îlots peuvent être exposés de façon chronique à de hauts taux de glucose provenant de l'absorption intestinale et de la glycogénolyse du foie et à des hautes doses de drogues (médicaments, immunosuppresseurs) [54-56]. De plus, il semble que la fonction des cellules α soit altérée, c'est-à-dire que la sécrétion de somatostatine lors d'épisodes hypoglycémiques est grandement diminuée, et ce, de façon spécifique aux îlots transplantés dans le foie [57, 58]. Les îlots sont également soumis à une basse tension d'oxygène comme pour le péritoine [59]. Finalement, des perturbations au niveau du foie ont déjà été rapportées lors de la transplantation d'îlots non encapsulés tels que la nécrose et la stéatose hépatique [60, 61]. Étonnamment, peu d'études ont démontré la faisabilité de la transplantation d'îlots

microencapsulés dans le foie. Plusieurs dangers reliés à la transplantation d'îlots microencapsulés peuvent être considérés, tels que la possibilité d'induire une réaction immunitaire contre la microcapsule, de créer des thromboses sévères ou encore d'augmenter de la pression portale à cause de la taille des microcapsules. Une étude publiée en 1999 par notre groupe a pourtant démontré la faisabilité d'une telle transplantation [41]. En effet, nous avons démontré, chez le rat, que la transplantation de microcapsules vides d'environ 315 μm de diamètre n'avait qu'un effet minimal et transitoire sur la pression portale [41]. Ces résultats ont été confirmés par un autre groupe, en 2003, avec la transplantantion des microcapsules vides de 400 μm de diamètre chez le porc [40]. Par contre, à ce jour, il n'existe aucune étude concernant la fonction ou la survie d'îlots microencapsulés dans le foie.

La capsule rénale comme site de transplantation d'îlots est également de plus en plus utilisée. Il s'agit d'un site intéressant puisqu'il est très vascularisé, avec un bon apport en oxygène et en nutriments et assurant une diffusion rapide de l'insuline [62]. Plusieurs succès ont d'ailleurs été rapportés lors de transplantations d'îlots non encapsulés [43-45]. De plus, en cas de problème grave avec la greffe, il est toujours possible de retirer le greffon par néphrectomie, ce que l'on ne peut faire pour la transplantation dans le foie. Par contre, ce site limite la taille du transplant puisque l'espace disponible dans la capsule rénale est restreint.

Dans le domaine de la transplantation d'îlots, qu'ils soient encapsulés ou non, les recherches pour un site de transplantation idéal sont de plus en plus nombreuses. Plusieurs sites, autres que ceux mentionnés ci-haut, sont présentement sous investigation tels que la rate [63-67], des sites intra-dermiques ou inter/intra-musculaires [36, 68], l'épiloon [69-71] et également des sites artificiels [72].

Microencapsulation cellulaire

Une des stratégies proposée afin de séparer les cellules de leur environnement est l'encapsulation dans des hydrogels. Ces hydrogels sont fabriqués à partir de polymères ayant la capacité de former des structures tridimensionnelles sous forme de gel pouvant absorber de grandes quantités de molécules d'eau. Comme certains de ces hydrogels peuvent être constitués jusqu'à 99% d'eau, ils créent une structure compatible avec la survie des cellules encapsulées à l'intérieur du gel tout en étant bien tolérés dans le corps humain. La formation de ces hydrogels offre un support tridimensionnel aux cellules tout en les isolant de l'extérieur grâce aux réseaux des chaînes de polymères qui empêchent tout ce qui a un haut poids moléculaire de pénétrer à l'intérieur du gel y compris les cellules.

Polymères utilisés en microencapsulation

Deux types principaux de polymères peuvent être utilisés afin d'immuno-isoler les cellules de leur environnement, soit les polymères naturels ou synthétiques. Des polymères naturels comme l'alginate, l'agarose [31, 73-76], le collagène [77, 78] et l'acide hyaluronique [79-82] sont présentement à l'étude comme système d'encapsulation des îlots de Langerhans. Le processus associé à la gélification de l'agarose est du type température-dépendant, c'est-à-dire qu'au dessus d'une température donnée, l'agarose se retrouve sous sa forme liquide alors qu'elle se solidifie si la température passe sous ce point. Les microcapsules d'agarose sont généralement obtenues par émulsion [74] ou encore par extrusion de l'agarose liquide [83], puis gélifiées en diminuant la température du système. Le collagène quant à lui, est une protéine connu comme faisant partie du tissu conjonctif et jouant un rôle structurel. La gélification du collagène est dépendante de deux variables : la température et le pH. Contrairement à l'agarose, le collagène gélifie plus rapidement s'il est maintenu à haute température mais formera un gel moins solide si la solution de collagène utilisé est à pH trop acide. L'encapsulation avec le collagène est surtout observée lors de l'encapsulation de cellules nécessitant un encrage pour leur survie [84-86]. Comme le

collagène est une composante naturelle de la matrice extracellulaire, c'est le polymère tout désigné pour ce type d'encapsulation. L'acide hyaluronique est un glycoaminoglycan normalement retrouvé dans le liquide synovial ou encore faisant partie de l'humeur vitreux de l'œil [87]. Il se retrouve également au niveau du tissu conjonctif. Ce polymère peut également être utilisé afin de fournir aux cellules encapsulées une structure tridimensionnelle à laquelle ils peuvent s'accrocher.

Il existe une multitude de polymères synthétiques pouvant servir à l'encapsulation cellulaire. Certains sont même de bons candidats car ils sont bien tolérés par le système immunitaire du receveur [88]. Les plus fréquents sont les dérivés du méthacrylate [89-96] comme le poly(methyl méthacrylate), poly(2-hydroxyéthyl méthacrylate), le poly(méthacrylique acide) et le populaire poly(2-hydroxyéthyl méthacrylate-co-méthyl méthacrylate) [P(HEMA-co-MMA)], pour ne nommer que ceux là. Que l'on utilise des polymères naturels ou synthétiques, chacun présentent ses avantages et désavantages. Les polymères naturels sont souvent considérés comme étant plus physiologique alors que les synthétiques ont une structure et des caractéristiques plus contrôlées à cause de leur synthèse en laboratoire. Cependant, les deux peuvent présenter des problèmes de biocompatibilité, l'un pouvant être contaminé par leur environnement et leur extraction, l'autre par les produits utilisés pour leur fabrication comme des monomères et des initiateurs de polymérisation.

Hydrogel d'alginate

De nos jours, l'alginate est le polymère le plus utilisé afin d'immuno-isoler les îlots de Langerhans. C'est vers la fin des années 40 que l'on s'intéresse aux propriétés particulières de l'alginate. On remarque alors que ce polymère est capable de séquestrer différents ions et de former des gels. Elle sera surtout utilisée à ce moment comme moule pour prendre des impressions dentaires [97-99], comme agent séquestrant de métaux lourds [100-102] et comme moyen de neutraliser l'acidité gastrique [103-105]. La première

utilisation de l'alginate afin d'encapsuler des cellules vivantes fût publiée en 1980 où Pilwat *et coll.* [106] ont démontré que l'immobilisation d'érythrocytes dans un gel d'alginate permettait d'améliorer leur conservation comparativement à une simple suspension cellulaire. Dans la même année, on rapporte l'encapsulation de protoplastes et également la toute première encapsulation d'îlots de Langerhans par le Lim et Sun [107]. Les caractéristiques qui distinguent l'alginate des autres polymères sont, entre autres, sa capacité à former facilement et rapidement un hydrogel dans des conditions compatibles avec la vie et sa biocompatibilité avec les cellules encapsulées. De plus, depuis maintenant le début des années 60, ce polymère a été hautement caractérisé en termes de composition, structure et polymérisation. Une panoplie d'outils de caractérisation et d'évaluation se rapportant à la formation d'hydrogel d'alginate a été développée au fil des années de même qu'un savoir-faire considérable.

Composition et structure de l'alginate

L'alginate est un polysaccharide qui est principalement extrait des algues marines mais qui peut également être produit par certaines bactéries comme le *Pseudomonas aeruginosa* [108] et l'*Azotobacter vinelandii* [109, 110]. Ce polysaccharide est un copolymère composé de deux acides uroniques, l'acide β-L-guluronique (G) et l'acide α-D-mannuronique (M) distribués de façon aléatoire (Figure 6, page 19). Trois types de blocs peuvent être ainsi formés le long de la chaîne : les blocs homopolymériques G composés d'acide guluronique, les blocs homopolymériques M composés d'acide mannuronique et les blocs MG alternant les deux acides. Ces blocs peuvent être de longueurs variables [111, 112]. Dans cette structure, ce sont les blocs G qui sont responsables de la gélification de l'alginate [113, 114]. Plus le nombre de blocs G est élevé ou encore, plus les blocs G sont longs, plus le gel formé sera rigide. Cette caractéristique origine du rôle que joue ce polysaccharide chez l'algue marine. L'alginate est une composante des parois cellulaires qui enrobent les cellules et leur offre un support structurel et l'on croit que cette paroi est également responsable de la flexibilité de l'algue [115, 116]. En effet, l'alginate extrait des

algues près des côtes est plutôt riche en blocs G car elle n'a pas besoin d'autant de flexibilité que les algues en courant fort où l'alginate extraite est plus riche en blocs M. En conséquence, toute un gamme d'alginate ayant différents ratios M/G peut être utilisée, dépendamment des caractéristiques voulues pour une application donnée.

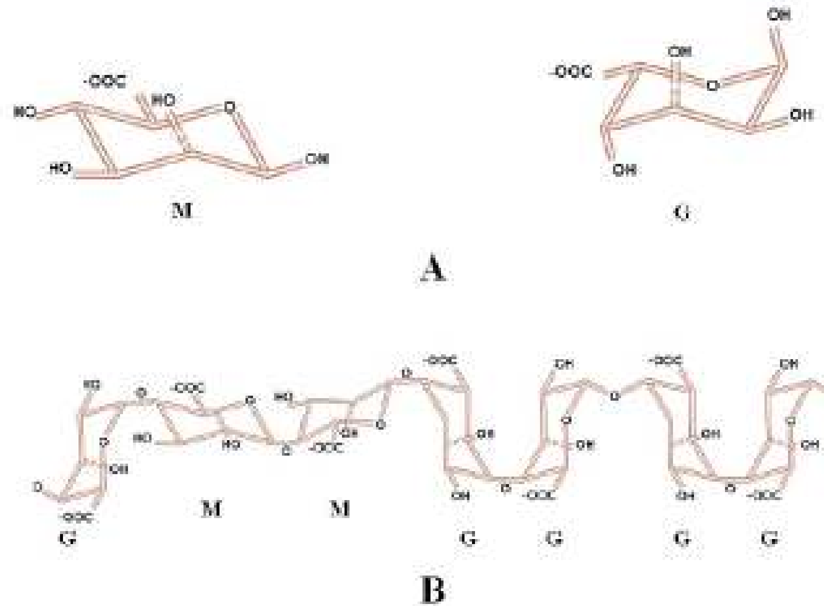


Figure 6 : Composition chimique de l'alginate. A. Acide mannuronique (M) et acide guluronique (G). B. Chaîne d'alginate contenant à la fois bloc G et bloc M [38].

Mécanismes de gélification

L'alginate a l'avantage de pouvoir gélifier et former un hydrogel dans des conditions qui sont compatibles avec la vie (pH et température constants). Elle est constituée de résidus polyanions et forme une chaîne chargée négativement lorsque dissoute dans une solution aqueuse ionique. L'hydrogel se forme lorsque les chaînes d'alginates rencontrent des cations divalents tels que le Ca^{2+} ou le Sr^{2+}. Cette interaction entre l'alginate et les cations divalents fut décrite pour la première fois en 1973 par Grant *et coll.* [113]. Il a été démontré que ces cations se lient de façon coopérative aux blocs G entraînant l'adoption d'une structure appelée modèle «egg-box» (Figure 7, page 21). Dans ce modèle, les unités G consécutives s'arrangent de façon tridimensionnelle pour créer un

«nest-like» structure où le cation divalent va se nicher [117]. Pour cette raison, ce sont les blocs G qui sont responsables de la gélification des chaînes d'alginate [113, 114, 118] dictant les propriétés mécaniques de l'hydrogel en fonction du contenu en bloc G de l'alginate. La rigidité de l'hydrogel n'est pas seulement déterminée par le ratio M/G de l'alginate utilisée mais également par le type de cation divalent utilisé lors de la gélification [111, 119]. L'alginate se lie préférentiellement à certain cations selon la charte suivante : Pb>Cu>Cd>Ba>Sr>Ca>Co=Ni=Zn>Mn [119]. Dans le contexte de l'encapsulation cellulaire dans l'alginate, on utilise principalement le baryum, le calcium et le strontium, quoique ce dernier soit moins populaire que les deux autres cations [112, 120]. Les hydrogels d'alginate de calcium sont très utilisés car ils sont connus pour ne pas avoir d'effet négatif sur la survie des cellules encapsulées. Par contre, comme les hydrogels de calcium forment des gels moins solides, ils peuvent être plus facilement déstabilisés par la présence d'autres cations tels que le sodium (Na^{+}) [121]. En effet, lorsque les hydrogels de calcium sont en contact avec de grandes concentrations de Na^{2+}, il peut y avoir échange d'un ion Ca^{2+} pour deux cations Na^{+}, affaiblissant les liens chimiques dans l'hydrogel et créant un appel d'eau vers l'intérieur de la microcapsule causant un gonflement de celle-ci [122]. Pour augmenter la résistance chimique et mécanique de ces microcapsules fait d'hydrogels de calcium, on utilise ces hydrogels en complexation avec des polyélectrolytes comme la poly-L-lysine (voir section Complexation de l'alginate avec les polycations23). On peut également augmenter la rigidité de l'hydrogel en remplaçant le calcium par le baryum qui possède une plus forte affinité pour l'alginate. Le baryum est donc moins sujet à être déplacé par d'autres ions. Il a également été démontré que les ions de baryum n'interagissaient pas seulement avec les blocs G mais aussi avec les blocs M [112]. Les concentrations de baryum utilisées pour la gélification doivent cependant rester basses car on craint que ces ions puissent inhiber les canaux potassiques dans les membranes cellulaires [123] et interférer avec la fonction cellulaire et la sécrétion d'insuline. Comme les hydrogels de baryum sont plus résistants que ceux au calcium, ceux-ci peuvent être

utilisés comme dispositif immuno-isolant sans complexation avec des polyélectrolytes [30, 124, 125].

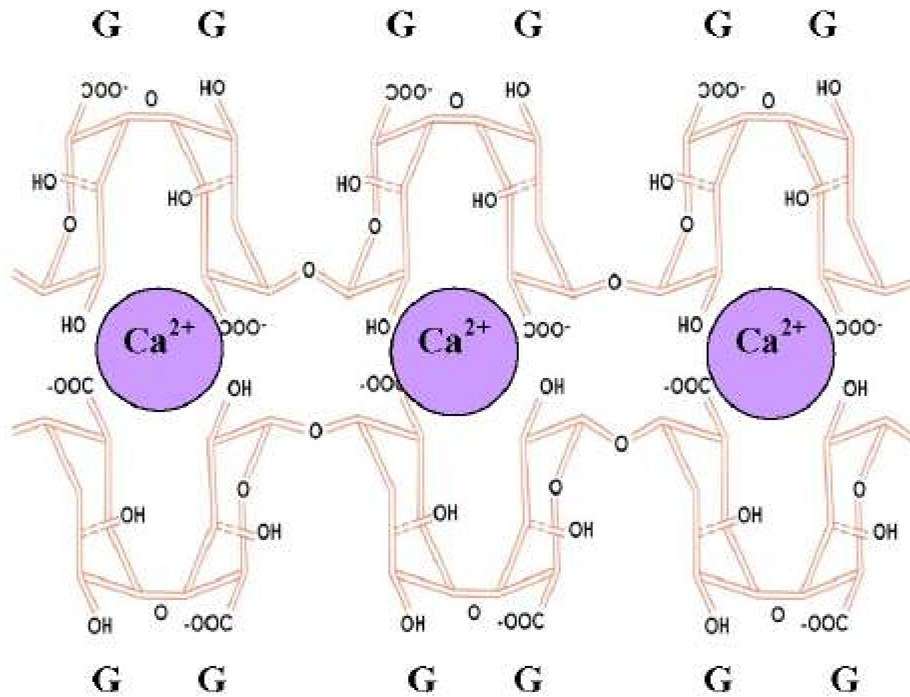


Figure 7 : Illustration du modèle «egg-box» [38].

Formation des microcapsules

Les microcapsules utilisées comme dispositif immuno-isolant sont formées à partir d'une solution d'alginate de sodium (Figure 8, page 23). Cette solution est extrudée à travers une aiguille afin de former des fines gouttelettes. Deux types d'appareils sont principalement utilisés pour diminuer la taille des gouttelettes d'alginate, soit un appareil à jet d'air qui aspire l'alginate hors de l'aiguille et détachent les gouttelettes de l'aiguille avant qu'elles n'atteignent leur pleine taille ou encore un générateur d'impulsions électrostatiques qui, à l'aide d'un courant électrique, détache les gouttelettes de la pointe de l'aiguille. En fait, le mécanisme d'action des charges électrostatiques est de diminuer la

tension de surface qui retient la gouttelette d'alginate à l'aiguille. Cette dernière technique est plus avantageuse, puisque grâce au courant électrique, les gouttelettes formées peuvent atteindre de plus petits diamètres. Une fois les gouttelettes formées, elles entrent en contact avec une solution riche en cations divalents qui gélifient instantanément l'alginate. La gélification rapide de l'alginate est un processus favorable puisqu'elle permet de conserver la forme sphérique parfaite que la gouttelette avait prise dans l'air lui donnant une surface lisse connue pour être un paramètre important pour la biocompatibilité du dispositif [126]. Les microcapsules ainsi formées peuvent être utilisées ainsi sans traitement supplémentaire, à condition qu'elles aient été gélifiées avec le baryum, les billes de calcium étant trop fragiles pour résister *in vivo* à long terme. Ces microcapsules sont en fait appelées billes d'alginate, le terme microcapsule étant plutôt réservé pour les microcapsules complètes complexées avec les polycations. Lorsque des cellules doivent être encapsulées, elles sont préalablement mélangées à la solution d'alginate avant l'extrusion à travers l'aiguille.

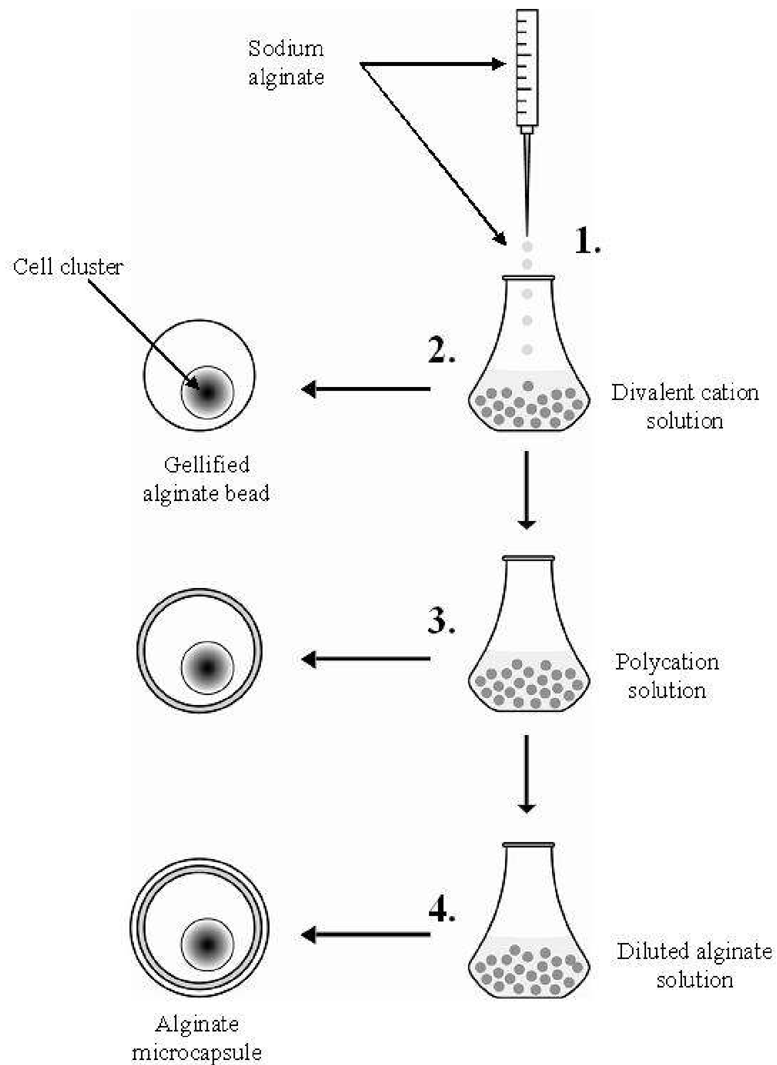


Figure 8 : Schéma de la fabrication des billes d'alginate et des microcapsules complètes. 1. Génération des gouttelettes d'alginate à l'aide d'un appareil électrostatique ou d'un système Air-jet. 2. Gélification des gouttelettes lors du contact avec la solution de cations divalents et formation de billes d'alginate. 3. Complexation des billes d'alginate avec le polycation. 4. Formation des microcapsules complètes suite à une incubation dans une solution d'alginate diluée [38].

Complexation de l'alginate avec les polycations

Une fois les billes d'alginate formées, elles peuvent ensuite être incubées dans une solution de polycations. C'est le polycation qui est responsable de la perméabilité restreinte

de la microcapsule puisqu'il formera un treillis moléculaire en interagissant avec l'alginate. Les billes d'alginate ont une charge nette négative nous permettant de les complexer avec un polycation comme la poly-L-lysine (PLL) (Figure 8, page 23). Grâce à des interactions électrostatiques entre l'alginate et PLL, les molécules de PLL pourront enrober les billes d'alginate. Les polycations sont connus pour interagir principalement avec les blocs M de l'alginate. Finalement, les billes enrobées de PLL sont ensuite incubées dans une solution diluée d'alginate afin de recouvrir correctement la PLL connue pour être immunogénique, en plus de neutraliser la charge nette de la microcapsule. Ces microcapsules alginate-poly-L-lysine-alginate peuvent également être appelées microcapsules APA.

L'alginate peut également être utilisée en combinaison avec d'autres polycations ou polymères pour former des microcapsules. L'énantiomère de la PLL, la poly-D-lysine (PDL) [127] ainsi que la poly-L-ornithine (PLO) [128], qui possède la même structure que la PLL mais qui a un groupement fonctionnel plus court, $(CH)_3$-NH_2 vs $(CH)_4$-NH_2, sont également utilisé dans la littérature. Des études comparatives entre des microcapsules fabriquées avec PLL, PDL ou PLO montrent que ces trois polycations engendrent des microcapsules ayant une morphologie et une biocompatibilité similaire [127]. Il semble que la PLO offre un avantage, comparativement à la PLL originalement utilisée, sur le plan de la résistance à divers stress [129]. Il est suggéré que le groupement fonctionnel plus court de la PLO soit en mesure d'interagir de façon plus efficace avec la bille d'alginate créant ainsi des microcapsules ayant une membrane semi-perméable plus épaisse et plus résistante aux stress. L'alginate peut également être complexée au chitosan, un polymère naturel extrait de l'exosquelette de crustacées [130]. Quoique moins utilisées que les microcapsules standards APA, la combinaison de l'alginate et du chitosan est connu pour être très stable, ce qui pourrait constituer un avantage non négligeable au niveau de la biocompatibilité puisque le possible relarguage de polycations dans l'environnement de la microcapsule, connu pour activer le système immunitaire, est diminué [131, 132]. Toujours afin d'améliorer la stabilité des microcapsules, Orive *et coll.* [133] ont proposé l'utilisation de poly(methylene-co-guanidine) (PMCG) comme polycation, son utilisation anciennement

décrite pour des microcapsules à base de cellulose-sulphate [134]. La complexation de l'alginate et du PMCG mène à la fabrication de microcapsules qui ont une stabilité plus grande que les microcapsules standards [133]. De nos jours, la quantité de polymères et de polycations disponibles est énorme et les possibilités de complexation entre les deux l'est d'autant plus. Il faut donc bien cerner au départ quels sont les besoins et les caractéristiques que les biomatériaux doivent démontrer pour pleinement remplir leurs fonctions.

Problématique

Tout d'abord, qu'est-ce que la biocompatibilité d'un biomatériau? Un biomatériau est biocompatible lorsqu'il effectue adéquatement la tâche pour laquelle il a été conçu. Dans l'optique de la transplantation d'îlots microencapsulés, le dispositif doit posséder certaines caractéristiques, entre autre de promouvoir la survie et la fonction des cellules encapsulées, protéger le greffon du système immunitaire de l'hôte, ne pas engendrer de réaction immunitaire contre le biomatériau lui-même, ne pas se dégrader prématurément, etc. Dans cette thèse nous avons abordé deux aspects reliés à la biocompatibilité des microcapsules APA.

Dans la première partie, nous nous sommes interrogés sur les interractions entre la surface des microcapsules et le système immunitaire de l'hôte et la relation existante entre la présence de contaminants dans l'alginate utilisée dans la fabrication des microcapsules et le développement d'une réaction immunitaire contre cette surface. Nous avons comparé quelques procédés de purification d'alginate ayant différents rendement et étudié la biocompatibilité de ces alginates *in vivo*. Notre hypothèse était que l'amélioration des procédés de purification afin de réduire au maximum les contaminants de l'alginate allait améliorer la biocompatibilité des microcapsules.

Dans la deuxième partie, nous nous sommes intéressés à la stabilité et la résistance des microcapsules APA et de l'effet de cette stabilité sur la protection du receveur. Notre

hypothèse était que l'amélioration de la stabilité des microcapsules APA allait augmenter sa biocompatibilité et également mieux protéger le receveur contre les cellules du greffon.

Afin d'aider à la compréhension, les deux parties (Purification d'alginate et Protection de l'hôte), ont été divisées et traitées de façon séparée possédant chacune une introduction et une discussion gérérale.

Partie I : Purification d'alginate et réaction immunitaire de l'hôte

L'implantation d'un biomatériau dans le corps humain engendre toute une cascade de phénomènes qui font partie de la réaction immunitaire face à l'introduction d'un corps étranger. Cette réaction s'effectue en plusieurs étapes :

1. **Procédure d'implantation.** Tout d'abord, la procédure d'implantation d'un biomatériau s'accompagne nécessairement d'un bris des tissus et/ou de vaisseaux sanguins au site d'injection, induisant dans un premier temps les phénomènes immunologiques normaux reliés à la cicatrisation d'une blessure [135, 136]. Il y a d'abord initiation de la coagulation sanguine et de la thrombose afin de former un caillot sanguin et freiner la perte de sang. Les plaquettes relâchent également des facteurs de croissance et des protéines faisant partie de la matrice extracellulaire qui favorise la migration et la prolifération de cellules comme les neutrophiles. Parallèlement à ce phénomène de cicatrisation, les protéines présentes dans l'environnement vont s'adsorber à la surface du biomatériau implanté. Selon l'effet Vroman, les protéines les plus mobiles interagiront rapidement avec la surface et seront graduellement remplacées par des protéines moins mobiles mais ayant une plus grande affinité pour la surface du biomatériau [137]. Le fibrinogène, la fibronectine, l'albumine, les γ-globulines et la vitronectine sont quelques unes des protéines connues pour s'adsorber facilement à la surface des biomatériaux [135, 136]. Ces protéines adsorbées forment en quelque sorte une matrice extracellulaire provisoire avec laquelle les cellules pourront éventuellement interagir. L'adsorption protéique à la surface des biomatériaux dépend entièrement des propriétés physico-chimiques de cette surface (hydrophilicité, rugosité, composition chimique, etc.) Ces propriétés détermineront quelles sont les protéines qui s'adsorberont mais également la conformation que ces protéines adopteront lors de l'adsorption.

   Parmi les protéines pouvant interagir avec la surface d'un biomatériau l'on retrouve également la famille des protéines de la cascade du complément [138]. Les mécanismes exacts par lesquels le complément entre en jeu dans la réaction à corps

étranger sont encore mal connus. Cependant, il y a quelques évidences dans la littérature qui démontrent que des protéines telles que le C3, le C3b et les IgG vont opsoniser la surface de biomatériau et éventuellement former les complexes enzymatiques C3 et C5 convertases qui vont fragmenter le C3 et le C5 [139]. Ces fragments pourront servir de boucle d'amplification du signal (adsorption de plus de C3 à la surface), de ligand pour les récepteurs du C5a à la surface des neutrophiles et macrophages [140] et de signal d'activation cellulaire par l'entremise de la formation du complexe d'attaque membrane (CAM) de la part du C5b [138].

2. **Phase aiguë de l'inflammation.** Les neutrophiles sont les principaux intervenants lors de la phase aiguë. Ils vont transmigrer de la circulation sanguine au site d'inflammation par chemotaxisme [141, 142]. Ils agissent comme des nettoyeurs au niveau de la plaie en phagocytant des débris cellulaires, dégradant les tissus endommagés à l'aide de protéases et ils débarrasseront le site de potentielles bactéries. Conjointement avec les mastocytes, ils vont sécréter de grandes quantités de chemokines et d'histamine afin de recruter les cellules phagocytaires au site du dommage [143].

3. **Phase chronique de l'inflammation.** L'inflammation, un processus normal de la cicatrisation, se poursuit avec l'arrivée de monocytes/macrophages et des lymphocytes [136]. Ces cellules auront pour tâche de phagocyter bactéries, débris tissulaires, cellules mortes et de sécréter des facteurs de croissance et cytokines afin d'éventuellement attirer les fibroblastes et de favoriser leur prolifération. Ils jouent également un rôle dans l'induction de l'angiogénèse. Lorsqu'un biomatériau est présent au site de la lésion, c'est à ce stade qu'il fera une différence dans les phénomènes normaux de cicatrisation et favorisera le développement d'une réaction immune à corps étranger. Les protéines adsorbées à la surface du biomatériau qui forment une matrice extracellulaire temporaire vont favoriser l'adhésion des

macrophages via leur intégrines, des récepteurs membranaires spécifiques aux molécules de la matrice extracellulaire. La liaison des intégrines du macrophage aux protéines adsorbées envoi des signaux intracellulaires lui indiquant de mettre en branle les événements pour favoriser son adhésion à la surface ainsi que son étalement grâce à l'induction de la formation de podosomes [144]. Une fois les macrophages adhérés, la sécrétion d'interleukine-4 (IL-4) et d'interleukine-13 (IL-13) qui augmente les quantités de récepteur au mannose, connu pour être impliqué dans la fusion cellulaire, en combinaison avec la proximité des cellules adhérées vont induire la formation de cellules géantes, spécifiques aux réactions immunes à corps étranger [145-147]. Les macrophages et les cellules géantes adhérées ne pouvant phagocyter le biomatériau subiront ce qui a été décrit comme le «frustrated phagocytosis» où les macrophages et les cellules géantes créeront un site particulier et où ils relâcheront de grandes quantités d'enzymes de dégradation, d'acides et de radicaux libres afin de tenter de dégrader et de détruire le biomatériau [148, 149].

4. **Fibrose.** Dans la dernière phase, les fibroblastes viendront s'accumuler au pourtour de la surface du biomatériau en plusieurs couches successives, ce que l'on appelle tissu granuleux, et ils se mettront à produire des protéoglycans, des fibres de collagène (particulièrement le collagène de type III) et des protéines de la matrice extracellulaire [135]. Il y aura finalement formation d'une capsule fibreuse tout autour du biomatériau afin de l'isoler du système et ainsi compromettre sa fonction.

La réaction immunitaire engendrée par l'implantation de microcapsules est appelée plus communément la Réaction de l'Hôte contre la Microcapsule (RHM) et, selon les données publiées dans la littérature, elle semble adhérer au patron décrit lors de réactions immunes à corps étranger (voir Figure 9, page 32). En effet, suite à une implantation *in vivo*, des protéines s'étant adsorbées à la surface de microcapsules APA furent identifiées par des immunobuvardage de type Western, telles que le fibrinogène, la fibronectine, le

plasminogène, la vitronectine, l'albumine, le facteur XI, XII, les IgGs, ainsi que des protéines de la cascade du complément comme le C3, C3a, C3b, les facteurs B, H et I [150]. L'adsorption protéique fut également confirmée par une technique physicochimique, le X-Ray Photoelectron Spectroscopy (XPS) et par la détection d'une augmentation du rapport azote/carbone dans les 7 premiers jours suite à l'implantation des microcapsules [151]. Les neutrophiles [152], les granulocytes et les basophiles [151], les érythrocytes [151] et les macrophages [151-155] ont été identifiés comme étant des joueurs importants dans la RHM à court terme (entre 0 et 7 jours). La présence inattendue d'érythrocytes pouvant probablement s'expliquer par le bris de vaisseaux sanguins lors de la procédure d'implantation. Comme mentionné plus haut, les cellules granulocytes comme les neutrophiles et les basophiles sont connues pour leur pouvoir de dégranulation et de sécrétion de cytokines et plus particulièrement de chemokines. Ces produits de sécrétions ont effectivement été détectés, comme l'interleukine-1β (IL-1β) et l'interleukine-6 (IL-6) [152]. Les macrophages, quant à eux, semblent jouer un rôle central puisqu'ils sont pratiquement détectés à tous les stades de la RHM, et il a été montré *in vitro* qu'ils pouvaient sécréter de grande quantité d'IL-1β et de Tumor Necrosis Factor-α (TNF-α) s'ils étaient incubés avec des microcapsules APA [156]. Dans un stade plus avancé de la RHM (entre 7 et 14 jours), les macrophages [151, 152, 154, 157] sont effectivement toujours présents en grandes quantités ainsi que les cellules géantes [151, 154], les fibroblastes [151, 154, 157] et les lymphocytes [152], quoique ces derniers ne soient pas toujours décelés [151]. Le profil de sécrétion de cytokines est également un peu différent puisque l'on retrouve en plus une sécrétion accrue de Tissue Growth Factor-β (TGF-β) une cytokine connue pour être impliquée dans le développement de la phase fibrotique [152, 158]. Finalement, dans le stade final du développement de la fibrose (entre 28 et 60 jours), la plupart des types cellulaires décris ci-haut ont disparu pour laisser la place en majorité aux fibroblastes, à quelques cellules géantes et également à des cellules mésothéliales [154, 159].

Le développement de la RHM a également deux effets majeurs sur la fonction et la survie des cellules encapsulées. Premièrement, cette RHM dans laquelle il y a sécrétion de cytokines et d'histamine par les cellules immunes du receveur, peut avoir un effet sur les cellules encapsulées. Comme les îlots sont entourés d'une membrane semi-perméable ayant une perméabilité restreinte, certaines cytokines de plus hauts poids moléculaires comme le TGF-β (~51 kDa) et TNF-α (~27 kDa) peuvent difficilement pénétrer la microcapsule alors qu'en théorie, les cytokines de faibles poids moléculaires comme les interleukines (~17 à 21 kDa) et l'histamine (~110 Da) pourraient avoir un effet négatif sur la survie des cellules encapsulées [160]. Il a également été démontré que, en comparant le profil de cytokines produites en implantant des microcapsules vides versus des microcapsules contenant des îlots, la nature des cytokines détectées étaient différentes, indiquant une probable production de cytokine de la part du greffon (telles que le Macrophage Chemotaxism Protein-1, Macrophage Inflammatory Protein, NO et IL-6) [161]. Il est suggéré, dans la littérature, que cette production de cytokines par le greffon serait en réponse aux cytokines produites par les cellules immunes du receveur [151, 162]. Afin de contrer l'effet des cytokines, une des stratégies mise de l'avant est la réduction de la perméabilité de la membrane semi-perméable des microcapsules. Il semble que la réduction de la perméabilité, même si cette perméabilité reste plus grande que la taille de l'IL-1β (~17 kDa) par exemple, réussisse à diminuer la diffusion de cette cytokine de 24 % à 6 %, tout en laissant l'insuline circuler librement [163, 164]. Il semble que pour que les cytokines influencent la survie des îlots, elles doivent être présentes en quantités suffisantes dans la microcapsule mais aussi en combinaisons spécifiques [161, 165]. Deuxièmement, la formation de la RHM, surtout lors de son stade fibrotique, détériore les échanges entre les cellules encapsulées et l'environnement extra-microcapsulaire [166, 167]. En effet, lorsqu'il y a développement d'une réaction péricapsulaire suite à une transplantation d'îlots microencapsulés dans des modèles animaux diabétiques, on constate une récidive de la maladie, c'est-à-dire que le greffon n'arrive plus à contrôler la glycémie [166, 168]. Deux phénomènes reliés à la RHM peuvent produire cette perte de contrôle. Tout d'abord,

l'accumulation de cellules immunes tout autour de la membrane semi-perméable des microcapsules et l'amoncèlement des fibres de collagène peuvent retarder la réponse des îlots au taux de glucose circulant mais également ralentir la diffusion de l'insuline hors de la microcapsule. D'une autre côté, la RHM peut également empêcher le passage de nutriments et de l'oxygène vers l'intérieur de la microcapsule et la sortie des déchets métaboliques hors de la microcapsule causant ainsi la mort prématurée des cellules encapsulées par nécrose et/ou hypoxie.

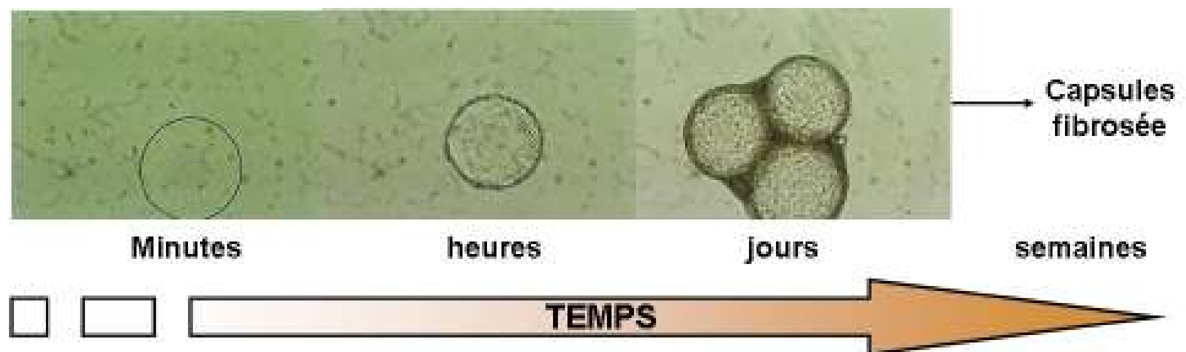


Figure 9 : Cinétique du développement de la réaction de l'hôte contre la microcapsule (RHM).

Afin d'améliorer la biocompatibilité *in vivo* des microcapsules et avant même de se questionner sur les propriétés de surface de celles-ci, il est important de s'interroger sur la pureté des polymères utilisés pour la fabrication des microcapsules. La purification d'alginate est donc la toute première étape vers le développement de microcapsules biocompatibles. Dans les années passées, les alginates qui étaient disponibles commercialement et utilisées à des fin d'encapsulation cellulaire étaient des alginates originalement préparées pour une utilisation alimentaire comme, par exemple, dans les crèmes glacées et les yogourts. Ces alginates n'avaient donc pas à subir de processus pointus de purification et n'étaient pas conçues pour servir à des fins de transplantation.

Comme l'alginate est un polysaccharide extrait des algues marines, elle a tendance à être particulièrement contaminée. De plus, les techniques industrielles employées pour extraire le polymère de la plante marine peuvent également contribuer à la contamination de l'alginate.

C'est au début des années 1990 que les premières constatations de l'influence de la purification de l'alginate sur sa capacité à activer ou non le système immunitaire on été publiées. En effet, à l'aide d'une technique d'électrophorèse «free-flow», Zimmermann *et coll.* [169] ont montré que l'alginate pouvait contenir jusqu'à un minimum de 10 à 20 fractions ayant une activité mitogénique et que l'alginate ayant subi une purification par électrophorèse avait une moins bonne capacité à activer la prolifération des lymphocytes *in vitro*, la prolifération lymphocytaire pouvant être un marqueur de mitogénicité. Il a également été montré que l'utilisation de l'alginate purifiée dans la fabrication des microcapsules diminuait la sévérité de la RHM *in vivo* [169]. L'électrophorèse «free-flow» est une technique où les analytes sont séparés selon leur mobilité électrophorétique (EPM) et leur point isoélectrique (pI) dans un système fluidique ne contenant pas de phase stationnaire (comme un gel). Le volume maximal de solution pouvant être injecté est de 10 ml. L'utilisation de la technologie d'électrophorèse afin de purifier l'alginate étant impensable à appliquer à grande échelle ou dans des processus industriels, les auteurs ont alors mis au point et publié une procédure complète de purification d'alginate. Cette alginate purifiée ne semblait pas induire de RHM sévère et ce 3 semaines après une implantation *in vivo* [170]. Les protocoles de purification d'alginate se sont ensuite multipliés dans la littérature et chaque groupe s'est mis à utiliser sa propre méthode [159, 169-173], avec des succès différents, rendant l'interprétation des résultats entre les groupes de recherches de plus en plus difficile.

Les trois principaux contaminants connus de l'alginate sont les endotoxines, les composés polyphénoliques et les protéines étrangères. Les endotoxines (ou lipopolysaccharides, LPS), qui sont une des composantes des membranes bactériennes

gram-négatives, sont déjà connues pour leur capacité à activer le système immunitaire. Le LPS est libéré dans le corps humain lorsque les bactéries sont détruites par le système immunitaire de la personne infectée et peut induire des manifestations telles qu'une hyperthermie ou un choc septique. Avec toutes ces caractéristiques, il n'est pas étonnant que la teneur en endotoxines de l'alginate ait été autant étudiée [170, 172-176]. Un groupe de recherche a même élaboré un protocole spécifique afin de retirer le plus d'endotoxines possible, mais celui-ci n'est pas directement divulgué à cause d'une demande de brevet [172]. Les composés polyphénoliques quant à eux sont contenus de façon naturelle dans les algues marines [177] et leur détection et leur quantification dans l'alginate furent décrites par Skjak-Braek [178]. La nature exacte de ces composés n'est pas connue, par contre, leur présence dans l'alginate est facilement détectable en spectrofluorométrie et la plupart des techniques de purification publiées réduisent efficacement ce contaminant, même s'il fut démontré plus tard que de petites quantité de polyphénols résiduelles n'affectaient pas la biocompatibilité des alginates [170]. Les contaminants protéiques, quant à eux, n'ont pas reçu d'attention avant le début des années 2000 [173].

Le système d'encapsulation cellulaire se situe à la frontière de deux disciplines différentes mais complémentaires, la biologie et l'ingénierie. La partie biologique fait référence aux cellules qui sont encapsulées ainsi que la réponse du receveur lors de l'implantation du biomatériau. La portion ingénierie quant à elle fait référence aux caractéristiques chimiques et physiques du biomatériau. Afin d'aller une étape plus loin dans l'analyse des alginates purifiées, nous avons fait appel à différentes techniques de génie biomédical. Ces techniques offrent deux avantages majeurs 1) elles sont non biaisées, c'est-à-dire que ce sont des méthodes d'analyse directes et, 2) elles nous donnent des renseignements sur le comportement de l'alginate suite aux processus de purification. Nous avons décidé d'utiliser les trois techniques suivantes, soit le XPS, l'ATR-FTIR et l'angle de contact. La technique de X-ray Photoelectron Spectroscopy (XPS), fait appel à un rayonnement X qui bombarde la surface à étudier (microcapsule alginate) pour en éjecter des photoélectrons. La vitesse à laquelle ces photoélectrons sont éjectés de la surface est

spécifique aux atomes dont ils proviennent, nous permettant ainsi de pouvoir les identifier. Cette technique nous renseigne sur la composition élémentaire de la surface étudiée ainsi que les différents liens chimiques qui unissent ces atomes entre eux. L'Attenuated Total Reflection Fourier-Transform Infrared spectroscopy (ATR-FTIR) consiste à bombarder la surface des microcapsules d'alginate que l'on veut étudier avec un rayonnement infrarouge ayant préalablement passé à travers un cristal. En fonction du mode vibratoire spécifique des différents groupes chimiques, l'ATR-FTIR nous permet de détecter quels sont ces différents groupements fonctionnels exposés à la surface (ex. C=C, COO-). Finalement, l'hydrophilicité de la surface d'alginate a été étudiée par angle de contact (Figure 10, page 35). Pour ce faire, une fine gouttelette d'eau est déposée à la surface du biomatériau à étudier. L'angle formé par cette gouttelette par rapport à la surface nous renseigne sur sa mouillabilité. Plus l'angle formé est grand, plus cette surface est hydrophobe et repousse la gouttelette d'eau alors que plus la surface est hydrophile, plus la gouttelette s'étendra sur la surface et plus l'angle formé sera petit.

