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Par

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Interpenetrating polymer networks hydrogels of silanized hydroxypropyl methylcellulose/methacrylated polysaccharides for biomedical applications

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«A me tremavano un po' le gambe; provavo paura retrospettiva,
e insieme una certa sciocca fierezza, per aver confermato un'ipotesi,
e per aver scatenato una forza della natura.
Era proprio idrogeno, dunque: lo stesso che brucia nel sole e nelle stelle,
e dalla cui condensazione si formano in eterno silenzio gli universi»

Il sistema periodico-Primo Levi

«I experienced retrospective fear
and at the same time a kind of foolish pride, at having confirmed a hypothesis
and having unleashed a force of nature.
It was indeed hydrogen, therefore: the same element that burns in the sun and stars,
and from whose condensation the universes are formed in eternal silence»

The periodic table- Primo Levi

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Abstract

Interpenetrating polymer networks hydrogels of silanized hydroxypropyl methylcellulose/methacrylated polysaccharides for biomedical applications

Guided tissue regeneration and guided bone regeneration are surgical procedures aiming the regeneration of lost components of periodontium. In these techniques a periodontal intraosseous defect is filled or not with a bone graft material and covered with a biocompatible membrane in order to prevent its colonization by soft tissues. In fact, during a physiological healing, it appears that the soft tissue migrates rapidly into the wound, avoiding the regeneration. The barrier membrane plays a key role to prevent undesirable tissue migration into the defective area, and consequently, it allows sufficient time for bone, cementum, and periodontal ligament regeneration.

Silanized hydroxypropyl methylcellulose (Si-HPMC) is a self-setting hydrogel that can be injected *in vivo* as a viscous solution and then, due to the condensation reaction, it builds a 3D network. It has been demonstrated that cross-linked Si-HPMC acted as a physical barrier against soft tissue invasion in periodontal defects in dogs. The main drawback of this self-setting hydrogel is an excessively slow crosslinking process, which is not suitable for clinical needs. Nevertheless, the results were quite promising due to the easy preparation and handling of polymer solution, which could lead to simplified periodontal treatment[1,2].

Therefore, the main objective of this thesis was the development and the characterization of two injectable interpenetrating polymer networks (IPNs) hydrogels composed of Si-HPMC and two polysaccharides: carboxymethyl chitosan (CMCS) or dextran. We grafted on CMCS and dextran methacrylate groups able to react under irradiation of standard polymerization dentist's lamp. We selected vitamin B2 or riboflavin as a photoinitiator.

The polymers were successfully synthetized and characterized. Two innovative IPNs were realized and the chemical and physicochemical properties were studied. In addition, *in vitro, in vivo* studies were performed to assess the cytompatibility and to study the suitability of these two innovative IPNs hydrogels membranes for guided tissue or bone regeneration.

The encouraging results need *in vivo* further investigations to characterize the biomaterials and confirm the potentiality as dental biomaterials.

Résumé

Réseaux interpénétrés d'hydrogels d'hydroxypropyl méthylcellulose silanisé/ polysaccharides méthacrylés pour des applications biomédicales.

La régénération tissulaire guidée et la régénération osseuse guidée sont des interventions chirurgicales visant la régénération des tissus perdus du parodonte et de l'os alvéolaire. Dans ces techniques, une membrane biocompatible est implantée autour de la lésion intra osseuse parodontale, remplie ou non d'une greffe osseuse, afin de prévenir sa colonisation par les tissus mous. L'hydroxypropyl méthylcellulose silanisée (Si-HPMC) est un hydrogel auto-réticulant qui peut être injecté sous forme de solution visqueuse puis, grâce à la réaction de condensation, construire un réseau 3D. Il a été démontré que le Si-HPMC réticulé agissait comme barrière physique contre l'invasion des tissus mous dans les défauts parodontaux chez le chien. Le principal inconvénient de cet hydrogel auto-réticulant est une cinétique de réticulation trop lente pour des applications cliniques optimales[1,2].

L'objectif principal de cette thèse était donc le développement et la caractérisation de deux réseaux interpénétrés d'hydrogels (IPNs) composés de Si-HPMC et d'un polysaccharide : soit le carboxyméthyl chitosane (CMCS), soit le dextrane. Nous avons greffé sur le CMCS et le dextrane des groupes méthacrylate capables de réagir sous irradiation d'une lampe à polymériser standard utilisée en dentisterie en présence d'un photo-initiateur composé de vitamine B2.

Les polymères ont été synthétisés et caractérisés avec succès. Deux IPNs innovants ont été réalisés et les propriétés chimiques et physico-chimiques ont été étudiées. De plus, des études *in vitro* et *in vivo* ont été réalisées pour évaluer la cytocompatibilité et pour étudier l'aptitude de ces deux membranes innovantes hydrogel IPN à promouvoir la régénération tissulaire ou osseuse guidée.

Les résultats sont encourageants et nécessitent d'autres investigations pour caractériser les biomatériaux et confirmer leur potentiel en tant que biomatériaux dentaires et implantaire.

Publications

In situ photochemical crosslinking of hydrogel membrane for Guided Tissue Regeneration. Pauline Marie Chichiricco, Raphaël Riva, Jean-Michel Thomassin, Xavier Struillou, Catherine Le Visage, Christine Jérôme and Pierre Weiss. **In press in Dental Materials.**

Communications

➢ Oral communications

- *Photo-crosslinkable membrane for Guided Periodontal Tissue Regeneration.* Pauline Marie Chichiricco, Raphaël Riva, Jean-Michel Thomassin, Xavier Struillou, Catherine Le Visage, Christine Jérôme, Pierre Weiss. **Dentistry and dental materials, Rome, IT, July 2018**
- *Photo-crosslinkable hydrogel for Guided Periodontal Tissue Regeneration*. Pauline Marie Chichiricco, Raphaël Riva, Jean-Michel Thomassin, Xavier Struillou, Catherine Le Visage, Pierre Weiss, Christine Jérôme**. Belgian Polymer Group (BPG), Blankenberge, BE, May 2018 Prize best oral presentation.**
- *Photo-crosslinkable Interpenetrated Polymer Network for guided periodontal regeneration*. Pauline Marie Chichiricco, Raphaël Riva, Jean-Michel Thomassin, Catherine Le Visage, Xavier Struillou, Pierre Weiss, Christine Jérôme. **European Society For Biomaterials (ESB), Athens, GR, Sept 2017**
- *Membrane photoréticulable pour la régénération tissulaire guidée du parodonte.* Pauline Marie Chichiricco, Raphaël Riva, Jean-Michel Thomassin, Catherine Le Visage, Xavier Struillou, Pierre Weiss, Christine Jérôme. **Societe francophone des biomateriaux dentaires (SFBD), Paris, FR, July 2017. Prize best oral presentation.**
- *Self-setting hydrogel for 3D bioprinting.* Pauline Marie Chichiricco, Benoit Rosa, Perrine de Villemagne,Lara Figuereido, Kevin Shakesheff, Jing Yang, Christine Jérôme, Jérôme Guicheux, Jean-Yves Hascoet, Catherine Le Visage Pierre Weiss. **3D bioprinting in cancer research, Nantes, FR, July 2017**
- *Photocrosslinked hydrogel for Periodontal Tissue Regeneration.* Pauline Marie Chichiricco, Raphaël Riva, Jean-Michel Thomassin, Xavier Struillou, Catherine Le Visage, Christine Jérôme, Pierre Weiss. **Journee Scientifique ED, Nantes, FR, Dec 2016. Prize best oral presentation.**
- *Photocrosslinked hydrogels for guided periodontal tissue regeneration.* Pauline Marie Chichiricco, Xavier Struillou, Pierre Weiss, Christine Jérôme. **International Automn NanoFar school, Nantes, FR, October 2015. Prize best oral presentation.**

➢ Posters

- *Photo-crosslinkable Interpenetrated Polymer Network for guided periodontal regeneration.* Pauline Marie Chichiricco, Raphaël Riva, Jean-Michel Thomassin, Catherine Le Visage, Xavier Struillou, Pierre Weiss, Christine Jérôme. **European Society For Biomaterials (ESB), Athens, GR, Sept 2017**
- *Self-setting hydrogel for 3D bioprinting.* Pauline Marie Chichiricco, Benoit Rosa, Perrine de Villemagne, Christine Jérôme, Jérôme Guicheux, Jean-Yves Hascoet, Catherine Le Visage Pierre Weiss. **3D bioprinting in cancer research, Nantes, FR, July 2017**

- *Photo-crosslinkable membrane for guided tissue regeneration.* Pauline Marie Chichiricco, Catherine Le Visage, Xavier Struillou, Pierre Weiss, Christine Jérôme. **European Chapter meeting of the Tissue Engineering and Regenerative Medicine International Society (TERMIS), Davos, CH, 2017**
- *Photo-crosslinkable membrane for guided tissue regeneration.* Pauline Marie Chichiricco, Catherine Le Visage, Xavier Struillou, Pierre Weiss, Christine Jérôme. **International NanoFar school, Notthingam, UK, April 2017**
- *Photo-crosslinkable membrane for guided tissue regeneration.* Pauline Marie Chichiricco, Catherine Le Visage, Xavier Struillou, Pierre Weiss, Christine Jérôme. **Belgian Polymer Group (BPG), Houffalize, BE, May 2017**
- *Polysaccharides interpenetrated polymer networks for biomedical applications*. Pauline Marie Chichiricco, Raphaël Riva, Jean-Michel Thomassin, Pierre Weiss, Christine Jérôme. **Belgian Polymer Group (BPG), Hasselt, BE, May 2016**
- *Photo-crosslinkable interpenetrated polymer network for biomedical application.* Pauline Marie Chichiricco, Raphaël Riva, Jean-Michel Thomassin, Catherine Le Visage, Xavier Struillou, Pierre Weiss, Christine Jérôme. **Bioregate Forum, Nantes, FR, Sept 2016**

Supervision activity

Marie Ramirez (January-July 2017. Stage Master 2 Ingénierie de la santé, parcours Biomatériaux et Dispositifs Médicaux, Université de Bordeaux). Elaboration d'hydrogels à base de polymères boroniques pour l'ingénierie tissulaire.

Noémie Brunelliere (June 2018. Stage TER, Master 1 Biologie-Sante, Université de Nantes). Caractérisation physico-chimique d'hydrogels pour applications biomédicales.

List of acronyms

CMCS: carboxymethyl chitosan

DexMA: dextran methacrylate

DMEM: dulbecco's modified Eagle's medium

DMSO: dimethyl sulfoxide

DN: double network

ECM: extracellular matrix

FT-IR: fourier transform infrared spectroscopy

GBR: guided bone regeneration

GMA: glycidyl methacrylate

GPC: gel permeation chromatography

GPTMS: 3-glycidoxypropyltrimethoxysilane

GTI: guided tissue induction

GTR: guided tissue regeneration

HA: hydroxyapatite

HEPES: (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid)

HES: hematoxylin, eosin Y, safranin

HGFs: human gingival fibroblasts

homo-IPN: homo interpenetrating polymer network

HPLC: high performance liquid chromatography

HPMC: hydroxypropyl methylcellulose

IPN: interpenetrating polymer network

MA-CMCS: methacrylated carboxymethyl chitosan

NMP: N-methyl-2-pyrrolidone

NMR: nuclear magnetic resonance

NPs: nanoparticles

OCT: optimal cutting temperature

PBS: phosphate buffered saline

PEG: poly(ethylene glycol)

PFA: paraformaldehyde

PGA: poly(glycolide)

PHEMA: (poly(2-hydroxyethylmethacrylate)

PIS: photoinitiator solution

PLA: poly(lactic acid)

PLGA: poly(lactic-co-glycolic acid)

PRF: platelet-rich fibrin

PTFE: polytetrafluroethylene

RGD: tripeptide Arg-Gly-Asp

ROS: reactive oxygen species

RP: Riboflavin 5'-phosphate sodium salt hydrate derivative

semi-IPN: semi interpenetrating polymer network

Si-HPMC: sylanized hydroxypropylmethyl cellulose

TCP: tricalcium phosphate

TEOHA: triethanolamine

Contents

Chapter 1 : introduction and objectives

I. Polymer composite hydrogel

1. Introduction

Hydrogels are three-dimensional polymer networks able to retain a large amount of water. These materials having physicochemical characteristics close to soft tissues find applications especially in the biomedical field. Currently, hydrogels are used for manufacturing contact intraocular lenses, as drug delivery systems in various pharmaceutical products, as scaffolds for tissue engineering or wound dressing, and also in hygiene and cosmetic products.

Hydrogels are composed of hydrophilic chains of either natural or synthetic polymers. Natural polymers, e.g. collagen, hyaluronic acid, alginate, are mostly appreciated due to their high biocompatibility and for polymer/tissue interaction. However, some limitations include the poor mechanical properties and the potential immunogenicity. On the opposite, synthetic polymers, e.g. poly(ethylene glycol) (PEG), (poly(2-hydroxyethylmethacrylate) (PHEMA), are inert in biological environments, but present better mechanical properties and controllable structure.

Polymers could be physically or chemically cross-linked in order to produce hydrogels. Physical interactions usually involve secondary bonds such as H-bonding or hydrophobic forces, and they are considered as reversible depending on environment stimuli (e.g. temperature, pH, ionic strength). Some examples are alginate or gellan gum[3–6], which cross-link in presence of divalent cations. The advantage of physically cross-linked hydrogels is the absence of chemical reactants, catalysts or the residual monomers in the 3D network. However, changing the environment could alter the network structure. Chemically cross-linked hydrogels, on the contrary, have a high stability due to the formation of covalent bonds after reaction such as PHEMA, or cross-linked collagen [7–9].

Unfortunately, the mechanical fragility of most hydrogels represents a strong limitation for their applications as a substitute of tissue-like biomaterial. In fact, the extra cellular matrix possesses extraordinary mechanical properties despite of the high water content compared to hydrogels[10,11].

Many strategies have been explored to increase the mechanical characteristics of hydrogels. Multicomponent hydrogels represent a strategy for improving mechanical properties of biomaterials or to tailor particular properties to meet specific needs. The design of hydrogels may lead to significant improvements of stimuli responsive behavior, exploiting the 'smart' properties of at least one of the polymer components. For example, they could be capable to release an entrapped drug in response to specific physiological triggers, at the appropriate time and site of action [3], or capable to enhance the cell/biomaterial interaction through mechanical properties modifications such as stiffness or relaxation [12–14], or modifying the bioadhesion properties[15–17].

Nanoparticles (NPs) are frequently used to enhance or modify hydrogels properties. Nanoparticles are prepared from different materials such as polymers, metals, minerals, semiconductors and with various shapes such as spheres, rods, tubes. The addition of NPs to a hydrogel system, according to their chemical and physical properties creates reinforced nanocomposite hydrogels. For example, introducing mechanically stiff fillers, such as nanoclays, in the hydrogel network, leads to reinforced composite hydrogels with improved mechanical strength thanks to multi-point crosslinking.

The simplest strategy to tailor the properties of a hydrogel system is to create a polymer-composite hydrogel, composed of two or more polymers, in which at least one is cross-linked. An overview of such polymer-composite hydrogels is presented in the following. Polymer-composite hydrogels have been developed to circumvent the limitation of polymer hydrogels especially to improve the mechanical

properties or to tailor particular properties to meet specific needs. There are two main types of composite hydrogels: hybrid networks and interpenetrating polymer networks (Figure 1).

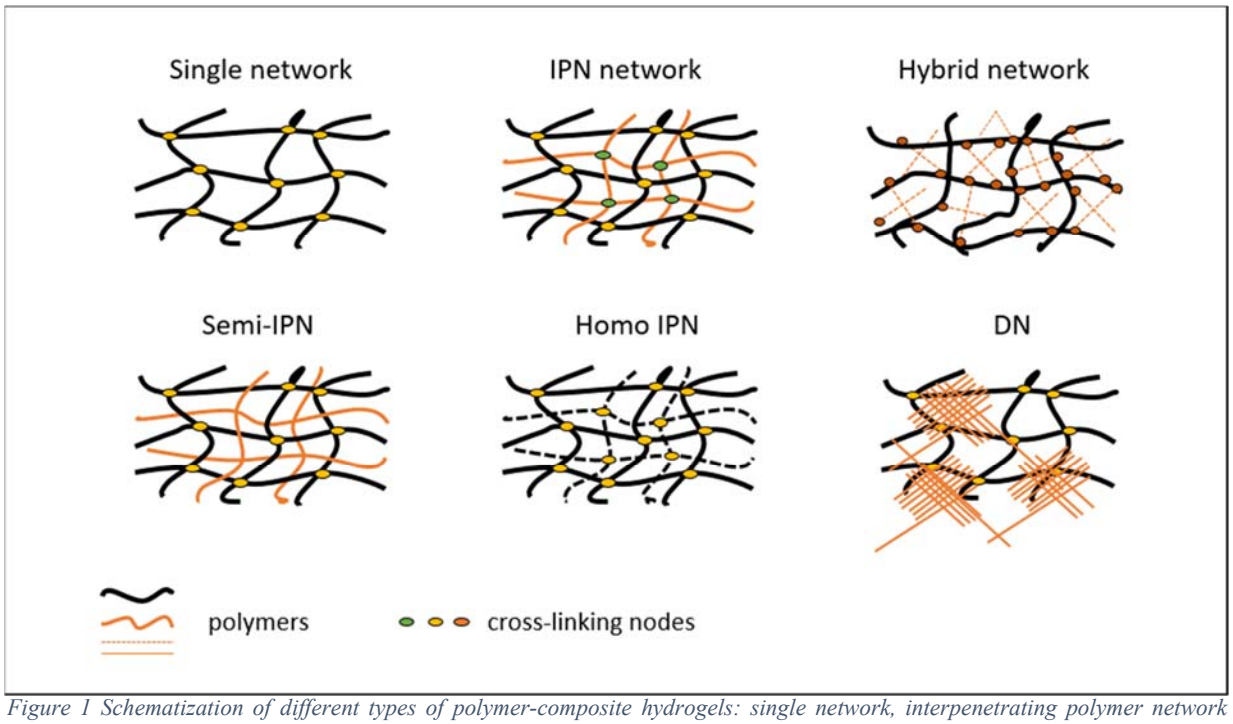

Figure 1 Schematization of different types of polymer-composite hydrogels: single network, interpenetrating polymer network (IPN), hybrid network, semi interpenetrating polymer network (Semi-IPN), homo interpenetrating polymer network and double network (DN).

2. Hybrid networks

Hybrid hydrogel networks are composed of two or more preformed polymers that are blended and then cross-linked together.

Hybrid polymer scaffolds composed of natural polymers and synthetic polymers, combined the potential to mimic the extracellular matrix (ECM) with bioactive moieties, such as RGD in peptides with the possibility to improve the mechanical robustness of the hydrogel network[18].

Covalently cross-linked hybrid hydrogels can be formed via various reactions, including free radical polymerization, and click chemistry reactions, such as thiol-ene addition. In radical polymerization multiple similar reactive groups are able to cross-link, such as vinyl and methacrylate moieties, that can react and form hybrid hydrogels in a one-pot reaction. An example reported in the literature by Elisseff *et al.* is a hybrid formulation composed of methacrylate hyaluronic acid cross-linked with methacrylated PEG under

UV irradiation. The photocrosslinking was performed transdermally after injection and the ratio between the two methacrylic polymers was varied to tune the gelation time and the elastic properties as required by the targeted application[19].

