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# Ingénierie tissulaire en chirurgie colorectale : du défaut pariétal au remplacement d'organe : étude in vitro et in vivo

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**THESE**

pour le

**DOCTORAT DE L'UNIVERSITE BORDEAUX**

**Ecole doctorale des Sciences de la Vie  
Mention : Sciences, Technologie, Santé  
Option : Biologie Cellulaire et Physiopathologie**

Présentée et soutenue publiquement le 24 octobre 2014

par **Mr Quentin DENOST**

Né le 9 juin 1980 à Angoulême

**INGENIERIE TISSULAIRE**

**EN CHIRURGIE COLORECTALE :**

**DU DEFECT PARIETAL AU REMPLACEMENT D'ORGANE**

*Etude in vitro et in vivo*

**Membres du jury**

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## **Résumé**

Ingénierie tissulaire colorectale : vers la régénération complète de la paroi

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L'ingénierie tissulaire représente un nouvel outil en chirurgie colorectale pour la prévention et le traitement des fistules et pour la substitution sphinctérienne et rectale après chirurgie d'exérèse. Ce travail comprend :

1) La sélection de la composante matricielle : *in vivo*, en remplacement d'un défaut de paroi colique, 2 matrices ont été comparées chez 16 lapins : groupe A, matrice de sous-muqueuse intestinale de porc décellularisée (SIS, BioDesign®), référente; groupe B, matrice de chitosane, innovante. Les animaux étaient sacrifiés à 4 et 8 semaines. A 8 semaines, une régénération épithéliale plus précoce, un meilleur contrôle de la réponse inflammatoire avec un rapport de fibrose plus faible et l'obtention de quelques îlots de cellules musculaires lisses sont obtenus dans le groupe B.

2) La confection d'un gel de délivrance cellulaire: *in vitro*, fibrine et chitosane ont été combinés selon différentes formulations pour sélectionner un gel composite fibrine-chitosane, caractérisé par des tests mécaniques, de viabilité et de prolifération cellulaires et l'étude de son ultrastructure.

3) Leur combinaison : *in vivo*, en remplacement d'un défaut de paroi colique, nous avons comparé chez 20 porcs la matrice acellulaire de chitosane à la matrice cellularisée par la fraction stromale vasculaire autologue, isolée et contenue dans le gel composite fibrine-chitosane. A 8 semaines, un recouvrement muqueux complet de la zone implantée était observé dans les 2 groupes avec une régénération *ad integrum* de la paroi colique, y compris des cellules musculaires lisses confirmées par immunohistochimie, et un rapport de fibrose significativement plus faible dans le groupe cellularisé (15% vs. 50%, p=0,01). Enfin, une matrice de chitosane circonférentielle cellularisée a remplacé un défaut colique de 2 cm de longueur chez 3 porcs avec succès.

Avec les critères de jugement tels que faisabilité technique, comportement matriciel et qualité de la régénération tissulaire, le chitosane présente un intérêt majeur pour la régénération tissulaire colorectale.

**Mots clés** : ingénierie tissulaire, colon, rectum, tissu colorectal, chitosane, fraction stromale vasculaire, cellules mesenchymateuses

## **Summary**

Colorectal tissue engineering: towards the comprehensive regeneration of the wall

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Tissue engineering is a new tool in colorectal surgery for the prevention and treatment of fistula and rectal sphincter substitution after surgery for resection. This work includes:

1) The selection of the matrix component : *in vivo*, 2 matrices were compared in 16 rabbits: Group A, matrix of decellularized swine intestinal submucosa (SIS, BioDesign ®), or reference matrix; Group B, a three layers matrix of Chitosan hydrogel, or new matrix. The animals were sacrificed at 4 and 8 weeks. At 8 weeks, earlier epithelial regeneration, better control of the inflammatory response with a lower fibrosis report and obtaining of some islets of smooth muscle cells are obtained in the B group.

2) The conception of an optimal delivering cells system: *in vitro*, fibrin and Chitosan were combined according to different formulations for Select a composite gel fibrin-Chitosan, characterized by mechanical tests, viability and cellular proliferation and the study of its ultrastructure.

3) Their combination: *vivo*, in lieu of a colonic wall defect, we compared, in 20 pigs, acellular Chitosan matrix to Chitosan matrix cellularized by autologous stromal vascular fraction isolated and contained in the gel composite Chitosan-fibrin. At 8 weeks, a full mucosal recovery was observed in the 2 groups with recovery *ad integrum* of the colonic walls, including smooth muscle cells confirmed by immunohistochemistry. The fibrosis ratio was significantly lower in the cellularized group (15% vs. 50%,  $p = 0,01$ ). Finally, a cellularized matrix of circumferential Chitosan has successfully implanted to replace a colonic defect of 2 cm in length in 3 pigs success. With end points such as technical feasibility, matrix behavior and quality of tissue regeneration, Chitosan has a major interest for the Colorectal tissue regeneration.

**Key words** : tissue engineering, colon, rectum, colorectal tissue, chitosan, stromal vascular fraction, stem cells

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# Liste des abréviations

AM : Acétoxyméthyl calcéine  
BMSCs : Bone marrow mesenchymal stem cells  
CE : Communauté européenne  
CML : Cellules musculaires lisses  
DA : Degré d'acétylation  
DMEM : Dulbecco's modified eagle medium  
DMSO : Diméthyl Sulfoxide  
DTT : Dithiothreitol  
EDTA: Ethylène Diamine Tétra Acétique  
ELB: Erythrosis lysis buffer  
EthD-1: Homodimer-1 d'éthidium  
hADSCs : Human adipose-derived stem cells  
HBSS : Hank's balanced salt solution  
HES : Hématoxyline-érythrosine-safran  
HMDS: Hexamethyldisilazane  
MM: Masse moléculaire  
MEB: Microscope électronique à balayage  
MTT : Méthyle thiazole tetrazolium  
pADSCs : Porcine adipose-derived stem cells  
PBS : Phosphate buffered saline  
ppm : partie par million  
RMN: Résonance magnétique nucléaire  
SIS : Small intestine submucosa  
SVF: Stromal vascular fraction

# PARTIE I

ETAT DE L'ART

&

OBJECTIFS

# INTRODUCTION GÉNÉRALE



Le génie tissulaire ou ingénierie tissulaire a été défini à la fin des années 80 comme l'application des principes et méthodes d'ingénierie et de sciences de la vie orientée vers le développement de substituts biologiques qui restaurent, maintiennent et améliorent la fonction tissulaire (Vacanti 1988) (Langer 1993). Les principes de l'ingénierie tissulaire reposent sur trois composantes principales i) un matériau naturel à partir duquel s'organise la matrice extracellulaire (scaffold) qui sera dégradé au fur et à mesure de la formation du néo-tissu; ii) des cellules, différenciées ou non, isolées etensemencées sur la matrice in vitro ; iii) des signaux hormonaux et mécaniques in vivo qui orientent le comportement de la matrice et des cellules. Quatre étapes fondamentales sont rapportées dans la genèse du néo-tissu (Rabkin 2002) : 1) Prolifération et différenciation cellulaires ; 2) Production et organisation de la matrice extracellulaire ; 3) Dégradation du biomatériau ; 4) Remodelage et croissance du néo-tissu. L'idée est de transplanter un organe « simple » autologue auquel le corps humain apporte des performances physiologiques additionnelles lorsqu'il l'assimile. C'est sur cette approche que repose notre travail avec l'idée que les cellules souches mésenchymateuses adultes, de par leur pluripotence<sup>4</sup> et leur effet paracrine (Psaltis 2008), vont pouvoir orienter le remodelage tissulaire, épithélial et musculaire, selon l'environnement dans lequel elles seront implantées (de Villiers 2009) (Baer 2011), l'hôte constituant le bioréacteur « idéal ». La finalité de notre travail de recherche est à terme l'élaboration d'un substitut rectal *ad integrum* par ingénierie tissulaire, les étapes présentées dans ce travail de thèse d'université sont des préambules indispensables qui permettront d'ouvrir sur cette perspective de remplacement d'organe.

L'ingénierie tissulaire peut en outre constituer un nouvel outil en chirurgie colorectale pour l'amélioration des processus de cicatrisation. Dans le cadre de ce projet à long terme de remplacement d'organe, chaque étape de la recherche a été pensée pour apporter une réponse à des besoins cliniques tels que le traitement et la prévention des fistules anastomotiques, pelviennes et périnéales ou le comblement de défaut pariétaux après exérèse locale d'une tumeur rectale.

Ce manuscrit se présente en 4 parties:

- La première partie, bibliographique, expose les notions importantes sur la paroi colorectale, l'état de l'art de l'ingénierie tissulaire colorectale (*Article Annexe 1*) et des informations sur le

biomatériau support que nous avons choisi d'exploiter : le chitosane. Enfin, cette première partie est ponctuée par la présentation des objectifs de ce travail de thèse.

- La deuxième partie présente les résultats sous forme d'articles issus des travaux réalisés au cours de ces trois années de doctorat (*Annexes 2, 3 et 4*). Chaque article est précédé d'un commentaire.

- Enfin la quatrième et dernière partie constitue une conclusion sur les résultats obtenus et ouvre sur les perspectives de ce travail.

# REVUE BIBLIOGRAPHIQUE

Article correspondant

Annexe 1

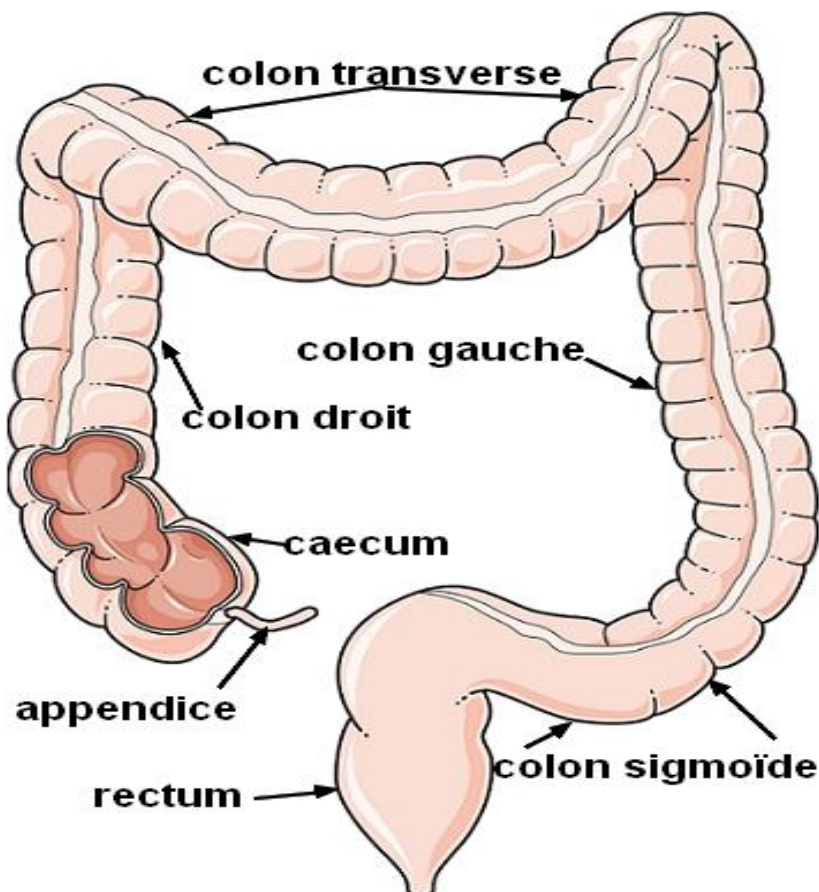
## 1- Le tissu colorectal

Ce tissu creux organisé en plusieurs couches est revêtu par une muqueuse qui le sépare du milieu extérieur (lumière intestinale) et par laquelle il est en contact avec les matières fécales. Il possède une structure histologique complexe dominée par les phénomènes de perte cellulaire et de renouvellement dans des structures hautement spécialisées.

### 1.1- Histologie du tissu colorectal

#### 1.1.2-Architecture macroscopique et histophysiologie colique

Le colon mesure entre 1 m et 1,5 m de long pour une surface de  $0,3\text{m}^2$  et est, sauf pour son extrémité distale, entièrement intrapéritonéal. Le rectum constitue sur 8 à 15 cm la portion extrapéritonéale du colon (Figure 1).



**Figure 1. Anatomie générale du colon et du rectum**

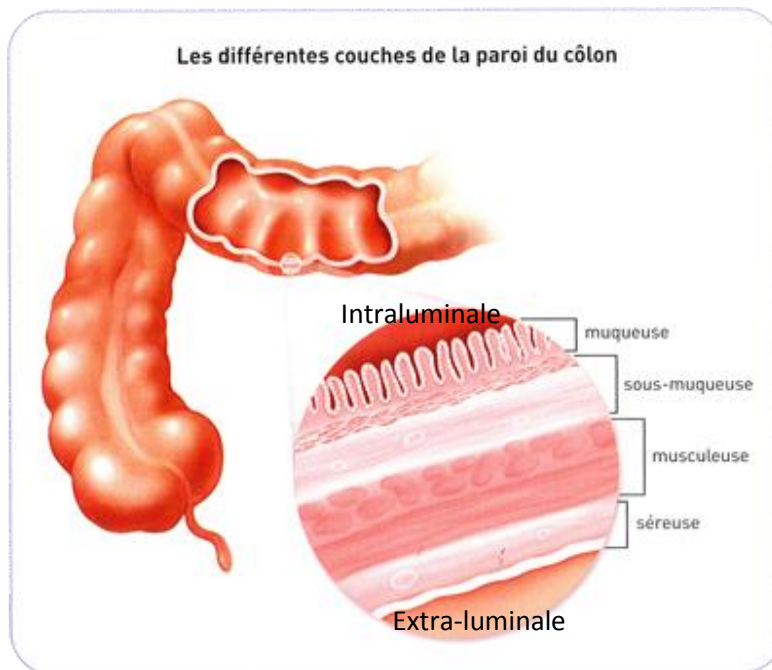
Le colon est fixé à la paroi abdominale postérieure par des plis péritonéaux et par des fascias. A travers la séreuse et la sous-séreuse qui constituent les couches externes de la paroi colique, les couches musculaires longitudinales de la tunique musculaire externe forment des

bandelettes distinctes, trois à droite et deux à gauche (par confluence de deux bandelettes). La vascularisation est assurée par des branches de l'artère mésentérique supérieure du caecum à la coudure hépatique, et par des branches de l'artère mésentérique inférieure pour le reste. Le rectum est en outre partiellement vascularisé par des branches des artères iliaques internes. Le retour veineux est assuré par le système porte, ainsi que par le plexus hémorroïdal pour certaines portions du rectum. Le drainage lymphatique se fait vers les ganglions épicoliques à la racine d'insertion de la séreuse, puis vers les ganglions paracoliques le long de l'arcade artérielle, pour aboutir dans les ganglions des groupes intermédiaires et principaux près des artères coliques. Les lymphatiques du rectum sont drainés par des ganglions hypogastriques et de l'antra mésentérique. Le colon est innervé par des branches parasymphatiques du nerf vague et par les nerfs sympathiques venant des ganglions nerveux mésentériques supérieurs, mésentériques inférieurs et pelviens. Les variations anatomiques selon les individus sont fréquentes, dues à des variations dans le développement. Le colon présente quatre fonctions principales :

- Fonction de motricité : stockage et brassage des matières grâce à des mouvements de contraction segmentaire et propulsion des matières vers le rectum par des mouvements longitudinaux
- Fonction d'absorption : résorption d'eau au niveau des entérocytes du colon droit
- Fonction de sécrétion : mucus des cellules caliciformes qui protège la muqueuse et facilite la progression des selles
- Fonction de digestion : assurée par la flore bactérienne

#### 1.1.3-Architecture microscopique :

Contrairement à l'épithélium de l'intestin grêle qui est organisé en deux compartiments fonctionnellement distincts, les cryptes de Lieberkühn qui s'invaginent dans le chorion de la sous-muqueuse, et les villosités intestinales, qui forment des digitations dans la lumière intestinale, le colon ne possède pas de villosités, mais un épithélium de revêtement où s'abouchent les cryptes. Pour le reste, la muqueuse colique présente le même degré de complexité que celle de l'iléon, et sa paroi comporte les mêmes couches tissulaires successives. L'épaisseur de la paroi colorectale varie de  $1\text{mm} \pm 0,3$  au niveau du colon à  $2,1\text{mm} \pm 0,5$  au niveau du rectum et dépend de l'âge et du poids (Nylund 2012). La paroi est constituée de 4 couches principales (Figure 2) :



**Figure 2. Schéma de la paroi colique**

-La muqueuse colique

La muqueuse colique (figure 3) est composée d'une couche épithéliale unique de cellules cylindriques hautes bordant la lumière et s'invaginant pour former les cryptes, dans un chorion ou lamina propria. L'ensemble est sous-tendu par une fine couche musculaire lisse, la musculaire muqueuse ou muscularis mucosae. Les cryptes s'ouvrent dans la lumière épithéliale le plus souvent par un abouchement unique, cependant un aspect ramifié peut parfois s'observer à l'état normal. Le tout est sous-tendu par une membrane basale qui est perméable aux ions, à l'eau et aux protéines, ainsi qu'aux mouvements de lymphocytes à partir de ou vers l'épithélium de surface. L'architecture en cryptes se retrouve tout au long du colon, sauf lorsqu'elle est interrompue par des nids de lymphocytes, ou dans la zone de transition de la valvule iléo-caecale, ou encore vers l'épithélium malpighien anal. L'épithélium qui revêt la muqueuse à la surface et le long des cryptes est complexe :

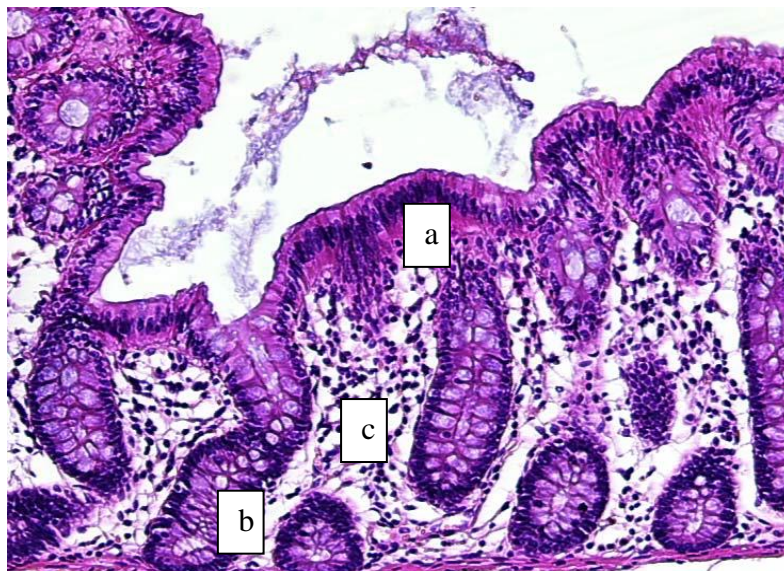
- Epithélium de surface :

Il est composé de cellules absorbantes, ou entérocytes, les plus nombreuses, et de cellules caliciformes. Les entérocytes assurent le transport de l'eau et des ions. Les cellules caliciformes, ou cellules en gobelet, ont un cytoplasme rempli au pôle apical de granules constitués par des glycoprotéines, des sialomucines et des mucopolysaccharides sulfatés qui

protègent la muqueuse colique. On trouve entre les cellules épithéliales des lymphocytes, quelques éosinophiles et des corps apoptiques.

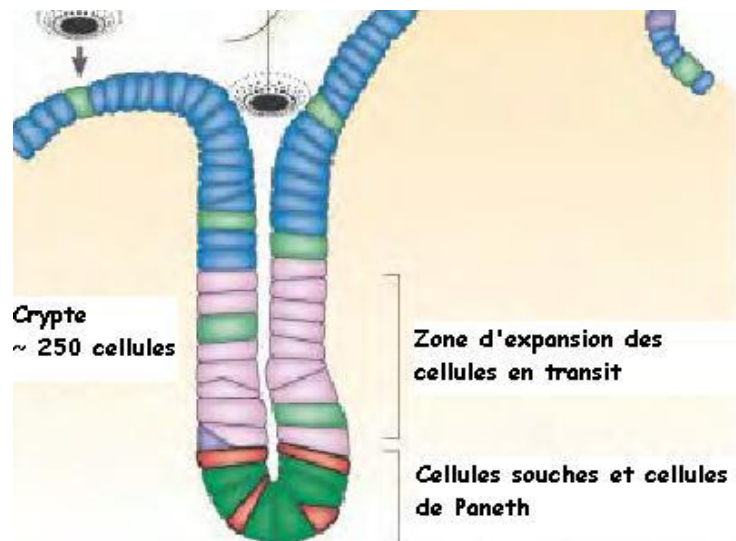
- Epithélium des cryptes :

La population cellulaire est plus hétérogène. Outre les entérocytes et les cellules caliciformes qui forment la population cellulaire la plus importante, on trouve des cellules endocrines et des cellules souches. Les cellules souches participent au renouvellement des cellules éliminées par apoptose. Le processus de différenciation se déroule le long de la migration des cellules vers le haut : les cellules migrent du tiers supérieur des cryptes vers l'épithélium de revêtement où les cellules différenciées subissent l'apoptose et s'exfolient dans la lumière. L'épithélium intestinal est ainsi renouvelé totalement tous les 3 jours chez la souris, tous les 5 jours chez l'homme. Cette durée correspond au temps de migration des cellules différenciées jusque vers la lumière. La moitié profonde des cryptes constitue une zone de prolifération alors que la zone luminale des cryptes constitue la zone de maturation, où les cellules ne peuvent plus se diviser, mais continuent à se différencier (Figure 4). Outre ces cellules spécialisées, l'épithélium des cryptes comporte des cellules impliquées dans les phénomènes immunitaires locaux. Les plus nombreuses de ces cellules sont les plasmocytes (lymphocytes B). Les lymphocytes T sont présents non seulement dans le chorion, mais aussi dans l'épithélium colique et dans la sous-muqueuse. Les fibroblastes et les macrophages sont nombreux.



**Figure 3. Muqueuse colique normale (HESx20) : section transversale :**

a : épithélium de revêtement ; b : crypte ; c : chorion.



**Figure 4. Schéma de mise en place fonctionnelle de la muqueuse intestinale**

D'après Crosnier C, Nat review 2006

La musculaire muqueuse (*muscularis mucosae*) sépare la muqueuse de la sous-muqueuse. Elle est constituée d'une mince couche de tissu musculaire lisse d'épaisseur variable. Outre les complexes lymphoglandulaires, elle est traversée par les réseaux vasculaires et nerveux.

#### -La sous-muqueuse

Elle comporte globalement les mêmes constituants que la musculaire muqueuse. Deux plexus nerveux occupent la sous-muqueuse, le plexus sous-muqueux de Meissner, constitué de neurones et de cellules gliales dispersés dans les éléments de soutien et situé immédiatement sous la musculaire muqueuse, et le plexus sous-muqueux profond (ou plexus d'Henle), vraisemblablement l'analogue du plexus d'Auerbach de l'étage aval. La vascularisation comporte des artérioles, des veinules et des vaisseaux lymphatiques.

#### -La couche musculaire

Elle est constituée par du muscle lisse organisé en une couche interne circulaire et une couche externe longitudinale, entre lesquelles le plexus nerveux d'Auerbach a une composition analogue à celle du plexus de Meissner. Elle est perforée par les vaisseaux sanguins et lymphatiques.



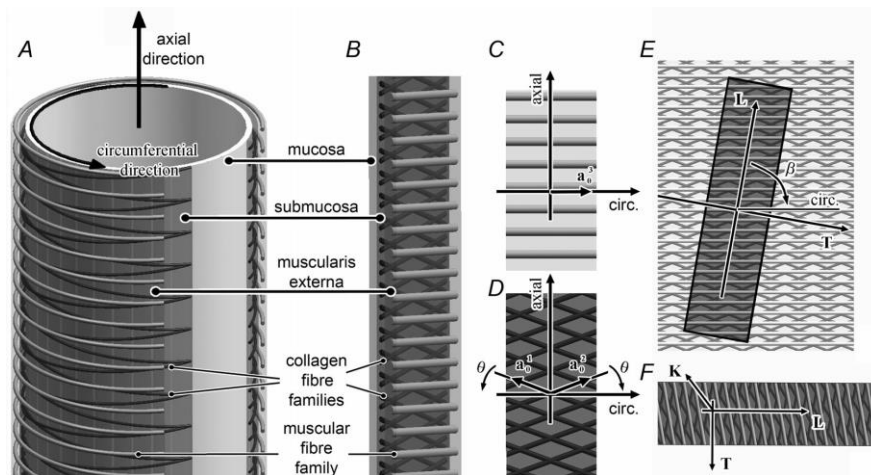
### -La séreuse

Elle comporte une sous-séreuse d'épaisseur variable, constituée de tissu conjonctif, qui entoure la couche musculaire, et une mince couche de cellules mésothéliales.

## **1.2-Propriétés mécaniques de la paroi colrectale**

Les propriétés mécaniques de la paroi colrectale sont directement liées à son organisation structurale (Figure 5). La distribution en sous-couche de la paroi colrectale est responsable du caractère non-linéaire des réponses mécaniques à la stimulation. En effet, la différence de résistance mécanique des différentes couches s'explique par leur composition :

- la muqueuse est constituée d'un réseau lâche de fibres de collagène,
- la sous-muqueuse est riche en fibres de collagène arrangées en 2 hélices principales, horaire et antihoraire,
- la couche musculaire est composée de cellules musculaires lisses longitudinales (taeniae coli) et circonférentielles
- la séreuse est constituée d'un tissu conjonctif lâche

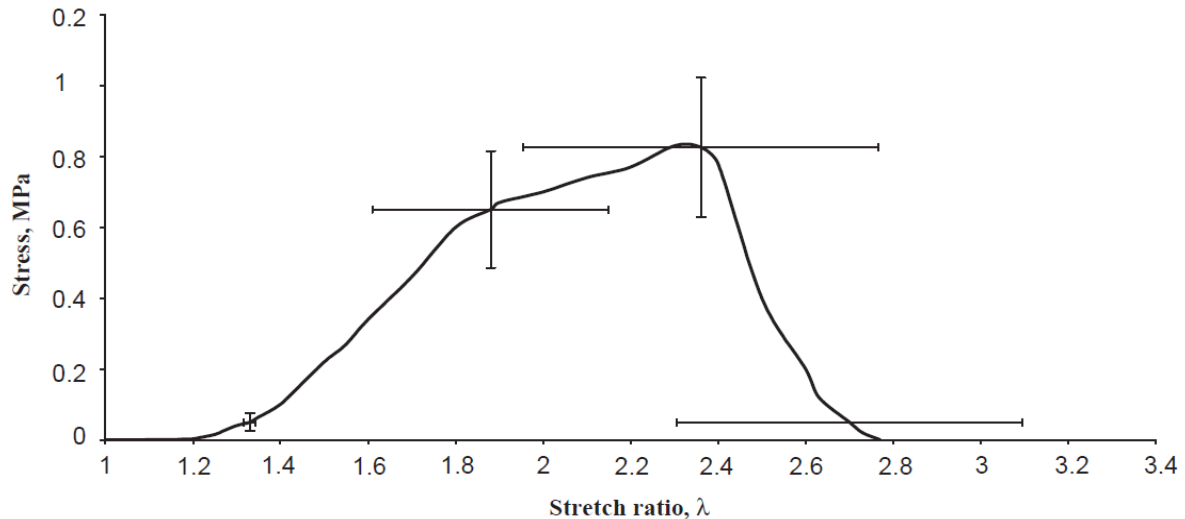


**Figure 5. Schéma de l'organisation structurale de la paroi colique**

D'après Carniel EL, Exp Physio 2014

La paroi colrectale soumise à une contrainte mécanique d'étirement longitudinal présente 2 pics successifs dits de stress mécanique avant la rupture (Figure 6) (Egorov 2002) (Massalou 2013). Le premier pic correspond à la rupture des couches externes, séreuse et musculaire

longitudinale. Le deuxième pic correspond à la rupture des couches internes, musculaire circulaire et sous-muqueuse. La couche sous-muqueuse et musculaire circulaire sont les deux couches assurant la résistance mécanique de la paroi colorectale.



**Figure 6. Profil de résistance mécanique à l'étirement de la paroi colique**

D'après Egorov VI, J Biomech 2002

La contrainte d'étirement maximale tolérée en traction avant rupture (« strength pressure ») est de 0,9 MPa ( $6,7 \cdot 10^3$  mmHg) dans la littérature sur colon humain de cadavre (Egorov 2002) (Massalou 2013). En présence d'une anastomose, cette valeur est divisée de 50% (Egorov 2002). La contrainte de distension maximale avant rupture (« bursting pressure ») varie de 47 à 50 kPa (352 à 375 mmHg) pour le colon de rat (Ji 2013).

### **1.3-La flore colorectale**

La flore colique est la plus riche et la plus variée des flores commensales du système digestif avec des concentrations atteignant  $10^{11}$  germes /gr notamment au niveau du caecum, du colon droit et du rectum. 99% des germes de la flore colique sont des anaérobies stricts (flore dominante), 1% sont des aéro-anaérobies (flore sous-dominante). Cette flore bactérienne évolue au sein d'un milieu faiblement acide puisque le pH du colon évolue entre 6 et 7 (Ewans 1988).

La flore colique a un rôle de défense en luttant contre des agents entéropathogènes extrinsèques et un rôle métabolique :

- Métabolisme des glucides :

De nombreux résidus glucidiques parviennent au colon : des résidus endogènes ( glycoprotéines, mucines, résidus de cellules desquamés) , des résidus exogènes alimentaires: sucres simples (ex:lactose) pour lesquels le grêle n'a pas l'équipement enzymatique nécessaire, soit des résidus d'amidon, des fibres ( pectine, cellulose, d'hémicellulose), non dégradés par les enzymes digestives. Ces glucides « résiduels » sont hydrolysés en sucres simples (glucose) pour partie utilisés par les bactéries comme substrat énergétique et pour partie transformés par fermentation : processus anaérobie de transformation des sucres simples par les enzymes bactériennes, aboutissant à la production de CO<sub>2</sub>, d'hydrogène, de méthane, et d'acides gras volatils (pyruvique puis acétique, lactique, propionique et butyrique).

- Métabolisme des lipides :

L'essentiel des triglycérides est normalement absorbé au niveau de l'intestin grêle, mais une faible partie de graisses neutres peut atteindre le colon. L'effet de la flore semble s'exercer surtout sur les acides gras désaturés qui une fois hydroxylés ont un effet laxatif.

- Métabolisme azoté :

Les résidus protidiques d'origine exogène (urée, protéines alimentaires non digérés) et endogènes (enzymes digestives, cellules desquamées, bactéries mortes), sont dégradés par les bactéries de la flore : processus de putréfaction qui aboutit à la production de : CO<sub>2</sub>, NH<sub>3</sub>, HS<sub>2</sub>, d'amines biologiquement actives ( histamine, tyramine, tryptamine qui ont des effets vaso-actifs), de polyamines notamment l'ornithine qui semble jouer un rôle trophique majeur sur la muqueuse intestinale.

## **2- Ingénierie tissulaire colorectale**

L'ingénierie tissulaire est considérée comme une voie prometteuse pour engendrer de nouveaux produits de santé et dispositifs médicaux. Malgré des progrès considérables réalisés au cours de ces dernières années dans le domaine de la substitution d'organes et de tissu, le remplacement de segments de tube digestif pose de nombreux problèmes irrésolus, notamment l'obtention d'une couche de cellules musculaires lisses (Hori 2002) (Araki 2009). Les segments de tube digestif les plus étudiés en ingénierie tissulaire sont l'œsophage (Badylak 2005) (Beckstead 2005) (Marzaro 2006), l'intestin grêle (Choi 1997) (Hori 2002) (Sala 2009) et l'estomac (Araki 2009) (Badylak 2005) (Beckstead 2005) (Marzaro 2006) (Choi 1997) (Hori 2002) (Sala 2009). En revanche peu de travaux ont été menés sur l'ingénierie tissulaire colorectale (Ueno 2007) (Grikscheit 2002) (Grikscheit 2003) (Fujie 2010) malgré des perspectives cliniques nombreuses, du comblement de défaut pariétal ou anastomotique au remplacement de tout ou partie du rectum répondant à une demande fonctionnelle.