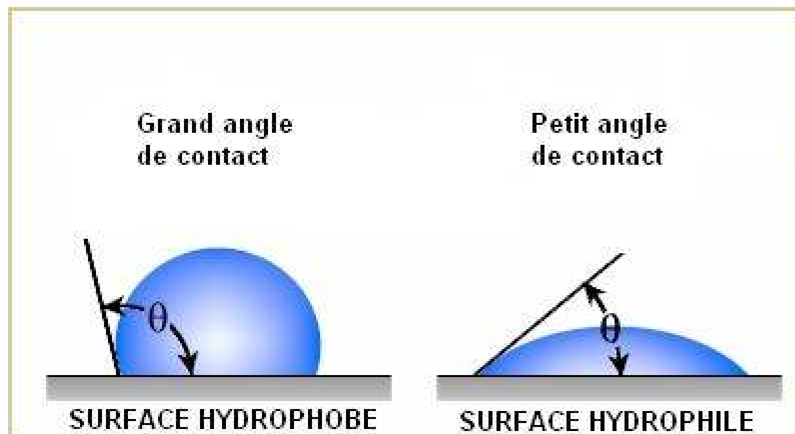


Figure 10 : Technique de l'angle de contact où l'angle formé sur une surface nous renseigne sur sa mouillabilité

Les articles qui suivent comparent tout d'abord l'efficacité des techniques de purification d'alginate déjà publiées et mettent en évidence que le rôle des contaminants protéiques a été jusqu'ici sous-estimé. Les protéines jouent un rôle important dans

l'initiation de la RHM. De plus, à l'aide de technologies de pointe, nous avons également étudié le comportement de l'alginate, c'est-à-dire ses propriétés physicochimiques, à la suite de la purification. Comme le but était d'étudier la biocompatibilité de l'alginate, toutes les expériences comprises dans la première partie ont été effectués sur des billes de baryum, sans enrobage, pour ne pas confondre la biocompatibilité du polymère avec celui de la microcapsule complète qui inclurait également la biocompatibilité de la PLL.

Evaluation of Alginate Purification Methods: Effect on Polyphenol, Endotoxin and Protein Contamination

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ABSTRACT

Alginate, a polysaccharide extracted from brown seaweed, is widely used for the micro encapsulation of islets of Langerhans, allowing their transplantation without immunosuppression. This natural polymer is known to be largely contaminated. The implantation of islets encapsulated using unpurified alginate leads to the development of fibrotic cell overgrowth around the microcapsules and normalization of the blood glucose is restricted to a very short period if it is achieved at all. Several research groups have developed their own purification method and obtained relatively good results. No comparative evaluation of the efficiencies of these methods has been published. We conducted an evaluative study of five different alginate preparations: a pharmaceutical-grade alginate in its raw state, the same alginate after purification according to three different published methods and a commercially available purified alginate. The results showed that all purification methods reduced the amounts of known contaminants, that is, polyphenols, endotoxins and proteins, although with varying efficiencies. Increased viscosity of alginate solutions was observed after purification of the alginates. Despite a general efficiency in decreasing contamination levels, all of the purified alginates contained relatively high residual amounts of protein contaminants. Because proteins may be immunogenic, these residual proteins may play a role in persisting microcapsule immunogenicity.

INTRODUCTION

The microencapsulation of islets of Langerhans within semi-permeable microcapsules has been proposed as a means to prevent the immune destruction of transplanted cells while avoiding the requirement for the use of immunosuppressive drugs[1]. The overall success of this approach has lagged behind initial expectations, one of the main reasons being a lack of reproducible biocompatibility of the biomaterials that are used to produce the microcapsules[2]. The consequences of an insufficient biocompatibility are serious, as graft failure is often associated with the development of pericapsular cell overgrowth[3-13]. Cytokines, such as interleukine-1 (Il-1), which are released by immune cells that surround the capsule, may diffuse through the microcapsule membrane and induce islet cell death[14,15]. Moreover, an immune reaction against the implant leads to the formation of fibrosis[16], which limits the diffusion of nutrients and also affects encapsulated cell viability.

Alginate remains the most widely used biomaterial for immobilizing cells to be transplanted, that is, for forming the microcapsule core, because of the many advantages it offers[17-19]. Firs, alginate has the capacity to gel under physiological conditions that are compatible with cell survival. The gelling reaction does not require toxic solvents and does not generate harmful byproducts. Second, the rapidity of the gelling process is a critical advantage. When islet-containing droplets of sodium alginate solution are extruded from a needle and dropped into a divalent cation bath, they gel instantaneously, allowing the formed calcium alginate beads to retain the perfectly spherical shape that the alginate droplet had upon extrusion. Although the suitability of other polymers is still under investigation (eg. agarose[20,21]), none has been as extensively studied nor has reached the same level of performance as alginate. As a natural polymer, alginate is limited by its tendency to be largely contaminated. Moreover, the industrial processes used for extracting alginates from seaweed could introduce additional contaminants into the raw materials. When unpurified alginate is used for islet encapsulation and transplantation into diabetic

animals, a severe reaction against the microcapsules occurs immediately after implantation, and blood glucose is not normalized or is normalized only for a few days[13,22]. However, alginate purification has led to successful encapsulated islet transplantation in terms of normalizing blood glucose in diabetic animals[23,24]. Despite this improvement, the poor biocompatibility of these transplants remains to be a problem. A low grade inflammatory reaction still develops around the microcapsules, and even though this reaction does not prevent the normalization of blood glucose in the short-term, it leads to long-term graft failure[23]. As a consequence, large amounts of islets are required and/or the duration of graft function is limited.

Some of the limitations associated with alginate contamination have been overcome, as several research groups have successfully developed their own in-house methods for alginate purification[23-28]. To ensure the clinical application of alginate-based microencapsulation, however, the variability between purification protocols applied by independent laboratories must be reduced and a purity standard that conforms to current health regulations must be defined. To our knowledge, no comparative studies of presently applied methods for alginate purification have been published. As a result, there is currently no consensus on which methods, or which procedural steps, are optimal for producing a transplant-grade alginate

The present article reports the results of a comparative study of five alginate preparations: a pharmaceutical-grade alginate in its non-purified state, the same alginate after purification according to three published in-house methods[23,25,26], and a commercially available "ultra-pure" alginate. The relative efficiencies of the purification methods were evaluated using two criteria, the contamination levels of the purified alginates and their solution viscosities. Contamination levels were measured in terms of their endotoxin, polyphenols and protein content. These three contaminant types were chosen as contamination indicators because they are most commonly detected in alginates. The effect of purification on the viscosity of the alginate solutions was evaluated because solution

viscosity plays a critical role in the microcapsule morphology[29] and microcapsule morphology is an important parameter influencing the development of an immune reaction[11,12,30]. In addition, others have observed that alginate purification induced a change in solution viscosity[25,31]. In the present study, we showed that the final purity level and viscosity of the alginates varied depending upon the applied purification protocol. Nevertheless, all of the evaluated methods reduced the levels of the three contaminants and increased the viscosity of the alginate solution. Moreover, we found that, despite a reduction in contamination levels, a certain amount of residual proteins remained in all of the purified alginates.

MATERIALS AND METHODS

Materials

A pharmaceutical-grade sodium alginate Protanal® LF 10/60 (65-75% guluronic acid, Mw 135 kDa, as specified by the manufacturer) and a commercially available industrially purified sodium alginate, Pronova™ UPLVG (ultrapure, low viscosity, 67% guluronic acid, Mw 160 kDa, as specified by the manufacturer) were purchased from FMC Biopolymers (Drammen, Norway). All other materials (chloroform, acetone, alcohol, acetic acid, sodium citrate) were of analytical grade, purchased from Fisher Scientific Ltd. (Pittsburgh, PA, USA), and were used without further purification. All glassware was washed (soaked for 10 minutes in Extran soap, then washed with distilled water, HCl 2N and sterile water) and submitted to autoclave or ethylene oxide gas sterilization in order to remove endotoxins. All manipulations were performed under sterile conditions whenever possible.

Alginate Purification

The Protanal® LF 10/60 alginate was separated into four batches: The first batch was not purified (Prot-raw), and the others were purified according to three different in-house purification procedures that were originally published by Prokop and Wang[25] (ProtpurP), de Vos *et al.*[23] (ProtpurD) and Klock *et al.*[26] (ProtpurK). Briefly, in de Vos *et al.*[23] alginate was dissolved, under constant stirring, in 1 M sodium ethyleneglycotetraacetic acid (EGTA) solution at 4°C into a 1 % solution and filtered over a 0.22 μm filters. Afterward, alginate solution pH was lowered to 2.2 under constant monitoring by addition of 2 N HCl plus 20 mmol/l NaCl. Next, the solution was filtered over a Buchner funnel (pore size 1.5 mm). The precipitate was brought in 0.01 N HCL plus 20 mmol/l NaCl, vigorously shaken, and filtered again over the Buchner funnel. This washing procedure was performed three times. Then, three extractions with chloroform/butanol was performed by suspending alginate in 100 ml of 0.01 N HCl with 20 mmol/l NaCl and supplemented with 20 ml chloroform and 5 ml 1-butanol. The mixture was vigorously shaken for 30 min and filtered over the Buchner funnel. Next, alginate was brought in water and slowly dissolved by gradually raising the pH to 7.0 by the slow addition of 0.5 N NaOH plus 20 mmol/l NaCl over a period of at least 1 hour. The alginate solution obtained was subjected to two chloroform/butanol extractions by the addition of chloroform (20 ml at each 100 ml alginate solution) and 1-butanol (5 ml at each 100 ml alginate solution) and vigorously shaken for 30 min. The mixture was centrifuged for 3–5 min at 3000 rev/min. The chloroform/butanol phase was removed by aspiration. Alginate was finally precipitated with ethanol (200 ml of absolute ethanol was added to each 100 ml of alginate solution) for 10 min, filtered over the Buchner funnel and washed two times with absolute ethanol. Finally, the alginate was washed three times with ethylether and freeze-dried overnight.

The original purification methods described by Klock *et al.*[26] was slightly modified (see table 1 for details). Briefly, 9 g of alginate powder was incubated in 400 ml of chloroform for 30 min and filtered under vacuum on Whatman #4 filtrating paper. This

chloroform extraction was performed three times. Then, alginate was dissolved in distilled water to a 1.5 % solution. Next, an equivalent alginate-weight of acid-washed activated charcoal was added to the solution and the mixture was stirred for 4 h. This procedure was repeated once using neutral charcoal. The solution was filtered on a 0.22 μm filters. Afterward, alginate beads were produced using a 50 mM $BaCl_2$ solution as the jellifying agent. Beads were washed with sterile water and then incubated three times into a 1 M acetic acid (pH 2.3) for 14 h. Beads were washed with sterile water between each medium change. Next, beads were incubated twice in a 500 mM sodium citrate solution (pH 8) for 8 h. Beads were washed with sterile water at each medium change. Then alginate beads were extracted twice with 50 % and 70 % ethanol (containing 5 % acetone) for 16 h each without sterile water wash. Afterward, beads were washed with a 20 mM $BaCl_2$ solution then extensively with sterile water. Beads were dissolved into an alkaline 250 mM ethylenediaminetetraacetic acid (EDTA) solution (pH 10) overnight. The recovered solution was filtered (0.22 μm) and dialyzed (membrane molecular weight cut-off 50,000 Da) against distilled water for 20 h with three medium changes. After the addition of 10 mM NaCl, alginate was precipitated with two volumes of 100 % ethanol and freeze-dried overnight. For the Prokop and Wang[25] purification protocol, alginate was purified the same way as Klöck purification procedure without the chemicals extractions on alginate beads. The Pronova™ UPLVG alginate (PronpurC) was purified by the supplier before its purchase according to an undisclosed protocol. No additional purification of this alginate was performed. The steps of each procedure are described and compared in Table 1.

Measurement of alginate contamination

Proteins quantification

The amount of protein contaminants present in alginate was measured using the commercially available Micro BCA Protein Assay Reagent Kit (Pierce Biotechnology,

Rockford, Illinois). Briefly, alginates were dissolved in sterile water at a concentration of 1% w/v and sterilized by sequential filtration (final filter pore size: 0.2 µm). Alginate solutions were diluted in water in ratios ranging from 1:1 to 1:500 and incubated for one hour at 60°C with the BCA reagent. Proteins were detected by the appearance of a purple color that results from the formation of a peptide-copper ion-BCA complex. Using a spectrophotometer (Spectronic 1001 plus, Milton Roy, Rochester, NY), the protein concentration was measured in terms of light absorbance at 540 nm, which is specific for the purple color. To quantify the results, light absorbance levels were compared to a standard curve that was produced using bovine albumin. For the presentation of the results, concentrations were converted to the equivalent mg of proteins per g of dry alginate.

Polyphenolic-like compounds quantification

The fluorescence spectra of alginate solutions (1% w/v in sterile water) were obtained using a spectrofluorimeter (LS-5 Luminescence spectrometer, Perkins-Elmer, Oak Brook, Illinois). An emission wavelength of 445 nm and an excitation wavelength of 366 nm were applied. Polyphenol-like compounds were detected by the appearance of a characteristic absorbance peak at 445 nm[32]. The relative quantities of polyphenol were measured in terms of arbitrary fluorescence units (AFU).

Endotoxin quantification

A commercial Limulus Amoebocyte lysate (LAL) assay (E-Toxare®, Sigma-Aldrich Inc., St-Louis, MO, USA) was used to determine the endotoxin content of the samples. Alginate solutions (1% w/v in sterile water) were diluted in water in ratios ranging from 1:10 to 1:5000 and incubated for one hour at 37°C with LAL reagent. Endotoxins were detected by a characteristic clotting reaction, which is marked by an increase in viscosity and opacity of the solutions. Quantification of endotoxin levels was performed by comparison of the sample viscosity with a standard endotoxin curve that was included in

the kit. For the presentation of the results, concentrations were converted to the equivalent endotoxin units (EU) per g of dry alginate.

Alginate viscosity measurement

Samples of alginate were dissolved in a saline buffer (NaCl 0.9 %) in concentrations ranging from 0.4 to 3 % w/v. For each alginate solution, the dynamic viscosity was measured at various concentrations using a Synchro-Lectric rotational viscometer (Brookfield Engineering Laboratories Inc., MA) operated at 25°C. A minimum of 10 rotations was done for each measurement in order to ensure an accurate reading.

Statistical Analyses

A Student *t*-test (unequal variance, two-tailed) was used to compare the results, a difference for which $p < 0.05$ was considered to be statistically significant. Results are presented as average value ± standard error of the mean.

RESULTS

Quantification of contaminants

Endotoxins, polyphenols and proteins, which are the most commonly found contaminants in alginate extracted from marine algae, were assayed for each sample. The endotoxin content in the alginates ranged from 8 to 7625 EU/g [Fig. 1(a)]. The amount of endotoxins [Fig. 1(a)] that was detected in three of the purified alginates, ProtpurD (763 EU/g), ProtpurK (8 EU/g), and PronpurC (8 EU/g), was significantly lower ($p < 0.05$) than

the amount measured in the non purified alginate Prot-raw (3813 EU/g). There was no significant difference in the level of endotoxins between Prot-raw and ProtpurP (7625 EU/g).

In terms of polyphenol-like compounds, all four purified alginate contained significantly lower amounts of this contaminant compared to the non purified alginate, Prot-raw ($p < 0.01$), as shown in Figure 1(b). Polyphenol levels were 82-97 % lower in the purified than in the non purified alginate, Prot-raw (13.83 AFU). The purified alginates ProtpurK and PronpurC contained the smallest relative amounts of polyphenols (0.47 AFU).

Although the purification procedures appeared to be efficient for the removal of endotoxins and polyphenols, these methods were less appropriate for the removal of foreign proteins [Fig. 1(c)]. There was no important decrease of measured protein contents in the case of two purified alginates ProtpurP (3.95 mg/g) and ProtpurD (6.38 mg/g), as the protein level in these alginates was not significantly different from that of the non-purified alginate, Prot-raw (5.88 mg/g). A significant reduction of proteins was observed in the case of the alginates ProtpurK and PronpurC, which contained the smallest quantities of this impurity (2.94 mg/g and 2.38 mg/g, respectively). Although the protein levels in these two purified alginates were significantly lower than that measured in the raw alginate ($p < 0.01$), the residual protein contaminants that remained after purification were present in concentrations that are high enough (0.3 % of the alginate's dry weight) to compromise the biocompatibility of the alginate.

Alginate viscosity

Plot curves displaying the variation of the viscosity of the alginate solutions as a function of the solution concentration for the different alginate preparations are shown in figure 2. For the creation of these plots, we compared only those alginate samples that derived from the same source (i.e. Protanal® LF 10/60). Purification of this

pharmaceutical-grade alginate resulted in an increase of the dynamic viscosity of the alginate solutions, which had the effect of shifting the curves toward the left. From our previous work, we determined that alginate solutions with a viscosity of 200 centipoise (cps) form microcapsules of with optimal morphological properties. Thus, we used the curve fits in order to calculate, for each purification protocol, the alginate solution concentrations required to obtain a viscosity of 200 cps. These were 1.90 % for ProtpurK, 2.15 % for ProtpruD, 2.15 % for ProtpurP and 2.5 % for Prot-raw.

DISCUSSION

In this study, we evaluated the efficiencies of three published, in-house purification protocols by comparing the contamination levels of purified alginates, including an industrially purified product. Although it is recognized that other purification protocols have been published[24,27,28,31,33,34], we decided not to include them in this study for economical and practicality reasons. Specifically, though Zimmermann *et al.*[34] published a purification method that employed free-flow electrophoresis, we chose not to reproduce the described protocol because the procedure is expensive and difficult to perform on a large scale. Furthermore, because of these inconveniences, the authors later abandoned the electrophoresis method in favor of a chemical extraction procedure, which was in fact evaluated in the present study[26]. Jork *et al.*[28] have also published a method for purifying alginates that are freshly collected from kelp. This procedure, however, is currently inaccessible and inconvenient for the average laboratory, and thus we did not attempt to reproduce it. We believe that the three purification methods that were selected for this study were sufficient to clearly demonstrate that in-house protocols can be quite variable in efficiency and that a purity standard for a transplant-grade alginate must be defined.

Alginate contamination by polyphenol-like compounds was first described by Skjak-Braek *et al.*[32]. In nature, aromatic compounds are the second most abundant organic

compounds after carbohydrates. They are particularly found in plants, seeds, flowers and fruit skin[35]. Polyphenols are a growth substrate for many organisms and a part of the lignin and tannin structure[36]. More specifically, polyphenols are responsible for the chemical defense against herbivores in brown seaweed[37]. Because they are naturally present in algae, one expects that this kind of contaminant can be found in alginate that is extracted from the algae. It is of great importance to remove these polyphenol-like compounds from alginates before their implantation since the World Health Organization reports that they can be dangerous for humans[38]. In addition, polyphenols are biorecalcitrants and can possibly accumulate in the body[39]. In our study, all of the purification procedures were found to significantly decrease the polyphenol content in the alginate. Even the simplest purification procedure, that is, that based on the protocol by Prokop and Wang[25], which involved only one chloroform and two charcoal treatments was efficient enough to significantly reduce the polyphenol content. This implies that only a minimum degree of chemical treatment is required to remove most of this contaminant from alginate.

Generally, endotoxins originate from the cell wall of gram negative bacteria[31]. Endotoxins are a part of the pyrogen family, meaning that they increase the body temperature when injected into the blood stream. The maximum allowable concentration of endotoxins is stated to be 5 EU/kg of body weight per hour for intravenous injections[40]. In terms of contamination limits for alginates used for encapsulation, Wandrey *et al.*[31] calculated this limit to be equivalent to 2,000 EU/g of polymer, while Prokop and Wang[25] estimated the maximum to be equivalent to 1,220 EU/g of dry alginate. Because we measured an endotoxin level of almost 4,000 EU/g in the non-purified alginate, it is clear that commercially available pharmaceutical-grade alginates are not suitable as purchased for cell encapsulation and transplantation purposes. Furthermore, we demonstrated that some published procedures are less efficient than others for removing endotoxins from the alginate. Specifically, the endotoxin content of the alginate ProtpurP was not significantly lower than that of the raw alginate. This result suggests that charcoal extraction of alginate powder is not a particularly effective step for decreasing endotoxin levels. However, the

purified alginates ProtpurD, ProtpurK and PronpurC all had endotoxin levels that were far below the estimated safety limit of 1,000-2,000 EU/g. Although all three of these alginates presented a significant decrease of endotoxins, only the latter two alginates had nearly undetectable levels of endotoxins. The major difference between the method published by Klöck *et al.*[26] and the two other alginate purification procedures is the inclusion of chemical extractions (acetic acid, sodium citrate and ethanol/acetone washings) of alginate barium beads. This implies that these supplementary steps are particularly effective for removing contaminating endotoxins.

Depending upon the brown seaweed source, the distribution of macrocomponents of algae can be up to 50 % of carbohydrates (alginate, carrageenan and agar) and up to 40% of proteins[41]. Because of the similar quantities of these two co-existing components, the contamination of the bulk alginate by proteins is not surprising. The presence of certain proteins in biomedical devices is known to provoke a strong host immune reaction[42,43], thus their removal from the alginate before implantation is crucial. The studied purification methods seemed to be less efficient for eliminating protein contaminants than polyphenol and endotoxin contaminants. The alginates PronpurC and the ProtpurK were the only ones that had a significantly lower protein level than found in the raw alginate. Even in these two cases, however, the remaining protein contaminants were presents in high enough concentrations (almost 3 mg/g of dry alginate) to compromise the biocompatibility of the alginates and microcapsules. Since immune reactions are frequently induced by proteins, this finding could, at least in part, explain the observed immunogenicity of microcapsules.

All of the studied in-house purification methods induced an increase in the viscosity of the alginate solutions. Two phenomena can hypothetically explain this change in viscosity. (1) Certain purification steps, such as dialysis (molecular weight cut-off: 50,000 Da), were likely to have removed small alginate chains. Chemical extractions of alginate beads could have also eliminated small alginate chains or remove the “non-gelling” chains. It is clear that removing small polymer chains in a solution increases the solution viscosity,

because the viscosity is known to be positively correlated with the mean molecular weight of the polymer chains. It is noteworthy that the removal of mannuronic acid and guluronic acid monomers and small oligomers is likely to have a beneficial effect, because they may be released from the implanted microcapsules and contribute to their immunogenicity[44]. (2) Contaminants contained within the starting material may have interfered with the natural alginate inter-chain interactions and the three dimensional organization of these polymer chains. During the purification process, the removal of such interfering contaminants could thus have led to an increase in the viscosity of the solution. Other groups have observed different trends in the variation of the viscosity of alginate solutions following purification. Prokop and Wang[25] observed a 10% to 30% decrease in the viscosity after applying the procedure described by Klöck *et al.*[26] They suggested that the multiple steps and long duration of this purification procedure can lead to polymer degradation. A decrease in alginate viscosity (~5 %) following a chemical purification was also noted by Wandrey *et al.*[31]. In these cases where a viscosity decrease was reported, however, either the purification procedure was not described in detail or the results of the viscosity measurements were not reported, thus an interpretation of the results is limited. In contrast, we observed that purification consistently induced an increase in alginate viscosity, indicating that, in our case, removing contaminants and/or small oligomers had a more important effect on viscosity than did polymer degradation.

It is difficult to perform an extensive comparative analysis between the in-house purification methods and the industrial purification process because the available information about the commercially purified alginate, PronpurC, is limited. This includes the details about the purification protocol that was applied and the source, or starting material, of this alginate. It is interesting to note, however, that the industrially purified alginate, PronpurC, had very similar qualities to the alginate that was purified in the laboratory following the protocol based on that described by Klöck *et al.*[26]. There is a possibility that the manufacturer has incorporated in its purification processes a number of procedures that are described in the published purification methods. Nevertheless, residual

proteins were also detected in the PronpurC alginate, in amounts that may be significant enough to compromise microcapsule biocompatibility. This indicates that improvements of industrial purification processes still need to be made.

In the present study, we chose to evaluate two commercially available alginates that are at opposite ends of the purity spectrum, an unpurified pharmaceutical grade alginate and an "ultrapure" biomedical grade alginate, in order to analyze a broad range of purity levels. It is recognized that these two samples are only representative of the many commercially produced alginates that are available for encapsulation purposes, and that the properties and purity levels other alginates may not be identical. That said, a difference in starting material can explain for some discrepancies between the endotoxin and protein values measured in the present study and those reported in other studies, despite the application of the same or similar purification methods. Specifically, the source of the alginate governs several properties, including the initial contamination level, the molecular weight (viscosity), and guluronic/mannuronic acid ratio, that may have an important impact on the final purity level of the product. Nevertheless, the results of this study clearly demonstrated that common impurity types are present in different alginates. Moreover, this comparative evaluation provided an important insight into our current ability to produce purified alginates that are suitable for implantation purposes. The observation that purified alginates contain significant and varying amounts of residual contaminants indicates a need to perform a systematic study that includes an analysis of all commercially available alginates, so that contamination levels can be precisely known and a purity standard for optimal biocompatibility may be developed.

In conclusion, we showed that currently applied purification procedures decreased the levels of common contaminants, namely polyphenols, endotoxins and proteins. The efficiency of the compared methods for removing each impurity, however, was variable, which emphasizes the need to define a standard purification protocol for transplant-grade alginates. The purification processes induced an increase in the viscosity of the alginate

solutions, which is an important effect to consider since solution viscosity has an impact on the morphology and biocompatibility of alginate-based microcapsules. Despite a general improvement in purity level, the results of the present study indicated that the existing purification methods are not efficient enough to completely remove foreign proteins. Efforts should be made to find an effective method to diminish or completely remove these proteins in order to decrease the immunogenicity of alginate. Finally, an improved knowledge of the effects and efficiencies of the different purification processes will lead to the production of a more standardized alginate that is suitable for the fabrication of immuno-isolating devices.

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FIGURES, TABLES AND LEGENDS

TABLE I
Detailed Purification Procedures ([a]Supplementary Steps)

Prot-raw	$Prot_{pur}P$[25]	$Prot_{pur}D$[23]	$Prot_{pur}K$[26]
	Three $CHCl_3$ extractions on alginate powder[a]		Three $CHCl_3$ extractions on alginate powder[a]
Alginate solubilization H_2O	Alginate solubilization H_2O (1.5%)	Alginate solubilization 1 m*M* EGTA (1%)	Alginate solubilization H_2O (1.5%)
	Acid-washed charcoal treatment (1:1)[a]	Sterilization by filtration (0.22 µm)	Acid-washed charcoal treatment (1:1)[a]
	Neutral charcoal treatment (1:1)	2*N* HCl/20 m*M* NaCl acidification/precipitation (pH 2)	Neutral charcoal treatment (1:1)
	Sterilization by filtration (0.22 µm)	Filtration (pore size 1.5 mm)	Sterilization by filtration (0.22 µm)
		Three solubilizations 0.01*N* HCl/filtration	Barium beads fabrication (50 m*M* $BaCl_2$)
		Three $CHCl_3$/butanol extractions (30 min)	Three 14 h 1*M* acid acetic treatments (pH ~2.3)
		Solubilization in H_2O + 0.5*N* NaOH/20 m*M* NaCl (pH 7)	Three 8 h 500 m*M* sodium citrate treatments (pH8)
		Two $CHCl_3$/butanol extractions	Two 16 h 50% ethanol treatments
		5 min centrifugation 3000 rev/min	Two 16 h 70% ethanol treatments
			Beads dissolution 250 m*M* EDTA (pH 10)
			Sterilization by filtration (0.2 µm)
	20 h dialysis H_2O (cut-off 50 kDa)		20 h dialysis H_2O (cut-off 50 kDa[a])
	Sterilization by filtration (0.22 µm)	100% alcohol precipitation	Sterilization by filtration (0.22 µm)
	100% alcohol excess precipitation	Three ethylether washes	100% alcohol excess precipitation
	Lyophilization	Lyophilization	Lyophilization
	Alginate dissolution	Alginate dissolution	Alginate dissolution
Sterilization by filtration (0.22 µm)	Sterilization by filtration (0.22 µm)	Sterilization by filtration	Sterilization by filtration (0.22 µm)

Steps of the three published in-house purification methods[23,25,26] evaluated in the present report ([a]supplementary steps added to the original protocol).

Table 1: Steps of the three published in-house purification methods[23,25,26] evaluated in the present report (* supplementary steps added to the original protocol).

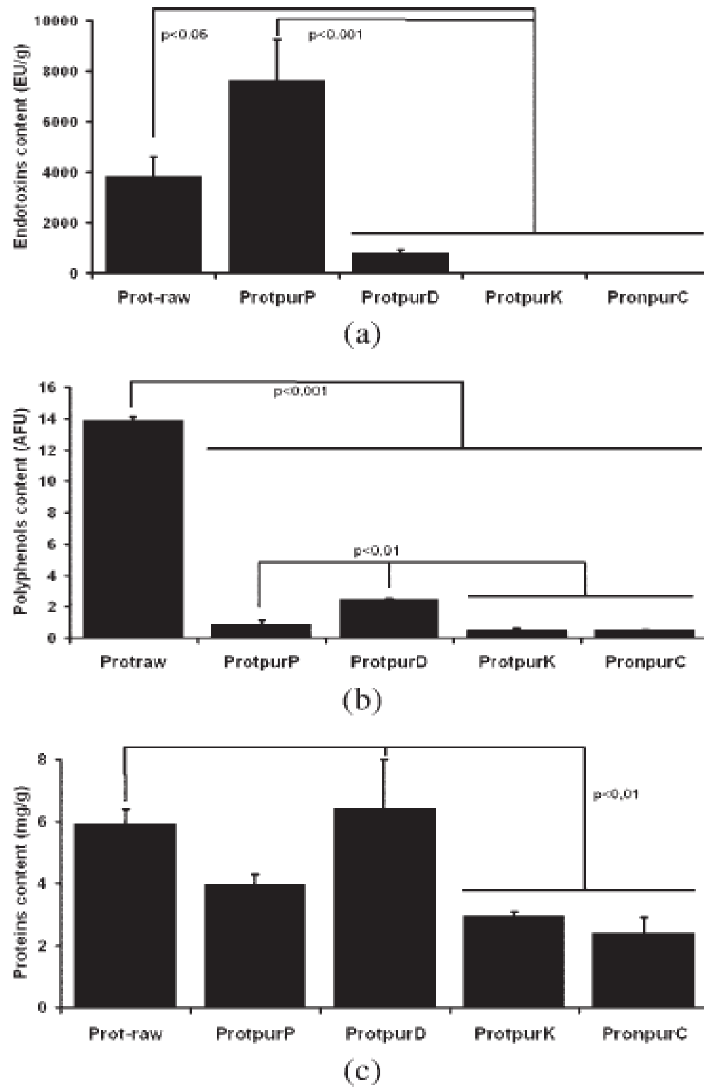

Figure 1: Quantitative evaluation of contaminants in sodium alginate purified by different protocols. A. Endotoxin quantification. Results are presented in endotoxin units (EU) per gram of dry alginate ± SEM. B. Polyphenol quantification. Results are presented in arbitrary units of fluorescence (AFU) ± SEM. C. Protein quantification. Results are presented in milligrams of protein per gram of dry alginate ± SEM.

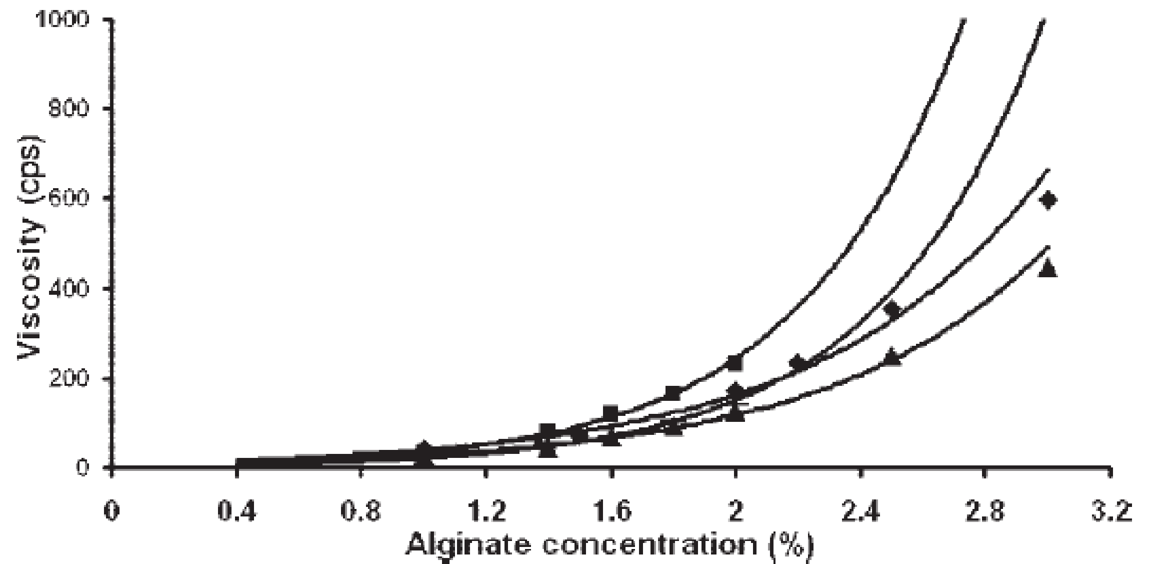

Figure 2: Dynamic viscosity of alginates solutions. The fitted curves represent the variation of alginate solution viscosity (cps = centipoises) as a function of alginate concentration (percentage w/v) for Protanal 10/60, non purified (Prot-raw) ▲, and purified according to different purification methods: ProtpurD ♦, ProtpurP – and ProtpurK ■.

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Impact of Residual Contamination on the Biofunctional Properties of Purified Alginates Used for Cell Encapsulation

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ABSTRACT

Alginate is frequently used for cell encapsulation, but its biocompatibility is neither optimal nor reproducible. Purifying the alginate is critical for achieving a suitable biocompatibility. However, published purification methods vary in efficiency and may induce changes in polymer biofunctionality. Applying X-Ray Photoelectron Spectroscopy, we showed that commercial alginates, purified by in-house and industrial methods, contained elemental impurities that contributed 0.41 – 1.73 % of their atomic composition. Residual contaminants were identified to be proteins (nitrogen/COOH), endotoxins (phosphorus), and fucoidans (sulphur). Studies using Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy suggested that trace contamination did not alter the alginate molecular structure. Alginate hydrophilicity increased by 19 – 40 % after purification, in correlation with a reduction in protein and polyphenol content. Solution viscosity of the alginate increased by 28 – 108 % after purification, in correlation with a reduction in protein content. These results demonstrate that commercial alginates contain potentially immunogenic contaminants that are not completely eliminated by current purification methods. Moreover, these contaminants alter the functional properties of the alginate in a manner that may compromise biocompatibility: Hydrophilicity may affect protein adsorption and solution viscosity influences the morphology of alginate-based microcapsules. These findings highlight the need to improve and better control alginate purity to ensure a reproducible biofunctionality and optimal biocompatibility of alginate and microcapsules.

INTRODUCTION

Alginate, a natural polymer composed of guluronic (G) and mannuronic (M) acid residues (Figure 1), is favourably used for the immobilization and encapsulation of therapeutic cells because of its unique ability to gel quickly under conditions that are compatible with living cells [1]. For the fabrication of alginate-polycation microcapsules, a frequently studied design that was originally described by Lim and Sun [2], alginate is applied as an outer coating as well as an immobilization matrix. The purpose of this outer coating is to render the capsule surface less attractive to immune cells and proteins by neutralizing and masking the charged polycation.

The reported biocompatibility of alginate and cross-linked alginate microcapsules is often inadequate and inconsistent between studies. Significant immune reactions are reported in some cases [3,4] while very little fibrotic overgrowth is observed in others [5]. To ensure the clinical application of alginate-based therapeutic microdevices, it is critical to prove that the alginate has a suitable and reproducible biocompatibility. This requires the establishment of strict standards and a highly controlled quality of the polymer. Current ASTM standards fail to be specific about the necessary requirements for a biomedical grade alginate so that it can safely be used for encapsulation purposes [6,7]. Improving current standards requires gaining a more in depth understanding of the properties that influence alginate bioreactivity.

To date, studies have clearly demonstrated that achieving a suitable level of biocompatibility requires, at minimum, a highly purified alginate [8-12]. Other factors that may influence the in vitro and in vivo response to alginate gels and capsules have also been investigated, including the M/G ratio [4,13,14] and molecular weight [15], but the resulting views have been conflicting [16]. Such debates have arisen, in part, because studies have failed to mention whether the alginates were properly purified. In fact, some authors have suggested that high-M alginates are more susceptible to contamination [10], so an

insufficient purity could explain the discrepancy between the immunogenicity of alginates ranging in M/G content. This view emphasizes the necessity to produce highly purified alginates as a priority in order to achieve a reproducible biocompatibility.

Having recognized the importance of purity, research groups began to purify alginates using in-house procedures [9,11,12,17,18]. Though noticeable improvements were made as a result of this precaution, alginate gels and capsules continue to display variable immunogenic properties, even in the absence of a polycation [8,11,19]. Moreover, we recently demonstrated that purification protocols, which differ between published studies, yield alginates of variable purity [20]. That is, we used standard biochemical assays to show that, depending upon the protocol applied, varying amounts of endotoxins, polyphenol-like compounds, and proteins continued to contaminate the alginates after they were purified. This observation raises concerns about the variable efficiency of current purification protocols and the lack of standardized criteria for a medical grade alginate suitable for encapsulation. In addition, it leads us to question whether other residual contaminants exist in the alginates and are unknowingly compromising the reproducibility of the polymer's biocompatibility.

Furthermore, we [20] and others [18,21] have observed that the purification process induces a change in the viscosity of alginate solutions. If this effect is a result of contaminants interfering with the inter-chain interactions of the alginate molecules, then it should be considered whether these contaminants are capable of altering the polymer biofunctionality, since this last parameter has a potentially large impact on the reactivity of alginate gels and capsules when they are placed in a biological environment. Otherwise stated, if the alginate biocompatibility is to be well controlled and reproducible, not only should the chemical composition (i.e. purity) be evaluated and standardized, but the effect of purification on the polymer biofunctionality should also be taken into account.

In the present study, we define the chemical composition of purified alginates using unbiased techniques, with the purpose of identifying residual contaminants

that are potentially immunogenic but may be overlooked using standard assays. We also investigate the relationship between the chemical composition of the alginate and its biofunctionality, which we define in terms of its structural properties, wettability, and viscosity. The results of this study are crucial for the establishment of a purification standard that is essential to produce alginates of an optimal and reproducible biocompatibility.

MATERIALS AND METHODS

Study Design

Two types of commercially available sodium alginates, each having high guluronic acid content, were used in this study. The first type, an ultrapure alginate that was purified by the supplier, was analysed as purchased. The second type, a pharmaceutical grade alginate, was separated into four batches: three of the batches were purified in our laboratory following independent protocols before they were analysed, and the last batch, which served as a control, was analysed as purchased.

X-Ray Photoelectron Spectroscopy (XPS) was used to detect all contaminating elements in the alginates. Compared to standard assays for purity, this technique is advantageous because it is unbiased, in the sense that all elements (except hydrogen) are detectable, and it does not require the preliminary identification of specific molecule(s) to target or the addition of a chemical marker. Furthermore, XPS can specify the chemical groups of the detected elements, provide quantitative results, and detect atomic concentrations of 0.1% and lower.

To investigate the effect of purity on the structural properties of the alginates and on the bonding behaviour of the polymer functional groups (COONa, C-OH), Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) was applied.

Using the contact angle technique, we investigated the influence of purity on the hydrophilicity of the alginates, a property that is generally sensitive to changes in chemical composition and functional group behaviour. There was a special interest in studying the wettability of the alginates because this property is known to play an important role in the interactions of biomaterial surfaces with proteins and cells [22-26].

The viscosities of the alginate solutions were measured to verify that purity influences the bulk properties of the alginate in the same manner that the surface properties of the samples are affected (XPS, ATR-FTIR, and contact angle are all surface sensitive techniques). Furthermore, the viscosity of the alginate solution has an important impact on the final morphology, and thus biocompatibility, of alginate-based microcapsules [27,28].

With the exception of the viscosity measurements, we chose to analyse the alginates in the form of thin films, cast from aqueous solutions of the alginates. This approach was adopted in order to homogenize the samples and therefore increase the reproducibility of the results. Furthermore, the use of films made the analytical results of the different alginate types more comparable since their powders varied in particle size (surface area), texture, and shape. This sample form was also suitable for all the applied techniques. Finally, compared to powders, films are more representative of the alginate as a microcapsule component, therefore the results of this study will be more relevant to the interpretation of alginate gel and capsule biocompatibility studies. Based on our preliminary tests, it was assumed that the film surfaces were homogeneous and represented the bulk characteristics of the alginate.

Alginates

Sodium alginate powders, Protanal® LF 10/60 (65-75% guluronic acid content, Mw = 135 kDa, as specified by the manufacturer) and Pronova™ UP LVG (67% guluronic acid content, Mw = 160 kDa, as specified by the manufacturer), were purchased from FMC Biopolymer (Drammen, Norway). Protanal® LF 10/60 is a pharmaceutical grade alginate while Pronova™ UP LVG is promoted as an ultrapure alginate that was developed for biomedical as well as pharmaceutical applications.

Alginate purification and preparation

The Protanal® LF 10/60 alginate was purified using one of three different protocols that were based on methods described in published studies: de Vos et al. [11], Prokop and Wang [18], and Klöck et al. [12]. The latter two methods were slightly modified from the published versions by adding a chloroform extraction of the alginate powders and an acid-washed charcoal treatment of the alginate solutions, since our preliminary results showed that the inclusion of these steps increases the efficiency of the purification process. All three protocols are described in more detail in the original publications [11,12,18] and in our previous study [20]. No additional purification of the Pronova™ UP LVG alginate was performed. Raw (i.e. unpurified) Protanal® LF 10/60 alginate was also included in the study as a control. Alginates that were not immediately used were stored in their dry powder/fibrous forms at 4°C. To prepare aqueous solutions for the XPS, ATR-FTIR, and the contact angle analyses, only sterile water was used to dissolve the alginates in order to avoid interference of the results by sodium; for the viscosity measurements, a saline buffer was used. All solutions were sterilized using a 0.2 μm filter before further manipulation.