3. Interpenetrating polymer networks (IPNs)

IPNs are systems composed of two or more polymer networks entangled at the molecular scale to each other, but not covalently bond to each other. In fact, a mixture of pre-formed hydrogels is not an IPN. If just one of the two polymers is cross-linked, the structure is called semi-IPN. Many example in literature reported the convenience to have a polymer trapped in a three-dimensional structure. Matricardi *et al.* studied a semi-IPN composed of alginate/scleroglucan polysaccharides as a drug delivery system. The addition of alginate to the system showed an increase in the elastic modulus of one order of magnitude and modification of the kinetics of a released drug [20]. Chitosan is one of the most abundant polysaccharides often used in IPN structures with ionic polymers. $-NH_3$ groups of the chitosan backbone are able to interact with polyelectrolyte as $–COO^-$, which increases the mechanical properties, but are reversible in response to variation of the pH[21]. This property makes it an interesting system for drug delivery. As example, Gu *et al.* reported on a thermo- and pH-responsive semi-IPN polyampholyte hydrogels prepared by using carboxymethyl chitosan and poly(2-(dimethylamino) ethyl methacrylate) with N N'-Methylenebisacrylamide (BIS) as crosslinking agent. It was found that the semi-IPN hydrogel shrunk mostly at the isoelectric point (IEP) and swelled when pH deviated from the IEP. [22].

IPNs could be built from monomers, polymers or both. IPNs can be prepared in simultaneous pathway, when the precursors are mixed together and cross-linked simultaneously, or prepared in a sequential pathway, in which a first polymer network is swollen in the second precursor and then cross-

linked in presence of first polymer network (Figure 2). If the sequentially prepared structure is composed of the same polymer network, they are called homo-IPN.

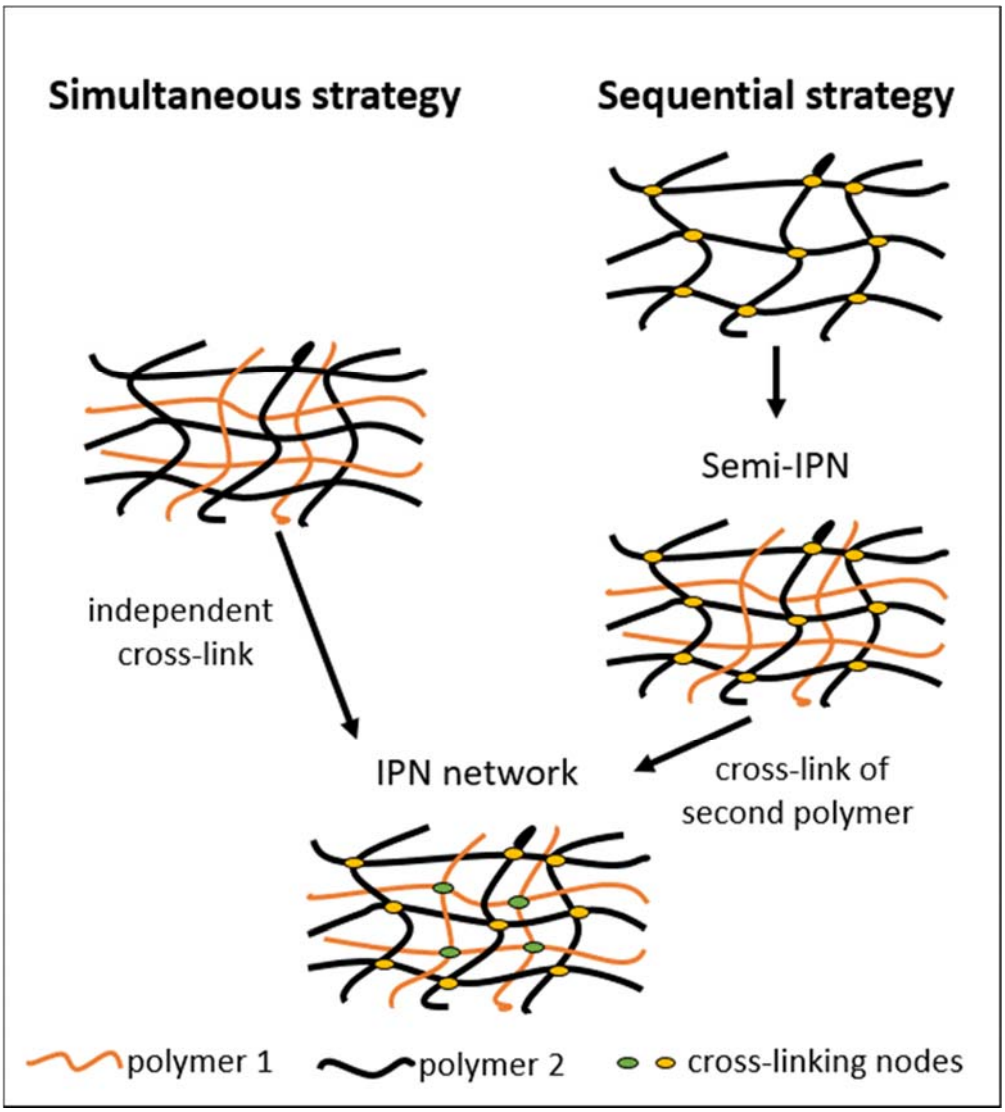

Figure 2 Schematic representation of IPN formation from two polymers. IPN can be obtained in a simultaneous or sequential pathway.

IPNs have been received many attentions in the biomedical and pharmaceutical fields because the mixture of two polymers can lead to a synergistic combination of favorable properties.

Khademhosseini *et al.* prepared an IPN composed of methacrylated gelatin and silk fibroin. The full IPN possessed tunable structural and biological properties compared to methacrylated gelatin or semi-IPN hydrogels[23]. Carboxymethyl chitosan was mixed with alginate and genepin to obtain a pH sensitive hydrogel suitable for the targeted drug delivery of a protein in the intestinal medium. At pH 1.2, the swelling ratio of the genipin cross-linked carboxymethyl chitosan /alginate hydrogel was limited due to the formation of hydrogen bonds between carboxymethyl chitosan and alginate. At pH 7.4, the carboxylic acid groups became progressively ionized and the hydrogel swelled more significantly due to the large number of force created by the electrostatic repulsion between the ionized acid groups releasing a significantly increased amount of the model protein BSA as compared to lower pH[24]. Felisberti and co-workers described an IPN of poly(1-vinyl-2-pyrrolidinone) and gelatin were obtained by casting of aqueous solution using potassium persulfate and glutaraldehyde as respective crosslinking agents. Due to the increase in crosslinking density, high elastic modulus was obtained maintaining a good results in cytocompatibility[25].

3.1 Double network (DN)

Within the IPNs class, double network materials described by Gong *et al.* [26] represent a unique class with high strength and toughness. These materials consist in a first network composed of polyelectrolyte polymer densely cross-linked, which is swollen in a monomer aqueous solution with a low quantity of crosslinking agent to obtain a second neutral loosely cross-linked network. In addition, the concentration of second polymer network should largely exceed the molar concentration of first network (by 20 or 30 times). The DN proposed by Gong was composed of poly (2-acrylamido-2-methylpropanesulfonic acid) (PAMPS) interpenetrated with poly (acrylamide) (PAAm) which presented up to 90% of water content and resistance to wear and high fracture strength up to 17 times higher than the single networks. DN hydrogels present mechanical characteristics comparable with those of soft load-

bearing tissues, such as tendon, dermis, connective tissue, muscle and human heel pad that have an elastic modulus in the range 0.1–10 MPa [10,27].

4. Summary

During the past decades, hydrogels have emerged as promising biomaterials supporting viability, proliferation and differentiation of cells. However, for some applications such as tissue engineering or regenerative medicine, hydrogels do not replicate the complexities of native human environment in its entirely. To overcome this limitation, multicomponent polymer-composites hydrogels try to combine different polymers for improving mechanical properties of biomaterials or to tailor particular properties to meet specific needs. The design of hydrogels may lead to significant improvements of stimuli responsive behavior, exploiting the 'smart' properties of at least one of the polymer components. Between this materials, IPNs have emerged as a category to their improved responsiveness and mechanical properties. Many examples exist in literature of IPNs systems, however the development of systems that fulfil unmet medical and pharmaceutical needs is still a challenging task.

II. Membranes for guided tissue regeneration of periodontal defects

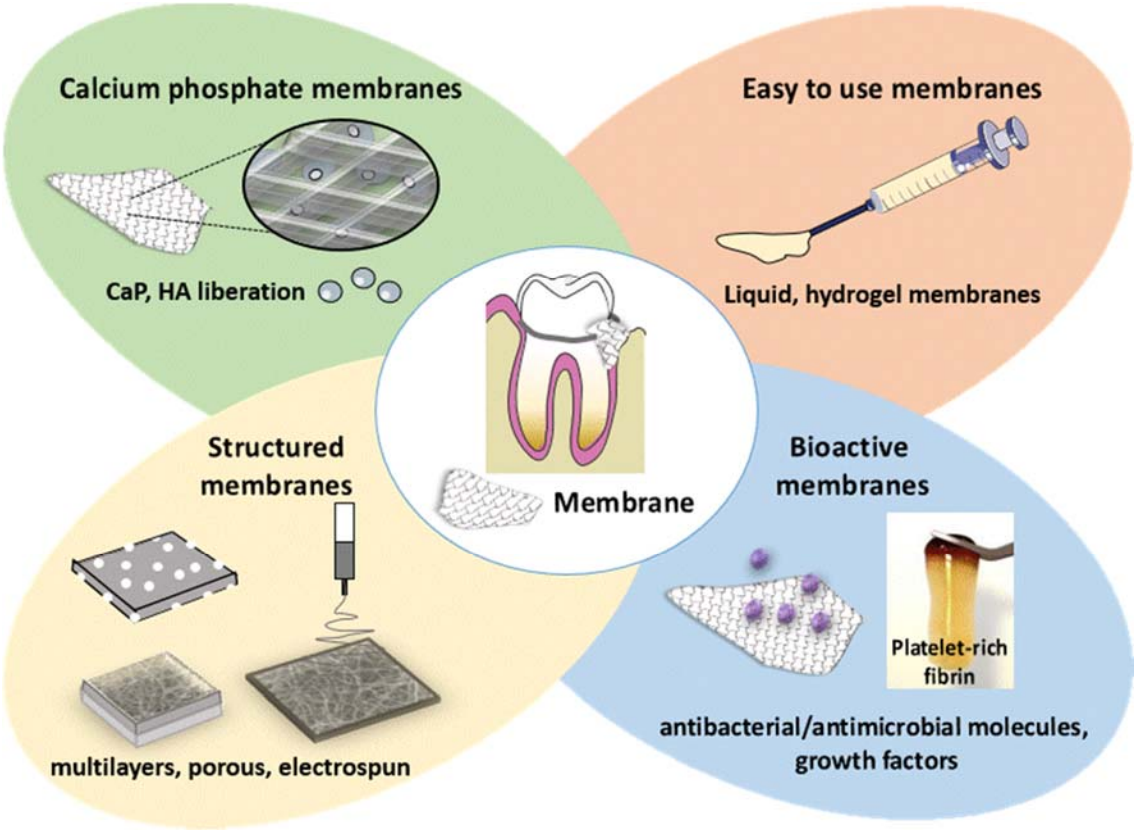

Figure 1 Main strategies used in membranes conception for guided regeneration techniques.

1. Periodontitis

Oral diseases, including dental caries and periodontitis, are among the most important global health burdens, affecting the majority of school-aged children and adults worldwide. Gingivitis is a reversible inflammation caused by dental plaque accumulating in gum and impact 50-90% of the populations[28]. Periodontitis are a set of inflammatory diseases resulting from the presence of oral bacteria biofilm in periodontal tissue, which provokes an immune-inflammatory response and destroys the tooth-supporting attachment apparatus[29]. According to Word Health Organization, periodontitis are identified as one of the most common cause of tooth loss in worldwide and severe periodontitis is found in 15-20% of middle aged adult (35-44 years)[30]. In Europe, the more severe forms of periodontitis affect 10% of the population[31].

The significant role of socio-behavioural and environment factors in oral disease and health is

evidenced in an extensive number of epidemiological survey. However, in industrialize countries, studies show that smoking is a major risk factor for adult periodontal disease, responsible of most cases of periodontitis in middle age group. Smokers have high risk to develop periodontitis and lesions of oral mucosa[32]. Furthermore, associations between periodontitis and alcohol consumption, stress, obesity and genetic factors[33–36] are described but also relations with systemic diseases such as cardiovascular disease or diabetes are reported [37,38].

2. Physiology

Periodontium refers to the specialized tissue that surround and support the teeth, which consists of the alveolar bone, gingiva, cementum and periodontal ligament. Some signs and symptoms of periodontal diseases include swollen red gum, gingival recession, halitosis, pocket formation between the teeth and gums, attachment loss and alveolar bone loss. At the gingiva/tooth interface two tissues are present: epithelial and connective. The epithelial tissue or junctional epithelium is a non-vascularized tissue, characterized by keratinized cells to protect the below parts. The connective tissue is found in two zone: the connective tissue supporting the epithelium and the connective tissue in contact with tooth surface. This latter is composed of collagen fibers, fibroblasts, blood vessels and nerves. The junctional epithelium and the connective tissue at tooth interface, also called biological width, play a primordial role assuring a barrier effect against the microorganism's penetration. When the periodontium is aggressed by physical, chemical, traumatic or infectious event, an inflammatory response is produced. Oral flora bacteria are composed mostly of gram positive bacteria; however, it was found that anaerobic gram negative bacteria are involved in etiopathogenesis of periodontal disease[29]. Porphyromonas Gingivalis is one of the most common bacteria found young adult and often a functional association in periodontitis among Porphyromonas Gingivalis and Agregatibacter Actynomycetemcomitans and Prevotella Intermedia is described. However, the composition of periodontal pocket could change in relation with patient's health situation (e.g. AIDS/HIV, diabetes) and habits (e.g. smokers).

We can divide the conducting evolution to clinical periodontitis in four steps. The first phase is the

clinically healthy gingiva, which correspond to a balanced situation with a gingiva without clinical sign of inflammation. Here, a perfect balance between the junctional epithelium barrier, the positive fluid to the gingival crevice which wash away the unattached microorganism, the phagocytic function of neutrophils and macrophages and microbial presence is reached. The clinical healthy gingiva, in fact, continuously produces a small inflammatory infiltrate involving junctional epithelium and subjacent connective tissue. However, the host-microbial interplay change if gingivitis is follow. The second and third phases is gingivitis in acute and chronic inflammation form. This inflammation existence is due to the bacterial presence and in particular due to the accumulation of plaque. In these phases clinical symptoms emerge soon. The gingiva appears red due to the alteration in vascular network. The exudation increases, which makes the tissue swollen. Inflammatory cells (T lymphocytes and macrophages) leave the vasculature and accumulate in connective tissue, meanwhile bacteria biofilm increase on tooth surface. At this time, just the connective tissue is concerned. Healthy tissue by the presence of inflammatory cells decrease in the collagen network (about 70%), connective fibers that support the junctional epithelium are the most degraded. Furthermore, gingival fibroblasts exhibit alterations of their cytoplasmic components (e.g. vacuolization of the reticulum endoplasmic) and their number probably decreases by apoptosis. This would promote the infiltration of leucocytes. Finally, an early proliferation of basal cells of the epithelium is observed. Epithelial loops are formed and penetrate the underlying infiltrated connective tissue. In absence of treatment, the inflammation is established. The continuous exposition to plaque enhances the inflammation. Collagen loss continue as the inflammatory cell infiltrate expands, resulting in collagen-depleted spaces, which become available for cell inflammatory infiltration. During this time the junctional epithelium is substituted by a pocket epithelium that is not attached to the surface. This, it allows further migration biofilm and has more permeability than the original one. The last phase is the periodontitis which consist in an advanced lesion. Here, the damage to the collagen fibers are extensive, the pocket epithelium migrates apically from the cemento-enamel junction. The inflammation can spread to lateral tissue and true the attachment apparatus. The destruction of alveolar bone is mediated by osteoclast in respond to inflammatory mediators in order to maintain a safety distance between healthy bone and inflammatory cell infiltrate.

After a surgical treatment of a periodontal defect, the body reacts with an inflammation reaction. The cicatrisation phase starts after a clot is formed. The clot protects the uncovered tissues and it acts as a matrix for neutrophils and macrophages migration. These cells are in charge to clean the wound and to secrete cytokines able to recruit others cells for inflammation, but also cells like fibroblasts able to produce extracellular matrix[39]. The inflammation phase is followed by a granulation phase and end with a cicatrisation. When natural cicatrisation occurs, we can obtain a reparation of wound for small size defect. However, a regeneration in which tissue and function are restored is not possible. In fact, in the first stage soft tissue rapidly proliferate in the wound to cover the clot and a long junctional epithelium is formed. It was proved that soft tissue is able to migrate between 0.5-1 mm per day, instead 0.05-0.06 mm per day by bone and ligament cells. Histologically, the reparation could be involved in long junctional epithelium, ankyloses and new attachment in which collagen fibers from connective tissue orientated in a parallel sense compare to junctional epithelium[40,41].

3. Guided Tissue Regeneration

In 1969 Melcher postulated the biological concept for full regeneration, according to his theory, the exclusion of soft tissue from tooth surface is necessary. In this way periodontal ligament stem cells could populate the defect and pursue their differentiation in cementoblasts, osteoblasts or fibroblasts. This means that new attachment to the tooth surface by collagen fibers to new cement is restored and new alveolar bone is regenerate[42].

Guided tissue regeneration (GTR) is a technique to regenerate lost components of periodontium. This technique is based on the cells epithelium and connective tissue isolation from the wound (clot) allowing attachment and alveolar bone restauration[43].

In 1980 Nyman and Karring were the first to describe guided tissue regeneration for periodontal tissue. These authors based their technique on the neuronal regeneration described in 50th by Murray. Experimental studies showed that a regeneration was possible using a physical barrier against cells invasion of cellulose membrane with 0.22 µm pore size. Nowadays GTR represents a well-known therapeutic approach able to regenerate periodontium in intrabony defect and class II furcation.