Parmi les matériaux testés en ingénierie tissulaire digestive, et notamment les matrices extracellulaires décellularisées d'origine biologique, celles obtenues à partir de la sous-muqueuse intestinale de porc (SIS ou Small Intestine Sub-mucosa) ont été les plus utilisées (Badylak 2005) et apparaissent aujourd'hui comme la référence dans ce domaine : œsophage (Badylak 2000), estomac (de la Fuente 2007), intestin grêle (Chen 2001), colon (Ueno 2007) et voies biliaires (Rosen 2002), uniquement chez l'animal. La structure tridimensionnelle des SIS facilite l'hébergement et le recrutement de cellules de l'hôte, elle contient des facteurs de croissance (Voytik-Harbin 1997), elle favorise l'adhésion et la prolifération cellulaires, notamment de cellules endothéliales, promouvant ainsi l'angiogénèse (Hodde 2001), elle est biocompatible et rapidement biodégradable réduisant ainsi la réponse inflammatoire et la fibrose. Les SIS ont déjà été testées dans la littérature pour l'ensemencement de cellules épithéliales (Wei 2009) et de cellules musculaires lisses (Kropp 1996). L'analyse du remodelage des matrices de SIS ensemencées de cellules musculaires lisses, dans l'évaluation du remplacement de paroi vésicale (Kropp 1996), révèle une augmentation de la compliance de la matrice avec le temps et l'acquisition d'une contractilité comparable au tissu vésical natif. Deux publications récentes rapportent l'utilisation de matrice de SIS ensemencée de cellules souches mésenchymateuses issues du tissu adipeux (Adiposed Derived Stem Cells, ADSC) pour l'ingénierie tissulaire de la trachée (Du 2012) et de l'albuginée (Ma 2012), avec dans les deux cas une activité fonctionnelle prometteuse.

En ce qui concerne la composante cellulaire, plusieurs types cellulaires ont été utilisés dans la littérature pour favoriser la régénération du tissu intestinal. Les meilleurs résultats ont été obtenus en utilisant les "organoïd units" composées d'un noyau de cellules souches stromales entouré d'un épithélium intestinal polarisé. Une étude a rapporté leur utilisation à partir du colon de rats nouveaux-nés (Grikscheit 2003): l'implantation d'une matrice tubulaire biodégradable préalablementensemencée par ces unités multicellulaires a induit la formation d'un néotissu colique de même structure histologique et doté des mêmes propriétés physiologiques d'absorption que le tissu natif. La perspective d'une application en clinique humaine d'un biomatériauensemencé par des cellules autologues n'autorise pas l'utilisation de ces unités cellulaires.

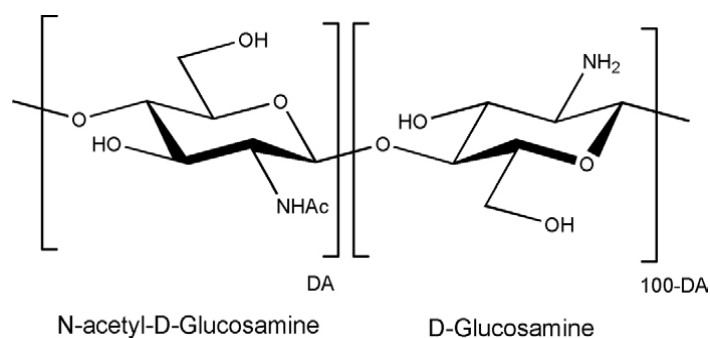
Les données existantes sur l'ingénierie colorectale ont été rapportées dans la revue de la littérature jointe en fin de première partie.

### 3- Le chitosane :

#### 3.1-Definition, structure et origine

##### 3.1.1-Structure

Le chitosane est constitué de résidus D-glucosamine (GlcN) et N-acétyl-D-glucosamine (GlcNAc) liés par des liaisons  $\beta$ -(1 $\rightarrow$ 4). De par sa structure, proche de l'acide hyaluronique notamment, le chitosane est considéré comme appartenant à la famille des glycosaminoglycanes (GAG), un sous-groupe de polysaccharides, présents dans la matrice extracellulaire des tissus conjonctifs des mammifères.



**Figure 7. Schéma de la structure chimique de la chitine et du chitosane.**

La chitine et le chitosane possèdent la même structure chimique riche en fonctions hydroxyle qui confèrent un fort caractère hydrophile au chitosane. Outre la masse moléculaire, ces structures se caractérisent et se différencient en première approximation par la proportion de groupements N-acétamide nommée « degré d'acétylation » ou « DA ».

Le DA est un paramètre important influant notamment sur la solubilité du chitosane et sur sa biodégradabilité. Ainsi, la chitine étant soluble pour un DA inférieur à 30-40%, et le chitosane est soluble si son DA est inférieur à 70%. Il est alors admis que la frontière chitine/chitosane se situe autour de cette valeur (Domard 2001).

##### 3.1.2-Source

Très peu présent dans la nature, sa principale source est la modification chimique de la chitine. La chitine est avec la cellulose, le polysaccharide le plus répandu dans la biosphère (Kaur and Dhillon 2013). Les deux principales sources sont la cuticule des arthropodes, ainsi que les parois cellulaires et la matrice extracellulaire de certains champignons, levures et algues (Muzzarelli 1988) (Kurita 2006). Le chitosane a pour origine la désacétylation de la chitine qui est contenue dans l'exosquelette des crustacés, en particulier des crevettes, issus

des déchets alimentaires. Cette production en solution alcaline offre le meilleur rendement et est adapté à une production à l'échelle industrielle.

### 3.1.3-De la chitine au chitosane

Le chitosane est obtenu par la désacétylation de la chitine qui en fonction de la source, peut être associée à des protéines, des minéraux, des lipides et des pigments. Ceci implique le recours à des traitements complexes de purification de la chitine pour obtenir le chitosane. Le traitement le plus couramment appliqué consiste en une succession de bains acides (HCl) puis basiques (NaOH) afin d'éliminer les carbonates et les protéines. La désacétylation est réalisée en présence de soude concentrée (35 à 50%) à hautes températures (80-140°C) (Domard 2001). Pour atteindre une désacétylation totale, les réactions doivent être réalisées en conditions contrôlées afin de limiter les mécanismes d'oxydation et donc la dégradation de la chaîne polymère. La désacétylation peut être menée en présence de thiophénol ou être complétée par des cycles de congélation/décongélation sous vide pour empêcher la dégradation des chaînes polymères due au traitement (Tolaimate 2000) (Lamarque 2004). Des molécules de chitosane de faible DA et de haute masse molaire (M) peuvent être alors obtenues avant leur éventuelle ré-acétylation.

## 3.2- Caractéristiques physico-chimiques du chitosane

### 3.2.1. Le degré d'acétylation

Le chitosane est obtenu par la désacétylation de la chitine mais si le DA de la chaîne polymère est inférieur à 70% on parle de chitosane. Il est ainsi possible d'acétyler le chitosane et de contrôler le DA par le procédé d'acétylation suivant. Les chaînes polymères étant sensibles aux températures élevées, l'acétylation du chitosane est réalisée à température ambiante, en milieu aqueux et en conditions homogènes. La réaction se fait en présence d'un réactif acétylant (donneur de groupe acétylé), tel que l'anhydride acétique et doit se dérouler en présence d'alcool (co-solvant) qui empêche l'acétylation des fonctions hydroxyles. Le méthanol et le propanediol sont les co-solvants les plus couramment utilisés.

### 3.2.2. Masse moléculaire

Au cours des traitements de purification et d'acétylation par exemple, la chaîne polymère peut être dégradée diminuant ainsi la masse moléculaire. La détermination de la masse moléculaire nécessite la mise en solution du polymère et peut se faire suivant plusieurs techniques : par

viscosimétrie capillaire ou dynamique à l'aide de l'équation de Mark-Houwink- Sakurada ; par diffusion statique de la lumière via la construction d'un diagramme de Zimm et enfin par chromatographie d'exclusion stérique couplée à la diffusion de la lumière qui constitue la technique la plus couramment utilisée pour la détermination de la masse moléculaire du chitosane. La masse moléculaire affecte de nombreux paramètres physico-chimiques tels que la solubilité ou la viscosité. Ainsi, le chitosane de masse moléculaire élevée occupera un volume hydrodynamique plus important et sera plus visqueux que pour de faibles masses moléculaires. En fonction de la masse moléculaire, au-delà d'une concentration critique dite d'entrecroisement, le polymère tend, par repliement, à former des liaisons de type hydrogène inter- et intra-chaines, diminuant la solubilité du chitosane. Cette concentration est inversement proportionnelle à la masse moléculaire du chitosane.

### 3.2.3. Méthodes de caractérisation

La pureté du chitosane est un critère prépondérant dans le cas d'une utilisation médicale. Avant son utilisation, le chitosane est caractérisé la masse moléculaire, le DA et par la détection éventuelle de traces de résidus protéiques. Pour cela, les méthodes les plus efficaces sont la RMN du proton (RMN 1H) et la spectroscopie infra-rouge à transformée de Fourier (IRTF). La caractérisation du chitosane par IRTF est obtenue par la transmission d'un faisceau de lumière infra-rouge à travers l'échantillon. Le spectre obtenu est composé de pics d'absorbance a des 59 longueurs d'ondes précises, chacun correspondant spécifiquement a une fonction (NH<sub>2</sub>, C=O,...) (Shigemasa 1996). Des études ont été menées sur le chitosane afin d'établir ces correspondances. Le DA est calculé en fonction de A<sub>1655</sub> et A<sub>3450</sub> qui sont les pics sortant respectivement à 1655 et 3450 cm<sup>-1</sup>. Cette technique est rapide et peu couteuse mais seuls les DA d'échantillons insolubles peuvent être déterminés par cette technique, peu adaptée aux chaînes polymères de faible DA (DA<50%). De plus, avant l'analyse, l'échantillon doit être purifié d'éthanol et de KOH avant d'être mélangé à du KBr pour l'analyse (Baxter 1992) (Duarte 2002) (Kasaai 2008) (Kasaai, Arul et al. 2008). La technique de caractérisation du chitosane la plus commune est la RMN 1H en phase liquide. Le spectre RMN 1H du polymère est la superposition de spectres individuels des résidus qui sont légèrement modifiés par leur liaison les uns aux autres. Une table a été établie par l'équipe de Kasaai établissant la correspondance entre le ppm auquel apparait le pic et la nature du proton. Ainsi, sur le spectre RMN du chitosane, le pic à 2,0 – 2,1 ppm représente 3 protons du groupe GlcNAc et le pic à 3,1 – 3,2 ppm représente le H<sub>2</sub> du groupe GlcN (Kasaai 2009). L'analyse RMN du chitosane se fait à partir d'une faible quantité d'échantillon lyophilisé,



dissout dans une solution de D<sub>2</sub>O/DCI, à 70°C afin de favoriser la solubilisation. La détermination du DA est réalisée suivant la méthode proposée par Hirai et al. (Hirai 1991) et consiste en la comparaison de l'intensité de résonance magnétique des protons H des résidus GlcNAc (2 ppm) avec celle des protons H<sub>2</sub> à H<sub>6</sub> des résidus GlcN et GlcNAc (3 – 4,2 ppm). Le DA est alors calculé selon la formule suivante :

$$DA (\%) = [(1/3 \times I_{CH3}) / (1/6 \times I_{H2-H6})] \times 100$$
 I = intensité de résonance

La spectroscopie RMN permet la caractérisation rapide, précise et reproductible du chitosane faisant d'elle la technique la plus couramment utilisée. Toutefois, elle n'est applicable que pour des DA auxquels le chitosane est soluble, soit inférieur à 60% (Lavertu 2003). La caractérisation du chitosane insoluble peut être alors réalisée par RMN 13C, qui constitue une bonne alternative.

### 3.3. Propriétés physico-chimiques du chitosane

#### 3.3.1. Solubilité

La solubilité d'un composé ionique ou moléculaire est la concentration maximale que l'on peut dissoudre dans un solvant à une température donnée. Le chitosane est un polyélectrolyte cationique naturel dont la solubilité est dépendante du DA et du pH. Le chitosane commercial 60 qui est faiblement acétylé (DA de 10-15%) présente un pKa voisin de 6 le rendant soluble uniquement dans les solutions aqueuses acides (pH < 6), par protonation des groupes amines. L'acide le plus couramment utilisé pour préparer une solution de chitosane est l'acide acétique dilué. Lorsque le DA augmente, le nombre de groupes amine potentiellement protonables diminue, réduisant ainsi la solubilité du chitosane. De plus, le DA impacte aussi le pKa du chitosane. L'augmentation du DA induit une augmentation du pKa du chitosane le rendant soluble quel que soit le pH de la solution (Sorlier 2001). Par conséquent, le pKa dépend fortement du DA.

#### 3.3.2. Propriétés rhéologiques

Des mesures de rhéologie en mode dynamique ont mis en évidence le caractère viscoélastique du chitosane en solution (Hamdine 2005). Il existe une transition sol-gel des solutions de chitosane (Iversen 1997). Ainsi, plus la concentration en chitosane est élevée, plus le

comportement du chitosane tend vers celui d'un gel. Ces propriétés rhéologiques apportent au chitosane un intérêt certain pour l'ingénierie tissulaire.

### 3.3.3. Modifications chimiques

De par ses nombreux sites réactifs, le chitosane peut facilement être modifié chimiquement. On distingue les réactions spécifiques faisant intervenir le groupe  $-NH_2$  en C2 des réactions non spécifiques sur les  $-OH$  en position C3 et C6 (Rinaudo 2006). Ainsi par greffage, il est possible d'améliorer la solubilité du chitosane ainsi que ses propriétés anti-bactériennes (Xie 2001).

## 3.4. Propriétés biologiques

### 3.4.1. Biocompatibilité

Le chitosane n'est pas toxique pour l'organisme. La DL50 est évaluée (chez la souris) à 16g/j et par Kg de masse corporelle, ce qui équivaut à celle du sucrose (Domard 2001). Depuis près de 30 ans, le chitosane est utilisé comme complément alimentaire visant à réduire l'absorption des graisses. De plus Behren et al. ont mis en évidence l'endocytose du chitosane par les entérocytes (Behren 2002). Le chitosane, notamment sous forme amine protonée, possède un caractère hémostatique reconnu (Domard 2001). Mais le mécanisme exact par lequel il participe à l'hémostase n'est pas connu. L'hypothèse la plus vraisemblable serait que des interactions de type électrostatique s'établiraient entre le chitosane et les protéines de surface des érythrocytes (Malette 1983) (Rao 1997). D'un point de vue cellulaire, de nombreuses études ont été menées sur des fibroblastes, des kératinocytes, des ostéoblastes et des cellules souches mésenchymateuses (Lahiji 2000) (Chatelet 2001) (Uygun 2009). Ces études ont montré que le DA bien que n'affectant pas la cytocompatibilité des films de chitosane, a un impact important sur l'adhésion et la prolifération cellulaires. Ainsi, ces deux paramètres sont meilleurs lorsque le DA est faible (Rami 2013). Des tests cliniques menés sur des biomatériaux à base de chitosane n'ont montré aucune réaction inflammatoire ou allergique après implantation, injection, application ou ingestion chez l'homme (Muzzarelli 1995).

### 3.4.2. Biodégradabilité

La biodégradabilité est une propriété indispensable à un substitut issu de l'ingénierie tissulaire. Le chitosane est facilement hydrolysable par de nombreuses chitinases et par des

enzymes protéolytiques non spécifiques appelées lysozymes, de manière plus ou moins efficace selon le DA (Hirano 1989) (Izume 1992). Les produits de dégradation obtenus sont des oligomères de chitosane dont les propriétés biologiques sont encore mal connues. Ainsi un matériau de chitosane faiblement acétylé peut subsister plusieurs mois in vivo (Suh 2000). Lorsque le chitosane est implanté chez l'homme, il est dégradé, et ce d'autant plus vite que le DA est élevé (Lee 1995). La biodégradabilité du chitosane au niveau colorectal est développée à la fin du chapitre sur le chitosane.

#### 3.4.3. Propriétés bactériostatiques et bactéricides

La propriété antibactérienne du chitosane repose sur son caractère polycationique. En solution, ses résidus GlcN (-NH<sub>2</sub>) sont protonnés (-NH<sub>3</sub><sup>+</sup>) acquérant ainsi un fort pouvoir attractant des bactéries dont la paroi et la membrane sont chargées négativement (Tayel 2010). Ces interactions entraînent la diminution de la stabilité osmotique, la destruction de la membrane et éventuellement la perte des éléments intra-bactériens. Par conséquent, la diminution du pH augmente l'activité antibactérienne du chitosane du fait de la protonation des amines (Devlieghere 2004). Le chitosane de faible MM traverse plus facilement la membrane des bactéries dont il inhibe la synthèse des ARNm et des protéines interagissant avec l'ADN bactérien (Qi 2005) (Ma YP 2008) la concentration minimale d'inhibition étant fonction de la masse moléculaire, du DA, de la viscosité, de la force ionique, du pH et de la température (No 2002) (Chung 2003). Le DA et la masse moléculaire influencent l'activité antibactérienne du chitosane (Rhoades 2000) (Mellegard 2011). Le chitosane de faible masse moléculaire et de faible DA sont plus bactéricides que ceux de haute masse moléculaire et fort DA (Tayel 2010). Mais l'utilisation du chitosane est limitée par sa faible solubilité dans l'eau (Pradip Kumar Dutta 2004). La chélation par le chitosane de certains ions est impliquée comme possible mode d'action antimicrobien (Rabea 2003). Une importante quantité d'ions dans le milieu peut diminuer l'efficacité du chitosane (Tsai 1999) car ceci diminue le nombre de site NH<sub>3</sub><sup>+</sup> disponibles pour interagir avec les bactéries.

#### 3.4.4. Bioactivité

Certaines études ont mis en évidence le caractère bioactif du chitosane en s'intéressant à ses capacités cicatrisantes notamment en favorisant la prolifération de fibroblastes (Okamoto 1995). Le chitosane accroît la fonction inflammatoire de cellules immunitaires telles que les macrophages, en induisant la production de cytokines et l'expression de marqueurs d'activation (Porporatto 2003). La bioactivité du chitosane est intimement liée au DA de la

chaîne macromoléculaire. In vivo plus le DA du chitosane est élevé plus la réponse inflammatoire est importante (Rami 2013).

### 3.5. Les hydrogels physiques de chitosane

On peut définir un gel comme « un réseau constitué de chaînes polymères interconnectées et gonflées par un solvant dont la concentration est supérieure à 90% » (Guenet 1993). On qualifie d'« hydrogels » les gels dont le réseau est gonflé par l'eau. Les hydrogels peuvent être chimiques ou physiques. L'élaboration des hydrogels chimiques nécessite l'emploi d'agents réticulant tels que le glutaraldéhyde, le glyoxal, le diéthyl suarate ou encore l'acide oxalique. Ce traitement induit la formation de liaisons covalentes entre les chaînes polymères rendant l'hydrogel ni thermoréversible ni solvoréversible (Berger 2004). A l'inverse, les chaînes polymères des hydrogels physiques interagissent par des interactions de faible énergie (liaisons hydrogènes, interactions de Van der Waals (liaisons hydrophobes), interactions ioniques,..) apportant le caractère réversible aux hydrogels physiques, selon la température ou le pH. Le chitosane peut former différents types de gels selon les interactions chimiques et les espèces chimiques mises en jeu. Les hydrogels physiques de chitosane peuvent être formés selon trois voies.

a) Complexes ioniques : ce mode de réticulation met en jeu les ions  $\text{NH}_3^+$  qui interagissent avec des anions ou des fonctions anioniques telles que le sulfate (Shu 2002). Des systèmes thermogélifiants à 37°C à base de  $\beta$ -glycérophosphates ont été décrits (Chenite, 2000) (Cho 2006).

b) Complexes électrolytiques : la formation de ces complexes est basée sur l'interaction entre la chaîne polymère de chitosane et un autre polymère. Les hydrogels élaborés selon cette voie associent au chitosane des polysaccharides qui présentent des groupes carboxyles  $\text{COO}^-$  tels que l'alginate (Kim, Lee 1999), la pectine (Yao 2002) ou encore l'acide hyaluronique (Rusu-Balaita 2003). Taravel et al. ont également mis au point un hydrogel physique de chitosane/collagène, permettant d'améliorer notamment l'adhésion cellulaire (Taravel 1996).

c) Complexes hydrophobes. Montembault et al. (Montembault 2005) ont décrit des gels exclusivement constitués de chitosane et d'eau. Le chitosane est solubilisé dans une solution à pH acide. Après évaporation, la solution est neutralisée par des vapeurs

### 3.6-Chitosane et tissu colorectal

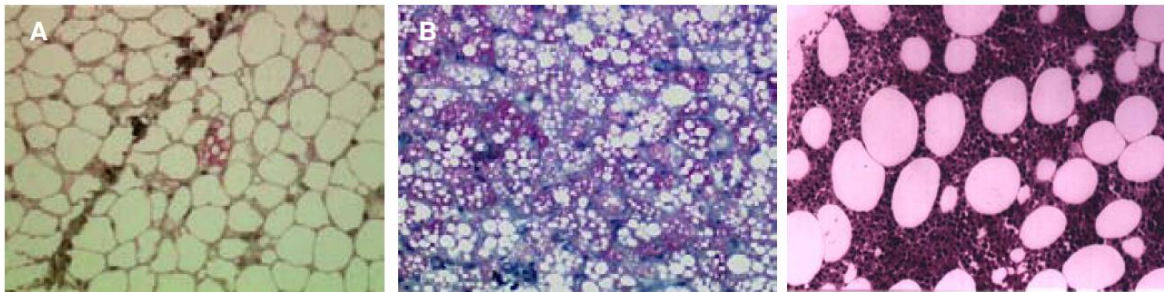
Le chitosane a fait la preuve de son efficacité dans la libération colique ciblée de nombreuses molécules bioactives (Yassin 2010) (Gulbake 2012). La dégradation du chitosane chez les vertébrés est assurée par le lysozyme et les enzymes bactériennes retrouvées de façon prédominante au niveau du côlon (Jain 2007) (McConnell 2008). En effet ce mécanisme a été rapporté chez le rat (Zhang 2002) et on sait par ailleurs qu'il existe une grande similitude entre la flore colique humaine et celle du rongeur (Gulbake 2012). Les bactéries hydrolysent les liaisons glycosidiques du polymère permettant ainsi la libération du composé actif à partir du chitosane. D'autre part, les mammifères ont gardé l'expression de trois familles de chitinases représentées par les AMCases, les di-N-acetylchitobiasés et les chitotriosidases toutes impliquées dans la digestion spécifique de la chitine et du chitosane (Funkhouser 2007). De plus, le mécanisme d'action colique du chitosane découlerait de ses propriétés mucoadhésives et de son aptitude à accroître la perméabilité colique. En effet, le chitosane faciliterait le transport paracellulaire à travers l'épithélium en permettant l'ouverture des « tight junction », canaux impliqués dans le transport de molécules hydrophiles (peptides et protéines) (Rosenthal 2012). La surface positive du chitosane interagirait avec la membrane cellulaire induisant une réorganisation du cytosquelette d'actine et des protéines constitutives des « tight junction ». Parmi les applications thérapeutiques du concept de « colonic drug delivery », la prise en charge des maladies inflammatoires chroniques de l'intestin a été étudiée à travers l'utilisation de nombreux médicaments (Tozaki 2002) (Oosegi 2008) (Kaur 2010) (Umadevi 2010) (Nath 2013) (Omwancha 2013). Une seule équipe a travaillé sur l'effet du chitosane sur la cicatrisation des anastomoses coliques chez le rat (Citgez 2012). Les auteurs ont rapporté une augmentation de la collagénisation de l'anastomose lors de la phase de cicatrisation, associée à une résistance mécanique plus importante.

#### **4- Le tissu adipeux : source « idéale » de cellules pour l'ingénierie tissulaire**

L'ingénierie tissulaire consiste à associer des cellules « réparatrices » à un biomatériau capable de véhiculer et de délivrer ces cellules au niveau du site lésé afin d'initier un processus de réparation tissulaire (Howard 2008). Les ADSCs sont des cellules de choix pour l'ingénierie tissulaire et la thérapie cellulaire (Kim 2014) (Trounson 2011) (Casteilla 2011) (Lopez 2011). Elles se définissent par leur capacité d'autorenouvellement, leur potentiel prolifératif et leur plasticité importante. Depuis quelques années, la communauté scientifique assiste à l'émergence de cette nouvelle source de cellules souches adultes (Bunnell 2008) (Zuk 2002). Le tissu adipeux peut être prélevé en grande quantité sous simple anesthésie locale, son prélèvement est moins invasif que la ponction de moelle osseuse. De plus, 1 g de tissu donne environ  $5 \times 10^3$  de cellules mésenchymateuses c'est à dire 500 fois plus que dans la moelle osseuse (Gimble 2007).

##### **4.1- Les différents types de tissus adipeux et leurs fonctions**

Chez les mammifères, trois types de tissus adipeux sont classiquement distingués morphologiquement et fonctionnellement (figure 8): le tissu adipeux blanc, le tissu adipeux brun et le tissu adipeux médullaire.



**Figure 8 : Aspects histo morphologiques des tissus adipeux blancs (A), bruns (B) et médullaires (C).**

Le tissu adipeux blanc existe sous une forme diffuse constituée de cellules plus ou moins isolées (par exemple le tissu adipeux intramusculaire), et sous forme de dépôts localisés dans différents sites anatomiques. Les dépôts les plus importants sont les dépôts sous-cutanés et profonds intra abdominaux (péri rénal, rétro péritonéal) ou péri gonadiques (épididymaire, péri ovarien). Le tissu adipeux blanc assume plusieurs fonctions. Les adipocytes blancs sont la principale réserve énergétique de l'organisme. Ils sont capables de stocker l'énergie sous forme de triglycérides durant les périodes d'excès calorique et de la

libérer sous forme d'acides gras libres en période de jeûne ou lorsque les dépenses énergétiques sont supérieures aux apports. Les adipocytes possèdent les voies métaboliques et les protéines de régulation associées aux processus de synthèse des triglycérides (synthèse *de novo* à partir de glucose, la lipogenèse, ou bien à partir d'acides gras captés dans la circulation sanguine) et de lipolyse (Kersten 2001). Le tissu adipeux a aussi une fonction sécrétoire et endocrine. Le tissu adipeux sécrète avant tout des acides gras, substrats énergétiques pour divers organes, mais également des peptides bioactifs, appelés adipokines, qui vont agir au niveau local par une action autocrine et/ou paracrine (cytokines, facteurs de croissance) ou au niveau systémique par une action endocrine (libération d'hormones). Le tissu adipeux blanc est aussi impliqué dans d'autres fonctions comme les fonctions neuroendocrines, l'immunité et l'inflammation (Kershaw 2004) (Fruhbeck 2001) (Lyon 2003).

Le tissu adipeux brun est localisé dans des sites bien déterminés tels que la région péri rénale, cervicale ou encore inter scapulaire chez les rongeurs. Il participe de manière active au phénomène de thermogénèse dite de non-frisson par opposition à la production de chaleur assurée par le frissonnement musculaire. La distribution efficace de la chaleur produite par ce tissu adipeux est assurée par une vascularisation importante. Les adipocytes bruns sont plus petits que les adipocytes blancs et possèdent plusieurs gouttelettes lipidiques ainsi que de nombreuses mitochondries. L'activité thermogénique du tissu est due à l'expression de la protéine découplante, UCP1 (Nicholls 1999). Chez l'homme, le tissu adipeux brun est principalement présent chez les nouveau-nés et disparaît au cours des semaines qui suivent la naissance. Cependant, il persiste à l'âge adulte chez les animaux hibernants et les petits rongeurs. Chez les grands mammifères, le tissu adipeux brun se convertit progressivement en tissu adipeux blanc au cours du développement (Gemmell 1972) (Vatnick 1987) (Casteilla 1989). Inversement, chez des animaux exposés au froid (la même observation a été faite chez l'espèce humaine (Huttunen 1981), des adipocytes bruns apparaissent en nombre important au sein des dépôts adipeux blancs (Cousin 1992) (Loncar 1991).

Le tissu adipeux médullaire n'est pas un tissu au même titre que les deux précédents. Il est plus approprié de parler d'adipocytes médullaires. En effet, de nombreux adipocytes sont présents au sein même de la moelle osseuse et font partie du stroma médullaire qui est le support de l'hématopoïèse. Au cours du vieillissement, la moelle rouge, dite active, évolue vers un phénotype de moelle jaune dans laquelle le processus hématopoïétique s'est arrêté. Cette moelle jaune est « envahie » par les adipocytes.

#### 4.2- Origine, développement embryonnaire et postnatal du tissu adipeux

Wasserman, en 1964, introduit le concept d' « organes adipeux » sur la base de l'observation anatomique de leur développement embryonnaire (Wasserman 1964). Au même titre que d'autres organes, les tissus adipeux émergent de groupes cellulaires primitifs clairement individualisés et localisés dans les sites des futurs dépôts adipeux. Des cellules mésenchymateuses prolifèrent autour d'un bourgeon vasculaire. Les capillaires se développent pendant que certaines cellules mésenchymateuses se détachent de ces capillaires pour donner naissance à des pré adipocytes. Ceux-ci commencent alors à accumuler des lipides. Les cellules les plus avancées dans la différenciation sont celles qui sont les plus éloignées des capillaires. La vascularisation joue un rôle primordial dans le développement du tissu adipeux : la densité des adipocytes est corrélée à la densité des capillaires sanguins (Hahn 1975). Il a été démontré que les cellules du lignage adipocytaire ont de fortes propriétés angiogéniques (Planat-Benard 2004), suggérant une relation de lignage entre les cellules adipeuses et les cellules endothéliales. Chez l'homme adulte, les tissus adipeux peuvent représenter de 10 à 50 % du poids corporel. Ces extraordinaires capacités d'expansion sont réversibles. Elles sont le résultat de l'hypertrophie des adipocytes et du recrutement et de la différenciation des précurseurs cellulaires localisés dans la fraction stromale du tissu adipeux (SVF). Les adipocytes libèrent des facteurs pro-angiogéniques comme la leptine et le VEGF (Song 2007) (Castello 1980) (Bouloumie 1998) (Sierra-Honigmann 1998). Le développement de la vascularisation semble être nécessaire au développement des tissus adipeux comme l'attestent deux études qui montrent que l'inhibition de l'angiogenèse permet de lutter efficacement contre le développement du tissu adipeux y compris chez des individus obèses (Kolonin 2004).

#### 4.3- Populations cellulaires des tissus adipeux

Un dépôt de tissu adipeux contient les adipocytes, cellules différenciées qui accumulent au sein de leur cytoplasme une énorme gouttelette lipidique, des cellules d'aspect fibroblastique (parmi lesquelles se trouvent les préadipocytes, cellules précurseurs des adipocytes), des capillaires sanguins, des terminaisons nerveuses, du tissu conjonctif et des macrophages (Ailhaud 1992). La séparation des cellules du tissu adipeux par digestion enzymatique permet d'isoler facilement les adipocytes (fraction cellulaire qui flotte en milieu liquide) du reste des cellules appelé fraction stromale vasculaire (SVF). La SVF représente la population cellulaire très hétérogène entourant les adipocytes dans le tissu gras. Cette fraction



SVF peut être mise en culture et permet d'obtenir les ADSCs sélectionnées par leur capacité d'adhérence au plastique (Figure 9). Le milieu de culture doit préserver le phénotype des cellules souches indifférenciées : l'expression des marqueurs de surfaces CD73, CD90, CD105, et absence d'expression de CD34 et de CD45 (Arthur 2009) (Chamberlain 2007) (Gronthos 1994). Le tissu adipeux est une source importante de cellules souches mésenchymateuses (Gimble 2007) (Bunnell 2008) (Zuk 2002). En effet, ce tissu contient des cellules mésenchymateuses possédant les mêmes propriétés que celles issues de la moelle osseuse.