X-Ray Photoelectron Spectroscopy

To improve the reproducibility of the measurements, alginate solutions (2% w/v) were first homogenized, by agitating them ultrasonically for 30 minutes using a Branson 3510 ultrasonic cleaner (Branson Ultrasonics Corp., Danbury, CT, USA) immediately before casting them onto 1cm2 squares of silicon wafer. To prevent the films from peeling under the high vacuum conditions (~10-9 Torr) during the spectral measurements, the films were dried slowly under atmospheric conditions for at least 24 hours before transferring them to the desiccator for another 24 hours. XPS spectra were obtained using an Escalab 3 MKII surface analysis system (VG Scientific, Beverly, MA, USA). An unmonochromated Mg Kα anode operated at 216 W (18 mA, 12kV) was used for X-ray generation. Survey spectra were recorded for 0 – 1200 eV binding energy range, at a pass energy of 50 eV. High resolution spectra of C1s, O1s, and Na1s peaks were recorded at 20 eV pass energy. To avoid sample degradation during analyses, exposure to X-ray radiation was limited by omitting high resolution scans of low intensity peaks and recording scans only once. Spectral analysis was performed using the software supplied by the company (Avantage, VG Scientific). Charge shift corrections were made by setting the C1s peak of saturated hydrocarbons to 285.0 eV. Peaks were fitted by fixing the full-width half maximum of the C1s, O1s, and Na1s peaks at 1.6 eV, 1.8 eV, and 1.7 eV, respectively, and setting the Gaussian/Lorentzian ratio to 50%.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy

Alginate solutions (2% w/v) were cast onto glass microscope slides and dried in a vacuum desiccator for at least 24 hours to form thin films. Two films of each sample were peeled from the slides and pressed onto each side of a germanium ATR crystal using a

metal clamp. Spectra were obtained using an Excalibur FTS 3000 FTIR Spectrometer (Digilab, Inc., Randolph, MA, USA). To avoid signal interference from water and CO_2 vapours in the atmosphere, the samples were analysed in a nitrogen-purged chamber at room temperature. Spectra were recorded for the range of 400-4000 cm-1 at a resolution of 8 cm-1. Each spectrum represents an average of 256 scans. Background spectra consisted of the bare Ge crystal under the same experimental conditions.

Contact Angle measurements

Alginate solutions (2% w/v) were cast on glass microscope slides and dried in a vacuum desiccator for at least 24 hours to form thin films. The left and right contact angles of water on the (unpeeled) films were measured using a VCA Optima System (AST Products, Inc., Billerica, MA, USA). A water drop of 1.0 μl volume was deposited onto the film surface using a mechanically controlled syringe, and a photograph was taken 10 s after contact. The contact angles were averaged over three spots on each film.

Viscosity measurements

The dynamic viscosities of the alginate solutions (2% w/v in a saline buffer) were measured using a Synchro-Lectric rotational viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA). Measurements were made at 25 °C, and a minimum of ten rotations were completed for each measurement.

RESULTS

For the presentation of the results, unpurified samples of Protanal® LF 10/60 sodium alginate are abbreviated as "Prot-raw". Meanwhile, the samples of Protanal® LF 10/60 sodium alginate that were purified using methods based on protocols published by de Vos et al. [11], Prokop and Wang [18], and Klöck et al. [12] are denoted as "ProtpurD", "ProtpurP", and "ProtpurK", respectively. The Pronova™ UP LVG sodium alginate, which was commercially purified before its purchase, is denoted as "PronpurC".

Detection and quantification of impurities using XPS

The elemental compositions of the alginates were calculated from the peak areas in the XPS spectra (Table 1). As expected from the molecular structure of sodium alginate (Figure 1), all samples contained high amounts of carbon and oxygen, as well as some sodium. In theory, a molecule of sodium alginate should have an atomic composition of 46% C, 46% O and 8% Na. Thus, the samples appeared to contain an excess of carbon, which was measured to be 53.4 at% for the raw alginate (Prot-raw) and 49.7 – 51.2 at% for the purified alginates. This excess carbon had the effect of lowering the relative amount of oxygen in the samples, which ranged from 37.0 to 40.2 at%. Sodium levels, which were measured to be 7.3 – 10.1 at%, were consistently close to the theoretical value.

The non-purified alginate, Prot-raw, was contaminated by small amounts of sulphur, phosphorus, nitrogen, and chlorine that, when added together, contributed 1.98 % of the sample atomic composition. After purification, all traces of chlorine were removed and the total quantity of impure elements decreased to 0.41 – 1.73 at%. The chlorine was regarded as a contaminant because sterile water, rather than saline (NaCl), was used to dissolve the alginate powders.

The nitrogen content amounted to 1.17 at% and 0.16 at% for two of the purified samples (ProtPurD and ProtPurP, respectively), while the concentration of this element was below the detection limit of the technique for the other two purified alginates (ProtPurK and PronPurC). The nitrogen was presumed to have originated from the amino acid groups of proteins that we previously determined to be contaminating the same alginates [20]. In support of this view, a strong correlation (R^2 = 0.95) between previously measured protein amounts and the atomic percentage of nitrogen was observed, as illustrated in Figure 2a.

The detected phosphorus, which contributed 0.03 – 0.12 at% of the purified samples, was attributed to the presence of endotoxins since phosphate is a main component of the endotoxin structure [29]. Furthermore, as shown in Figure 2b, phosphorus concentrations correlated (R^2 = 0.69) with the previously measured endotoxin content of the same alginates [20].

The contaminating sulphur that was detected in the purified alginates, in amounts ranging from 0.28 – 0.67 at%, possibly originated from sulphur-containing proteins, but a lack of correlation with the protein content of the alginates does not support this scenario. It is much more likely that this element originated from fucoidans, i.e. sulphuric polysaccharides, that would have co-existed with the alginate within the cell walls of the brown algae from which alginate was extracted [30,31].

During the XPS measurements, other foreign elements were detected, but these were not considered to be contaminants in the alginates. Specifically, for a single trial, fluorine was detected in one of the laboratory purified alginates (ProtpurP). Since this was an isolated case, it was assumed that this element contaminated the alginate during sample preparation or handling, plausibly from Teflon® tweezers that were in the vicinity of the samples. Furthermore, traces of silicon were detected in a few trials, contributing up to 0.15 % of the measured atomic composition. This result was attributed to exposed portions of the silicon wafer substrate that were incompletely covered by the alginate film.

In order to identify the chemical groups that involved the detected carbon, oxygen, and sodium atoms, the C1s, O1s, and Na1s peaks in the XPS spectra were deconvoluted at high resolution. Figure 3 shows an example of the high-resolution spectrum for the Pronova™ UP LVG alginate (PronpurC). Peaks at similar binding energies were observed in the spectra for all other alginate samples (data not shown).

The chemical groups associated with each of the deconvoluted peaks were identified by the characteristic binding energies [32,33] and quantified by the peak areas (Table 2). In addition to the peaks that are associated with the theoretical structure of sodium alginate (C-OH, C-O-C, O-C-O, NaO-C=O), several contaminating peaks were identified. Three of the five detected contaminants were attributed to adventitious hydrocarbons (C-C), carbon dioxide (CO_2), and water vapour (H_2O) that presumably adsorbed onto the sample surface from the atmosphere during sample preparation and handling. Such surface contamination is commonly detected by XPS.

The carboxylic group (COOH) was identified as a contaminant within the alginates. The possibility that this chemical group belonged to segments of alginic acid was excluded because the dissociation constants (pKa) for mannuronic and guluronic acids are 3.38 and 3.65, respectively, and the sample solutions that were used to produce the analysed films were of neutral pH. Rather, its presence was attributed to contaminating proteins since COOH exists in all amino acids. In support of this hypothesis, a strong correlation (R^2 = 0.85) between the atomic percentage of COOH and previously measured protein levels in the same alginates [20] was observed, as shown in Figure 2c.

A sodium-containing impurity (Na-?) corresponding to a peak near 1072.4 eV (Figure 3c) was also detected, though it could not be identified. It is unlikely that this impurity originated from NaCl because the binding energy is about 1 eV higher than expected for NaCl [33] and chlorine was not detected in the purified alginates (Table 1).

Rather, this contaminant plausibly adsorbed onto the film surface during sample handling or atmospheric exposure since sodium is abundant in our environment.

Evaluation of the effect of contaminants on structural properties and specific bonds using ATR-FTIR

The effect of chemical composition, or purity, on the molecular structure of the alginates was verified using ATR-FTIR; peaks in the absorbance spectra of a sample represent the presence of molecular bonds with characteristic vibrational frequencies. Spectral ranges that contain absorbance peaks that are characteristic for alginate are compared in Figure 4. There were no obvious differences between the spectra of the five alginate samples. This observed similarity demonstrates that impurities were not abundant enough to create detectable absorption bands in the spectra and, more importantly, the basic molecular structure of the alginate was not significantly affected by changes in the chemical composition of the samples.

Subtle differences in the shape and intensity of the peaks associated with the intermolecular/intramolecular hydrogen bonding of the alginate functional groups (Figure 4a) and with the bonds of the carbohydrate ring (Figure 4c) were observable. The shifts in peak shape and intensity, however, were not consistent between trials (data not shown for other two trials), which suggests that an outside factor was influencing these specific bonds, rather than an intrinsic property of the alginates. This effect was thus attributed to varying interactions of the alginate hydrophilic groups (COONa, C-OH) with humidity in the atmosphere because the humidity level is difficult to keep constant, despite the use of a nitrogen-purged chamber during the measurements.

Evaluation of the effect of contaminants on wettability using the contact angle technique

The contact angle of water droplets on the surface of the alginate films was measured because this parameter is sensitive to changes in the sample chemical composition and provides insights into the behaviour of the functional, or hydrophilic, groups of the polymer. The values of the left contact angles are compared in Figure 5. Due to the fact that the surface was horizontal, the right contact angles had identical values (data not shown).

The contact angles for each of the purified samples, which ranged from 30.9° to 41.6°, were 19 – 40 % lower than the contact angle for the raw alginate, Prot-raw (51.5°). This result suggests that the contaminants reduced the intrinsic hydrophilicity of the alginate (or equivalently, induced a hydrophobicity). This effect could be explained if the impurities were hydrophobic and/or they occupied the hydrophilic groups (COONa, C-OH) of the alginate molecules to prevent them from interacting with the water. The latter explanation, however, is not directly supported by the results of the ATR-FTIR analyses, which demonstrated that the hydrophilic groups of the alginate are not significantly altered by the presence of contaminants (Figure 4a-b).

Of the three contaminant types that were previously detected in the alginates [20], any of these could have hypothetically contributed to the increased hydrophobicity of the samples since proteins, polyphenols, and endotoxins can each contain both hydrophobic as well as hydrophilic regions. However, as shown in Figure 6, there existed a correlation between the increased hydrophobicity of the alginates and each of their polyphenol and protein content (R^2 = 0.88 and R^2 = 0.68, respectively) that did not exist for the endotoxin contents, implying that the latter did not play an important role in reducing the alginate wettability.

It was also noted that the increased hydrophobicity of the samples tended to correlate with the amount of adventitious hydrocarbons and water vapour that were adsorbed on the samples surfaces and detected during the XPS analyses (Table 2). This was expected since the drive for atmospheric species to adsorb to surfaces is to lower the surface energy, and this phenomenon has the consequence of lowering the surface wettability.

Evaluation of the effect of contaminants on solution viscosity

The measured values of the dynamic viscosity of the alginate solutions (2 % w/v in a saline buffer) are compared in Figure 7. Purification of the pharmaceutical grade alginate resulted in a 28 – 108 % increase in the solution viscosity. The industrially purified alginate, PronPurC, had a solution viscosity that was 3.7- to 6-fold that of the other purified samples. The relatively high viscosity of this alginate, however, was attributed to its greater molecular weight (160 kDa vs 135 kDa) rather than to its purity level. While it is possible that the industrial purification process induced an increase in solution viscosity, this hypothesis could not be confirmed since the viscosity value of the alginate before its purification was not provided by the company.

In the case of the pharmaceutical grade alginate (PronPurC was excluded here because its source differs from that of the other four samples), a decrease in solution viscosity correlated strongly (R^2 = 0.93) with the measured levels of the carboxylic group that contaminated the alginates (Figure 8a). Although this chemical group was most likely to be a component of contaminating amino acids, there was only a slight correlation (R^2 = 0.53) between the solution viscosity and the reduction of proteins in the samples (Figure 8b). On the other hand, no correlation at all was observed between the viscosity and the levels of the other identified contaminants. This result suggests the possibility that the

contaminating proteins, but not the other impurities, interfered with the intermolecular interactions between alginate chains.

DISCUSSION

In this study, the chemical details of purified alginates were defined using high-performance techniques that have only recently been introduced to the field of microencapsulation [34,35]. In verifying their complete chemical composition, we demonstrated that, even after purification, commercially available alginates are contaminated by four chemical species: nitrogen, carboxylic groups, phosphorus, and sulphur. While the detection of the first three impurity types reinforced our previous observation of contamination by proteins and endotoxins [20], the sulphuric impurity, which was detectable in this study due to the unbiased nature of the applied techniques, has previously been overlooked by standard assays and is not commonly measured in implantable alginates. This impurity was presumed to contaminate the bulk of the alginates because sulphur-containing species are not known to adsorb from the atmosphere and the sulphur levels decreased after purification. It is very plausible that this element originated from sulphated polysaccharides, termed fucoidans, that co-existed with the alginate within the algae cell walls and contaminated the alginate during the extraction process [30,31]. Contamination by fucoidans has also been suggested by others who have detected trace amounts of sulphur in purified alginates extracted from fresh brown algae [19,36]. These authors concluded that, in their case, the sulphur concentration was too low (370 mg sulphur/kg dried alginate) to induce fibrosis or apoptosis. In the more general case, however, sulphated polysaccharides and fucoidans have displayed a large range of biological activities that are currently under investigation [37,38], and their specific effects on the biocompatibility of alginate has yet to be clearly determined.

The XPS technique proved to be very suitable for identifying elemental contamination at low concentrations and in an unbiased manner, but there are limitations to this technique. In particular, being a surface sensitive technique with an analytical depth of 50-100 Å, XPS readily detects adventitious hydrocarbons and other species that adsorb onto the sample from the atmosphere. Since these adsorbed species are organic, they contain many of the same chemical groups (C-O, for example) as the alginate molecule and as the impurities in the alginate. As a consequence, their associated peaks overlap in the spectra, cannot be easily deconvoluted, and a clear interpretation of the data becomes challenging. On the other hand, XPS was proven to be valuable for the quantification of low concentrations of contaminating elements. We were able to determine that the total amount of impure elements (S, N, P, Cl) was 1.98% of the atomic composition of raw alginate, and this sum was reduced to as low as 0.41% after purification. Furthermore, even though XPS may not display the same sensitivity as certain assays that are specific for the quantification of one contaminant type (e.g. microBCA protein assay [20]), this unbiased analytical technique provided the important advantage of allowing us to study the complete chemical composition of the alginates and thus scan for all possible contaminants.

The purity, or chemical composition, of the alginates did not have an observable effect on the structural properties nor the functional groups of the alginates when analysed using ATR-FTIR. On the other hand, the chemical composition of the samples, and particularly the content of polyphenol-like compounds and proteins, had a significant impact on the alginate wettability. If these contaminants lowered the wettability of the alginate by occupying the hydrophilic groups (COONa, C-OH), this should have induced observable peak shifts in the infrared spectrum. The fact that it didn't may be a reflection of the differing sensitivities of each technique to variations in alginate properties. That is, the contact angle technique is generally sensitive to variations in the physical forces as well as the chemical composition at the surface, while the ATR-FTIR technique measures only chemical modifications. Alternatively, perhaps the proteins and polyphenol-

like compounds were themselves hydrophobic enough that simply their presence disrupted the natural hydrophilic/phobic balance of the alginate. In either case, the observed effect of purity on the hydrophilicity of the alginate is very important because, for the first time, it leads us to question whether these contaminants are directly immunogenic, or if they indirectly compromise the alginate biocompatibility by altering its natural hydrophilicity. In fact, Morra et al. have emphasized that hydrophilicity is a key characteristic of alginate that allows it to resist the adsorption of proteins that can mediate cell adhesion, at least in the context of surface coatings [39,40]. It should be mentioned, however, that the same authors suggested that the ability of alginate to resist cell adhesion may be dependent on the extent at which the polymer can bind water, more so than its wettability as evaluated by the contact angle of water. In the case of other biomaterial types, the effect of wettability and water-binding properties on protein adsorption patterns has been extensively investigated and debated [22-26]. From such studies, it is clear that surface wettability plays an important role in the overall biofunctionality and biocompatibility of an implanted device. Given this insight, further studies will be performed in order to clarify the specific relationship between alginate purity, hydrophilicity, and protein adsorption/cell adhesion to alginates and microcapsules.

We observed that purification of the alginate induced an increase of the solution viscosity. This viscosity increase may be explained if the contaminants were interfering with the intermolecular interactions between alginate chains before they were removed during the purification process. While this view is only hypothetical at this point, it is supported by an inverse correlation between the protein content (more specifically, the amount of COOH groups) and the viscosity of the alginate solution. Our observation that proteins also appear to interfere with the alginate hydrophilicity further suggests that these contaminants are capable of altering other intrinsic properties of the polymer, including its inter-chain interactions. Alternatively (or additionally), the viscosity increase may have resulted from the removal of low molecular weight oligomers during the filtration steps of the purification process, which would have essentially increased the average molecular

weight of the solution [20]. Interestingly, other researchers have reported that purification resulted in a decrease of solution viscosity and suggested that the lengthy purification process leads to polymer degradation [18,21]. Despite this disagreement with our observations, any alterations of the solution viscosity, whether it is an increase or a decrease, that is induced by the purification process is nevertheless an important effect to consider when preparing alginates for encapsulation purposes. Viscosity is a key parameter influencing the final morphology of alginate-polycation microcapsules and, in turn, morphology has been demonstrated to have a significant impact on the microcapsule biocompatibility [27,28].

CONCLUSIONS

In this study, we detected and characterized residual contaminants in purified alginates and investigated their effect on the biofunctionality of the polymer. We determined that 1.98 at% of a pharmaceutical grade alginate consisted of contaminating elements; after purification, this proportion diminished to as little as 0.41 at%. The detection of nitrogen, COOH, and phosphorus confirmed that the alginates were contaminated by proteins and endotoxins. Traces of sulphur were also detected; this was attributed to contaminating fucoidans that may inadvertently compromise the reproducibility of alginate biocompatibility. The presence of proteins and polyphenols correlated with an increase in alginate hydrophobicity, leading to the suggestion that contaminants compromise the biocompatibility of the alginate by reducing its intrinsic wettability. An increase in solution viscosity correlated with a reduction of protein content, implying their role in interfering with the interactions between the polymer chains, which can consequently have an important effect on the morphology and biocompatibility of alginate-based microcapsules. This study clearly demonstrates that an improved control of

alginate purity is essential to achieve a reproducible functionality, and thus biocompatibility, of the alginate.

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FIGURES, TABLES AND LEGENDS

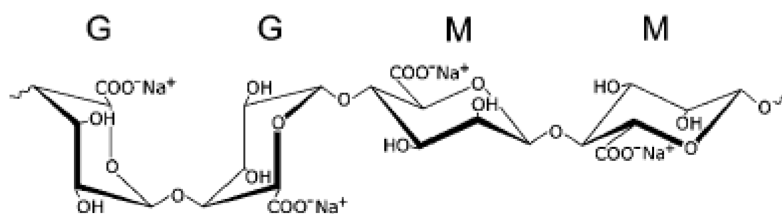

Figure 1: Schematic of the structure of a GGMM segment of the sodium alginate molecule. G = guluronic acid residue, M = mannuronic acid residue.

Table 1
Elemental compositions of sodium alginates that were subjected to different purification protocols

	C	O	Na	S	P	N	Cl	F	Si
Prot-raw	53.54 ± 0.79	36.98 ± 1.19	7.34 ± 0.87	0.78 ± 0.23	0.06 ± 0.03	1.03 ± 0.47	0.11 ± 0.06	—	0.15 ± 0.15
$Prot_{pur}D$	49.95 ± 1.05	39.20 ± 1.86	9.04 ± 0.39	0.48 ± 0.05	0.08 ± 0.07	1.17 ± 0.63	—	—	0.09 ± 0.09
$Prot_{pur}P$	50.49 ± 1.87	40.21 ± 0.31	8.63 ± 2.05	0.28 ± 0.17	0.12 ± 0.12	0.16 ± 0.08	—	0.12 ± 0.12	—
$Prot_{pur}K$	49.67 ± 3.58	40.16 ± 0.37	9.41 ± 3.41	0.67 ± 0.55	0.02 ± 0.02	—	—	—	0.07 ± 0.07
$Prot_{pur}C$	51.21 ± 0.13	38.31 ± 0.60	10.07 ± 0.78	0.37 ± 0.13	0.03 ± 0.02	—	—	—	—

Values represent the mean atomic percentage ± standard error of the mean for three trials, as calculated by XPS spectral peak areas. The symbol '—' indicates that the element was not detectable in any of the trials.

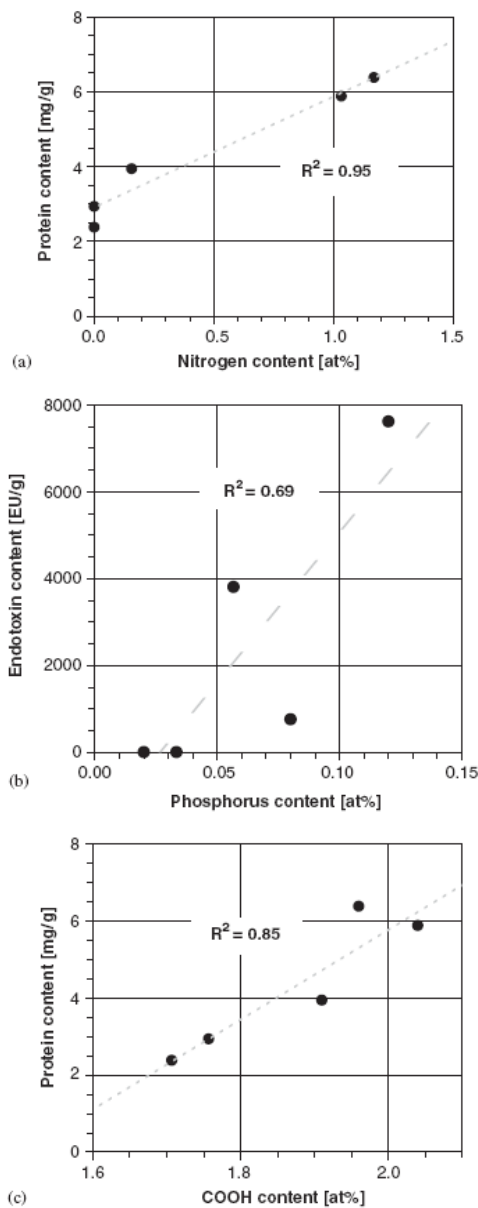

Figure 2: Correlations between the elemental compositions (as measured by XPS) and contamination levels of sodium alginates that were subjected to different purification protocols. Graphs represent the correlation between (a) nitrogen levels and protein content, (b) phosphorus levels and endotoxin content, and (c) COOH levels and protein content. EU = Endotoxin Unit.

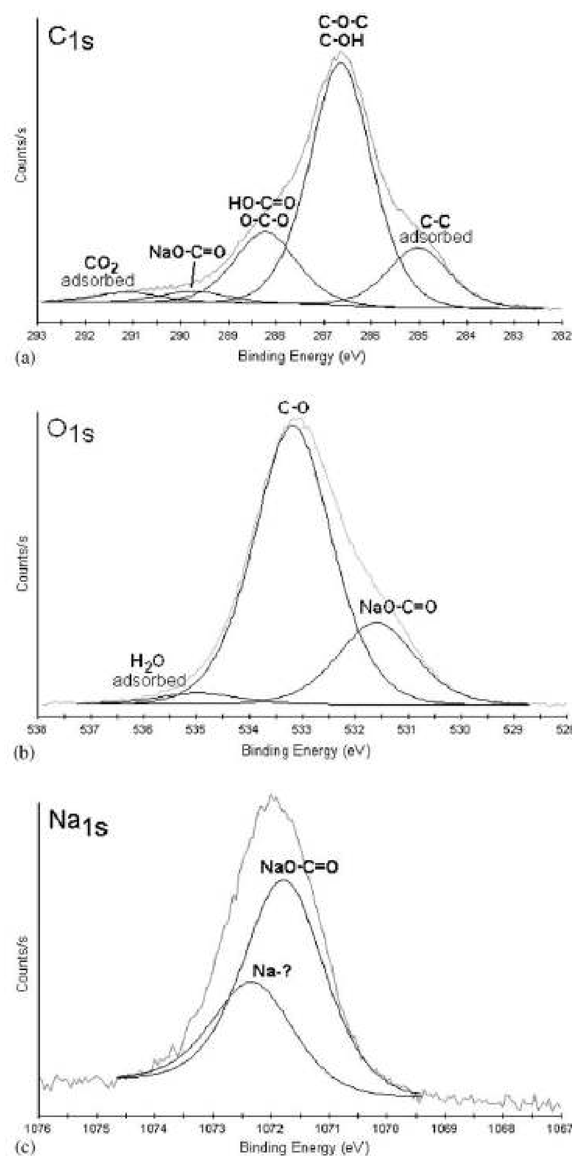

Figure 3: Chemical groups involving (a) carbon, (b) oxygen, and (c) sodium atoms that are present in Pronova™ UP LVG sodium alginate ($Pron_{Pur}C$). Chemical groups (labelled above the peaks) are identified by the characteristic binding energies of the C1s, O1s, and Na1s peaks the XPS spectra. The spectral peaks for the other four alginates were similar in shape and binding energy (not shown).

Table 2
Chemical groups involving carbon, oxygen, and sodium atoms, that were detected in sodium alginates subjected to different methods of purification

	C1s					O1s			Na1s	
	C–C adsorbed	C–O–C, C–OH	O–C–O COONa	COOH	CO_2 adsorbed	COONa	C–O	H_2O adsorbed	COONa	Na-?
Prot-raw	15.1±1.8	27.0±2.3	10.3±0.6	2.0±0.3	1.5±0.3	10.4±0.8	24.9±2.5	2.6±0.9	4.6±0.3	1.6±0.5
$Prot_{pur}D$	10.5±2.8	26.2±2.4	12.0±0.7	2.0±0.2	1.7±0.1	12.8±0.8	26.0±2.5	1.3±0.1	6.2±0.4	1.3±0.1
$Prot_{pur}P$	10.2±1.5	27.8±3.7	10.6±0.7	1.9±0.5	1.6±0.4	10.6±1.8	28.5±2.7	1.8±0.7	6.3±1.6	0.7±0.1
$Prot_{pur}K$	8.5±0.8	29.1±4.2	10.1±0.1	1.8±0.3	1.6±0.3	10.3±1.8	29.1±2.2	1.2±0.5	6.9±2.8	1.1±0.3
$Prot_{pur}C$	11.7±1.9	26.4±3.1	11.1±0.8	1.7±0.1	1.8±0.3	11.8±1.4	26.6±2.1	1.1±0.1	6.2±1.1	1.7±0.2

Values represent the mean atomic percentage±standard error of the mean for three trials, as calculated by XPS spectral peak areas.

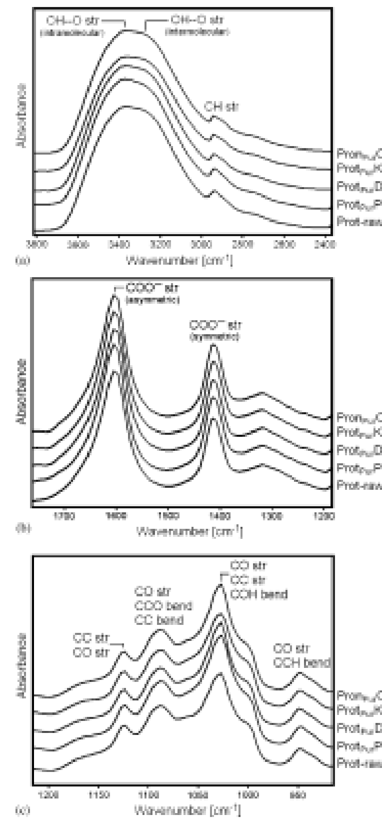

Figure 4: Infrared absorption spectra of sodium alginates that were subjected to different purification protocols. Magnified views of the spectral regions containing peaks that represent (a) intermolecular and intramolecular hydrogen bonding (C-OH), (b) the carboxyl group (COO^-Na^+), (c) various bonds of the carbohydrate ring. The spectra shown are from one of three trials; the spectra from the other two trials contained similar peak shapes and intensities (not shown). str = stretching, bend = bending.

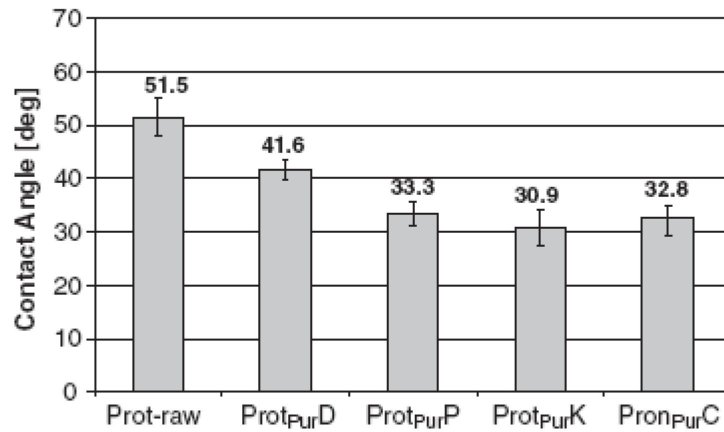

Figure 5: Left contact angle of water droplets measured on films of sodium alginates that were subjected to different purification protocols. The values are expressed as the mean ± standard error of the mean (error bars).

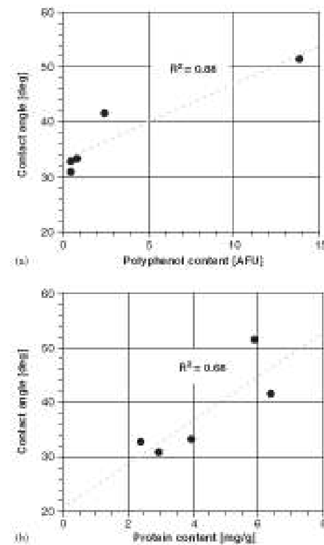

Figure 6: Correlations between contamination levels and the wettability of sodium alginates that were subjected to different purification protocols. Graphs represent the relationship between the contact angle of water on the alginate film and (a) the polyphenol content or the (b) protein content of the alginates. AFU = Arbitrary Fluorescence Unit.

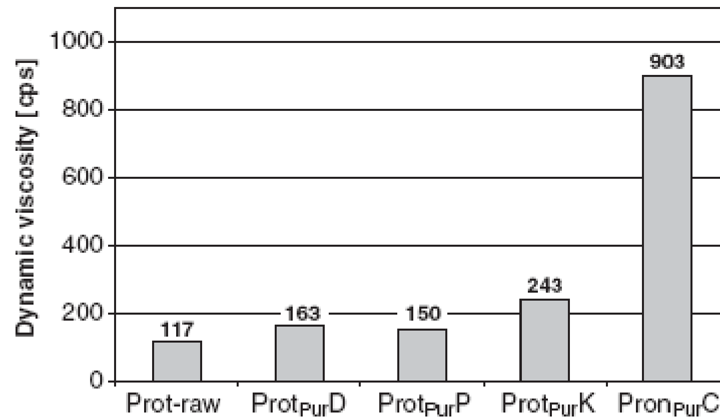

Figure 7: Dynamic viscosities of solutions (2% w/v) of sodium alginates that were subjected to different purification protocols. cps = Centipoise.

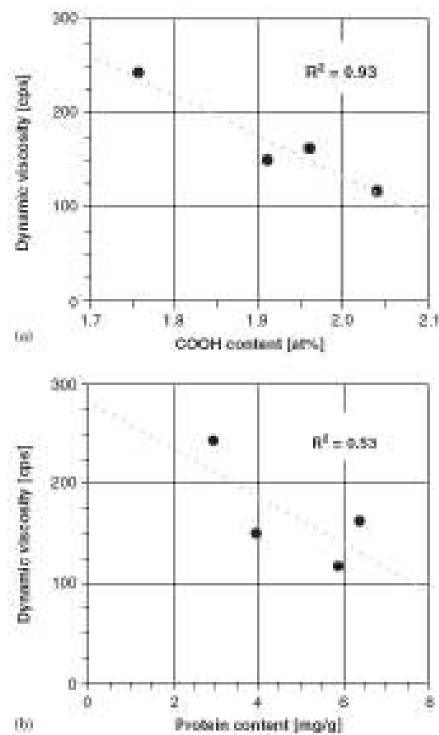

Figure 8: Correlations between the solution viscosity and (a) the COOH levels or (b) the protein content of a pharmaceutical grade sodium alginate that was subjected to different purification protocols. cps = Centipoise.

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In Vivo Behaviour of Microcapsules Using Alginate of Varying Composition

En preparation pour soumission dans JBMR.

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ABSTRACT

Islet microencapsulation within alginate gel is considered as one of the most promising technique to provide a bioartificial pancreas for people suffering from Type I Diabetes. Even if tremendous work has been done to increase alginate biocompatibility, in terms of polymer purification, and if some successful encapsulated islet transplantation were reported, reproducibility and standardization of alginate purification processes has to be addressed. A better understanding of the links between alginate contaminations and the induction of a host immune reaction against microcapsules can be the key in alginate and microcapsules biocompatibility. Here we present an analytical study where different alginate types were assessed for their in vitro and in vivo biocompatibility in function of their alginate contamination levels. We found that the quantities of foreign proteins contained into alginate strongly correlated with the in vivo behavior of microcapsules. Until now, the importance of alginate foreign proteins on microcapsule biocompatibility was underestimated. Though, for the first time, we raise suspicions against the presence of residual foreign proteins as potential responsible for the induction of a host reaction.

INTRODUCTION

Bioartificial organs can be an alternative to regain an essential function from a defective original organ. The biological part of such a device is composed of cells secreting the appropriate therapeutic agent while the artificial component is made with synthetic or natural polymer. In the case of type I diabetes, islets (the biological part) are encapsulated within a hydrogel made with alginate, a natural polysaccharide (the artificial part). This hydrogel has the primary function of protecting the encapsulated cells from the hostile environment of the recipient by the formation of a semi-permeable membrane between them.

Since it is a natural polymer harvested from seaweed and it has to pass through an industrial extraction procedure, alginate has the disadvantage to be highly contaminated. Impurities such as polyphenolic compounds [1, 2], endotoxins [3-5] and foreign proteins [6, 7] were described to be major material contaminants influencing the overall biocompatibility. Fortunately, much progress has been reported from the time when alginate immunogenicity was questioned in the early 90's. Several alginate purification protocols have been described [3, 8-14]. They were shown to be efficient in lowering the levels of contaminants and improving biocompatibility of commercially available alginates. Purification on freshly harvested algae was also depicted and seems to increase alginate quality [12]. Moreover, methods for the evaluation of alginate immunogenicity were developed such as the quantification of in vitro macrophages activation using RT-PCR [15] or the induction rate of apoptosis in cells for a sensitive screening of the potential presence of fibrosis-inducing impurities in alginate [16]. In spite of this progress, improved alginate purification procedures continue to be published [17] as well as evidence of the negative effects of impurities on encapsulated cells [17-19], illustrating the need for further analysis. Persisting concerns pertain to the lack of standardisation between laboratories, the complexity of some purification protocols or the availability of the starting materials (fresh seaweed) and the discrepancy between reported results. Improving the alginate purification procedures requires the identification of the contaminants that are triggering the immune reaction. Most researches have focused on two major contaminants, polyphenols and endotoxins. In a previous study, we have shown that some purification methods remove polyphenols and endotoxins efficiently but that they do not eliminate protein contaminants. Here we report a complete study on the foreign body response to implanted microcapsules made from alginate with varying levels of purity and for the first time, we raise suspicions against the presence of residual foreign proteins as potential responsible for the induction of a host reaction.

MATERIAL AND METHODS

Materials

Two batches of sodium alginate, a commercially purified alginate (Pronova^{TM} UPLVG, Ultrapure, 67% guluronic acid, Mw: 160 kDa) and a non purified alginate (Protanal® LF10/60, 65-75% guluronic acid, Mw: 135 kDa) were purchased from FMC Biopolymers (Drammen, Norway). Solvents used (chloroform, acetic acid, acetone, alcohol) were of analytical grade and purchased from Fisher Scientific (Ottawa, Canada). All manipulations were done under sterile and endotoxin-free conditions.

Animals

C57Bl/6J mice and Wistar rats were purchased from The Jackson Laboratories (15-20g, Bar Harbor, USA) and Charles-River (250-300g, St-Constant, Canada). All animal manipulations were conformed to and approved by the Animal Protection Committee of the Maisonneuve-Rosemont Hospital Research Centre.

Alginate purification

Crude alginate Protanal® LF10/60 (AlgNP) was purified following three in-house protocols described by Prokop and Wang [8] (Alg1), de Vos *et al.* [10] (Alg2) and Klöck *et al.* [3] (Alg3). Details of these purification protocols are available in the original papers and were also described and compared in Dusseault *et al.* [7]. A commercially purified alginate Pronova^{TM} UPLVG was used in comparison (Alg4) with no further purification steps. Briefly, in the purification procedure by de Vos *et al.* [10], alginate was dissolved, under constant stirring, in 1 M sodium ethyleneglycotetraacetic acid (EGTA) solution at 4°C into a 1% solution and filtered over a 0.22 μm ilter. Afterwards, the alginate solution pH was lowered to 2.2 under constant monitoring by addition of 2 N HCl plus 20 mM NaCl. Next, the solution was filtered over a Buchner funnel (pore size 1.5 mm). The precipitate was

brought in 0.01 N HCL plus 20 mM NaCl, vigorously shaken, and filtered again over the Buchner funnel. This washing procedure was performed three times. Then, three extractions with chloroform/butanol was performed by suspending alginate in 100 mL of 0.01 N HCl with 20 mM NaCl and supplemented with 20 mL chloroform and 5 mL 1-butanol. The mixture was vigorously shaken for 30 minutes and filtered over the Buchner funnel. Next, alginate was brought in water and slowly dissolved by gradually increasing the pH to 7.0 by the slow addition of 0.5 N NaOH plus 20 mM NaCl over a period of at least 1 h. The alginate solution obtained was subjected to two chloroform/butanol extractions by the addition of chloroform (20 mL at each 100 mL alginate solution) and 1-butanol (5 mL at each 100 mL alginate solution) and vigorously shaken for 30 min. The mixture was centrifuged for 3–5 min at 3000 rev/min. The chloroform/butanol phase was removed by aspiration. Alginate was finally precipitated with ethanol (200 mL of absolute ethanol was added to each 100 mL of alginate solution) for 10 min, filtered over the Buchner funnel, and washed two times with absolute ethanol. Finally, the alginate was washed three times with ethyl ether and freeze-dried overnight.

The original purification methods described by Klöck *et al.* [3] were slightly modified. Briefly, 9 g of alginate powder was incubated in 400 mL of chloroform for 30 min and filtered under vacuum on Whatman no. 4 filtrating paper. This chloroform extraction was performed three times. Then, alginate was dissolved in distilled water to a 1.5% solution. Next, an equivalent alginate-weight of acid-washed activated charcoal was added to the solution and the mixture was stirred for 4 h. This procedure was repeated once using neutral charcoal. The solution was filtered on a 0.22 μm filter. Afterward, alginate beads were produced using a 50 mM $BaCl_2$ solution as the jellifying agent. Beads were washed with sterile water and then incubated three times into a 1 M acetic acid (pH 2.3) for 14 h. Beads were washed with sterile water between each medium change. Next, beads were incubated twice in a 500 mM sodium citrate solution (pH 8) for 8 h. Beads were washed with sterile water at each medium change. Then alginate beads were extracted twice with 50 and 70% ethanol (containing 5% acetone) for 16 h each without sterile water

wash. Afterward, beads were washed with a 20 mM $BaCl_2$ solution then extensively with sterile water. Beads were dissolved into an alkaline 250 mM ethylenediaminetetraacetic acid (EDTA) solution (pH 10) overnight. The recovered solution was filtered (0.22 µm) and dialyzed (membrane molecular weight cut-off 50,000 Da) against distilled water for 20 h with three medium changes. After the addition of 10 mM NaCl, alginate was precipitated with two volumes of 100% ethanol and freeze-dried overnight. For the Prokop and Wang [8] purification protocol, alginate was purified the same way as the Klöck *et al.* [3] purification procedure without the chemical extractions on alginate beads. The Pronova™ UPLVG alginate (Alg4) was purified by the supplier before its purchase according to an undisclosed protocol. No additional purification of this alginate was performed.

Quantification of alginate contaminants

The most commonly known alginate contaminants are endotoxins, polyphenols and foreign proteins. The quantities of these particular contaminants in alginate batches used in the present study were previously determined [7] and are reported in Table 1. Endotoxins were detected using a commercial limulus amoebocyte lysate (LAL) assay (E-Toxate®; Sigma-Aldrich Inc., St-Louis, USA). Polyphenolic compounds were revealed by the fluorescence spectra of alginate and quantified using a spectrofluorimeter (emission wavelength of 445 nm and excitation wavelength of 366 nm; LS-5 Luminescent Spectrometer; Perkin-Elmer, Oak Brook, USA) while foreign proteins were measured using a MicroBCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, USA) accordingly to the manufacturer specifications.

Microcapsules fabrication

Microcapsules were prepared using the following protocol. Briefly, a sterile solution of sodium alginate of varying purity was extruded using an electrostatic droplet generator [20] into a bath of 100 mM calcium lactate buffered with HEPES, using a syringe fitted with a 22G needle. The calcium alginate microcapsules were allowed for complete

jellification for 30 minutes. Finally, microcapsules were washed twice with Ringer solution (150 mM NaCl, 5.6 mM KCl, 1.7 mM $CaCl_2$ and 10 mM HEPES) for 3 minutes. Microcapsules were always used the day after their fabrication.

Microcapsule implantation and explantation

Five hundred microcapsules were suspended in Ringer solution and injected through a 22G catheter into the peritoneal cavity of anesthetized mice (Isoflurane, 5% for 30 seconds and then 3% for a maximum of 5 minutes, CDMV, Ste-Hyacinthe, Canada) (n=6). Two or fourteen days following microcapsule implantation, mice were sacrificed and microcapsules were retrieved. To do so, a thin incision was cut into the middle-line of the abdomen and maintained open with surgical haemostatic grips. Then, the peritoneal cavity of the mouse was washed carefully with approximately 30 ml of Ringer solution. The recovered solution containing microcapsules was passed through a 200 μm sieve, where microcapsules were retained and separated from free floating immune cells of the peritoneal fluid. The number of these floating cells was determined using a hemacytometer (HY LITE, Hausser Scientific, Illinois, USA). Non injected as well as saline injected animals were used as controls for the quantification of free floating cells (n=6).

Determination of the pericapsular reaction index

All recovered microcapsules from the peritoneal cavity were classified according to the degree of pericapsular reaction [21], using a scale of 0 to 2.0: Score 0: microcapsules showing less than ten adhered immune cells; score 1: microcapsules showing more than ten immune cells but not a uniform layer and score 2: microcapsule showing at least one uniform layer of immune cells surrounding the entire surface. The index of pericapsular reaction was calculated as the mean score of all retrieved microcapsules. The maximal index value for an implantation is 2 and this is reached when all of the explanted microcapsules are covered with a layer of cells.

Pericapsular reaction thickness

Once the index of pericapsular reaction was determined, half of the recovered microcapsules were fixed in 10 % neutral buffered formalin (VWR, Mississauga, Canada), embedded in paraffin and cut into 4 μm slices using a Leica RM2165 microtome. Slices were stained with Wright-Giemsa (Sigma-Aldrich Inc, Oakville, Canada) using the following protocol. First, slides were deparaffined for 2 hours at 60oC and then incubated 30 sec in methanol 100%, 5 minutes in Wright-Giemsa staining solution and finally 7 minutes in water. Slides were rinsed under running distillated water, dried and mounted using Eukitt (ESBE, Montreal, Canada). For each stained microcapsule, four measurements of adheres cell/tissue thickness were made at the microcapsule azimuths (see Figure 2B) and an average value was calculated.