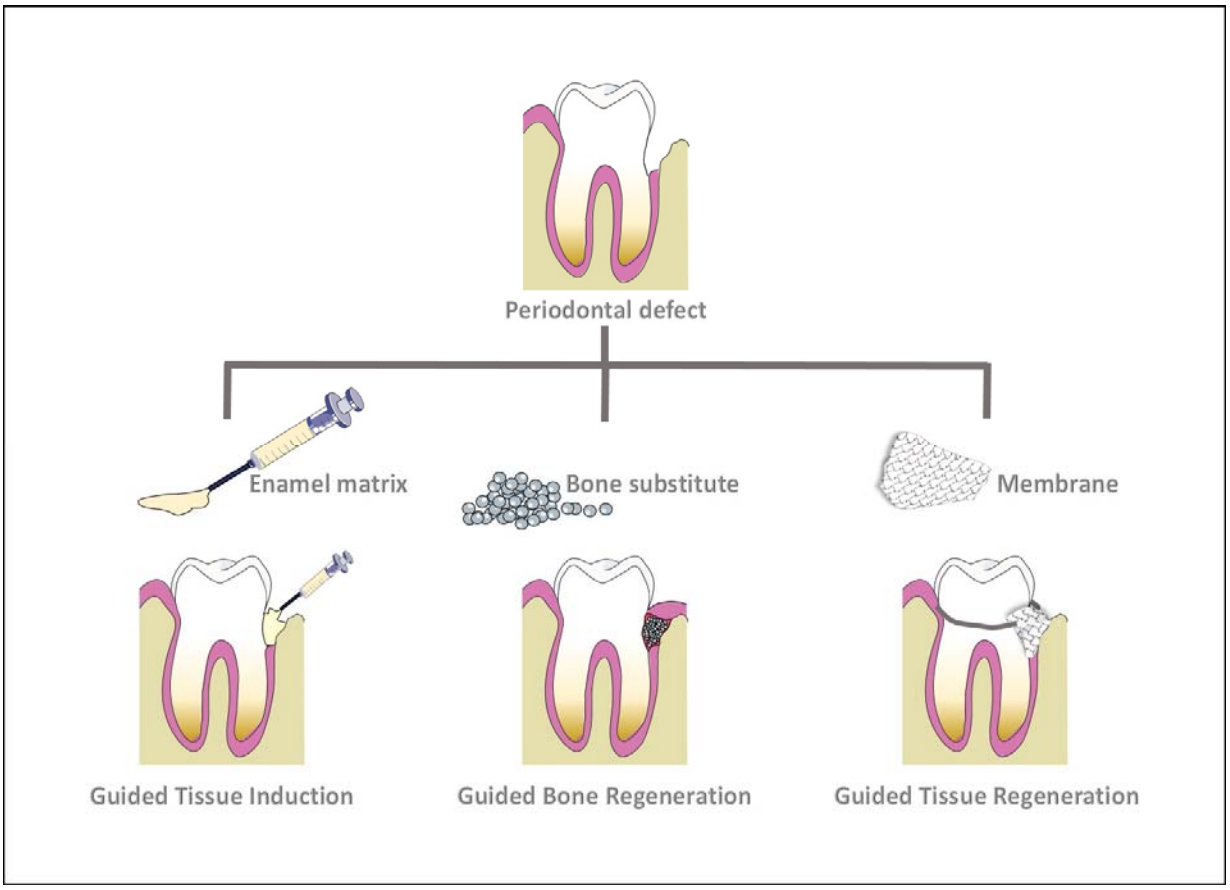

Figure 3 Surgical procedures after peridontal defect formation. Guided tissue induction uses enamel matrix, guided bone regeneration eses bone substitutes and guided tissue regeneration uses membranes.

Today the concept of GTR is used as a generic regeneration concept, however we have to distinguish the guided tissue regeneration (GTR) from the guided tissue induction (GTI) and guided bone regeneration (GBR) (Figure 1). The most commonly used term is GTR, while the term GTI has appeared more recently, although the GTR covers the scope of the GTI. In fact, the use of the enamel matrix derivatives (GTI) contributed to differentiate the concept of mechanical insolation by a membrane (GTR) of the biological induction membrane a regenerative healing process (GTI). However, there are situations where it is possible

to combine both a membrane with derivatives of the enamel matrix, or even alternative biomaterials bone. The principle of GBR is directly inspired by GTR, in which a bone graft biomaterial is implanted into the wound cavity to trigger bone regeneration and above a membrane is deposited avoiding soft tissue invasion. The choice of membrane depends on material resorbability, size of defect, operator preference and patient acceptance.

4. Guided Tissue Induction

GTI is a technique using some derivate of porcine enamel matrix (Straumann Emdogain®, Straumann USA LLC). Hammarstrom *et al.* demonstrated the possibility to mimic the cementum formation using enamel matrix proteins. His work was based on the possibility to imitate the embryologic periodontal development plan. This material (Amelogenin), in fact, was found in human teeth developing in the area of cementogenesis and cementum-like tissue formed. This mixture contains proteins with low molecular weight able to recruit and stimulate mesenchymal cells differentiation. It contains also TGF-ß able to induce stimulation and production of BMPs, endothelial proliferation and chemoattract mesenchymal stem cells. Moreover, proteins are adsorbed on radicular surface, helping the cementogenesis[44,45]. Different preclinical and clinical studies showed a periodontal regeneration. Melloing *et al*. and Sculean *et al*. evidenced in histological section from animal model, the neocemuntum in cellular and acellular cementum. Clinical study results indicated significant attachment improvement, pocket depth reduction, and bone gain compared to periodontal surgery alone, demonstrating that today this material constitutes an alternative of membrane uses in GTR[46]. On the other side, the animal source of the material and the complex matrix composition remain a question mark.

5. Guided Bone Regeneration

The main objectives of the GBR are to treat bone defects and promote the new bone formation. In this technique, only one tissue has to be regenerated: the bone. Consequently, the biological challenge is easier in GBR compare to GTR techniques. GBR treatment advocates that regeneration of osseous defects

is predictably attainable via the application of an occlusive membrane in combination with a bone fill material. GBR is commonly used to treat ridge defects before placing dental implants or fixed prosthodontics. This technique uses a soft tissue membrane, as in GTR, but focused on just bone regeneration using a bone substitute biomaterial in the wound cavity. The membrane protects the defect space for better stabilization of blood clot and bone substitute biomaterials for higher bone formation. The bone substitute biomaterial is used as a scaffold for new bone production. Today on the markets, a big panel of bone substitutes are available.

The material available as a bone substitute could be from different origins: autograft and allograft, from human bone, xenograft, when the bone derived from animals and alloplastic graft when the material is synthetic. The biomaterial has to be biocompatible, non-immunogenic, with a degradation rate and porosity comparable with the human bone to allow the substitution with new bone and permit neovascularization[47,48].

Autograft is considered as the gold-standard material because of its osteogenicity, osteoinductivity and osteoconductivity. Fresh autograft contains alive cells that help the cells colonization and vascularization. However, this procedure need a second surgical intervention with a non-negligent morbidity.

The term allograft is used when the biomaterial derived and are implanted in the same species but from another body. Bone allograft are distributed by specialized companies (biobank). The tissue is available on powder, blocks or particles. It could be demineralized or not, fresh, fresh-frozen and freeze-dried forms. During its preparation, all the cellular elements are disrupted, however some biological factors, like bone morphogenetic proteins could give it the osteoinductive and osteoconductive potential. In addition, the structure is well adapted to facilitate the regeneration of lost tissue[49,50].

Xenograft are heterologous graft derived from animals. For periodontal defect most common materials used are from bovine and coral. Bovine grafts are really common product (*e.g.* Bio-Oss®,

Geistlich, Lubboc®, OST). It is treated thermally and chemically to make it safe avoiding transmission of pathogens agents. The resorbability is quite slow, but it assures stability inside the wound and thanks to its structure assures cells colonization. Preclinical and clinical study, however, present discordant results about a periodontal ligament regeneration [51]. Coral xenograft (*e.g*. Biocoral®), are thermally treated to maintain the mineral architecture of calcium carbonate. This structure with high porosity is completely resorbable giving space to new bone formation[52].

Alloplastic materials are obtained from chemical synthesis. They are available in tricalcium phosphate (ßTCP/ ß Ca_3PO_4) or in hydroxyapatite (HA/ $Ca_{10}(PO_4)_6(OH)_2$) material. TCP degrades realising calcium and phosphate ions useful for new bone formation. However, the clinical results are not predictable, due to rapid kinetics of resorption. HA-based substitutes are considered non-resorbable or poorly resorbable, making them important space maintaining during regeneration. The two alloplastic materials could be used alone or together, in a combination called biphasic calcium phosphate (BCP), in which varying the HA/TCP ratio it has been possible to modulate the degradation and regeneration. The chemical procedures could be adapted as a function of granulation, shape, porosity and final resistance desired of biomaterial. BCP are used in a 60/40 or 80/20 ratio with granulometry between 80-200 µm and 0.5-1mm. The combination of the biomaterials represents a common procedure for periodontal surgery as it allows a stable space maintenance with good bioactivity[53–56].

Bioactive glasses are presented as silicate granules also represents an alternative in bone substitutes biomaterial. Ones in bone defect, they release a critical concentration of soluble silicium, calcium, phosphorus and sodium ions inducing a cellular response between the biomaterial surface and cells. Hydroxyapatite, with similar chemistry and mechanical properties, are produced at the granules interphase. Bioactivity is related to the capacity to develop a connection between the organic structure of connective tissue and bone tissue and to induce the mineralization with the activation of osteoblast and with mineral substances furnished. This material present high biocompatibility, osteoconductivity and in some cases osteoinductivity was shown [57,58]. In 1 or 2 wall defect, the granules present some limitation in stability

and space maintenance. The addition of matrix delivery could help the cohesion and homogeneity between the material and the space during cicatrisation. Some clinical studies are realized with this type of materials show contrasting results[57,59].

6. Combined technique: GTR/GBR and bone filling materials

Resorbable membranes compared to non-resorbable membranes could have some limitation in rigidity and so in space maintaining. Combined techniques are structured to combined membranes with bone substitutes. The use of bone substitute allows osteoconduction properties and sustained the membrane under the pression of soft upper tissue. This practice is indicated in large and non-supportive osseous defects (one or two walls defects). In these specific defects the stability of the clot and bone fill material cannot be assumed by the anatomy of the defect, the use of a membrane allows an initial stability and during all the time of the wound healing. However, data obtained in animals and in humans suggested that bone substitute do not influence negatively new attachment formation. Van Dyke *et al.* treated sixty-four patients with 93 of 2- or 3-wall intrabony defect treated with resorbable membrane in combination with autogenous bone graft alone or in combination with bovine-derived xenograft or HA/ßTCP. Successful regeneration of intrabony defects were demonstrated in all the clinical group with a significantly better outcome for autograft material added with bovine-derived xenograft or HA/ßTCP compared to the autograft alone[60].

7. Membranes

Membranes were developed with the function to act as a physical barrier to avoid gingival cell invasion led to the development of GTR/GBR. These material has to present good integration with hosting tissue, avoiding inflammations reaction, but also good mechanical properties against compressive force of overlying soft tissue and maintain the space for tissue regeneration[43,61,62]. In addition, clinical handling by the physicians when applying the membrane is also of great importance[63,64].

A different panel of membranes are available on market. From early days of using membranes with the only aim to be functional and avoiding host tissue reaction, an evolution on membrane's characteristics

is observed. We can divide the membrane in three generations. The first includes the non resorbable membranes, the second ones the resorbable membranes and the third generation, which includes all the membranes with bioactive roles, specific structure or easy to handle (table 1). The most common membranes available on market are summarized on table 2.

Table 1 Classification of barrier membranes for GTR/GBR

Class	Materials	Advantages	Disadvantages
1st generation	e-PTFE[1], d-PTFE[2], Titanium reinforced PTFE	Good space maintainer, stiff	Non-resorbable (need second surgical intervention), difficult to apply
2nd generation	Natural: collagen	Resorbable, tunable mechanical properties, wide range of degradation, good biocompatibility and tissue integration	Degradation not always predictable, low mechanical properties, low bioactivity (synthetic)
	Synthetic: PLA,PGA		
3rd generation	I, II and new III generation materials	Easy to manipulate membrane, bioactive membranes: antimicrobial material (*e.g.* ZnO), release biological active molecules (*e.g.* growth factors), multilayer membrane (compartmentalization for regeneration)	Controlled delivery, Long process for commercialization for bioactive membranes

[1] Expanded polytetrafluoroethylene
[2] Dense polytetrafluoroethylene

8.1 Non-resorbable membranes

Non-resorbable membranes present a high strength and rigidity that could be augmented such as in titanium reinforced membrane. However, these membranes are difficult to use requiring ability and experience to be perfectly applied upon the defects. The major drawback is that they require a second surgery to their removal after a healing period of 3 to 6 months, increasing the cost and time of intervention and high post-operative morbidity for patient. Among the non-absorbable membranes, expanded (e-PTFE) was extensively used in the past. This membranes present a microporous structure enhancing host-tissue interaction but that can also represent an exposition to local bacteria[65]. Today, high density tetrafluoroethylene (d-PTFE) based membranes (e.g., Cytoplast® TXT-200, Osteogenics Biomedical) are widely used representing the «gold standard» (table 2). Previous authors have reported that d-PTFE completely blocks the penetration of food and bacteria, and thus, even if it is exposed to the oral cavity, it is still acts as an appropriate membrane barrier. This represent an advantageous for large intrabony defect of peri-implantitis treated with GBR. However, as a consequence of the necessity of second intervention for removing, the use of non-degradable membranes is quite limited to GBR. [66–68])

8.2 Resorbable membranes

Resorbable membranes require one-pot intervention in patient, limiting the impact of soft tissue and have to degrade progressively allowing periodontal tissue regeneration. The critical time for soft tissue cells migration has been reported at 14 days [69], in which the membrane has to be intact during this time for wound healing. These membranes can be made in natural and/or synthetic material. Most common natural membranes are made on collagen (porcine, bovine, calf). Nevertheless, the main drawback of resorbable membranes is their poor predictability in the resorption time largely influenced by the characteristics of the patient. Most resorbable membranes used until now are characterized by a too rapid absorption kinetics after implantation. In fact, these membranes do not ensure, for a period of 8 weeks or more, the regenerating process intact below the barrier. With the purpose to improve their properties, commercially available membranes are generally made of cross-linked polymer. Indeed, the presence crosslinking nodes delays the

loss of barrier properties. Marques *et al.* reported the difference in bone gain in crosslinked and linear collagen. The results in bone ingrown were comparable, instead post-operative complications suggested a better results for cross-linked collagen membrane [70,71]. BioMend® (Zimmer, USA) membrane, for example, is made of type I bovine collagen, degrades in 6-8 weeks, instead of BioMend Extend® with same composition but cross-linked with formaldehyde which degrades in 18 weeks. To improve the efficacy of membranes, association between collagen others materials are available on market like with chondroitin sulphate or hydroxyapatite (e.g. Paroguide ® Acteon, UK). Gelatin is a partially denaturized collagen which present biocompatibility, biodegradability, promotes cells adhesion and has low cost. However, this material exhibits low mechanical properties and fast degradation. To overcome its limits is always studied in association with other polymers or cross-linked. Zhang *et al.* overcome the limits of fast degradation, they fabricated PCL/gelatin hydrid nanofibers: the incorporation of natural polymer enhanced cell adhesion and proliferation compare to PCL alone[72].

One of the most investigated natural polymer for membrane realization is chitosan. Chitosan is a nontoxic, biodegradable, and biocompatible polysaccharide of b(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine. This versatile polymer is the object of many studies in GTR for its attractive properties such as hemostatic and antimicrobial activity. It has been reported to inhibit the growth of Gram-positive and Gram-negative oral pathogens and modulate inflammatory responses in human gingival fibroblasts (HGFs) [73–76]. Chitosan is obtained from deacetylation of chitin in alkali condition. In nature, chitin is the major component of insects and crustaceans where it represents the major component of their exoskeleton, but is also present in the cell wall of some mushrooms [77–79]. Chitosan polymer offers many groups for chemical modification of the chain or cross-linking. Chitosan membrane where obtained with treatment with NaOH as a gelating agent and with Na5P2O10 and Na2SO3 as a crosslinking reactive. The membranes were tested in dental defect in rats for 4 weeks. Histological evaluation, exhibit a better membrane integrity than the control and with chitosan-NaOH 30% of new bone formation was obtained[80]. Chitosan membrane were studied prepared with fibers, in association with bioceramics or in a multilayers structure in combination

with other polymers[81–85]. In multilayer chitosan membrane it was shown to enhance adhesion and proliferation of HGFs avoiding the invasion and antibacterial properties were shown against most important periodontal pathogens found in periodontitis and new bone formation in beagle animal model were observed[16].

Most of synthetic membranes available on the market are made of aliphatic polyesters like polylactic acid (PLA) or polyglycolic acid (PGA) as well as their copolymers. These synthetic materials are inert and degrade into pyruvic acid and lactic acid with a degradation rate modifiable changing their composition. Example of available membrane are Resolut® (Gore Inc., USA) made of a copolymer PGA/PLA and Tisseos® (Biomedical Tissues, France) composed of a dense layer bound to a micro-fibrous layer. Karring *et al.* reported a 5-years case report in which intrabony defect were treated with GTR using bone substitute (Bio-Oss®) and PLA/PGA membrane (Resolut®). All surgical sites were treated without problems. A precocious exposure of the membrane into the oral was a common event, however, no associated adverse event was related and none of them was removed. The results after one year showed a clinical and radiographical improvement [86]. *In vivo* study were performed in rat calvaria defect treated with PLA membrane (Atrisorb®, Atrix, USA). Histological observation was performed after 3, 5, 7 and 12 months. After 12 months, it can be observed residues of biomaterial with evidence of foreign body reaction[87]. Too long degradation, in fact, could negatively impact regenerative process with inflammation and bone resorption. A clinical study comparing degradable membrane in PLA (Guidor®, Sunstar, Janpan) and non-degradable one in e-PTFE (Gore-Tex Periodontal Material®, Gore Inc., USA) in 19 patient's defects were analyse after one year. The clinical depth and bone fill in patients treated with PLA membrane, show results at least equivalent to the results obtained with non-resorbable membrane, avoiding the second unnecessary trauma of intervention to remove the membrane which might negatively influence wound regeneration[88].

8.3 Third generation membranes

8.3.1 Membrane with specific structure

Membrane porosity is an important key point in order to assure a selective diffusion of oxygen, nutrients and others important elements for tissue development, but avoiding the bacterial or soft tissue ingrowth. Many studies analysed the pore size impact on membrane efficacy. Asymmetrical membranes, like multilayers or gradient materials, are developed to ensure one layer with microporous to prevent tissue ingrowth and a layer with bigger porous to allow cells adhesion and proliferation for new tissue formation[83,89–91]. However, increasing the pore size seems may results in a decrease of mechanical properties[92]. Fibrous structure, like electrospinning fabricated materials, seems to be promising, creating porous interconnected structure with high surface area. Thomsen *et al*. analysed in a review of literature a comparison between e-PTFE with high density versus PTFE porous material. Reinforced or non-porous materials have been reported to protect defect space and clot with good bone formation, however membranes with microporosity (20-25 μm or 100 μm) presents a better bone formation after six weeks, instead of macroporous (100-300 μm) in which the defect presents soft-tissue invasion.[93,94].

8.3.2 Membrane with antibacterial/antimicrobial material

The presence of bacteria is required for periodontitis development. Antibiotics like Tetracycline, Metronidazole and Amoxicillin are the most commonly prescribed antibiotics against major periodontal pathogens. The possibility to product antibacterial membranes were investigated in different studies. Metronidazole and Amoxicillin were loaded in the materials to produce electrospun fibers or directly integrated in a layer of the membrane [43,95,96] inhibiting bacterial growth. Also non antibiotics antimicrobial agents were investigated like zinc- or silver-based material, lauric acid or chlorhexidine[97–103]. These composite membranes revealed antimicrobial activity versus Porphyromonas Gingivalis and others oral major pathogens bacteria. Ag^{+} has been reported to cause bacteria inactivation by binding microbial DNA and sulphydryl groups of metabolic enzymes of the bacterial electron transport chain[104].

Yihong *et al.* demonstrated that a mineralization with ZnO of Resolut® Membrane (Gore Inc., USA) can inhibit oral bacteria colonization and prevent inflammation[105].