#### 4.4- Plasticité et potentiels des cellules souches mésenchymateuses du tissu adipeux

Comparativement, la SVF et les ADSC représentent une source alternative de cellules progénitrices pluripotentes, possédant des potentiels de différenciation similaires vers les lignages mésodermiques (Zuk 2002) (Erickson 2002) (Safford 2002). Il a aussi été décrits que les ADSC peuvent assurer *in vivo* le support de l'hématopoïèse (Kim 2005) et jouer un rôle immunomodulateur (Yañez 2006). Cependant, même si les MSCs de la moelle osseuse, du tissu adipeux ou même du muscle squelettique, semblent apparentées, une littérature documentée établie des différences notables quant à leur phénotype, leur profil d'expression génétique, leurs caractéristiques en culture, leurs besoins en facteurs de différenciation (Lee 2004) (Izadpanah 2006). Actuellement ces populations de MSCs issues de ces divers tissus mésodermiques sont donc considérées comme distinctes (Gronthos 2001) (De Ugarte 2003) (Lee 2004) (Liu 2007). La plasticité des cellules issues de tissu adipeux a été étendue à de multiples autres types cellulaires. Les ADSCs sont d'origine mésodermale, elles peuvent se différencier préférentiellement en adipocytes, ostéoblastes, chondrocytes, muscle squelettique (Zuk 2001). Mais elles sont capables de se différencier également en cellules d'autres lignages non mésodermiques : neuronal (Fujimura 2005), endothélial (Planat-Benard 2004), épithélial (Brzoska 2005), hépatocytaire (Banas 2007), cellules pancréatiques (Timper 2006).

#### 4.5- Utilisation clinique du tissu adipeux

Depuis très longtemps les tissus adipeux sont utilisés en chirurgie plastique et reconstructrice (Mojallal 2004). La première utilisation de graisse remonte à 1889 où des fragments de tissus adipeux autologues ont été transplantés, servant de matériel de comblement pour reconstituer des volumes. Depuis ces premières greffes d'adipocytes, des injections de préadipocytes (cellules de la SVF) ont été décrites de même que celles de cellules adipocytaires préalablement cultivées (Van 1982) (Huss 2002) (Patrick 2001).

Utilisés pour des indications chirurgicales variées (malformations, traumatismes, lipoatrophies, cancers, esthétiques,...) ces travaux antérieurs fournissent des résultats intéressants avec un suivi de plusieurs années des patients. Alors que la greffe de fragments de tissus adipeux tend à diminuer avec le temps, la greffe de fractions issues de lipoaspiration présente une meilleure survie à plus long terme avec revascularisation de la greffe. À ce jour, ces pratiques restent associées à une absence de tumorigénicité. L'injection sous-cutanée de cellules humaines de la fraction stromale vasculaire de tissus adipeux dans des souris immunocompétentes n'induit pas de développement de cancer sur un suivi de plusieurs mois (Huss 2002) (Patrick 2001) (Gavrilova 2000). Des essais cliniques utilisant la SVF sont actuellement en cours, menées notamment par Cytori Therapeutics avec le Celution™ System (permettant la préparation automatisée de SVF en 1h) dans le domaine cardiovasculaire (PRECISE Trial, Espagne – APPOLO trial, Pays-Bas), ainsi que dans le domaine des reconstructions mammaires après mastectomie avec ou sans radiothérapie, au Japon (RESTORE I & RESTORE II).

La thérapie cellulaire basée sur l'utilisation d'ADSCs autologues fait ses preuves dans des essais de phase II dans plusieurs applications et notamment dans le traitement des fistules liées ou non à la maladie de Crohn (de la Portilla 2013) (Guadalajara 2012) (Herreros 2012) (Garcia-Olmo 2009, 2008, 2005) ou encore pour le traitement des affections cardiovasculaires (Madonna 2009) (Mazo 2008) (Li 2007). Ces études réalisées dans le traitement des fistules périnéales, liées ou non à la maladie de Crohn, indiquent que les ADSCs semblent être très efficaces pour le traitement de l'inflammation et l'amélioration du processus de guérison.

Enfin, l'ingénierie tissulaire basée sur l'ensemencement d'ADSCs sur matrice de SIS a récemment été rapportée sur 2 modèles animaux, le rat et le lapin, pour respectivement l'albuginée (Ma L 2012) et la trachée (Du 2012).

Le tissu adipeux s'avère être une source tissulaire abondante, riche en cellules mésenchymateuses. Les ADSCs remplissent tous les critères d'une cellule souche « idéale » décrits par Gimble (Gimble 2007) (Gimble 2003). Leurs nombreuses qualités en font des cellules de choix pour l'ingénierie tissulaire colorectale.



**Figure 9 : Procédure d'extraction des ADSCs à partir du tissu adipeux.**

D'après Seong JM, Biomed Mater 2010

## **Objectifs de la recherche**

L'objectif général de ce travail de thèse était l'élaboration d'un substitut colorectal circulaire qui tente de mimer la structure et la fonction de la paroi colorectale native. Le travail a été scindé en trois étapes:

- La sélection d'une matrice d'origine biologique optimale à partir d'études *in vivo* et *in vitro* dans son aptitude à favoriser la régénération tissulaire colorectale. (Article Annexe 2 - soumis à Journal of Tissue Engineering and Regenerative Medicine)
- La confection d'un système composite de confinement et de délivrance des cellules répondant à des critères d'évaluation *in vitro*. (Article Annexe 3 – soumis à Tissue Engineering Part C)
- L'implantation et la cellularisation autologue peropératoire de la matrice sélectionnée plane et circulaire. (Article Annexe 4 – en cours de correction avec les différents co-auteurs)

La dernière étape établit un lien fondamental entre les étapes initiales de sélection matricielle et de confection d'un système de confinement et de délivrance cellulaire, et l'ouverture sur l'élaboration d'un substitut rectal cellularisé.

## Article – Annexe 1



**Title:** Colorectal tissue engineering: prerequisites, current status and perspectives

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## **ABSTRACT**

Gastrointestinal tissue engineering has emerged over the past 20 years and was often focused on oesophagus, stomach or small intestine, whereas bioengineering researches of colorectal tissue is scarce. However, some promising results have been obtained in animal models. Colorectal tissue engineering still evolves refinements with scaffold and cell sources selection to allow muscle layer regeneration. Indeed, synthetic and natural polymers such as small intestinal submucosa and collagen sponge seeded with organoid units or smooth muscle cells did not allow smooth muscle regeneration. In this goal, mesenchymal stem cells derived from adipose tissue seeded on composite scaffold could represent an interesting way. This article reviews potential indications, current status and perspectives of tissue engineering in the area of colorectal surgery.

**Key words:** Tissue engineering, colon tissue, rectal tissue, allograft, scaffold, bowel replacement

## INTRODUCTION

Tissue engineering was defined as the application of the principles and methods of engineering and of the life sciences towards the fundamental understanding and the development of biological substitutes to restore, maintain or improve functions of tissue [1]. This is a relatively new interdisciplinary field of research and one of the first stocktaking exercises took place in France in 1988 [2]. The underlying principles of tissue engineering consist of three components: (1) the synthetic or natural biomaterial from which the extracellular matrix (ECM) is organized in neotissue, (2) the cells seeded in vitro on the biomaterial, and (3) the humoral and mechanical signals which regulate the behaviour of the first two. The key processes occurring during the in vitro and in vivo phases of tissue formation and maturation are thus (1) cell proliferation, sorting and differentiation ; (2) ECM production and organization ; (3) degradation of the scaffold ; (4) remodelling and potentially growth of the tissue. Current clinical applications of tissue-engineered constructs include engineering of bladder [3], skin and cartilage with tissue-engineered products such as Apligraf® (Novartis AG, Basle, Switzerland) and Dermagraft® (Advanced BioHealing Inc., New York, NY) for the skin and Carticel® (Genzyme Corporation, Cambridge, MA) for the cartilage. This article reviews potential clinical applications, current status and future perspectives in the field of colorectal tissue engineering.

## CURRENT STATUS

The tissue engineering triad comprises scaffolds, cells and bioreactors [4-6]. Data regarding colorectal tissue engineering are scarce. However, some promising results have been obtained in animal models but none was tested in clinical practice yet. *In vivo* assessments of colon tissue engineering are summarized in Table 1.

### Scaffolds

In tissue engineering, biomaterials should replicate the biological and mechanical properties of the native ECM providing a three-dimensional space for cell colonization. The ECM influences the phenotype, migration, and proliferation of its resident cells, and serves as a medium for signal transfers between cells [7-10]. For colorectal tissue engineering, scaffolds should be suturable, antimicrobial, biocompatible, nontoxic and biodegradable. Also, the main requirements is probably the suturability and antimicrobial effect of the scaffold because colonic tract is a bacterial contaminated system and the colonic wall represents an interface between the organism and its environment.

#### *Synthetic polymers used for colorectal tissue engineering*

Initial reports on the use of nonabsorbable materials such as Dacron [11] or expanded polytetrafluoroethylene (ePTFE) [12] to repair full-thickness wall defects in the colon respectively in rats and dogs models appeared to be a poor choice for tissue ingrowth. Materials acted as a bridge and were extracted into the lumen by the bowel. Pioneers in the area of small bowel tissue engineering [13-17] used a 2 mm thick nonwoven polyglycolic acid tubes (PGA) in colon tissue bioengineering [18,19]. The scaffold was seeded with previously harvested mesenchymal and epithelial cells derived from autologous full thickness colon wall swab and referred to as organoid units. Constructs exhibited the same mucosal architecture and electrophysiological parameters than native tissue with a complete absorption of the biomaterials in 8 weeks. However, PGA mesh was previously used to re-inforce colon anastomoses in rat models and seemed to be associated with motility disorders, delayed healing and leakage followed by peritonitis [20].

#### *Natural scaffolds used for colorectal tissue engineering*

Reports on the use of connective tissue patches consisting of collagen-fibronectin matrices [21], double layered collagen matrices [22,23] or collagen-fibrin matrices [24] supported the



use of collagen biomaterial in colonic anastomoses. Small Intestine Submucosa (SIS) is comprised of fibrillar collagens and adhesive glycoproteins and is an acellular xenogenic ECM harvested from the porcine small intestine. SIS is commercially available for human application as a scaffold for abdominal wall repair and has been assessed in tissue engineering experiments with animal models in esophagus [25], biliary tract [26], and stomach [27]. The feasibility of using SIS as a bioscaffold for a 1 cm colonic full-thickness wall defect in rats model have been also assessed [28]. Granulation tissue and inflammatory cells covered the defect at 3 weeks whereas the region of the graft was completely covered at 6 months by regenerated tissue including all layers of the native caecum wall. More recently, SIS scaffold was used to cover bigger full-thickness wall defects in the colon (4.5×1.5 cm) in pigs model [29] with less promising outcomes. Contraction of the reconstructed area and limited architectural regeneration of the bowel wall suggest limitation of morphologic regenerative capacities in colorectal regeneration.

#### *Mechanical properties*

Limitations in clinical evaluation of scaffolds are due to their lack either on cell specific signal or on adequate mechanical strength. It is considered that natural polymer can facilitate cell adhesion and proliferation but with physical strength capacities lower than synthetic polymers. Moreover, the mechanical behaviour of scaffolds changes during the process of in vivo remodelling [30], and such changes are dependent on factors such as the local tissue microenvironment, the rate of scaffold degradation, forces present within the mechanical environment, and the rate and extent to which the infiltrating cells deposit new extra cellular matrix. The most studied scaffold during the in vivo remodelling process is SIS for which degradation kinetic comprise several phases. In the early phase of remodelling, degradation occurs rapidly, with nearly 50% of the scaffold degraded by 1 month and complete degradation by 3 months [31], before newly deposited extra cellular matrix has the opportunity to fully organize, thus the initial decrease in scaffold strength. However, once the infiltrating cells have colonized the graft and begun to produce site-specific extra cellular matrix, rapid scaffold remodelling occurs, with an increase in the strength and site-appropriate mechanical behaviour corresponding to an increase of compliance for the digestive tract [32]. SIS has a break strength of 2-6 Mpa [33] whereas synthetic polymers, such as Poly-Lactic-coated-Glycolic Acid (PLGA) have a greater flexibility in tailoring the mechanical properties and degradation characteristics [34, 35]. This last polymer lack bioregulating signals that can direct cell proliferation and differentiation [36]. Combining natural and synthetic polymers

could be considered such as one way to take advantages of both types of materials, i.e. biological and mechanical properties [37].

### **Cell sources**

Cells are the crucial part of any tissue engineering efforts. The combination of biomaterial with cells was shown to reduce contraction and shrinkage of the graft and to improve its integration [25,38,39]. Cells should be nonimmunogenic, highly proliferative, easy to harvest and have the ability to differentiate into application-specific cell types with specialized functions [4]. One of the basic concept of tissue engineering is to use autologous cells to avoid graft rejection by the host.

#### *Colon epithelial cells*

In initial attempts to engineer small bowel, fetal and adults enterocytes were seeded on biodegradable polymer tubes to generate stratified epithelium [40]. Primary epithelial cells culture from colon was used as a model for the colon epithelium in physiological, toxicological and oncological studies in vitro [41-43]. However, long-term cultures of primary colon epithelial cells remain a great challenge [44]. In our knowledge, none study had reported the use of colonic epithelial cells to engineer colon tissue.

#### *Organoid Unit*

Organoid units are multicellular units, containing mesenchymal cell cores surrounded by a polarized epithelia derived from full-thickness sigmoid colon dissection from rat model [18, 19]. Organoid units were seeded on PGA/PLA scaffold and constructs were implanted into the omentum. Tissue-engineered colon (TEC) which was placed in lieu of end-ileostomy had major physiologic functions of native colon with a particularly longer transit time and a lower stool moisture content. Moreover, weight loss and electrophysiological troubles were significantly higher in end-ileostomy group [19]. Despite promising outcomes, this technique is limited by clinical concerns. Indeed, the fact that the engineered colon derives from a full-thickness wall dissection of the colon and the large numbers of organoid units which are necessary to achieve a bowel tissue-engineered construct makes this procedure not convenient in clinical practice. Moreover, in patients with abnormal epithelial colon cells (inflammatory

or oncologic diseases) this bioengineered colon derived from autologous tissue precludes its use for clinical applications.

#### *Smooth muscle cells (SMCs)*

One of the most difficult parts of bowel tissue engineering is to accomplish regeneration of a smooth muscle layer. Isolation and culture of SMCs were established from stomach [45], small bowel [46], sigmoid colon [47] or internal anal sphincter [48] to engineer small bowel wall [45,46] and internal anal sphincter [47,48]. SMCs have been seeded on SIS [46] and collagen [45] scaffolds. The longitudinal muscle constructs could maintain smooth muscle phenotype and electrophysiological properties [47] but results between studies are controversial. In our knowledge, none study had reported the use of SMCs to engineer colon tissue.

## **EXPERT COMMENTARY**

Tissue engineering of colorectal tissue is at an early stage and has to prove clinical suitability. Colon and rectum exhibits various anatomical and physiological features. Moreover, important work remains to be done regarding to the vascularization, motility, absorption and innervation of bioengineered colon. However, regenerative medicine technology may provide new surgical tool for alternative therapy to conventional colorectal surgery in electives indications.

### *Local excision for rectal cancer*

Radical rectal excision is the standard in rectal cancer treatment. However patients with early stage of tumour, or with good response after radiochemotherapy [49] can be treated by local excision to avoid the morbidity of radical rectal surgery [50-52]. Wound complications are more frequently observed after conservative surgery in the neoadjuvant setting reaching 70% [53,54]. Rectal tissue engineering could be used as replacement of the rectal wall mainly to decrease the wound dehiscence rate.

### *Anastomotic fistula*

Despite technical improvements in stapling anastomosis procedure [55], anastomotic leakage is the most important surgical complication after rectal cancer surgery varying from 10 to 16% [51,56]. Pre-existing disorders such as low collagen I and III distribution and high expression of matrix metalloproteinase (MMP 1, MMP 2, MMP9) in the extracellular matrix of colon play a role in the pathogenesis of anastomotic leakage after colorectal surgery [57]. After radical surgery for rectal cancer, large bowel tissue engineering could be used to reinforce colorectal anastomosis in patients with high risk of fistula.

### *Perineal fistula*

Therapeutic options for perineal fistula associated or not to Crohn's disease are limited despite many data available in the literature [58]. Randomized phase I and II trials in which authors injected adipose derived stem cells (ADSC) with fibrin glue into rectal wall showed a healing rate of 71% [59,60]. Tissue engineering with ADSC seeded on a bioscaffold could be used in perineal fistula with large defect of the digestive wall.

### *Total colectomy*

The surgical substitution of an ileal pouch to replace the colon presents serious complications including severe pouchitis in 50% of cases over 10 years [61,62], high stool frequency, megapouch and pelvic sepsis as a result of pouch leak [63]. Ileal mucosal adaptation in the pouch by colonic metaplasia and the presence of chronic inflammation has been postulated to increase long-term neoplasia [64]. After total colectomy, large bowel tissue engineering could be used as replacement of the ileal pouch [18, 19].

## **FIVE-YEAR VIEW**

The use of organoid units by Vacanti and colleagues has proven to be a successful method in the generation of bowel well-differentiated mucosa. The technical success of this procedure is probably based on the presence of stem cells in the organoid units identified in small bowel [65] and in the colon [66]. Adult stem cells can be found in others sites, particularly in bone marrow (BM-MSC) and adipose tissue (ADSCs) [67]. Recent studies have suggested that BM-MSC play a role in the processes of intestinal repair [68], angiogenesis [69], and healing of ischemic colonic anastomoses [70]. However, only two studies using BM-MSC have been reported in small bowel tissue engineering [71,72] with promising outcomes. In the field of digestive tract disease, experimental and clinical researches have grown exponentially during the last decade [73] with several investigations about adipose-derived stem cells in the area of inflammatory, ischemic, fistulising and functional diseases. Stem cells, and more precisely adipose-derived stem cells (ADSCs), may provide a new promising cell source for colorectal tissue engineering by their characteristics: very abundant and accessible under local anesthesia with a low morbidity, effective yield of extracted tissue with pluripotent and replicative capacity similar as BM-MSC. ADSCs were also used for complex perineal fistulas treatment associated or not with Crohn disease in randomized phase I and II trials [59,60] with promising outcomes. Moreover, ADSCs seeded on SIS were recently reported in two studies to engineer tracheal tissue [74] and tunica albuginea [75] with good functional outcomes.

Advances in the area of biomaterials may provide a higher-ordered tissue structure. In particular, a better scaffold design is needed to produce a structured layer of smooth muscle. Until now, none of the scaffold used have shown a significant superiority. New biomaterials such as chitosan [76] which have shown to be useful in regenerative medicine should be tried for bowel tissue engineering and compared with PGA/PLA, SIS and collagen. Chitosan has been reported to be biocompatible, biodegradable, and non-toxic with antimicrobial and wound healing accelerating effects. In experimental *in vivo* study, chitosan powder applied to colonic anastomosis had a positive effect in the process of collagenization in colonic anastomosis healing [77]. Composite structure PLGA/chitosan mimicking SIS were constructed and show potential as a tissue engineering material [37]. Moreover, adhesive film based on chitosan integrated [78] or not with SIS [79] have been already assessed and bonded to calf intestine. Recently, the enhancing effect of chitosan on transcellular and paracellular mechanism in the intestinal epithelial barrier was reported *in vitro* [80]. Moreover, composite

collagen/chitosan scaffold appeared to be suitable to support in vitro bioengineered smooth muscle intestinal constructs [81].

In conclusion, success of tissue engineering relies on the selection of the appropriate type of biomaterials and cells source. It still represents a promising approach to generate functional colorectal substitutes which could have numerous clinical applications. Composite scaffold such as collagen/chitosan matrix which could combine optimal mechanical and biological properties seeded by clinically accessible pluripotent stem cells such as ADSCs may provide a new way for the next five-year of colorectal tissue engineering.

## KEY ISSUES

- Colorectal bowel tissue engineering is still at his first stage.
- Appropriate type of scaffold and cells source was not already found, but some data provide promising outcomes in animal models.
- Scaffold reported: 1) Synthetic polymers appeared to be unusable for tissue ingrowth and induced a significant contraction of the construct; 2) Small intestinal submucosa (SIS), widely used for tissue engineering, allowed tissue ingrowth and regeneration of the native tissue with a significant contraction of the graft; 3) Collagen sponge allowed mucosal layer regeneration without muscle layer.
- Scaffold in five-year view: composite collagen/chitosan scaffold could support smooth muscle regeneration with physiological functionality.
- Cells source reported: 1) Organoid units derived from neonatal rat intestine had allowed interesting perspectives for the generation of bowel well-differentiated mucosa but were not convenient for clinical practice because of their low yield; 2) No successful results about the use of colonic epithelial cells, adult enterocytes, intestinal stem cells or smooth muscle cells were reported.
- Cells source in five-year: Mesenchymal stem cells derived from adipose tissue (ADSC) could represent an interesting cell lineage for bowel tissue engineering inducing angiogenesis and host cells colonization via their paracrine effect.
- Colorectal tissue engineering could offer alternative therapy in numerous indications: anastomotic or perineal fistula, rectal wall replacement after local rectal cancer excision, replacement of the ileal pouch after total colectomy.



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## **REFERENCES WITH INTEREST\***

[18] [19] In these two studies from Boston, Polymer tubes were seeded by colon organoid units and placed into the omentum. Tissue-engineered colons had architecture and major function of native colon. Full-thickness wall colon dissection required for organoid units isolation is limited by clinical concerns. However, these outcomes were very promising and highlight the crucial association of scaffold and cells for colon tissue engineering.

[28] In this study, whole defect (1 cm) of the cecal wall was repaired with SIS patched without cells seeded. SIS scaffold was often used for digestive tract tissue engineering but for the first time in colon with this study.

[29] After Ueno, *et al.* authors assessed SIS scaffold to repair a larger defect of colon. Contraction of the patched area of 77% after 30 days and more than 90% after 60 days is due to the size of the defect and suggest the crucial part of cells in any tissue engineering efforts.

## **TABLE LEGEND**

**TABLE 1:** Summary of in vivo assessment of colon tissue engineering

## **FINANCIAL DISCLOSURE**

There is no ethical problem, conflict of interest or financial support with regard to this manuscript.

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We are working currently on colorectal tissue engineering in our laboratory and this work is supported by the Fondation pour l'Avenir in the program of Recherche Médical Appliquée.

# PARTIE II

## RESULTATS

Projets d'articles correspondants

Annexe 2

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# CHAPITRE I

Article correspondant

Annexe 2

Brevet académique n°1363099

## **Ingénierie tissulaire colorectale : Etude matricielle comparative sous-muqueuse intestinale (SIS) vs. hydrogel de chitosane**

L'objectif de la première année du projet d'ingénierie colorectale était de sélectionner une matrice d'origine biologique à partir d'études *in vivo* et *in vitro* dans son aptitude à favoriser la régénération tissulaire colorectale. La matrice destinée à remplacer un défaut pariétal colorectal doit être biocompatible, biodégradable, non-immunogène, et non toxique. Elle doit aussi avoir un degré d'élasticité autorisant une reprise d'activité péristaltique, et des propriétés mécaniques autorisant une manipulation facile et une suture par le chirurgien. Elle doit enfin posséder des propriétés bactériostatiques afin de permettre son intégration au contact de la flore bactérienne intestinale, immunomodulatrices afin de maîtriser l'inflammation et pro-angiogéniques afin de favoriser la vascularisation du néo tissu. Par ailleurs, sa composition et sa structure tri dimensionnelle doivent permettre l'ensemencement et une colonisation cellulaire trans-matricielle.

Les hydrogels de chitosane présentent de nombreux avantages et constituent de remarquables biomatériaux pour l'ingénierie tissulaire. Ils sont biodégradables, biocompatibles, bactériostatiques et miment la matrice extracellulaire. Outre ses propriétés répondant au cahier des charges de la matrice visée, ce matériau est modulable selon 4 paramètres physico-chimiques : la concentration en chitosane, le degré d'acétylation, la voie de gélification et la voie de neutralisation (Rami 2013). Cette « modulabilité » est une avancée considérable dans le domaine de l'ingénierie tissulaire car elle permet de tenir compte de la complexité structurelle des organes, dans notre cas de la paroi colorectale.

Les différents matériaux déjà testés en ingénierie tissulaire colorectale sont rapportés dans l'article de revue bibliographique (*Annexe 1*), avec leurs limites. Brièvement, il s'agit de PolyTetraFluoroEthylene (PTFE), d'Acide Polyglycolique (PGA), de collagène porcine ou humaine, de la sous-muqueuse intestinale de porc décellularisée (SIS). Cette dernière matrice a été retenue comme matrice référence d'une part car elle a été la plus utilisée en ingénierie du tractus digestif et d'autre part car ses particularités biochimiques associées à sa structure tridimensionnelle non réticulée en font un substrat propice à la régénération tissulaire et à une application chirurgicale. Le SIS est biocompatible, biodégradable, non toxique, non-immunogène, suturable, imperméable et possède une résistance à l'étirement supérieure au

tissu colique. Le SIS est commercialisé (BioDesign<sup>®</sup>, COOK) sous la forme de plaques mono- ou multicouches et son application est validée en clinique humaine dans le renfort de paroi.

Nous avons donc réalisé une étude comparative, *in vivo* et *in vitro*, comparant la matrice référente SIS à une matrice innovante d'hydrogel de chitosane afin de sélectionner la matrice permettant une régénération optimale de la paroi colorectale. Le modèle lapin a été retenu comme modèle d'expérimentation *in vivo* du fait de sa taille, de sa disponibilité et de son coût. En effet, le rat apparaissait être un modèle trop petit pour évaluer une taille de défaut pariétale suffisante, et le modèle porcine trop onéreux et peu éthique étant donné l'état d'avancée des travaux. La matrice à base d'hydrogel de chitosane a permis d'obtenir *in vivo* une régénération épithéliale plus précoce, un meilleur contrôle de la réponse inflammatoire avec un rapport de fibrose plus faible et l'obtention d'îlots de cellules musculaires lisses à 8 semaines. La matrice de SIS montrait *in vitro* de meilleurs résultats en termes d'attachement, de prolifération et de colonisation matricielle des hADSC (ADSC humains) et des cellules de la SVF, sans que cette différence soit considérée comme significative. Au vu de ces résultats, l'hydrogel physique de chitosane a été choisi pour ses propriétés *in vivo* de régénération tissulaire et de contrôle de l'inflammation permettant une régénération tissulaire supérieure à celle du SIS. *In vitro*, bien qu'ayant des résultats en terme d'adhésion et de prolifération cellulaires inférieurs à ceux des matrices de SIS, les hydrogels de chitosane étaient colonisés par les ADSC de façon durable avec préservation du phénotype indifférencié des cellules.

La matrice à base d'hydrogel de chitosane a donc été sélectionnée pour la suite du programme de recherche en ingénierie tissulaire colorectale.

## Article – Annexe 2

(Soumis à Journal of Tissue Engineering and Regenerative Medicine)

**Title:** Colorectal tissue engineering: a comparative study between porcine Small Intestinal Submucosa (SIS) and chitosan hydrogel patches.

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**Meeting presentation:** Presented as oral communication at the congress of International Federation for Adipose Therapeutics and Science (IFATS), New-York, November 21-24<sup>th</sup>, 2013 and at the congress of European Society of Coloproctology (ESCP), Belgrade, September 25-27<sup>th</sup>, 2013.

## ABSTRACT

**Objective:** Regenerative medicine technology may provide new surgical tools for colorectal surgery in elective indications. The aim of this study was to compare porcine Small Intestinal Submucosa (SIS) and chitosan hydrogel patches so as to define an optimal scaffold for colorectal tissue engineering.

**Methods:** We compared two bioscaffolds with *in vitro* and *in vivo* experiments: porcine Small Intestinal Submucosa (SIS) vs. chitosan hydrogel matrix. *In vitro*, we assessed nontoxicity of scaffold by using human Adipose Derived Stem Cell (hADSC). The readouts of *in vitro* experiments were rates of adhesion and proliferation and capacity to maintain mesenchymal stem phenotype after seeding. *In vivo*, a 1x2 cm colonic wall defect was performed in 16 rabbits. Animals were randomly divided into two groups: rabbits with cecum defects given porcine SIS graft (SIS group, n=8) and rabbits with cecum defect given chitosan hydrogel graft (Chitosan group, n=8). Graft area was explanted at 4 and 8 weeks. The endpoints of *in vivo* experiments were technical feasibility, scaffold behavior, *in situ* putative inflammatory effect and quality of tissue regeneration, in particular smooth muscle layer regeneration.

**Results:** *In vitro*, ADSC attachment and proliferation were obtained on both scaffolds without significant difference. After proliferation, ADSC kept their mesenchymal stem cell characteristics. *In vivo*, one animal died in each group after postoperative occlusion and postoperative leakage in SIS and Chitosan group, respectively. After 8 weeks implantation, chitosan scaffold allowed to obtain a better wound healing compared to SIS scaffold with a more effective inflammatory activity control and an ad integral regeneration of the colonic wall including smooth muscle cells layer around the fibrosis scar.

**Conclusion:** The outcomes of *in vitro* experiments did not differ significantly between the 2 groups. However, macroscopic and histological findings revealed a better wound healing of

the colonic wall in the Chitosan group. These results suggest that the chitosan hydrogel matrix could be the scaffold of choice for colorectal tissue engineering probably due to its antimicrobial effect and to its specific multilayered structure inducing partial colonization and biodegradability in colonic location.

## INTRODUCTION

Tissue engineering is an interdisciplinary field of research for the development of biological substitutes to restore, maintain or improve the functions of living tissue.<sup>1</sup> It may provide new surgical tools for alternative therapy to conventional colorectal surgery in elective indications such as treatment of anastomotic or perineal fistula, rectal wall healing after local excision for cancer, ileal pouch substitution after total colectomy.<sup>2</sup> The underlying principles of tissue engineering consist in the design of substitutes with two components: material scaffold for neotissue reorganization and cells seeded on the scaffold. The evolution of these two components changes during the *in vivo* phases of tissue formation and maturation in accordance with their location since they are specifically submitted to various chemical, mechanical and physical stimuli. Indeed, humoral and mechanical signals received by the scaffold and cells *in vivo* are different in oesophagus, stomach, small intestine or colorectal tissue. Thus, such a biomaterial concept underlines the specificity of scaffold/cell associations to the particular biological context of implantation. For colorectal tissue engineering, the patches, matrix, grafts or scaffolds should prevent the leaks of the colorectal lumen content to the abdominal cavity, be suturable and ideally, antimicrobial, nontoxic and biodegradable. The main requirement is probably the antimicrobial effect of the scaffold because colonic tract is a bacterial contaminated system and the colonic wall represents an interface between the organism and its external environment. Several studies have shown the activity of chitosan in the healing process with an antimicrobial effect and biodegradability due to colonic microflora used for colonic-specific drug delivery.<sup>3, 4</sup> For these reasons, chitosan could be a promising scaffold for colorectal tissue engineering. However, bioengineering researches of colorectal tissue are scarce and chitosan has never been specifically used for the reconstruction of the colonic wall. In accordance with literature, decellularized porcine Small

Intestinal Submucosa (SIS) can be considered as a reference scaffold for tissue engineering of the gastrointestinal tract.<sup>5-9</sup>

Thus, the aim of the present study was to compare chitosan scaffold with SIS scaffold using *in vivo* and *in vitro* approaches in order to evaluate the interest of chitosan physical hydrogel patches for colorectal tissue engineering.