Collagen staining

A second set of microcapsule slides was assessed for a specific collagen staining. Microcapsules slides were deparaffined in toluene (2 x 3 minutes), in absolute ethanol (2 x 3 minutes) and rehydrated in water for 5 minutes. Then, microcapsules were stained for 30 minutes in Fast Green solution (2.5 mM in 1.5 % w/v picric acid, AnaSpec, San Jose, USA), rinsed for 5 minutes in water, stained for 30 minutes in 1:1 Fast Green/ Sirius Red (Sirius Red 0.5 mM in 1.5 % w/v picric acid, Atomergic Chemetals Corp., New York, USA) and rinse for 5 minutes in water. Collagen fibers are specifically stained in red.

Quantification of adhered cells

Half of the recovered microcapsules from explantation were incubated with an enzymatic cocktail of collagenase type V (8.3 mg/ml in PBS, Sigma-Aldrich Inc, Oakville, Canada), DNAse I (0.28 mg/ml in PBS, Sigma-Aldrich Inc., Oakville, Canada) and trypsin (2.8 mg/ml in PBS, Sigma-Aldrich Inc., Oakville, Canada) for 15 minutes at 37°C. To totally digest the pericapsular reaction and separate adhered cells, microcapsules were

vortexed vigorously every 3-4 minutes. To stop the enzymatic digestion, test tubes were put on ice and the supernatant containing the freed cells was collected, washed in HBSS (Invitrogen, Burlington, Canada) and centrifuge twice for 10 minutes at 200g at 4^oC. These cells were quantified using a hemacytometer.

Lymphoid activation of splenocytes

Mitogens are known to activate splenocytes and to induce their proliferation. To investigate their mitogenic potential, alginates were incubated with splenocytes and the proliferation of cells was monitored by the incorporation of [Methyl-3H]thymidine into these cells. First, splenocytes were isolated from a rat spleen. Briefly, the spleen was decapsulated and crushed using a glass rammer. Cells in the supernatant were filtered on a 70 μM cell strainer (BD Falcon, Mississauga, Canada) and washed twice with RPMI (Invitrogen, Burlington, Canada) and centrifuged at 200 g for 10 minutes. Then, erythrocytes were discarded using a NH_4Cl lysis solution (150 mM NH_4Cl, 10 mM $KHCO_3$ and 90 mM EDTA) for 1 minute. The lyses solution was neutralized with the addition of RPMI (Invitrogen, Burlington, Canada) containing 10 % new calf serum (Invitrogen, Burlington, Canada), 1 % penicillin-streptomycin (Invitrogen, Burlington, Canada) and centrifuged at 200 g for 10 minutes. Finally, cells were counted using a hemacytometer, suspended in RPMI containing 0.3 μM of β-mercaptoethanol (Schwarz/Mann Biotech, Cleveland, USA) and plated 0.8 x 106 splenocytes per well (in a volume of 175 μl) into a 96-wells plate. 25 μl of diluted alginate was added into wells and cultivated for 168h. Six hours before the end of the culture period, 10 μl of [Methyl-3H]thymidine (2 μCi/well, Perkin-Elmer, Woodbridge, Canada) was added in wells. Finally, splenocytes were collected, mixed with 3 ml of scintillation liquid and the [Methyl-3H]thymidine incorporation rate in cells was evaluated using a Wallace 1409 β-counter (Perkin-Elmer, Woodbridge, Canada). Lipopolysaccharide (LPS) (10 μg/ml, Sigma-Aldrich Inc., Oakville,

Canada) was added as a positive control. Splenocytes cultivated in RPMI only (no alginate nor LPS) was used has a negative control.

Statistical analysis

A student t test (unequal variance, two-tailed) was performed to compare all results except for the collagen staining where a contingency table and a Chi-square test with Yate's correction (two-sided) were done. All results are presented as mean ± standard error of the mean. A correlation between detected quantities of contaminants and the in vivo behavior of the implanted microcapsules was performed to see if one of these contaminants can predict the bioperformance of the implant. To do this, a linear stepwise regression between in vivo pericapsular index after 2 days of implantation and each of the quantified contaminant was analyzed in a multivariable analysis. Results are presented as a partial R-square, reflecting the relative influence of each contaminant on the in vivo index score.

RESULTS

In vivo biocompatibility of microcapsules

Microcapsules made with alginates that underwent different purification processes were assessed for their *in vivo* biocompatibility in mice. After 2 or 14 days of implantation into the peritoneal cavity of mice, microcapsules were explanted and pericapsular reaction against the microcapsules surface was characterized by the evaluation of several parameters. First, a visual evaluation of the immune reaction was performed and an index of the pericapsular reaction was obtained (Figure 1). At both post-implantation periods, microcapsules made from non purified alginate showed the higher pericapsular reaction with 1.39 ± 0.04 at day 2 (Figure 1A) and 1.84 ± 0.03 at day 14 (Figure 1B). All purified alginates induced a significantly lowered pericapsular reaction (Alg1: 1.09 ± 0.07, Alg2:

0.93 ± 0.12 and Alg3: 0.88 ± 0.06) while the commercially purified alginate Alg4 showed the lowest index (0.33 ± 0.05), which was statistically different from the other purification methods. At day 14, the index value increased for all alginates in comparison with day 2 and this time, only the Alg3 purification process with 1.38 ± 0.1 and the commercially purified alginate Alg4 with 1.42 ± 0.1 were statistically lower than the non purified control, AlgNP (1.84 ± 0.03).

Since the in-house alginate purification method Alg3 and the commercially purified Alg4 showed the best in vivo biocompatibility, the following pericapsular reaction characterization set of experiments were only performed on these alginates in comparison with the non purified one AlgNP. The thickness of the accumulated cells around microcapsule surface was measured and is reported in figure 2. After a short implantation period of 2 days, a variation of microcapsule pericapsular reaction was observed. Microcapsules made from AlgNP alginate showed the more significant pericapsular reaction reaching 73.7 ± 18.1 μm of thickness comparatively with the Alg3 purified alginate of 48.0 ± 10.0 μm of thickness. No pericapsular reaction was seen on microcapsules surface when commercial Alg4 was used. The results obtained for a longer post-implantation time, at 14 days, illustrated a different pattern. In all alginate categories, the pericapsular reaction thickness was increased compared to the results at 2 days of implantation; however, no significant difference was detected between AlgNP, Alg3 and Alg4 with thicknesses of 89.4 ± 9.7 μm, 78.5 ± 12.3 μm and 85.8 ± 13.9 μm respectively.

Afterwards, the number of adhered cells on microcapsule surface was quantified (Figure 3). We found that the number of adhered immune cells on microcapsules made from the commercial Alg4 of 204.8 ± 130.9 cells per microcapsule was significantly lower compared with all other microcapsules prepared from purified or crude alginate, the number of adhered cells ranging from 1795.4 ± 154.1 to 2187.4 ± 392.7 cells per microcapsule.

Collagen content of fibrosis surrounding explanted microcapsules

The nature of the immune reaction around microcapsule surface was further analyzed and monitored for the development of a fibrotic reaction by the deposition of collagen fibers. Fourteen days post-implantation, purified alginate (either in-house Alg3 or commercial Alg4) presented similar scores (Figure 4A). Approximately half of microcapsules found on microscope slide showed collagen fibers accumulation while all of the microcapsules made with crude alginate were positively stained (Figure 4C and E). No collagen positive staining was observed surrounding all groups of microcapsules 2 days after implantation (Figure 4B and D).

When microcapsules were retrieved from mice peritoneum, free floating cells into the peritoneal fluid were also recovered and quantified (Table 2). The amount of floating cells was significantly elevated (ranging from 3.31 ± 0.47 to 4.45 ± 1.42 million of cells) when microcapsules made from any alginate, purified or not, was implanted compared to the saline injections (0.18 ± 0.18) or the non injected controls (0.49 ± 0.09). The number of free floating cells decreased significantly by 14 days (ranging from 0.46 ± 0.13 to 1.43 ± 0.41 million of cells) for most of microcapsule categories, reaching the non injected basal level of cells. There was no significant difference in free floating cells due to the type of alginate used to fabricate the microcapsules.

Finally, the relative capacity of each type of alginate to activate the immune system was evaluated in terms of lymphocyte activation and proliferation. The quantity of incorporated [Methyl-^{3}H]thymidine within rat splenocytes, which is a measure of splenocytes activation and proliferation, was high for the crude alginate AlgNP (38.0 ± 8.4 cpm) and for two in-house purification protocol Alg1 (37.4 ± 1.8 cpm) and Alg2 (35.5 ± 8.1 cpm) and similar to the positive control using LPS (54.7 ± 3.9). Again, activation levels

for the same alginate obtained from in-house purification Alg3 (27.2 ± 0.5 cpm) or the commercial Alg4 (13.5 ± 2.7 cpm) were significantly lower than the LPS positive control. Moreover, activation by the commercially purified alginate Alg4 was also lower than the non purified alginate AlgNP.

Relationship between the immune response and alginate contaminants

Correlation studies between quantified contaminants and the in vivo biocompatibility of alginate microcapsules (pericapsular reaction index 2 days post-implantation) gave the partial R-square results where proteins (R^2 = 0.5957) demonstrated a higher R-square than the two other contaminants polyphenols (R^2 = 0.0499) and endotoxins (R^2 = 0.0332), meaning that the amount of protein contamination in alginate is the main factor predicting the in vivo bioperformance of microcapsules.

DISCUSSION

In this paper, by studying different aspects of the host immune reaction against microcapsules of varying purity, we showed that the in vivo fate of alginate microcapsule is strongly related to the contaminants levels and that foreign proteins contained into alginate seems to play a major role in that immunogenicity.

First, the analysis of the index of the pericapsular reaction against microcapsules gave us a more general picture on what's going on when microcapsules made from different alginate are implanted in vivo. After only two days of implantation, representing the acute phase of the host reaction against microcapsule, it was seen that when contamination quantities of endotoxins, polyphenols and proteins were lowered with purification (Alg1, 2, 3 and 4), the host reaction against microcapsules was also reduced. Moreover, an even more reduced reaction was measured with the less contaminated

alginate Alg4. This result was supported with other measurements of the foreign body response such as the number of adhered immune cells per microcapsules that where diminished for the Alg 4 and the average thickness of the accumulated cells around microcapsules's surface that were reduced or even not detected when alginate was purified.

In the late phase of the foreign body response, 14 days after implantation, the index of the pericapsular reaction was greater for all alginate but the two less contaminated alginate Alg 3 and 4 where their index are still significantly different from the crude alginate. To know if implanted microcapsules were subjected to fibrosis phenomenon, a reflect of a more chronic phase of the foreign body response, the deposition of collagen fibres around microcapsules was assess and again, purified alginate were inducing a less severe host reaction with a decreased collagen positive microcapsules. All microcapsules were collagen negative 2 days following the implantation because it was too early in the foreign body response. However, no difference between studied alginate was observed in term of pericapsular reaction thickness. This can be easily explained by the fact that the analysis of the cellular thickness is less representative of the reaction because only a few microcapsules are analysed comparatively to the pericapsular reaction index where all retrieved microcapsule are analysed and classified, leading to a more representative and precise result. This can also explain why the pericapsular reaction thickness was zero for Alg4 at day 2 since only 32% of the retrieve microcapsules were showing a reaction.

It should be also noticed that the quantification of adhered cells per microcapsules was only done 2 days following the implantation since at 14 days, the fibrosis reaction has already begun with the deposition of collagen fibres, making cell count almost impossible.

Quantification of free floating cells present in the peritoneal cavity following microcapsule implantation was taken has an indication of inflammation. Two distinct phenomenons could be observed in the acute phase of the inflammation (2 days). First, the only injection of a saline vehicle raised the quantity of free floating cells into in the peritoneum, and second, the addition of microcapsules re-increased these cells quantities,

meaning that the implantation procedure may contribute to the initiation of the foreign body reaction. This effect was also reported elsewhere [22]. Surprisingly, no differences were detected between all alginates, including the non purified one. This means that the quantities of contaminants into alginate are not responsible for the increase of the number of cells into the peritoneum. After 14 days, all cell amounts came back to the basal level.

Activation and proliferation of lymphocytes was already used in other publication [4, 6] to assess the mitogenic activity of alginate e.i. to induce lymphocytes mitosis and proliferation. We observed that if fewer contaminants were detected in alginate, the lowest was the lymphocytes activation. This is agreement of phenomenon reported by others. Here, measurements were made on diluted alginate sample and not with microcapsules. In a 2005 publication [6], it was shown that same tendencies are obtained with both techniques.

Finally, we were interested to know if one of the known contaminant, namely endotoxins, proteins and polyphenols, can correlate with the in vivo behaviour of microcapsules and we unexpectedly found that the quantities of foreign proteins contained into alginate strongly correlated with the pericapsular reaction index of microcapsules. We are not stating that proteins are the only factor influencing the biocompatibility of microcapsules and that endotoxins and polyphenolic compounds cannot cause a foreign body reaction because it is well known, for example, that endotoxins can activate macrophages via the Toll-like receptor 4. However, until now, the importance of alginate foreign proteins on microcapsule biocompatibility has been underestimated.

We are aware that new alginates and some new commercially purified alginate are now available. Further analysis on these starting materials remains to be done to see if they can be used safety in vivo and also in cell encapsulation paying attention to the levels of remaining proteins. We unexpectedly found that the quantities of foreign proteins contained into alginate strongly correlated with the pericapsular reaction index of microcapsules. We are not stating that proteins are the only factor influencing the biocompatibility of microcapsules and that endotoxins and polyphenolic compounds cannot cause a foreign

body reaction because it is well known, for example, that endotoxins can activate macrophages via the Toll-like receptor 4. However, until now, the importance of alginate foreign proteins on microcapsule biocompatibility has been underestimated.

FIGURE, TABLES AND LEGENDS

Table 1 : Alginate specifications and contamination.

Label	Source	Purification procedure	Endotoxins (EU/ml)	Polyphenols (AUF/ml)	Proteins (mg/ml)
AlgNP	Protanal®LF10/60	No	87.69 ± 8.13	31.81 ± 0.55	135.33 ± 8.51
Alg1	Protanal®LF10/60	Prokop and Wang(ref)	162.41 ± 16.3	1.78 ± 0.68	84.08 ± 8.96
Alg2	Protanal®LF10/60	De Vos *et al.*(ref)	15.56 ± 1.63	4.96 ± 0.18	130.21 ± 40.26
Alg3	Protanal®LF10/60	Klöck *et al.*(ref)	0.13 ± 0.02	0.82 ± 0.31	51.46 ± 6.66
Alg4	Pronova™UPLVG	Commercially purified[a]	0.10 ± 0.02	0.61 ± 0.04	30.99 ± 0.08

[a] purification protocol not provided by the manufacturer, no further purification step was performed.

Table 2: Amount of free floating cells into mice peritoneum.

Alginate type	2 days post-implantation ($1x10^6$ cells ± SEM)	14 days post-implantation ($1x10^6$ cells ± SEM)
AlgNP	3.71 ± 1.42 $^{\psi}$	0.69 ± 0.27 ***
Alg1	3.29 ± 0.49 $^{\delta}$	1.43 ± 0.41 *,ψ
Alg2	3.31 ± 0.47 $^{\delta}$	0.39 ± 0.09 ***
Alg3	3.84 ± 1.37 $^{\psi}$	0.87 ± 0.35
Alg4	4.45 ± 1.42 $^{\psi}$	0.46 ± 0.13 *
Saline	1.18 ± 0.18 $^{\gamma}$	0.50 ± 0.10 **
No injection	0.49 ± 0.09	

ψ p<0.05 vs saline and non injected
δ p<0.001 vs saline and non injected
γ p<0.01 vs non injected

*** p<0.005 vs 2 days
** p<0.01 vs 2 days
* p<0.05 vs 2 days

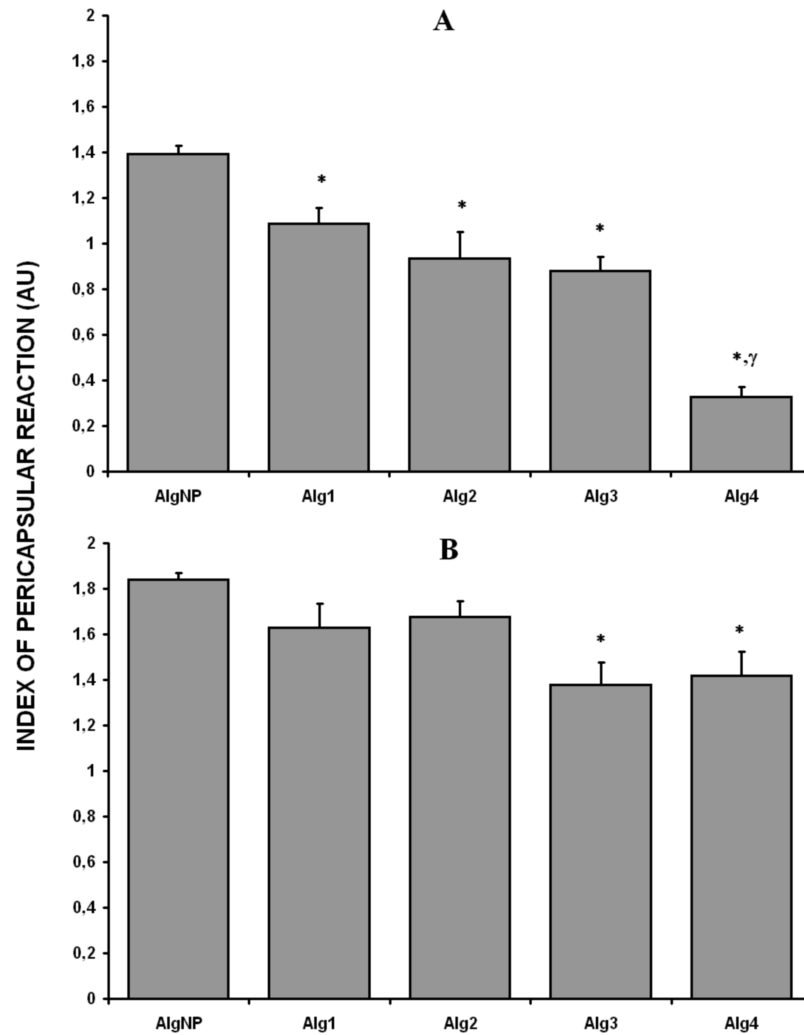

Figure 1: In vivo biocompatibility of microcapsules. Microcapsules made from alginates of varying purity were retrieved from mice peritoneal cavity at A. two and B. fourteen days post-implantation. Results are presented in term of index of pericapsular reaction arbitrary units (AU) ± SEM (n=6). * $p<0.01$ vs. AlgNP and γ □$p<0.001$ vs. Alg1, Alg2 and Alg3.

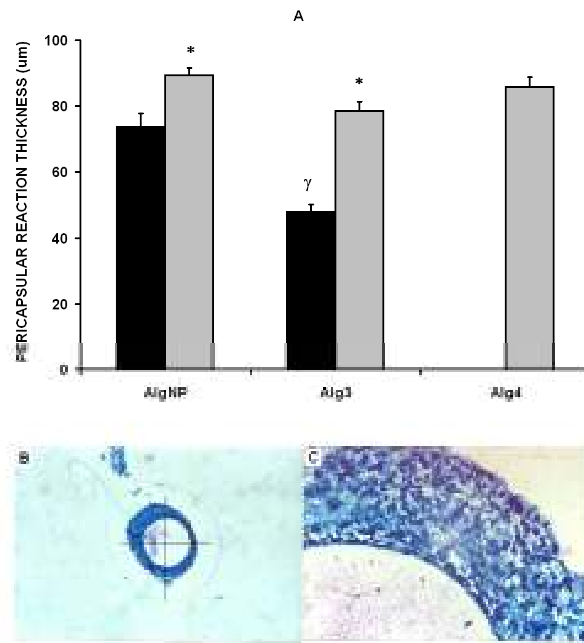

Figure 2: Pericapsular reaction severity. A. Thickness of pericapsular reaction was measured on histological blades and is presented in micrometers of cells ± SEM. Black columns: 2 days post-implantation, grey columns: 14 days post-implantation. * $p<0.01$ for 2 vs. 14 days and γ $p<0.001$ AlgNP vs. Alg3. Photomicrographs at B. 32X and C. 63X of Wright-Giemsa histological staining.

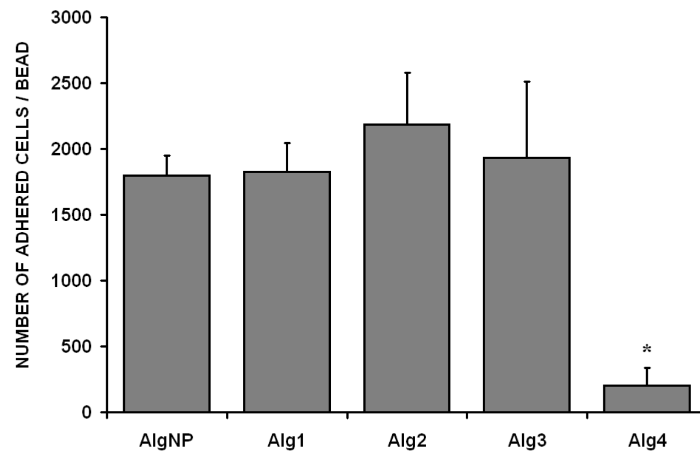

Figure 3: Measurement of cell adhesion. Results are presented in number of cells per microcapsules ± SEM (n=6). Adhered to microcapsules after 2 days of implantation into the peritoneal cavity of mice * p<0.05 vs. AlgNP.

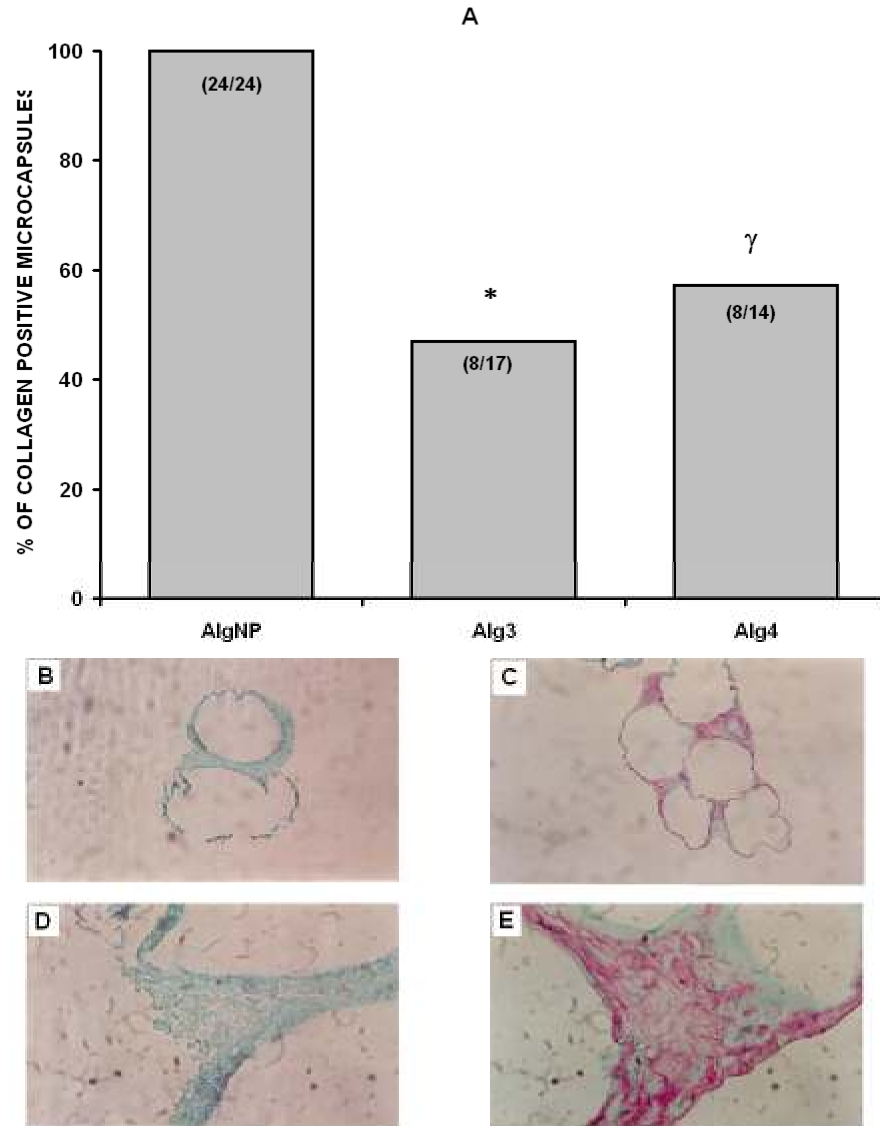

Figure 4: Formation of fibrosis. Pericapsular reaction surrounding microcapsule surface, 14 days after implantation, showing collagen deposits were quantified. A. Results are presented in percentage of collagen positive microcapsules (%). * $p<0.001$ and γ $p<0.01$ vs. AlgNP. Photomicrographs of collagen negative microcapsules at B. 10X and D. 63X and collagen positive microcapsules at C. 10X and E. 63X.

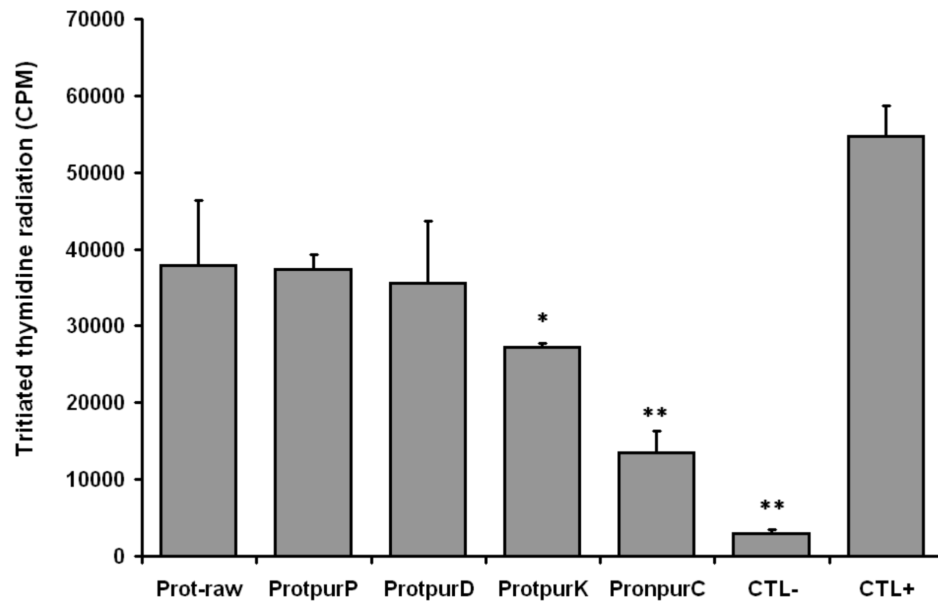

Figure 5: Lymphoid activation. Crude and purified alginate was assessed for lymphocytes activation. Results are presented as the rate of tritiated thymidine incorporation by cells in CPM ± SEM (n=3). * $p<0.05$ and ** $p<0.01$ vs. CTL+.

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Role of Protein Contaminants in the Immunogenicity of Alginates.

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No benefit of any kind will be received either directly or indirectly by the authors.

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Abbreviated title: Immunogenicity of protein contaminants in alginate.

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ABSTRACT

Alginate is widely used for cell microencapsulation and transplantation. There is a lack of standardization of alginate purity and composition. In a previous study, we compared different alginate purification methods and concluded that polyphenol and endotoxin contaminants were eliminated efficiently but residual protein contaminants persisted with all of the methods under evaluation. The objective of the present study was to test the hypothesis that residual proteins play a role in the immunogenicity of certain alginate preparations. Using preparative size exclusion chromatography (SEC) and a large scale purification protocol that was derived from the findings obtained with SEC, we substantially decreased the protein content of alginate preparations. When implanted into mouse peritoneum, barium alginate beads made of alginates that were purified using SEC or the derived large scale protocol induced significantly less pericapsular cell adhesion than those made with control alginates. Conclusion: These results suggest that removing residual protein contamination may decrease the immunogenicity of certain alginate preparations. The measurement of proteins could be used as a screening method for evaluating alginate preparations.

Key words: Microencapsulation, islets of Langerhans, alginate purification, cell transplantation

INTRODUCTION

Microencapsulation of therapeutic cells within semipermeable membranes is investigated as a means of immunoprotection that allows transplantation without immunosuppression (1-4). Alginate remains the most widely experimented polymer for immobilizing cells and forming the microcapsule core (2,5-8). This choice is justified by the capacity of alginate to be jellified in physiological conditions, without the use of toxic solvents or the generation of harmful by-products that would damage living cells (9,10). The rapidity of the gelling process is also a critical advantage (9,10). When cell-containing sodium alginate droplets fall into a divalent cation solution, they gel instantaneously, allowing the resulting polymerized alginate beads to keep the spherical shape the droplets have taken in the air.

A major hurdle to successful application of microencapsulated cell transplantation is the immunogenicity of the biomaterials that are used to fabricate microcapsules (2,5,6,11-15). Alginate, a natural polymer that is extracted from seaweeds, is highly susceptible to contamination. Moreover, the industrial processes that are used to extract alginate may introduce additional contaminants. When islets of Langerhans that are encapsulated using unpurified alginates are transplanted into diabetic animals, an inflammatory reaction develops around the microcapsules. The immune cells release cytokines that are toxic for encapsulated cells and attract fibroblasts leading to pericapsular fibrosis, which interfere with the diffusion of nutrients and hormones (16). In this case, the normalization of blood glucose lasts only a very short period, if it is achieved at all (11,12,16).

A number of research groups have developed their own in-house methods for purifying alginates (17-20). The use of purified alginate has allowed successful encapsulated islet transplantation in terms of normalizing blood glucose in different animal

models of diabetes (19,21). Despite these encouraging results, there is a lack of standardization of alginate preparations and the immunogenicity of these transplants remains a problem (2,5,6,22,23). Alginate immunogenicity is affected by a number of variables, which respective role is poorly defined. These include the starting material (i.e. alginate batches), the method of purification, the nature and quantity of residual contaminants and their respective impact and the chemical composition of alginate, particularly the guluronic acid/mannuronic acid ratio. By optimizing both the alginate purity and composition, it has been possible to produce microcapsules that have induced minimal cell adhesion for up to two years after peritoneal implantation (24). As a means to improve the standardization of the alginate starting material, a purification procedure starting from freshly harvested algae was also described (25). Purified alginate obtained from this purification process showed interesting results (12,26) but is restricted to laboratories having an easy access to algae. The poor reproducibility of the results within and between laboratories, including the limited duration of graft survival, which varies from a few days to over one year (18,19,22,23,27-29), indicates that more knowledge is needed to allow a better standardization of alginate preparations for clinical use.

In order to standardize alginate preparations for transplantation applications and to obtain reproducible results, it is mandatory to better understand the mechanisms involved and to characterize the alginates that are less versus more immunogenic. We published the first comparative study (30) of the different in-house alginate purification methods. We found that the methods under study decrease polyphenol, endotoxin and protein contaminants with various efficiencies. However, with the alginate batch that was used as starting materials, a significant residual protein contamination was observed after purification with all of these methods (30).

The objective of the present work was to verify the hypothesis that residual proteins play a role in the immunogenicity of certain purified alginates.

MATERIALS AND METHODS

Materials

A pharmaceutical-grade sodium alginate Protanal® LF10/60 (65-75% guluronic acid, Mw 135 kDa, as specified by the manufacturer, lot# S13636) and a commercially available industrially purified sodium alginate, Pronova UPLVG™ (ultrapure, low viscosity, 67% guluronic acid, Mw 160 kDa, as specified by the manufacturer, lot# FP-303-02) were purchased from FMC Biopolymers, Drammen, Norway. All other materials (chloroform, acetone, alcohol, acetic acid, sodium citrate, potassium bromide) were of analytical grade, purchased from Fisher Scientific Ltd. (Pittsburgh, PA, USA), and were used without further purification. All glassware was washed (soaked for 10 minutes in Extran soap, then washed with distilled water, HCl 2N and sterile water) and submitted to autoclave or ethylene oxide gas sterilization in order to remove endotoxins. Manipulations were performed under sterile conditions whenever possible.

Alginate purification

The Protanal® LF10/60 alginate was purified using three different in-house purification procedures that were derived from a protocol that was originally published by Klöck et al. (18,30).

Size Exclusion Chromatography Procedure

Size exclusion chromatography (SEC) was performed on a Waters 600 system equipped with SEC columns (Shodex Ohpak SB-2003, exclusion limit: 100 kDa and SB-2004 ,exclusion limit: 400 kDa, 20 x 300 mm) in series and in-line with a Waters 2414 refractive index detector and a Water 486 UV detector. Alginate elution was detected using the refractive index while proteins were detected by their characteristic absorbance at 280 nm. Distilled water or 150 mM KBr solutions were used as the eluent at a flow rate of 2 mL/min. Poly(ethylene glycol)s of 300, 40 and 4 kDa (Sigma-Aldrich Inc., St-Louis, MO, USA) were used as calibration standards (R^2 0.9935).

Alginate purification using the Klöck procedure and saline dialysis (K+SD)

The studies using SEC revealed that when alginate is dissolved in water, as is usually done for alginate purification, some proteins stick to alginate and are not eliminated during the purification process. The SEC studies also showed that by modifying the salt content of the eluent, the electrostatic links between the two polymers are neutralized, allowing their separation. However, the SEC procedure is not appropriate for large scale production. Therefore, a step of dialysis in saline was added to the Klöck procedure. This was followed by the usual dialysis against water for the removal of salts and other potential contaminants.

The Pronova UPLVG^{TM} alginate was purified by the supplier before its purchase according to an undisclosed protocol. No additional purification of this alginate was performed.

Measurement of alginate contamination

Protein quantification

The amount of protein contaminants present in alginate was measured using the commercially available Micro BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, Illinois). Alginates were dissolved in sterile water at a concentration of 1% w/v and sterilized by sequential filtration (final filter pore size: 0.22 µm). Using a spectrophotometer (Spectronic 1001 plus, Milton Roy, Rochester, NY), the protein concentration was measured in terms of light absorbance at 540 nm, which is specific for the purple color. To quantify the results, light absorbance levels were compared to a standard curve that was produced using bovine albumin (R^2 of 0.9886 and 0.9917). For the presentation of the results, concentrations were converted to the equivalent mg of proteins per g of dry alginate (n=2).

Quantification of polyphenol-like compounds

The fluorescence spectra of alginate solutions (1 % w/v in sterile water) were obtained using a spectrofluorimeter (LS-5 Luminescence spectrometer, Perkins-Elmer, Oak Brook, Illinois). An excitation wavelength of 366 nm was applied for an emission at 445 nm in wavelength. Polyphenol-like compounds were detected by the appearance of a characteristic absorbance peak at 445 nm (10). The relative quantities of polyphenol were measured in terms of arbitrary fluorescence units (AFU) (n=3).

Quantification of endotoxins

A commercial Limulus Amoebocyte lysate (LAL) assay (E-Toxare®, Sigma-Aldrich Inc., St-Louis, MO, USA) was used to determine the endotoxin content of the samples. Alginate solutions (1 % w/v in sterile water) were diluted in water in ratios ranging from 1:10 to 1:5.000 and incubated for one hour at 37 ºC with LAL reagent.

Endotoxins were detected by a characteristic clotting reaction, which is marked by an increase in viscosity and opacity of the solutions. Quantification of endotoxin levels was performed by comparison of the sample viscosity with a standard endotoxin curve that was included in the kit. For the presentation of the results, concentrations were converted to the equivalent endotoxin units (EU) per g of dry alginate (n=4).

Alginate viscosity measurement

Samples of alginate were dissolved in a saline buffer (NaCl 0.9 %) in concentrations ranging from 0.4 to 3 % w/v. For each alginate solution, the dynamic viscosity was measured at various concentrations using a Synchro-Lectric rotational viscometer (Brookfield Engineering Laboratories Inc., MA, USA) operated at 25 ºC. A minimum of 10 rotations were done for each measurement in order to ensure an accurate reading.

Beads formation and in vivo implantation

Alginate beads were prepared according to a previously published method (31) with the following modifications. A sterile solution of 1.3 to 2.3 % sodium alginate was extruded using an electrostatic droplet generator (32) into a bath of 10 mM barium chloride buffered with HEPES, using a syringe fitted with a 22G needle. The barium alginate gelled beads were washed in two successive 3 min saline bath. Five hundred alginate beads were then implanted via a 16G catheter into the peritoneal cavity of C57Bl/6 mice anesthetized with Isoflurane (Abbott Laboratories Limited, Montreal, Canada). Protocols were reviewed and stated to conform to the ethical guidelines of the Canadian Council for Animal Care by the animal care ethics committee of the Centre de recherche de l'Hôpital Maisonneuve-Rosemont. These guidelines were observed throughout the study.

Immunogenicity of alginates

Two days following peritoneal implantation, alginate beads immunogenicity was evaluated. Beads were retrieved by peritoneal washings. Briefly, mice were euthanized and a small ventral incision was made. Saline was injected, taking care to irrigate all exposed organs, and transferred into sterile propylene tubes using a pipette. Beads were separated from peritoneal fluid using a 100 μm nylon mesh sieve, and washed three times with saline. The recovered beads were evaluated under an inverted-light microscope and classified into three different categories using the following scoring system: Score 0: no pericapsular cell adhesion, score 1: from more than 10 cells to less than a complete layer of cells coverage, score 2: one uniform layer of cells or more. A scoring system chart is shown in Figure 1. A total score of 2 means that all retrieve microcapsules were totally covered with immune cells while a score of 0, means that all retrieved microcapsules were free of immune cells (n=6).

Statistical Analyses

A Student *t*-test (unequal variance, two-tailed) was used to compare the results. A difference for which $p<0.05$ was considered to be statistically significant. Results are presented as measured value ± standard error of the mean.

RESULTS

An alginate purification protocol that reduces the residual protein contaminants was developed. Since the Klöck purification method has yielded the lowest contaminant levels

and immunogenicity among the in-house alginate purification protocols that were evaluated in our previous study 30, this pre-purified alginate was used as the starting materials and one of the controls for the experiments of the present study.

Preparative Size Exclusion Chromatography (SEC).

During the initial experiments, distilled water was used as eluent, and a 2 ml alginate (1 % w/v) sample was applied onto the columns. The resulting chromatogram (Figure 2A) shows the formation of two major protein peaks (absorbance at 280 nm). The first peak at an elution time (t_e) of 22 min corresponds to a molecular weight of 400 kDa. The second peak was eluted at 34 min (50 kDa). A clear overlap was observed between the alginate peak (t_e: 30 min, 120 kDa) and the second protein peak (absorbance at 280 nm). These results suggested that alginate-protein complexes formed in a water solution. The presence of such complexes severely limits the efficiency of alginate purification with distilled water as the eluent.

To reduce the electrostatic protein-alginate, a salt solution (150 mM KCl) was used as the eluent. Under this condition, the chromatogram (Figure 2B) showed the formation of only one major protein peak at a lower molecular weight (15 kD). It also demonstrated a clear separation between the alginate (t_e: 29min, 135 kDa) and the protein peaks (t_e: 43min). This separation allowed the purification of alginate by pooling the fractions collected between 25 and 38 minutes. Fifty runs were required to obtain the quantity of purified alginate that was required for the completion of the present study.

Quantification of the contaminants

Protanal® LF10/60 alginates that were purified by the SEC and the saline dialysis methods were assayed for their polyphenol, endotoxin and protein contents. Raw Protanal® LF10/60 alginate, the same purified according to the Klöck protocol and UPLVG^{TM}, an alginate commercially marketed as an ultrapure alginate for pharmaceutical purpose, were used as controls.

The alginates that were purified by the SEC and the saline dialysis methods contained similar amounts of proteins (0.52 and 0.53 mg/g), which were significantly less ($p<0.05$ vs UPLVG and $P<0.001$ vs crude and Klöck) than those found in all of the control alginate preparations (Figure 3A). Both new purification methods reduced by more than five fold the alginate protein content compared to the Klöck protocol (2.90 mg/g) and by more than ten fold compared to the non purified alginates (5.15 mg/g). The commercially purified UPLVG^{TM} alginate contained three fold more proteins (1.51 mg/g) than the alginates purified by the SEC or the saline dialysis method.

The polyphenol (1.58 to 2.22 AFU) (Figure 3B) and endotoxin (Figure 3C) (8.3 EU/g) content of the alginates purified by the saline dialysis methods was not significantly different from that of the alginate purified by the Klöck method (polyphenol: 1.39 AFU and endotoxin: 7.6 EU/g), while it was slightly but significantly higher with the SEC method. These results demonstrate that the additional step of dialysis in saline does not adversely modify the effectiveness of the method for polyphenol and endotoxin elimination.

Alginate viscosity

We (30,33) previously showed that the viscosity of alginate solutions is directly affected by the type of purification processed on alginate. That is, when the same starting

material is used, higher levels of contaminants correlated with lower dynamic viscosity of the solutions. In the present experiments, the purification also resulted in the increase of the dynamic viscosity of alginate solutions, which had the effect of shifting the viscosity-concentration curves toward the left from the non-purified to the SEC purified alginates (Figure 4). The results showed that, among the alginates prepared using Protanal® LF10/60 as the starting material, the alginate purified by the SEC method (355 CPS at 2 % w/v) had the highest viscosity followed by the alginate purified by the saline dialysis (280 CPS at 2 % w/v) and the Klöck methods (229 CPS at 2 %). The raw Protanal LF10/60 alginate presented the lowest viscosity with 127 CPS at 2% w/v. Because viscosity is mainly affected by the average molecular weight of the alginate molecules, we ensured that all of the alginate preparations used in this study, except for UPLVG™, were purified from the same starting material, Protanal® LF10/60. Therefore, the viscosity can be used as an indirect measure of the relative purity and/or of a variation in the alginate average molecular weight that is obtained using each of the purification methods. In contrast, the viscosity obtained with UPLVG™ cannot be used to compare the purity of this preparation to the other ones.

Alginate immunogenicity

To investigate the impact of the different purification methods on alginate immunogenicity, we evaluated the pericapsular cell adhesion score on barium alginate beads retrieved 2 days after implantation into mouse peritoneal cavities. Two days after implantation (Figure 5), similar cell adhesion scores were obtained when the beads made with alginates purified by the SEC and the saline dialysis methods were used (0.38 AU vs 0.33 AU). These scores were significantly ($p<0.001$) lower than those obtained with alginate beads made with the control alginates (1.54 AU). The pericapsular cell adhesion scores were reduced by up to 66 % as compared to the score obtained with the Klöck

protocol (0.99 AU). The cell adhesion score of the beads made using the commercially purified UPLVG^{TM} alginate (0.52 AU) was 38 % and 58 % higher ($p< 0,05$ for both), respectively than those obtained with the beads made using alginates purified by the SEC (0.38 AU) and the saline dialysis methods (0.33 AU).

DISCUSSION

We previously conducted a comparative evaluative study of different published in house alginate purification methods, initially focusing on the capacity of these methods to eliminate or reduce the amount of known alginate contaminants (30). It was found that all of the methods under study decrease the polyphenol, endotoxin and protein contents, though with variable efficiency. The most effective methods almost completely eliminated polyphenols and endotoxins. However, significant residual amounts of proteins were found in purified alginate preparations. Additional preliminary studies (unpublished data) suggested that there was a possible relationship between residual proteins and the immunogenicity of alginates.