8.3.3 Platelet-rich fibrin membrane –an autologous membrane

Choukroun *et al.* used for first time platelet-rich fibrin (PRF) in oral and maxillofacial surgery. It is consisting of an autologous matrix of fibrin, prepared before use. The preparation consists in a fresh blood centrifugation in glass tube. After that three layer are formed: the upper consist in cellular plasma, the middle in PRF clot and the latter in red corpuscles base. Platelets are trapped in fibrin meshes. The scientific rationale is linked in the content of α granules in many growth factors stimulate and attract stem cells, promoting cell mitosis, angiogenesis and osteogenesis. The clot could be transformed in membrane using specific sterile compression tool and used alone or in association with others dental biomaterials[106,107]. However, the concentration of these different growth factors is notoriously insufficient in comparison with those needed to obtain regeneration. A systematic clinical review, based on a several clinical studies, has revealed a very strong heterogeneity of the results obtained with the use of PRF, in the treatment of infra-osseous periodontal defects. Current scientific data therefore do not allow to validate the PRF as a safe and reproducible adjuvant in order to obtain a periodontal regeneration at the level of infra-osseous defects or impairments of furcation[108].

8.3.4 Membrane loaded with growth factors

To control the quantity of growth factor release, some researchers, reported growth factor loaded in polymer matrix. In these membranes, bioactive molecules can be locally deliver, controlling the quantity released. One of the most studied molecules are bone morphogenetic proteins (BMPs) and rhPDGF (platelet derived growth factor). These molecules are already associated with bone substitute substances available on market for GBR, on the contrary the integration in membrane are still object of research.

8.3.5 Membrane with bioactive CaP incorporation

Bioactive ceramics, such as hydroxyapatite (HA) or tricalcium phosphate (TCP), have osteoconductive properties which can enhance the biological response but also mechanical properties to

avoid the collapsing into the defect and ensuring clot protection and the regeneration process. Several works, reported the incorporation of bioactive material in membrane structure. In particular multilayer membranes seems promising for a better tissue integration and calcium deposition[81,84,109–111]

Davies *et al.* analysed a membrane of PLGA/calcium phosphate in class II furcation defects in dogs. This membrane revealed an adapt stiffness to not collapse in defect and retain blood clot. *In vivo* experiment shown new cementum, bone and periodontal ligament fibre insertion only in the treated group compare to the control (no biomaterial)[110].

Table 2 The most commonly used barrier membranes

Material		Commercial Name	Resorption time
e-PTFE		Gore-Tex ® (Gore Inc., USA)	No
		CYTOFLEX® TEFGUARD® (Unicare Biomedical, USA)	
d-PTFE		Cytoplast GBR-200 & GBR-200 Singles ® (Osteogenics Biomedical, USA)	No
d-PTFE reinforced with titanium		Cytoplast Ti-250® (Osteogenics Biomedical, USA)	No
		OpenTex® (Purgo biologics, Gyeonggi-do, South Korea)	
Animal collagen	Porcine	Bio-Gide ® (Geisttlich, Switzerland)	24 weeks[112]
		EZ Cure ® (Biomatlante, France)	12 weeks[113]
		GuidOss® (Nibec, UK)	NA[114]
		Ossix ® Plus (Datum Biotech Ltd, Israel)	3-5 weeks[115]
	Bovine	BioMend ® (Zimmer, USA)	8 weeks[116]
		CopiOs ® (Zimmer, USA)	24 weeks [117]
	Equine	Paroguide ® (Acteon, UK)	4-8 weeks[118]
Liquid PLA and N-methyl-2-pyrrolidone		Atrisorb ® (Atrix, USA, not available)	16 weeks[119]
PLA or PGA or PLGA		Resolut ® (Gore Inc., USA)	4-6 weeks
		Tisseos ® (Biomedical Tissues, France)	4 weeks [91]
		Vicryl ® (Ethicon, USA)	4 weeks[120]
		Guidor ® (Sunstar, Japan)	4-6 weeks
Modified PEO		Membragel ® (Straumann, not available)	16-24 weeks[121]
Cadaveric human acellular skin		Allo Derm GBR® (BioHorizons, USA)	16 weeks [122]
Human amniotic-tissue		BioXclude ® (Snoasis Medical, USA)	NA [123]

8. Future perspective

Today periodontal diseases represent the most common cause of tooth loss. Guided tissue regeneration (GTR) is a technique to regenerate lost components of periodontium. This technique is based on the cells epithelium and connective tissue isolation from the wound (clot) allowing attachment and alveolar bone restauration. For that, a membrane is used as a physical barrier. The choice of membrane depends on material resorbability, size of the defect, morphology of the defect, operator preference and patient acceptance.

Many efforts have been done on the optimization of GTR membranes, starting from non-degradable PTFE membranes to the bioactive membranes. An ideal membrane is described as a biocompatible material, to avoid tissue response, preferably biodegradable to avoid second surgical intervention, acting as a soft tissue physical barrier, space maintaining for tissue regeneration and with clinical handling. The compartmentalisation of biomaterials, also seems to have more than more attention, with double- multi-layers' material, including different porosity or loaded with bioactive molecule. All the membranes present some limitations, however according to the clinical cases, some characteristics are preferable to others and to get the achievement of property some techniques are required. As evidenced from bibliographic review, different techniques are often used together to try to combine properties and achieve a synergism of properties. New materials and fabrication techniques appear from biomaterials and biological research.

Hydrogels represent an interesting material to design GTR membranes. Hydrogels are 3D network highly hydrated and mechanically supported environment mimicking the ECM. Due to is similarity with human tissues they are used in tissue engineering, drug delivery and cell therapy. Between the crosslinking strategy to create hydrogel, *in situ* gelling materials represents an interesting strategy for membranes or bone substitutes conception. To increase the manageability, i*n situ* forming hydrogels, able to fill complex defects and quickly form solid membranes upon injection, are particularly appealing owing to their ease of handling and cost- and time-saving features. A large panel of membranes are available today on the market. According to our knowledge only two kind of liquid membranes have been commercialized: Atrisorb® (Tolmar, USA) and Membragel® (Straumann, Austria), but they are no longer available according to the

manufacturers [43]. The first one is composed of poly(DL-lactide) (PLA) dissolved in *N*-methyl-2-pyrrolidone (NMP)[124]. The second one was composed of multiarms PEG with thiol end-groups and acrylate end-groups that react forming a hydrogel membrane via Michael-type addition[125].

Other techniques that could be applied to design *in situ* barrier are thermo-responsive polymers, which could be applied mainly for controlled delivery of biologicals agents, or pH sensitive gel that making use of variation. Chemical *in situ* reaction are also studied like Michael-type addition or Shiff base crosslinking. The photocrosslinking also represent an interesting option to be explored[126]. This method is already used by dentists in photopolymerizable dimethacrylate resins, filled with silica particles, to create tooth-composite restorations in the treatment of dental caries as an alternative to mercury amalgam fillings[127].

Polymer-composites hydrogels represent a technique to increase mechanical properties of the final systems. In addition, the possibility to add charge like calcium phosphate could increase the new bone formation via the osteoconductive activity.

Among the techniques for materials manufacturing, 3D bioprinting are more and more used in tissue engineering and regenerative medicine. This technique, in fact, is an additive manufacturing that allows the production of 3D object with complexity in composition and architecture. Indeed, the realization of compartmentalization of tissue healing could be facilitate via multilayer membrane. In addition, thanks to the computer-aided transfer process, this technique is characterized of an high reproducibility of realization[63,128,129]. Thanks to this additive manufacturing and with the intervention of medical imaging, biomanufacturing represents the possibility for personalized clinical adapted multilayers polymers membrane. In addition, new generation of combined bioprinter consents the production of stiff construct, coupling the classical bioprinting with thermoplastic polymer and electrospun fiber[13,14,130].

The development of an ideal material to design a membrane for guided tissue regeneration remain a challenge and it required more investigations in hybrid and multiphasic material combining biological effect and mechanical properties.

III. Objective of the thesis

The main theme of this thesis is the development and the characterization of two interpenetrating polymer network hydrogels (IPNs) based on Si-HPMC and two polysaccharides: dextran and carboxymethyl chitosan. We will explore the suitability of these novel systems as formulation for the development of *in situ* IPN hydrogel membrane material for GTR/GBR.

Si-HPMC is a self-setting hydrogel that has been reported in the literature for many biological applications. It has the advantage of being injected as a viscous solution and then, due to the condensation reaction, it builds a 3D network *in situ*. This material was demonstrated to be biocompatible and slowly degraded in a rabbit model[131–134]. In addition, it has been demonstrated the capability of the cross-linked biomaterial to act as a physical barrier against cell invasion[60]. The main drawback of this self-setting hydrogel is an excessively slow crosslinking process for clinical needs. Nevertheless, biomaterial was easy to use and simplified the process of the cover periodontal lesion, leading to a simplification of periodontal treatment. The results were quite encouraging, but a stable barrier with suitable mechanical properties, quick and controlled time of setting is required[60].

In our work, we developed two original mixture of biomaterials that could be used as a liquid formulation, precursor of an interpenetrated polymer network hydrogel membrane formed by *in situ* under irradiation of a standard polymerization dentist's lamp. Indeed, photo-curing appears as the most appropriate technique for this application thanks to its both shape and curing time control [127,135–137].

Chapter 2 reports the preparation and characterization of *in situ* forming IPN hydrogels based on Si-HPMC and dextran methacrylate (DexMA) networks. The polymers are synthetized. The DexMA solution are characterized. Crosslinking kinetics and the injectability of these systems are studied. Cytocompatibility are assessed by neutral red uptake assay. The potentiality as an *in situ* IPN hydrogel membrane is studied in calvaria defects in GBR animal model.

Chapter 3 describes the preparation and the characterization of IPN based on Si-HPMC and methacrylate carboxymethyl chitosan (MA-CMCS). The polymers are synthetized. The physicochemical and mechanical properties of these hydrogels are measured. Cytocompatibility are assessed by neutral red uptake assay. *In vitro* and *ex vivo* experiment using human gingival fibroblasts and human gingival explant are performed to study the barrier effect of IPN hydrogel membrane.

Finally, in **Chapter 4** the findings of this thesis are summarized and the potential future applications of these IPNs are discussed

Chapter 2 : Interpenetrating polymer network hydrogel membrane of silanized hydroxypropylmethyl cellulose/methacrylated dextran

Rational of the study

POsTURE project : PhOtocrosslinked hydrogels for guided periodontal TissUe Regeneration

POsTURE is a three-year project funded by EuroNanoMed II, which is a research funding program with the goal of creating and funding collaborative research and innovation projects that can convert research in regenerative medicine, diagnostics and targeted delivery systems into practical gains in medicine. The multidisciplinary project is directed by Dr. Catherine Le Visage and is developed between four European academia partners' countries (France, Latvia, Portugal, Italy) and an industrial partner (Hygitech, France). Periodontitis, a recognized chronic disease worldwide, is a serious gum infection, characterized by a chronic inflammation affecting of all components of the tooth-supporting system (gingiva/ligament/alveolar bone/cement) and results in loss of tooth-supporting alveolar bone, loss of clinical attachment and formation of periodontal pockets. Regenerative periodontal procedures aim to reverse this damage by using both a bone graft and a membrane to obtain if possible a complete tissue reconstruction. The multidisciplinary POsTURE project aims to develop an innovative periodontal regeneration device based on: (i) a photocrosslinked interpenetrating polymer network (IPN) that will be applied as a viscous solution and cured *in situ* under irradiation, as a membrane to prevent excessive proliferation of gingival tissue and (ii) a self-setting injectable bone grafting material containing Sr, Mg or Si substituted calcium phosphate (CaP) nanoparticles with enhanced bioactivity. Hence, in the project three axis are distinguished: the development of IPN hydrogel membrane, the bone substitute material and the proof of concept of photocrosslinked IPN combined with injectable bone graft. Both biomaterials are based on silanized hydroxypropyl methylcellulose hydrogel (Figure 1).

Si-HPMC is a self-setting hydrogel developed and studied by our group extensively in biomedical applications such as cartilage repair, bone regeneration or drug delivery for intervertebral disc [5,60,138,139]. This material was studied in an injectable association with biphasic calcium phosphate

(BCP) for treating periodontal defects in dog . The polymer solution was added to deliver BCP and to act as a barrier against soft tissue invagination to promote periodontal tissue regeneration. Histological results demonstrated the ability of this cross-linked biomaterial to act as a physical barrier against cell invasion, however the bone ingrowth was not also distinctive in superficial defects where the biomaterials appear unstable[2]. In fact, the main drawback of this self-setting hydrogel is an excessively slow crosslinking process for clinical needs. Nevertheless, the biomaterial was easy to use and simplify the process of filling the periodontal lesion, which could lead to simplified periodontal disease treatment. In addition, the cross-linked Si-HPMC was demonstrated to be slowly degraded by phagocytosis in implantation experiment in

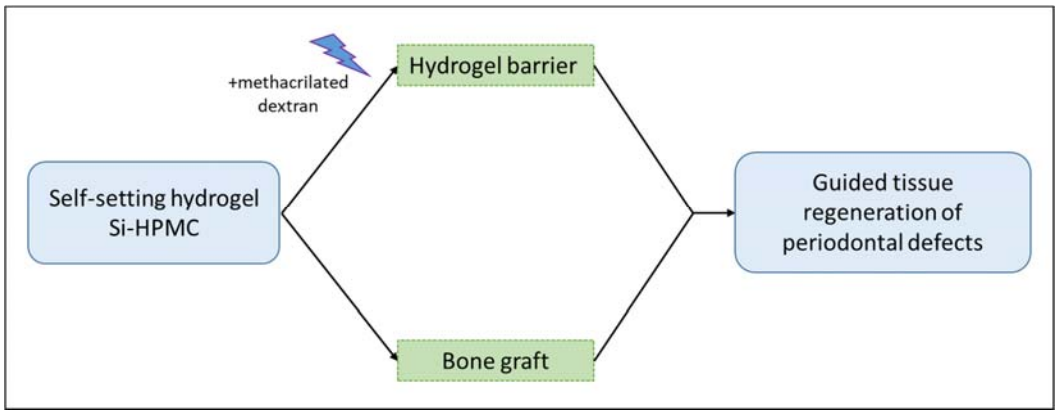

Figure 1 Schematization of POsTURE project products to obtain guided tissue regeneration of periodontal tissue.

bone defects in rabbits[140]. These results were quite promising, but a stable barrier with suitable mechanical properties, quick and controlled time of setting is required [8].

For that we decided to focus our research to design a membrane which possess barrier properties that can quicky form a solid membrane. The photocrosslinking technology appears as to be the most appropriate technique for this application given both its shape and the control of the curing time over the crosslinking process. In addition, polymerisation lamp is already used by the dentists and a large panel of photoinitiators are described in literature and are available on the market.

The IPN hydrogel membrane will be described following. For that we selected two grafted polymers: Si-HPMC and dextran methacrylate.

2.1 Hydroxypropylmethyl cellulose (HPMC) and silanized HPMC

Cellulose ethers are water-soluble polymers obtained by modifying the chemical structure of a natural product, the main constituent of wood, paper and cotton: cellulose.

Cellulose is a linear polysaccharide (or rather a polyglucoside) consisting of monomeric units of D-glucopyranose bound together by a glycosidic β(1→4) bond. Each glucose unit contains three hydroxyl groups (OH) arranged at regular intervals. The hydroxyl present can be replaced by different etherifying reagents (alkyl halides) resulting in cellulose ethers (or alkylated cellulose).

HPMC is a non-ionic water-soluble and pH insensitive cellulose ether, stable within pH 3-13. It largely used in pharmaceuticals as a modified release and film coating, tablet binding and thickening agent[141].

The first generation of injectable calcium phosphate ceramics suspensions is composed of a mixture of HPMC solution and biphasic calcium phosphate granules[142–144]. These materials exhibit good biocompatibility, and have convenient rheological properties for injection. However, the viscous suspension shows a tendency to flow after implantation *in vivo*. A chemical modification of HPMC allows to obtain a material which gelify *in situ* under the influence of pH and without any further chemical reactive. This property will be obtained thanks to the introduction of silanized groups on backbone. This polymer has a sufficient stiffness to be molded and to maintain its shape in contact with human tissues. Alkoxysilane group is grafted onto the backbone of HPMC (19-24% methoxyl and 7-12% hydroxypropyl, Mw=4.4x105) polymer using 3-glycidoxypropyltrimethoxysilane (GPTMS). The dissolution of this silated polymer is achieved in a strong basic medium (pH > 12.3) to induce the formation of silanolates (SiO−Na+). A decrease of the pH using an appropriate buffer triggers the formation of reactive silanols (SiOH) that polycondensate into covalent silane bonds to form a 3D hydrogel (Figure 2)[145–147]. Therefore, the Si-HPMC products suppress the long-term flow through easily controllable crosslinking. Si-HPMC is non-toxic and biocompatible polymer, so that it can be widely used in biomedical areas, such as scaffold for cell culture,

cell encapsulation, drug delivery, cartilage model and implanted in bone defects[2,5,138,148]. Si-HMPC was used in tissue engineering as a scaffold for 3D culture of osteogenic cells, which would be suitable for both *in vitro* culture and *in vivo* injection. Si-HPMC hydrogel with calcium phosphates support osteoblastic survival, proliferation, and differentiation when it is used as a new scaffold, and represents a potential basis for an innovative bone repair material[133,149,150]. In addition, an IPN was investigated with calcium-alginate network demonstrating the stiffness increase compare to alginate network alone and the multicomponent material was proved to maintain cells viable in 2D and in 3D during the observation period[151].

2.2 Dextran and methacrylate dextran

Dextran is a bacterial polysaccharide consisting of consecutive α-1,6 linked D-glucopyranose units in their major chains (more than 50% of the total linkages) with some side chains stemming from α-1,2, α-1,3

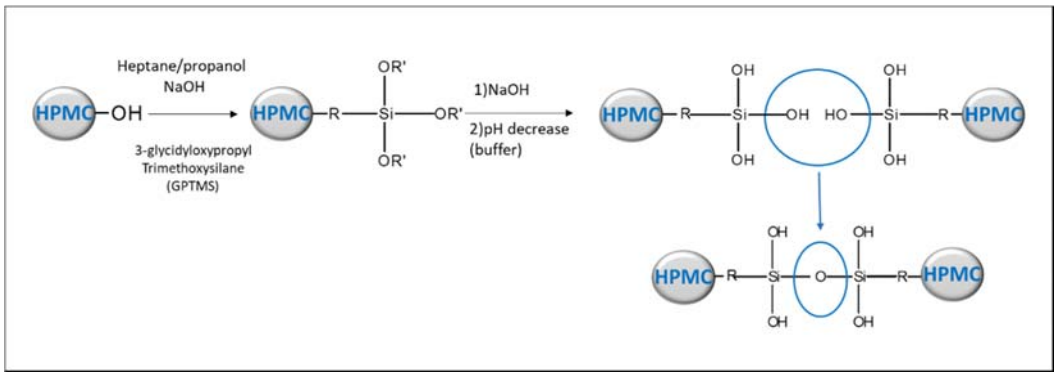

Figure 2 Grafting reaction and neutralization of Si-HPMC.

or α-1,4 branch linkages. The structure of each type of dextran depends on the specific microrganism from which it is extracted. The action of the dexane-sucrase enzyme, present in various bacteria, transforms sucrose into a gummy mass that constitutes the raw dextran. The purified dextran is white in color and solid to the touch. The viscosity of dextran solutions is generally a function of concentration, temperature and mean molecular weight. The free hydroxyl groups of dextran are often ideal points for chemical derivatization, particularly in molecules with few ramifications.

Dextran is used in medicine as an antithrombotic agent to reduce blood viscosity, and as a plasma expander[152]. The dextran, in fact, being a hydrophilic polymer, is able to exert an osmotic pressure inside the blood vessels similar to that of the plasma by recalling water from the compartment outside the vessels: it is used in all cases where it is necessary to restore or maintain the volume of blood and therefore in the case of hemorrhagic shock or burns.