## **METHODS**

### **1- Preparation of Small Intestine Submucosa (SIS) patches**

In accordance with the manufacturer's recommendation (Biodesign® 4-layers Tissue Graft, Cook Medical, Charenton Le Pont, France), the sterile SIS patches were allowed to rehydrate by immersion for 10 min in sterile Ringer's solution before use.

### **2- Chitosan Purification and characterization**

Chitosan, purchased from Mahtani Chitosan (Veraval, India), was extracted from squid pens and then purified before use as previously described.<sup>10</sup> The degree of acetylation (DA) of chitosan corresponding to the molar fraction of acetyl units within the polymer chains was determined by <sup>1</sup>H NMR spectroscopy,<sup>11</sup> according to the method developed by Hirai et al.<sup>12</sup> The weight-average molecular weight  $M_w$  of chitosans and I polydispersity index  $I_p$  were determined by size exclusion chromatography coupled on line with a multi-angle laser light scattering detector.<sup>11</sup>

### **3- *In vitro* assessment**

#### **3.1- Isolation, characterisation and culture of human Adipose Derived Stem Cells**

human Adipose Derived Stem Cells (hADSC) were isolated and cultured as previously described<sup>13</sup> with minor modifications.<sup>14</sup> Briefly, human adipose tissue was obtained from bariatric patients undergoing 67eotissue67ds67ie. Tissue samples were minced and digested in a solution of 0.1% (w/v) collagenase type I (Worthington) for 1h30 at 37°C with vigorous

shaking. After filtration and centrifugation, the top lipid layer was removed and the remaining Stromal Vascular Fraction (SVF) was treated for 10 minutes with ELB (Erythrocyte Lysis Buffer; 155 mM NH<sub>4</sub>Cl (Sigma), 5.7 mM K<sub>2</sub>HPO<sub>4</sub> (Sigma), 7.4 mM K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O (Sigma), 0.1 mM EDTA (Sigma)) and then centrifuged. The pellet was resuspended in basal medium, DMEM F12 medium (Invitrogen) supplemented with 10% (v/v) Fetal Bovine Serum (Lonza) and sequentially filtered through 100, 70 and 40 µm cell strainer (BD Falcon). The resulting cells were plated (10<sup>4</sup> cells/cm<sup>2</sup>) and cultivated at 37°C in 5% CO<sub>2</sub> atmosphere. Culture medium was replaced every three days when hADSC reached 80% of confluence. Flow cytometric analyses (BD Accuri™ C6 flow cytometer) were performed to characterize cells before and 7 days after seeding. Briefly, trypsin-EDTA solution (0,125 % (w/v) – 0,0625 % (v/w), Sigma-Aldrich) was used for isolation of cells. The following antibodies were used: CD 34, CD 45, CD 73, CD90 and CD105 (eBiosciences®).

### 3.2- Seeding procedure

The sterilized scaffolds, in the shape of patches of 1cm<sup>2</sup>, were immersed into the DMEM-F12 culture media (Invitrogen) 24 hours before cell seeding. Culture media contained 10% of Fetal Bovine Serum (Lonza) for SIS sheet and platelet lysate for chitosan sheet. Confluent hADSC were harvested after the 3<sup>rd</sup> passage with trypsin-EDTA solution (0,125 % (w/v) – 0,0625 % (v/w), Sigma-Aldrich). Cell pellet obtained after centrifugation was re-suspended in culture media with 10% Fetal Bovine Serum (Lonza) or platelet lysate (Cryocheck™) for SIS and chitosan patches, respectively. DMEM-F12 culture media (Invitrogen) which immersed each scaffold was settled and hADSC in suspension were seeded onto SIS and chitosan patches with a density of 50.10<sup>3</sup> cells per cm<sup>2</sup>. The culture media was changed every 2 days.

### 3.3- Adhesion and proliferation assays

Cells adhesion and proliferation rate onto SIS was assessed with the dimethylthiazol-diphenyltetrazolium bromide (MTT) assay (Sigma) via spectrophotometry (Dynex MRX<sup>®</sup>). Cells adhesion and proliferation on the chitosan scaffold was determined by fluorescence microscopy after nuclear staining (Cyquant<sup>®</sup> Cell Proliferation Assay – Invitrogen C7026). Results were expressed in percentages after normalization with the values of adhesion and proliferation on plastic wells. Times of assessment were 6 hours for the adhesion assessment and 48 hours and 168 hours for the proliferation assessment. Four independent experiments were performed.

#### **4- *In vivo* assessment**

Sixteen New-Zealand white rabbits (weight of 3 kg) were randomly divided into two groups: rabbits with cecum defect were given porcine SIS grafts (SIS group, n=8) and rabbits with cecum defect were given chitosan hydrogel grafts (Chitosan group, n=8). All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of Bordeaux University.

##### **4.1- Animal and anesthesia**

Each animal was restricted from any oral intake except for water 24 h before surgery. Rabbits were anesthetized with a combination of acepromazine (5mg/mL) and ketamine (100mg/mL) administrated intramuscularly. Anesthesia was maintained by propofol (2%) administrated intravenously during the operative procedure. Antibioprophylaxy was performed by TERRAMYCINE<sup>®</sup> LA (50mg/Kg) administrated intramuscularly at the end of the procedure.

##### **4.2- Operative procedure and postoperative management**



A transversal laparotomy was performed, and the proximal colon was identified and gently mobilized with atraumatic forceps. A 1×2-cm full-thickness wall defect was created on the antimesenteric side wall of the colon with scissors. The fixation of matrices was done with absorbable polyglycolic acid 6/0 single knot suture. Stitches were placed at a distance of 2 mm to the edge of the SIS patch which overlapped the serosa for this distance. Two single non-resorbable Prolene 4/0 sutures were placed at 1 cm from the margins of the matrices as markers for a later identification of the patch location. The abdominal cavity was closed in layers with absorbable sutures. In the postoperative period, the rabbits were examined daily by the surgeon for signs of wound infection, fever, abdominal pain, behavior, bowel movements, stool, and eating behavior.

#### 4.3- Graft specimen examination

Animals in each group were scheduled to be sacrificed for the evaluation of reconstructed colon at 4 and 8 weeks after transplantation with respectively 4 per time and per group. Immediately after sacrifice, laparotomy was performed and the grafted area was removed with surrounding tissues. The samples were fixed with 10% formalin then embedded in paraffin. The slides were stained with hematoxylin and eosin and examined with the light microscopy by the same pathology specialist blinded to the groups. Inflammatory cell infiltration and fibroblast activity were assessed. A fibrosis ratio was calculated and corresponded to the ratio of the fibrosis scar thickness to the native colonic wall thickness around the graft. Immunohistochemistry with staining for alpha-smooth muscle actin (Dako®) and caldesmon (Dako®) was performed to assess smooth muscle cells layer regeneration.

### **5- Statistical analysis**

All data were expressed as mean  $\pm$  standard deviation (SD). The difference between the two groups was compared by using the Mann-Whitney test. A p value  $<0.05$  was considered statistically significant.

## RESULTS

### 1- Formation of the three-layer chitosan hydrogel patch

For this study, a symmetrical three-layer patch composed by different chitosan physical hydrogels was developed as schematized in Figure 1.<sup>15</sup>

The processing physico-chemical conditions of the gel layer located in the central part of the matrix were optimized to provide high mechanical properties and to obtain a suturable final patch (final polymer concentration in the central layer: 7% (w/w); characteristics of chitosan: DA =  $1.5 \pm 0.3$  %,  $M_w = 420,000 \pm 40,000$  g/mol,  $I_p = 1.6 \pm 0.3$ ). This rigid gel was generated from a hydroalcoholic chitosan solution.<sup>11</sup> Thus, after complete dissolution of chitosan in an aqueous acetic acid solution, alcohol was added acting as a bridging agent by the establishment of hydrogen bonds with chitosan.<sup>16</sup> The following hydro-alcoholic medium was chosen: 50% water, 30% of glycerol and 20% of 1,2-propanediol. By evaporating the water and acid from the hydroalcoholic chitosan solution (evaporation in an oven at 50°C for 12h), the decrease of the apparent charge density of chitosan favours hydrogen bonding and hydrophobic interactions leading to the formation of an hydro-alcoholic chitosan gel.

In contrast, soft gel layers on each side were developed in order to ensure cell colonization and neo-tissue formation. Modulating physico-chemical parameters of chitosan physical hydrogel processing conditions was found to impact their biological properties.<sup>17, 18</sup>

Thus, an aqueous chitosan solution (chitosan concentration: 3% (w/w), DA =  $3.0 \pm 0.6$  %,  $M_w = 487,000 \pm 49,000$  g/mol,  $I_p = 1.1 \pm 0.2$ ) was prepared by dissolving a chitosan lyophilizate with the stoichiometric amount of acetic acid respectively to the amine groups of chitosan. Acetic acid was then further added in this solution (around 1  $\mu$ L acid / g of aqueous chitosan acetate solution). The rigid gel of the central part was then covered on both sides with this

acidic solution and it was superficially re-dissolved, inducing chain interdiffusion between the aqueous solution layers on both sides and the central hydrogel one.<sup>19</sup> After 90 minutes, the system was neutralized in 1M NaOH solution for 75 minutes. During this step, soft gels formed on both sides, and were adherent to the central layer.

The final 3-layered patch was then washed thoroughly with water to eliminate alcohols, NaOH in excess and salts. It was then sterilized by autoclaving at 121°C for 20 minutes before use. The thickness of the resulting patch was around 1 mm.

## **2- *In vitro* assessment**

### *Cell characterization*

hADSC cultures were successfully obtained from bariatric patients undergoing 73eotissue73ds73ie with a population doubling time of 2 days. Confluent cells were harvested after 3<sup>rd</sup> passage. Before seeding on SIS and chitosan scaffolds, flow cytometric analysis showed ADSC phenotype: positive expression for CD73 (84.7%), CD90 (97.9%), and CD105 (93.9%), but exhibited negative expression for CD34 (1.9%) and CD45 (0.8%) (Figure 2). Moreover, confluent cells were harvested from scaffolds 7 days after seeding and flow cytometric analysis showed that cells kept their hADSC phenotype in 90% of cases on both matrices (data not shown).

### *Cell adhesion and proliferation*

Cell adhesion and proliferation was quantified on both matrices and was assessed 6 hours after seeding. Live-dead staining allowed to observe a large majority of viable cells 6 hours after seeding on both scaffolds (see Figure 3). However, ADSC were more likely organized in clusters on the surface of chitosan scaffold while the distribution of ADSC on the surface of SIS scaffold appeared more homogeneous. ADSC adhesion and proliferation rate on the SIS

scaffold was 61%  $\pm$ 29% while it was 59%  $\pm$ 18% on the external chitosan hydrogel layer. This difference was not statistically significant ( $p=0.85$ ).

Seven days after seeding live-dead analyses for both scaffolds allowed observing a large majority of viable cells. ADSC adhesion and proliferation rates were 80%  $\pm$ 31% vs. 53%  $\pm$ 10% ( $p=0.01$ ) at 2 days and 51%  $\pm$  18% vs. 66%  $\pm$  17% ( $p=0.15$ ) at 7 days, respectively for SIS and chitosan scaffolds (Figure 4).

As a conclusion, from *in vitro* experiments, ADSC attachment and proliferation were obtained on both scaffolds without significant difference after 7 days. Moreover, after proliferation on both scaffolds, ADSC kept their mesenchymal stem cell phenotype without differentiation.

### **3- *In vivo* assessment**

#### *Surgical procedure and follow-up*

Both scaffolds were successfully implanted (Figure 5) with one death in each group due to bowel obstruction in SIS group and intra-abdominal abscess in chitosan group. There was no other postoperative complication. Animals tolerated their oral intake without difference between the two groups on the weight gain 60 days after implantation (Table 1). None of the 14 surviving animals showed clinical signs of illness due to colonic leakage, intra-abdominal infection or wound infection.

#### *Macroscopic assessment*

Macroscopic detection of the patches from the serosal view was difficult in both groups at 60 days due to complete or partial coverage of the patched area by reconstructed tissues. However, at 60 days, we observed less peritoneal adhesion in chitosan group suggesting a better control of inflammation for this scaffold. Macroscopic examination (Figure 6) showed a graft area contraction at 30 and 60 days in both groups without significant difference. Initial scaffolds grafted had an elliptic area of 1.57 cm<sup>2</sup> in both groups. Graft areas were 0.305  $\pm$

0.156 vs.  $0.245 \pm 0.277 \text{ cm}^2$  ( $p=0.72$ ) at 30 days and  $0.085 \pm 0.064$  vs.  $0.075 \pm 0.078 \text{ cm}^2$  ( $p=0.85$ ) and 60 days, respectively for SIS group and chitosan group.

#### *Microscopic assessment*

Infiltration by inflammatory cells and foreign body reactions surrounding the graft areas were evaluated. Chitosan fragments partly degraded by inflammatory cells (macrophages) were observed on the serosal side of the colonic wall. Moreover, the fibrosis ratio calculated for both scaffolds is presented in Table 1. The fibrosis ratio at 30 days and 60 days did not vary for SIS while in the same time it decreased 3-fold for chitosan.

Moreover, although non-significant, there is a tendency to a low fibrosis ratio in chitosan group compared with SIS at 60 days. Optical microscopy with hematoxylin and eosin staining showed a faster regeneration of the epithelial cell layer in chitosan group with a more organized architecture at 30 days. Regeneration of the epithelial cell layer was obtained in both groups at 60 days. Finally, at 60 days, partial regeneration of smooth muscle cells layer was observed around the fibrosis scar in the chitosan group and not in the SIS group (Figure 7). Immunohistochemical examination confirmed this smooth muscle layer regeneration. Indeed, an alpha-smooth muscle actin-positive layer and a caldesmon positive layer located in the inner portion of the graft and corresponded to the muscularis mucosae were observed in both groups. In contrast, a muscularis propria with smooth muscle cells was highlighted in chitosan group but not in SIS group where only fibrosis was observed (Figure 8).

Thus, from *in vivo* experiments, chitosan scaffold allowed to obtain a better tissue repair compared to SIS scaffold with a more effective inflammatory activity control and an *ad integrum* regeneration of the colonic wall around the fibrosis scar.

## DISCUSSION

Our study is the first colorectal tissue engineering evaluation comparing two scaffolds: chitosan hydrogel patches and SIS matrices. We report better outcomes with the un-cellularized chitosan scaffolds especially regarding inflammation control and the *muscularis propria* layer regeneration after *in vivo* experiments whereas no significant difference was evidenced by *in vitro* experiments.

Tissue engineering requires a reliable source of cells for neotissue formation. The cells that invade the scaffold play naturally a critical role in tissue engineering. They can be recruited and differentiated locally as tissue specific differentiated cells and/or from stem cells. The combination of biomaterials with cells was shown to reduce shrinkage of the graft.<sup>5, 20, 21</sup> Vacanti *and coll.* Seeded synthetic polymer tubes by colon organoid units and placed them into the great omentum.<sup>22, 23</sup> Organoid units are multicellular units, containing mesenchymal cell cores surrounded by a polarized epithelia derived from full-thickness sigmoid colon dissection from rat model. In this way, tissue-engineered colons exhibited architecture and major function of native colon. However, even if full-thickness wall colon dissection required for organoid units isolation is limited by clinical concerns, these outcomes highlight the crucial need for the association of scaffold and cells for colon tissue engineering. Moreover, one of the most difficult parts of bowel tissue engineering is to accomplish regeneration of a smooth muscle layer (SMC). Isolation and culture of smooth muscle cells were performed to rebuild a small bowel wall<sup>24, 25</sup> and internal anal sphincter.<sup>26, 27</sup> SMCs have previously been seeded on SIS<sup>25</sup> and collagen<sup>24</sup> scaffolds. The results from different studies are still controversial. From our knowledge, this work is the first report of the use of SMCs to engineer colon tissue. Ideally, the cell source used to seed the developing tissues should be non immunogenic, highly proliferative, easy to harvest and have the ability to differentiate

into application-specific cell types with specialized functions.<sup>28</sup> In the field of digestive tract disease, experimental and clinical researches have grown exponentially during the last decade<sup>29</sup> with several investigations about adipose-derived stem cells in the area of inflammatory, ischemic, fistulising and functional diseases. Adipose-derived stem cells may indeed provide a new promising cell source for colorectal tissue engineering. Moreover, ADSCs were also used for complex perineal fistulas treatment associated in randomized phase I and II trials with promising outcomes.<sup>30, 31</sup> Finally, ADSCs seeded on SIS were recently reported in two studies for the reconstruction of tracheal tissue<sup>32</sup> and tunica albuginea<sup>33</sup> with good functional outcomes. For these reasons, we compared ADSC adhesion and proliferation on both bioscaffolds to assess their cytocompatibility during *in vitro* experiments. We showed that ADSC attachment and proliferation were obtained with both scaffolds without significant difference. Moreover, after proliferation on both scaffolds, ADSC kept their mesenchymal stem cell phenotype.

SIS patches have been used in sheet form in multiple *in vivo* settings as a graft material in animal model, including esophagus,<sup>5</sup> stomach,<sup>6</sup> small intestine,<sup>7</sup> colon<sup>9</sup> and biliary duct.<sup>8</sup> Moreover, this matrix has been approved by the Food and Drug Administration (FDA) for abdominal wall repair. SIS is a rigid, cell-free collagen matrix comprising primarily fibrillar collagens and adhesive glycoproteins which serve as a scaffold onto which cells can adhere, migrate and multiply. The feasibility of using SIS as a *77eotissue77ds* for a 1 cm colonic full-thickness wall defect has been firstly assessed in a rat model.<sup>9</sup> Granulation tissue and inflammatory cells covered the defect at 3 weeks whereas the region of the graft was completely covered only at 6 months by a regenerated tissue including all layers of the native caecum wall. However, more recently, SIS scaffold was used to cover bigger full-thickness wall defects in the colon (4.5×1.5 cm) in pig model<sup>34</sup> with less promising outcomes. Contraction of the reconstructed area and limited organization of the *77eotissue* bowel wall



suggests strong limitations in colorectal wall regeneration. Authors suggested that bacterial contamination at the implantation site and other site-specific factors could be the reason for these findings which are in accordance with our outcomes regarding the SIS group. In contrast, chitosan has been reported to have antimicrobial and wound healing properties.<sup>35</sup> This polymer is a partially or fully deacetylated form of chitin and exhibits a range of physico-chemical behaviours as a function of DA.<sup>36</sup> In addition, it has been shown that modulating the physico-chemical parameters of chitosan physical hydrogel elaboration induces various biological response, a possibility that appears essential for tissue engineering applications.<sup>17, 18</sup> Moreover, mechanical properties of this kind of hydrogels depend mainly on chitosan concentration, DA<sup>11, 37</sup> and the neutralization kinetics. In experimental *in vivo* study, chitosan powder applied to colonic anastomosis had a positive effect in the process of collagenization in colonic anastomosis healing<sup>38</sup> and composite collagen/chitosan scaffold appeared to be suitable to support *in vitro* bioengineered smooth muscle intestinal constructs.<sup>39</sup> Tissue engineering of the colorectal tissue involves the regeneration of the smooth muscle layer. Our data confirm the capacity of chitosan scaffold to support smooth muscle layer regeneration *in vivo*. Moreover, chitosan scaffold biodegradability due to colonic microflora activity and to colonic enzymatic activity<sup>3</sup> may allow to control bacterial activity and inflammatory activity at the implantation site. A fibrosis ratio rate close to one at 60 days is related to a graft colonic wall thickness similar to those of native colonic walls. These findings reinforce the concept of tissue or organ specificity for the scaffold and should be confirmed by further experimental works.

Chitosan multilayered or multi-hydrogel matrix could be more suitable for colorectal tissue engineering than SIS scaffold and could be defined as the scaffold of choice for this field of tissue engineering. The soft hydrogel layers promote cell invasion and efficient tissue reconstruction of the entire structure of the colon wall, whereas the internal membrane

enables to close the defect avoiding colon leaks in the short term. Thus, the multigel architecture of the chitosan patch is essential in the quality of the reconstructed tissues and the mechanism of colon wall reconstruction itself. However, the transfer of these findings to a larger animal model with *in vivo* chitosan hydrogel patch implantation seeded or not by autologous ADSC is mandatory and in progress.

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## **AUTHOR DISCLOSURE STATEMENT**

No competing financial interests exist.

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## TABLES AND FIGURES LEGENDS

**Table 1:** Clinical and histological results (in vivo experiments)

**Figure 1:** Scheme of the Chitosan patch, made of three layers of chitosan hydrogels. The central layer can be sutured, whereas the external layers can be invaded by cells and act as a scaffold for tissue reconstruction.

**Figure 2:** Flow cytometry before seeding confirmed stem cells phenotype.

**Figure 3:** Live-dead 6 hours after seeding.

**Figure 4:** ADSC adhesion and proliferation at 6 hours (H6), day 2 (D2) and day 7 (D7).

**Figure 5:** Operative picture of scaffold implantation.

**Figure 6:** Macroscopic assessment of the graft area.

**Figure 7:** Microscopic assessment of the graft area.

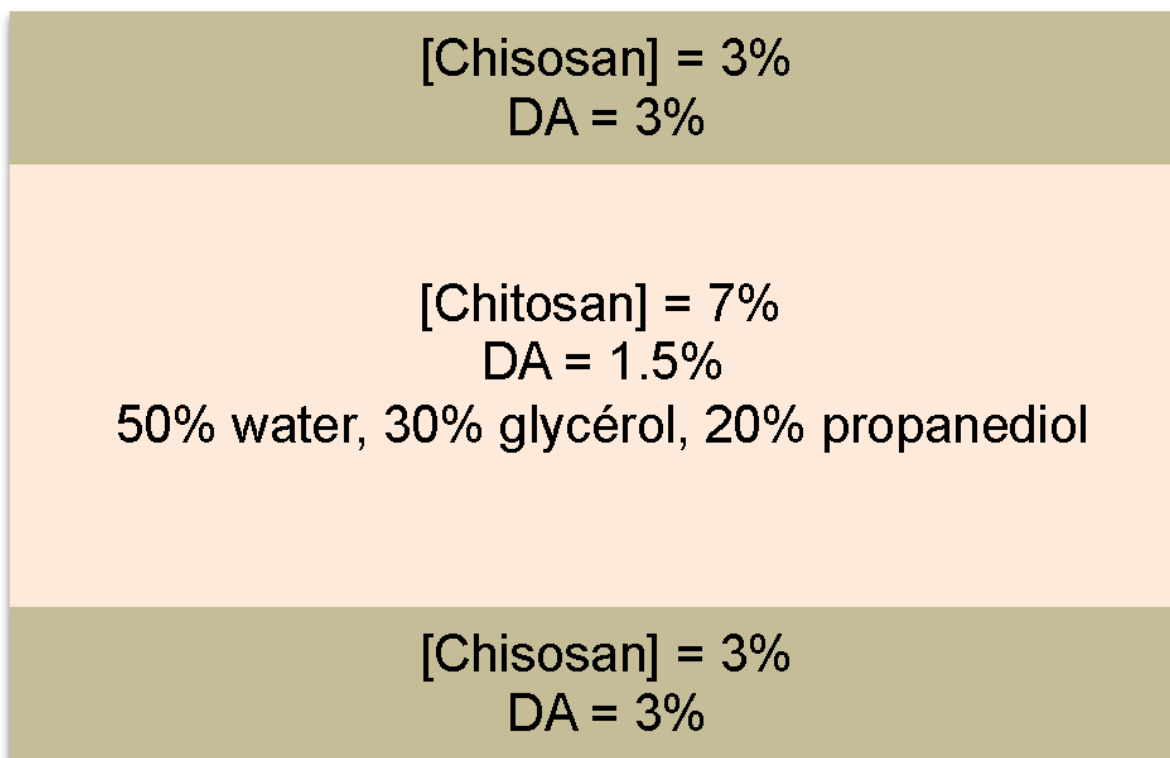
**Figure 8:** Immunohistochemistry assessment of the graft area with staining for alpha-smooth muscle actin and for caldesmon.



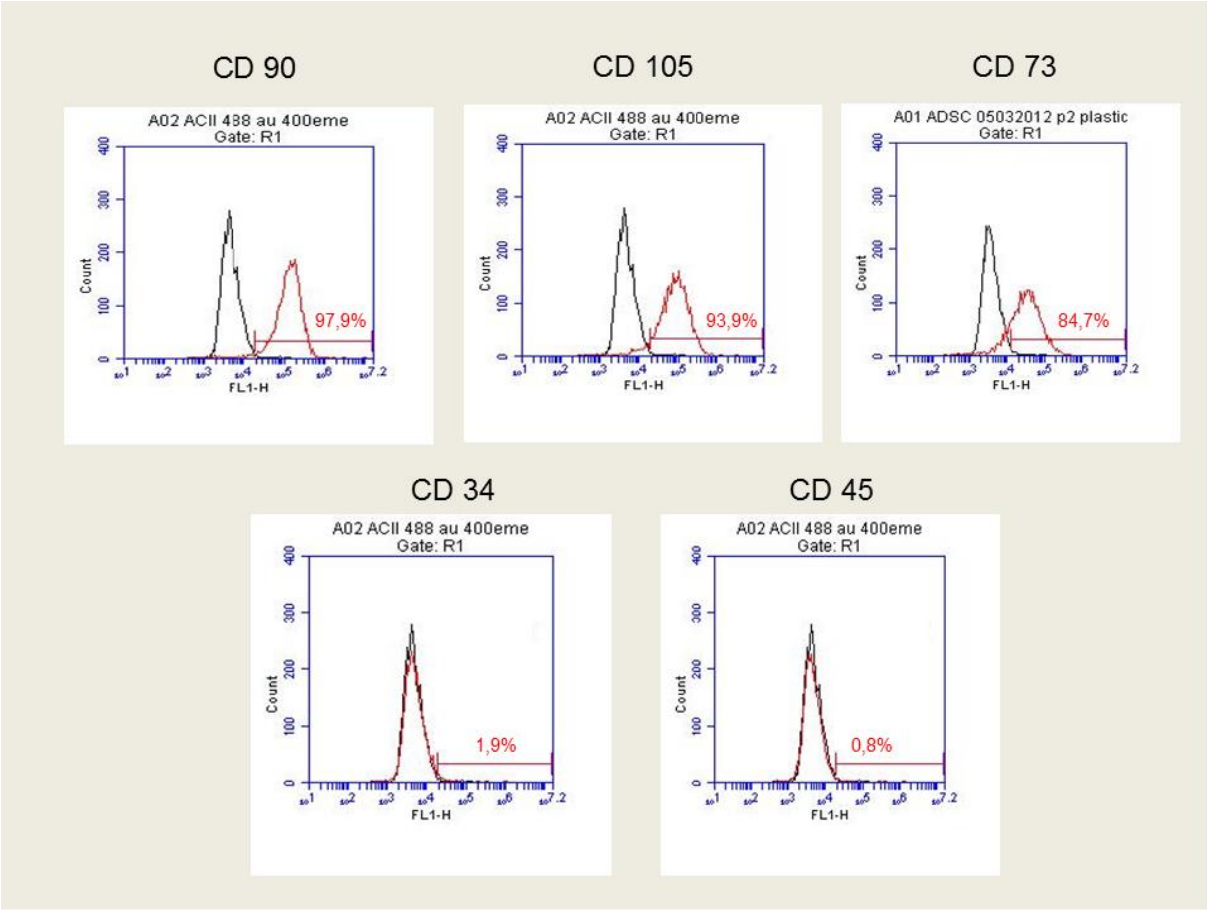
**Table 1. Clinical and histological results (in vivo experiments)**

	SIS	Chitosan	p
Death, n	1	1	-
Surviving, n	7	7	-
Weight gain at 60 days (g)	349 ± 137	494 ± 163	0.22
Graft area (cm <sup>2</sup> )			
- 30 days	0.305 ± 0.156	0.245 ± 0.277	0.72
- 60 days	0.075 ± 0.064	0.085 ± 0.078	0.85
Fibrosis ratio			
- 30 days	3.82	2.96	0.36
- 60 days	3.70	1.10	0.07

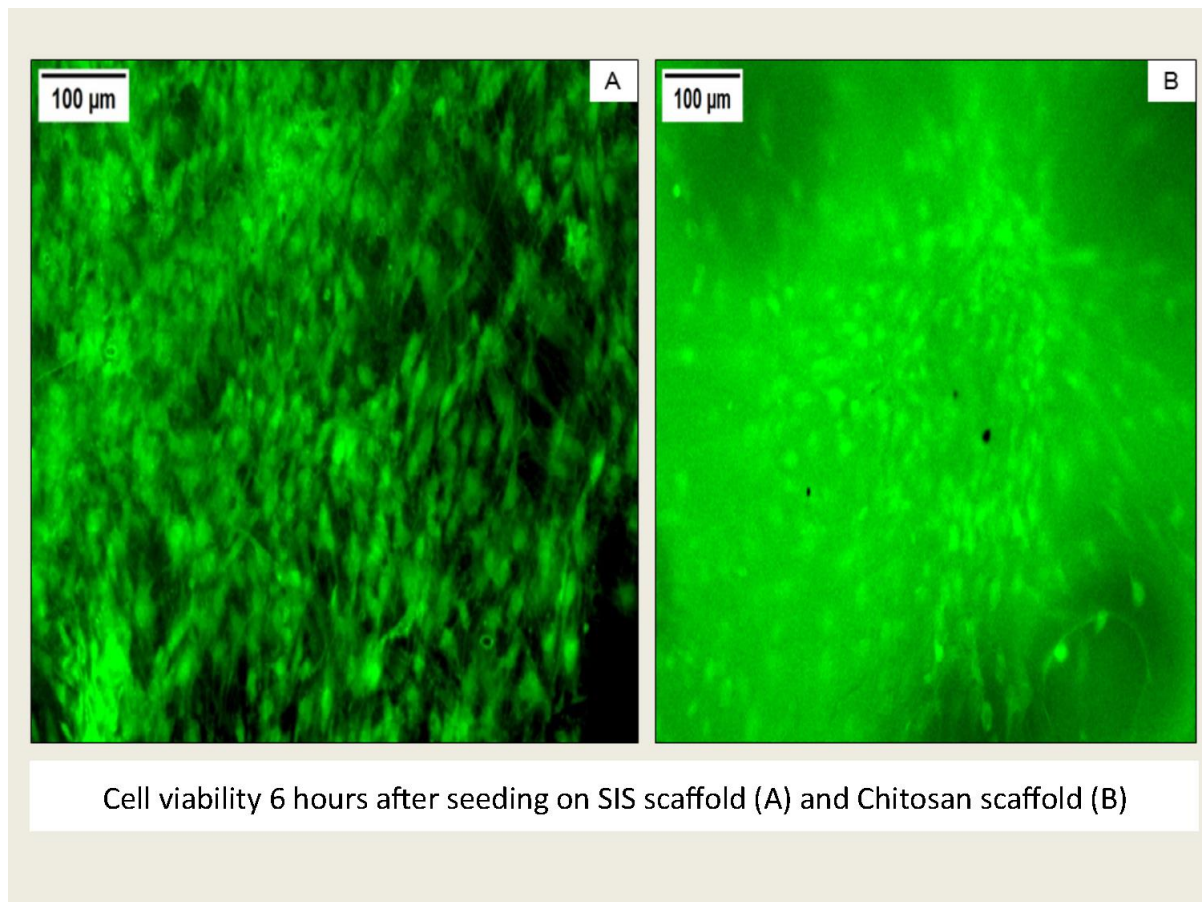
**Figure 1:** Scheme of the Chitosan patch, made of three layers of chitosan hydrogels. The central layer can be sutured, whereas the external layers can be invaded by cells and act as a scaffold for tissue reconstruction.