The present study was undertaken to test the hypothesis that residual proteins play a role in the immunogenicity of alginates. We investigated methods to remove the residual protein contaminants, using as the starting material a Protanal® LF10/60 alginate sample that was purified by the Klöck procedure. In previous experiments (30), the latter was found to be the most effective in-house purification protocol for reducing the contaminant levels (including the proteins) and the immunogenicity of Protanal LF10/60® alginate. This purified alginate preparation as well as UPLVG™, a commercially available alginate marketed as "Ultrapure" for pharmaceutical purpose, which provided similarly good results were both used as controls in the present study. Raw Protanal LF10/60® alginate was used as a non purified control.

In a first series of experiments, using size exclusion preparative chromatography (SEC), we observed that a protein peak of the chromatographic profile could not be separated from the alginate peak, suggesting that some proteins were sticking to alginate. This problem was overcome by modifying the salt content of the eluent used for chromatography. Thereafter, the alginate and protein peaks could be separated, and we succeeded in decreasing the protein content of raw alginate (90 % decrease), as well as of the alginate purified according to the Klöck method (82 % decrease) and the commercially purified UPLVG™ (65 % decrease). Using this new insight, we further modified the Klöck procedure by adding a step of dialysis against a saline solution, followed by the usual step of dialysis against distilled water, which remained required to eliminate the concentrated salts and potentially other contaminants. It is noteworthy that, in all of the published purification techniques that include a dialysis step, only distilled water has been previously used for dissolving and dialyzing alginate.

All of these alginate preparations were evaluated for their contaminant content, as well as for their viscosity and immunogenicity. The alginate that was prepared using the SEC method presented lower protein contaminant content, higher viscosity and lower immunogenicity than the previously used alginates. Alginate that was prepared using the saline dialysis method presented equal or even better results, for the three parameters, than that produced using SEC, while allowing the preparation of larger quantities of materials. The principal determinant of alginate viscosity is the average molecular weight. When the same starting material is used, an increased viscosity/concentration ratio represents an overall estimation of the efficiency of the procedure to eliminate small alginate oligomers and possibly other contaminants. There is possibilities that, upon implantation, some of these oligomers, which are not involved in the alginate gelling process, are released into the environment and trigger an immune reaction. The viscosity/concentration ratio was higher for the alginates purified with the SEC and the Klöck+saline dialysis methods than for other alginates prepared with Protanal LF10/60® alginate as the starting material. This

observation can be explained by two distinct phenomena. First, it is obvious that in both techniques low molecular weight alginate chains are discarded. The 50 kDa molecular weight cut-off dialysis membrane used in the first purification procedure is known to specifically removed contaminants such as EDTA but also discriminated small alginate chains with a molecular weight less than 50 kDa. In the same way, it is clearly seen in SEC chromatograms that the removal of proteins contaminants also retrieved a non negligible quantity of low molecular weight alginate under 100 kDa, corresponding to the cut-off of the chromatographic column. Second, we cannot exclude that the possible interaction between alginate chains and protein contaminants can interfere with the physicochemical characteristic, leading to an increased viscosity when these proteins are removed. These phenomena have previously been described (30).

Whereas in vitro methods were used to characterize different features of alginate preparations, in vivo studies were required to estimate the clinical impact of these features. The effect of the different alginate preparations on microcapsule immunogenicity was evaluated using intraperitoneal microcapsule implantations and recovery, followed by histological analysis. The results of these studies showed a decreased immunogenicity of alginate beads made with the SEC method. Since the fractions removed by this method contained important amounts of proteins, this suggests that the removal of residual proteins substantially decreases alginate immunogenicity. Beads made of purified alginate that using the saline dialysis method, which contained minimal amounts of proteins, also induced a weaker immune reaction than that made of control alginates. Since large quantities of alginate can be processed with this method, it could be potentially used in the manufacturing process.

Polyphenols, endotoxins and proteins are the most abundant contaminants of alginate. Skjak-Braek *et al.* (10) first described alginate contamination by polyphenol-like compounds. Their removal from alginate has been shown to improve biocompatibility (10). All of the alginate purification methods that were evaluated in our previous comparative

study (30), even the simplest procedures, were found to effectively decrease the polyphenol content. Endotoxins are components of the cell wall of gram-negative bacteria and are part of the pyrogen family. The maximum allowable concentration of endotoxins is 5 EU/kg of body weight per hour for intravenous injections (34). Wandrey and Vidal (35) calculated that the contamination limit for alginates used for microencapsulation is equivalent to 2,000 EU/g of polymer, whereas Prokop and Wang (20) estimated the maximum to be equivalent to 1,220 EU/ g of dry alginate. In our comparative study, nearly undetectable levels of endotoxins were found in the commercially purified alginate UPLVG^{TM} and alginate that was purified using the Klöck method. Finally, foreign proteins found in crude alginate were also shown to induce an immune reaction (10,25). In our comparative study, UPLVG^{TM} and alginate that was purified according to the Klöck method contained the smallest amount of proteins (30). However, with the starting material that was used (30), all of the purification methods yielded a residual amount of proteins that could potentially trigger an immune reaction (30). It is noteworthy that different raw alginate batches that are used as the starting material before purification may have quantitatively and/or qualitatively different protein contents. This might explain the different results obtained by different laboratories using the same purification procedures. Nevertheless, the present study confirms the potential harmful effect of residual proteins in purified alginate.

CONCLUSION

Approximately 90% reduction in residual protein contaminants was obtained using size exclusion chromatography or a derived method applicable to large scale alginate purification. The alginates that were produced using either method presented lower immunogenicity than the control alginates. These results suggest that protein contaminants may play a role in alginate immunogenicity. Therefore, when evaluating new batches of alginate, the measurement of protein contaminants should be used as a simple screening

procedure. The biocompatibility of alginate preparations can be improved by selecting batches with low protein contaminant content and/or by eliminating protein contaminants.

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FIGURES, TABLES AND LEGENDS

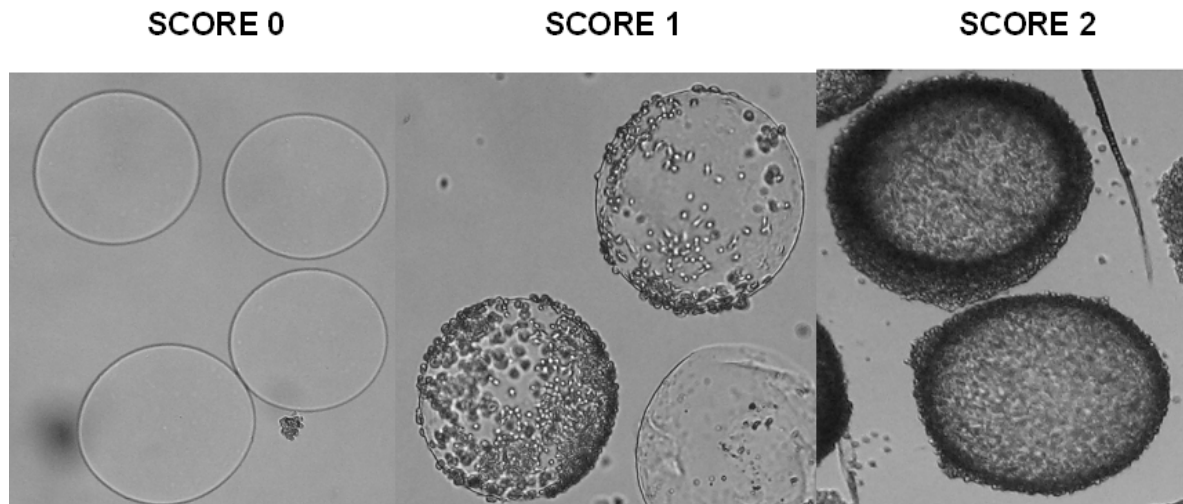

Figure 1. Representative photomicrographs of retrieved microcapsules for each categories of the cell adhesion scoring system.

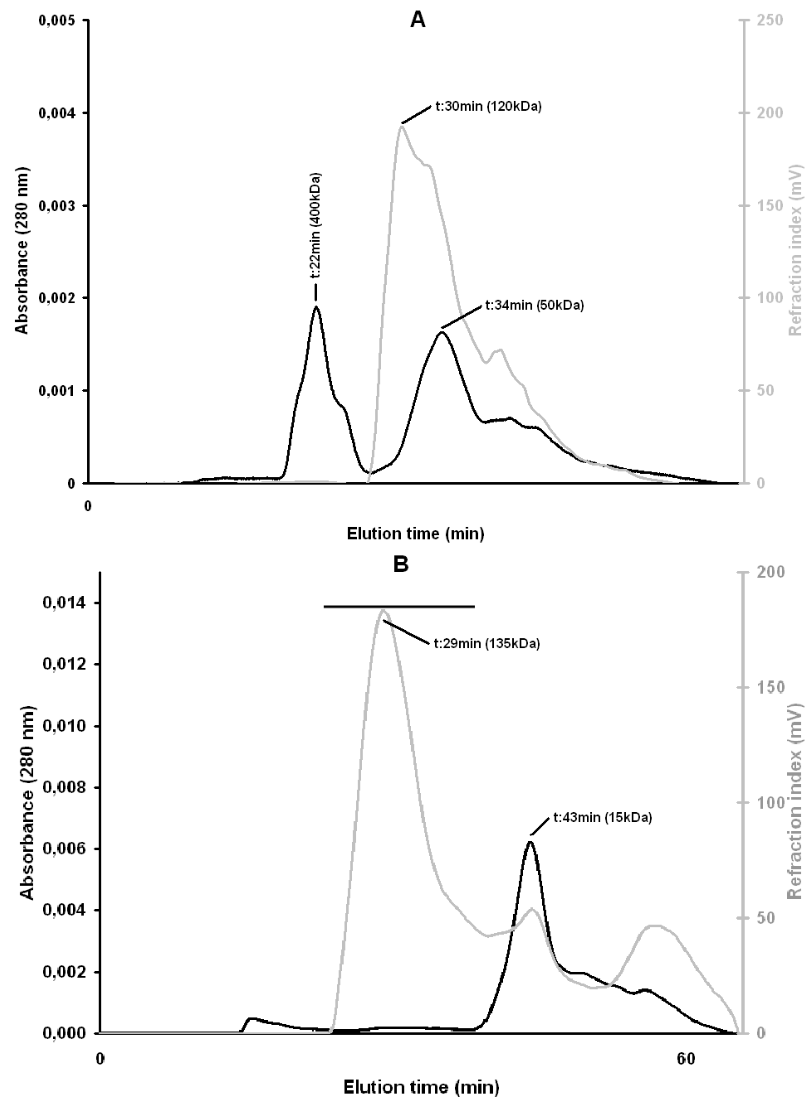

Figure 2. Size exclusion chromatography (SEC) elution profile of Protanal LF10/60 alginate purified using A. Klöck protocol or B. Klöck protocol modified with the addition of a saline dialysis step. The bold line corresponds to the pooled fractions used for subsequent experiments. The proteins are measured by absorbance at 280 nm (black line) and alginate is measured by its refractive index (grey line).

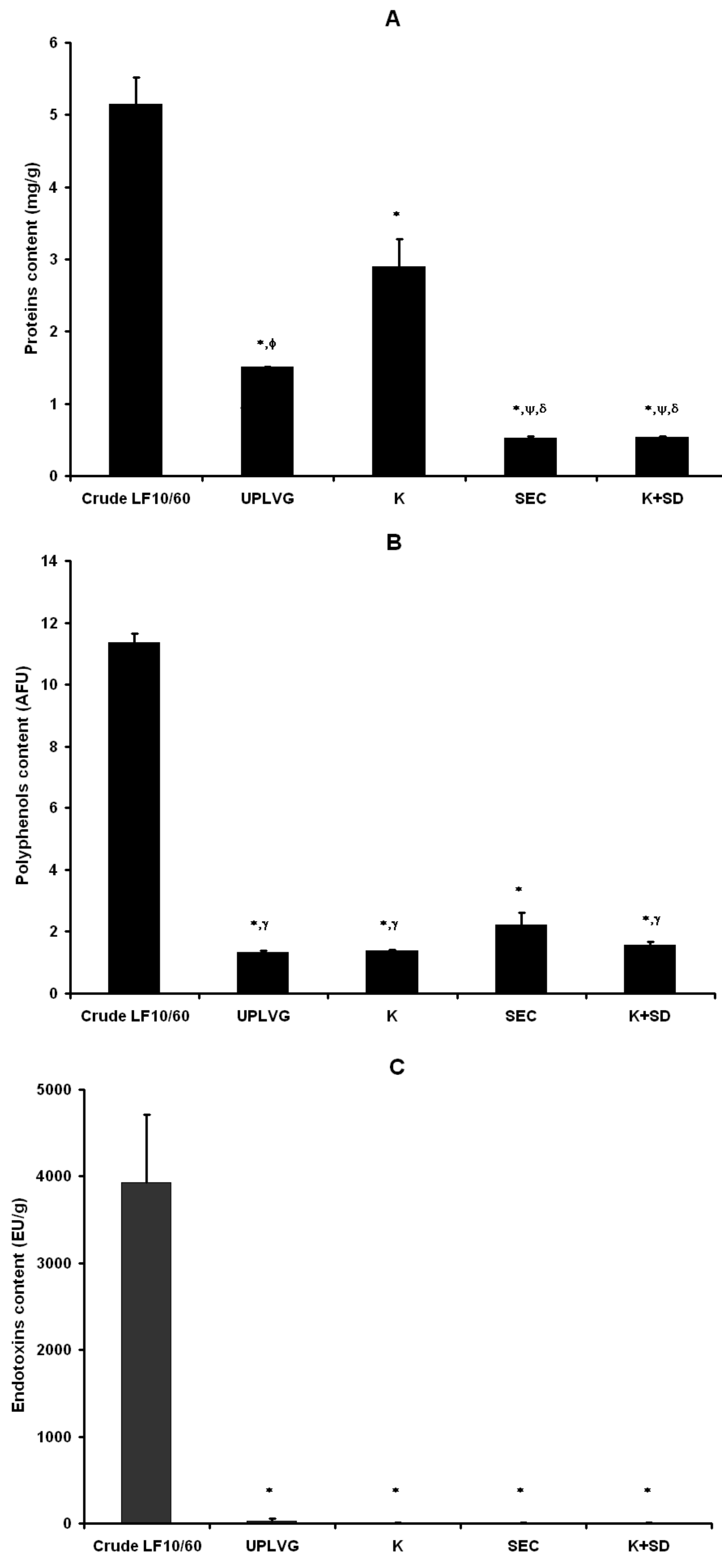
A
Proteins content (mg/g)
6
5
4
3
2
1
0
*,ϕ
*
*,ψ,δ
*,ψ,δ
Crude LF10/60
UPLVG
K
SEC
K+SD
B
Polyphenols content (AFU)
14
12
10
8
6
4
2
0
*,γ
*,γ
*
*,γ
Crude LF10/60
UPLVG
K
SEC
K+SD
C
Endotoxins content (EU/g)
5000
4000
3000
2000
1000
0
*
*
*
*
Crude LF10/60
UPLVG
K
SEC
K+SD

Figure 3. Detection of majors alginate contaminants in different alginate preparations (Crude LF10/60: raw Protanal® LF10/60 alginate; K: Protanal® LF10/60 alginate purified using Klöck purification protocol; UPLVG: commercially purified Pronova™ UPLVG alginate; SEC: Protanal® LF10/60 alginate purified using size exclusion chromatography; K+SD: Protanal® LF10/60 alginate purified using Klöck-modified method with a saline dialysis). A. Protein content measured using the Micro BCA Protein Assay. Results are presented in mg of proteins per g of alginate (mg/g) (n=2). B. Polyphenols content detected by the appearance of a characteristic absorbance peak at 445 nm. Results are presented in arbitrary fluorescence units (AFU) (n=3). C. Endotoxin content determined using the Limulus Amoebocyte lysate (LAL) assay. Results are presented in endotoxin units per g of alginate (EU/g) (n=4). (* : $p<0,001$ vs Crude LF10/60, Ψ : $p<0,001$ vs K, δ : $p<0,05$ vs UPLVG, ϕ : $p<0,01$ vs K, γ : $p<0,01$ vs SEC.)

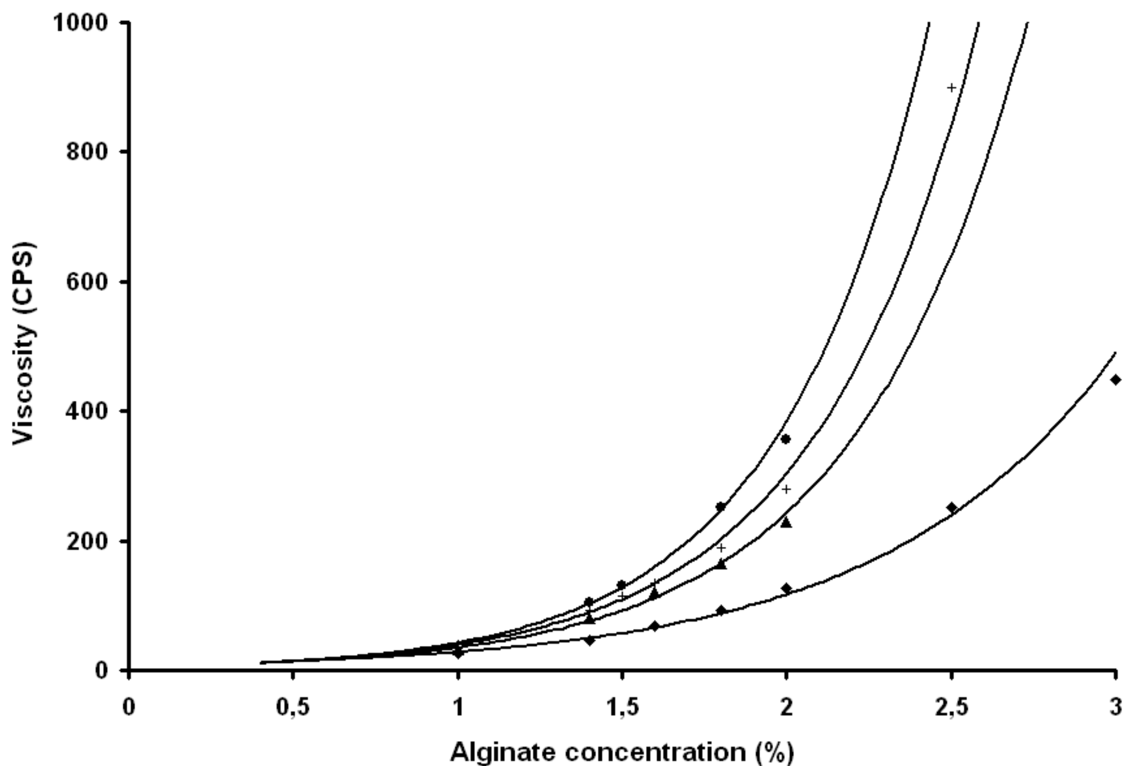

Figure 4. Viscosity/concentration curves of the different alginate preparations. The viscosity was measured using a Synchro-Lectric rotational viscometer operated at 25°C. The results are expressed as centipoise (CPS). ♦: Raw Protanal® LF10/60 alginate; ▲: Protanal® LF10/60 alginate purified using Klöck purification protocol; +: Protanal® LF10/60 alginate purified using size exclusion chromatography; ●: Protanal® LF10/60 alginate purified using Klöck-modified method with a saline dialysis).

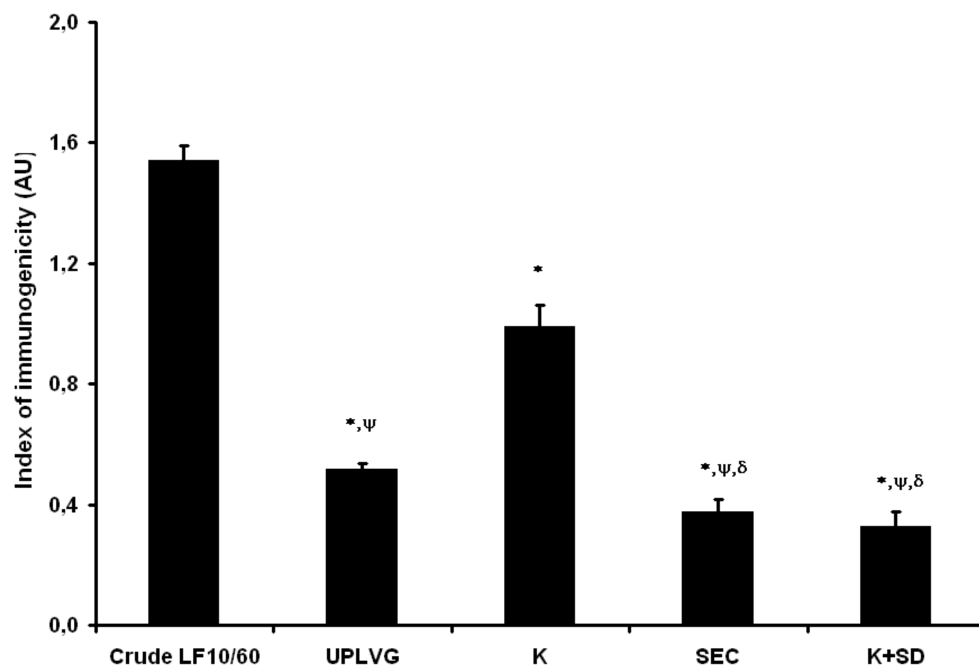

Figure 5. Index of pericapsular cell adhesion (immunogenicity) of barium alginate beads prepared with different alginates two days following in vivo implantation into mice peritoneum (n=6). Results are presented in arbitrary units of pericapsular index (AU). (Crude LF10/60: raw Protanal® LF10/60 alginate; K: Protanal® LF10/60 alginate purified using Klöck purification protocol; UPLVG: commercially purified Pronova™ UPLVG alginate; SEC: Protanal® LF10/60 alginate purified using size exclusion chromatography; K+SD: Protanal® LF10/60 alginate purified using Klöck-modified method with a saline dialysis). (* : $p<0,001$ vs crude LF10/60, ψ: $p<0,001$ vs K and δ : $p<0,05$ vs UPLVG).

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Discussion générale (Partie I)

Afin d'améliorer les processus de purification de l'alginate et d'améliorer sa biocompatibilité, nous avons :

- Comparé 3 techniques de purification d'alginate publiées dans la littérature et une purification commerciale relativement à leur efficacité à réduire les quantités de contaminants connus, nous avons diagnostiqué que :

    - Toutes les techniques étudiées sont efficace pour éliminer les polyphénols.
    - La plupart des techniques réduisent efficacement les endotoxines.
    - Les techniques diminuent les niveaux de protéines mais les quantités résiduelles de protéines sont encore relativement élevées.

    - ➢ Donc, les différentes techniques de purification d'alginate ont des rendements variables, mais l'alginate purifiée commercialement ou l'alginate purifiée selon le protocle de Klöck sont les meilleurs choix.
    - ➢ Il existe un problème de reproductibilité et un manque de standardisation des alginates purifiées.

- Quantifié que les contaminants de l'alginate non purifiée représentent 2% de la composition atomique, mais seulement 0,41% lorsque l'alginate est purifiée.

- Observé que la purification d'alginate n'a pas d'effet sur ses propriétés structurelles ou ses groupements fonctionnels.

- Observé que la présence de contaminants, plus particulièrement les polyphénols et les protéines, affectent l'hydrophilicité (ou mouillabilité) de l'alginate en masquant/interagissant avec les groupements COO-Na et C-OH.

- Détecté la présence d'un contaminant, les fucoïdans, encore peu décrit dans la littérature.

- Corrélé mathématiquement la biocompatibilité des alginates en fonction de leur taux de contaminants protéiques, le rôle des protéines ayant été négligé jusqu'à présent.

- Amélioré les processus de purification d'alginate afin de réduire au maximum les niveaux de contaminants protéiques.
- Obtenus une alginate purifiée montrant une biocompatibilité améliorée.

Dans le domaine de la transplantation d'îlots microencapsulés, il est devenu de plus en plus ardu de comparer les résultats entre les différents laboratoires car l'alginate utilisée peut provenir de différentes sources ou encore subir différents traitements de purification. Comme mentionné dans l'introduction de cette section, ce n'est pas d'hier que la pureté de l'alginate fait l'objet de recherches. Afin d'améliorer la pureté du polymère, plusieurs processus de purification d'alginate ont été proposés dans la littérature et chaque groupe utilise sa propre «recette». Il y a donc un problème de reproductibilité et de standardisation des alginates utilisées pour l'encapsulation cellulaire. Pour la première fois depuis la fin des années 90, nous avons décidé d'étudier et de comparer trois techniques de purification d'alginate les plus utilisées, ou encore montrant des résultats intéressants. Les résultats de cette étude sont clairs et confirment ce que l'on soupçonnait : il est difficile de mettre en commun tous les résultats concernant l'encapsulation et la transplantation des îlots de Langerhans puisque les différents processus de purification ne montrent pas la même efficacité à se débarrasser des contaminants.

Une des conclusions importantes tirée des études présentées est que les contaminants protéiques jouent un rôle majeur dans l'initiation de la RHM contre les microcapsules, rôle qui avait jusqu'ici été minimisé. Nous avons observé qu'il y avait une corrélation mathématique plus importante entre la RHM et la présence des protéines résiduelles qu'avec les autres contaminants. De plus, nous avons montrés qu'en diminuant encore plus les quantités de contaminants protéiques par l'amélioration du processus de purification, on réduisait du même coup la sévérité de la RHM. La présence des protéines résiduelles dans l'alginate suite à la purification a également été remarqué par d'autres [179]. En effet, seulement 65% des contaminants protéiques avaient été extraits de l'alginate. C'est ce que nous démontrent les profils d'élution obtenus suite à une analyse de

l'alginate par chromatographie par exclusion de taille (SEC). En effet, en absence de sels, le pic principal correspondant aux protéines contaminantes élu au même moment que le pic de l'alginate, indiquant une possible interaction entre les deux. Le pic secondaire de protéine correspond probablement à des protéines aggrégées expliquant le de haut poids moléculaire de ce pic. Trois mécanismes différents peuvent être proposés afin d'expliquer comment les protéines résiduelles peuvent influencer de développement de la RHM.

1) Les contaminants protéiques résiduels pourraient favoriser ou faciliter l'adsorption d'autres protéines provenant du receveur, à la surface des microcapsules, par des interactions protéines-protéines. Bien que ce scénario soit possible, il est très peu probable qu'il ait une influence majeure puisque les quantités de protéines résiduelles sont assez faibles et elles ne se retrouvent pas nécessairement à la surface mais sont plutôt distribuées dans toute la structure tridimensionnelle de la bille d'alginate.

2) Les protéines résiduelles distribuées à l'intérieur de la bille d'alginate diffusent hors de la bille pour aller interagir et activer les cellules du système immunitaire du receveur, ayant pour effet d'amplifier la réaction à corps étranger. Même si nous ne possédons aucune donnée pour soutenir ce mécanisme, il est tout à fait possible que des protéines de poids moléculaire inférieur à 30 à 50 kDa (qui correspond à la perméabilité des microcapsules) puissent diffuser librement comme le font l'insuline, le glucose et les autres nutriments.

3) Les protéines résiduelles dans l'alginate sont en quantités suffisantes pour modifier ses propriétés physico-chimiques et influencer son comportement avec les protéines du receveur. Les données obtenues avec les analyses de génie biomédical soutiennent cette hypothèse. En effet, à l'aide des analyses d'angle de contact, nous avons pu observer que la purification d'alginate modifie l'hydrophilicité de celle-ci et que cette modification est corrélée mathématiquement avec les quantités d'azote, correspondant au signal des

protéines. Plus les quantités de protéines résiduelles sont faibles (plus l'alginate est purifiée), plus l'angle de contact est petit (plus la surface est hydrophile). Il a d'ailleurs déjà été démontré que, dans d'autres systèmes que les microcapsules APA, les surfaces modifiées pour augmenter leur hydrophilicité réduisaient leur capacité à adsorber les protéines [180, 181]. De plus, les résultats acquis sur la viscosité dynamique montrent que les protéines peuvent également influencer cette propriété de l'alginate. En résumé, nous croyons qu'il est possible que les protéines résiduelles affectent le développement de la RHM en modifiant les propriétés physico-chimiques de l'alginate, comme son hydrophilicité.

À la lumière de ces constatations, nous pensons que les dosages de protéine devraient être effectués de façon systématique suite aux procédés de purification, quel que soit le procédé utilisé, puisque ces dosages pourraient être utilisés comme un outil diagnostique afin prédire si une alginate sera ou non capable d'induire une RHM.

Avec les techniques d'ingénierie, il nous a été possible, par X-ray Photoelectron Spectroscopy (XPS), de détecter et quantifier les fucoïdans, qui sont en fait des polysaccharides sulfatés. Ces fucoïdans sont présent naturellement dans les algues marines [182, 183] et ils ont déjà été décrits comme contaminants dans l'alginate [184, 185]. Dans notre étude ainsi que dans d'autres [182, 183], l'implication des fucoïdans dans le développement de la RHM n'a pas été démontrée. Par contre Stevan *et al.* ont rapportés que les polysaccharides sulfatés pouvaient avoir un effet cytotoxique sur des cellules HeLa encapsulées, dans un contexte autre que l'encapsulation cellulaire [186]. Les fucoïdans ne semblent pas avoir d'effet sur le receveur mais il serait intéressant de savoir s'ils peuvent avoir des conséquences sur les cellules encapsulées. Nous avons également observé une corrélation intéressante ($R^2 = 0.69$) entre les concentrations en phosphore et le contenu en endotoxines dans les alginates. Un tel coefficient de corrélation indique une corrélation modérée, qui ne nous permet pas d'exclure le fait que des molécules autres que les endotoxines puissent contribuer à la contamination au phosphore.

Un des phénomènes observés lors des études décrites dans cette partie est que les processus de purification étudiés semblent modifier la viscosité de l'alginate. La plupart des publications sur la purification d'alginate dont celle de Klöcl *et coll.* rapportent que les procédés appliqués pour purifier l'alginate ont eu pour effet de diminuer la viscosité [172]. L'hydrolyse des chaînes polymériques d'alginate due aux différents traitements chimiques qui ont été utilisés est l'explication fournis par les auteurs. Il existe deux raisons pour lesquelles nous observons plutôt une augmentation de la viscosité. Tout d'abord, certaines techniques utilisées lors de la purification de l'alginate comme l'utilisation d'une dialyse avec une perméabilité de 50,000 Da qui élimine les monomères ou les petites chaîne d'alginate en combinaison avec des étapes de purification sur des billes d'alginate au baryum (le baryum étant utilisé comme agent gélifiant au lieu du calcium) qui contribuent à se débarrasser des petites chaînes d'alginate qui ne participent pas au processus de gélification, font en sorte que l'on augmente la viscosité de l'alginate. Ces étapes constituent le principal avantage de cette technique. De plus, il est connu que les contaminants contenus dans l'alginate peuvent perturber les interactions et la structure qui existent normalement entre les chaînes d'alginate. La réduction des ces contaminants dans l'alginate peut alors influencer la viscosité de celle-ci. Il est certain qu'à coups sûrs, l'utilisation d'un protocole de purification comme celui de Klöck *et coll.* [170] aura pour effet d'augmenter la viscosité de l'alginate. On peut cependant obtenir des alginates ayant différentes viscosités en utilisant une alginate de départ composée de chaines d'alginates plus ou moins grandes (et donc une viscosité de départ différente), en fonction de la viscosité finale désirée.

Il est également important de noter que les conclusions tirées des résultats que nous avons obtenus proviennent d'un type d'alginate en particulier, la Protanal® LF10/60, qui est connue pour être une alginate riche en blocs G (65%-75%) et ayant une faible viscosité. De plus, afin de pouvoir comparer tous les résultats de ces études, le même lot a été utilisé lors de ces études. Plusieurs alginates disponibles commercialement peuvent présenter des

caractéristiques très différentes en termes de composition (ratio M/G) et de viscosité intrinsèque et ces paramètres peuvent influencer le rendement de certaines étapes de purification. De plus, il est connu que pour une même alginate, les taux de contaminants de départ peuvent varier d'un lot à l'autre. Il n'est donc pas exclu que les résultats décrits et leurs conclusions respectives auraient pu être différentes si une autre alginate ou un lot différent avait été utilisé. Ce problème de variabilité entre les lots d'une même alginate ainsi que le questionnement sur la réelle provenance et l'uniformité des algues utilisées dans l'industrie ont amené un groupe de recherche à proposer d'effectuer l'extraction et la purification d'alginate directement sur les algues fraichement récoltées, plutôt que sur l'alginate en poudre disponible commercialement [173, 185]. Cette technique montre d'excellents résultats de biocompatibilité mais reste tout de même restreinte aux laboratoires pouvant se procurer facilement les algues fraîches.

Depuis la publication de nos résultats, la purification d'alginate fait encore l'objet d'études, signe évident que la purification d'alginate reste un problème d'actualité puisqu'aucun consensus ne ressort de la littérature. Entre autre, le groupe d'Edmonton, qui publia la désormais célèbre étude de transplantation d'îlots humain [9], qui s'intéresse maintenant à la technique de microencapsulation, propose à leur tour une procédure de purification d'alginate [187]. Beaucoup d'études concernant l'influence de la purification d'alginate sur la RHM ont été publiées par le passé mais maintenant, il y a également un intérêt pour la relation entre la purification d'alginate et la viabilité et la survie des cellules encapsulées [187-189]. Il a entre autre été démontré que l'utilisation d'une alginate purifiée pour encapsuler les îlots pouvait améliorer leur survie ainsi que leur fonction *in vitro*. Ces îlots ont également montrés une meilleure réponse suite à une stimulation au glucose[189]. Ailleurs, il a été démontré que les endotoxines pouvaient se trouver en quantités suffisantes dans l'alginate pour réussir à activer les macrophages à produire du NO et ainsi endommager la survie et la fonction des îlots encapsulés [188]. Il y a également eu quelques développements du côté industriel. Des alginates dites purifiées et stériles sont maintenant disponibles sur le marché. Jusqu'à tout récemment, Medipol, une compagnie

pharmaceutique spécialisée en encapsulation, offrait sur le marché, une alginate purifiée, de même que NovaMatrix Ultrapure Polymers (FMC Biopolymers), dont une alginate stérile et purifiée (Pronova SLG/SLM alginates) est maintenant commercialisée. Quoique très intéressantes, ces alginates n'ont toutefois pas été testées dans le contexte de transplantation d'îlots de Langerhans et, à cause de brevets, les protocoles de purification de ces alginates ne sont pas divulgués par les compagnies.

L'application chez l'être humain de la technique d'encapsulation des îlots de Langerhans afin de recréer un pancréas bioartificiel doit, comme tout les autres dispositifs biomédicaux, être approuvé par les organismes régulatoires tels que la «Food and Drug Administration» (FDA) aux États-Unis, et de Santé Canada au Canada. Aux yeux de la FDA, la recherche sur les îlots encapsulés est chapeautée plus spécifiquement par l'Office des Produits Combinaisons, c'est-à-dire un produit qui combine aux moins deux des composantes suivantes : une drogue et/ou un dispositif et/ou un produit biologique. Dans le cas des îlots encapsulés, l'on combine une composante biologique, l'îlot, et un dispositif, la microcapsule. L'approbation doit alors se faire à trois niveaux selon le diagramme suivant :

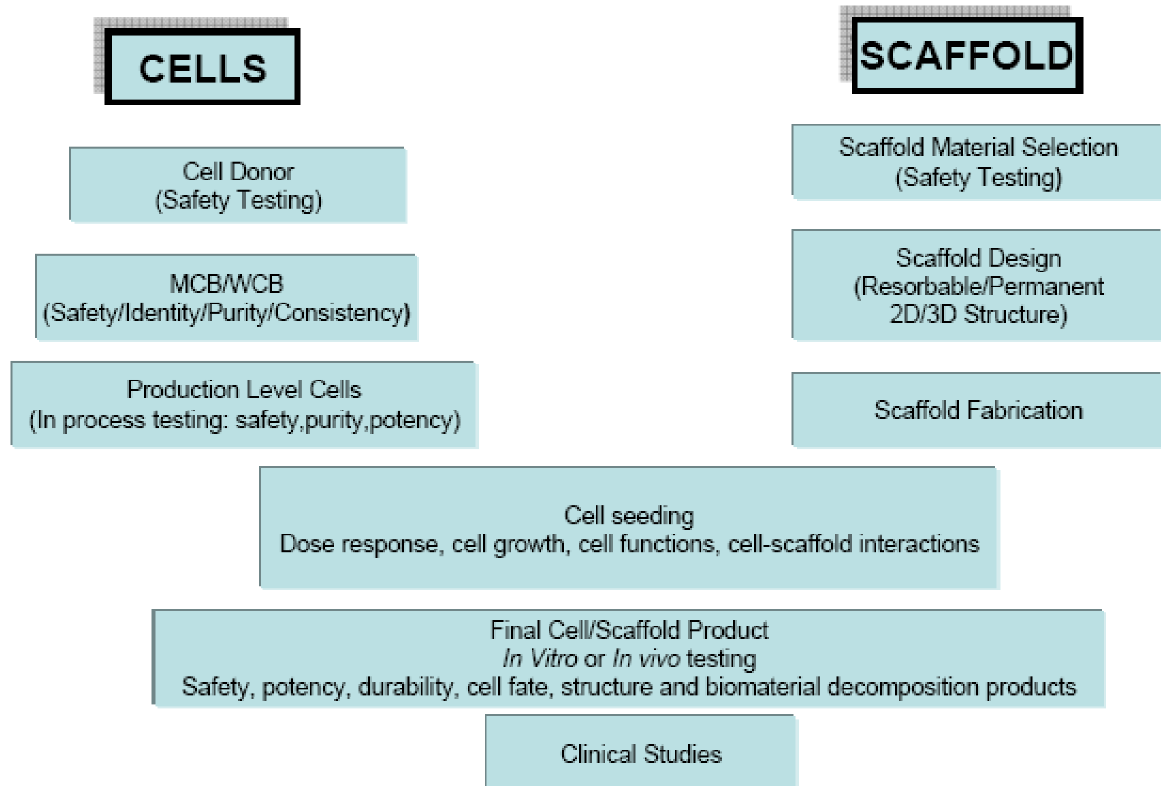

Figure 11 : Étapes du développement d'un produit de combinaison pour approbation par la FDA. Tiré de «FDA Perspective/Review of Cell Scaffold Products» 2007.

La première approbation concerne la partie biologique, c'est-à-dire l'îlot. Grâce à l'effort soutenu et aux nombreux succès des centres d'isolement d'îlots humains, il existe déjà une approbation et un guide concernant l'obtention des îlots humains, le «Guidance for Industry Considerations for Allogeneic Pancreatic Islet Cell Products» qui a récemment fait l'objet d'une mise à jour (Septembre 2009). Ce guide décrit toutes les recommandations et les opinions de la FDA sur les étapes nécessaires pour isoler adéquatement les îlots et pour mener à bien les études précliniques et cliniques. Il est même stipuler que les îlots obtenus de cette façon pourraient éventuellement servir à des fins d'encapsulation.

En ce qui concerne la microcapsule, il n'existe pas encore de tel guide. On doit alors suivre la procédure illustrée à la figure 11 sous la rubrique «Scaffold» et caractériser le matériel, le dispositif et la procédure de fabrication:

1) le matériel de base sélectionné pour le dispositif : l'alginate. Pour ce faire, il existe des normes dictées par le «American Society for Testing and Materials» (ASTM) sur les procédures et les techniques à employer pour caractériser l'alginate adéquatement. Il s'agit du document «Standard Guide for Characterization and Testing of Alginates as Starting Materials Intended for Use in Biomedical and Tissue-Engineered Medical Products Application» (#F2064-00) publié en 2000 et mise à jour en 2006, dans lequel on retrouve, entre autres, des tests standards que l'on devrait effectuer pour obtenir des informations sur l'identité de l'alginate (par FT-IR) et ses propriétés physiques et chimiques (par RMN et viscosimétrie). Dans ce document, la question des contaminants de l'alginate est également abordée. Ils recommandent entre autres de porter une attention particulière et quantifier les endotoxines, les niveaux de protéines, la quantité de métaux lourds et des possibles contaminants microbiologiques comme les bactéries, levures et moisissures. Dans nos études, les endotoxines ont été dosées avec la méthode proposée, soit le LAL test alors qu'une quantification différente a été utilisée pour quantifier les protéines. À l'aide du ToF-SIMS, nous avons pu déterminer qu'il n'y avait pas de métaux lourds dans les alginates étudiées et finalement, comme recommandé par les standards ASTM, nous avons stérilisé les alginates, par filtration, pour éliminer toutes traces de microorganismes. Il est à noter que les normes ASTM ne font aucunes mentions des contaminants polyphénoliques même si ceux-ci sont connus depuis 1989.
2) le dispositif lui-même : la microcapsule. Il faut ensuite caractériser les microcapsules fabriquées avec l'alginate. L'ASTM a également un guide standardisé intitulé «Standard Guide for Immobilization or Encapsulation of Living Cells or Tissue on Alginate Gels» (#F2315-03), dans lequel on décrit les techniques standards de

gélification de l'alginate, des différentes propriétés des microcapsules (homogénéité, perméabilité, les propriétés mécaniques et stabilité, l'utilisation de polycations) ainsi que les différentes considérations pour la biocompatibilité des microcapsules.

3) les procédés employés pour fabriquer le dispositif : formation des microcapsules. Évidement, il faut que la procédure de fabrication des microcapsules soit également standardisée. D'ailleurs, une description des différents approches pouvant être utilisées pour la fabrication des microcapsules est incluse dans le document #F2315-03 ci-haut mentionné, dont l'utilisation d'un appareil électrostatique, d'un système d'injection d'air coaxial, de l'émulsion ainsi que des systèmes vibratoires ou rotatifs.

Une fois la partie biologique et la partie dispositif bien caractérisées, il faut ensuite connaître le comportement des cellules à l'intérieur du dispositif (Figure 11 «Cell seeding»), c'est-à-dire la survie et la fonction des îlots à l'intérieur des microcapsules d'alginate. Dans une étape subséquente, l'on doit évaluer la performance *in vitro* et *in vivo* du produit de combinaison et s'assurer d'être conforme avec les règles ISO 10993 qui sont une série de standards pré-établis pour évaluer la biocompatibilité d'un dispositif médical avant de pouvoir passer aux études cliniques :

- ISO 10993-1:2003 Biological evaluation of medical devices Part 1: Evaluation and testing
- ISO 10993-2:2006 Biological evaluation of medical devices Part 2: Animal welfare requirements
- ISO 10993-3:2003 Biological evaluation of medical devices Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity
- ISO 10993-4:2002/Amd 1:2006 Biological evaluation of medical devices Part 4: Selection of tests for interactions with blood
- ISO 10993-5:2009 Biological evaluation of medical devices Part 5: Tests for in vitro cytotoxicity
- ISO 10993-6:2007 Biological evaluation of medical devices Part 6: Tests for local effects after implantation
- ISO 10993-7:1995 Biological evaluation of medical devices Part 7: Ethylene oxide sterilization residuals
- ISO 10993-8:2001 Biological evaluation of medical devices Part 8: Selection of reference materials
- ISO 10993-9:1999 Biological evaluation of medical devices Part 9: Framework for identification and quantification of potential degradation products

- ISO 10993-10:2002/Amd 1:2006 Biological evaluation of medical devices Part 10: Tests for irritation and delayed-type hypersensitivity
- ISO 10993-11:2006 Biological evaluation of medical devices Part 11: Tests for systemic toxicity
- ISO 10993-12:2007 Biological evaluation of medical devices Part 12: Sample preparation and reference materials (available in English only)
- ISO 10993-13:1998 Biological evaluation of medical devices Part 13: Identification and quantification of degradation products from polymeric medical devices
- ISO 10993-14:2001 Biological evaluation of medical devices Part 14: Identification and quantification of degradation products from ceramics
- ISO 10993-15:2000 Biological evaluation of medical devices Part 15: Identification and quantification of degradation products from metals and alloys
- ISO 10993-16:1997 Biological evaluation of medical devices Part 16: Toxicokinetic study design for degradation products and leachables
- ISO 10993-17:2002 Biological evaluation of medical devices Part 17: Establishment of allowable limits for leachable substances
- ISO 10993-18:2005 Biological evaluation of medical devices Part 18: Chemical characterization of materials
- ISO/TS 10993-19:2006 Biological evaluation of medical devices Part 19: Physico-chemical, morphological and topographical characterization of materials
- ISO/TS 10993-20:2006 Biological evaluation of medical devices Part 20: Principles and methods for immunotoxicology testing of medical devices

Partie II : Protection du receveur

Comme décrit précédemment dans l'introduction de cette thèse, certains paramètres des microcapsules, comme sa biocompatibilité, doivent être étudiés et optimisés afin de pouvoir les utiliser pour d'éventuelles transplantations d'îlots de Langerhans chez l'humain. La biocompatibilité peut être reliée à la résistance et la stabilité des microcapsules. En effet, il a été démontré que le bris d'une faible proportion de microcapsules transplantées *in vivo* pouvait activer le système immunitaire du receveur et ainsi affecter la bioperformance de tout le transplant à cause d'un mauvais recouvrement et une exposition de la PLL, connue pour être un activateur du système immunitaire. De plus, le bris de microcapsules peut permettre aux cellules encapsulées de s'échapper et d'activer le système immunitaire de l'hôte par la relâche d'immunogènes de la part de ces cellules.