After oral absorption, the low molecular weight dextran has a half-life of 8 h and is secreted by the kidneys. High molecular weight dextran exhibits longer half-life and are subsequently degraded by the reticuloendothelial system. In addition, dextran is metabolized by different dexanases in various parts of the body, including liver and colon.

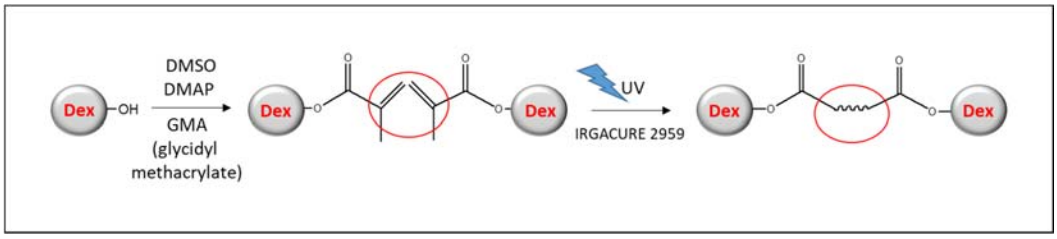

Figure 3 Grafting reaction and photocrosslinking of dextran methacrylate using UV irradiation and Irgacure 2959 as a photoinitiator.

In recent years, dextran derivatives are described in literature for biomedical and pharmaceutical field. Chemical modification of dextran could be make on the three hydroxyl groups available for repeating sugar unit. Dextran can be modified in several ways to obtain chemically crosslinkable polymers[153]. One of the most used chemical modifications consists in the introduction of methacrylic groups in the dextran molecule: a type of synthesis of this derivative consists in making the dextran react with glycidyl methacrylate in the presence of 4-(N,Ndimethylaminopyridine) using dimethylsulphoxide as aprotic solvent. The degree of dextran substitution, determined by the 1H NMR technique, can be varied by controlling the molar ratio between glycidylmethacrylate and dextran[154]. The functionalization of the dextran chains with methacrylate moieties (DexMA) allows hydrogel formation by radical polymerization initiated chemically or by UV irradiation[154]. Dextran hydrogels can be used for the release of biologically active molecules.

It has been shown that the rate of release of these molecules depends on the initial water content and the degree of cross-linking of the hydrogels[155,156].

First article: injectable *in situ* IPN hydrogel membrane for guided bone regeneration

In the present study, we hypothesized that combining Si-HPMC with DexMA, we could induce the formation of an innovative IPN hydrogel. We wanted to demonstrate that Si-HPMC/DexMA solution can be injected directly *in situ* and upon visible light irradiation, a hydrogel can be quickly obtained to use as a membrane for GBR. All the results obtained will be described in the article ***Injectable in situ IPN hydrogel membrane for guided bone regeneration*** The analysis of *in vivo* experiments in calvaria defect in rabbit are still on going. The results will bring further information about the safety, tissue integration and bone regeneration on the use of this novel IPN hydrogel membrane.

Injectable *in situ* IPN hydrogel membrane for guided bone regeneration

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Keyword: photocrosslinking, visible light photopolymerization, silanized hydroxypropyl methylcellulose, dextran methacrylate, riboflavin, calvaria bone regeneration

Abstract Injectable *in situ* forming hydrogels have been developed for minimally invasive surgical procedures. In recent years, multicomponent hydrogels such as interpenetrated polymer networks (IPNs) have emerged as innovative biomaterials due to the synergistic combination of favorable properties of each network. In the present study, we hypothesized that combining Si-HPMC with DexMA, we could induce the formation of an innovative IPN hydrogel. This IPN was composed of self-setting Si-HPMC and photochemically cross-linked DexMA. The Si-HPMC/DexMA solution was demonstrated to be injectable

through a syringe equipped with an 18 G needle. The synergistic effect obtained by the interpenetration of the two polymer networks improve the physicochemical properties and the gel point under visible light lamp is reached instantaneously. Neutral red uptake on L929 cells confirmed the cytocompatibility of the IPN and its components. Finally, we compared the *in situ* obtained IPN hydrogel to a commercial collagen membrane, in a relevant guided bone regeneration (GBR) animal model. Calvaria critical size defects (⌀ = 8 mm) in rabbits were filled with synthetic biphasic calcium phosphates bone graft granules in conjunction with experimental membranes: Si-HPMC, DexMA, Si-HPMC/DexMA and a control group with commercial collagen solid membrane. Si-HPMC solution was injected *in situ*, instead Si-HPMC/DexMA and DexMA solutions were injected and photocrosslinked for 120 s using visible light lamp. The injectable membranes demonstrated handling properties and to facilitate the control over the shape of the membrane, preventing flowing of the material during the application. After sacrifice and the calvaria bones with periosteum were analysed. The surgical area was studied by histology and micro-computer tomography analysis. Quantitative assessment of mineral deposition after 8 weeks amounted to 34.30±8.17% for collagen, 33.74±9.94% for Si-HPMC, 35.19±6.74% for DexMA and 30.49±5.23% for IPN. The results in IPN test group were comparable to collagen membrane. Histological analysis is still on going to determine the morphology and quantification of mineral and soft tissue in the defects.

1 INTRODUCTION

Injectable *in situ* forming hydrogels have been studied aiming to develop minimally invasive surgical procedures. These 3D networks, composed of biocompatible polymers and reagents, are usually formed in mild conditions (body temperature, aqueous environment) [1,2].

In recent years, multicomponent hydrogels such as interpenetrated polymer networks (IPNs), which are systems composed of two or more networks, have emerged as innovative biomaterials. The success of the IPN strategy is due to the synergistic combination of favorable properties of each of the polymer networks. [3–5].

Polysaccharides represent a class of macromolecules of particular interest for IPN design, as they are usually abundant and available from renewable sources. In addition, they have a large variety of compositions and properties, and they allow appropriately tailored chemical modifications to tune the biological/mechanical properties of the resulting network [6–8].

Guided bone regeneration (GBR) is an established clinical procedure in which a bone graft biomaterial is implanted into the wound cavity to trigger bone regeneration and above a membrane is deposited avoiding soft tissue invasion and enhance the stability of bone graft material [9–11]. The membrane plays a key role preventing undesirable tissue migration in the defective area, and consequently it allows sufficient time and space for osteogenic cell invasion and proliferation.

Today, a large panel of membranes are commercially available. Among them, high density polytetrafluoroethylene (d-PTFE) -based membranes (e.g. Cytoplast® TXT-200, Osteogenics Biomedical) are widely used and are considered the «gold standard». Natural membranes are mostly represented by porcine or bovine collagen with products such as Bio-Gide® (Geistlich, Switzerland), EZ Cure® (Biomatlante, France) or BioMend® (Zimmer, USA). These materials must avoid inflammatory reactions and display good load-bearing capacities against compressive force of overlying soft tissue, while

maintaining a sufficient space for tissue regeneration [12–14]. In addition, clinical handling by the physicians when applying the membrane is also of great importance [15,16]. To this end, injectable formulations, able to fill complex defects and quickly form solid membranes upon injection, are particularly appealing owing to their ease of handling and cost- and time-saving features [13,17,18].

We previously reported the silanization of hydroxypropyl methycellulose by addition of alkoxysilane groups [19–21], enabling the formation of Si-HPMC hydrogels by silanol condensation. This self-setting hydrogel was extensively studied for various applications such as cartilage repair, bone regeneration or drug delivery for intervertebral disc [8,22–24]. In addition, we demonstrated the ability of this cross-linked polymer to act as a physical barrier against cell invasion in a periodontal defect in dogs [24,25]. However, for GBR application, the main drawback of this self-setting hydrogel is its slow crosslinking process that impairs the *in situ* membrane formation.

In situ photopolymerizable formulations are already used to prepare methacrylate resins for the treatment of dental decays. This technique provide numerous benefits such as a spatio-temporal control over the crosslinking process [26–29]. Dextran methacrylate (DexMA) photopolymer, which undergoes crosslinking in presence of a photoinitiator, upon exposition to UV or visible irradiation, forms hydrogels that have been studied for multiple biomedical applications [30–33].

In the present study, we hypothesized that combining Si-HPMC with DexMA, we could induce the formation of an innovative IPN hydrogel. We want to demonstrate that Si-HPMC/DexMA can be injected directly *in situ* as a membrane for GBR and upon visible light irradiation, a hydrogel can be quickly obtained. The synergistic effect obtained by the interpenetration of the two polymer networks could improve the physicochemical properties and the dense network of IPN ensures the barrier effect against soft tissue invasion and delayed the hydrogel resorbability.

We synthetized several DexMA and we used riboflavin as a photoinitiator and a standard dentist's lamp to cure the hydrogel. We selected the IPN formulation of which we further assessed its biological and

physicochemical properties. Finally, we compared the *in situ* obtained IPN hydrogel to a commercial collagen membrane, in a relevant GBR animal model. Calvaria critical size defects (⌀ = 8 mm) in rabbits were filled with synthetic biphasic calcium phosphates bone graft granules in conjunction with experimental membranes to evaluate the biocompatibility and the efficacy for GBR techniques. Quantitative analysis of newly formed bone were performed using micro-computed tomography system and histological stain was used to visualize soft tissue in presence of membranes.

2 MATERIALS AND METHODS

2.1 Materials

List of acronyms

List of acronyms	
Si-HPMC	silanized hydroxypropyl methylcellulose
Dex40MA20	dextran 40 kDa with theoretical methacrylation degree of 20%*
Dex100MA20	dextran 100 kDa with theoretical methacrylation degree of 20%*
Dex500MA20	dextran 500 kDa with theoretical methacrylation degree of 20%*
PIS	photoinitiator solution
IPN	interpenetrating polymer network
BCP	biphasic calcium phosphate

** methacrylation degree is defined as the number of methacrylated groups per 100 glucopyranose residues of dextran*

Dextran 40, 100 and 500 kDa were purchased from Fluka (Milwaukee, United States). Riboflavin 5'-phosphate sodium salt hydrate (RP), triethanolamine (TEOHA), glycidyl methacrylate (GMA), 4-(Dimethylamino)pyridine (DMAP), dimethyl sulfoxide (DMSO), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and neutral red (NR) were all obtained from Sigma Aldrich (Saint Louis, MO, USA). Hydroxypropyl methylcellulose (HPMC) was furnished by Colorcon-Dow Chemical (Harleysville, PA, United States). Fetal bovine serum (FBS) was purchased from PAN biotech GmbH (Aidenbach, Germany). Penicillin, streptomycin, trypsin–EDTA, DMEM and phosphate buffered saline (PBS) were purchased from Gibco (Invitrogen, Carlsbad, CA, USA) and L929 cells from ATCC (Manassas, VA, USA). Biphasic calcium phosphate bone graft (MBCP+®) was purchased from Biomatlante (Vigneux de

Bretagne, France) with the following characteristics: granules of 0.5-1 mm, ratio of 20/80 in weight of HA/β-TCP (hydroxyapatite/β-tricalcium phosphate).

2.2 Dextran methacrylate synthesis

Synthesis of dextran derivatives was carried out according to the procedure described in literature [30,32,34-35]. We prepared three methacrylate dextran derivatives, by using dextran of different molecular weight (M_w) of 40, 100 and 500 kDa. In a typical synthesis, 20 g of dextran and 5 g of DMAP were solubilized in 220 mL of DMSO. In order to obtain a dextran with 20% of methacrylation degree, 3.47 mL of GMA were added to the solution which was kept in the dark for 48 h at room temperature under magnetic stirring. Finally, the reaction was stopped by adding HCl 0.1 M until pH 8 was reached. The solution was dialyzed (dialysis membrane cut off 12-14 kDa, Spectrapor®), against distilled water, then freeze-dried. The degree of methacrylation of the final freeze-dried powder, defined as the number of methacrylated groups per 100 glucopyranose residues of dextran, was determined by ^{1}H NMR in D_2O using a Bruker AC-400 spectrometer.

2 3 Synthesis of Si-HPMC and hydrogel preparation

Si-HPMC was synthesized as previously described [21]. Briefly, HPMC was solubilized in a heptane/propanol/NaOH solution and grafted with 3-glycidoxypropyltrimethoxysilane (GPTMS). After reaction, the polymer was purified by dialysis and freeze-dried. HPMC was modified by the addition of silanol groups with a degree of substitution of 0.6% (w/w) as determined by inductively coupled plasma atomic emission spectroscopy [19–21]. Si-HPMC powder was dissolved in 0.1 M NaOH overnight and sterilized by autoclave (121 °C for 20 min). Si-HPMC hydrogel was obtained by mixing the basic Si-HPMC solution with an acidic buffer in a 2:1 (polymer solution: acid buffer) ratio to reach a final pH of 7.4. The acidic buffer consisted of 0.06 M HCl, 1.8% (w/v) NaCl and 6.2% (w/v) HEPES. The buffer was sterilized by autoclave (121 °C for 20 min) prior to use.

2.4 Dextran methacrylate hydrogel formation

We first determined the gelation and shape properties of dextran derivatives as a function of molecular weight, concentration and the photoinitiator concentration. Dextran methacrylate 40, 100 or 500 kDa was dissolved at the concentration of 5, 15 and 30% (w/v). One milliliter of each polymer solution was prepared in distilled water under magnetic stirring in a glass vial. A stock photoinitiator solution (PIS) composed of 4.2 mM of riboflavin phosphate and 4.2 M of TEOHA was prepared. HCl 6M was added to the solution to adjust the pH to balance the basicity of TEOHA at neutral pH [34]. The PIS was added to the dextran solution at 1.5, 5 and 50 µL/mL (µL for each mL of polymer solution) and the mixture was stirred for 5 minutes until a homogeneous solution was formed. Then, the magnetic stirrer was removed and the solution was irradiated directly in the glass vial at 1 cm distance. Irradiation was performed 30 to 240 s from one side, 30 s for each time, with a dentistry lamp BA-Optima 10 curing light 1200mw/cm^2, 420-480nm (B.A. International). Hydrogel formation was assessed by the visual inspection (vial tilting method)[36,37]. The gel point was considered when the solution/gel stops to flow and the gelation was considered completed when the whole hydrogel could be lifted with a spatula without any fluid flowing. After gelling, hydrogels were demolded and visually inspected. The capacity of the hydrogel to maintain the original shape was scored from ++ very good, + good, +/- fair, - bad, -- very bad. All further experimentations were then performed with Dex500MA20, named DexMA.

2.5 DexMA filter sterilization

To sterilize the DexMA, a 30% (w/v) solution in distilled water of Dex500MA20 was filtered on 0.22 µm filter. DexMA solution was filtered through a EMD Millipore Steriflip™ sterile disposable vacuum filter unit using a diaphragm vacuum pump N-022-AN.18 (KNF). The filtration was completed in about 15 min. The filtered solution was diluted (final concentration of about 5 mg/mL) then freeze-dried. The freeze-dried polymers, were solubilized overnight (under gentle magnetic stirring) in the eluent composed of $NaNO_3$ 0.1 M with NaN_3 0.01% (w/v) at a concentration of 1-2 mg/mL, and filtered before injection (0.22 µm). The solution was analyzed by GPC Viscotek TDA 305 Triple Detector using 3 column TSKgel GMPWXL

(Tosoh) (100-1000 Å) with flow rate of 0.6 mL/min. For comparison, dextran and unfiltered DexMA were analyzed according to the above described procedure.

2.6 DexMA polymer solution stability

To assess the stability of a concentrated polymer solution as a function of time, DexMA of 500 kDa was solubilized in distilled water to obtain a 30% (w/v) solution (stock solution). The stock solution was filter on 0.22 µm filter and kept at 4°C avoiding the contact with light using an aluminum sheet. Stock solution and solution with addition of methacrylic acid were analyzed at day 0,1,7 and 30. To detect the hydrolyzed groups, some samples were added of 15.225 ppm, 10.15 ppm, 5.075 ppm, 2.03 ppm of methacrylic acid[38]. At different time points, 10 µL of the solutions were injected in an HPLC-UV (MERK with DAD) equipped with SunFire column (C18, 4,6x150 mm, 5 µm particles). The eluent was H2O/MeOH (60/40) with flow rate of 1 mL/min.

2.7 IPN hydrogel preparation

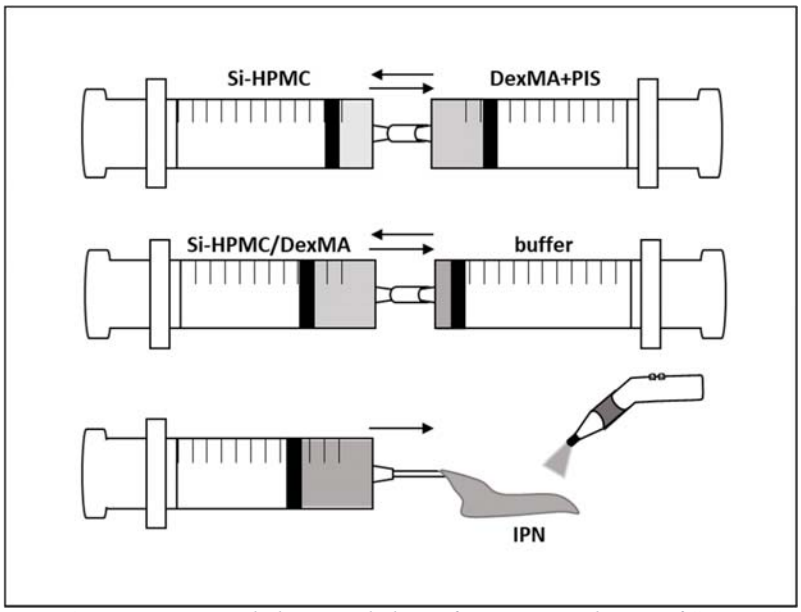

Figure 1 - Preparation and photocrosslinking of IPN. First, solutions of Si-HPMC and DexMA containing the PIS are mixed together through two syringes connected with a luer-lock. Then, the acidic buffer is added. The IPN solution is ready to be injected and photocrosslinked (420-480nm). Si-HPMC: silanized hydroxypropyl methylcellulose, DexMA: dextran methacrylate, PIS: photoinitiator solution, IPN: interpenetrating polymer network.

Si-HPMC/DexMA solution was obtained mixing the three following solutions. (1) Si-HPMC was solubilized in NaOH 0.1 M at 4% (w/v), (2) Dex500MA20 was solubilized at 30% (w/v) in distilled water with 5 µL of photoinitiator for each final mL of IPN solution. HCl 6M was added to the solution to adjust the pH to balance the basicity of TEOHA, at neutral pH [34]. (3) Acid buffer was prepared with 0.06 M HCl, 1.8% (w/v) NaCl and 6.2% (w/v) HEPES. To obtain the Si-HPMC/DexMA solution we mixed 1 volume of solution (1) with 2 volumes of solution (2) through two syringes connected with a luer-lock. The resulting solution was mixed with 0.5 volume of solution (3) (fig. 1).

The final solution contains 1.14% (w/v) of Si-HPMC and 17.15% (w/v) of DexMA (18.29% (w/v) total amount of polymer). The resulting Si-HPMC/DexMA solution was photocrosslinked to obtain the IPN, as described for DexMA hydrogel, with a standard dentist's visible light lamp (B.A. International).

2.8 Rheological analysis

Rheological measurements were performed on ARES G2 rheometer (TA Instruments). We analyzed the Si-HPMC 1.14% (w/v), DexMA 17.15% (w/v) and Si-HPMC/DexMA (Si-HPMC 1.14% (w/v), DexMA 17.15% (w/v)), with or without irradiation. The data were collected using the TRIOS software. Time sweep experiments were performed (1% strain, 6.28 rad/s) using a parallel plate geometry (20 mm diameter) with an upper transparent plate in quartz, allowing the material irradiation with a lamp to follow the gelation (λ = 450 nm, 430mW, 350mA, Royal blue).