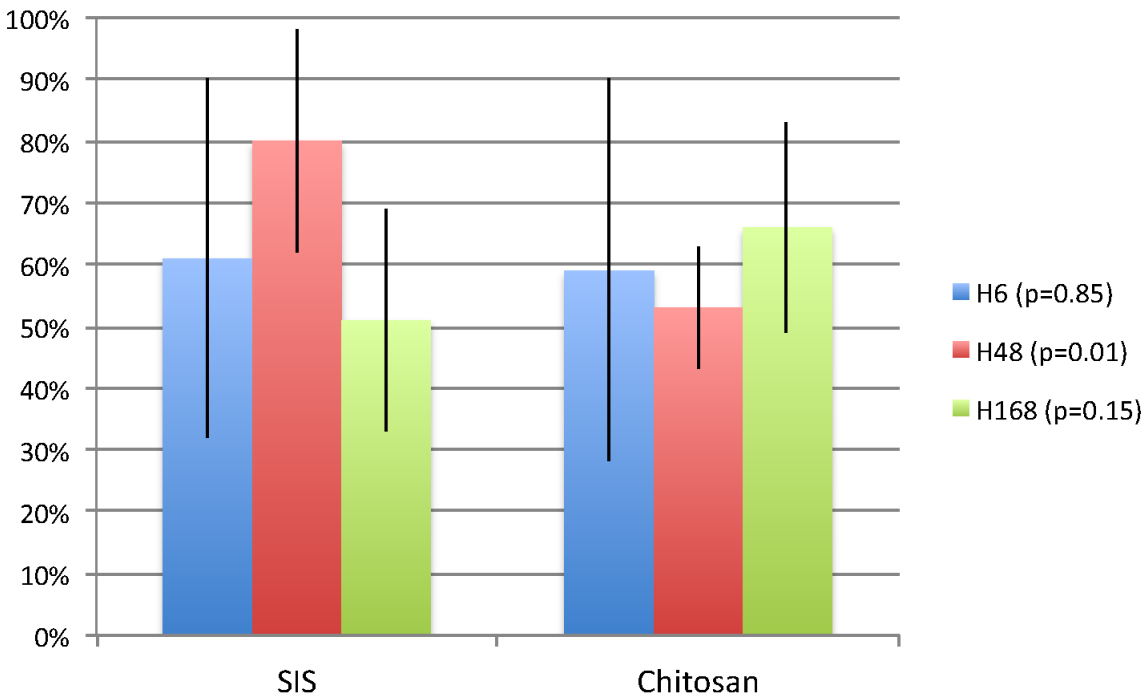
**Figure 2:** Flow cytometry before seeding confirmed stem cells phenotype.



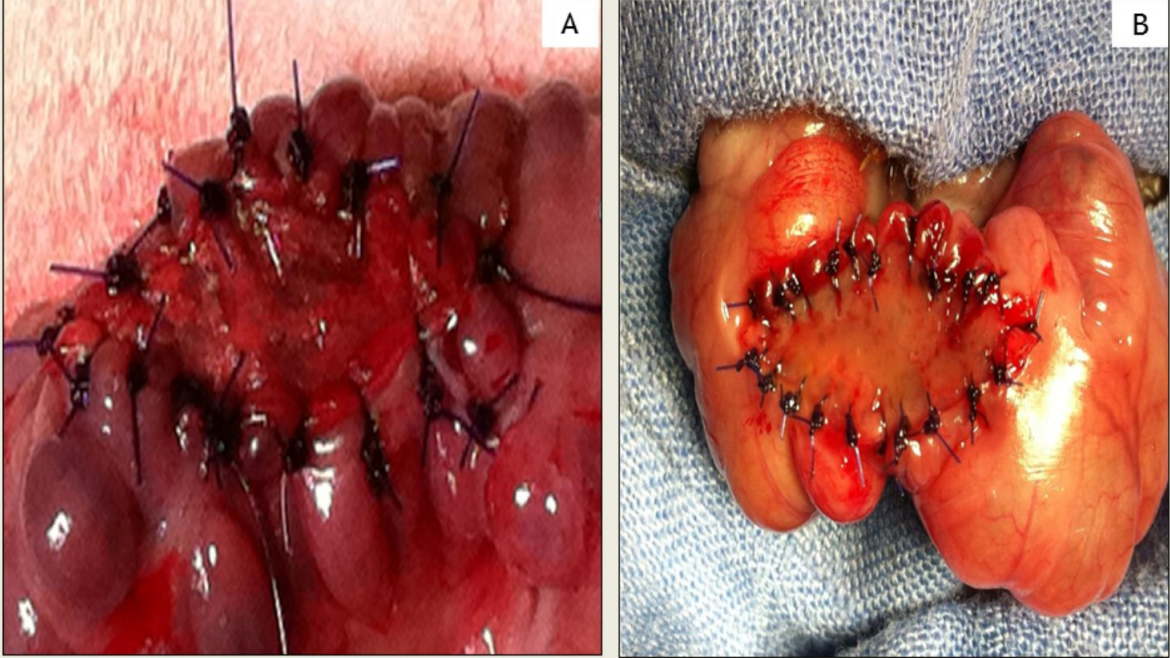
**Figure 3:** Live-dead 6 hours after seeding.



**Figure 4:** ADSC adhesion and proliferation at 6 hours (H6), day 2 (D2) and day 7 (D7).

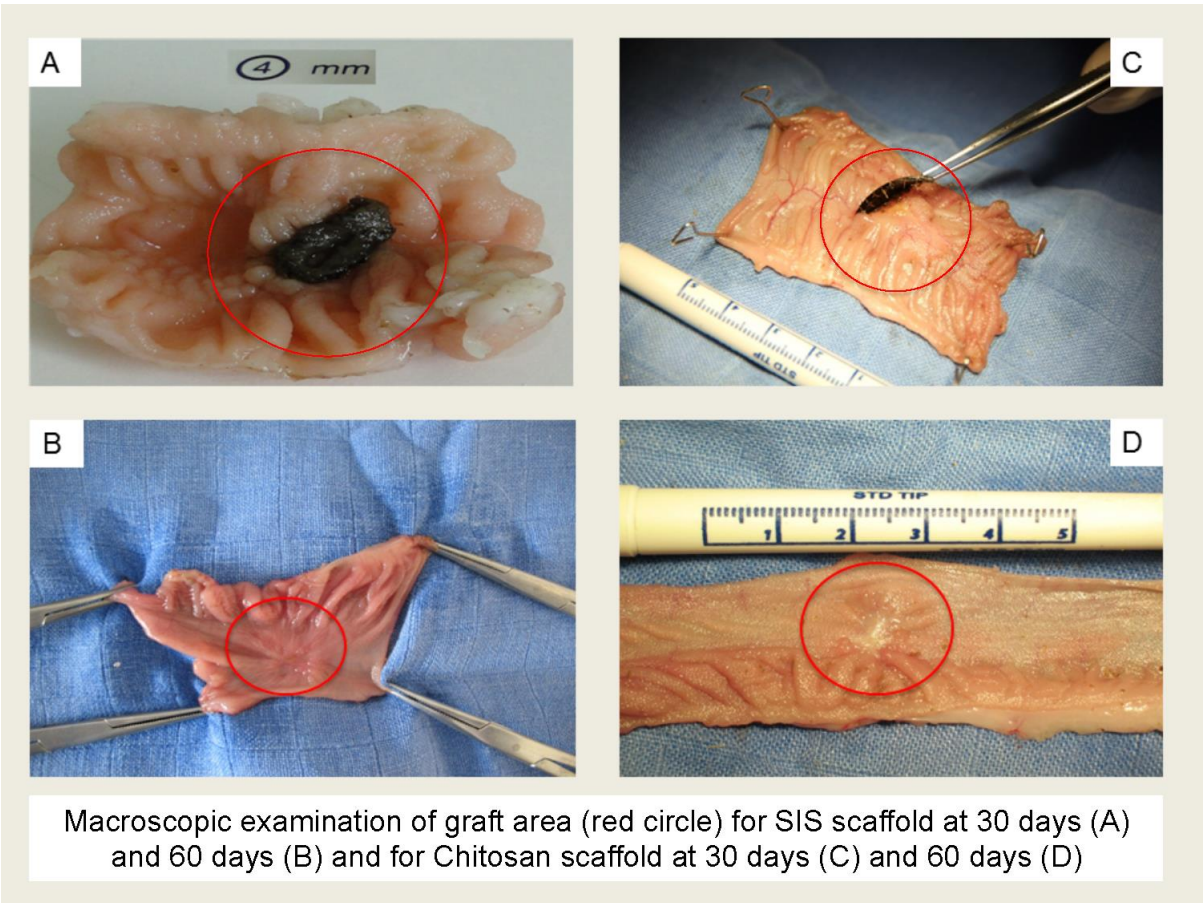


**Figure 5:** Operative picture of scaffold implantation.

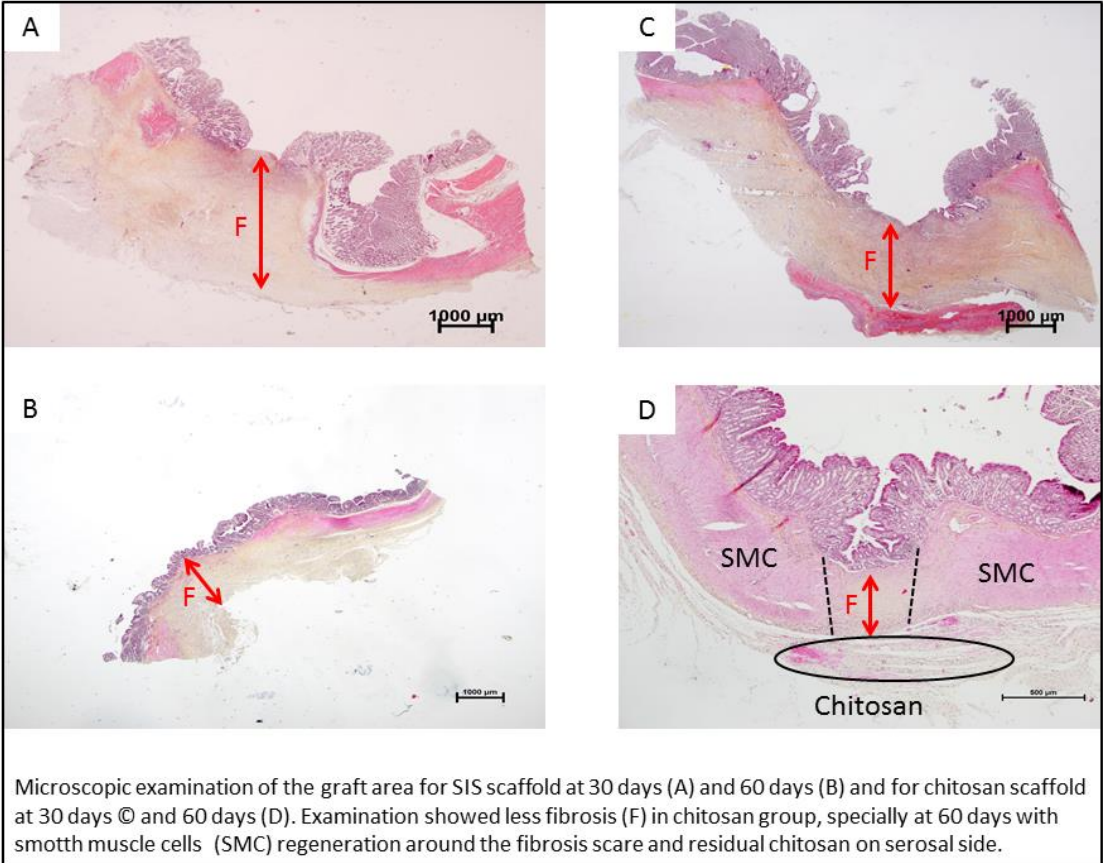


Intraoperative view of scaffold implantation: SIS (A), Chitosan (B)

**Figure 6:** Macroscopic assessment of the graft area.

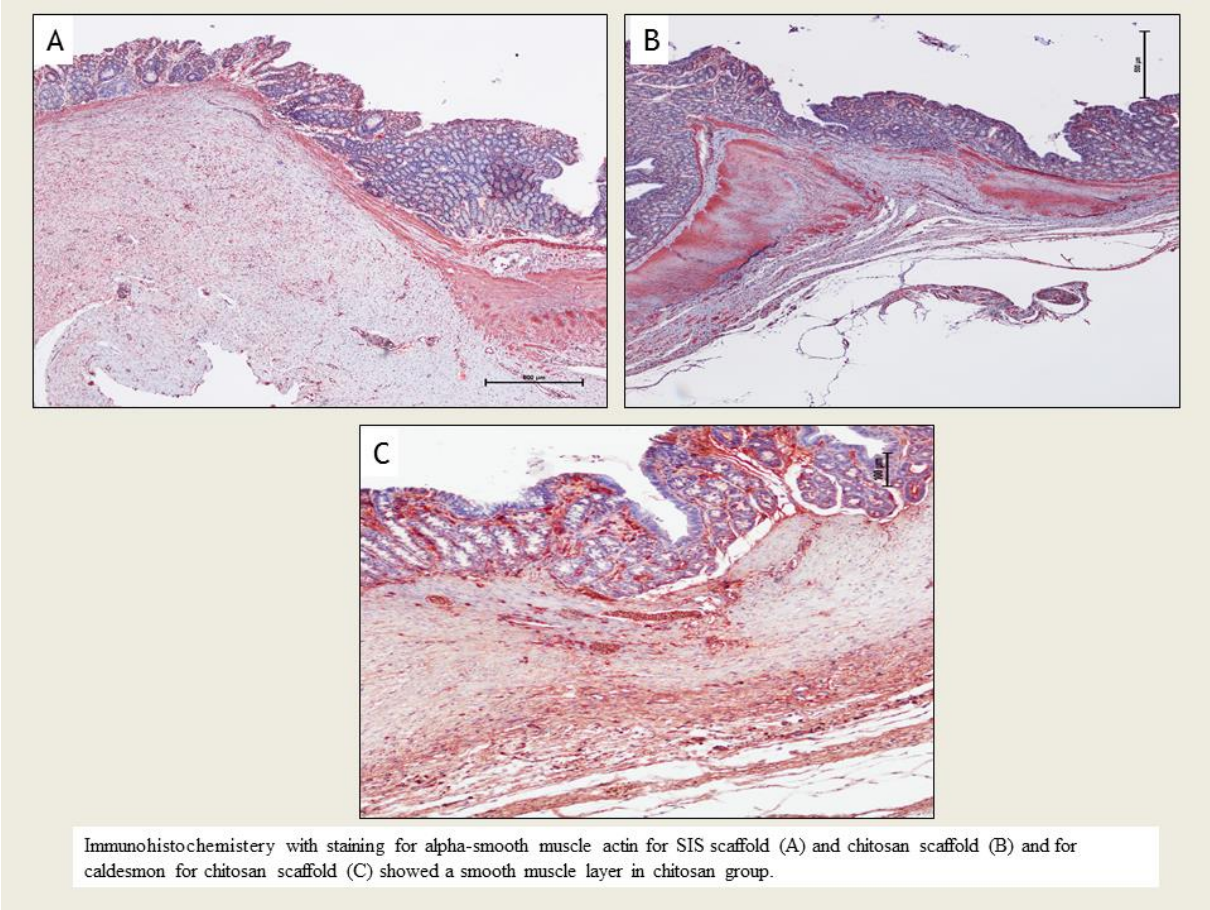


**Figure 7:** Microscopic assessment of the graft area.





**Figure 8:** Immunohistochemistry assessment of the graft area with staining for alpha-smooth muscle actin and for caldesmon.



# CHAPITRE II

Article Correspondant

Annexe 3

# **Colle composite Chitosane-Fibrine :**

## **Etude préliminaire pour une application**

### **en ingénierie tissulaire colorectale**

L'ingénierie tissulaire est née du mariage entre l'ingénierie des biomatériaux et la biologie cellulaire et moléculaire. Elle repose sur trois composantes principales : un matériau naturel ou synthétique biorésorbableensemencé de cellules différenciées ou non, créant alors un organoïde dont les conditions de culture humorales et mécaniques au sein d'un bioréacteur orientent le comportement en structure tissulaire tridimensionnelle. Au cours de la phase de culture de l'organoïde, des phénomènes biologiques et physico-chimiques complexes interviennent et interagissent entre eux à divers échelles de temps et de longueur. La complexité de ces phénomènes du vivant tend à faire penser que le seul bioréacteur permettant de transmettre les signaux humoraux et mécaniques nécessaires à la maturation de l'organoïde reste le site d'implantation *in vivo* du matériau. En outre, les contraintes de temps propres à la culture cellulaire et à l'ensemencement matriciel apparaissent non adaptées à la démocratisation future de la pratique clinique de l'ingénierie tissulaire. Le choix des cellules ensemencées et de la procédure de cellularisation matricielle doit permettre une utilisation per opératoire reproductible répondant à ces deux contraintes principales. Ainsi, la fraction stromale vasculaire (SVF) issue du tissu adipeux présente l'avantage de ne pas nécessiter le passage préalable par une culture cellulaire *in vitro*. Des dispositifs médicaux automatisés, dont le système Celution® (Cytori Therapeutics, San Diego, Etats-Unis), ont ainsi été développés dans le but de faciliter l'isolement et l'utilisation extemporanée de la fraction vasculaire stromale au bloc opératoire.

La confection d'un système de confinement et de délivrance des cellules sur le site d'implantation de la matrice nous est donc apparu nécessaire pour évoluer dans cette voie du « tout per opératoire » en ingénierie tissulaire.

Nous avons mis au point une colle biologique conjuguant la fibrine avec une solution de chitosane afin d'obtenir un gel original dans sa capacité à diriger et maintenir localement l'ensemencement cellulaire grâce aux propriétés structurelles et biochimiques des deux composés. Les propriétés structurales et biochimiques de la fibrine en font une matrice biologique naturelle idéale en médecine régénératrice. Biodégradable par fibrinolyse, elle est

une matrice bioactive dans sa capacité à lier des facteurs de croissance spécifiques et à interagir avec de nombreuses intégrines présentes à la surface des cellules. Dans ce cadre, les différents avantages, limites et applications des colles biologiques de fibrine sont discutés dans l'article en annexe 3.

La préparation de la solution de chitosane a nécessité le choix d'une formulation (DA et concentration) permettant d'obtenir sa neutralisation tout en préservant au maximum les propriétés biologiques du polymère. Celles-ci sont essentiellement une activité bactériostatique et une stimulation de la prolifération cellulaire.

Différentes formulations de fibrine et d'hydrogels physiques de chitosane ont été élaborées en modulant 2 paramètres physico-chimiques : les concentrations de chacun des composants (thrombine, fibrinogène et chitosane) et le degré d'acétylation du chitosane. La maniabilité, la viabilité et la prolifération cellulaires, l'ultrastructure et les qualités rhéologiques de chaque formulation ont été évaluées permettant de définir une formulation de la colle composite Chitosane-Fibrine répondant au cahier des charges.

## Article – Annexe 3

(Soumis à Tissue Engineering – Part C)

**Title:** Composite Chitosan-Fibrin glue in a preliminary study for colorectal tissue engineering application

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## **AUTHOR CONTRIBUTIONS**

**Study concept and design:** Quentin Denost, Arnaud Pontallier, Laurence Bordenave

**Acquisition of data:** Quentin Denost, Arnaud Pontallier, Alexandra Montembault, Robin Siadous, Reine Bareille

**Analysis and interpretation of data:** Quentin Denost, Arnaud Pontallier, Marlene Durand, Laurence Bordenave

**Drafting of the manuscript:** Arnaud Pontallier, Quentin Denost, Laurence Bordenave

**Critical revision of the manuscript for important intellectual content:** Quentin Denost, Arnaud Pontallier, Laurence Bordenave, Alexandra Montembault, Laurent David

**Statistical analysis:** Quentin Denost, Arnaud Pontallier

**Obtained funding:** *Fondation de l'avenir*

## INTRODUCTION

Tissue engineering uses biological substitutes to restore, maintain or improve functions of tissue<sup>1</sup>. It may provide alternative therapy to conventional colorectal surgery in electives indications such as treatment of anastomotic or perineal fistula and rectal wall healing after local excision for cancer<sup>2</sup>. This approach involves the use of three components: (1) a synthetic or natural biomaterial from which the extracellular matrix (ECM) is organized in neotissue, (2) cells seeded *in vitro* on the biomaterial, and (3) humoral and mechanical signals which regulate the behaviour of the first two<sup>3</sup>. However, cell seeding requires an adequate time of cell adhesion to obtain optimal biomaterial implementation. *In vitro* step in tissue engineering process is not relevant for routine clinical application. Seeding and implementation should be carried out simultaneously ensuring a more efficient and less time-consuming procedure. Fibrin glue may be a good candidate allowing cell growth and biomaterial implementation during the same operative procedure. Fibrin in combination with various cell sources was effective in a variety of tissue-engineering applications<sup>4</sup>. However, experience in colorectal bioengineering is scarce including only chronic intestinal fistulae healing with moderate success rate<sup>5</sup>. Therefore, we have assessed in this study new biological composite glue able to deliver cells on a selected scaffold during the operative course of colorectal tissue engineering.

Two components, chitosan and fibrin, have been combined in various formulations and concentrations. Firstly, the structural and biochemical properties of the fibrin polymer and its suitability as a cell delivery system make it a promising candidate. Secondly, the chitosan is a partially or fully deacetylated form of chitin, composed of glucosamine and N-acetyl glucosamine units linked by  $\beta$  (1–4) glycosidic bonds. The degree of acetylation (DA) of chitosan corresponds to the molar fraction of acetyl units within the polymer chains. Its unique biological properties including biocompatibility, biodegradability, low toxicity, antimicrobial and haemostatic properties as well as its versatility in chemical modification are very interesting for tissue engineering<sup>6</sup>. Moreover, its biodegradability due to colonic microflora activity and to colonic enzymatic activity<sup>7,8</sup> used in colonic specific drug delivery reinforce the concept of tissue or organ specificity<sup>9-11</sup>. For these reasons, chitosan could be a promising scaffold for colorectal tissue engineering. Thus, in the present study, we combined a fibrin sealant able to sustain cell growth and a chitosan solution providing specific biologic properties for cell delivery in colorectal tissue engineering. We assessed cells viability, ultrastructure and mechanical properties of this composite glue.



## MATERIALS AND METHODS

### 1- Composite glue components and preparation

#### 1.1 Chitosan component

Purified chitosan was N-acetylated with acetic anhydride in homogeneous medium to obtain polymers with different DA. The reaction was performed in a hydroalcoholic medium (water-propanediol). After reaction, chitosans were neutralized with dilute ammonium hydroxide, rinsed with deionized water, and then freeze-dried. The DA of chitosan was determined by  $^1\text{H}$  NMR spectroscopy. The weight-average molecular weight ( $M_w$ ) and the molar-mass dispersity were measured by gel permeation chromatography coupled with a multiangle laser light scattering (MALLS) instrument. Chitosan was dispersed in de-ionized water and acetic acid was added to achieve the stoichiometric protonation of the  $-\text{NH}_2$  sites. Then, the system was kept under magnetic stirring at room temperature until complete dissolution of chitosan (minimum 12 h of stirring). In order to ensure viability of seeded cells, it was necessary to use a neutral chitosan solution close to physiological pH value. However, chitosan is insoluble in most solvents at neutral or high pH, except in organic acids.

The solution homogeneity and viscosity at pH 7 were studied in the case of various formulations (concentration and DA) of chitosan. The solutions were alkalinized with dilute ammonia (pH 10.5) up to a constant pH close to 7.

The pH of the samples was measured directly with a contact electrode (IQ 240, IQ Scientific Instruments).

#### 1.2 Fibrin component

Fibrin gel was prepared using a Tissucol<sup>®</sup> kit (Baxter Healthcare, Bioscience Division), according to the manufacturer's instructions. The major components are fibrinogen, thrombin, calcium chloride, factor XIII and aprotinin. Components are formulated as two solutions, which are mixed together in equal volumes to mimic the final phase of the coagulation cascade. Fibrin gels were identified by their respective fibrinogen/thrombin concentration (FC and TC, respectively). In accordance with the literature, thrombin concentration was fixed at 100 U/mL<sup>12</sup>:

- Formulation A: [FC] = 90 mg, [TC] = 100 U/mL
- Formulation B: [FC] = 40 mg, [TC] = 100 U/mL
- Formulation C: [FC] = 20 mg, [TC] = 100 U/mL
- Formulation D: [FC] = 10 mg, [TC] = 100 U/mL
- Formulation E: [FC] = 5 mg, [TC] = 100 U/mL

### **1.3 Composite glue preparation**

Fibrinogen and thrombin solutions were diluted to the appropriate concentration (formulation A to E), using Phosphate Buffer Saline solution (PBS) and chitosan solution, respectively. Fibrinogen solution was added to the thrombin-chitosan solution at 1:1 ratio to form the composite glue.

## **2- Assessment criteria**

### **2.1 Cells isolation, culture and characterization for *in vitro* experiments**

We have used human Adipose-Derived Stem Cells (hADSC) which may provide a new promising cell source for tissue engineering. hADCs were isolated and cultured as previously described by Bunnell et al<sup>13</sup> with minor modifications<sup>14</sup>. Briefly, human adipose tissue was obtained from bariatric patients undergoing abdominoplasty. Tissue samples were minced and digested in a solution of 0.1% (w/v) collagenase type I (Worthington) for 1h30 at 37°C with vigorous shaking. After filtration and centrifugation, the top lipid layer was removed and the remaining Stromal Vascular Fraction (SVF) was treated for 10 minutes with ELB (Erythrocyte Lysis Buffer; 155 mM NH<sub>4</sub>Cl (Sigma), 5.7 mM K<sub>2</sub>HPO<sub>4</sub> (Sigma), 7.4 mM K<sub>2</sub>HPO<sub>4</sub>-3H<sub>2</sub>O (Sigma), 0.1 mM EDTA (Sigma) and then centrifuged. The pellet was resuspended in basal medium, DMEM F12 medium (Invitrogen) supplemented with 10% (v/v) Fetal Bovine Serum (Lonza) and sequentially filtered through 100, 70 and 40 µm cell strainer (BD Falcon). Cells were plated (10<sup>4</sup> cells/cm<sup>2</sup>) and cultivated at 37°C in 5% CO<sub>2</sub>. Culture medium was replaced every three days when hADSC reached 80% of confluence. Flow cytometry analysis was used to determine specific markers on the cultured cells before seeding. ADSCs in suspension were mixed with fluorochrome-conjugated antibodies against CD73, CD90, CD105, CD31, CD34 et CD45 (eBiosciences®).

### **2.2 Cells viability and proliferation**

To analyze the effects of variations of the fibrinogen concentrations on cell viability, composite glue was prepared using formulations A to E. Upon cell isolation with trypsin, the incorporation of resulting ADSCs into the gel was achieved by re-suspending 0.1 X 10<sup>6</sup> cells in 150 µL of thrombin-chitosan solution. For each formulation, fibrinogen solution (150 µL) was added to each well of a 24-well plate simultaneously with 150 µL of the cell-thrombin-chitosan solution. In order to avoid cell adhesion to the plastic plate, agarose gel 2% (p/v) was deposited under all composite glues. Fibrin-chitosan clot with the entrapped ADSCs was allowed to solidify at room temperature for 2 hours. Afterward, the 3D fibrin-chitosan clots were washed with basal medium, each given 1 mL of growth medium, and then incubated at

37°C in 5% humidified CO<sub>2</sub> incubator. Growth medium was changed every 2 days until the day of harvest. As a control, ADSCs were cultured on tissue-culture dishes. A Live/Dead staining (Molecular Probes) was performed at days 1, 3, 7 to label live and dead cells. Four plates per concentrations were analyzed. The procedure was performed three times. At each time point, culture medium was removed from the 24-well plates. Fibrin-chitosan gels were washed with 1 mL of warm serum-free medium and labeled with the live/dead dye solution at room temperature and in the dark for 50 min. Five microliters of 2 microM ethidium homodimer-1 and 1.25 of 4 microM calcein was added to 5 ml of PBS. The fibrin-chitosan clots were then rinsed with serum-free medium to wash away residual stain and observed with an inverted fluorescent microscope equipped with a digital imaging acquisition system.

We compared Human ADSCs proliferation in fibrin glue and 3D composite glue using various fibrinogen concentrations from 10 to 40 mg/mL. We used the Cyquant® Cell Proliferation Assay (Invitrogen). Cell incorporation into the fibrin glue was achieved by re-suspending  $0.1 \times 10^6$  ADSCs in 100µL of diluted thrombin-hank's solution. For composite glue, cells were incorporated into diluted thrombin-chitosan solution. Six composite glue samples were prepared for each fibrin formulation. Times of assessment were 1 and 4 days. We used control fibrin glue seeded with cells, control composite glue (no cells) for gel background fluorescence measurement, and control plastic with cells. The procedure was performed 2 times. At each time point, culture medium was removed from the 24-well plates and gels were washed with HBSS. Then, plates were frozen at -80°C until needed for experimentation. All plates were then thawed at room temperature, and 300µL of the Cyquant GR dye lysis buffer was added to each sample well. The samples were incubated in darkness for 2 to 5 minutes at room temperature. The fluorescence of each sample was measured with a CytoFluor 2350 fluorescence microplate reader PerSeptive Biosystems, Bedford, ME with 480 nm excitation and 520 nm emission filters. The fluorescence emission of the dye–nucleic acid complexes correlated linearly with cell number.

### **2.3 Scanning electron microscopy**

Seeded composite glues were allowed to form for 2 hours at room temperature before adding a 2.5% glutaraldehyde solution with cacodylate 0.1M and allowing the clots to fix for 2 hours at room temperature and then overnight at 4°C.

The clots underwent a graded ethanol series consisting of 5-min steps of 30%, 50%, 70%, 80%, 90%, and 100% ethanol. Next the ethanol was replaced with 100% hexamethyldisilazane (HMDS) under the hood. Finally the clots were air dried overnight, mounted, sputter coated with gold and visualized under a scanning electron microscope. Microphotographs were taken at 3.500X and 7.000X magnifications. Various formulations of the composite glue were assessed.

## **2.4 Mechanical properties**

Dynamic rheological measurements were performed with a controlled stress rheometer (ARES, TA Instruments), using parallel plates of 25 mm with a gap varying from 1 to 1.5 mm, depending on the samples. We studied the evolution of shear storage (or elastic) modulus and shear loss (or viscous) modulus respectively  $G'$  and  $G''$ , through time sweep measurements with a fixed strain of 5%. Immediately after preparation, glues were placed between the plates. Frequency sweep tests from 100 to 0.1 rad/s were also carried out with a strain of 5%. The temperature was 22 °C for all rheology measurements. Composite glues were prepared using fibrinogen concentration from 10 to 20 mg/mL.

## **3- Statistical analysis**

All data were expressed as average value. The difference between groups was compared by using the Mann-Whitney test. A p value <0.05 was considered statistically significant.

Moreover, composite glue formulations from A to E were selected gradually step by step with assessment criteria previously described. Thus, all formulations were not assessed in each step but only those selected in the previous one.

## RESULTS

### 1- Composite glue preparation

Before mixing chitosan with fibrin glue component, chitosan concentration and DA were shown to drastically affect the chitosan solubility at pH 7. Polymer precipitation occurred as soon as the chitosan concentration increased over 1% and DA decreased below 38% (Table 1). Moreover, solutions with polymer concentration higher than 1% had a viscosity unsuitable for optimal fibrin polymerization. In accordance with these macroscopic outcomes, composite glue preparation was performed with chitosan concentration varying from 0.5% to 1% and DA varying from 38% to 48%.

Upon mixing chitosan with fibrinogen, we observed an immediate process of emulsification with aggregates formation and subsequent heterogeneous solution unsuitable for composite glue preparation. This reaction increased with fibrinogen concentration. It was more relevant to mix chitosan with thrombin (Table 2). The solutions were homogeneous whatever thrombin concentration varying from 100 to 500 U/mL.

### 2- Cell viability and proliferation assessment

ADSC cultures were successfully obtained from bariatric patients undergoing abdominoplasty with a population doubling time of 2 days. Flow cytometric analysis showed hADSC phenotype: positive expression for CD73 (84.7%), CD90 (97.9%) and CD105 (93.9%) and negative expression for CD34 (1.9%) and CD45 (0.8%).