Dans le cas des microcapsules standards APA et des tous les dispositifs d'encapsulation utilisant l'alginate complexée à un polycation, la stabilité dépends des interactions électrostatiques qui existent entre les deux polymères [118] comme, dans l'exemple qui nous intéresse, entre l'alginate qui porte une charge nette négative et la PLL qui porte une charge nette positive. Ce type d'interaction est toujours en compétition avec les autres ions présents dans l'environnement [121, 122, 190]. De plus, Thu *et coll.* ont démontré que la seconde couche d'alginate (couche périphérique) qui entoure la microcapsule n'est pas stable et que jusqu'à 80% de cette alginate se détache de la surface, laissant la PLL exposée à la surface des microcapsules [114].

Plusieurs stratégies peuvent être utilisées pour améliorer la résistance des microcapsules avec différents succès. Il est possible de modifier la stabilité des microcapsules en faisant varier des éléments intrinsèques aux microcapsules tels que la concentration de PLL, son poids moléculaire ainsi que le temps d'incubation des microcapsules dans la PLL [118, 191, 192]. Ces variations sont connues pour moduler les propriétés des microcapsules mais avec des effets très limités. Une autre façon d'augmenter

la stabilité des microcapsules est de remplacer les interactions électrostatiques qui existent entre les composantes de la microcapsule par des liens covalents qui sont plus stables.

Dans cette deuxième partie, nous présentons nos travaux sous la forme de deux articles scientifiques dans lesquels nous décrivons une technique que nous avons développée au laboratoire afin d'améliorer la résistance mécanique et chimique des microcapsules APA à l'aide d'un agent réticulant photoréactif qui remplace une certaines proportions des liens électrostatiques entre l'alginate et la PLL par des liens covalents. De plus, ces nouvelles microcapsules pourraient améliorer la protection du receveur lors de transplantations de cellules souches, de cellules modifiées génétiquement ou de cellules immortalisées.

En effet, la combinaison du manque de donneurs cadavériques de pancréas et le fait que l'on doit transplanter de 2 à 4 pancréas par patients pour atteindre une insulino-indépendance [9, 13], on fait en sorte que la recherche de source alternatives de cellules β s'est accentuée. Des approches comme l'utilisation de cellules souches [193-195], de précurseurs de cellules β [196], de cellules immortalisées [197, 198] ou modifiées génétiquement [199] afin de remplacer la sécrétion d'insuline sont présentement sous investigation. Cependant, l'utilisation de ces cellules n'est pas sans risques et ces types cellulaires ont le potentiel de subir une transformation maligne [195, 200-204]. Il est certain que ce risque est difficile à quantifier mais il constitue une crainte majeure de la part des agences régulatrices comme la Food and Drug Administration (FDA) aux États-Unis et Santé Canada dans le développement d'études cliniques utilisant ces types de cellules.

Depuis le développement et la caractérisation de la première lignée de cellules souches embryonnaires (CSE) par Thomson *et coll.* [205], l'intérêt pour l'utilisation des cellules souches comme source alternative de cellules pouvant remplacer les cellules défectueuses dans les désordres endocriniens s'est accrue. Les ECSs sont des cellules dérivées de la masse interne des blastocystes et possèdent trois caractéristiques: 1) elles

peuvent proliférer indéfiniment, 2) elles ont la capacité de rester dans un état non différentié et 3) il est possible d'induire et de diriger leur différentiation. Cependant, à cause de ces caractéristiques uniques, ces cellules sont potentiellement dangereuses. En effet, il a déjà été rapporté que les CSE pouvaient subir une transformation maligne [202, 205]. Dans le cas particulier du diabète de type I, l'induction de la différentiation des CSE en cellules pouvant sécréter de l'insuline en réponse au glucose est un champ d'étude en pleine expansion. Il a entre autre été rapporté que la transplantation de cellules productrices d'insuline dérivées des ECSs avait le potentiel de renverser le diabète [193, 201, 202], mais ces succès restent limités. De façon répétitive, la formation de structures «tumor-like» 3 semaines seulement post-transplantation a été observée [193, 201], allant même jusqu'à la mort des souris suite à la formation de tératomes 6 semaines suite à la transplantation [193]. De plus, il semble que la culture à long terme des ECSs favorise l'accumulation de défauts chromosomaux, pouvant peut-être expliquer la caractéristique tumorigène de ces cellules [206, 207].

La transplantation de cellules immortalisées pouvant sécréter de l'insuline est une autre approche étudiée en tant que source alternative de cellules pouvant remplacer la fonction des cellules β chez des animaux diabétiques. Les cellules qui subissent une immortalisation augmentent leur capacité proliférative ainsi que leur danger de subir une prolifération incontrôlée [208, 209] comme ce fut le cas pour les cellules β immortalisées avec l'antigène SV40 [210]. Dans ce cas particulier, il a été possible de corriger la glycémie des souris diabétiques suite à la transplantation de ces cellules d'insulinôme immortalisées mais rapidement, les animaux traités ont ensuite montrés des épisodes d'hypoglycémie sévères. Une analyse subséquente a démontré que la prolifération excessive des ces cellules ainsi que la formation de tumeurs pouvait expliquer ce phénomène [208].

L'utilisation de microcapsules plus résistantes et plus stables afin de confiner les cellules potentiellement malignes est explorée et discutée dans le deuxième article.

Microencapsulation of Living Cells in Semi-Permeable Membranes with Covalently Cross-Linked Layers.

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ABSTRACT

Microencapsulation in semi-permeable membranes protects transplanted cells against immune destruction, and facilitates separating cells from supernatants in bioreactors. Microcapsule strength is critical. We describe a method to microencapsulate living cells in alginate-poly-L-lysine(PLL)-alginate membranes with covalent links between adjacent layers of microcapsule membranes, while preserving the desired membrane molecular weight cut-off (MWCO) and microencapsulated cell viability. A hetero-bifunctional photoactivatable cross-linker, N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS), was used to create covalent links between PLL and alginate from both the core bead and the outer sheet. The N-hydroxysuccinimide ester group ANB-NOS was covalently linked to PLL. Islets of Langerhans were immobilized in alginate beads, incubated in PLL-ANB-NOS and again in alginate. Upon illumination with UVA, covalent links were created between the phenyl azide residue of ANB-NOS and alginate from both the core bead and the outer coating. Covalently linked microcapsules remained intact after 3 years in a strong alkaline buffer (pH 12), whereas standard microcapsules disappeared within 45 seconds in the same solution. A standardised mechanical stress broke 22-fold more standard than covalently linked microcapsules. The MWCO and microencapsulated cell viability were similar with standard and covalently linked microcapsules. These microcapsules, extremely resistant to chemical and mechanical stresses, will be useful in numerous applications.

INTRODUCTION

Immuno-isolation of cells in semi-permeable membranes has been proposed as a means to prevent their immune destruction following transplantation [3,4]. However,

several fundamental issues remain to be addressed before considering clinical application of this method. One such issue is the resistance of microcapsules to chemical and mechanical degradation. Strong microcapsules will obviously increase the durability of the transplant. It is also likely to improve long-term biocompatibility of microcapsules, since a strong pericapsular reaction always develops around broken or damaged capsules [5,6]. Moreover, the strength and durability of microcapsule membrane are critical issues for the transplantation of stem cell derived cells, immortalized cell lines and bioengineered cells. The major concern related to these approaches is the risk of malignant cell transformation and dissemination [7]. Microcapsules that can hardly be destroyed in conditions compatible with life would provide a safe method of transplanting stem cell derived cells, bioengineered cells or immortalized cells.

The use of microencapsulated cells or enzymes in bioreactors represents a set of potential biomedical industrial applications for extremely resistant microcapsules [9-11]. These include microencapsulation of probiotic bacteria for the fermentation of dairy products [8], and of bacteria, yeast and cells for the industrial production of a large number of drugs by DNA recombinant technologies [9,10]. When the desired products or reactions are obtained, cell-containing microcapsules are easily separated from the supernatant. However, the shearing forces encountered in bioreactors may break microcapsules, releasing micro-organisms or cells. This contamination decreases the purity of the products and may jeopardize the safety, efficacy and usefulness of the method [1,2].

Formation of electrostatic complexation between the negatively charged polyanion alginate and positively charged polycations such as poly-L-lysine (PLL) to form alginate-poly-L-lysine-alginate (APA) microcapsules is the most widely used method to microencapsulate cells [11-14]. Microcapsules are constructed by a simple three steps procedure [15]. First, the cells are entrapped in a bead formed by the ionic cross-linking of alginate by divalent electrolytes such as calcium [16]. The second step comprises coating the alginate beads with a polycations, such as PLL, which forms a peripheral membrane

ensuring a good control of the molecular weight cut-off (MWCO) and increasing membrane stability. Finally, the third step consists to coat the microcapsules with a dilute alginate solution for insuring biocompatibility. The two latter reactions rely on the electrostatic interactions between the polyanionic alginate and the polycationic polymer of lysine [17,18].

Efforts have been made to improve microcapsule strength [19-23]. Others [18,24,25], and we [26] have evaluated the effect on microcapsule strength of modulating PLL molecular weight, concentration and incubation time and the mannuronic/guluronic acid ratio of alginate. The formation of neutral capsules by the introduction of a new coating agent [27] has also been investigated. Following these experiments, tighter binding between PLL and alginate was obtained. Nevertheless, the electrostatically linked alginate and PLL still competes with other charged molecules in the environment [16,18,28], and the microcapsule strength improvement is limited. A prolonged incubation in solutions with high concentrations of Ca^{2+} or Sr^{2+} has showed a displacement of the alginate-poly-L-lysine bounds [29,30]. In addition, Thu has observed the progressive loss of the external sheet of alginate from microcapsules within days or weeks [18].

Another approach has been to create covalent links between molecules of one microcapsule compound. Hertzberg *et al.* [31] have increased the stability of uncoated (i.e. without PLL) alginate beads by introducing covalent links between alginate molecules. This may be useful in situation wherein a controlled MWCO is not required. Lu *et al.* [32,33] have designed a method, for use in bioreactors, which consists of grafting a photodimerizable reactive group on the polycationic polymer forming the semi-permeable membrane of microcapsules. With this method, a covalent network is formed between the molecules of the cationic polymers, but there are no covalent links with adjacent layers.

We developed a method [34] to covalently link PLL with alginates from both the core and the outer sheet of microcapsules, without affecting microencapsulated cell viability or membrane MWCO. We used N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-

NOS), a photoactivatable heterobifunctional cross-linker [35,36], comprising a N-hydroxysuccinimide ester group and a phenyl azide group. Firstly, the N-hydroxysuccinimide ester group was used to covalently link ANB-NOS and PLL under conditions that might be harmful for living cells, but before the cells are involved in the process (Figure 1A). Then, cell-containing alginate beads were coated with the PLL-ANB-NOS compound and with a second biocompatible alginate layer. When illuminated with UVA, the phenyl azide residue creates covalent links between the PLL-ANB-NOS compound and alginate from both the core bead and the outer coating. The latter reaction is not harmful for living cells (Figure 1B). We, herein, describe the method and its effect on microcapsule characteristics and functions, using a model of microencapsulation of islets of Langerhans (islets), which contains insulin-producing cells. The results of the study confirmed that a covalent link was formed, providing considerable improvement of microcapsule resistance to chemical and mechanical degradation, and showed that the procedure did not modify microcapsule membrane permeability and encapsulated cell viability.

METHODS

Material and reagents

Sodium alginate (Keltone LVCR 60% mannuronic acid) was obtained from ISP ALGINATES (San Diego, CA) and purified according to Klock's procedure [37], poly-L-Lysine was from Sigma Chemical Co. (St-Louis, MO) and photoactivatable cross-linkers sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate (Sulfo-SANPAH) and N-5-Azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) from Pierce (Rockford, IL). Wistar rats were purchased from Charles-River (St-Constant, QC, Canada). Protocols involving animal use were reviewed and stated to conform to the ethical guidelines of the Canadian Council

for Animal care by the animal care ethic committee of Guy-Bernier Research Centre of Maisonneuve-Rosemont Hospital. These guidelines were observed throughout the study.

Preparation of covalently-linked poly- L -lysine and cross-linkers

Experiments using photoactivatable cross-linkers were performed in a dark room until UVA illumination. Two cross-linkers were evaluated: 1) Sulfo-SANPAH and 2) N-5-Azido-2-nitrobenzoyloxysuccinimide (ANB-NOS). Sulfo-SANPAH or ANB-NOS dissolved in dimethylsulfoxide (DMSO) were mixed with PLL dissolved in 0.2 M borate buffer pH 8,4. PLL at a ratio cross-linker:lysine of 1:20, remained 3 hours at room temperature and was kept overnight at 4 °C (Figure 1A). This insured a level of derivation ~5%. PLL-cross-linkers were purified from residual reaction products by dialysis against NaCl 0.9 % (w/v), using a 6,000-8,000 Da molecular weight cut-off membrane (Spectrum Laboratories, CA), at 4°C for two days with four medium changes. The modified poly- L -lysine was quantified with MicroBCA Protein Assay (Pierce, Rochford, IL) and diluted with saline at the appropriate concentration. The resulting preparation was kept in dark until its use for microcapsule coating.

Microcapsule preparation

Microcapsules were prepared according to a previously published method [15] with the following modifications. A sterile solution of 1.8% sodium alginate was extruded using an electrostatic droplet generator [38] into a bath of 100mM calcium lactate buffered with HEPES, using a syringe fitted with a 22G needle. The calcium alginate gelled beads were incubated into a 0.05% w/v PLL solution for 5 minutes, washed with saline and incubated 5 minutes into 0.18% w/v sodium alginate. Finally, microcapsules were washed twice with saline. Steps that involved the use of modified PLL were performed under dim light to avoid a premature activation of the photoactivatable cross-linking groups. Microcapsules were then submitted to a UVA lamp (Blacklight 25W #F25T8-BL, UV

product, Upland, CA) at 2 kJ/m^2 or 23 kJ/m^2 to covalently cross-link PLL with the alginate beads (Figure 1B). Microcapsules prepared without cross-linker were used as controls.

Demonstration of covalent links and evaluation of resistance to chemical stress.

A suspension of microcapsules in saline was adjusted to pH 12 by adding 1/20 volume of 2M Glycine buffer pH 12 (2M glycine, 14mM NaCl, 2N NaOH). Microcapsules were incubated in this solution for at least 5 minutes and up to three years. Morphological changes were followed by microscopic examination.

Evaluation of microcapsule physical and functional characteristics

Microcapsule mechanical resistance was evaluated, using a quantitative method previously reported [26]. Briefly, microcapsules were prepared with sodium alginate containing 0.2% w/v 2,000 kDa FITC-labelled dextran. These large size dextrans have been shown to be withhold inside intact microcapsules indefinitely [26]. Measurement of fluorescence in the supernatant is linearly and very accurately correlated with the number of broken microcapsules [26]. The sensitivity of this measurement is such that one single broken microcapsule dissolved in 10 ml of solution can be detected [26]. A suspension of 1000 of these microcapsules in saline was mixed with 225 borosilicate glass beads of 3 mm diameter in 15 ml polystyrene tubes. They were submitted to continuous agitation on a rotator vertical agitator at 35 rpm for 72 hours. At this relatively slow speed, the centrifuge force is weak, and while the tube turns upside down, the glass beads and microcapsules move rapidly from one end of the tube to the other, submitting microcapsules to thousands of strokes and crushes between beads and the tube wall. To calculate the percentage of broken microcapsules, the amount of FITC labelled dextran in the supernatant and in intact microcapsules was quantified using a LS-5 spectrofluorometer (Perkin-Elmer Canada, Laval, QC; excitation 490 nm and emission 520 nm). For this purpose, standard microcapsules were dissolved in 20 ml of 0.9 % NaCl buffered to pH12 with 0.1 M glycine as described. The following modifications to the original techniques were made. Since this

method could not break cross-linked microcapsules, the latter were digested (or dissolved) by the addition of HCl 1.2 N and incubated at 100 ^{o}C for 18 minutes. This process does not break the covalent links but breaks links between atoms within the alginate or PLL molecules. For normalization, the samples of the standard curve were digested in same conditions. A control sample without agitation was included to establish that there is no leak-out of dextran from unbroken microcapsules. Another control, agitated without glass beads, was designed to detect any inadequate microcapsule preparation that would contain very fragile microcapsules, a very rare event.

Evaluation of the microcapsule molecular weight cut-off (MWCO)

Membrane MWCO was evaluated using size exclusion chromatography as previously described [39,40]. Microcapsules under study were used as the column matrix. FITC-dextran (2,000 kDa) was used to measure the column dead or void volume (Vo) and glucose (180 kDa) was used to determine the column total volume (Vt). Markers of different molecular weight (MW) and viscosity radius (Rη) were sequentially run on the column: dextrans (MW: 2,000 kDa, 19 kDa, 4.4 kDa; Rη 34.2 nm, 3.4 nm, 1.7 nm, respectively); proteins: bovine serum albumin (MW: 66 kDa; Rη 3.4 nm), ovalbumine (MW: 45 kDa; Rη 2.9 nm) and carboxipeptidase (MW: 35.2 kDa; Rη 2.7 nm). For each marker, a chromatographic partition coefficient (Ksec) was calculated using the formula: Ksec = (Ve – Vo) / (Vt – Vo), where Ve is the marker retention. Freely permeable markers have a Ksec value near 1. Excluded markers have a Ksec value of 0.

Islet isolation and encapsulation

Islets from Wistar rats (300-400g) were isolated using a published method [41]. Briefly, pancreases were injected via the bile duct with a Hank's Balance Salt Solution at 4°C and removed from the animal. Then, the pancreatic tissues was cut in small pieces and digested with 7.5 mg/ml collagenase type V (Sigma, St-Louis, MO) and 0.25

mg/mL DNase I (Sigma, St-Louis, MO) at 37°C under agitation. Islets of Langerhans were separated from acinar tissue on a discontinuous Ficoll gradient (Pharmacia, Upsala, Sweden). After purification, islets were handpicked under an inverted microscope. Pancreatic islets were cultured overnight in RPMI 1640 (Invitrogen Life Technologies, Burlington, ON Canada) supplemented with 10% v/v foetal calf serum and 1mM penicillin-streptomycin-glutamine at 24°C, 5% CO_2. For microencapsulation, islets were suspended in 1.8% w/v alginate solution at approximately 3000 islets/ml and encapsulated using standard or cross-linked derived PLL. Covalent cross-linking under UVA lamp of microcapsules containing cells were performed on ice to minimise heat damage from UVA. Controls included non encapsulated islets submitted to the same UVA radiation exposure. All groups were then cultured in CMRL 1066 medium supplemented with 10% foetal calf serum and 1mM Penicillin-Streptomycin-Glutamine at 37°C, 5% CO_2 with medium changes performed every second days.

Effect on islet cell survival

The survival of microencapsulated islet cells was evaluated in vitro using dual staining and observation under a fluorescent microscope [42,43]. Briefly, an aliquot of approximately 100 islets is handpicked and stained with 0.01 mg/ml orange acridine and 1 mg/ml propidium iodide (Sigma-Aldrich Ltd, Oakville, ON, Canada) for 10 minutes at room temperature and protected from light. Then, islet viability was observed under fluorescent microscope and classified in distinct categories. Viability was evaluated for standard and cross-linked encapsulated islets as well as standard encapsulated islets submitted to the same doses of UVA radiations used for cross-linked microcapsules.

Fresh (unstained) islets were examined under a stereomicroscope (Labovert FS, Leizt, Leica Canada, St-Laurent, QC, Canada), and the number (%) and size of islets with necrotic centers were recorded as well as the diameter of the necrotic area [44].The necrotic

center is characterized by the formation of a dark structure with sharply demarcating borders in the middle of the pancreatic islet.

Statistical analysis

Results are expressed as mean ± SEM. The differences between experimental groups were analyzed by unpaired Student's t-test with p values less than 0.05 considered significant.

RESULTS

Demonstration of covalent links and evaluation of resistance to chemical stress.

The presence of covalent bonds was confirmed by incubation in a strong alkaline solution. In standard alginate-PLL microcapsules, the positively charged amino group of the side chain of the lysine molecule interacts with the negatively charged carboxyl and hydroxyl groups of the uronic acid (basic unit of alginate). This interaction can be broken by neutralizing one of the charged groups. The pK value of the side chain amino group of the lysine is approximately 10.54. Therefore, microcapsules were incubated in a strong alkaline solution at pH 12 (i.e. a pH at which most side-chain amino group are neutralized) to determine if the membrane could resist to this treatment. Standard electrostatically linked microcapsules, used as controls (Figure 2A), rapidly dissolved and disappeared from the solution (Figure 2B and C). In less than 45 seconds, no intact microcapsules could be found under microscopic examination (Figure 2C). In contrast, microcapsules prepared with PLL derived with ANB-NOS were able to resist to an incubation in this alkaline solution with a swelling percentage of approximately 39% (Figure 2D and E), and 96.5 ± 4.3 % were retrieved from this incubation. After 3 years in the alkaline buffer, they have remained intact, retaining a normal round shape and a smooth surface.

Effect of the length of the cross-linker on alginate-PLL cross-linking efficiency

Initially, using the alkaline dissolution test, two similar photoactivatable cross-linkers were evaluated (Figure 3A and B). ANB-NOS and Sulfo-SANPAH are two heterobifunctional cross-linkers made of an ester of N-hydroxysuccinimide linked to an phenyl azide group. The only difference between these molecules is the length of the chain joining the two reactive groups. Sulfo-SANPAH possesses 6 carbon atoms and one nitrogen atom forming a spacer arm of 18.2 Å. Since ANB-NOS has no spacer arm, the distance between the two reactive residues is only 7.7 Å. When microcapsules made with sulfo-SANPAH-derived PLL were submitted to the alkaline dissolution test, they completely disappeared from the solution within a few minutes (data not shown).

Evaluation of microcapsules resistance to mechanical stress

Microcapsules were submitted to a mechanical stress test [26]. During this test, microcapsules, containing 2,000 kDa FITC labeled dextran, are submitted to more and stronger mechanical stresses than they are likely to encounter during a lifetime in an implantation site such as the peritoneum and in bioreactors. The percentage of broken microcapsules (Figure 4A) was 22-fold lower for microcapsules cross-linked using ANB-NOS (4.4 %) than for standard microcapsules (53.3 %). All controls showed (Figure 4B and C) negligible amounts of dextran in the supernatant, confirming that there was no dextran leaking out from unbroken microcapsules.

Effect on microcapsule membrane permeability.

Studies of microcapsule membrane molecular weight cut-off (MWCO), using a previously published method [39], showed that covalent cross-linking of APA membranes has no significant effect on membrane permeability. Both covalently cross-linked and standard microcapsules excluded [partition chromatographic coefficient (Ksec) $\approx$ 0.1] dextrans with a MW $\geq$ 19 kDa [viscosity radius (Rη) $\geq$ 3.4 nm] and proteins with a MW $\geq$

45 kDa ($R\eta \geq 2.9$ nm), whereas dextrans with a MW ≤ 4.4 kDa ($R\eta \leq 1.7$ nm) and proteins with a MW ≤ 35.2 kDa ($R\eta \leq 2.7$ nm), diffused freely ($K_{sec} \approx 1.0$) through both types of membranes (Table 1). The apparent discrepancy between the permeation of carbohydrates and proteins is due to the fact that dextrans are linear neutral molecules whereas proteins are globular charged molecules [45]. Dextrans have a larger molecular volume to mass ratio than proteins [46]. It is noteworthy that the Rη provides a better relative estimation of molecule diameters than the MW [47].

Microencapsulated cell viability

The key step of the process is the generation of covalent links by UVA illumination. A wavelength of 320-350 nm, known to induce minimal cell damage, was selected. Potential cell damages were evaluated using a double stain viability test and the observation of necrotic centres on fresh islets under a stereomicroscope. In initial studies, a 23 kJ/m^2 UVA dose, with or without ANB-NOS, decreased islet cell viability (Figure 5A), whereas ANB-NOS alone had no direct cytotoxicity. The cell damage was partially explained by the UV radiation thermal effect, since the preparation warmed up despite the fact it was on ice. To investigate the hypothesis that the harmful effect is dose related, covalently linked microcapsules were produced, using a large range of UVA doses. A dose of 2 kJ/m^2, 11.5 fold smaller than the initial dose (23 kJ/m^2), was equally effective in generating covalent links and improving microcapsule resistance. This small dose was used (and compared with the high dose where indicated) for all the experiments reported in the present article, except for the MWCO studies. The latter were not repeated, since even the high dose had no effect on this parameter. The smaller dose was as effective as the large dose in improving chemical and mechanical resistance. The effect does not seem progressive (Figure 4); therefore, it suggests that the smaller dose induces the same level of covalent links as the larger dose. The results of the dual staining viability tests (Figure 5A) and the evaluation of necrotic centres (Figure 5B) showed that the illumination of encapsulated islets with a 2 kJ/m^2 dose of UVA, with or without ANB-NOS, did not affect islet cell viability as

compared with standard microcapsules (~ 90 % viability and 15 % necrotic centres for each of the 3 conditions).

DISCUSSION

The objective of this work was to develop a method for producing very strong microcapsules with controlled MWCO, by creating covalent links between the different layers of microcapsule membranes. The first constraint was that most methods that induce such a link would severely affect cell viability. Photoactivatable cross-linkers presented a potential solution because the reaction induced by UVA illumination of the phenyl azide group was less likely to induce cell damage. The second constraint was that the cross-linker had to allow reactions in two steps, the second reactive group not being reactive during the first reaction. The selected cross-linkers comprise a N-hydroxysuccinimide residue that initially reacted with PLL, creating covalent links, while the phenyl azide group reacted only when exposed to UVA light after the compound was used to build the microcapsules. Two heterobifunctional photoactivatable cross-linkers, which differ only by the length of the spacer arm included in their structure, were investigated. The results showed that sulfo-SANPAH-derived PLL was ineffective in producing covalent links with alginate whereas such links were induced using ANB-NOS. This suggests that the spacer arm length is critical.

Our evaluation confirmed that ANB-NOS derived microcapsules are very resistant to chemical and mechanical stresses and unlikely to be affected by competition with charged molecules in the environment. The breakage of covalent links usually requires extreme conditions of pH or temperature that are incompatible with cell survival. Therefore, the covalent links between the different layers of the microcapsule membrane ensure a very strong structure. The evaluative experiments confirmed that these

microcapsules would be virtually indestructible under conditions encountered in the living body.

The use of a photoactivatable cross-linker such as ANB-NOS to create covalent links within the microcapsule membrane is advantageous. The procedure does not require harsh conditions and does not produce toxic by-products. Hydroxysuccinimide is easily and simply removed by a dialysis step. Short wavelength ultraviolet radiation, such as UVB and UVC, can provoke rapid and severe damages in living cells. The classical aryl azide functional group used for the formation of covalent bonds is generally activated with light possessing this kind of short wavelength (260-280 nm). Therefore, a phenyl azide group was preferred. The presence of the nitro group on the aryl azide group allowed the use of safer long wavelengths corresponding to UVA and even visible light for activating the cross-linking reaction [35,36]. The viability experiments showed no cytotoxic effects of the total procedure, except for microencapsulated islets exposed to high doses of UVA radiation.

Hertzberg *et al.* [31] have attempted to increase microcapsule resistance by introducing covalent links within the structure of the alginate beads. In microcapsules made exclusively with alginate, the addition of a photosensitive polyvinyl alcohol into the alginate solution has covalently linked the alginate molecules one to another to form a rigid core. This method has increased the stability of alginate beads. However, for microcapsules comprised of an alginate bead core subsequently coated, the covalent links would only solidify the inside of the microcapsules with no effect on the stability of semi-permeable membrane or of the outer biocompatible coats. There would be no control of the MWCO, which would not ensure the same protection as in PLL-ANB-NOS derived microcapsules. Lu *et al.* [32,33] have designed a method for use in bioreactors, which consists of grafting a photodimerizable reactive group on the polycationic polymer forming the semi-permeable membrane of microcapsules. This functional reactive group has the capacity to dimerize when exposed to light allowing poly (allylamine) (or eventually PLL) to form a covalent

network between the molecules of the cationic polymers, but no links with adjacent layers. However, the remaining problem is that only weak electrostatic bonds still link the microcapsule membranes layers to one another. These microcapsules have been submitted to a mechanical stress test similar to the one used in the present study, but without glass beads in the test tubes. Therefore, the mechanical stress has consisted only of microcapsules bumping on the tube walls. In our test, glass beads submitted microcapsules to extreme shearing, crushing and abrasive stress. In spite of this milder stress test, the method proposed by Lu *et al.* [32,33] has decreased only by 50 % (i.e. a factor of 2) the number of broken microcapsules as compared to standard microcapsules, and after 48 hours of this stress test, 17 % of microcapsules have been broken. In the present study, the new method decreased the number of broken microcapsules by a factor of 22, and after 72 hours of the extreme stress test, only 4.4 % of covalently cross-linked microcapsules were broken. Moreover, we are aware of no reports of microcapsules being submitted to a severe chemical stress, such as incubation in a strong alkaline buffer (pH 12), which ANB-NOS derived microcapsules resisted to, for more than three years.

Another important advantage of the method described in the present article is that it can be used to covalently bind to the microcapsule any molecule that would be identified or developed for improving microcapsule biocompatibility. Alginate from the core bead could also be replaced by another material for embedding living cells. The only essential requirement is that such molecules, to be used for outer coating or for the inner bead, comprise carbon-carbon or carbon-hydrogen bonds, which react with the phenyl azide residue of ANB-NOS. With the methods described by Hertzberg *et al.* [31] and Lu *et al.* [32,33], covalent links are induced between molecules of the same type but not with adjacent layers.

The extremely strong microcapsules with a controlled MWCO, produced with the method described in the present article, will be useful for many applications. In the field of islet transplantation, which is our primary interest, it will allow a safer use of stem cell

derived islets, immortalized insulin-producing cell lines or bioengineered insulin-producing cells. It will also allow a safer use of islets or cells co-encapsulated with islets, transfected with the genes of molecules that promote islet cell survival or immunoprotection [48].

In conclusion, the use of PLL derived with the photoactivatable cross-linker ANB-NOS, associated with a low dose UVA illumination, induced covalent links between PLL (MW: 29.3 kDa) and alginate. This method considerably enhanced microcapsule resistance to severe chemical and mechanical stresses, while preserving the desired membrane MWCO. These microcapsules are very unlikely to be damaged or destroyed in the environment found in the living body or in bioreactors. They resisted, for over three years, in extreme chemical conditions, such as pH 12, whereas standard microcapsules dissolved within 45 seconds in the same conditions. They proved to be extremely resistant to a very challenging mechanical stress test. The method did not affect membrane permeability or microencapsulated cell viability. It will be useful for the safe transplantation of stem cell derived, immortalized or bioengineered cells, and for numerous applications in bioreactors. Finally, this method may enable a safer use of viral vectors for gene therapy though further studies are required to investigate this issue. It is noteworthy that we actually propose a new role for microencapsulation: in addition to protecting the transplant from the recipient immune system, it will protect the recipient from the risks associated with the transplant.

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FIGURES AND LEGENDS

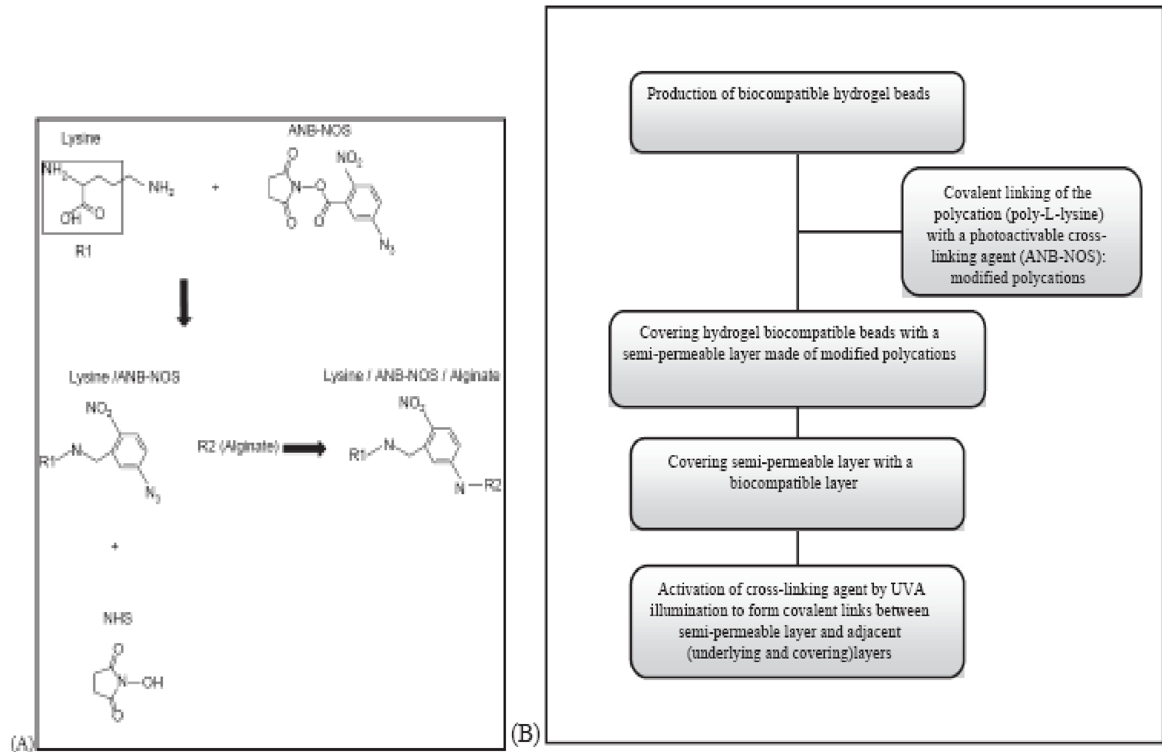

Figure 1: Description of the microencapsulation method. **A.** Details of the chemical reaction of the ANB-NOS on the primary amine of PLL, which is performed before the cells are involved in the encapsulation procedure. **B.** Schematic steps of cell encapsulation using the photoactivatable cross-linker and PLL.

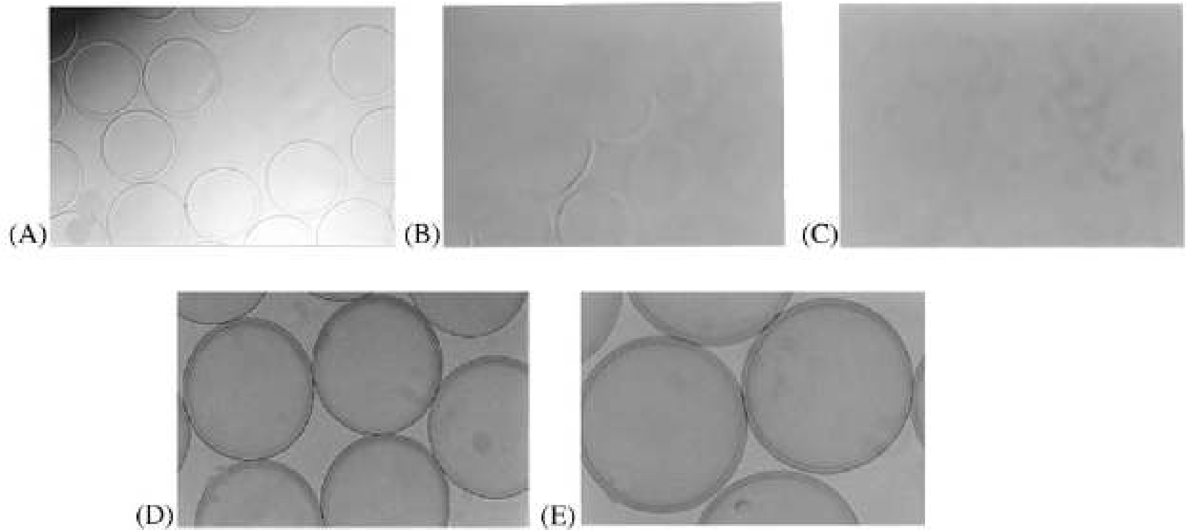

Figure 2: Microcapsule resistance to chemical degradation (alkaline dissolution test). Standard and ANB-NOS cross-linked microcapsules were incubated into strong alkaline glycine buffer (pH = 12). **A.** Standard microcapsules, before the incubation (63 X). **B.** Standard microcapsules into glycine buffer pH 12, a few seconds after the addition of the alkaline buffer (63 X). **C.** Standard microcapsules into glycine buffer pH 12 after 45 seconds (63 X). **D.** ANB-NOS cross-linked microcapsules before the incubation (100 X). **E.** ANB-NOS cross-linked microcapsules after two years in glycine buffer pH 12 (100 X). Since the pK value of the side chain amino group of the lysine is 10.54, at pH 12, the electrostatic interactions between PLL and alginate are neutralised. This experiment confirms that ANB-NOS and UVA illumination induce the formation of covalent links that are chemically resistant to strong pH variations.

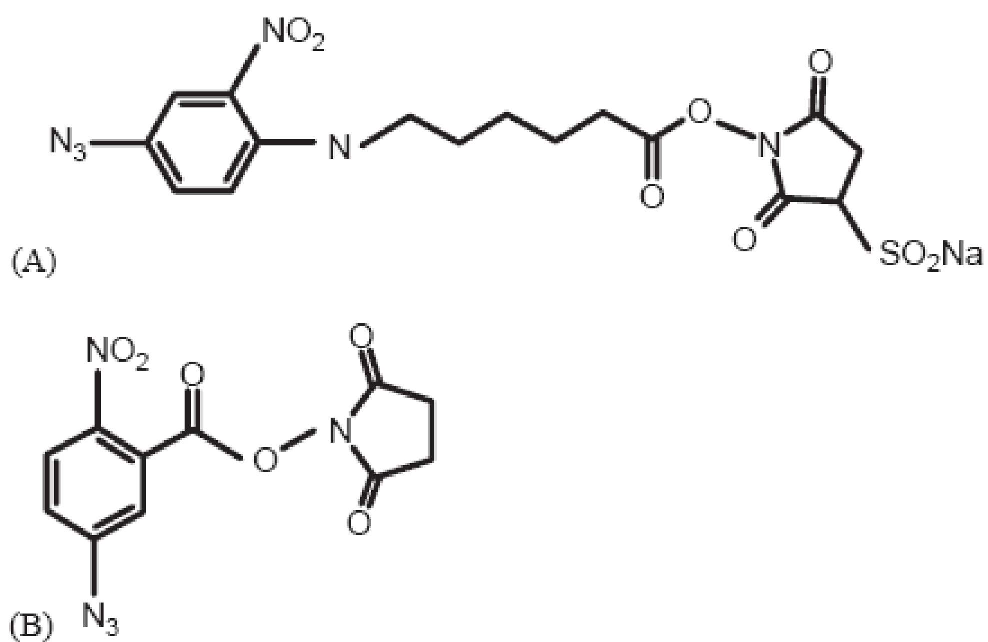

Figure 3: Cross-linker chemical representation. **A.** Sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate (Sulfo-SANPAH). **B.** N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS).

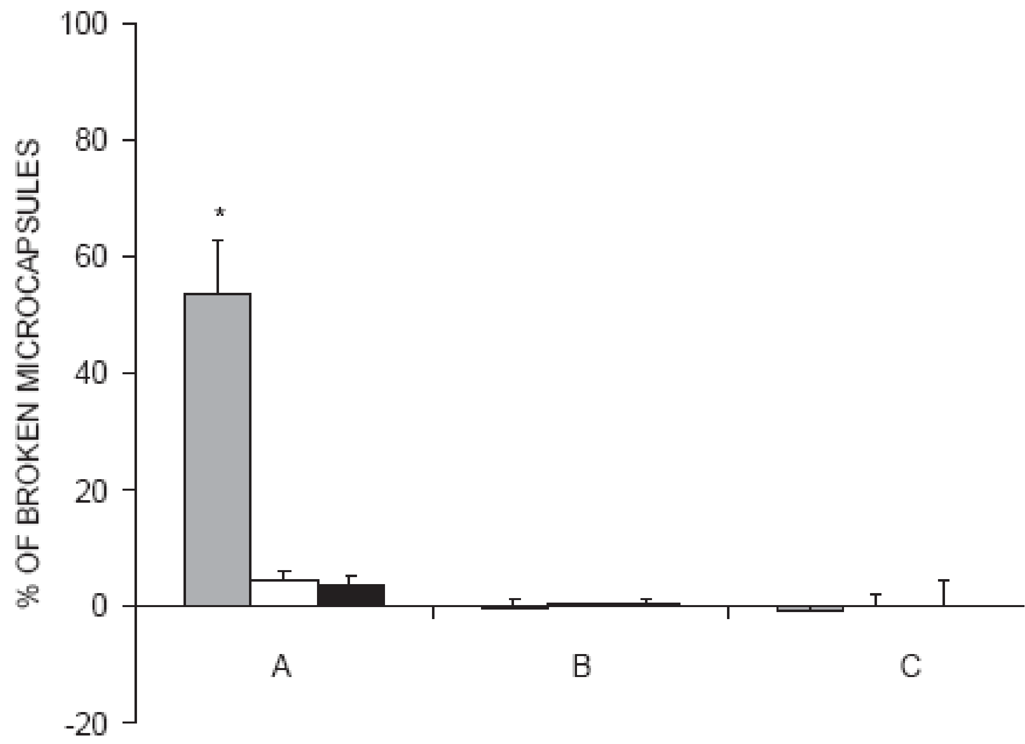

Figure 4: Microcapsule resistance to mechanical stress. The percentage of broken microcapsules was quantified after 72 hours of agitation with borosilicate beads. **A.** Standard microcapsules (hatched columns) and ANB-NOS cross-linked microcapsules made using the low (white columns) and high (black columns) UVA doses. **B.** Controls containing microcapsules and borosilicate beads without agitation are shown as a control of the microcapsule preparations. **C.** Agitated microcapsules without borosilicate beads are included as a control to detect potential inadequate preparation with unusually fragile microcapsules (a rare event). Data are presented as average ± SEM. * $p < 0.001$ ($n = 3$).