2.9 Injectability

Injectability of Si-HPMC 1.14% (w/v), DexMA 17.15% (w/v) and Si-HPMC/DexMA solution (Si-HPMC 1.14% (w/v), DexMA 17.15% (w/v)) were analysed using Texture Analyzer (TA DH plus) with a cylindrical aluminium probe of 25 mm, at 0.1mm/s (compression rate) using a trigger force of 0.05 g. We ejected 0.5 mL of sample through a 3 mL syringe with an 18 G needle containing 1 mL of solution. The plots of force/displacement were recorded and the mean force of injectability was taken from the plateau of the curve. The experiments were performed in triplicate and the results statistically analyzed using Kruskal-Wallis test on GraphPad 6.

2.10 Hydrogels apposition on BCP

To simulate the implant in GBR model, hydrogel membranes were cross-linked on top of BCP (MBCP+®, Vigneux de Bretagne, France) Cylindrical PLA molds were fabricated using DiscoEasy 200 printer (Dagoma, Roubaix, France) with 8 mm of internal diameter and 1.5 mm of height. The molds were filled with BCP and covered with freshly prepared polymer solutions. The solutions were injected (0.1 mL) through a syringe equipped with an 18 G needle on the BCP. Si-HPMC/DexMA and DexMA solutions were photocrosslinked for 120 s. A control group of Si-HPMC (not irradiated), was observed after 1 h of crosslinking. Samples were preserved at room temperature prior to be observed with Macroscope AxioZoom (Zeiss).

2.11 Cytocompatibility

A neutral red (NR) uptake assay was carried out on murine fibroblasts (L929, ATCC, VA, USA) expose to (1) DexMA extract and (2) PIS, Si-HPMC, DexMA, Si-HPMC/DexMA solutions/hydrogels.

The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 mg/mL glucose, 10% fetal bovine serum and 1% penicillin–streptomycin. Cells were detached by 0.2% trypsin and seeded at 16,000 cells/mm^2 in multiwell plates 24 h before each test.

To perform the NR uptake assay, a NR solution was prepared 24 h before the test (0.04 mg/mL in PBS) and incubated at 37°C. Before being added to cells, the solution was centrifuged to remove any crystals. Culture medium was discarded and 100 µL of NR solution was added to cells and incubated for 3 h. After incubation, the NR solution was removed and a destain solution (50% ethanol 96%, 49% deionized water, 1% glacial acetic acid) was used to solubilize the dye trapped in living cells. The optical density was read at 550 nm using a microplate reader (Victor3V, PerkinElmer, Wellesley, MA, USA). The averages of optical density units were calculated after blank subtraction. All the tests were performed in triplicate. Results of NR uptake were expressed as the ratio (%) between the absorbance and the absorbance of untreated cells.

To evaluate the cytocompatibility of DexMA polymer extract, cells were seeded at 16,000 cells/mm^2 in a 96-well plate for 24 h. Extracts in distilled water of DexMA (Dex500MA20) were prepared according to the ISO 10993-5. A polymer solution of 30% (w/v) was inserted in a dialysis tube (dialysis membrane cut off 1 kDA, Spectrapor®). The tube was introduced in a Falcon tube with 10 mL of distilled water under stirring at room temperature for 72 h. The extract was sterilized on a 0.22 µm filter and used for testing the effect of leach-out product on cell viability. The culture medium was discarded from cells and replaced with medium containing 10% (v/v) polymer extract. To examine the effect of irradiation (120 s), visible light lamp was used to expose cells in the wells containing or not containing polymer extract. Any plates not being exposed to visible light lamp, were covered to prevent any unregulated light exposure. The control well consisted in cell with culture medium without polymer extract and not irradiated, named untreated cells. After 24 h of incubation, the medium was removed, the cells rinsed with sterile PBS and neutral red solution added to each well to perform the assay.

To evaluate the cytocompatibility in presence of hydrogels, 48-well plates with inserts (polycarbonate 8 µm pore size) were used. The cells were seeded at 16,000 cells/mm^2 on wells and 24 h later Si-HPMC, DexMA and Si-HPMC/DexMA solutions were deposited on the inserts. In order to examine the effect of irradiation, visible light curing was used to expose cells and polymer solutions for 120 s with visible light lamp. In parallel the viability in presence of PIS was evaluated, adding the PIS to cells medium at the same concentration than in hydrogels. Any plates not being exposed were carefully covered to prevent any unregulated light exposure. All the plates were returned to the 37°C incubator for 24 h. Control well consisted of cells without PIS and not irradiated, named untreated cells. After 24 h, the medium was removed, the cells rinsed with sterile PBS and neutral red solution added per each well to perform the NR assay.

The experiments were performed in triplicate and the results statistically analyzed using Kruskal-Wallis test on GraphPad 6.

2.11 *In vivo* experiment: animal model and study design

Studies were conducted on 12 White New Zealand male rabbits (3.39±0.13 Kg body weight) of 13 weeks of age. Rabbits were obtained from Granja San Bernardo (Navarra, Spain). All procedures were carried out under project license (nº010532/2018) approved by the National Competent Authority for animal research, named Direção-Geral de Alimentação e Veterinária (DGAV, Lisbon, Portugal). The animals were kept in a purpose-designed room for experimental animals, one rabbit for cage and were fed with standard laboratory diet. All experimental procedures were performed in accordance with the European Directive 2010/63/EU and with National Law (DL n° 113/2013) on the protection of animals used for scientific purposes, and were locally approved by the Animal Welfare Committee of University of Trás-os-Montes e Alto Douro.

Four calvaria critical size defects of 8 mm diameter and the depth comprised the full thickness, according to the individual specimen, were surgically created in each animal. Therefore, the defects were filled with BCP consisting of granules with 0.5-1 mm in size with 20% hydroxyapatite and 80% β-tricalcium phosphate (MBCP+®, Biomatlante, Vigneux de Bretagne, France). The defects in 12 rabbits were filled with BCP and randomly treated with Si-HPMC, DexMA, Si-HPMC/DexMA or collagen membrane as a control (EZ Cure® Biomatlante, Vigneux de Bretagne, France). DexMA and Si-HPMC/DexMA solutions once applied *in situ* were photocrosslinked for 120 s. Because of the good adaptation of the membranes, no further stabilization by wound sutures was necessary. In order to confirm the incapacity of the 8-mm wound to heal without treatment (critical size), we decided to substituted one BCP with Si-HPMC hydrogel condition to add an empty defect to be analyzed after 8 weeks from surgery to validate the incapacity of the 8 mm wound to heal without treatment. Bone repairing and histological processing were analyzed at 2 (6 rabbits) and 8 (6 rabbits) weeks from implantation.

2.13 Surgical protocol

The animals were sedated with an intramuscular injection of diazepam 1 mg/kg. They were anesthetized with an intramuscular injection of ketamine 30 mg/kg and xylazine 5 mg/kg supplemented with intraoperative analgesia with buprenorphine 0,03 mg/kg. The animals were first shaved and disinfected for

aseptic surgical conditions. An incision was made along the middle of cranium and a full thickness flap was preformed to expose the calvaria. Once the calvaria exposed, the entire flap was elevated and four defects were realized using a trepans of 8 mm diameter under abundant saline irrigation. The defects were filled with BCP previously mixed with few drops of sterile saline solution. The four membranes conditions previously described were randomly assigned to the defect created. Subsequently, the flaps were repositioned and closed with wound sutures. Postoperative management included the subcutaneous administration of antibiotics (enrofloxacin 10mg/kg) and buprenorphine (0,01 mg/kg). The surgery was successfully realized (fig. 1). Four and 8 weeks later, the rabbits were sedated with barbiturates and sacrificed by an overdose of Ketamine.

2.14 Clinical observation

Animals were carefully examined for inflammation, allergic reactions, and other complications around the surgical site through the entire healing period.

2.15 Micro-computed tomography analysis

Qualitative analysis of newly formed bone was performed using the SkyScan-1072 high resolution X-ray micro-computed tomography system (SkyScan 1172, Kontich, Belgium). The following settings were used: 0.5 and 0.058 aluminum and copper filter, rotation step 0.71°, resolution at 24 μm. Three-dimensional reconstructions were built using NRcon® software (Skyscan) for each implant to assess bone formation in control (BCP with collagen solid membrane) and treated animals. Volume of interest (VOI) was defined for each defect represented by the defect borders and 76 sectional images in the coronal dimensions. CTAn® was used for calculation of new bone formation volume and BCP biomaterial volume. New bone formation was represented by the mineralized tissue subtracted from the BCP volume within the individual VOI. The lower grey value was set at 30 and the upper one at 255. The results statistically analyzed using Kruskal-Wallis test on GraphPad 6.

2.16 Histological processing

The calvaria were separated from cranium and the specimens were fixed in 4% paraformaldehyde and then dehydrated through a graded series of ethanol treatments (70-100%). Non-decalcified bone specimens were infiltrated and embedded using Technovit® 9100 (Heraeus Kulzer, Wehreim, Gemany). Blocks were cut into 100 µm slices with a circular diamond saw (Microtome 1600, Leica, Frankfurt am Main, Germany) for staining. Only section in the center of defects were selected. Sections were stained with HES (hematoxylin eosin safranin) and Goldner's staining and examined using a light microscope (Axioplan 2; Zeiss, Darmstadt, Germany).

3 RESULTS

3.1 Polymer synthesis

Si-HPMC was successfully modified by the addition of silanol groups with a degree of substitution of 0.6% (w/w) as determined by inductively coupled plasma atomic emission spectroscopy as described elsewhere [19]. Si-HPMC 4% (w/v) sterile stock solution was prepared in NaOH at as previously described [20,21].

	Theoretical values	**Experimental data**
Polymer	**% MA**	**% MA**
Dex40MA20	20	18
Dex100MA20	20	17
Dex500MA20	20	19

Table 4 - Theoretical and experimental degree of methacrylation (%MA) of Dex40MA20, Dex100MA20 and Dex500MA20. The degree of methacrylation, defined as the number of methacrylated groups per 100 glucopyranose residues of dextran, was determined by proton NMR 300 MHz. Dex40MA20: Dextran 40 kDa with theoretical methacrylatation degree of 20%, Dex100MA20: Dextran 100 kDa with theoretical methacrylatation degree of 20%, Dex500MA20: Dextran 500 kDa with theoretical methacrylatation degree of 20%.

Dextran methacrylate using polymers with M_w of 40,100 and 500 kDa were synthetized. Table 1 summarizes the values of the theoretical and the measured degree of methacrylation. NMR results show a percentage of 18, 17 and 19 of methacrylate groups grafted along the polymer chains.

3.2 Dextran methacrylate hydrogel formation

Polymer*	Conc. % (w/v)	Photoinitiator concentration (µL/mL)					
		1.5			5		
		Gel point (min)	Gel time (min)	Shape	Gel point (min)	Gel time (min)	Shape
Dex100MA20	**5**	/	/	/	/	/	/
	15	/	/	/	/	/	/
	30	150	210	+	90	150	-
Dex500MA20	**5**	210	240	--	30	150	--
	15	90	150	--	30	120	+
	30	60	60	++	30	90	++

*Table 2 - Gelation and shape properties of dextran methacrylate. Dex100MA20 and Dex500MA20 at 5, 15 and 30 % (w/v) with 1.5 or 5 µL/mL of photoinitiator solution were analyzed. The sol-to-gel transition was characterized by vial tilting method. The gel point was considered when the solution/gel stops to flow and the gelation was considered completed (gel time) when the whole hydrogel could be lifted without any fluid flowing. After gelling the demolded hydrogels were visually inspected and the capacity to maintain the original shape was scored from ++ very good, + good, +/- fair, - bad, -- very bad. / : symbol indicateds that no hydrogel formation occurred. Dex100MA20: Dextran 100 kDa with theoretical methacrylatation degree of 20%, Dex500MA20: Dextran 500 kDa with theoretical methacrylatation degree of 20%. * No Dex40MA20 hydrogel was obtained, and gelation and shape properties could not be recovered.*

To select the starting dextran methacrylate solution to combine with Si-HPMC, we determined the gelation and shape properties as a function of dextran molecular weight, polymer concentration and photoinitiator concentration. The sol-to-gel transition was characterized by vial tilting method. The gel point was considered when the solution/gel stops to flow and the gelation was considered completed (gel time) when the whole hydrogel could be lifted without any fluid flowing. After gelling, the demolded hydrogels were visually inspected at room temperature and their capacity to maintain the original shape was scored (table 2).

We analyzed dextran methacrylate of 40, 100 and 500 kDa (Dex40MA20, Dex100MA20 and Dex500MA20) at 5, 15 and 30 % (w/v) with 1.5, 5 or 50 µL/mL of PIS. We irradiated the solutions from 30 to 240 s, 30 s each time. With Dex40MA20, no hydrogel was obtained, whatever the polymer and the PIS concentration. Dex100MA20 formed hydrogels only at high concentration (30% (w/v)) with 1.5 or 5 µL/mL of PIS. Dex500MA20 solutions formed hydrogels at every concentration tested with 1.5 or 5 µL/mL of PIS. Dex100MA20 and Dex500MA20 did not form hydrogels any with 50 µL/mL of PIS, whatever the concentration. With Dex500MA20 15 and 30% (w/v), at the end of irradiation, the material was not homogeneous, with a cross-linked polymer layer on top of a liquid layer. On the other hand, Dex500MA20

with low PIS concentration (1.5 µL/mL) formed hydrogels, but the gel point was delayed in time compare to 5 µL/mL.

Dex500MA20 is able to form hydrogel with 5 µL/mL concentration of PIS in 30 s. However, hydrogel of Dex500MA20 at 30 and 15% (w/v) have a better shape maintaining after demolding compare to 5% (w/v). The polymer solution at 30% (w/v) with molecular weight 500 kDa with 5 µL/mL of PIS was selected as a starting solution for IPN formulation due to the capacity of quickly form solid upon irradiation and for good shape maintaining. All further experimentations were performed with Dex500MA20 named DexMA.

3.3 DexMA filter sterilization

To determine the impact of filtration of a 30% (w/v) solution of DexMA, the polymer was filtered and successively freeze-dried to be analyzed by HPLC-GPC. For comparison, dextran and no filtered DexMA were treated with the same described procedure.

	Dextran	**DexMA**	**Filtered DexMA**
M_w (kDa)	500	702.8	803.4
M_n (kDa)	52.9	144.3	171.5
PD	9.453	4.869	4.684

Table 3 - Molecular weights and molecular weights distributions of dextran, DexMA and DexMA filtered on 0.22 µm filter. The polymers solutions were analyzed by HPLC-GPC results. Dextran with theoretical methacrylatation degree of 20%. DexMA: dextran methacrylate, PD: polydispersion index.

The addition of methacrylate groups along the dextran backbone increased the molecular weight of the polymer (table 3). The filtration process of DexMA was able to cause an augmentation in molecular weight values compare to the unfiltered polymer. On the contrary, the polydispersity index decreased for DexMA as compared to dextran and for sterile DexMA as compared to unfiltered DexMA.

3.4 DexMA polymer solution stability

Stability study of DexMA in aqueous solution was performed, since methacrylic acid could be released. Stability of DexMA 30% (w/v) stock solution, was analyzed at day 0,1,7 and 30. The solution was stored at 4°C and preserved from light. To detect possible hydrolyzed groups, at every time points, the solutions

were analyzed on HPLC-UV. During these analyses, no trace of methacrylic acid was detected in the samples for these analysis conditions.

3.5 Rheological analysis

Gelation kinetics of Si-HPMC at 1.14% (w/v), Si-HPMC/DexMA at 1.14% (w/v)/17.15% (w/v) and DexMA at 17.15% (w/v) were measured. Elastic and viscous moduli as a function of time are reported in figure 2.

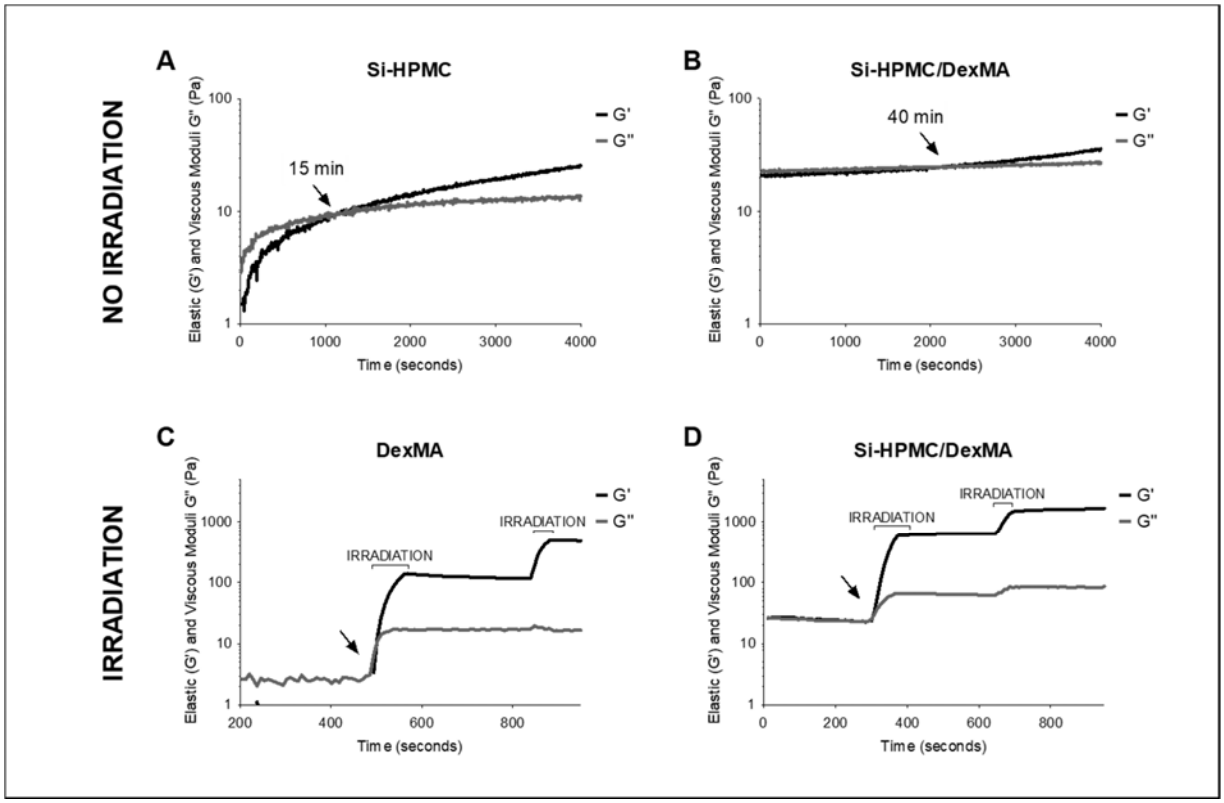

Figure 2 - Elastic (G') and viscous moduli (G'') as a function of time (seconds) of (A) Si-HPMC and (B) Si-HPMC/DexMA without irradiation, (C) DexMA and (D) Si-HPMC/DexMA under a first irradiation of 80 s and a second irradiation of 40 s. Gel point is illustrated by arrow. Polymer concentration: Si-HPMC at 1.14% (w/v), Si-HPMC/DexMA at 1.14% (w/v)/17.15% (w/v) and DexMA at 17.15% (w/v). Data recorded at 6.8 rad/s frequency, 1% strain, room temperature, the lamp used for irradiation has λ = 450 nm. Si-HPMC: silanized hydroxypropyl methylcellulose, DexMA: dextran methacrylate, Si-HPMC/DexMA silanized hydroxypropyl methylcellulose/dextran methacrylate

Elastic and viscous moduli of Si-HPMC (fig. 2A) increased slowly and the gel point is reached after 15 minutes (arrow), when the values of the moduli are around 10 Pa. After the gel point, the elastic modulus gradually increases as a consequence of the crosslinking by silanol condensation.