Live-dead analysis of ADSCs stained in the clot showed mainly viable cells (calcein) and a negligible amount of dead cells (ethidium) for all fibrinogen formulations using a chitosan solution with a DA of 38%. Addition of neutral chitosan solution in fibrin glue did not alter cell viability assessed by Live-dead test (Figure 1). However morphology differed depending on the fibrinogen concentrations (Table 3). Immediately after encapsulation, ADSCs were identifiable as round individual cells when observed with contrast microscopy. In clots containing low fibrinogen concentrations (FC 5, 10, 20 mg/mL), cells were found homogeneously distributed and started to spread and exhibit a fibroblast morphology as early as day 1. Interconnected network within the gel was formed as soon as day 4 for these lower fibrinogen concentrations. In the clot containing medium fibrinogen concentration (FC 40 mg/mL), we observed morphologies ranging from rounded to fusiform-shaped cells. However, majority of cells exhibited round morphology with few interconnected network on day 4. Finally, in clots containing higher fibrinogen concentration (formulation A), cells failed to spread and we observed most round or only "star shaped" cells (Figure 1). Cells behavior was optimal with low fibrinogen concentrations (FC 5, 10, 20 mg/mL). However, when using lowest fibrinogen concentration (FC 5 mg/mL), fibrin construct was poorly handled and

prematurely degraded during in vitro culture. Thus, cell proliferation tests in composite glue were performed with intermediate and low fibrinogen concentrations (FC 40, 20, 10 mg/mL) to assess objectively cells behavior over time.

We examined ADSCs proliferation within fibrin and composite glues (Figure 2). The proliferation rate varied depending on the fibrinogen concentration. ADSCs tended to proliferate into the fibrin glue and composite glue at day 4 except for the fibrin glue formulation B (40 mg/mL). Proliferation rate increased with lower fibrinogen concentrations (FC 10 and 20 mg/mL) at day 4 in the composite glue compared to higher concentrations (FC 40 mg/mL). The difference was statistically significant ( $p=0.002$ ). Chitosan addition resulted in an increasing proliferative pattern compared with fibrin alone for all fibrinogen concentrations ( $p<0.001$ ).

### **3- Scanning electron microscopy**

To investigate the formulation effects on fibrin ultrastructure, we examined the structure of the 3D composite clots with electron microscopy. Analysis revealed significant structural differences at various fibrinogen concentrations. As fibrinogen concentrations decreased (FC 10 and 20 mg/mL) fiber size increased and a more open pore structure was observed (Figure 3. A D, B E). Higher fibrinogen concentration (FC 90 mg/mL) resulted in the formation of highly interconnected network with finer fiber structure and smaller pores (Figure 3. C F). Whatever the fibrin formulation, chitosan addition did not seem to affect fibrin ultrastructure. The embedded cells were homogeneously distributed throughout the composite glue with low fibrin formulation (FC 10 mg/mL). The porous structure of the fibrin allowed cell adhesion (Figure 4).

### **4- Mechanical properties**

The rheology measurement of the composite glue showed the typical shear thinning behavior of a gel with a storage moduli ( $G'$ ) more than five times higher than loss moduli ( $G''$ ) at a wide range of frequency (Figure 5 A). A continuous measurement was performed to investigate the evolution of the moduli when fibrinogen concentration increased (FC from 10 to 20 mg/mL) and when a chitosan solution was added to the fibrin clot (Figure 5 B). Due to the very fast fibrin polymerization reaction, we could not observe the gel point defined by the crossover of the two moduli ( $G'$  and  $G''$ ) variations curves. Concerning the influence of the initial fibrinogen concentration, the values of  $G'$  increased with this concentration (Table 4). Whatever the fibrin formulation used, chitosan addition (concentration varying from 0.5% to 1%, DA 38%) did not modify rheological properties of fibrin glue.

## DISCUSSION

We have developed a new composite glue by combining a fibrin sealant able to sustain cell growth and a chitosan solution providing specific biologic properties to create an efficient vehicle for cell delivery usable in colorectal tissue engineering.

Several investigations about fibrin glue with or without stem cells have been performed in the area of inflammatory, ischemic, and fistulising diseases<sup>5,15,16</sup>. The management of complex perianal fistulas in crohn's and non crohn's disease remains challenging. Many patients do not respond to conventional treatments and tolerance may be poor. The use of fibrin glue alone has recently emerged as a simple procedure for the treatment of cryptogenic perianal fistulas<sup>17</sup>, but the experience is still scarce with low short-term success rate<sup>18-20</sup>. In a randomized clinical trial, fibrin glue was effective for the treatment of simple perianal fistulas in patients with Crohn's disease. However, the success rate appeared very low in complex fistulas especially in ano- or rectovaginal tracts<sup>21</sup>. In a phase II study comparing administration of ADSCs and fibrin glue with instillation of fibrin glue only in Crohn's and non Crohn's-related perianal fistulas, Garcia-olmo et al achieved higher healing rates with ADSCs plus fibrin glue<sup>22</sup>. Despite these promising results, non statistically differences were found in the randomized phase III trial when the 2 groups were compared<sup>5</sup>. These results may suggest a current lack of efficacy of fibrin glue to promote cell delivery in colorectal tissue engineering and highlight the need for further improvement. Fibrin contains growth factors and cell adhesion molecules including fibronectin stimulating cell proliferation and angiogenesis<sup>23,24</sup>. In addition fibrin naturally expresses inherent binding sites for various types of integrins<sup>25-27</sup> which results in increased cell adhesion and migration<sup>28</sup>. Several studies indicate that fibrin supports cell adhesion and favors cellular proliferation of various cell types<sup>29,30</sup>: endothelial cells<sup>31</sup>, chondrocytes<sup>32,33</sup>, myoblasts<sup>34</sup>, vascular smooth muscle cells<sup>35</sup>, Schwann cells<sup>36</sup> and mesenchymal stem cells (preadipocytes<sup>37-39</sup>, BMSCs<sup>12,23,29,40,41</sup>). Retaining and concentrating freshly formed extracellular matrix and desirable factors secreted by seeded cells are another property attributed to fibrin<sup>37</sup>. Fibrinogen structure, thrombin activation and fibrin polymerisation have been largely investigated<sup>25,42</sup>. It has been established that the viscoelastic properties of the gel depends on polymerisation conditions<sup>41</sup>. Fibrin-based materials can be obtained from commercial sources or generated from autologous blood including the platelet rich plasma (PRP) or the platelet rich fibrin (PRF). We used a commercial fibrin in order to avoid the isolation and extraction procedures, difficult to implement when clinical practice will be concerned.

Chitosan properties highlight the concept of tissue or organ specificity for the scaffold. Indeed biodegradability due to colonic microflora activity and to colonic enzymatic activity<sup>7</sup> may control bacterial activity and inflammatory activity. Moreover, in experimental *in vivo* study, chitosan powder applied to colonic anastomosis had a positive effect in the process of collagenization in colonic anastomosis healing<sup>43</sup>. We used a pure chitosan only constituted of polymer chain and water. This material is interesting since it can partly mimics extracellular matrix. Chitosan formulations were selected in order to obtain a pH-neutral solution enabling cell survival. DA and molecular weight directly affect the chemical and biological properties of the polymer. Chitosan, in its crystalline form is insoluble in most solvents at neutral or high pH, except in organic acids, which markedly limits its applications. The soluble–insoluble transition occurs at its pKa value around pH between 6 and 6.5. In dilute acids, the free amino groups get protonated making chitosan a water-soluble cationic polyelectrolyte<sup>44</sup>. On the other hand, as the pH increases, chitosan loses its charge and usually becomes insoluble in aqueous solution. The amount of acid needed depends on the quantity of chitosan to be dissolved and the concentration of protons needed is at least equal to the concentration of NH<sub>2</sub> units involved (DA). The solubility is related to the pH, DA and ionic concentration. Thus, we chose a chitosan concentration of 0.5 to 1% with a DA of 38 to 48% to obtain a homogeneous neutral solution. It was necessary to use a formulation able to maintain biological properties of the polymer: antimicrobial and cellular activity. The polycationic structure of chitosan is a prerequisite for antimicrobial activity. As environmental pH is below the pKa of chitosan, electrostatic interaction between the polycationic structure and the predominantly anionic components of the microorganism surface (such as Gram-negative lipopolysaccharide and cell surface proteins) plays a primary role in antimicrobial activity<sup>45</sup>. Concerning cellular activity, the stimulation of chondrocytes appears more important for chitosans with a DA located in the range within 30-50% than chitosans with low DAs<sup>46</sup>. The hypothesis is that weak electrostatic interactions may occur between chitosan and the negative surface charge of cells in this intermediate zone. For DAs below 25-30%, the chitosan acts as a polyelectrolyte of high charge density responsible for a too strong stress of living cells. Over 50%, the chitosan acts as a polymer bearing isolated charges with a hydrophobic environment increasing with DA. These results were in contrast with those reported by Prasitsilp et al who described a more important cell growth (fibroblast-like cells and hamster kidney cells) for chitosan with a low DA<sup>47</sup>. Thus, the influence of DA may depend on the cell type. In the present study, we have used adipose-derived stem cells (ADSCs) for cell viability and proliferation assessment. ADSCs are very abundant and accessible under local anesthesia with a low morbidity, effective yield of extracted tissue with pluripotent and replicative capacity similar as BM-MSC<sup>48</sup>. Moreover, ADSCs were also used for complex perineal fistulas treatment associated or not with Crohn disease<sup>5</sup>.



In a randomized phase III trial, Garcia-olmo et al did not find statistically difference between fibrin glue plus ADSCs compared to fibrin glue alone<sup>5</sup>. As in other studies, they used a commercial fibrin glue. Given the high concentration of fibrinogen (90 mg/mL) and thrombin (500 IU/mL) in commercial kit, such a glue formulation might not be effective to promote cell growth. Self-assembly of fibrin fibers is influenced by the concentration of fibrinogen, calcium and thrombin<sup>42</sup>. Recent evidence has shown that fibrinogen is the key determining factor that modulates the overall structural properties of the matrix<sup>12,23,24,29,49</sup>. Decrease in fibrinogen concentration is associated with an open pore structure and structural homogeneity while matrices prepared with high fibrinogen concentrations have closed pores and structural heterogeneity<sup>23</sup>. Whatever the fibrin formulation, ADSCs were viable and proliferated within the fibrin gel and composite gel. Changes in matrix structure modified cellular processes such as morphology, migration and proliferation. It was observed that the number of cells retaining a spherical morphology after 4 days increased with increasing fibrinogen concentration. At lower fibrinogen concentration, cells exhibited a fibroblast morphology with increasing proliferative pattern.

Reported limitations of this composite glue are the shrinkage and the weakness of the construct. The poor mechanical properties of fibrin render it susceptible to contraction/compaction by cells<sup>50</sup> and rapid degradation by proteases. Consequently, cell-seeded fibrin gels often shrink during *in vitro* culture<sup>35</sup> and/or degrade prematurely prior to or soon after transplantation<sup>51</sup>. Chitosan did not improve the mechanical properties of fibrin gels. However, our objective was not to improve mechanical stability of the fibrin sealant. As the gel only serves to obtain initial uniform cell distribution and more efficient seeding, the gel is allowed to degrade while seeded cells produce extracellular matrix and paracrine effect<sup>52</sup>. However, it is necessary to obtain a clot sufficiently handled and moldable to be shaped onto the surface to be repaired. Obtaining the ideal three dimensional gel structure needs to manage the delicate balance between porosity and mechanical strength. While larger pores may allow for increased cell growth rates, gel structural integrity must be preserved, risking weaker scaffold unsuitable for adhesive strength on the scaffold. Thus, decreasing the fibrinogen concentration below 10 mg/mL (Formulation E) may negatively affect the shear strength and hydrogel durability *in vivo* whereas increasing concentration above 20 mg/mL does not promote satisfactory cell proliferation (formulation A and B).

When chitosan was mixed with fibrinogen, we observed an immediate process of emulsification with aggregates formation and subsequent heterogeneous solution unsuitable for optimal fibrin polymerisation. Chitosan solubilized in an acid solution is a polycationic structure (as environmental pH is below the pKa) and may interact with the predominantly anionic components of fibrinogen sealant by electrostatic interaction. It is known that chitosan activates blood coagulation by offering binding sites to platelets that in turn trigger

the primary hemostasis by binding via GPIIb/IIIa to the adsorbed fibrinogen<sup>53</sup>. When chitosan was first mixed with thrombin, the formed solution was more homogenous. Moreover, we observed that chitosan addition resulted in an increasing proliferative pattern compared with fibrin alone for all fibrinogen concentrations. These findings highlight in *in vitro* experiments the biological effect of chitosan and confirm the theoretical interest of this composite chitosan-fibrin glue to deliver cells on the selected scaffold during the operative course for tissue engineering procedure.

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## TABLES AND FIGURES LEGEND

**Table 1:** Macroscopic aspect of chitosan solutions after alcalinisation depending on DA and concentrations.

**Table 2:** Macroscopic aspect of composite glue according to mixing procedure.

**Table 3:** Morphology of ADSCs seeded inside composite gel with various fibrin formulations.

**Table 4:** Influence of fibrinogen concentration (10 and 20 mg/mL) and chitosan addition ( $C_{\text{chitosan}} = 0.5$  and 1%) on the evolution of  $G'$  versus time (800s) during the gelation of the fibrin clot or composite glue.

**Figure 1:** Proliferation images of calcein-labeled hADSCs inside composite glue at days 1, 4, and 7 using a 0,5% chitosan DA 38%. Morphology and proliferation rates differed based on fibrinogen concentration between 5 mg/mL and 90 mg/mL.

**Figure 2:** Proliferation of ADSCs loaded either within fibrin or composite glue (\*  $p < 0.05$ , \*\*  $p < 0.01$ ), FC= fibrinogen concentration.

**Figure 3:** Scanning electron microscopy images of composite glue with varying fibrinogen concentrations: (A, D) 10 mg/mL, (B, E) 20 mg/mL, (C, F) 90 mg/mL. Fibrin pore structure appeared more open at the lower fibrinogen concentration (10 and 20 mg/m).

**Figure 4:** Scanning electron microscopy image revealed cell attachment of ADSCs throughout the composite glue ([FC]=10 mg/mL, [TC]=100UI/mL,  $C_{\text{chitosan}} = 0.5\%$  DA = 38%).

**Figure 5:** (A) Evolution of  $G'$  and  $G''$  versus the frequency (100 to 0.1 rad/s) for a composite Glue ([FC] = 20/100,  $C_{\text{chitosan}} = 0.5\%$  DA = 38%). (B) Evolution of  $G'$  and  $G''$  through time sweep measurement ([FC] = 20 mg/mL, [TC]=100UI/mL,  $C_{\text{chitosan}} = 0.5\%$  DA = 38%).

**Table 1:** Macroscopic aspect of chitosan solutions after alcalinisation depending on DA and concentrations.

Chitosan			[Fg] mg.ml <sup>-1</sup>	[Th] UI.ml <sup>-1</sup>	Macroscopy
Concentration (%)	DA (%)	pH			
0.5	4	6.1	90		Precipitate
0.5	38	7	10		Microprecipitate
0.5	48	7	10		Microprecipitate
0.5	48	7	20		Microprecipitate
0.5	4	6.1		250	Homogeneous
0.5	48	7		500	Homogeneous
0.5	38	7		250	Homogeneous
0.5	38	7		100	Homogeneous
0.5	48	7		100	Homogeneous
1	38	6.7		100	Homogeneous



**Table 2:** Macroscopic aspect of composite glue according to mixing procedure.

Chitosan				
Concentration (%)	DA (%)	pH/solution	pH/Alcalinisation	Macroscopy
0.5	4	4.9	6	Heterogeneous
0.5	15	5.14	6	Heterogeneous
0.5	28	-	7	Heterogeneous
0.5	38	5.3	7.1	Homogeneous
0.5	48	5.5	7	Homogeneous
1	4	5.6	6.1	Heterogeneous
1	28	-	6.7	Heterogeneous
1	38	-	6.7	Homogeneous
3	4	High viscosity		
3	48			

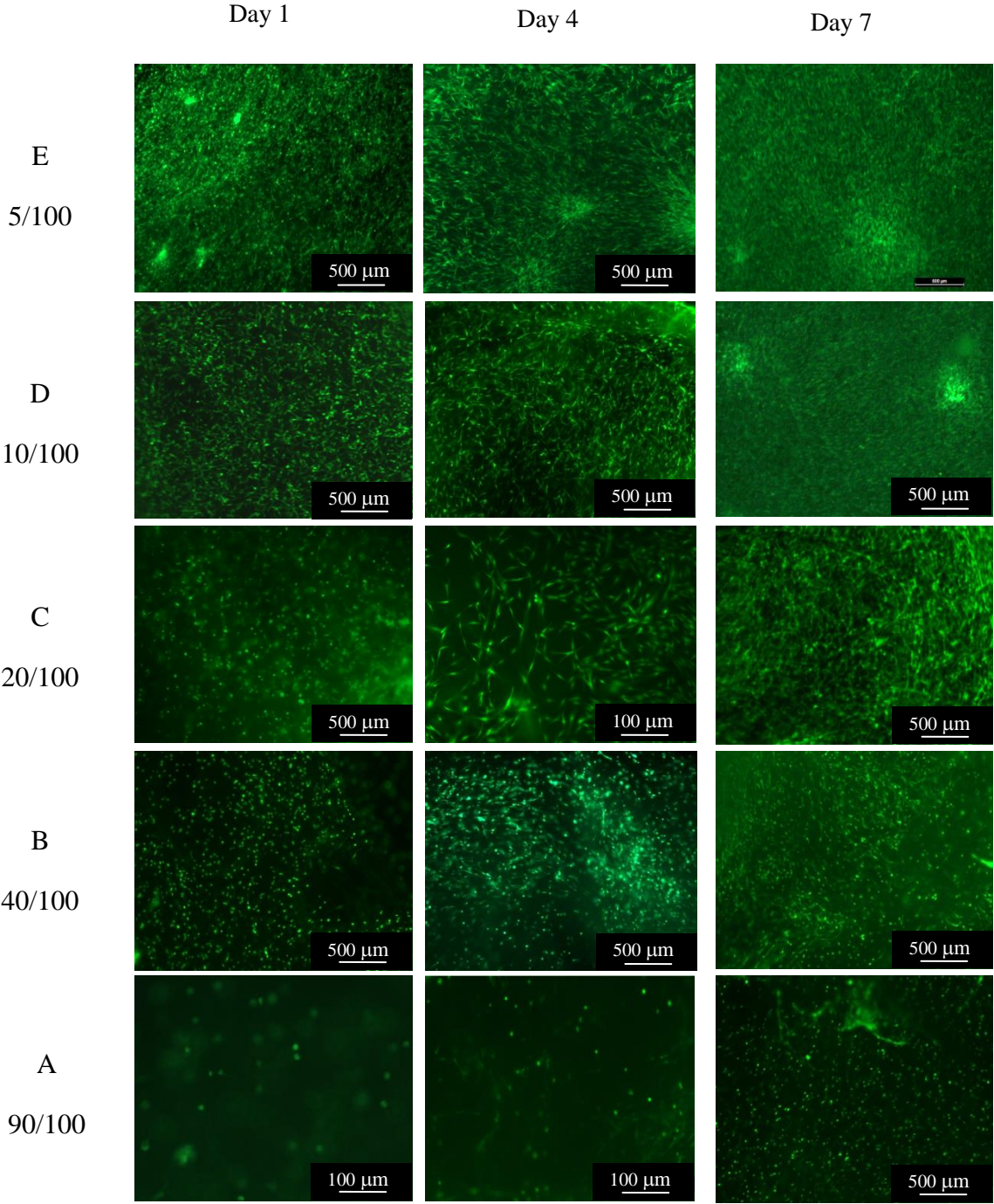
**Table 3:** Morphology of ADSCs seeded inside composite gel with various fibrin formulations.

Formulation	Day 1	Day 4	Day 7
A : 90/100	Rounded cells	Rounded cells	Rounded cells
B : 40/100	Rounded cells	Rounded cells Cell-to-cell contact	Rounded cells Cell-to-cell contact
C : 20/100	Some cells start to elongate	Cell-to-cell contact	Interconnected network
D : 10/100	Cells start to elongate	Cell-to-cell contact Interconnected network	Interconnected network
E : 5/100	Cells start to elongate	Interconnected network	Interconnected network

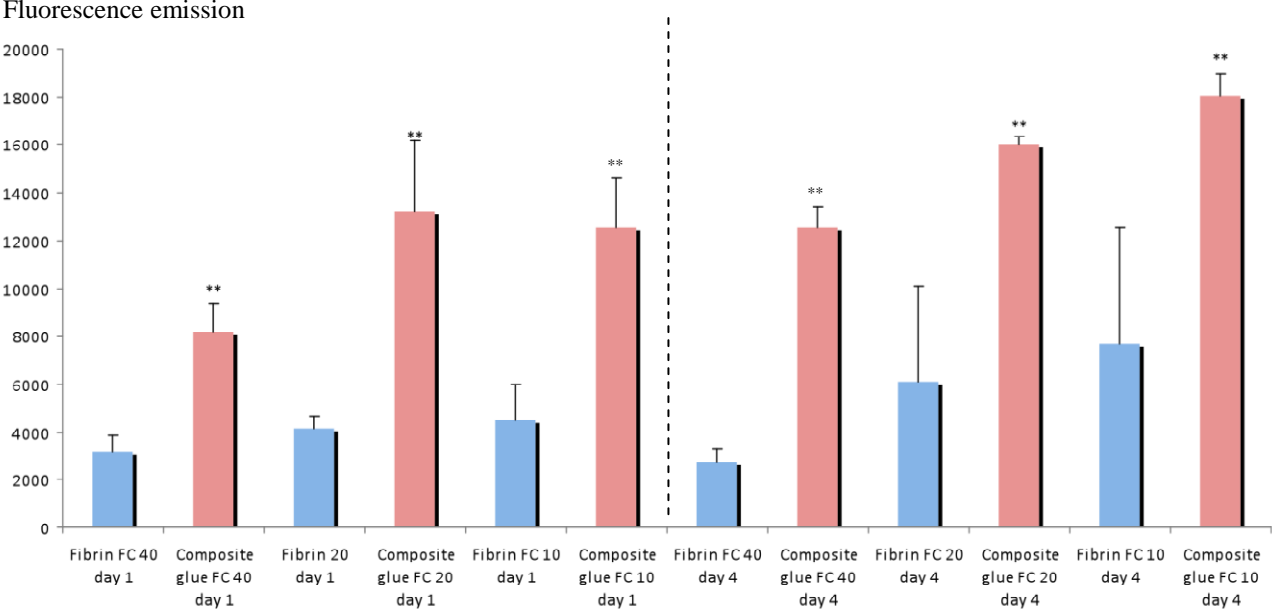
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<b>Fibrin glue</b>	$G'$ (Pa) (800 s)
10/100	50
20/100	150
<b>Fibrin-Chitosan glue</b>	
10/100 1-38	65
20/100 0.5-38	170
20/100 0.5-38	140
20/100 1-38	135

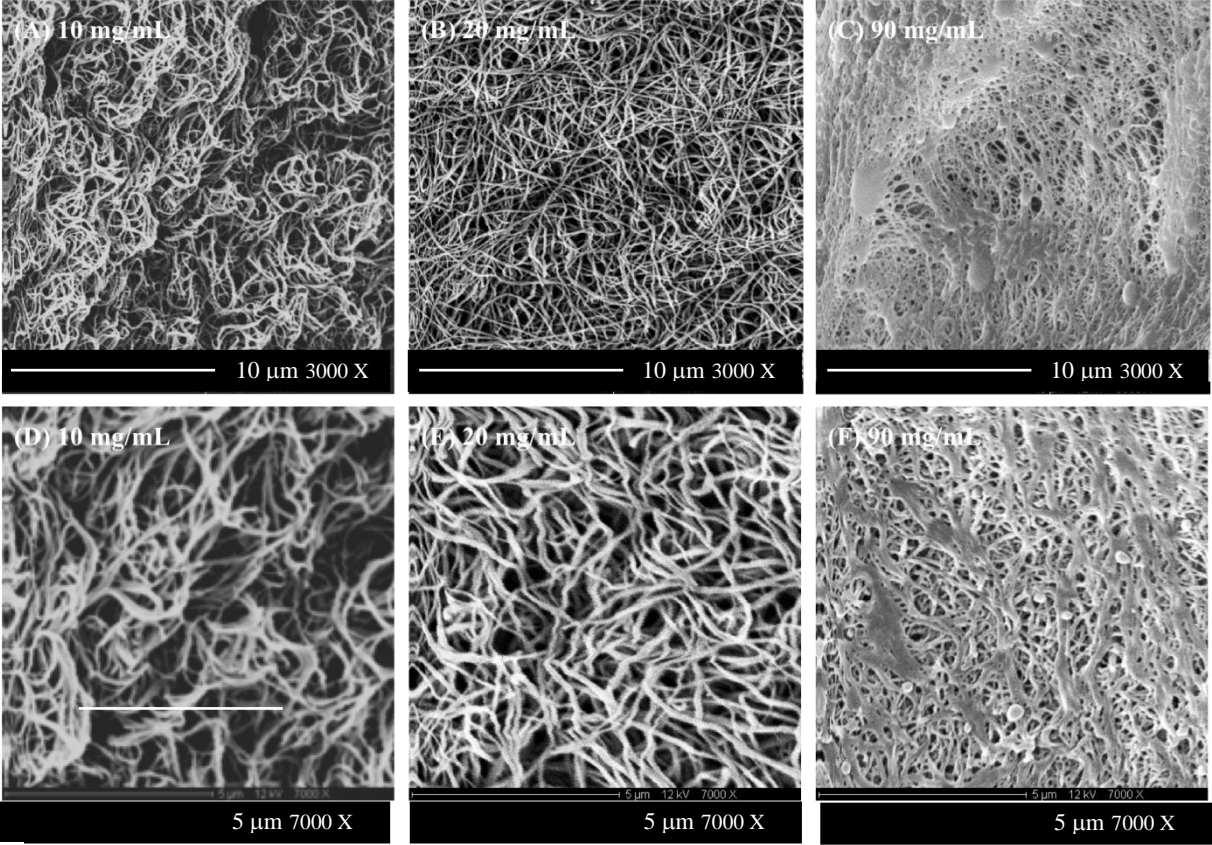
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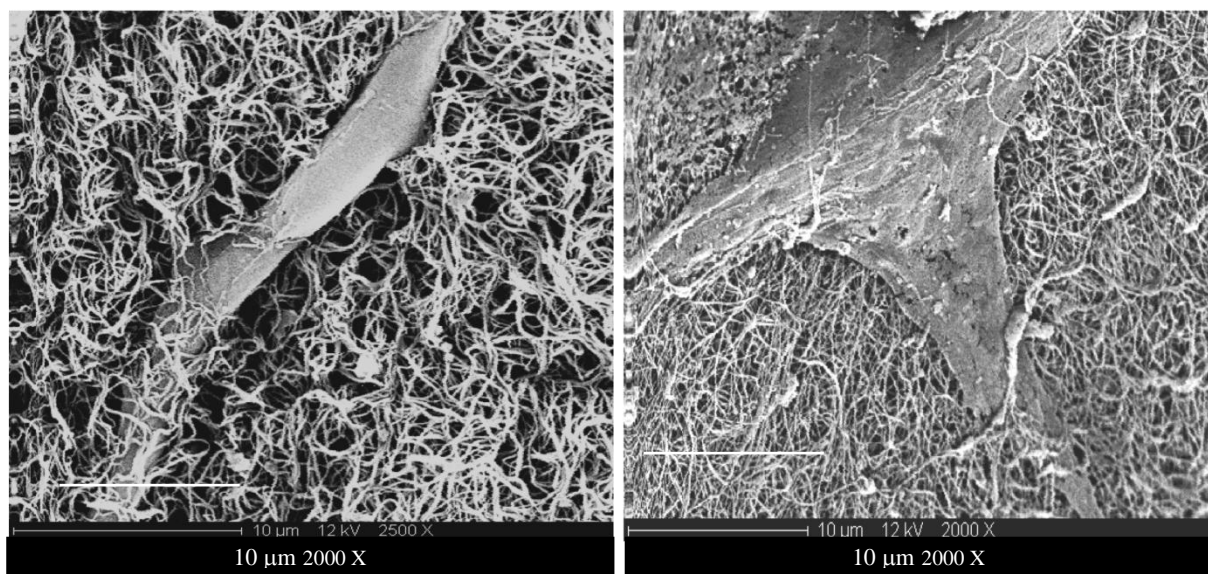


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**Figure 3**

**Figure 4:** Scanning electron microscopy image revealed cell attachment of ADSCs throughout the composite glue ([FC]=10 mg/mL, [TC]=100UI/mL,  $C_{\text{chitosan}} = 0.5\%$  DA = 38%).



**Figure 4**

**Figure 5:** (A) Evolution of  $G'$  and  $G''$  versus the frequency (100 to 0.1 rad/s) for a composite Glue ([FC] = 20/100,  $C_{\text{chitosan}}$  = 0.5% DA = 38%). (B) Evolution of  $G'$  and  $G''$  through time sweep measurement ([FC] = 20 mg/mL, [TC]=100UI/mL,  $C_{\text{chitosan}}$  = 0.5% DA = 38%).