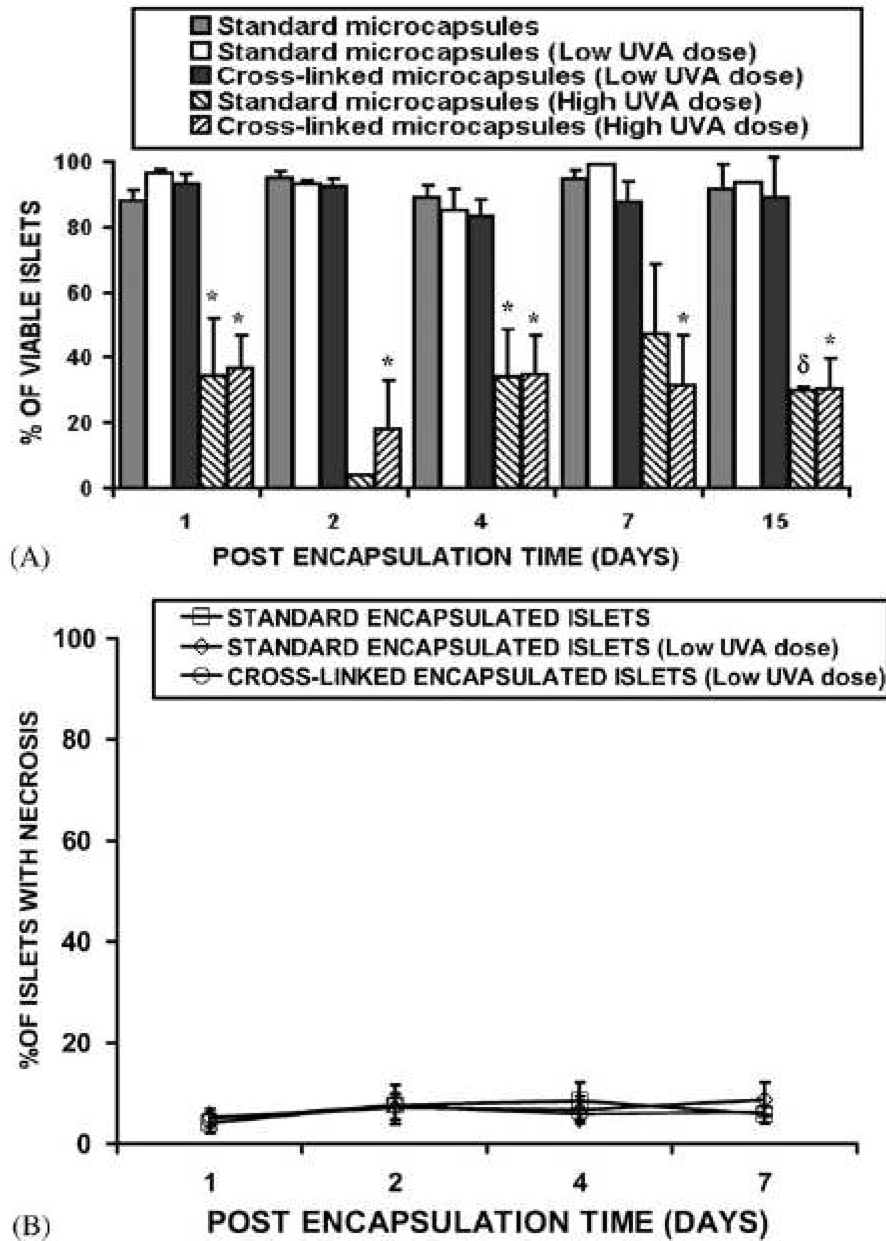

Figure 5: A. Microencapsulated islet cell viability. Isolated pancreatic islets were encapsulated into standard and ANB-NOS cross-linked microcapsules and submitted to 2 or 23 kJ/m^2 of UVA irradiation. At 1, 2, 4, 7 and 15 days after microencapsulation, islets were double stained with 0.01 mg/ml acridine orange (living cells, green) and 1 mg/ml

propidium iodide (dead cells, red), and were classified under fluorescence microscopy. Data are presented as average ± SEM. * $p<0.01$ vs. Same encapsulation process (standard or cross-linked) but low UVA dose and standard encapsulated islets, δ $p<0.01$ vs. Standard encapsulated islets without UVA irradiation. These data show that UVA irradiation does not affect cell viability, except when a large (23 kJ/m^2) UVA dose is used. **B.** Microencapsulated islet necrotic centers. Isolated pancreatic islets were encapsulated into standard microcapsules or covalently cross-linked microcapsules using low UVA dose only. At 1, 2, 4, and 7, days after microencapsulation, the presence of necrotic centers in microencapsulated islets was observed under optical microscopy. The cytotoxicity was expressed as the percentage of islets with a necrotic center. Results are presented as the average ± SEM.

Table 1
Microcapsule membrane molecular weight cut-off

Molecules	M_w (Da)	$R\eta$	Standard microcapsules		Cross-linked microcapsules	
			K_{sec}	Elution profiles	K_{sec}	Elution profiles
Dextran 2,000,000[a]	2×10^6	34.2	0.0	Excluded	0.0	Excluded
Dextran 19,000	19,000	3.4	0.0	Excluded	0.1	Excluded
Bovine serum albumin	66,000	3.4	0.0	Excluded	0.1	Excluded
Ovalbumine	45,000	2.9	0.0	Excluded	0.1	Excluded
Carboxipeptidase	35,250	2.7	0.9	Freely diffuses	1.0	Freely diffuses
Dextran 4400[b]	4400	1.7	1.0	Freely diffuses	1.0	Freely diffuses

Molecules were classified according to their relative viscosity radius ($R\eta$).
Molecules showing a K_{sec} near 1 are considered to freely diffuse through microcapsule membranes, while molecules with a K_{sec} around 0 are considered to be excluded from microcapsules.
[a] Excluded molecule used as void volume.
[b] Freely diffusing molecule used for the evaluation of total volume.

Table I: Microcapsule membrane molecular weight cut-off. Molecules were classified according to their relative viscosity radius (Rη). Molecules showing a Ksec near 1 are considered to freely diffuse through microcapsule membranes, while molecules with a Ksec around 0 are considered to be excluded from microcapsules.

The Effect of Covalent Cross-links Between the Membrane Components of Microcapsules on the Dissemination of Encapsulted Malignant Cells

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One-sentence summary

All recipients of 250,000 EL-4 thymoma cells microencapsulated in covalently cross-linked membranes were living and disease-free, 150 days post implantation, whereas all recipients of ≥20,000 EL-4 cells nonencapsulated or encapsulated in standard microcapsules died with widespread metastasis within 26.3 ± 1.0 and 35.2 ± 2.2 days, respectively.

CONTRIBUTIONS RESPECTIVES DES AUTEURS

Julie Dusseault (70%): Conception du projet, planification et exécution des expériences, analyse des résultats, écriture, correction et soumission de l'article.

Geneviève Langlois (2,5%): Participation aux expériences, correction de l'article.

Marie-Christine Meunier (2,5%): Planification des expériences, correction de l'article.

Martin Ménard (10%): Conception du projet, participation aux expériences, correction de l'article.

Claude Perreault (5%): Conception du projet, correction de l'article.

Jean-Pierre Hallé (10%): Conception du projet, analyse des résultats, écriture et correction de l'article.

ABSTRACT

Stem cells and immortalized cells have considerable therapeutic potential but present risks of malignant transformation. Cell microencapsulation allows transplantation without immunosuppression. We have developed a method for microencapsulating living cells within covalently cross-linked membranes that are extremely resistant. We provide herein direct evidence that these microcapsules can prevent malignant cell dissemination. When 20,000 or more nonencapsulated EL-4 thymoma cells were implanted intraperitoneally in mice, all recipients died with widespread metastasis within 26.3 ± 1.0 days. All recipients of 250,000 EL-4 cells microencapsulated in covalently cross-linked membranes were living and disease-free, 150 days post implantation. Encapsulation in standard microcapsules only slightly delayed the recipient death. Pancreatic islets transplanted using either type of microcapsule presented similar survival. We conclude that microencapsulation in covalently cross-linked membranes prevents malignant cell dissemination.

INTRODUCTION

Stem cell-derived cells or tissues (1-4), immortalized cell lines (5-7) and bioengineered cells (4, 8, 9) are considered potentially therapeutic for the treatment of many diseases including those with deficient hormone production, such as insulin in type 1 diabetes, erythropoietin in anemia (10, 11), factors VIII and IX in haemophilia (8, 12) and growth hormone in dwarfism (13). Moreover, delivery of therapeutic products from nonautologous engineered cell lines can also be used in cancer therapy (9). However, these three types of cell present risks of malignant transformation. Stem cells, particularly

embryonic stem cells and embryonic germ cells, have the ability to improperly differentiate into tumors in animals (2, 14-16). Other sources of cells for transplantation include cells that are genetically modified to produce a therapeutic agent or existing secreting cells that are immortalized for prolonged in vitro storage and/or in vivo therapeutic action. These bioengineered and immortalized cells can also undergo malignant transformation (4, 17). All efforts should be made to minimize potentially severe consequences.

Microencapsulation of cells or tissues, such as islets of Langerhans, in semipermeable alginate-poly-L-lysine-alginate (APA) membranes has been investigated as a means of protecting transplanted cells from the host immune system and avoiding the requirement for immunosuppression (18, 19). For this purpose, therapeutic cells are immobilized in calcium alginate beads that are sequentially incubated in solutions of polycation, such as poly-L-lysine (PLL), to form a semipermeable membrane, and in diluted alginate to improve the microcapsule biocompatibility. The strength of such microcapsules is dependent upon electrostatic interactions between alginate (negatively charged) and polycation (positively charged). Since these charged polymers are in competition with any charged molecules in their environment, the stability of standard microcapsule is limited (20). We previously developed a method for microencapsulating living cells in semipermeable membranes with covalent cross-links between the molecules of the microcapsule semipermeable layer and between such molecules and the microcapsule core bead and outer coating molecules (21). In covalent links, one electron is shared by two atoms; breaking such links usually requires very high energy and extreme conditions (temperature and pH) that are incompatible with cell survival. Thereby the membranes of such covalently cross-linked microcapsules are considerably strengthened. Most methods for creating covalently cross-linked microcapsules have been faced with the hurdle of incompatibility with cell survival. To overcome this, we have used a photoactivatable heterobifunctional cross-linker, N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS), comprising an N-hydroxysuccinimide ester group and a phenyl azide group that react in sequence (21). Firstly, the N-hydroxysuccinimide residue is used to covalently

link ANB-NOS with PLL in conditions that could be harmful for cells, but before the cells are involved in the process. Then, the PLL-(ANB-NOS) compound is used to encapsulate cells in alginate-PLL(ANB-NOS)-alginate microcapsules. At the end of the procedure, upon illumination of the final product with UVA light, the nitrophenyl azide residues create covalent cross-links between PLL(ANB-NOS) and molecules that have double bonds, C-H or N-H sites. Therefore, there are numerous covalent links within the semipermeable layer (i.e. between PLLs) as well as between the semipermeable layer and the other components of the membrane. This procedure is not harmful for living cells. In previous in vitro studies (21, 22), we showed that these microcapsules are extremely resistant to chemical and mechanical stresses. They remained intact in extreme conditions, such as after more than one-year incubation (now 3 years) in a strong alkaline buffer, at pH 12, whereas standard microcapsules were completely dissolved after 45 sec. in the same solution. They were 22-fold more resistant than standard microcapsules to mechanical stress. Moreover, in vitro experiments showed that the viability of islet cells that are encapsulated in either standard or covalently cross-linked microcapsules is similar (21, 22).

We now report a proof-of-concept study that tested the hypothesis that encapsulation in microcapsules with covalent cross-links between the different components of the microcapsule membrane prevents the dissemination of malignant cells. In addition, the absence of harmful effect on cell viability of the method to produce covalent cross-links was demonstrated in vivo with an islet transplantation study. For the first purpose, EL-4 thymoma cells, encapsulated within standard or covalently cross-linked microcapsules, or not encapsulated, were transplanted intraperitoneally into B6.SJL mice. EL-4 cells, a very malignant cell line, are derived from C57BL/6 mice and their phenotype differs from the recipient phenotype by only one antigen (CD 45.2 vs CD 45.1, respectively). Using fluorescence activated flow cytometry, such an almost syngeneic model allowed for the identification of the cells derived from implanted cells compared to the recipient cells, while avoiding allogeneic reactions. All recipients of nonencapsulated EL-4 cells died of widespread leukemia/lymphoma within an average of 26.3 ± 1.0 days, whereas all

recipients of EL-4 cells microencapsulated within covalently cross-linked membranes were living, without any signs of disease, at 150 days post-implantation. In contrast, the encapsulation of EL-4 cells within standard microcapsules delayed slightly, but did not prevent recipient death. Moreover, flow cytometry analysis of organs from the animals that were sacrificed at different times post-implantation revealed the presence of EL-4 cells in the peritoneal fluid, the epididymal fat pads and the inguinal lymph nodes of the recipients of nonencapsulated EL-4 cells but not of EL-4 cells encapsulated in covalently cross-linked microcapsules. These results support the concept that encapsulation in microcapsules with covalent cross-links between the different molecules forming the membrane prevents leakage of cells from the microcapsules. In addition, the islet transplantation study confirmed that microencapsulation in such covalently cross-linked membranes has no detrimental effect on in vivo islet cell survival and function.

MATERIALS AND METHODS

Materials

A commercially available purified sodium alginate, PronovaTM UPLVG (ultrapure, low viscosity, 67% guluronic acid, Mw: 160 kDa) was purchased from FMC Biopolymers (Drammen, Norway). All other materials (chloroform, acetone, alcohol, acetic acid, sodium citrate) were of analytical grade and purchased from Fisher Scientific Ltd. (Nepean, Canada).

Animals

Male B6.SJL mice (B6.SJL.PtprcaPep3b/BoyJ, Jackson Laboratories, Maine, USA) were used as recipients in EL-4 cell implantation studies. Male Wistar rats (250-400 g body weight; Charles River, St-Constant, Canada) were used for islet isolation. Male scid mice

(B6.CB17-Prkdcscid/szJ (Charles River, St-Constant, Canada) were used as recipients in islet transplantation experiments. Protocols were reviewed and stated conformed to the ethical guidelines of the Canadian Council for Animal Care by the Animal Care Ethics Committee of the Maisonneuve-Rosemont Hospital Research Center. These guidelines were observed throughout the study.

Tumor cells

EL-4 thymoma cells of C57BL/6 origin, were purchased from the American Type Culture Collection (ATCC® Number: TIB-39) and cultured in Dulbecco modified essential medium (DMEM) supplemented with 10% horse serum, 1% penicillin-streptomycin-glutamine (Invitrogen Life Technologies, Burlington, Canada). EL-4 cells are derived from a C57BL/6 mouse; their phenotype differs from the phenotype of B6.SJL mouse cells by only one antigen [CD 45.2 (Ly-5.2) versus CD 45.1 (Ly-5.1), respectively].

EL-4 cell staining and flow cytometry

Fluorescein isothiocyanate (FITC)-labeled anti-CD45.2 (Ly-5.2) and phycoerythrin (PE)-labeled anti-CD90.2 (Thy-1.2) were obtained from PharMingen. Cells were analyzed on a FACSscan using CellQuest (Becton Dickinson).

Pancreatic islet isolation

Islets from rat pancreases were isolated and purified as previously described [23]. Briefly, pancreases were infused via the common bile duct with 1 mg/ml Type V collagenase (Sigma-Aldrich Ltd. Oakville, Canada) in Hank's balanced salt solution

(HBSS) and digested 30 min at 37ºC. Islets were first purified on 800μ and 250μ consecutive filters and then on discontinuous Euroficoll gradient (Mediatech Inc., Herndon, USA). Islets were handpicked under an inverted light microscope and cultured overnight in RPMI-1640 medium (Invitrogen Life Technologies, Burlington, Canada) with 11 mM glucose supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Burlington, Canada), 1% penicillin-streptomycin-glutamine 100X solution (Invitrogen Life Technologies, Burlington, Canada) supplemented with 500 ng/ml IGF-II [24] at 37oC in humidified air atmosphere containing 5% CO2.

Cell microencapsulation

EL-4 thymoma cells or islets were encapsulated in APA microcapsules as previously described [25]. Briefly, cells were mixed with a 1.3% sodium alginate solution, which was then extruded through a 20-G needle, using an electrostatic droplet generator [26], into a 100-mM calcium lactate solution. The calcium, a divalent ion, cross-linked the alginate to form gel beads. The microcapsule membrane was formed by successively soaking the alginate beads in 0.05% w/v PLL (Mw: 29.3 kDa, Sigma-Aldrich Ltd., Oakville, Canada) and 0.13% w/v alginate solutions. Microencapsulated cells (microcapsule diameter: 280-320 μm) were cultured overnight in serum-free medium (DMEM for EL-4 thymoma cells or Ultraculture for islets) supplemented with 1% penicillin-streptomycin-glutamine 100X solution at 37oC in humidified air containing 5% CO2. After islets were microencapsulated, 500 ng/ml IGF-II was added to the medium [24]. The same procedure was used for encapsulating islets in covalently cross-linked microcapsules, except for the use of PLL that was previously derived with the photoactivatable cross-linker ANB-NOS and illumination of the final product with UVA light (2 kJ/m2), as previously described [27].

Cell viability

Encapsulated EL-4 cell viability was evaluated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, USA) [28]. An aliquot of 100 cell containing microcapsules was incubated in HBSS with 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) in a 96-well plate for 3 h at 37oC in humidified air containing 5% CO2. MTS is metabolized by mitochondria of viable cells to soluble formazan that can be detected with an ELISA plate reader (measure wavelength: 492 nm; reference wavelength: 620 nm).

Microencapsulated cell transplantation

Mice were anesthetized with Isoflurane. Microencapsulated or nonencapsulated cells (EL-4 thymoma cells or islets) were transplanted via a 16-G catheter into mouse peritoneal cavity.

Induction and follow-up of diabetes

Diabetes was induced in mice by intraperitoneal injection of streptozotocin (STZ; 185 mg/kg body weight in sodium citrate buffer, pH 4.5; Sigma-Aldrich Ltd, Oakville, Canada). The blood glucose of all mice raised > 20 mM within 2 days after STZ injection. Subcutaneous Ultralente insulin (Humulin U®, 2U/d/mouse, Eli Lilly, Indianapolis, USA) injections were used to maintain mice alive when necessary. Using a minimal mass model, 500 microencapsulated rat islet equivalents were transplanted intraperitoneally into diabetic mice (blood glucose >20 mM for 2 weeks) □14 d post STZ. Control groups included diabetic mice transplanted with the same number of nonencapsulated islets. The last insulin injection was given at the time of transplantation. Recipient mice had free access to water

and food (Mouse colony chow 5018; Agribrands Purina, Woodstock, Canada). Blood glucose levels were measured (Accu-Chek) on samples obtained from the mouse tail vein every day during the first week post-transplantation and then at least once per week. Mice were considered normoglycemic when glucose levels were <11 mM on two consecutive days.

Statistical analysis

All results are expressed as mean ± S.E.M. The differences between each experimental group were analyzed by unpaired Student's t-test with P values less than 0.05 considered significant.

RESULTS

Characterization of the model

The specificity of the EL-4 thymoma cell marker CD 45.2 was evaluated *in vitro* (Fig.1). EL-4 cells and B6.SJL mouse spleen cells were stained with antibodies against CD 45.2 and the common T-cell marker CD 90.2, labeled with fluorescein isothiocyanate (FITC) and phycoerythrin (PE), respectively. As expected, fluorescence activated flow cytometry analysis showed that approximately 23% of the cells isolated from mouse spleen were CD 90.2 positive, but none of these cells was positive for CD 45.2 (Fig.1A). Because cultured EL-4 cells were positive for both markers (Fig. 1B), they were able to be distinguished from the recipient T cells. This also allowed for following localization of cells derived from implanted EL-4 cells.

Thereafter, we injected B6.SJL mice intraperitoneally and estimated recipients' survival (Table I) and the pattern of tissue infiltration by EL-4 cells (Fig. 2). Mice implanted with 250,000 nonencapsulated EL-4 thymoma cells died within 22.3 ± 1.0 days after intraperitoneal implantation (Table I). Autopsy revealed that cancer cells and solid tumor-like cell proliferations were spreading into the peritoneal cavity. These solid masses contained nearly 100% EL-4 cells (unpublished data). In a second set of experiments, recipients of 250,000 nonencapsulated EL-4 cells were sacrificed and autopsied 2, 8, 15 and 20 days after implantation (Fig. 2). At day 15 post-implantation, flow cytometry analysis showed that 81.9 ± 3.1% of cells from the peritoneal fluid and 35.4 ± 4.2% from the epididymal fat pads were EL-4 cells (CD 45.2-positive). The appearance of EL-4 cells in the inguinal lymph nodes was slightly delayed. Nonetheless, on day 20 post-intraperitoneal implantation, 21.9 ± 7.1% of cells from the inguinal lymph nodes were positive for the CD 45.2 antigen.

We determined the minimal number of EL-4 cells that would lead to recipient death. Graded numbers of nonencapsulated EL-4 cells, ranging from 80 to 250,000 cells, were implanted into the peritoneal cavity of B6.SJL mice (Table I). With the maximal number of EL-4 cells, all mice died at an average of 22.3 ± 1.0 days post-implantation. Decreasing the number of implanted EL-4 cells slightly delayed the animal death. Nevertheless, all mice transplanted with ≥20,000 EL-4 cells died within a period of 26.5 ± 0.9 days. Out of 8 mice transplanted with 10,000 EL-4 cells, 5 survived. All mice transplanted with ≤2,000 nonencapsulated EL-4 cells survived.

Microencapsulated EL-4 cells viability was evaluated in vitro (Fig. 3) over a 100 day period. Cell viability appears to be higher for EL-4 encapsulated in covalently cross-linked than standard microcapsules (Fig.3A). Cell proliferation and tumor-like structures were observed within the microcapsules. However, the proliferation pattern of EL-4 cells encapsulated in standard (Figure 3B and D) versus covalently cross-linked (Figure 3C and E) microcapsules was different. EL-4 cells slipped out of standard microcapsules and

proliferated in the culture media (Figure 3D); therefore the total amount of cells within microcapsules was lower than with covalently cross-linked microcapsules. EL-4 cells remained inside covalently cross-linked microcapsules (Figure 3E).

Microencapsulation in covalently cross-linked membranes prevents EL-4 thymoma cell dissemination.

To test the hypothesis that covalently cross-linked microcapsules can prevent malignant cell dissemination, 250,000 EL-4 thymoma cells, nonencapsulated or encapsulated within standard or covalently cross-linked microcapsules were implanted intraperitoneally into B6.SJL mice. Mice implanted with nonencapsulated EL-4 cells died at a mean time of 22.3 ± 1.0 days after implantation (Fig. 4A). When 250,000 EL-4 cells encapsulated within standard microcapsules were implanted, the average survival time of recipients was prolonged to 35.2 ± 2.2 days (Fig. 4A). The key finding was that all mice (9/9) that were implanted with 250,000 EL-4 cells encapsulated within covalently cross-linked microcapsules were still alive with no sign of disease at 150 days post-implantation (Fig. 4A). Two mice that remained healthy 150 days after implantation with 250,000 EL-4 cells encapsulated in covalently cross-linked membranes were then re-implanted with 250,000 nonencapsulated EL-4 cells. These mice died with widespread metastasis 22 and 23 days, respectively, after the nonencapsulated EL-4 cell implantation. All other recipients of EL-4 cells encapsulated in covalently cross-linked microcapsules are still alive without any signs of disease 250 days after implantation.

EL-4 thymoma cell dissemination and spreading from standard and covalently cross-linked microcapsules were also evaluated by an autopsy and flow cytometry analysis of different organs of mice sacrificed 28 days following EL-4 cell implantations. With standard microcapsules, high numbers of EL-4 positive cells were found in the peritoneal fluid (41.5%), the epididymal fat pad (41.1%) and the lymph nodes (9.1%). However, EL-4

positive cells were absent in the same organs of mice implanted using covalently cross-linked microcapsules. These results clearly show that cancer cells can be confined within covalently cross-linked but not within standard microcapsules

Microencapsulation in covalently cross-linked membranes has no harmful effect on in vivo islet cell survival and function.

We previously showed (21, 22) that microencapsulation of islets in APA membranes covalently cross-linked using the heterobifunctional photoactivatable cross-linker ANB-NOS, does not decrease in vitro islet cell viability. To evaluate the in vivo survival and function of these encapsulated islet cells, we conducted an islet transplantation study (Fig. 5). Streptozotocin-induced diabetic mice, which had blood glucose over 20 mmol/L for at least two weeks, were transplanted with 500 islet-equivalents encapsulated within standard or covalently cross-linked microcapsules or nonencapsulated. No mice that were transplanted with nonencapsulated islets normalized their blood glucose (Fig. 5). In the recipients of islets encapsulated in either standard or covalently cross-linked microcapsules (Fig. 5), average blood glucose decreased rapidly to normal levels (~5 mmol/L) and continues to remain at this level six months after transplantation. Throughout the study, there were no significant differences between the blood glucose levels of the two groups. This confirmed that the method for microencapsulating islets within covalently cross-linked membranes has no harmful effect on islet cell survival.

DISCUSSION

Cell microencapsulation has been proposed to protect transplanted cells from the recipient's immune system (18, 23, 24). In most clinical applications microcapsule stability

is a critical issue (20). We reported *in vitro* studies (21, 22) demonstrating that our novel covalently cross-linked microcapsules are extremely resistant to chemical and mechanical stresses. We now report in vivo studies confirming that these microcapsules are so strong they can confine very malignant EL-4 cells, while presenting no adverse effect on cell survival. On the other hand, both *in vitro* and *in vivo* experiments showed that EL-4 cells escape from standard microcapsules. Based on our results, we propose a novel complementary application for microencapsulation: to protect the recipient from the untoward proliferation of transplanted cells, particularly cells that present a risk of malignant transformation. Examples of such cells include stem cells and immortalized cell lines.

As proof of this concept, a worst case scenario experiment was designed: the intraperitoneal implantation of 250,000 very malignant EL-4 thymoma cells in three conditions: nonencapsulated, encapsulated in standard and in covalently cross-linked microcapsules. Microencapsulation in covalently cross-linked membranes completely prevented the dissemination of EL-4 cell metastasis. All recipients were living without any signs of disease at 150 days post-implantation, whereas all recipients of the same number of nonencapsulated EL-4 cells died at an average of 22.3 ± 1.0 days post-implantation. Moreover, all recipients of EL-4 cells encapsulated in covalently cross-linked microcapsules that were not sacrificed for special studies are still alive and disease-free at ≥250 days post-implantation. Encapsulation in standard microcapsules did not prevent but only delayed recipients' death from 22.3 to 35.2 ± 2.2 days post-implantation. In addition, using flow cytometry analysis, EL-4 cells were found, from day 15 post-implantation and thereafter, in the peritoneal fluid, the epididymal fat pads and the lymph nodes of recipients of nonencapsulated EL-4 cells but not in the recipients of EL-4 cells microencapsulated in covalently cross-linked membranes. The results of the in vitro and in vivo studies demonstrate that proliferating EL-4 cells can break standard microcapsule membranes and disseminate into different organs. In our study, only covalently cross-linked membranes prevented extravasations of encapsulated cells in host tissues. We previously (21, 22)

showed that these microcapsules are 22-fold more resistant to an extreme mechanical stress than standard microcapsules. Moreover, they remained intact in extreme conditions, such as one-year incubation (now 3 years) in a strong alkaline buffer (pH 12), whereas standard microcapsules were completely dissolved after 45 sec. in the same solution (22). We are aware of no reports of microcapsules that resist such severe mechanical and chemical stresses.

To better characterize the model, a dose-response study was conducted by implanting different numbers of nonencapsulated EL-4 cells. All recipients of ≥20,000 nonencapsulated EL-4 cells died of metastasis spreading within 26.5 ± 0.9 days post-implantation. These results confirmed that the implantation of 250,000 EL-4 cells is actually a worst case scenario. A potential explanation for the protective effect of encapsulation in covalently cross-linked microcapsules could be the development of an immune response against EL-4 cells. To evaluate this possibility, at 150 days post-implantation of EL-4 cells microencapsulated in covalently cross-linked membranes, two mice that presented no signs of disease, received a second implantation, this time using nonencapsulated EL-4 cells. These mice died at 22 and 23 days, respectively, after the implantation. This duration of survival is similar to that found in naïve mice after nonencapsulated EL-4 cell implantation. This demonstrates that the immunization of the recipients does not account for the absence of EL-4 cell dissemination in mice transplanted with EL-4 cells encapsulated in covalently cross-linked microcapsules.

A major constraint in designing a method to produce microcapsules with covalent cross-links is the incompatibility with cell survival of most techniques that are used to create such links. The use of the heterobifunctional photoactivatable cross-linker ANB-NOS allowed us to overcome this problem. This method does not require harsh conditions and no toxic by-products are released. For activating ANB-NOS, we chose to use safe, long wavelengths in the UVA and even visible light range (25, 26). Illumination by light in these wavelengths is not harmful for cells. We showed previously (21, 22), that this method does

not affect the membrane permeability or the encapsulated cell viability. However, the effect on cell viability was evaluated only in vitro (21, 22). It was, therefore, important to evaluate the effect of the method on in vivo islet cell survival and function. In the present study, the transplantation of islets into diabetic mice decreased and maintained the recipient blood glucose at similar (normal) levels when islets were microencapsulated in either standard or covalently cross-linked membranes. Moreover, photomicrographs of encapsulated EL-4 cells in standard (Figure 3B and D) or covalently cross-linked microcapsules (Figure 3C and E) clearly showed that the encapsulation process was not harmful to EL-4 cells, since these cells strongly proliferated. These studies confirm that the method that was developed to create microcapsules with covalent cross-links does not negatively affect the survival and function of encapsulated cells.

This cross-linking technology can be applied using other polymers. Since the N-hydroxysuccinimide ester residue of ANB-NOS reacts with primary amino groups, other polyamines, such as poly-L-ornithine could be used instead of PLL (24). Alginate could also be replaced by any molecule comprising double-bonds, C-H or N-H sites, which the photoactivatable nitrophenyl azide residue of ANB-NOS reacts with.

This method may have a large number of useful applications. In islet transplantation, which is our primary research interest, one challenge is finding alternative sources of insulin producing cells. This challenge comes from the fact that the supply of allogeneic islets from human cadavers would provide enough islets to treat less than 1% of potential recipients (27, 28). Recent developments have been reported with the use of embryonic (2, 3, 29) and adult (1, 30-32) stem cell derived islets. As well, bioengineered cells can be used in a wide range of applications in the field of islet transplantation. Islet cells can be genetically modified, for example to promote their survival by the expression of anti-apoptotic and/or immunoregulatory genes (17). Cells from immortalized cell lines may also be genetically modified to produce molecules that promote islet cell survival or immune protection, and then be co-encapsulated with islets. Moreover, totally

bioengineered insulin secreting cells and immortalized cell lines such as RINm5F (7), K-cells (33), and other types of cells (34) have been investigated. However, all these therapeutic cells may present a risk of malignant transformation (14, 15). The present study supports the concept that encapsulating these cells within covalently cross-linked microcapsules would enhance the safety of such therapeutic approaches. This strategy has the potential to be applied to a large number of other diseases. Further studies are required to explore the potential use of these resistant microcapsules for the prevention of interspecies viral transmission in islet xenotransplantation.

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FIGURES AND LEGENDS

Table 1
Dose–response to peritoneal implantations of nonencapsulated EL-4 cells

Number of implanted EL-4 cells	Survival: % of mice (*N*)	Length of survival (days±SEM)
80	100 (4/4)	–
400	100 (4/4)	–
2000	100 (4/4)	–
10,000	62.5 (5/8)	29.0±0.0
20,000	0 (0/4)	26.5±0.9
30,000	0 (0/4)	24.0±0.4
40,000	0 (0/4)	22.5±0.5
50,000	0 (0/4)	22.8±0.7
250,000	0 (0/12)	22.3±1.0

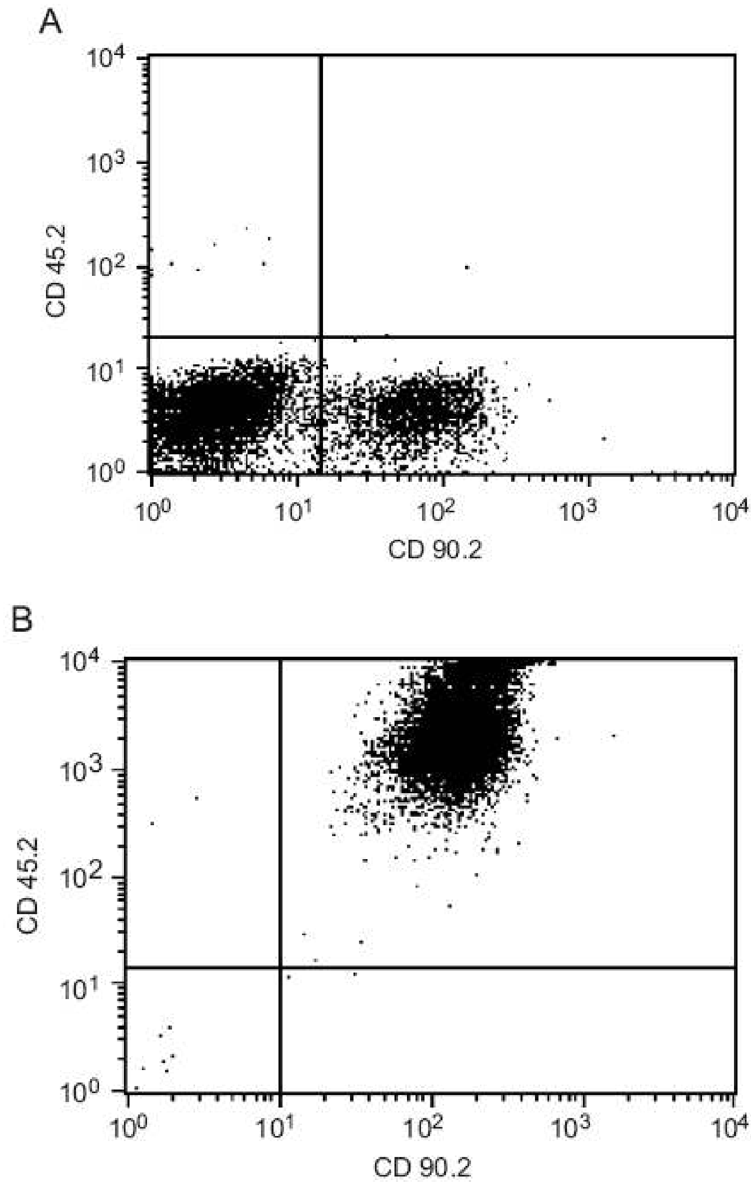

Figure 1. Specificity of cell marker antibodies. Fluorescence activated flow cytometry analysis of cells stained with antibodies specific for CD 45.2 and CD 90.2. **A.** Spleen cells from B6.SJL mice. **B.** EL-4 cells cultured in vitro.

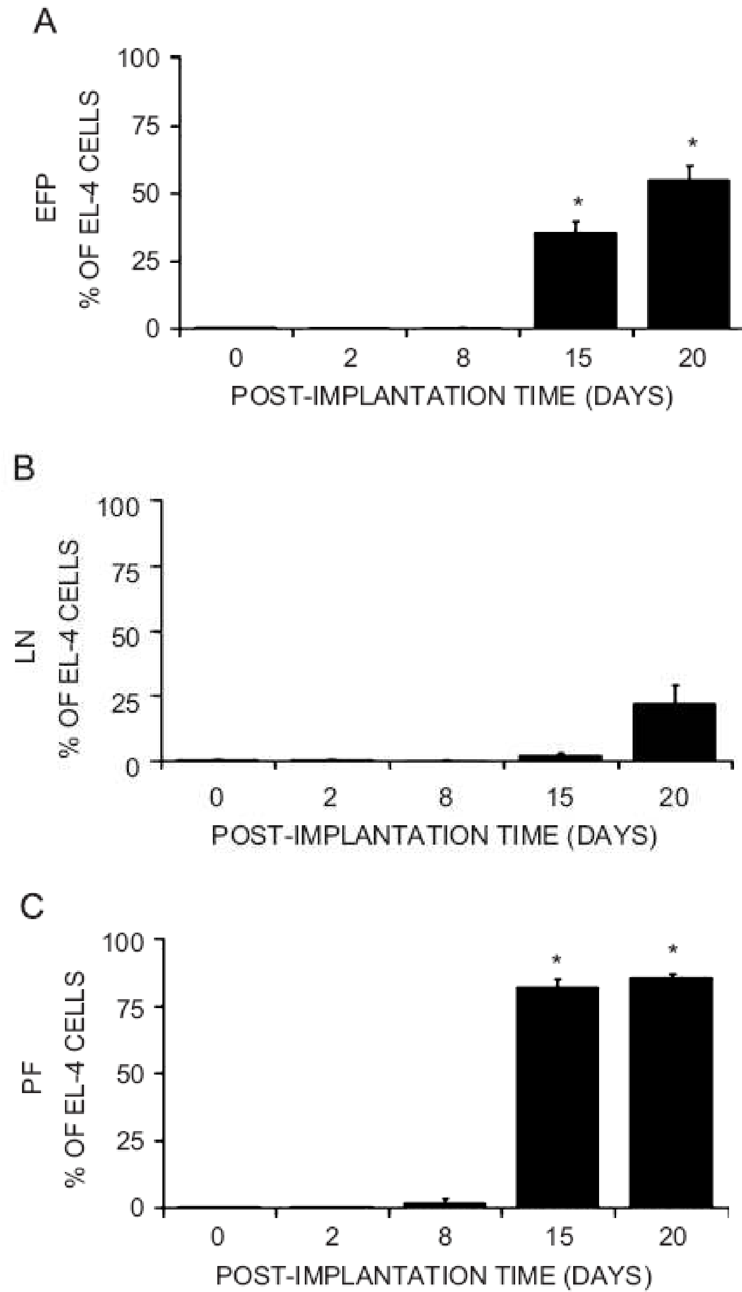

Figure 2. Time course of EL-4 thymoma cell dissemination. Fluorescence activated flow cytometry analysis of cell suspensions from B6.SJL mouse organs, **(A)** EFP: epididymal fat pad, **(B)** LN: lymph nodes and **(C)** PF: peritoneal fluid, at various times post EL-4 thymoma cell implantation into the peritoneal cavity. *p <0.001 vs no EL-4 implantation.

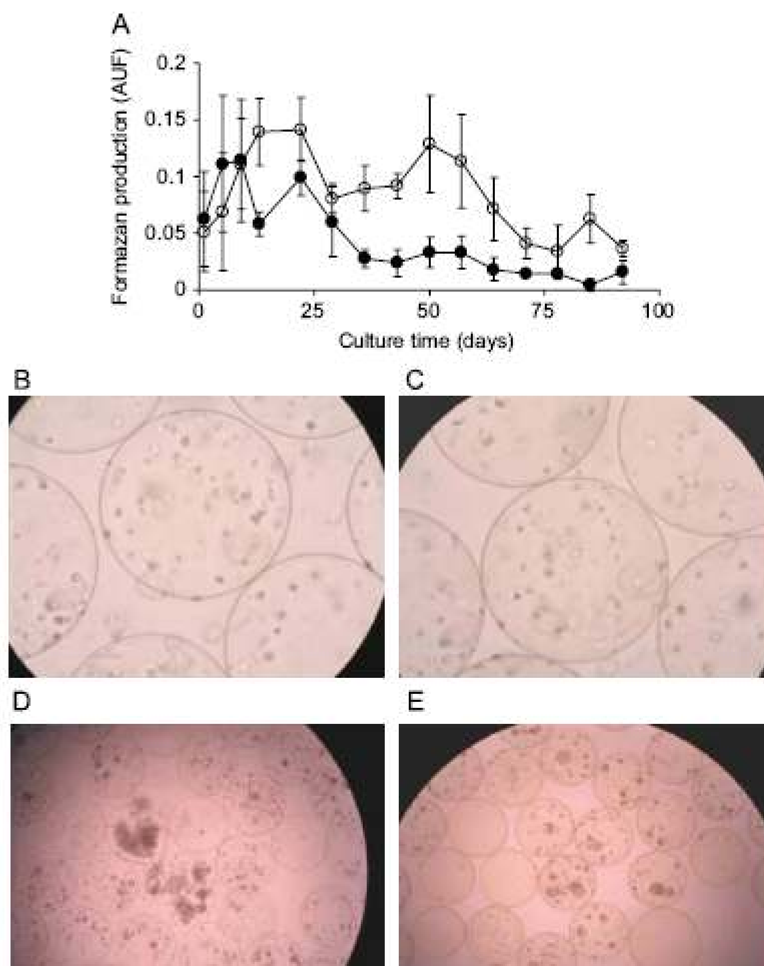

Figure 3. In vitro study of the viability of El-4 cells encapsulated in standard and covalently cross-linked microcapsules. **(A)** Viability (MTS test) of EL-4 cells (N=4) encapsulated in covalently cross-linked microcapsules (open circles) or standard microcapsules (closed circles). **(B)** Photomicrographs of EL-4 cells encapsulated in standard microcapsules or **(C)** covalently cross-linked microcapsules, the day of the encapsulation (magnification 100X). **(D)** Photomicrographs of EL-4 cells encapsulated in standard microcapsules or **(E)** covalently cross-linked microcapsules, after 12 d in culture following encapsulation (magnification: 63X).

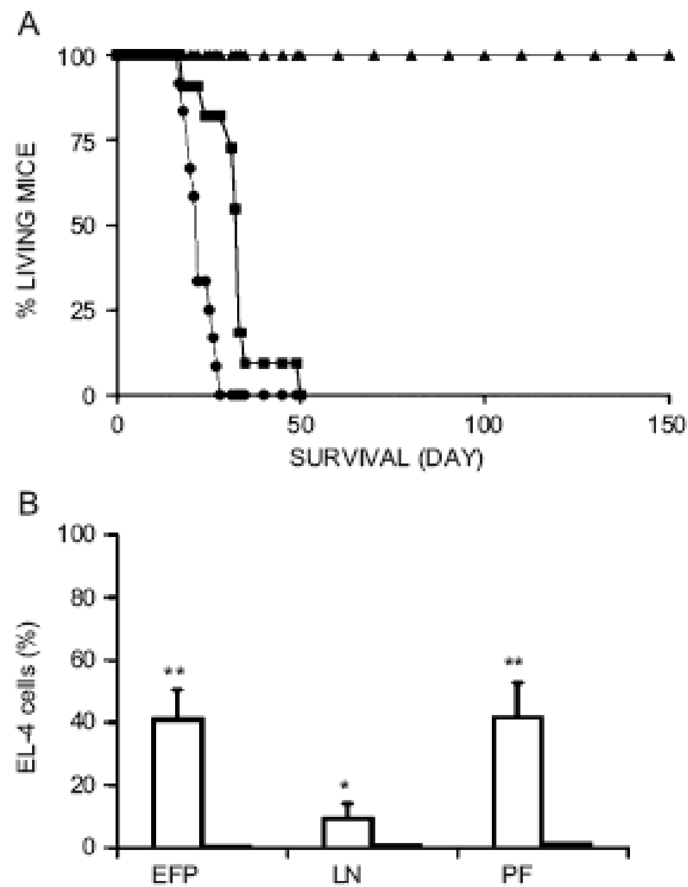

Figure 4. Prevention of EL-4 thymoma cell dissemination by microencapsulation in covalently cross-linked membranes. **(A)** Follow-up of B6.SJL mice survival (%) after EL-4 implantation. Circles: nonencapsulated EL-4 cells (N=12); squares: EL-4 cells microencapsulated in standard membranes (N=11); triangles: EL-4 cells microencapsulated in covalently cross-linked membranes (N=11). **(B)** EL-4 thymoma cell dissemination from standard and covalently cross-linked microcapsules 28 days following the implantation in mice. Fluorescence activated flow cytometry analysis of cell suspensions from B6.SJL mouse organs. (EFP: epididymal fat pad, LN: lymph nodes, PF: peritoneal fluid).).White: standard microcapsules. Black : covalently cross-linked microcapsules. **p <0.001 and *p <0.05 vs covalently cross-linked microcapsules.