For Si-HPMC/DexMA (fig. 2B), analyzed without irradiation, the elastic and viscous moduli have an initial similar values. The gel point is reached after 40 minutes (arrow) and subsequently a small increase of the elastic modulus is observed.

DexMA solution (fig. 2C) was first irradiated with visible light lamp for 80 s. The curve shows an instantaneous gel point (arrow) and a sharp and immediate increase of the elastic and viscous moduli. After light exposure, the moduli values are stabilized. During a second irradiation of 40 s, the elastic modulus increase to reach the value of 485 Pa, while the viscous modulus remains stable.

Si-HPMC/DexMA initial values of elastic and viscous moduli are higher of one order of magnitude as compared to the once of each polymer solution separately. It is worth noting that while non irradiated gels present similar values of elastic and viscous moduli, irradiated gels of DexMA and Si-HPMC/DexMA presented higher elastic moduli of 800 Pa and 1000 Pa respectively.

When Si-HPMC/DexMA (fig. 2D) solution is exposed along 80 s upon irradiation, the gel point is reached instantaneously and the elastic and viscous moduli rapidly increase. As observed for DexMA solution, the crosslinking is dependent from light lamp exposure and an increase in elastic modulus is observed after a second irradiation of 40 s to 1500 Pa.

3.6 Injectability

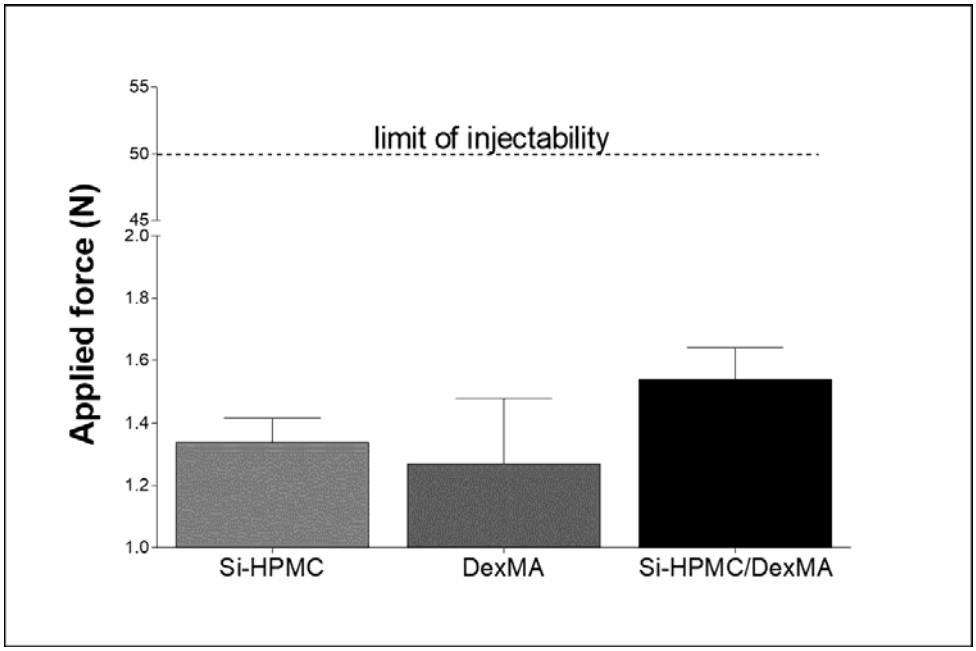

Figure 3 - Injectability of Si-HPMC, DexMA, Si-HPMC/DexMA solution. Required force to inject 1 mL of each polymer solution through a 3mL syringe loaded with 1mL of polymer solution using an 18 G needle. Results are expressed as a mean value ±SEM (n=3). Si-HPMC: silanized hydroxypropyl methylcellulose, DexMA: dextran methacrylate, Si-HPMC/DexMA silanized hydroxypropyl methylcellulose/dextran methacrylate

The force necessary to inject a solution through a syringe with needle was measured with texture analyzer. For all the solutions the plots of force/displacement were recorded and the mean force of injectability was taken from the plateau of the curve. Si-HPMC, DexMA and Si-HPMC/DexMA possess values of injectability force of the same order of magnitude between 1.4 and 1.6 N (fig. 3). All the measured forces are under the limit of injectability suggested in ISO 7886-1.

3.7 Hydrogels apposition on BCP

To simulate implants in GBR conditions, BCP were inserted in a mold and covered with Si-HPMC, DexMA and Si-HPMC/DexMA solutions. The solutions were injected through a syringe equipped with an 18 G needle. Si-HPMC/DexMA and DexMA solutions were photocrosslinked for 120 s and Si-HPMC (not irradiated), was cross-linked for 1 h at room temperature.

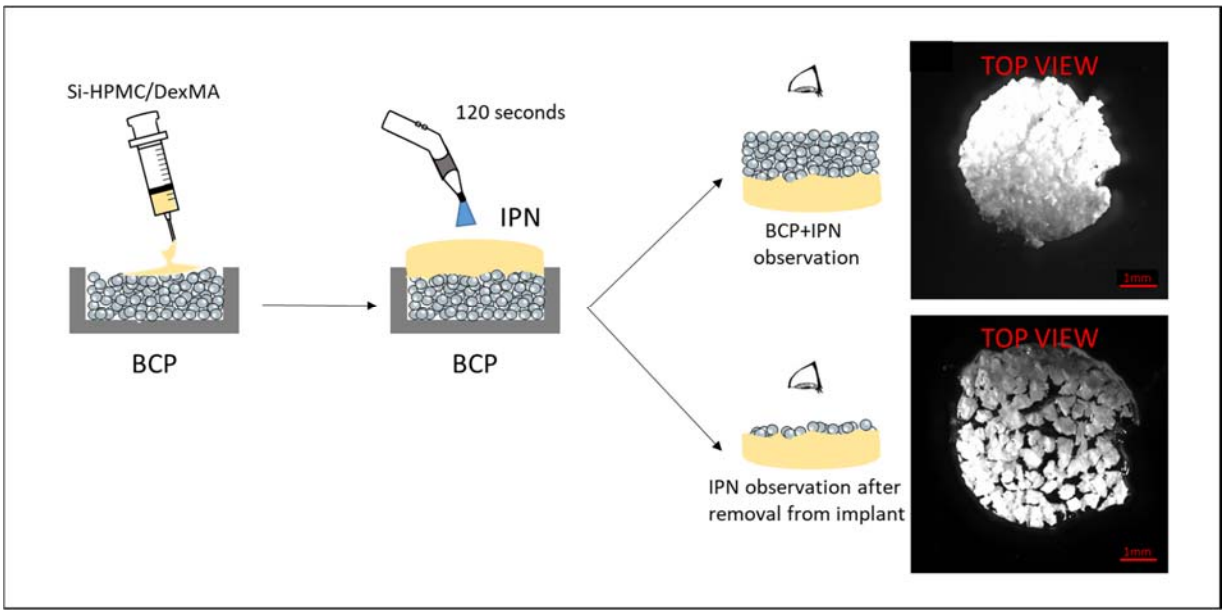

Figure 4 - Scheme of implant preparation composed of BCP and IPN hydrogel membrane. Cylindrical mold was filled with BCP. Si-HPMC/DexMA solution was injected on BCP surface. The solution was photocrosslinked for 120 s with visible light lamp to form the IPN. BCP with IPN and IPN after removing from BCP were observed with microscope. Mold of 8 mm diameter, 1.5 mm height. Si-HPMC: silanized hydroxypropyl methylcellulose, DexMA: dextran methacrylate, Si-HPMC/DexMA: silanized hydroxypropyl methylcellulose/dextran methacrylate, BCP: biphasic calcium phosphate, IPN: interpenetrating polymer network.

Si-HPMC is a transparent hydrogel poorly visible above the BCP (not shown). The kinetics to reach the gel point seems increase the capacity of the polymer to infiltrate the BCP. After crosslinking, the system composed of Si-HPMC and BCP was analyzed. Part of the hydrogel can be removed from BCP. The structure is composed of two-layer material: BCP and a cohesive interface composed of dense surface of BCP embedded in Si-HPMC.

Photocrosslinked DexMA is a yellowish hydrogel (not shown) and consequently it is easy to identify, due to the presence of riboflavin (yellow color). The bilayer structure composed of the hydrogel and BCP is discernible. Observing the interface of the hydrogel, after BCP removing, some BCP granules are trapped on it.

The IPN composed of Si-HPMC/DexMA polymers, appeared transparent, translucent but slightly yellowish due to the riboflavin presence like in DexMA alone. The bilayer structure composed of IPN on BCP is observable. The photocrosslinking technology combined with a second viscous polymer system seems decrease the infiltrating of the formulation in BCP compare to DexMA or Si-HPMC. The BCP appeared a

compact matrix with IPN. However, after IPN removing from BCP layer, the interface observation revealed that only few granules were embedded on it (fig. 4).

3.8 Cytocompatibility

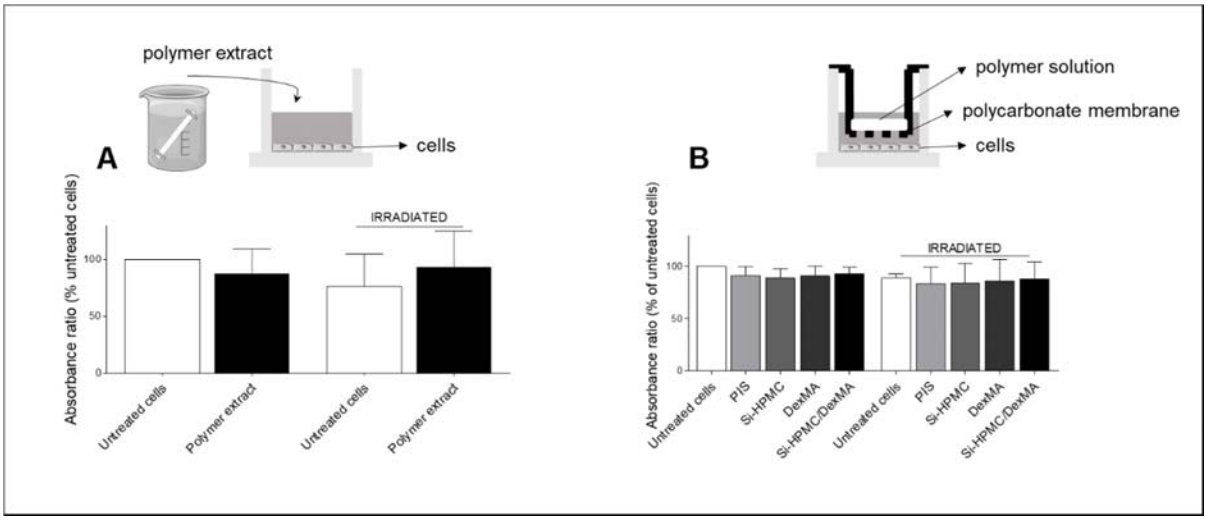

Figure 5 - Cytocompatibility assessed by neutral red uptake on L929 cells seeded at 16,000/cm^2 after (A) 24 h in presence of DexMA polymer extract, (B) 24h in presence of 5 µL/mL of PIS and Si-HPMC, DexMA, Si-HPMC/DexMA. In order to examine the effect of irradiation, visible light lamp was used to expose the solutions for 120 s. Results are presented as mean value ± SEM (n=3) and analyzed with 2-way ANOVA with the Bonferroni post test. Si-HPMC: silanized hydroxypropyl methylcellulose, DexMA: dextran methacrylate, Si-HPMC/DexMA: silanized hydroxypropyl methylcellulose/dextran methacrylate, PIS: photoinitiator solution.

In order to evaluate the cytocompatibility, neutral red uptake (NR) assay was carried out with the exposure of murine fibroblasts (L929, ATCC, VA, USA) to (1) DexMA extract and (2) PIS, Si-HPMC, DexMA, Si-HPMC/DexMA solutions/hydrogels.

After synthesis of DexMA polymer, an extract was made to assess the cytocompatibility. The polymer extract, containing the polymer leach-out products, was added to culture medium at 10% (v/v). Cytocompatibility results of cells exposed to polymer extract are reported on figure 5A. There were a non significative difference in the absorbance ratio of untreated cells compare to cells in presence of polymer extract. In addition, the cells irradiated for 120 s with visible light lamp did not show a significative difference in the absorbance ratio compare to the untreated cells.

Cytocompatibility of cells were investigated in presence of PIS, Si-HPMC, DexMA and Si-HPMC/DexMA. In order to examine the effect of irradiation, visible light curing was used to expose cells and PIS/polymer

solutions for 120 s (fig. 5B). The effect irradiation along 120 s, also in presence of PIS, did not show any difference in absorbance ratio compare to the untreated cells. In addition, no significant differences were found between the absorbance ratio of cells cultured, irradiated or not irradiated, in presence of Si-HPMC, DexMA, Si-HPMC/DexMA or PIS.

3.9 Surgical procedure and clinical observation

The surgery was successfully realized (fig. 6). No post-operative complication occurred.

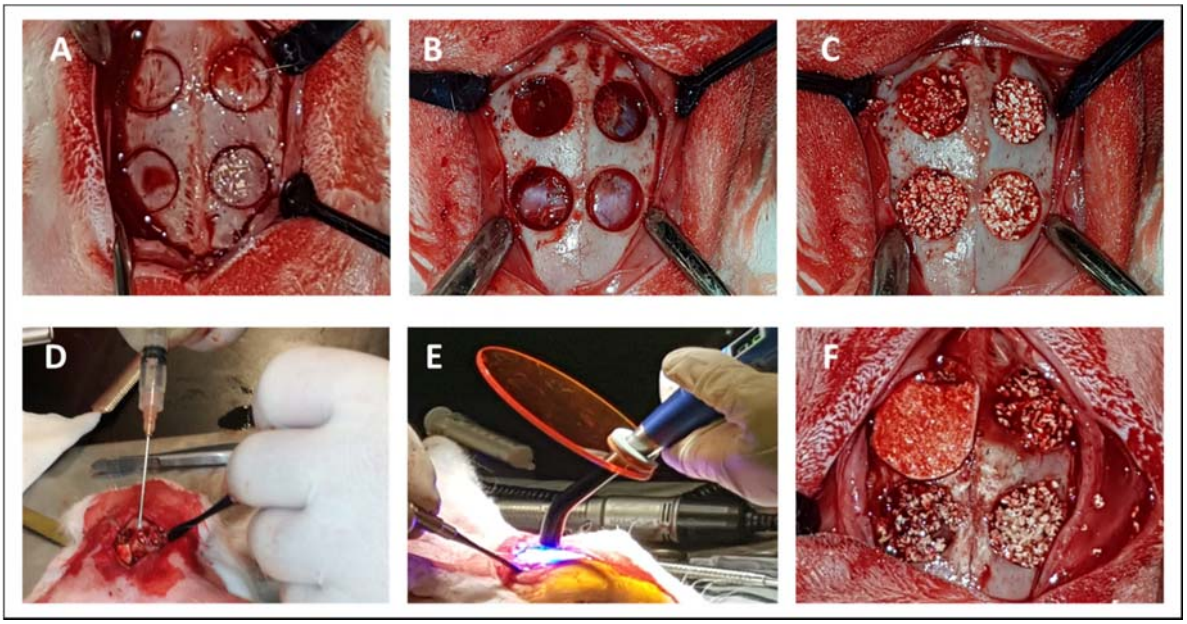

Figure 6 - Surgical procedure on rabbit calvaria. (A) Surgical calvaria incisions. (B) Four defects (Ø= 8 mm) were created in each calvaria of 12 male White New Zeeland rabbits and (C) the defects were filled of BCP. (D) Si-HPMC/DexMA application using a syringe with 18 G, (E) Si-HPMC/DexMA photocrosslinking with standard dentistry lamp along 120 s to form the IPN membrane. (F)Random of assignment membranes apposition on defect, clockwise from top left: control collagen membrane, Si-HPMC, IPN, DexMA. Si-HPMC: silanized hydroxypropyl methylcellulose, DexMA: dextran methacrylate, Si-HPMC/DexMA: silanized hydroxypropyl methylcellulose/dextran methacrylate, IPN: interpenetrating polymer network.

Animals were carefully examined for inflammation, allergic reactions, and other complications around the surgical site. None of animals exhibited adverse reactions or complications to the presence of the implants through the entire healing period. No significant reductions in body weights were noted, and no postoperative infections were observed. All animals behaved normally during the healing phase.

3.10 Macroscopic examination of implant after sacrifice

After 2 and 8 weeks from surgery, the animals were sacrificed. The calvaria with periosteum were separated from cranium and observed. Macroscopic examination was performed prior to micro-computer tomography

analysis and histological processing. Most of samples exhibited a regular morphology and color. Only one defect condition presented a clot formation, which correspond to the empty defect condition (supplementary data S.1). Periosteum appeared uniform in every sample and adherent to the calvaria. None of sample presented perforation, or membrane/BCP exposition. No BCP granules were found around the defects and the membranes are not visible observing the internal or external side of calvaria after 2 weeks from surgery. After 8 weeks from surgery some BCP granules are visible after around the defects, not related with a specific condition.

3.11 Micro-computed tomography analysis

Images and quantification of calvaria defects healing after two and height weeks from surgery were studied. Representative pictures of implants composed of BCP covered with commercial collagen solid membrane (control), Si-HPMC, DexMA and IPN hydrogel membranes from coronal and axial plane and quantitative analysis of mineral tissue are reported after two (fig. 7) and height weeks (fig. 8) from surgery. We decided to add an empty defect to be analyzed after 8 weeks from surgery to validate the incapacity of the 8-mm wound to heal without treatment.

Figure 7A shows representative picture of the collagen, Si-HPMC, DexMA, IPN group condition after 2 weeks from surgery. The borders of all defects group can be identified in every condition. All the conditions appeared filled of mineral bone graft and the quantity of BCP seems homogeneous in every defect. No empty spaces are visible from picture. Axial images confirm the homogeneous BCP distribution in the volume. No qualitative difference where noticed among the conditions. Quantitative analysis of total mineralized content, new bone and BCP was performed in the volume of interest using CTAn® software (fig. 7C). The quantification was expressed as a percentage of the mineral volume (MV) normalized by the total volume (TV) of defect. The results show a homogeneous BCP distribution in experimental membranes comparable with the control group of around 15%. The amount of new bone was respectively to 27.01±7% for collagen, 33.67±4.60% for Si-HPMC, 33.60±11.8% for DexMA and 27.71±14% for IPN. The amount of new mineral tissue deposition did not differ from the collagen membrane control material. However, none

of the defects were completely recovered at this stage and mineralized volume material represented around 40% of total volume, indicating that the healing process was not completed.