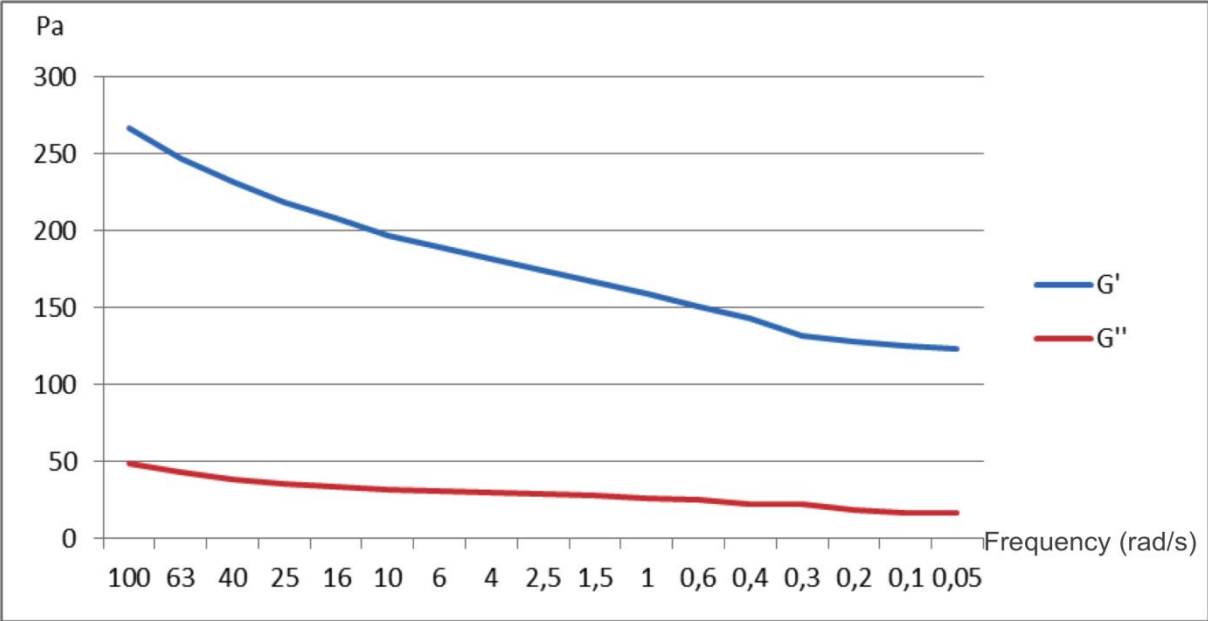


Figure 5A

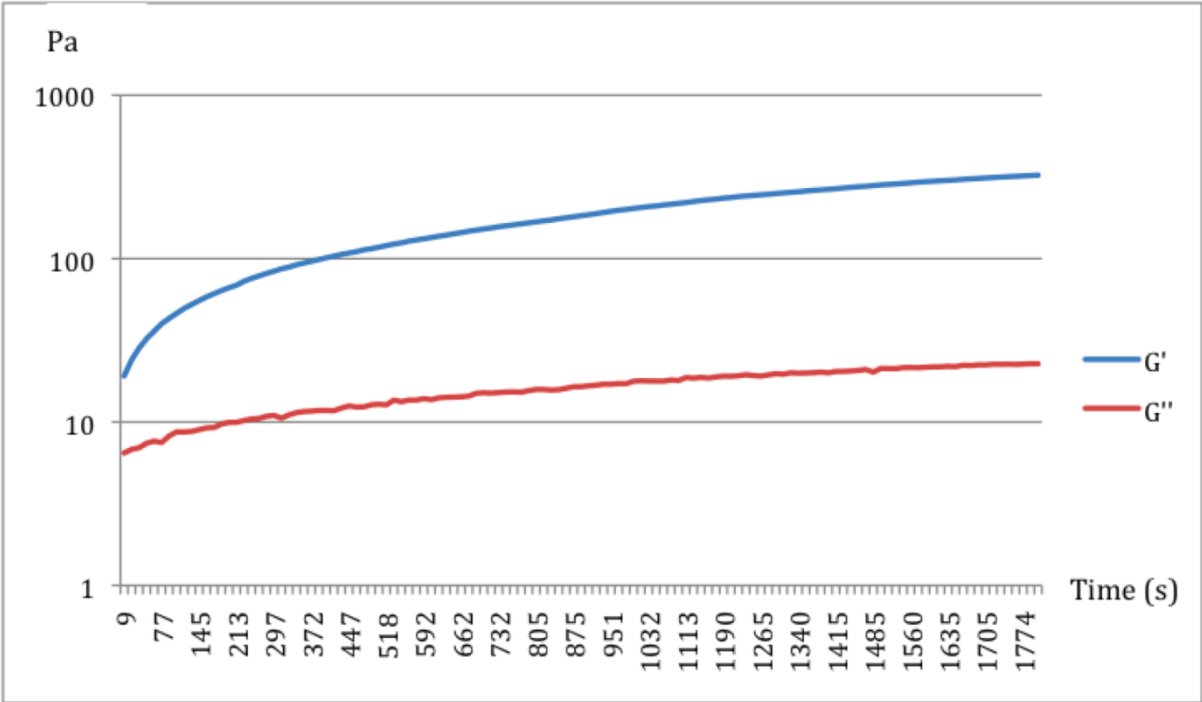


Figure 5B



# CHAPITRE III

Article correspondant

Annexe 4

## **Ingénierie tissulaire :**

### **Vers la construction d'une paroi colorectale**

L'élaboration d'un substitut rectal nécessite comme nous l'avons vu plusieurs étapes. La première étape de ce travail a permis de déterminer la composition physico-chimique idéale des hydrogels constituant une structure mono-lamellaire servant de support à la recolonisation cellulaire et à l'organisation tissulaire colorectale. La deuxième étape de ce travail a permis de définir la composition d'un système composite de confinement et de délivrance cellulaire dans une perspective d'ensemencement matriciel peropératoire.

Cette dernière partie du travail de thèse est scindée en 2 étapes, d'une part une étude comparative évaluant l'intérêt de la cellularisation autologue per opératoire d'un hydrogel de chitosane en remplacement d'un défaut pariétal colique de 2x3 cm, et d'autre part une étude de faisabilité sur l'implantation d'une matrice cellularisée circulaire en remplacement d'un défaut colique circonférentiel. La cellularisation de la matrice de chitosane par de la fraction stromale vasculaire du tissu adipeux est totalement novatrice dans ce domaine de l'ingénierie tissulaire. Le modèle porcin a été choisi sur les arguments suivants :

- la structure anatomique et cellulaire, ainsi que la physiologie de l'animal, sont très proches de l'homme (Kubiak 2011),

- la validité du modèle pour l'étude des fistules coliques ou colorectales dans sa capacité à mimer la réaction du modèle humain face à la même agression (Pommergaard 2011) (Hoepfner 2009),

- l'isolement et la mise en culture d'ADSCs pour d'autres applications chez le porc (Kumar 2012), dont les caractéristiques sont semblables à celles rapportées chez l'homme (Bunnell 2008) et validées dans notre laboratoire (Lalande 2001).

De plus, dans la perspective de concevoir une étude clinique pilote pour valider l'utilisation de ces matrices cellularisées comme tissu d'interposition dans le traitement des fistules recto-vaginales à l'issue de ce travail, le modèle porcin devrait constituer le pont entre le modèle animal lapin et l'étude chez l'homme.

Le modèle expérimental, défaut colique circonférentiel, permettra d'évaluer la capacité de la matrice circonférentielle à répondre aux phénomènes ischémiques et inflammatoires locaux et à orienter le processus de cicatrisation. Le principal écueil du transfert des acquis du

défect pariétal inférieur à 180° au remplacement circonférentiel est le risque de sténose du néo-colon par ischémie liée à la section des artères marginales coliques sur le site de résection. Aucune matrice n'a été rapportée dans la littérature à notre connaissance en remplacement circonférentiel colique.

Les travaux préliminaires *in vitro* sur l'isolement et la culture des ADSC porcins, ainsi que la méthode et les résultats de l'étude *in vivo* menée sur 23 porcs, 20 répartis en 2 groupes dans l'étude comparative et 3 dans l'étude de faisabilité, sont rapportés dans l'article en Annexe 4.

A l'issue de cette 3ème étape, nous avons validé la formulation de la matrice de chitosane et du système composite de confinement et de délivrance des cellules en ingénierie tissulaire colorectale. Il s'agit d'une étape majeure en ingénierie tissulaire colorectale qui entérine la faisabilité de la voie du « tout peropérateur », souligne le concept de spécificité d'organe de la matrice et ouvre la porte vers la complexification de la structure matricielle et l'élaboration d'un substitut rectal cellularisé.

## Article – Annexe 4

(Article en cours de correction avec les co-auteurs)

**Title:** Tissue engineering: towards the colorectal wall regeneration.

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## ABSTRACT

**Purpose:** Tissue engineering may provide a new surgical tool for colorectal surgery. It consists mainly of two components: scaffold from which the extracellular matrix is organized in neotissue and cells seeded on the scaffold. We have selected in previous experimental studies chitosan hydrogel matrix and Adiposed Derived Stem Cells as the optimal partnership for colorectal tissue engineering. Aims of the current experimental work were firstly to assess the interest of seeding chitosan hydrogel patches for colorectal tissue engineering in an *in vivo* comparative study, and secondly to assess the accuracy of segmental colonic replacement in an *in vivo* feasibility study.

**Methods:** Swine model has been selected to carry out both comparative and feasibility studies. In the comparative study, a 2x3 cm colonic wall defect was performed in 20 swines. Repair was done by suturing a chitosan hydrogel patch. Animals were divided in 2 groups: acellular chitosan hydrogel (Group A, n=10) vs. chitosan hydrogel seeded with autologous Stromal Vascular Fraction (SVF) from adipose tissue (Group B, n=10). Cells' seeding was carried out by spraying a composite chitosan-fibrin sealant adjusted in preliminary *in vitro* experiments. In the feasibility study, a 2 cm length chitosan hydrogel tube was implanted in 3 pigs. Graft areas were explanted at 8 weeks. Histological and immunohistochemical examination were performed. A fibrosis ratio was carried out to assess inflammation activity. Endpoints were technical feasibility, scaffold behavior and quality of tissue regeneration.

**Results:** All scaffolds were successfully implanted. One animal died in Group A without graft area leakage. Animals tolerated their oral intake without difference about the gained weight between the 2 groups. A complete coverage of the patched area was observed with an ad integrum regeneration of the colonic wall including smooth muscle cells layer around the fibrosis scare. Fibrosis ratio was significantly lower with cellularized scaffold: 13% vs. 55% (p=0.013). Immunohistochemical examination confirmed the smooth muscle layer regeneration.

**Conclusion:** Our data confirmed in a large animal model the healing effect of chitosan on colorectal tissue. The very low rate of the fibrosis ratio in the cellularized group emphasizes inflammatory control effect of the chitosan hydrogel and SVF association. It allows controlling inflammation required for healing process and managing cells recruitment, proliferation and organization. This experimental step in large animal model was mandatory to promote future bioengineered colon or rectum.

## INTRODUCTION

Tissue engineering uses biological substitutes to restore, maintain or improve functions of tissue [Langer R, 1993]. It may provide alternative therapy to conventional colorectal surgery in elective indications such as treatment of anastomotic or perineal fistula and rectal wall healing after local excision for cancer [Denost Q, 2013]. This approach involves the use of three components: (1) a synthetic or natural biomaterial from which the extracellular matrix (ECM) is organized in neotissue, (2) cells seeded *in vitro* on the biomaterial, and (3) humoral and mechanical signals which regulate the behaviour of the first two [Rabkin E, 2002].

Bioengineering researches for colorectal tissue engineering are scarce and neither scaffolds nor cells assessed until now have allowed to obtain a neotissue similar than the native tissue. In accordance with literature, decellularized porcine Small Intestinal Submucosa (SIS) can be considered as a reference scaffold for tissue engineering of the gastrointestinal tract [Badylak S 2000] [de la Fuente SG 2003] [Chen MK 2001] [Rosen M 2002] [Ueno T 2007]. However, several studies have shown the activity of chitosan in the healing process with an antimicrobial effect and biodegradability due to colonic microflora used for colonic-specific drug delivery [Zhang H, 2002] [McConnell EL, 2008]. For these reasons, we previously carried out a comparative study between SIS and chitosan hydrogel matrix and showed that chitosan scaffold was better for colorectal tissue engineering [Denost Q, *J Tissue Eng Regen Med* 2014, *In process*] in a rabbit experimental model.

Tissue engineering requires a reliable source of cells for neotissue formation. The cells that invade the scaffold play naturally a critical role in tissue engineering. In the field of digestive tract disease, experimental and clinical researches have grown exponentially during the last decade [Trebol Lopez J, 2011] with several investigations about adipose-derived stem cells in the area of inflammatory, ischemic, fistulising and functional diseases. Adipose-derived stem cells (ADSCs) may indeed provide a new promising cell source for colorectal tissue engineering. Moreover, ADSCs were also used for complex perineal fistulas treatment associated in randomized phase I and II trials [Garcia-Olmo D, 2008] [Garcia-Olmo D, 2009] with promising outcomes. One of the main issues in tissue engineering field is that cell seeding requires an adequate time of cell adhesion to obtain optimal biomaterial implementation. The previous *in vitro* step for cell colonization in the tissue engineering process is not relevant for routine clinical application. Seeding and implementation should be carried out simultaneously ensuring a more efficient and less time-consuming procedure. Fibrin glue in combination with various cell sources was effective in a variety of tissue-

engineering applications [Wu X, 2012]. However, experience in colorectal bioengineering is scarce including only chronic intestinal fistulae healing with moderate success rate [Herrerros MD, 2012]. As we have previously prepared and assessed an original composite biological glue made of fibrin sealant (able to sustain cell growth) and chitosan solution (providing specific biologic properties for cell delivery in colorectal tissue engineering) [Pontallier A, *Tissue Engineering 2014, In process*], we checked the latter for its ability to functionalize chitosan hydrogel patches with autologous ADSCs embedded in the composite glue.

Thus, aims of the current experimental work were firstly to assess the interest of seeding chitosan hydrogel patches with embedded cells in colorectal tissue engineering in a comparative study with unseeded patches, and secondly to assess the feasibility of replacing a colonic segment with tubular functionalized hydrogel.



## METHODS

### 1- *In vitro* experiments

#### 1.1- Chitosan purification and characterization

Chitosan, purchased from Mahtani Chitosan (Veraval, India), was extracted from squid pens and then purified before use as previously described [Montebault A, 2006]. The degree of acetylation (DA) of chitosan corresponding to the molar fraction of acetyl units within the polymer chains was determined by  $^1\text{H}$  NMR spectroscopy [Montebault A, *Biomaterials* 2005], according to the method developed by Hirai *et al* [Hirai A, 1991]. The weight-average molecular weight  $M_w$  of chitosans and their polydispersity index  $I_p$  were determined by size exclusion chromatography coupled on line with a multi-angle laser light scattering detector [Montebault A, *Biomaterials* 2005].

#### 1.2- Chitosan patches and tubes

For the comparative study between cellularized and non-cellularized scaffold of full-thickness colonic wall defect patch, a monolayer chitosan hydrogel patch was carried out. The processing physico-chemical conditions of the gel layer were optimized to provide high mechanical properties and to obtain a suturable final patch (final polymer concentration: 7% (w/w); characteristics of chitosan:  $\text{DA} = 1.5 \pm 0.3 \%$ ,  $M_w = 420,000 \pm 40,000 \text{ g/mol}$ ,  $I_p = 1.6 \pm 0.3$ ). This rigid gel was generated from a hydroalcoholic chitosan solution [Montebault *Biomaterials* 2005]. Thus, after complete dissolution of chitosan in an aqueous acetic acid solution, alcohol was added acting as a bridging agent by the establishment of hydrogen bonds with chitosan [Desorme M, 2013]. The following hydro-alcoholic medium was chosen: 50% water, 30% of glycerol and 20% of 1,2-propanediol. By evaporating the water and acid from the hydroalcoholic chitosan solution (evaporation in an oven at  $50^\circ\text{C}$  for 12h), the decrease of the apparent charge density of chitosan favours hydrogen bonding and hydrophobic interactions leading to the formation of a hydro-alcoholic chitosan gel.

For the feasibility study of segmental colonic replacement, a monolayer chitosan hydrogel tube was carried out. Physico-chemical characteristics of the chitosan layer was similar than the chitosan patch final polymer concentration: 7% (w/w); characteristics of chitosan:  $\text{DA} = 1.5 \pm 0.3 \%$ ,  $M_w = 420,000 \pm 40,000 \text{ g/mol}$ ,  $I_p = 1.6 \pm 0.3$ .

#### 1.3- Composite glue preparation

As previously described [Pontallier A, *Tissue Engineering* 2014, *In process*], composite glue preparation was performed with 1% (w/v) chitosan concentration and 38%

DA. Chitosan was firstly mixed with thrombin (100 U/mL), then fibrinogen (20 mg/mL) was added at the end of the procedure.

#### 1.4- Pig Stromal Vascular Fraction (pSVF) and ADSC isolation

##### 1.4.1-Isolation and culture of pADSC

Porcine adipose tissue was obtained from pigs'neck in the beginning of the surgical procedure. The procedure of pSVF isolation is similar than that for human procedure (*Bunnell BA 2008*). Tissue samples were minced and digested in a solution of 0.1% (w/v) collagenase type I (Worthington) for 1h30 at 37°C with vigorous shaking. After filtration and centrifugation, the top lipid layer was removed and the remaining SVF was treated for 10 minutes with ELB (Erythrocyte Lysis Buffer; 155 mM NH<sub>4</sub>Cl (Sigma), 5.7 mM K<sub>2</sub>HPO<sub>4</sub> (Sigma), 7.4 mM K<sub>2</sub>HPO<sub>4</sub>-3H<sub>2</sub>O (Sigma), 0.1 mM EDTA (Sigma)) and then centrifuged. The pellet was resuspended in basal medium, DMEM F12 medium (Invitrogen) supplemented with 10% (v/v) Fetal Bovine Serum (Lonza) and sequentially filtered through 100, 70 and 40 µm cell strainer (BD Falcon). The resulting cells were plated (10<sup>4</sup> cells/cm<sup>2</sup>) in culture wells and cultivated at 37°C in 5% CO<sub>2</sub> atmosphere. Culture medium was replaced every three days when pADSC reached 80% of confluence.

##### 1.4.2-Characterization of pADSC

Flow cytometric analyses (BD Accuri™ C6 flow cytometer) were performed to characterize cells 3 days after beginning of the culture. Briefly, trypsin-EDTA solution (0.125 % (w/v) – 0.0625 % (w/v), Sigma-Aldrich) was used for isolation of cells. The following antibodies were used: CD 11a, CD 29, CD 45 and CD90 (eBiosciences®) in accordance with data in the literature [*Casado, 2012*] [*Valina 2007*] [*Qu 2007*].

##### 1.4.3- Cell seeding procedures

The two final components, thrombin-chitosan containing pADSC in one side and fibrinogen in the other side, were put in their respective syringes. The syringes were connected to a common Duploject plunger for simultaneous application of glue components. Two different ways for delivering the composite cellularized Fibrin-Chitosan sealant were assessed *in vitro*: for applying or for spraying. For spraying this composite sealant, the DuploSpray (Baxter Healthcare) applicator was used. Cells viability and proliferation on the plastic wells was determined by fluorescence microscopy after nuclear staining (Cyquant® Cell Proliferation Assay – Invitrogen C7026). Times of assessment were 1 and 4 days. Four independent experiments were performed.

##### 1.4.4- Isolation of pSVF for the *in vivo* experiments

First steps of the procedure were similar than described in 1.3.1 but after treatment by ELB, the pellet was resuspended in composite chitosan-thrombin sealant.

## 2- *In vivo* experiments

### 2.1- Operative procedures:

All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of Poitou-Charente Region.

#### 2.1.1- Comparative study

Twenty large white pigs (weight of 45 kg) were randomly divided into two groups: pigs with colonic defect were given either cellularized chitosan hydrogel patches (Cellular Group CG, n=10) or non-cellularized chitosan hydrogel patches (Acellular Group AG, n=10). A medial laparotomy was performed. A 2×3-cm full-thickness wall defect was created on the antimesenteric side wall of the colon with scissors. The fixation of chitosan patch was done with absorbable polyglycolic acid 6/0 single knot suture. Stitches were placed at a distance of 2 mm to the edge of the patch (Figure 1A). The delivery of the composite cellularized Fibrin-Chitosan sealant was carried out in both luminal and external sides of the patch. Two single non-resorbable Prolene 4/0 sutures were placed at 3 cm from the margins of the patch as markers for a later identification of the patch location. The abdominal cavity was closed in layers with absorbable sutures.

#### 2.1.2- Feasibility study

Three large white pigs (weight of 45 kg) underwent a medial laparotomy. Colon was dissected and transected. A chitosan hydrogel tube was sutured to the colonic edges by two overcast stitches in both ends of the prosthesis (Figure 1B). All chitosan hydrogel tubes were cell seeded by spraying on both luminal and external sides. Two single non-resorbable Prolene 4/0 sutures were placed at 3 cm from overcast. The abdominal cavity was closed in layers with absorbable sutures.

#### 2.1.3- Animal anesthesia and postoperative follow-up

Each animal was restricted from any oral intake except for water 24 h before surgery. Premedication (acepromazine 5mg/mL) was administrated intramuscularly. Anesthesia was maintained by sevoflurane gaz during the operative procedure. Antibioprophylaxy was performed by TERRAMYCINE<sup>®</sup> LA (50mg/kg) administrated intramuscularly at the end of the procedure. In the postoperative period, the pigs were examined daily by the surgeon for signs of wound infection, fever, abdominal pain, behavior, bowel movements, stool, and eating behavior.

## 2.2- Graft specimen examination

All animals were scheduled to be sacrificed for the evaluation of reconstructed colon at 8 weeks. The samples were fixed with 10% formalin then embedded in paraffin. The slides were stained with hematoxylin and eosin and examined with the light microscopy by the same pathologist blinded to the groups. Inflammatory cell infiltration and fibroblast activity were assessed. A fibrosis ratio was calculated after Masson staining and corresponded to the ratio of the fibrosis scar thickness in the graft area. Immunohistochemistry with staining for alpha-smooth muscle actin (Dako<sup>®</sup>) and caldesmon (Dako<sup>®</sup>) was performed to assess smooth muscle cells layer regeneration.

## 3- Statistical analysis

All data were expressed as mean  $\pm$  standard deviation (SD). The difference between the two groups was compared by using the Mann-Whitney test or Wilcoxon rangs test when necessary. A p value  $<0.05$  was considered statistically significant.

## RESULTS

### 1-Chitosan Hydrogel

For the comparative study, the chitosan hydrogel patches have the following characteristics: 3 cm for the length, 2 cm for the width and from 1 to 1.5 mm for the thickness

For the feasibility study, chitosan hydrogel tubes were performed by punch to obtain the following characteristics: 25 mm for the inner diameter, 20 cm for the length and from 1 to 2.5 mm for the thickness.

### 2- Cell culture and seeding

pADSC cultures were successfully obtained with a population doubling time of 2 days (Figure 2). Confluent cells were harvested after 2<sup>nd</sup> passage. Flow cytometric analysis showed pADSC phenotype CD90+, CD29+, CD45-, CD11a-. Analysis has been performed after 2<sup>nd</sup> passage with cells harvested from three different pigs (Figure 3).

Cells viability and proliferation were similar between applying and spraying procedure for seeding (Figure 4). However, cells distribution was more homogeneous with spraying procedure at day 1 in Live-dead (Figure 5). For this last reason, seeding procedures during surgical procedure have been performed by spraying.

### 3- Surgical procedures and follow-up

The first step of the surgical procedure for CG in the comparative study and 3 pigs in the feasibility study was the adipose tissue harvesting. Mean time of this surgical procedure was 15 minutes  $\pm$  5 minutes.

#### 3.1- Comparative study

Mean time of surgical procedure for implantation of the chitosan patch was 50 minutes  $\pm$  10 minutes in AG and 60 minutes  $\pm$  8 minutes in CG. All scaffolds were successfully implanted. One death was observed in the AG but the autopsy did not reveal any graft area leakage or intra-abdominal abscess. None of the 19 surviving animals showed clinical signs of illness due to colonic leakage, intra-abdominal infection or wound infection. Animals tolerated their oral intake without significant difference between the two groups on the weight gain (Graph 1A) (Graph 1B).

#### 3.2- Feasibility study

Mean time of surgical procedure for implantation of the chitosan tube with spraying cells was 100 minutes  $\pm$  13 minutes. All scaffolds were successfully implanted. There was no postoperative morbidity or mortality. Animals tolerated their oral intake (Graph 2).

#### 4- Graft area assessment

##### 4.1- Comparative study

##### 4.1.1- Macroscopic analysis

At 8 weeks, we did not observe abscess or colonic stenosis of the graft area. Soft peritoneal adhesions were detected in 50% of pigs in AG whereas none was observed in the CG. The two single non-resorbable Prolene 4/0 sutures placed around matrix during the implantation of the chitosan patch allowed macroscopic detection of the graft area from the serosal view due to coverage of the patched area by reconstructed tissues in CG. In The AG, the serosal examination showed little round fibrosis scars almost invisible.

In the mucosal side, thin fibrosis scar were observed in pigs of the AG whereas in the CG only three pigs had a thin fibrosis scar and seven had no scar macroscopically (Figure 6). Remaining hydrogel chitosan was not detected macroscopically in both groups.

##### 4.1.2-Microscopic analysis

Microscopic analysis after hematoxylin and eosin staining showed a complete regeneration of the mature mucosal layer and the muscularis mucosae in 8 pigs in the AG versus 9 pigs in the CG. Partial regeneration of smooth muscle cells layer was observed around the fibrosis scar in all pigs in the AG whereas smooth muscle cells layer was interrupted only in half pigs in the CG. Chitosan fragments degraded by inflammatory cells (macrophages) were observed on the serosal side of the colonic wall.

Fibrosis ratio (Figure 7) was significantly lower in the CG than in AG: 55% *versus* 13% ( $p=0.01$ ), respectively (Table 1).

##### 4.1.3- Immunohistochemical examination

An alpha-smooth muscle actin-positive layer and a caldesmon positive layer located in the inner portion of the graft and corresponded to the muscularis mucosae were observed in both groups. In contrast, a muscularis propria with smooth muscle cells was more developed in CG group (Figure 8).

#### 4.2- Feasibility study

##### 4.2.1- Macroscopic analysis

At 8 weeks, we did not observe abscess on the graft area. Soft peritoneal adhesions were detected. The serosal examination showed a complete regeneration (Figure 9). The inner diameters of the graft area (Figure 10) were 16 mm, 14 mm and 5 mm. There was one strong stenosis without clinical issue. In the mucosal side, a very thin fibrosis scar was observed in

one pig (Figure 11), whereas in the two others pigs, there were one moderate and one major fibrosis area. Remaining hydrogel chitosan was not detected macroscopically in both groups.

#### 4.2.2- Microscopic analysis

Microscopic analysis after hematoxylin and eosin staining or Masson staining confirmed the macroscopic assessment with a large fibrosis area in two pigs. A complete coverage of the mucosal side was observed in one pig. A partial regeneration of smooth muscle cells layer was observed around the fibrosis scar in this pig (Figure 12).

#### 4.2.3- Immunohistochemical examination

The partial regeneration of smooth muscle cells layer observed after hematoxylin and eosin staining was confirmed by the caldesmon positive staining in the inner portion of the graft (Figure 14). This corresponded to the muscularis mucosae.

## DISCUSSION

Colorectal tissue engineering is at an early stage and important work remains to be done regarding to the vascularization, motility, absorption and innervation of bioengineered colon. However, regenerative medicine technology may provide new surgical tool for alternative therapy to conventional colorectal surgery in electives indications. We have already reported the strong interest of chitosan hydrogel matrix for colorectal tissue engineering [Denost, *J Tissue Eng Regen Med* 2014, *In process*] and the current study confirms preliminary outcomes. Moreover, the composite chitosan-fibrin sealant, which exhibited *in vitro* promising results [Pontallier, *Tissue Engineering* 2014, *In process*], has been used in *in vivo* experiments for the first time and practice underlines a very convenient formulation for clinical use. This experimental and comparative work is the first that highlights the requirement of cells seeding in colorectal tissue engineering and circumferential replacement has never been reported previously in this field of tissue engineering. We shall discuss firstly chitosan hydrogel characteristics compared to other matrices used in colorectal tissue engineering, secondly cells requirement for tissue engineering and properties of SVF and ADSC, and finally findings of circumferential replacement already reported in other gastrointestinal tract hollow organs comparing to ours.

Chitosan is a partially or fully deacetylated (DA) form of chitin and exhibits a range of physico-chemical behaviours as a function of DA and molecular weight [Schatz C, 2003]. In addition, it has been shown that modulating the physico-chemical parameters of chitosan physical hydrogel elaboration induces various biological response, a possibility that appears essential for tissue engineering applications [Rami L, 2013] [Malaise S, 2014 *In Press*]. Moreover, mechanical properties of this kind of hydrogels depend mainly on chitosan concentration, DA [Montebault A, *Biomaterial* 2005] [Montebault A, *Biomacromolecules* 2005] and the neutralization kinetics. Chitosan has been reported to have antimicrobial effects due to its polycationic characteristics [Tayel AA, 2010] and wound healing properties by stimulating fibroblastic cells proliferation and macrophages functions [Kong M, 2010] [Okamoto Y, 1995] [Porporatto C, 2003]. Chitosan properties highlight the concept of organ specificity for colorectal tissue engineering. These properties have already made use for colonic specific drug delivery but never in the field of colorectal tissue engineering. The high biodegradability properties reported in our previous experiments [Denost, *J Tissue Eng Regen Med* 2014, *In process*] and the current study, are probably due to colonic microflora and enzymatic activity [Zang H, 2002]. In experimental *in vivo* study, chitosan powder applied to



colonic anastomosis had a positive effect in the process of collagenization in colonic anastomosis healing [Citgez B 2012] and composite collagen/chitosan scaffold appeared to be suitable to support *in vitro* bioengineered smooth muscle intestinal constructs [Zakhem E 2012]. Tissue engineering of the colorectal tissue involves the regeneration of the smooth muscle layer. Our data confirm the capacity of chitosan scaffold to control the inflammatory reaction and to support smooth muscle layer regeneration.

Tissue engineering requires a reliable source of cells for neotissue formation. The combination of biomaterials with cells was shown to reduce shrinkage of the graft [Badylak S 2000] [Badylak 2005] [Wei RQ 2009]. Vacanti *and coll.* seeded synthetic polymer tubes by colon organoid units and placed them into the great omentum [Grikscheit TC 2002] [Grikscheit TC 2003]. Tissue-engineered colons exhibited architecture and major function of native colon but the full-thickness wall colon dissection required for organoid units isolation is limited by clinical concerns. Isolation and culture of smooth muscle cells were performed to rebuild a small bowel wall [Nakase Y 2006] [Qin 2011] and internal anal sphincter [Raghavan S 2010] [Raghavan S 2011]. In the field of digestive tract disease, experimental and clinical researches have grown exponentially during the last decade [Trebol Lopez J 2011] with several investigations about adipose-derived stem cells in the area of inflammatory, ischemic, fistulising and functional diseases. Adipose-derived stem cells may indeed provide a promising cell source for colorectal tissue engineering. ADSC exhibit high cell plasticity [Zuk PA, 2001] [Fujimura J, 2005] [Planat-Benard V, 2004] [Brzoska M, 2005] [Banas A, 2007] [Timper K, 2006], immune activity [Yañez R, 2006]. ADSC secrete a variety of cytokines and growth factors that have both paracrine and autocrine activities. These secreted bioactive factors suppress the local immune system, inhibit fibrosis (scar formation) and apoptosis, enhance angiogenesis, and stimulate mitosis and differentiation of tissue-intrinsic reparative or stem cells [Caplan AI 2006]. Our data regarding the graft area in the comparative study confirmed that scaffold seeding by ADSC allowed to obtain a better tissue repair compared to acellular scaffold with a significantly more effective inflammatory activity control and an *ad integrum* regeneration of the colonic wall. The very low rate of the fibrosis ratio in the cellularized group, only 13%, emphasizes the immune activity and inflammatory control of ADSC, but also the optimal selection of both scaffold and cells which work in the same way to control inflammation required for healing and to manage cells proliferation and organization. The issue of the neotissue cells origin remains unclear if either the seeded cells expand and differentiate or if cells from neighboring tissues migrate into the

developing neo-organ. Data in both sides of the debate have been provided. Firstly, in one study on regenerated vaginal replacement, labeled cells seeded with fluorochromes comprised over 85% of the regenerated neo-organ three months post-surgery [De Filippo RE, 2008]. In the opposite, when a neo-vessel scaffold seeded with human bone marrow-derived cells was implanted into immunodeficient mice, loss of all seeded human cells was observed within days of implant and repopulation occurred first with mouse monocytes, followed by mouse SMCs and endothelial cells recruited [Roh JD, 2010]. Several studies regarding the interest of MSC therapy for heart disease reinforce this last point of view reporting that the frequency of MSC engraftment and differentiation in the heart is low compared to the robust functional recovery observed. This suggests that soluble paracrine factors secreted by MSC have been postulated to contribute to endogenous cardiomyogenesis and angiogenesis. Our experiments were not designed to address this issue.

Two main approaches have been developed for hollow organ tissue engineering, firstly the *de novo* reconstitution of a functional hollow organ by tissue engineering and secondly the transplantation of the bioengineered donor organ [Alberti C, G Chir 2009]. We have chosen the *de novo* reconstitution approach because of the increase shortage of donor organs on the one side and the improvement in the research field of bioscaffolds on the other side. In our approach, bioengineered colon is made of autologous SVF seeded onto chitosan hydrogel matrix without their own microvascular network. When implanted *in vivo*, nutrients and oxygen, should diffuse from neighboring tissues. This approach have been already reported for oesophagus [Nakase, Y. 2008] [Wei, R.Q. 2009] [Doede, T. 2009], stomach [Araki, M. 2009] [Sala, F.G. 2009] and small intestine [Sala, F.G. 2009] [Nakase, Y. 2007] [Nakase, Y. 2006] but never for colonic tissue in our knowledge. All these studies highlighted the cells seeding requirement but none of them underlined the concept of scaffold organ specificity. The current feasibility study is therefore the first report of circular colonic replacement by tissue engineering and should open the field of rectal organ replacement by tissue engineering.