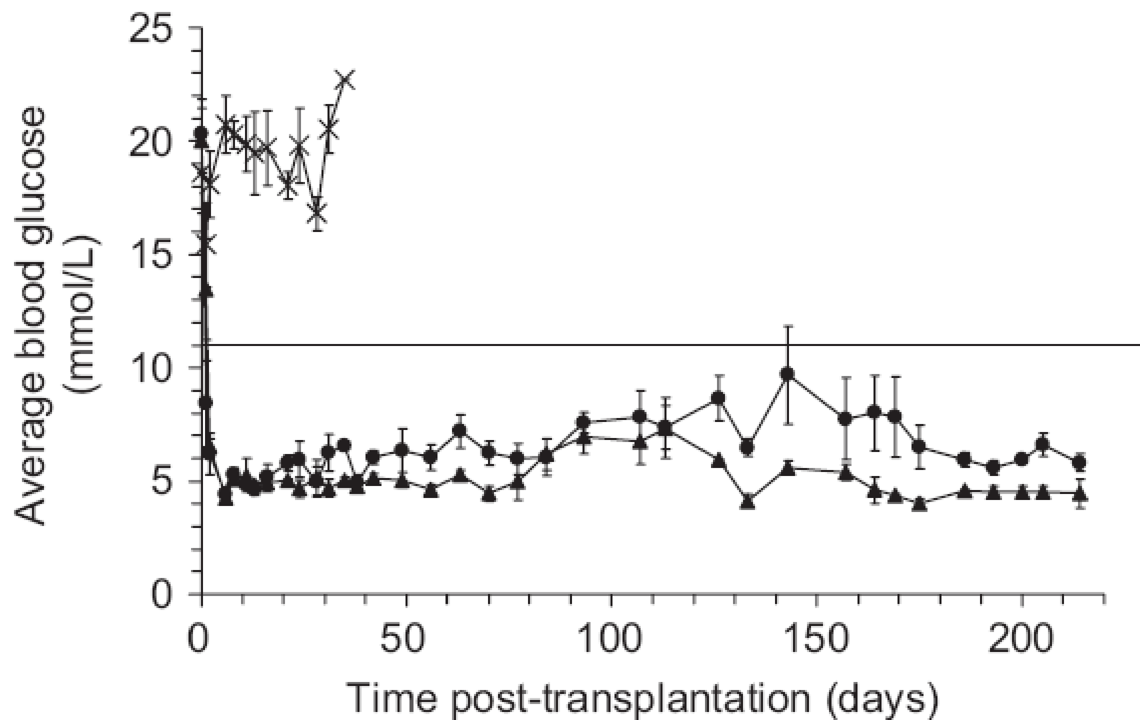

Figure 5. In vivo microencapsulated islet survival and function in covalently cross-linked membranes. Average blood glucose (mmol/L) of diabetic mice transplanted with: cross: nonencapsulated islets (N=3); closed circles: islets encapsulated in standard microcapsules (N=5); triangles: islets encapsulated in microcapsules with covalently cross-liked membranes (N=4).

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Discussion générale (Partie II)

Dans le but de d'améliorer un des paramètres important des microcapsules APA, soit leur solidité et leur intégrité, nous avons :

- Décrit une technique innovatrice dans laquelle nous avons remplacé dans une certaine proportion les liens électrostatiques préexistants dans la membrane semi-perméable des microcapsules par des liens covalents à l'aide de l'agent réticulant photoréactif ANB-NOS.

- Démontré que les microcapsules ainsi réticulées étaient plus résistantes aux stress chimiques.

- Démontré que les microcapsules réticulées étaient 22 fois plus solides que les microcapsules standards suite à un test de résistance mécanique extrême.

- Évalué que la perméabilité des microcapsules standards et réticulées est similaire à l'aide d'une technique de chromatographie à exclusion de taille inversée.

- Prouvé que l'encapsulation de cellules dans des microcapsules réticulées ne semble pas affecter leur survie (évaluation de la viabilité et du taux de nécrose des îlots).

- Démontré que des cellules potentiellement dangereuses pour le receveur peuvent être circonscrites à l'intérieur de microcapsules réticulées (utilisation d'un modèle de cellules malignes encapsulées).

- Confirmé que l'encapsulation d'îlots de Langerhans dans les microcapsules réticulées n'affecte pas leur survie ni leur fonction (expériences de transplantation d'îlots encapsulés chez des souris diabétiques).

L'amélioration de la stabilité des microcapsules a déjà fait l'objet de recherches. Comme mentionné précédemment, il est possible, dans une certaine mesure de faire varier les propriétés mécaniques des microcapsules en jouant avec les conditions utilisées lors de la fabrication de celles-ci. Les paramètres pouvant être explorés sont reliés soit à la PLL,

soit à l'alginate et sont résumés dans le Tableau 1 (page 218) [38]. Entre autre, il est possible d'augmenter la résistance des microcapsules en diminuant le poids moléculaire de la PLL, en augmentant la concentration de PLL ou d'alginate utilisée, en prolongeant la durée de l'incubation des billes d'alginate dans la PLL ou encore en utilisant un cation divalent ayant une plus grande affinité pour l'alginate (tel que le baryum) ou en augmentant la concentration de ce cation divalent [118, 191, 192]. Toutes ces interventions ont, par contre, un effet limité. De plus, la variation ne serait-ce que d'un de ces paramètres influence non seulement la résistance mais également d'autres paramètres des microcapsules tels que leur perméabilité, l'épaisseur de la membrane semi-perméable formée ainsi que le taux de gonflement de la microcapsule qui sont toutes des caractéristiques inter-reliées [38]. Par exemple, l'utilisation d'une PLL de plus faible poids moléculaire pour la formation de la membrane semi-perméable des microcapsules a la possibilité de pénétrer plus profondément à l'intérieur du gel d'alginate, formant ainsi une membrane plus épaisse et augmentant se résistance mécanique mais diminuant du même coup sa perméabilité [119]. Les mêmes phénomènes sont observés quand des concentrations ou des temps d'incubation plus élevés de PLL sont utilisés. D'un autre côté, lorsque l'on change les paramètres reliés à l'alginate, le phénomène est différent. Comme ce sont les blocs G qui sont responsables de la gélification de l'alginate, la teneure en résidus G ainsi que la longueur relative des blocs G peuvent également influencer la résistance des microcapsules formées [113, 118]. Par contre, comme la PLL est connue pour se lier préférentiellement aux résidus M de l'alginate, une alginate riche en résidus M favorisera donc la formation d'une membrane plus stable. Les stratégies ci-mentionnées ont toutefois un effet limité car la nature des interactions entre l'alginate et la PLL demeure une interaction électrostatique qui peut être déstabilisée par les ions ou des molécules/polymères chargés présents dans leur environnement.

INCREASING PARAMETERS	MICROCAPSULE PHYSICAL CHARACTERISTICS			
	Membrane thickness	Mechanical resistance	Permeability	Swelling
PLL molecular weight	↓ (43, 77, 79)	↓ (43)		↑ (79)
PLL concentration	↑ (43, 77, 79)	↑ (43, 77)	↓ (77)	↓ (77, 79)
PLL incubation time	↑ (43, 77, 79)	↑ (43, 77)	↓ (77)	↓ (79)
Alginate concentration	↑ (77, 79)	↑ (43)		↓ (79)
Alginate M composition	↑ (79)			↓ (79)
Cation concentration	↑ (43)	↑ (43)		

Tableau 1 : Influence des paramètres intrinsèques de l'alginate et de la PLL sur les propriétés des microcapsules APA [38].

Comme mentionné précédemment dans l'introduction, lors de la formation de la membrane semi-perméable des microcapsules, à pH physiologique, les billes d'alginate qui ont une charge nette négative attirent les chaines de PLL qui, à cause de leurs résidus amine, possèdent une charge nette positive. En solution, les chaines de PLL vont s'associer avec l'alginate afin de neutraliser leurs charges respectives. La seconde couche d'alginate vient donc neutraliser le restant de charges positives du système. Afin de pouvoir augmenter de façon plus convaincante la stabilité des microcapsules et de diminuer sa possible déstabilisation par les charges environnantes, il est possible d'introduire des liaisons covalentes dans la fabrication des microcapsules. Les liaisons covalentes sont des liaisons fortes où, lorsque deux atomes ayant une électronégativité similaire, partagent un électron de valence afin de diminuer leur état d'énergie. Les techniques traditionnelles de chimie de synthèse pour créer des liens covalents entre un saccharide et une protéine ne peuvent être facilement appliquées dans un contexte biologique car ils font appel à des conditions de réaction souvent incompatibles avec la vie (haute température, pH extrêmes, solvants, parfois production de sous-produits de réaction toxiques, ect.). Par exemple, ceci peut être accompli par la réduction d'une amine libre (contenu par exemple dans la lysine) par les extrémités des chaînes de polysaccharide réductrices. Ces extrémitées réductrices

sont en équilibres avec leur forme ouverte en configuration aldéhyde et cet aldéhyde peut créer une imine, souvent appelé base de Schiff, avec une amine primaire. Cet imine étant ensuite réduit pour former un lien covalent stable entre le polysaccharise et la protéine. Ce type de réaction chimique requiert par exemple de hautes températures et l'utilisation de solvant comme le benzène, tous deux étant négatifs pour la survie cellulaire. Dans le contexte de la formation d'une microcapsule, comme la liaison de la PLL à l'alginate doit s'effectuer alors que la bille d'alginate contenant des cellules est déjà formée, l'utilisation de telles procédures serait néfaste pour la survie et la fonction des cellules encapsulées. Fort heureusement, d'autres stratégies ont été élaborées afin de pouvoir créer des liens covalents dans la structure de la microcapsule sans endommager les cellules à l'intérieur de celles-ci. L'introduction sécuritaire de liens covalents peut se faire de plusieurs façons (voir Figure 12, page 220):

1. À l'intérieur de la bille d'alginate (alginate-alginate)
2. Entre les composantes de la membrane semi-perméable (PLL-PLL)
3. Entre les différentes composantes de la microcapsule (Alginate-PLL-Alginate)

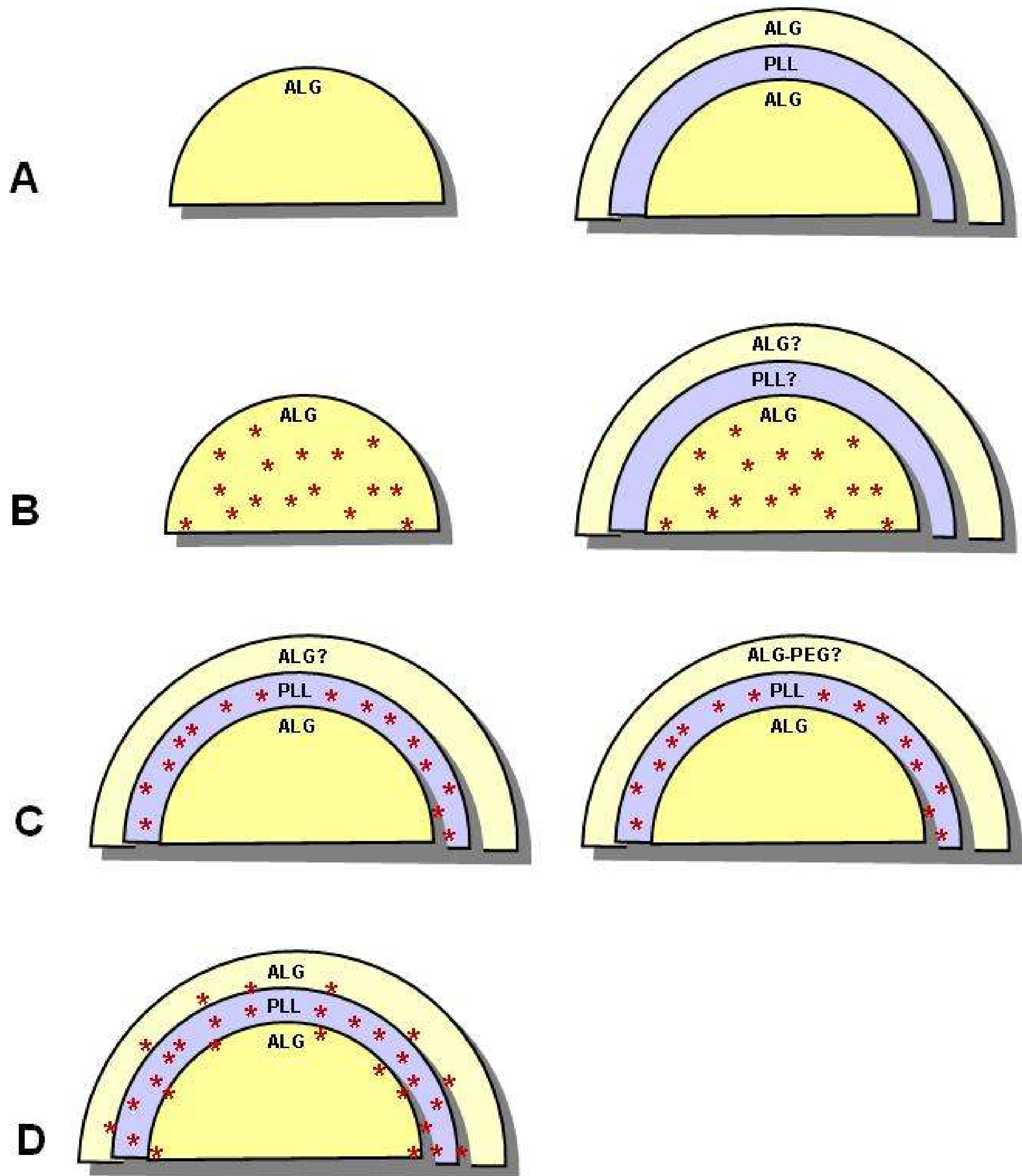

Figure 12 : Modèles de réticulation covalente des microcapsules. A. Bille et microcapsule APA standard sans réticulation covalente. B. Réticulation covalente de la bille d'alginate seulement. C. Réticulation covalente de la membrane semi-

perméable seulement. D. Réticulation covalente entre toutes les composantes de la microcapsule APA.

La formation de liaisons covalentes à l'intérieur même du cœur d'alginate fut proposée pour la première fois, en 1995, par Herztberg *et coll.* [211] (voir Figure 12B, page 220). La stratégie utilisée était simple. Les billes d'alginate sont fabriquées avec un mélange constitué d'alginate et de polyvinyl alcool (PVA). Dans un premier temps, la solution d'alginate est gélifiée de façon standard à l'aide de cations divalent, puis, comme le PVA avait été préalablement modifié avec un groupe photosensible (stilbazolium) [212] celui-ci est polymérisé suite à une exposition à la lumière. Ces billes modifiées ont démontré une meilleure tolérance suite à un test de compression ainsi qu'une plus faible déstabilisation par des hautes concentrations d'ions non gélifiants (Na^+). Plus récemment, deux groupes ont proposé l'utilisation de groupements méthacrylates [190, 213]. Cette fois-ci, c'est l'alginate qui est modifiée par l'introduction de groupement méthacrylates sur environ 5% des groupements hydroxyles des acides mannuroniques (M) composant l'alginate. Une fois les billes d'alginate gélifiées, l'activation de photo-initiateurs permet la dimérisation des groupements méthacrylates formant ainsi des liens covalents entre les chaînes d'alginate. Ces billes d'alginate ont montré une plus grande résistance lors d'incubations prolongées dans des solutions d'ions non gélifiants ou contenant des chélateurs de calcium. Toutes ces études démontrent que l'introduction de liens covalents à l'intérieur des billes d'alginate améliore leur stabilité et est compatible avec la survie des cellules encapsulées. Cependant, lors de ces études, les auteurs n'ont pas discuté de l'utilisation des ces billes modifiées pour la fabrication de microcapsules complètes avec membrane semi-perméable. Afin de restreindre la porosité des microcapsules et ainsi mieux protéger les cellules encapsulées du système immunitaire de l'hôte, on recouvre les billes d'alginate avec un polycation comme la PLL pour former la membrane semi-perméable. Dans ce système, l'utilisation des stratégies décrites ci-haut où les liens covalents se retrouvent à l'intérieur de la bille d'alginate n'ont aucun effet bénéfique sur la stabilité de la

membrane. Le cœur de la microcapsule sera plus résistant mais la stabilité des composantes de la membrane (polycation et seconde couche d'alginate) sera toujours dépendante des conditions ioniques de l'environnement.

La seconde tactique pouvant être utilisée pour produire des microcapsules plus stables est d'introduire les liens covalents à l'intérieur même de la membrane semi-perméable (voir Figure 12C, page 220). Pour ce faire, on doit modifier préalablement le polycation (PLL ou poly(allylamine)) avec un résidu photodimérisable tel que le cinnamate [214-217]. Les microcapsules sont ensuite fabriquées de façon standard avec un polycation modifié dans la membrane semi-perméable. Les microcapsules complètes sont ensuite exposées à un rayonnement lumineux spécifique qui induira la dimérisation des polycations entre eux suite à une cycloaddition [2+2] des résidus cinnamate. Dans ce cas, les liens covalents se retrouvent entre les molécules de PLL. La formation de ces liens covalents dans la membrane s'est avérée efficace pour augmenter la résistance mécanique des microcapsules, en conservant sa perméabilité d'origine (sans liens covalents) tout en étant viable pour les cellules encapsulées. Afin d'aller une étape plus loin et d'améliorer également la biocompatibilité de ces microcapsules, Chang *et coll.* [215] ainsi que Lee *et coll.* [216] ont ensuite enrobé ces microcapsules d'une couche d'alginate préalablement modifiée avec du polyéthylène glycol (PEG), connu pour avoir le pouvoir de repousser les cellules du système immunitaire. Finalement, cette stratégie, comparativement à la précédente, permet d'augmenter la stabilité de la microcapsule par l'entremise de sa membrane semi-perméable. Par contre, la dimérisation des polycations de la membrane n'implique d'aucune façon les molécules d'alginate de la seconde couche qui restent encore une fois en compétition avec les entités ioniques présentes dans leur environnement. Ceci est également vrai pour la couche supplémentaire d'alginate modifiée avec le PEG pouvant améliorer la biocompatibilité des microcapsules.

Le système décrit et proposé dans nos article est donc original et plus complet que tous ceux proposés jusqu'à maintenant afin d'augmenter la stabilité des microcapsules.

L'agent réticulant que nous proposons, l'ANB-NOS, possède un groupement photoréactif connu pour s'insérer, de façon non spécifique, dans des liaisons carbone-carbone ou carbone-hydrogène suite à une exposition lumineuse. Dans un système tridimensionnel comme une microcapsule, les résidus photoréactifs greffés sur le polycation peuvent donc former des liens covalents à la fois avec d'autres molécules de polycations ou encore avec les chaînes d'alginate du cœur de la microcapsule ou de la seconde couche (voir Figure 12D, page 220). Cette possibilité de créer des liens entre toutes les composantes des microcapsules est le système le plus efficace pour conserver l'intégrité de la membrane tout en augmentant la stabilité.

La résistance chimique et mécanique des microcapsules produites par notre méthode ont été évaluées à l'aide de méthodes plus poussées que celles utilisées par les autres groupes. Par exemple, pour l'évaluation de la résistance mécanique, nous avons utilisé un essai simple dans lequel les microcapsules sont incubées et agitées de façon rotative dans des tubes contenant également des billes de verre. Les microcapsules subissent ainsi un stress mécanique intense lorsqu'elles frappent les parois du tube mais également de la part des billes de verre qui s'entrechoquent et écrasent les microcapsules contre la paroi du tube. Avec cet essai, nous avons déterminé que les microcapsules possédant des liaisons covalentes étaient 22 fois plus résistantes que les microcapsules fabriquées de façon standard. Les tests de résistance mécanique utilisés par d'autres groupes sont beaucoup moins contraignants (microcapsules dans des tubes agités de façon orbitale), et les différences détectées sont moins grandes, indiquant que le système que nous avons élaboré pour l'introduction de liens covalents est nettement plus efficace [214, 217]. L'évaluation de la perméabilité des microcapsules constitue un autre exemple. La grande majorité des expériences de perméabilité rapportées par les autres groupes utilisent la quantification de la diminution de protéines dans le surnageant suite à une incubation avec des microcapsules. Cette technique a la faiblesse de ne pas tenir compte d'une possible adsorption des protéines étudiées à la surface des microcapsules au lieu d'une réelle pénétration des protéines étudiées à l'intérieur des microcapsules. L'utilisation de la

chromatographie à exclusion de taille inversée par contre, nous a permis de discriminer les deux phénomènes en fonction des différents profils d'élution obtenus où l'on peut distinguer la perméabilité ou la non-perméabilité d'une protéine ainsi que sa possible adsorption.

L'utilisation de rayonnements UVA afin de créer les liens covalents peut être questionnée quant à sa possible génotoxicité pour les cellules. Il est connu que l'exposition prolongée aux UVA peut causer des dommages à l'ADN, et ce, par l'entremise de dommages oxidatifs. En effet, contrairement aux rayons UVB qui sont directement absorbés par l'ADN et qui endommagent directement l'ADN, les rayons UVA quant à eux peuvent exciter des chromophores endogènes ou exogènes capables ensuite de modifier la structure chimique de l'ADN [218-220]. L'effet du nouveau processus d'encapsulation et de la dose d'UVA utilisée sur la survie et la fonction des îlots ont donc été évalués. Nous avons pu montrer, *in vitro*, que l'utilisation des microcapsules réticulées avec une faible dose d'UVA n'affectait pas la viabilité des îlots, alors qu'une dose 10 fois plus grande faisait chuter la viabilité d'environ 50%. Le taux de nécrose des îlots soumis à une faible dose d'UVA est comparable à celui des îlots encapsulés dans des microcapsules standards sans UVA. Finalement, la fonction des îlots, encapsulés dans des microcapsules standards ou réticulées, a été évaluée par des expériences de transplantations dans un modèle animal diabétique et le taux de normalisation de la glycémie est identique pour les deux types de microcapsules et ce, pour plus de 200 jours. Supporté par toutes ces données, nous pouvons donc conclure que la dose d'UVA choisie n'affecte pas ou très peu la survie et la fonction des îlots encapsulés dans les microcapsules réticulées.

Depuis la publication de ces études, d'autres techniques permettant l'augmentation de la stabilité des microcapsules ont été décrites. Entre autres, afin de créer des liens covalents dans la structure de la microcapsule, certains groupes ont pensé introduire un polyanion dans le système standard APA [221, 222]. Ce polyanion, le poly(methacrylic acid, sodium salt-*co*-2-[methacryloyloxy]ethyl acetoacetate) ou A70, est composé d'acide méthacrylique et d'acétoacétate et a la particularité de réagir fortement avec des résidus

amines comme ceux contenus dans la PLL afin de former des complexes polyélectrolytes liés de façon covalentes. Cette réaction se déroule de façon spontanée lors du contact entre le groupement réactif acétoacétate du A70 et de l'amine de la PLL. Le A70 peut être utilisé de deux façons. Il peut, dans un premier temps, être pré-mélangé à l'alginate liquide avant la gélification [221]. Lorsque les billes d'alginate sont ensuite formées avec le cation divalent, le A70 est alors distribué de façon uniforme à l'intérieur de la bille d'alginate. De cette façon, il est possible de contrôler le niveau de réticulation, c'est-à-dire que si l'on utilise une PLL de faible poids moléculaire, celle-ci pénètrera plus profondément dans le gel d'alginate et formera des liens covalents avec le A70 à l'intérieur même de la microcapsule d'alginate au lieu d'être seulement à la surface, comme le ferait une PLL de plus haut poids moléculaire n'ayant pas la possibilité de diffuser dans le gel d'alginate. Dans un deuxième temps, Shen *et coll.* [222] ont utilisé le polyanion A70 en combinaison avec le C70 (un mélange d'aminoéthyl méthacrylate et de (méthacryloyloxyéthyl)triméthylammonium) dans un système qu'il ont appelé «4-layers microcapsules», lequel système est composé d'un cœur d'alginate, une couche de polycation C70, une couche de polyanion A70, une couche de polycation PLL et une couche finale d'alginate. Le A70 réagit alors avec les groupements amines contenus à la fois sur la PLL et le C70 pour créer des liens covalents à l'intérieur de la membrane. Ces microcapsules modifiées sont effectivement plus résistantes que les microcapsules standards APA, leur perméabilité est légèrement diminuée mais il semblerait que le processus de formation de ces microcapsules soit un peu plus dommageable pour la survie des cellules encapsulées. Par contre, tel que mentionné dans les objections plus haut, ce système ne permet pas de lier de façon covalente la couche périphérique d'alginate. Cependant, les auteurs ont effectué des études sur la biocompatibilité des microcapsules «4-layers» et il ne semble pas y avoir de différence avec les microcapsules standards au niveau de la biocompatibilité *in vivo*.

Les caractéristiques uniques des microcapsules que nous avons produites nous ont amené à vérifier une nouvelle application potentielle pour des microcapsules liées de façon covalente, soit la protection de receveur contre les cellules encapsulées.

L'utilisation des cellules souches, de précurseurs de cellules β, de cellules immortalisées ou modifiées génétiquement comme source alternative de cellules pouvant sécréter de l'insuline est une option intéressante dans la mesure où l'on peut empêcher ces cellules potentiellement dangereuses de s'échapper des microcapsules et de migrer et proliférer chez le receveur. Il est clair que l'utilisation de l'encapsulation cellulaire afin de confiner ces cellules potentiellement malignes à l'intérieur des microcapsules pourrait permettre une protection additionnelle du receveur. C'est ce que nous avons tenté de démontrer dans notre second article en encapsulant des cellules déjà malignes ayant un fort potentiel prolifératif et en tentant de vérifier si elles peuvent s'échapper de la microcapsule réticulée et proliférer chez le receveur. Nos résultats montrent que les cellules malignes ne peuvent s'échapper des microcapsules réticulées, du moins en quantités insuffisantes pour provoquer le développement de tumeurs, puisqu'on ne détecte aucune infiltration cellulaire chez les animaux implantés et ce pour une longue période (plus de 150 jours). De plus, la transplantation d'îlots encapsulés dans les souris diabétiques démontre que le système d'encapsulation avec les microcapsules réticulées est biocompatible pour les cellules encapsulées et permet d'atteindre la normalisation de la glycémie. Il est à noter que les cellules utilisé dans l'étude sont reconnues pour être extrêmement malignes et que le nombre de cellules utilisées représente une condition extrême qui a peu de chance de se produire spontanément lors des transplantations. Comme l'étude le démontre, les microcapsules réussissent avec succès à contenir ces cellules malignes ce qui nous permet de croire qu'elles seront également aptes à retenir des cellules malignes moins agressives et en moins grand nombre.

Mais qu'advient-il des cellules malignes encapsulées après 150 jours *in vivo*? Malheureusement, lors de la planification de l'étude sur les cellules malignes, les

microcapsules implantées n'ont pas été récupérées. Par contre, nous pouvons émettre quelques hypothèses sur le devenir de ces cellules. Tout d'abord, comme ces microcapsules réticulées ont été implantées dans des souris B6.SJL, qui sont immunocompétentes, et que l'alginate utilisée à l'époque n'était pas totalement biocompatible, on peut croire qu'une RHM ait pu se développer autour du greffon. Ceci renforce alors les données obtenues puisque même si le transplant induit une réaction immune, les cellules EL-4 ne s'échappent tout de même pas des microcapsules réticulées, soutenant l'hypothèse selon laquelle les microcapsules réticulées sont extrêmement résistantes *in vivo*. Par contre, s'il y a effectivement développement d'une RHM, nous ne pouvons exclure le fait que les cellules malignes implantées sont peut-être mortes, à long terme, suite à un manque de nutriments et d'oxygène.

Il est certain que notre intérêt premier face à l'encapsulation est son application dans le diabète de type I. Cependant, ce qui augmente la valeur de ces études est que les microcapsules réticulées qui possèdent une stabilité plus grande que les microcapsules standards APA peuvent être facilement utilisées dans des applications autres que le diabète, pour lesquelles le confinement des cellules à l'intérieur de la microcapsule est important, ce qui est le cas, par exemple, dans le traitement de tumeurs cérébrales [223]. Afin de traiter efficacement ces types de tumeurs, il est important que l'agent thérapeutique utilisé agisse de façon locale et ce, pour une période de temps contrôlée [224]. Une des méthodes utilisée pour rencontrer ces deux exigences est l'encapsulation et l'implantation de cellules modifiées génétiquement afin de produire et de sécréter l'agent thérapeutique voulu [223-225]. L'encapsulation de ces cellules offre la possibilité de pouvoir les enlever du site d'implantation une fois le traitement terminé. L'un des problèmes rencontrés lors de l'utilisation de cette stratégie est que certaines cellules peuvent s'échapper des microcapsules et s'infiltrer au niveau du cerveau. L'utilisation des microcapsules réticulées, dans ce contexte, pourrait donc confiner les cellules sécrétrices à l'intérieur des microcapsules et minimiser les dangers pour le receveur.

Les microcapsules réticulées de façon covalente pourraient également bénéficier aux chercheurs utilisant l'encapsulation cellulaire à l'intérieur de bioréacteurs comme par exemple lors de l'encapsulation d'enzymes, comme l'α-acétolactate décarboxylase, pour la fermentation de la bière [226] ou de levures et de bactéries dans le cas des vins mousseux [227]. Dans ce cas particulier, l'encapsulation est utilisée car elle facilite la séparation des levures/bactéries/enzymes encapsulées du produit de fermentation. La résistance des microcapsules utilisées dans ces bioréacteurs est importante car il n'est pas souhaitable que le produit obtenu soit contaminé par l'introduction d'une trop grande quantité de levures/bactéries/enzymes car cela diminue la qualité du produit obtenu.

D'un point de vue purement technique, l'agent réticulant photoréactif choisi, l'ANB-NOS, pour la formation des liens covalents présente également des avantages. Premièrement, le groupement réactif N-hydroxysuccinimide ester de l'ANB-NOS que l'on greffe dans un premier temps aux molécules de PLL réagit de façon spécifique avec les groupements amine primaire permettant à l'expérimentateur de pouvoir également utiliser d'autre molécules possédant un résidu amine primaire au lieu de la PLL, comme la poly-L-ornithine (PLO), l'utilisation de la PLO étant favorisée par certains groupes. Deuxièmement, le groupement photoréactif nitrophényl azide de l'ANB-NOS, quant à lui, réagit de façon non spécifique en s'insérant dans les liens C-H ou C-C. C'est cette non spécificité qui nous permet de créer des liens covalents entre toutes les composantes de la membrane semi-perméable des microcapsules et c'est également elle qui pourrait aussi permettre de créer des liens covalents avec nombreuses molécules, contenant des liens C-C ou C-H. Pourvu qu'il y ait d'abord une interaction électrostatique pour amener ces molécules près de la surface des microcapsules celles-ci pourraient servir à l'enrobage des microcapsules. Cette application unique des microcapsules réticulées avec l'ANB-NOS pourrait faire l'objet d'une étude ultérieure qui aurait pour but d'améliorer la biocompatibilité *in vivo* des microcapsules en utilisant la non-spécificité de l'ANB-NOS à notre avantage. Il serait maintenant plus facile d'utiliser les percées faites au niveau de la

biocompatibilité des polymères et de les utiliser pour recouvrir la microcapsule APA. Des polymères comme le polyéthylène glycol (PEG) et la phosphorylcholine (PC) pourraient être facilement greffés à la surface des microcapsules. Le PEG est, entre autres, connu pour son pouvoir à diminuer l'adsorption de protéines et l'adhésion de cellules immunes à la surface de biomatériaux [228]. Il aurait également la propriété de diminuer l'adhésion plaquettaire, réduisant du même coup les risques de thrombose. Les longues chaînes inertes de PEG, pouvant parfois même être ramifiées, sont responsables leur comportement anti-immunogène. Les chaînes de PEG ont un effet d'encombrement stérique, empêchant l'accès aux protéines et aux cellules à la surface des implants [228-230]. La phosphorylcholine, quant à elle, est un phospholipide, principal composant des membranes cellulaires. Comme ce phospholipide a la propriété d'être électriquement neutre, il ne favorise pas l'adhésion des plaquettes sanguine ni des protéines à la surface des biomatériaux sur lesquels il a été greffé [231]. En fait, la PC attachée à une surface imite en quelque sorte les membranes bilipidiques et crée une barrière thermodynamique hautement hydratée à cause de la propriété très hydrophile de la PC [232, 233]. Le PEG et la PC ne sont que deux exemples de molécules candidates pouvant être utilisées dans le recouvrement de la surface des microcapsules APA afin d'en améliorer leur biocompatibilité *in vivo*.

Finalement, nous avons réussi à produire des microcapsules stables et nous avons démontré qu'elles étaient assez résistantes pour empêcher des cellules malignes de s'échapper de la microcapsule, mais qu'en est-il des virus? Dans une étude subséquente, il serait fort intéressant d'étudier la capacité des microcapsules à empêcher les virus ou les particules virales de s'échapper d'une microcapsule réticulée. Cette étude revêt une grande importance, particulièrement dans le domaine de la xénotransplantation. En effet, une autre alternative proposée au manque de donneurs d'îlots humains, autre que l'utilisation des cellules humaine modifiées ou cellules souches, est la transplantation d'îlots d'origine animale, entre autre les îlots de porcs [33, 234-236] ou encore de poisson [237-240]. Malheureusement, l'utilisation des îlots d'origine animale rencontre de problèmes éthiques

en plus de comporter plusieurs risques, dont la transmission possible de virus d'origine animal vers l'humain. Il est vrais que de façon générale, les virus sont espèce-dépendants et ne se transmettent que très peu d'une espèce à l'autre. Cependant, ces virus peuvent subir des mutations ou des recombinaisons génétiques permettant des contaminations croisées entre les espèces et rapidement causer des pandémies [241]. Le virus de la grippe porcine A(H1N1) (transmission porc-humain) qui fait les manchettes mondiales depuis quelques mois, celui de la grippe aviaire (transmission volaille-humain) et le virus de la panleukopénie (transmission félin-canin) sont de bon exemples de contaminations croisées. Le porc est un cas un peu particulier puisqu'il possède des rétrovirus endogènes (PERVs). Ces PERVs sont pour la plupart inactifs mais il a été démontré *in vitro* que ces rétrovirus pouvaient infecter des cellules humaines [242, 243]. La transmission du virus suite à la transplantation d'un organe porcin n'a pas encore été prouvée [244]. Il est difficile de prédire la performance des microcapsules réticulées à retenir des virus ou des particules virales puisque ceux-ci sont beaucoup plus petits qu'une cellule et l'on ne sait pas si elles peuvent facilement diffuser dans un gel d'alginate et traverser la membrane semi-perméable. Par contre, la solidité des microcapsules réticulées diminue les risques associés à une relâche de virus suite à un bris des microcapsules. La démonstration qu'une microcapsule réticulée puisse réduire ou empêcher les risques de transmission de virus d'origine animale pourrait permettre une utilisation plus sécuritaire de la transplantation de cellules animales chez l'humain.

Conclusion

Il y a maintenant plus de 40 ans, le concept d'encapsulation cellulaire voyait le jour. L'enthousiasme face à ce développement technologique était énorme puisque l'on comprenait que cette technique allait trouver application dans une foule de domaines. L'utilisation de la microencapsulation à des fins d'immuno-isolation lors de la transplantation d'îlots de Langerhans était une idée ingénieuse et faisait appel à un concept simple qui semblait facile à réaliser. Beaucoup d'années se sont écoulées depuis et, mise à part quelques essais cliniques marginaux, nous sommes encore assez loin de la coupe aux lèvres. Les scientifiques du domaine réalisent maintenant que les défis pour arriver à une application chez l'humain ont été grandement sous-estimés.

Cette thèse a le mérite de répondre en partie à certains de ces défis, et l'un de ces défis est la biocompatibilité des microcapsules, plus spécifiquement la biocompatibilité de l'alginate qui compose la microcapsule ainsi que la biocompatibilité du greffon avec le receveur. En effet, dans cette thèse nous avons, dans un premier temps, essayé de mieux comprendre les phénomènes d'initiation de la RHM en fonction de la teneure de l'alginate en différents contaminants. Sur cet aspect, nous avons fait le constat que les processus de purification de l'alginate n'ont pas tous la même efficacité de débarrasser l'alginate de ses principaux contaminants, nous avons mis en lumière le fait que les contaminants protéiques de l'alginate jouent un rôle important dans la RHM et finalement nous avons amélioré le processus de purification d'alginate afin de réduire significativement les traces de contaminants protéiques dans l'alginate.

Dans un deuxième temps, nous avons tenté d'améliorer la stabilité des microcapsules afin de mieux protéger le receveur du greffon et ainsi augmenter la biocompatibilité du greffon face au receveur. Sur cet aspect, nous avons créé un nouveau type de microcapsules dans lequel se trouvent des liaisons covalentes qui augmentent drastiquement leur stabilité et nous avons également démontré que ces nouvelles

microcapsules avaient le pouvoir de contenir des cellules potentiellement malignes et ainsi offrir une protection supplémentaire au receveur.

Ceci résume en quelque sorte l'apport de cette thèse au domaine de l'encapsulation et de la transplantation d'îlots de Langerhans microencapsulés. Afin de s'approcher encore plus près de l'application clinique, il faut aller au-delà de ce projet de recherche, exploiter les découvertes qui y sont présentées et poser de nouvelles questions de recherche. Dans cette thèse nous avons réussis à obtenir de l'alginate ayant un degré de pureté élevé nous permettant de fabriquer des billes d'alginate biocompatibles. Par contre, les microcapsules complètes, recouvertes de PLL et d'alginate diluée, présentent toujours un problème de biocomaptibilité. Deux avenues peuvent être explorées.

Première avenue : Peut-on utiliser simplement des billes d'alginate, sans recouvrement, à des fins de transplantation quand on sait que premièrement, les billes d'alginate ont une perméabilité plus grande que les microcapsules complètes et laisser ainsi entrer plus facilement des molécules toxiques de plus haut poids moléculaires, comme des cytokines et que deuxièmement, les billes d'alginates sont plus fragiles que les microcapsules complètes pouvant donc se détériorer *in vivo* et laisser s'échapper les cellules encapsulées?

Deuxième avenue : Est-il possible d'améliorer la biocompatibilité des microcapsules complètes? C'est actuellement l'avenue qu'emprunte le laboratoire du Dr Jean-Pierre Hallé, c'est-à-dire comprendre quels sont les facteurs physico-chimiques de la surface des microcapsules (charge, morphologie, composition, hydrophilicité) qui font en sorte que ces microcapsules engendrent une réaction immune? Également, quels sont les protéines du receveur qui vont interagir avec cette surface afin d'induire la réaction immune? Peut-on modifier les propriétés de la surface des microcapsules afin de changer le profil des protéines qui adhéreront à la surface et diminuer la réaction immunitaire? C'est à ce stade que les microcapsules réticulées présentées dans cette thèse pourraient être utilisées puisqu'elles nous permettent de modifier le type de polymère exposé à la surface et donc de varier les propriétés de surface des microcapsules de façon illimitée.

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Annexe I

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A) Déclaration des coauteurs d'un article
Lorsqu'un étudiant n'est pas le seul auteur d'un article qu'il veut inclure dans son mémoire ou dans sa thèse, il doit obtenir l'accord de tous les coauteurs à cet effet et joindre la déclaration signée à l'article en question. Une déclaration distincte doit accompagner chacun des articles inclus dans le mémoire ou la thèse.

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Nom de l'étudiant :
Julie Dusseault
Sigle et titre du programme, en indiquant l'option s'il y a lieu :
No Programme : 348410, Sciences biomédicales

2. Description de l'article:
Dusseault J, Tam SK, Ménard M, Polizu S, Jourdan G, Yahia L'H, Hallé JP. *Evaluation of alginate purification methods: effect on polyphenol, endotoxin and protein contamination.* **J Biomed Mat Res A (1 Feb 2006)**, 76(2): 243-51.

3. Déclaration de tous les coauteurs autres que l'étudiant
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Susan K Tam
Coauteur

Martin Ménard
Coauteur

Stéfania Polizu
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Dusseault J, Tam SK, Ménard M, Langlois G, Bilodeau S, Polizu S., Yahia LH, Hallé JP. *In vivo behaviour of microcapsules using alginate of varying composition.* En préparation et sera soumis à la revue Artificial Organs.

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Coauteur

Robert Robitaille
Coauteur

Guillaume Jourdan
Coauteur

Josée Tessier
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Martin Ménard
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Nathalie Henley
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Curriculum Vitae du candidat

JULIE DUSSEAULT

ACADEMIC TRAINING

Jan 2003-Aug 2009 **Ph.D. Biomedical Sciences**
Université de Montréal (Director : Jean-Pierre Hallé, Co-director : L'Hocine Yahia)
Project title: Alginate microcapsule biocompatibility: alginate purification, host reaction, immuno-camouflage and host protection.

Career Interruptions:
Maternity leaves from Jan 2005 to Aug 2005 and from Jun 2006 to Dec 2006, for a total of 14 months.

Sept 2000-Aug 2002 **M.Sc. Biomedical Sciences**
Université de Montréal (Director : Jean-Pierre Hallé)
Project title: Bioartificial endocrine pancreas: Development and evaluation of methods for the improvement of alginate-poly-l-lysine-alginate microcapsule resistance.

Sept 1997-Aug 2000 **B.Sc. Biochemistry**
Université de Montréal
Maisonneuve-Rosemont Hospital Research Centre **Summer Trainee**
Project title: The effect of Ramipril, an angiotensin conversion enzyme inhibitor, on the expression of the «connective tissue growth factor» (CTGF) in diabetic nephropathy.

TRAINNING GRANT

- **2006 / 2009 - Doctoral training grant, Fonds de la Recherche en Santé du Québec (FRSQ)**
- **2002 – Summer trainee grant, Association Diabète Québec**
- **2001 – Summer trainee grant, Association Diabète Québec**
- **1999 – Summer trainee grant, Hoechst Marion Roussel**

PROFESSIONNAL EXPERIENCE

Oct 2000-Apr 2003 **Clinical Research Associate (part-time)**
Diabetes Clinical Research Department
Maisonneuve-Rosemont Hospital
Clinical studies: Exubera (Pfizer), Dream/Epidream (Aventis Pharmaceuticals), HOE901 (Aventis Pharmaceuticals), AVD105248/AVD105720 (GlaxoSmithKline), CV138-062 (BristolMyers Squibb).

- In charge of the communications between pharmaceutical companies and the internal ethics committee (clinical studies start-up, informed consent approval, adverse events report)

- Patient data acquisition (blood pressure, weight, glucose challenge, quality of life questionnaire, electrocardiogramme)
- Clinical research form filling
- Recruitment
- Patient teaching (insulin injection, glycemia testing)

HONORS AND DISTINCTIONS

- **Feb 2009 – CIHR travel award for the 8th World Biomaterial Congress (Amsterdam, The Netherlands)**
- **Sept 2008 – BRG travel award for the Annual Meeting of the Bioencapsulation Research Group (Vienna, Austria)**
- **Apr 2007 – Best poster presentation award, Journée de la Recherche, Maisonneuve-Rosemont Hospital**
- **2002 – Best poster presentation award, Journée de la Recherche, Maisonneuve-Rosemont Hospital**
- **2001 – Best oral presentation award, Association Diabète Québec, Annual Congress**

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