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Table 1 Statistical analysis of fibrosis ratio

Graph 1A Weekly animal weight in kilograms acellular group

Graph 1B Weekly animal weight in kilograms cellular gro



Figure 1A Chitosan patch on colonic defect

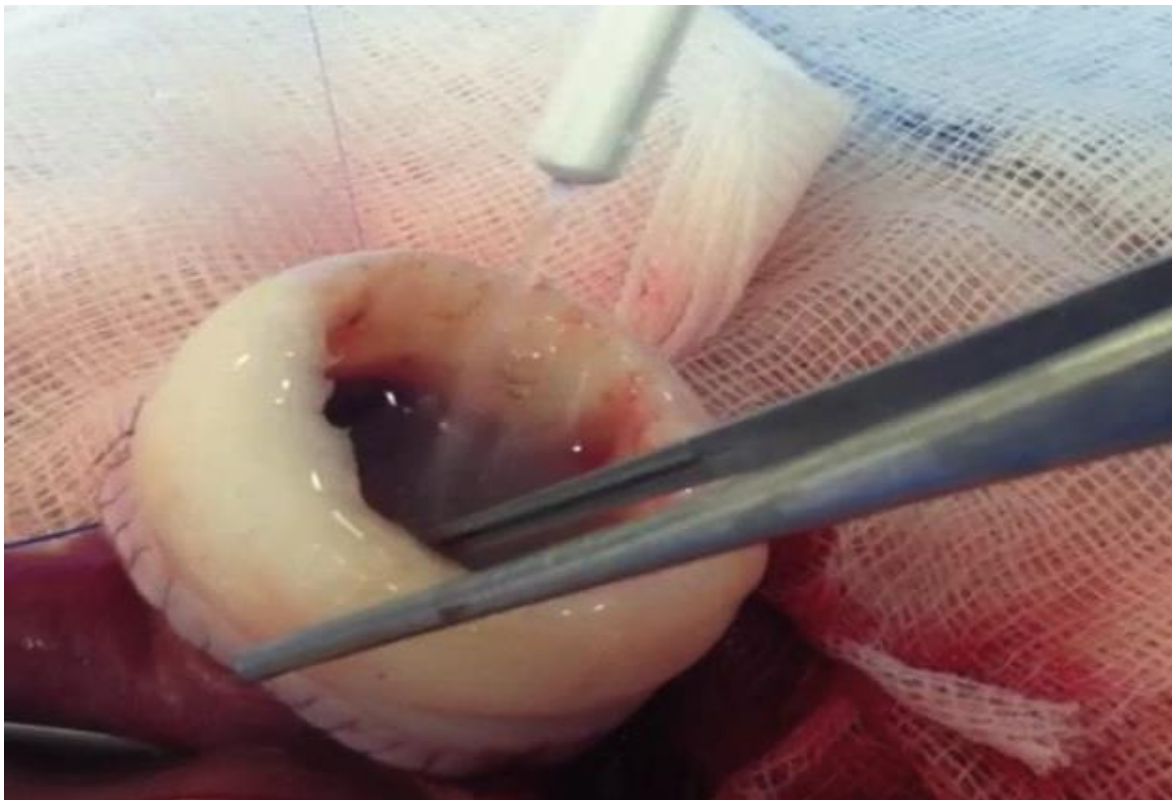


Figure 1B Chitosan hydrogel tube with composite glue application

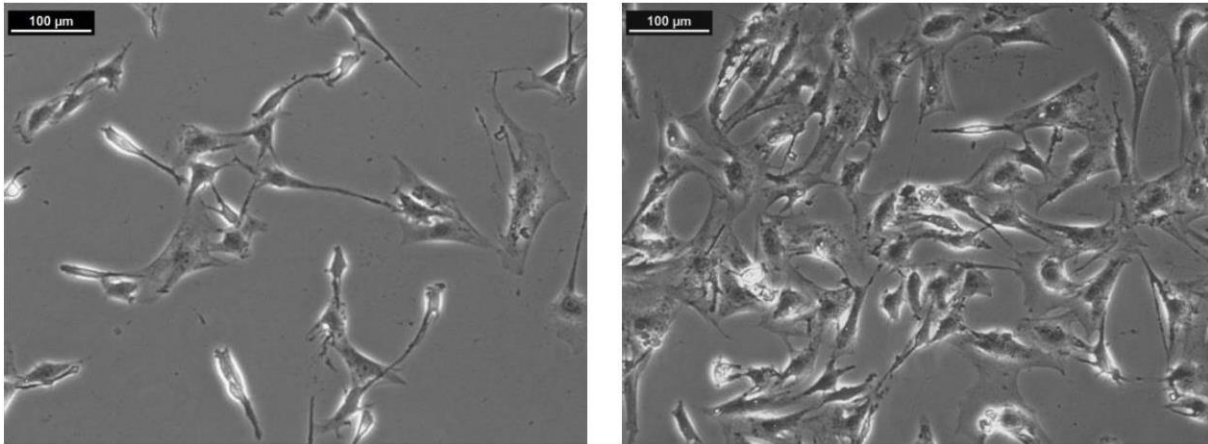


Figure 2 Observation under a phase contrast microscope pADSC culture a) 48 hours b) 4 Days

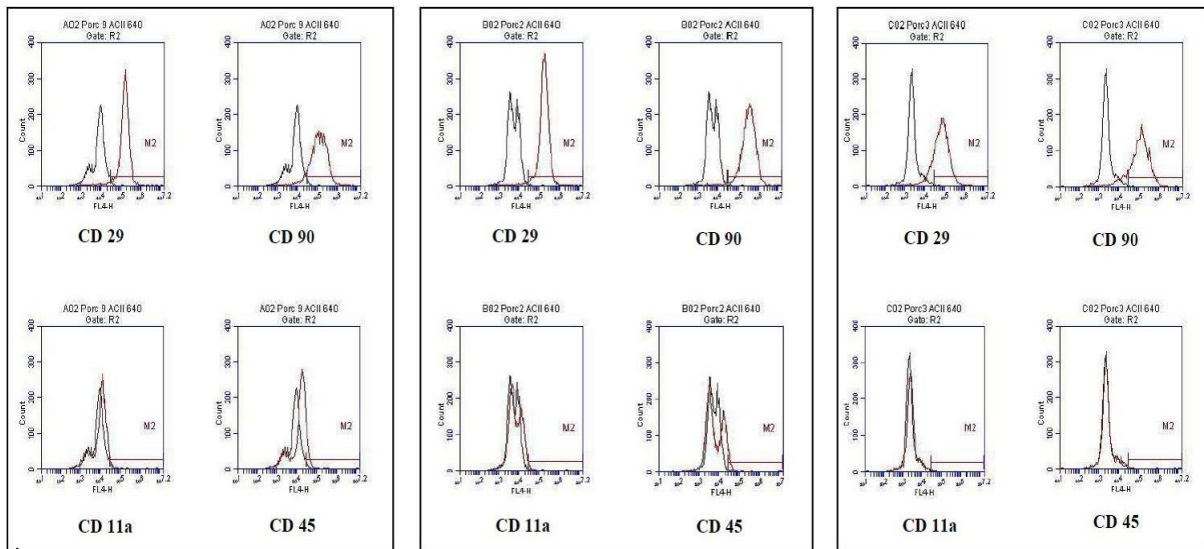


Figure 3 pADSCs's flow cytometric analysis isolated from three separate individuals

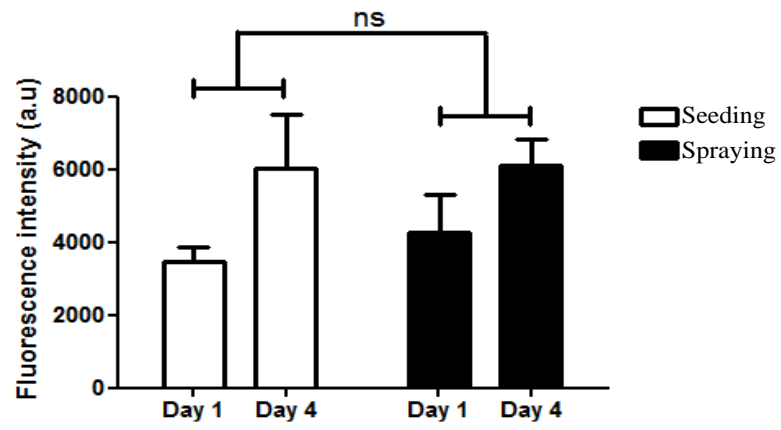


Figure 4 Cy-quant cell proliferation assay Statistical analysis

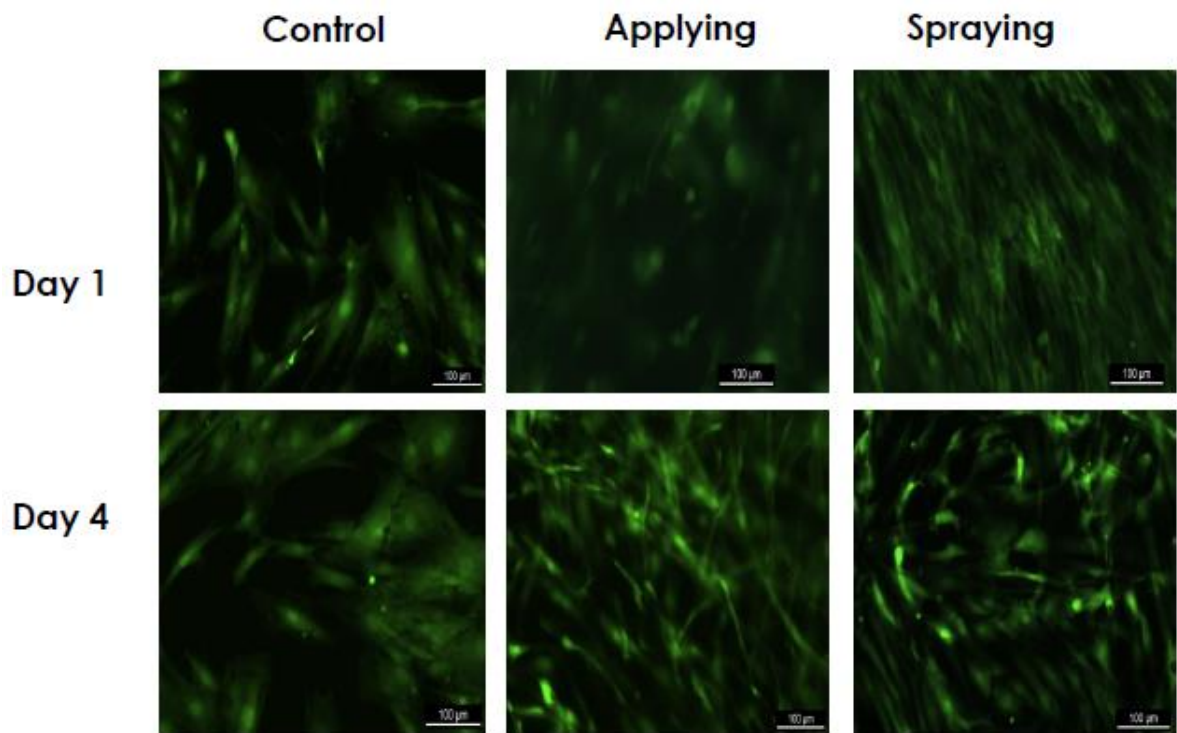


Figure 5 Cell viability test live/dead cells distribution seemed to be more homogeneous with spraying procedure at day 1



Figure 6 Macroscopic comparative analysis Acellular group on the left cellular group on right

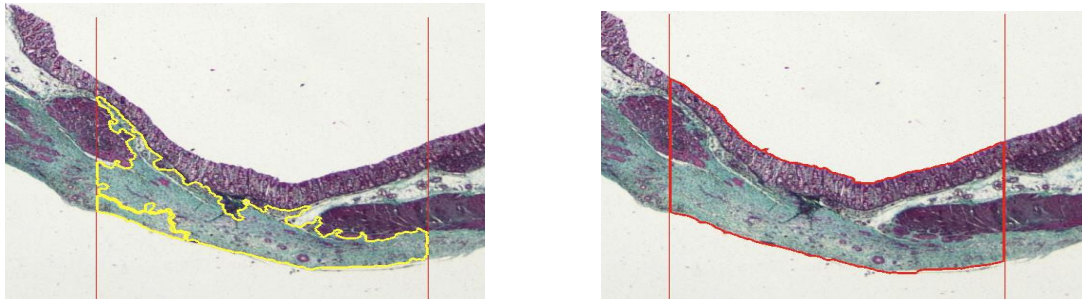


Figure 7 Masson staining fibrosis ratio :a) fibrosis area b) total tissue scar area

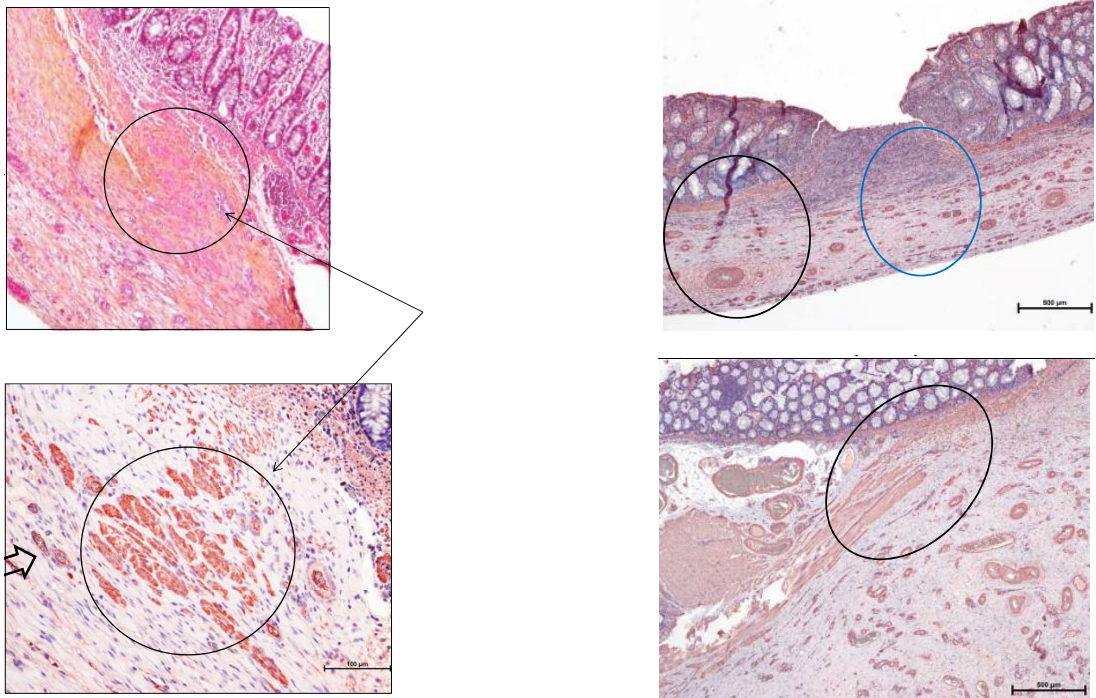


Figure 8 Immunohistochemistry with staining for smooth muscle a)caldesmon b)actin



Figure 9. Macroscopic view 8 weeks after circular matrix implantation



Figure 10. Inner diameter of the circular graft area

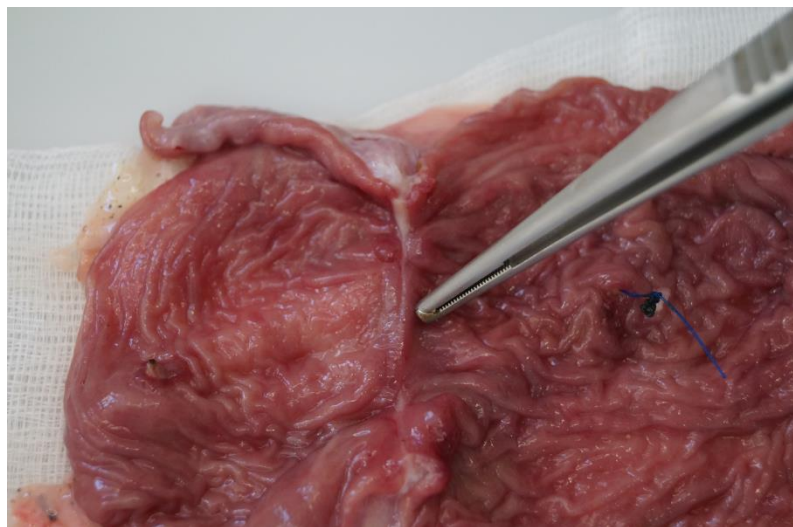


Figure 11. Fibrosis scar of the circular graft area





Figure 12. Microscopic view after Masson staining.

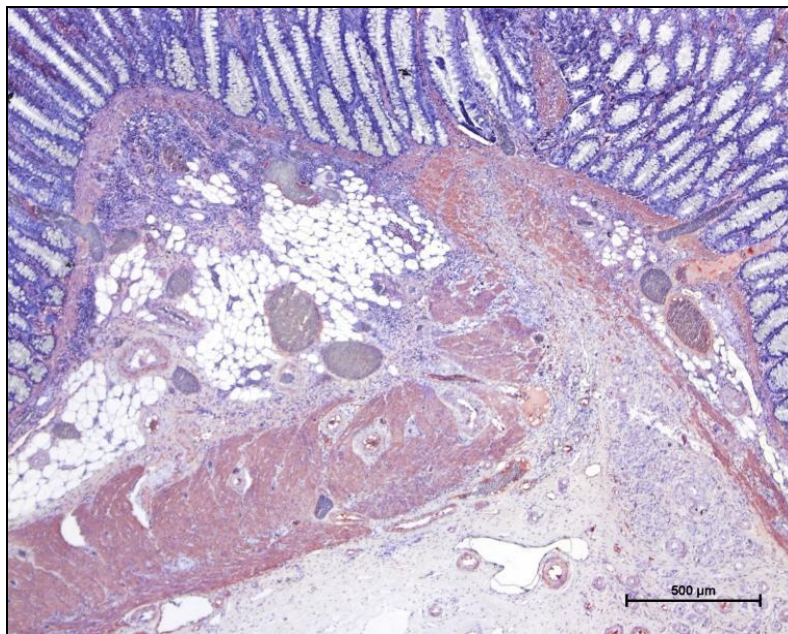
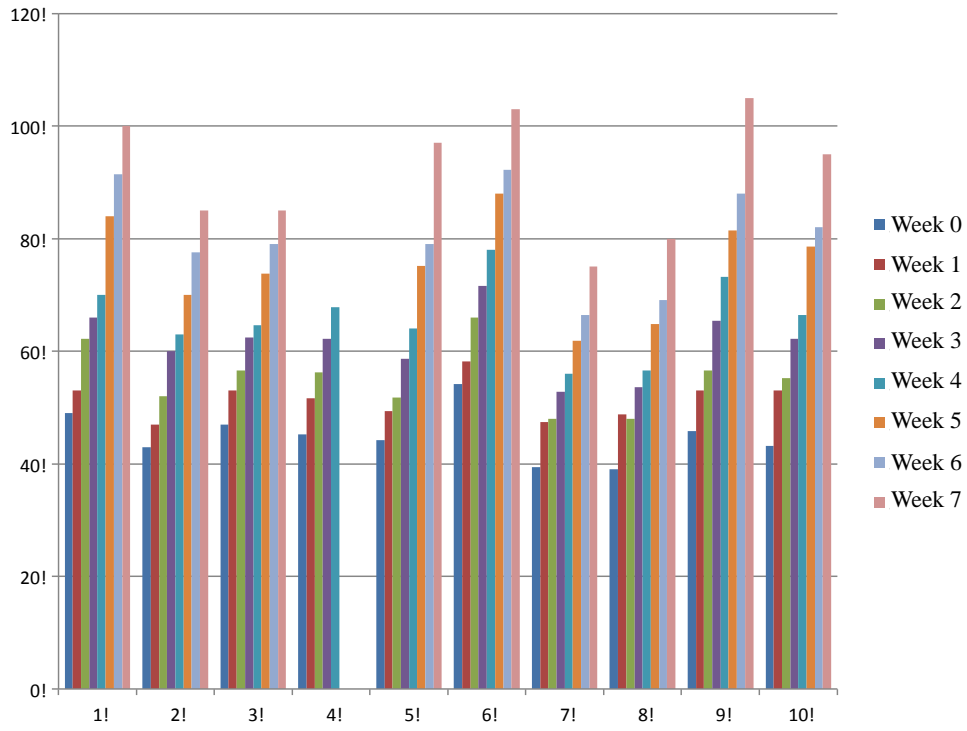


Figure 13. Positive caldesmon staining

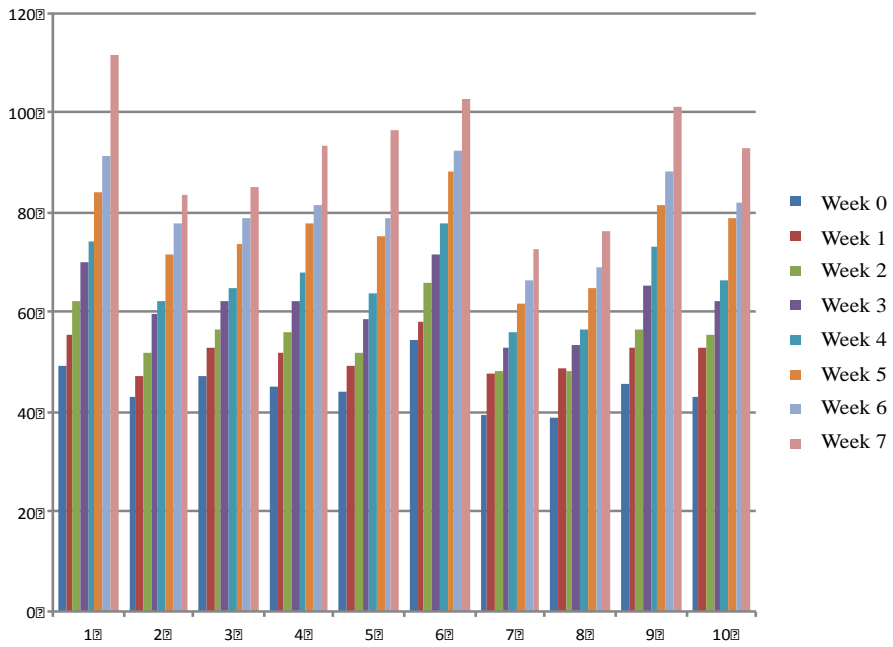
<b>Fibrosis ratio</b>	<b>Acellular Group (n=9)</b>	<b>Cellular Group (n=10)</b>	<b>p value*</b>
	Median (min-max)	Median (min-max)	
Ratio	0.55 (0.22-1.00)	0.13 (0.00-0.95)	<i>0.013</i>

- Wilcoxon rank test

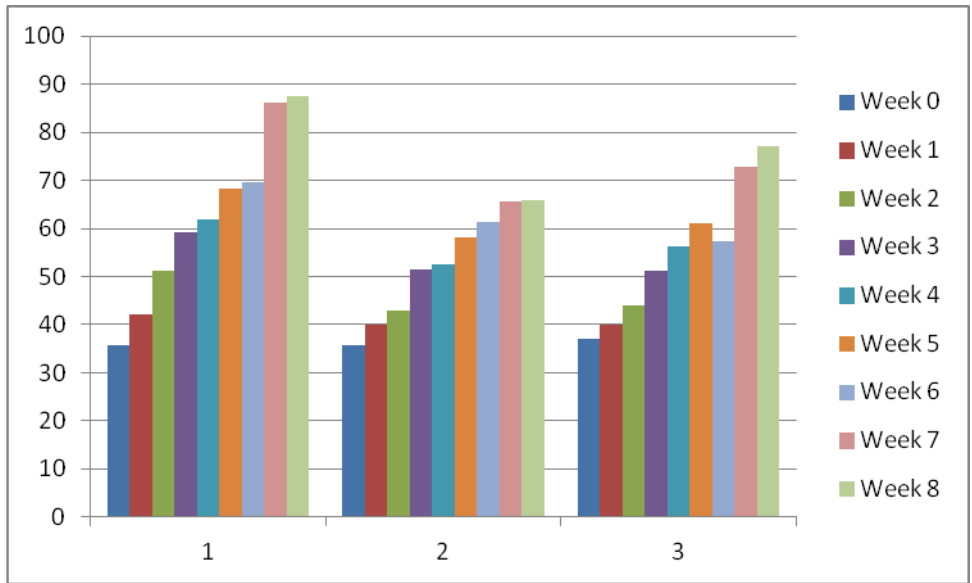
Table 1 Statistical analysis of fibrosis ratio



Graph 1A Weekly animal weight in kilograms acellular group



Graph 1B Weekly animal weight in kilograms cellular group



Graph2. Weekly animal weight in kilograms in feasibility study

# PARTIE III

CONCLUSION

DISCUSSION

PERSPECTIVES

L'objectif général de ce travail de thèse était l'élaboration d'un substitut colorectal circulaire devant mimer la structure et la fonction de la paroi colorectale native selon les principes de l'ingénierie tissulaire.

Dans l'article 2, la matrice à base d'hydrogel de chitosane a permis, comparativement à la matrice de SIS, d'obtenir *in vivo* chez le lapin une régénération épithéliale plus précoce, un meilleur contrôle de la réponse inflammatoire avec un rapport de fibrose plus faible ( $p=0,07$ ) et l'obtention d'ilots de cellules musculaires lisses à 8 semaines. *In vitro*, la viabilité et la prolifération cellulaires ont été obtenues avec les 2 matrices, sans différence significative.

Dans l'article 3, les tests mécaniques, de viabilité et de prolifération cellulaires ont permis de sélectionner un gel composite fibrine-chitosane avec une concentration de fibrinogène de 20 mg/mL, une concentration de chitosane de 1% et un degré d'acétylation (DA) de 38%. Cette composition a permis d'obtenir un taux de viabilité et de prolifération cellulaires significativement plus élevés que les autres formulations ( $p<0,001$ ).

Dans l'article 4, après succès de l'implantation du patch de chitosane chez les 20 porcs, un recouvrement muqueux complet de la zone implantée était observée dans les 2 groupes avec une régénération *ad integrum* de la paroi colique, y compris des cellules musculaires lisses confirmées par l'analyse immunohistochimique, et un rapport de fibrose significativement plus faible dans le groupe cellularisé (15% vs. 50%,  $p=0,01$ ).

Nos données sont les premières à confirmer l'intérêt pressenti du chitosane pour la régénération tissulaire colique. Le développement du gel composite et les résultats fructueux de l'implantation d'une matrice cellularisée autologue chez le porc sont un préalable au développement de l'ingénierie tissulaire colorectale chez l'homme. L'implantation d'une matrice circonférentielle, objectif général de ce travail de thèse, a pu être effectuée dans le cadre d'une étude de faisabilité sur un nombre restreint de porcs avec une qualité de reconstruction de la paroi colorectale variable. Cette variabilité sur un faible échantillon d'animaux souligne le caractère difficilement reproductible, à l'heure actuelle, des matrices circonférentielles de chitosane en termes d'épaisseur notamment. Cependant, ces résultats préliminaires apparaissent prometteurs et autorisent, comme perspective à ce travail, une ouverture sur l'élaboration d'un substitut rectal cellularisé.

Nos travaux sur la matrice d'hydrogel de chitosane ont évalué la bioactivité et la biodégradabilité du polymère au sein du tractus digestif bas. Le chitosane a une action dite « bio-stimulante » sur la cicatrisation tissulaire car il favorise une augmentation du nombre d'histiocytes et une stimulation des fibroblastes qui produisent une quantité abondante de collagène-3, indispensable dans les phénomènes de cicatrisation (Okamoto Y 1995) (Muzzarelli 2005) (Shi 2006). Ces données corroborent celles de Citgez, et al. (Citgez 2012) qui rapporte, lors de l'application de chitosane en périphérie d'une anastomose colique chez le rat, une augmentation de la collagénisation de l'anastomose associée à une augmentation des résistances mécaniques anastomotiques. Nous n'avons pas évalué le taux de collagénisation de la zone de greffe lors de nos expériences *in vivo*, mais nos données ont permis de valider les propriétés bio-stimulantes du chitosane sur la cicatrisation au niveau de la zone de greffe colorectale. Les propriétés bactériostatiques ou bactéricides du chitosane apparaissent d'une importance majeure au regard du site d'implantation. La biodégradabilité est une propriété indispensable à un substitut issu de l'ingénierie tissulaire. Le chitosane est facilement hydrolysable par de nombreuses chitinases et des enzymes bactériennes retrouvées de façon prédominante au niveau du côlon (Jain 2007) (McConnell 2008). Il est difficile d'estimer scientifiquement la biodégradabilité d'un matériau. Cependant, nos constatations macroscopiques et microscopiques, quel que soit le modèle animal (lapin ou porc), ont permis de mettre en évidence une dégradabilité importante du polymère sur le site d'implantation puisque seulement des fragments phagocytés ont pu être mis en évidence au sein de macrophages. L'activité enzymatique de la flore bactérienne et la présence de chitinases pourraient expliquer la biodégradabilité importante du chitosane sur le site de la greffe, soulignant le concept de spécificité d'organe de la matrice.

Nous avons réalisé, en parallèle de l'évaluation des propriétés de bioactivité et de biodégradabilité du chitosane, des tests mécaniques (échantillon de 0,5 cm x 1 cm mis en traction par un poids croissant jusqu'à rupture de la matrice) permettant d'estimer la résistance à l'étirement de la matrice de chitosane. Ces tests (n=4) ont été réalisés comparativement à du tissu colique natif porcin. La résistance à l'étirement pour l'hydrogel de chitosane était évaluée à 98 ( $\pm 10$ ) g/f versus 258 ( $\pm 80$ ) g/f pour le tissu colique. Il apparaît donc primordial dans les perspectives à apporter à ce travail d'améliorer les propriétés mécaniques du polymère. La réalisation de tube colique nécessite la réalisation future de tests biomécaniques pour l'évaluation de la résistance à l'étirement, mais aussi à la distension.

Les ADSC sont issues de la mise en culture de la fraction vasculaire stromale qui renferme une population cellulaire hétérogène dont les ADSC représentent 5%. La fraction vasculaire stromale est également employée en clinique pour les reconstructions mammaires (Mestak 2014). Cette application a l'avantage de ne pas nécessiter le passage préalable par une culture cellulaire *in vitro*. Des dispositifs médicaux, dont le système Celution® (Cytori Therapeutics, San Diego, Etats-Unis), ont d'ailleurs été développés pour permettre l'isolement et l'utilisation extemporanée de la fraction vasculaire stromale au bloc opératoire (Daniels 2007). L'ensemble de ces éléments, associés à nos résultats sur la cellularisation peropératoire des matrices, laisse penser que l'utilisation du site de greffe comme bioréacteur, peut être une alternative au développement de l'ingénierie tissulaire avec culture cellulaire *in vitro*.

Les forces de ce travail sont représentées par :

- le développement de modèles expérimentaux en chirurgie colorectale,
- la validation des techniques de prélèvement, d'amplification et de caractérisation des ADSC porcins,
- le développement d'un gel de confinement cellulaire,
- la validation de l'intérêt du chitosane en ingénierie colorectale.

Ce travail ouvre, de par son originalité, sur des opportunités de financements de nouveaux travaux et de collaboration avec l'IMP, laboratoire expert pour le chitosane. Il suscite par ailleurs l'intérêt des industriels dans le cadre de perspective d'applications cliniques.

Les perspectives de ce travail de thèse résident :

- d'une part dans l'élaboration d'un substitut rectal cellularisé dans le cadre de partenariat institutionnel. Les travaux à réaliser devraient tendre vers l'optimisation des propriétés mécaniques du polymère et la recherche d'une taille limite, ou critique, au remplacement colique segmentaire ;

- et d'autre part dans le développement clinique de matrice d'hydrogel de chitosane dans le cadre de partenariat institutionnels-industriels, le polymère présente les propriétés idéales pour le soutien de la cicatrisation du tissu colorectale en situation préventive et curative.



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