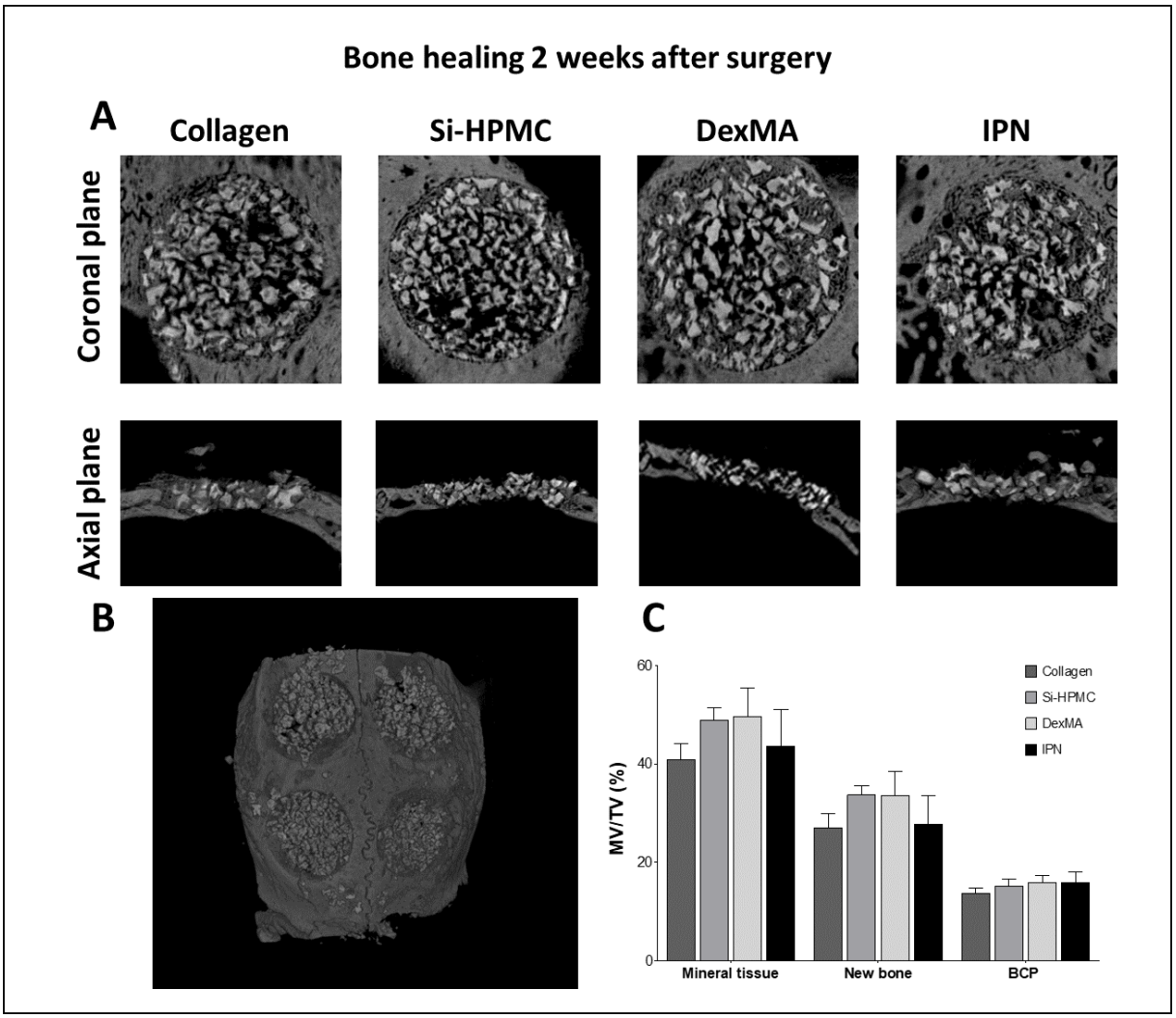

Figure 7 - Bone healing evaluation after 2 weeks from implantation (A) Micro-CT imaging of calvaria defects of 8 mm diameter (B) 3D reconstruction of external view of calvaria. Clockwise from top left: control collagen membrane, IPN, DexMA, Si-HPMC. (C) Quantification of mineral volume on total volume (MV/TV) % of total mineral tissue, new bone and biphasic calcium phosphate granules (BCP) in the volume of interest. Error bars represent the standard deviation of the mean (n=6). Si-HPMC: silanized hydroxypropyl methylcellulose, DexMA: dextran methacrylate, IPN: interpenetrating polymer network.

Images and quantification of calvaria defects healing after 8 weeks from surgery are reported on figure 8. In order to confirm the critical size of the defects created in calvaria, we added an empty condition (no BCP, no membrane), that we analysed after 8 weeks from surgery. Coronal and axial view reported on figure 8 shows an empty defect with no mineralization inside the volume of interest. This confirm the incapacity of the empty defect to heal in 8 weeks. Collagen, Si-HPMC, DexMA and IPN conditions after 8 weeks from surgery, presented in most of samples empty spaces (no BCP, no bone). These spaces seem not be related to a particular condition or to a specific position of the defect in the calvaria. Quantitative analysis shows a

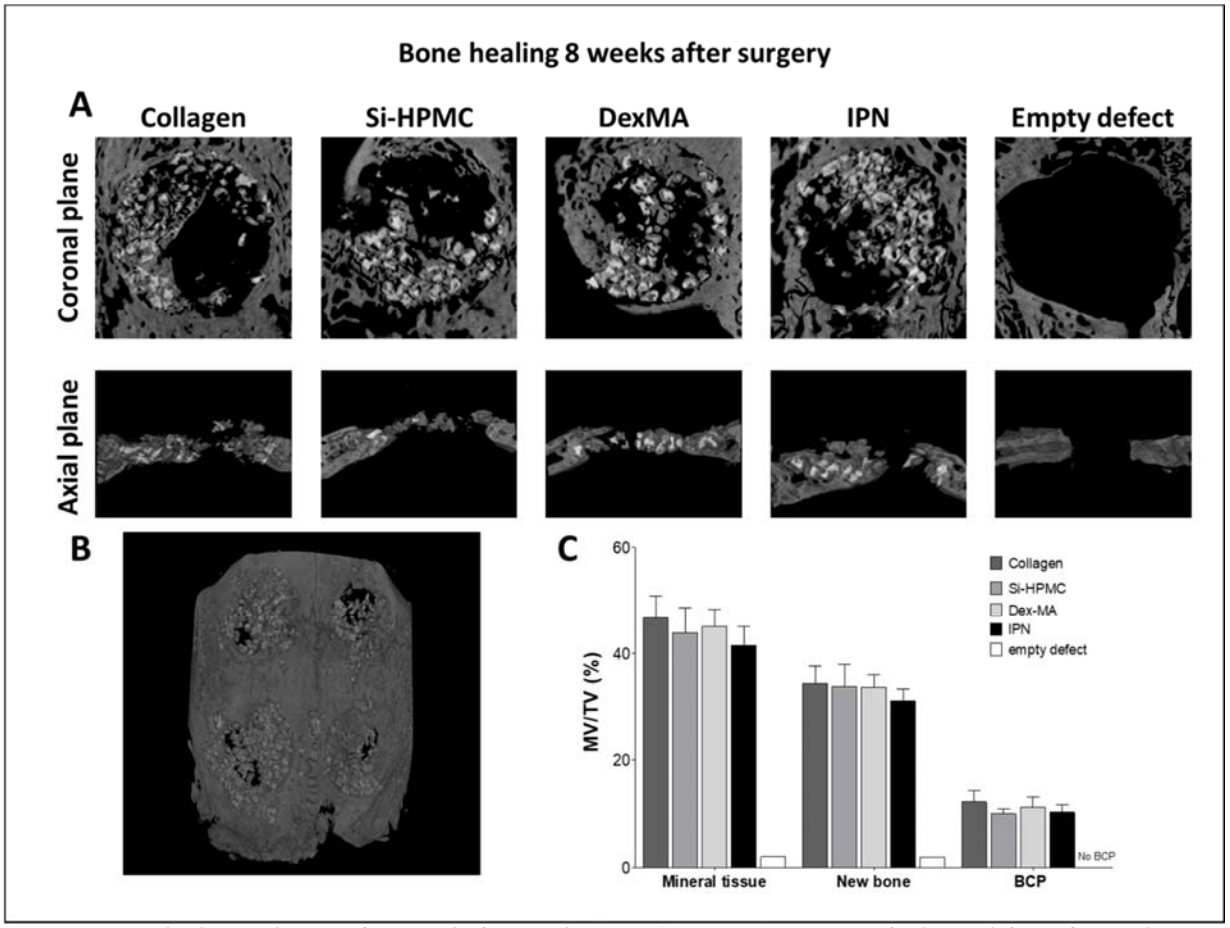

Figure 8 - Bone healing evaluation after 8 weeks from implantation (A) Micro-CT imaging of calvaria defects of 8 mm diameter (B) 3D reconstruction of external view of calvaria. Clockwise from top left: control collagen membrane, Si-HPMC, IPN, DexMA. (C) Quantification of mineral volume on total volume (MV/TV) % of total mineral tissue, new bone formation and biphasic calcium phosphate granules (BCP) in the volume of interest. Error bars represent the standard deviation of the mean, collagen n=6, Si-HPMC n=5, DexMA n=6, IPN n=6, empty defect n=1. Si-HPMC: silanized hydroxypropyl methylcellulose, DexMA: dextran methacrylate, IPN: interpenetrating polymer network.

total mineral tissue of around 44% and BCP distribution of 11%. The amount of new bone after 8 weeks from surgery was 34.30±8.17% for collagen, 33.74±9.94% for Si-HPMC, 35.19±6.74% for DexMA and 30.49±5.23% for IPN.Quantitative analysis of the control commercial collagen membrane, Si-HPMC, DexMA and IPN exhibit nonsignificant variation in the amount of new mineral tissue and BCP content.

3.12 Histological processing

After sacrifice, the skull containing all four craniotomy sites was removed and placed in 4% paraformaldehyde. Samples were dehydrated with ethanol treatments (70-100%) and embedded using Technovit® 9100 (Heraeus Kulzer, Wehreim, Gemany). Blocks were cut into 100 μm slices for HES staining. Histological analysis and quantification are still on going to determine the morphology and quantification of mineral and soft tissue in the defects.

4 DISCUSSION

Interpenetrating polymer networks (IPNs) are systems composed of two or more polymer network intercalated at the molecular scale to each other, but not linked between them. *In situ* simultaneously IPN are often described in literature for minimally invasive surgery for biomedical applications. These interventions, in fact, are associated to a reduced morbidity and post-operative pain[39].

In the present study, we hypothesized that combining Si-HPMC with DexMA, we could induce the formation of an innovative IPN hydrogel. We prepared a Si-HPMC/DexMA solution that could be injected directly *in situ* and quickly obtain a membrane for GBR upon visible light irradiation.

Si-HPMC self-setting hydrogel was successfully synthetized and stock sterile polymer solution was prepared as previously described[19,20]. Three different dextran methacrylate derivatives of 40, 100 and 500 kDa with theoretical methacrylatation degree of 20% were prepared. The NMR results show a percentage of methacrylation coherent with the theoretical values.

To photocrosslinked the methacrylate polymer, we selected a photoinitiator based on riboflavin used with triethanolamine as co-initiators[40,41]. To enhance the biocompatibility of the process it was preferred to

use a visible light lamp (λ 420-480 nm) to crosslink the solution than an UV lamp[42,43]. Specifically, we chose a standard dentistry lamp, very common today in dental clinical practice. To select the starting dextran methacrylate solution to combine with Si-HPMC, we determined the gelation and shape properties as a function of DexMA molecular weight, polymer concentration and the photoinitiator concentration. An inverse proportionality was evidenced between the polymer concentration and the time necessary to reach the gel point. In fact, concentrated solutions, probably due to the higher presence of methacrylate moieties, presented reduced time to reach the gel point. In addition, the increase in molecular weight also reduce the time to reach the gel point. In particular, DexMA at the lower molecular weight (40 kDa) was unable to form a solid in the observed time of irradiation. To initiate the crosslinking a precise photoinitiator concentration is necessary in the solution. In fact, increasing the photoinitiator concentration (50 µL/mL) no hydrogel was observed in each sample. This phenomenon might be attributed to the increased opacity of the hydrogel precursor solution at higher photoinitiator content, which could hinder the penetration of the visible light to the precursor solution. Riboflavin, in fact is a vitamin, present in food and plants, which impart yellow color as a function of solution concentration. On the other hand, DexMA with photoinitiator at low concentration (1.5 µl/mL) forms hydrogels but the gel point is delayed compare to 5 µL/mL. From these results, we selected as a starting solution DexMA of 500 kDa at 30% (w/v) with 5 µl/mL of PIS as a starting solution for the IPN realization.

Chu *et al.* described a DexMA cross-linked in presence of different amount of riboflavin with L-arginine as a co-initiator. The study described a solution of 25% in weight of dextran (64-75 KDa) poorly substituted with methacrylic anhydride (%MA 0.287). The solution was photocrosslinked with a flurescent lamp till 40 min. They showed that only few amount of riboflavin was necessary to cross-link the polymer (0.01-0.5%) and that bigger amount of PIS did not lead to a cross-linked biomaterial, confirming the observation described in our work[44]. Menzel investigated the photocrosslinking of dextran modified with hydroxyethyl methacrylate groups with visible light lamp and using camphorquinone as a photoinitiator with different types of co-initiators. They found hydrogel formation after 120 s of lamp irradiation, using a

polymer solution between 10-30% of 35-45 KDa and placing the light source directly over the polymer solution. However, for this study, due to the poor solubility of camphorquinone, the crosslinking was necessarily performed in presence of different amount of DMSO[43]. In our study, we selected riboflavin, and in particular its phosphate derivative,as a photoinitiator, because of its water-solubility avoiding the use of organic solvent in the final formulation.

The selected DexMA solution was sterilized by filtration on 0.22 μm filter. The filtered and unfiltered polymer were analysed by HPLC-GPC. The filtration process is able to cause a significant increase in molecular weight values, probably because exposure to light and the formation of radicals due to the presence of oxygen, leads to an initial polymerization of methacrylic groups, even in the absence of the photoinitiator. As evidenced by the reduction of polydispersion of filtered methacrylate dextran samples compared to unfiltered dextran samples, this phenomenon probably concerns more the shorter polymer chains that react to each other. In addition, we assessed the stability of methacrylate grafted group on dextran chain in solution along one months. The stability was studied at 4°C and the solution was preserved from heat and light source. The analysis did not evidence any free methacrylate chain, confirming the possibility to store the polymer in the described conditions.

We realized the IPN combining the two preformed solution to obtain a final polymer concentration contains 1.14% (w/v) of Si-HPMC and 17.15% (w/v) of DexMA. The IPN formation is based on the possibility of each polymer to form an independent network in presence of each other. The reason why these multicomponent hydrogels have emerged in recent years, is because of the synergistic combination of favourable properties of each polymer network. Rheological analysis of Si-HPMC/DexMA solution confirmed the Si-HPMC crosslinking in presence of DexMA. DexMA polymer was also able to crosslink and the presence of Si-HPMC did not seems to modify the kinetics of photocrosslinking. This phenomenon is probably due to the low number of crosslinking nodes of Si-HPMC after mixing which facilitate the diffusion and reaction of radicals species. Si-HPMC/DexMA initial values of elastic and viscous moduli are higher of one order of magnitude as compared to the once of each polymer solution separately and after

irradiation we reached an elastic moduli of 1500 Pa. In addition, as shown on figure 2, on IPN and DexMA hydrogel, just after photocrosslinking, two strong gels are obtained (G'>>G''), compare to Si-HPMC in which after the cross over (G'>G''), elastic moduli increase progressively but slowly at this concentration.

IPN solution injectability was investigated using a 3 mL syringe with an 18 G needle. Our data demonstrated that combining Si-HPMC with DexMA, the resulting solution is still injectable, as for each polymer solutions and are under the regulatory limit of norm, making them adapt of *in situ* applications. The injectable formulations, able to fill complex defects and quickly form solid membranes upon injection, are particularly appealing owing to their ease of handling.

The synergistic effect obtained by the interpenetration of the two polymer networks improved the physicochemical properties and the dense network could assure the barrier effect against soft tissue invasion and delayed the hydrogel resorbability. Most resorbable membranes, such as polylactic acid or collagen, used until now are characterized by a rapid resorption kinetics after implantation. In fact, these membranes do not ensure, for a period of 8 weeks or more, the regenerating process below the barrier. With the purpose to improve their properties, commercially available membranes are generally made of cross-linked polymers. Indeed, the presence of physical or chemical crosslinking nodes delays the loss of barrier properties[45]. Cross-linked membranes are commercially available to delay the resorption, for example, BioMend® (Zimmer, USA) is made of linear type I bovine collagen, degrades in 6-8 weeks, instead of BioMend Extend® with same composition but cross-linked with formaldehyde which degrades in 18 weeks[46]. Marquez *et al.* reported in a systematic review the comparison between the clinical outcomes of cross-link and linear collagen resorbable membranes in terms of regenerated bone volume and postoperative complications during bone regeneration procedures. The results in bone ingrown were comparable, instead post-operative complications suggested a better results for cross-linked collagen membrane after 4-6 months from surgery[45,47].

Hydrogel are three dimensional network able to retain a large amount of water. These hydrophilic construct are able to facilitate the passage of nutriments assuring the viability of cellular microenvironment, instead

the density of double cross-linked polymer network could block the cell invasion through the hydrogel membrane. In previous experiments, we used Si-HPMC as a self-setting hydrogel for cell encapsulation. The *in vitro* results confirm the ability of Si-HPMC to act as a physical barrier trapping cells inside the hydrogel. In addition, cell viability was demonstrated up to 21 days and diffusion experiments confirm the possibility of nutrient diffusion, essential for cell survival[8,48–50]. Due to the presence of Si-HPMC, the cells cannot cross the hydrogel barrier, maintaining cell viability. These physical barrier properties are also confirmed in several *in vivo* studies[24,25,50]. Si-HPMC was also previously described to form an IPN with calcium-alginate polymer. The addition of calcium-alginate network increased the stiffness of Si-HPMC, while maintained the cell viability of SW1353 cell line cultivated in 2D or encapsulated inside the hydrogel up to 7 days[51]. Dextran hydroxyethyl methacrylate was described in an IPN with calcium-alginate. The polymer mixture increased instantaneously their mechanical properties under UV irradiation and assured the chondrocytes survival encapsulated in IPN hydrogel matrix[35].

In this work the evaluation of cytotoxicity was performed by using neutral red assay on murine fibroblasts according to standard ISO 1993-5. Neutral red uptake test was performed on cells in presence of polymer extract or in presence of polymer solutions/hydrogels. Cell viability tests effectuated did not show a decrease in neutral red uptake compare to the untreated cells. In addition, no effect was evidenced due to the photoinitiator used or the visible light lamp irradiation.

We performed an *in vivo* study to assess the efficacy and biocompatibility of our experimental injectable *in situ* hydrogel membrane in conjunction with BCP. We used commercial collagen solid membrane as a positive control of our experimentation. The importance of membrane in GBR in calvaria defects was already evidenced in literature. The presence of membrane enhanced new bone formation by preventing the infiltration of soft tissues and stabilizing the graft particles during surgical manipulation. [52] Rabbit calvaria defects, treated just with BCP granules, have been shown a low percentage of new bone regeneration of around 10% after 21 days, compare to other anatomical defect such as femur or tibia of 50 and 20% [53]. Hawake *et al.* treated 8-mm defect in rabbit calvaria with BCP granules. After six weeks quantitative

analysis showed new bone formation of 17.2%[54]. Another study described rat calvaria defects treated with human bone morphogenetic protein-2 with or without the addition of collagen membrane. After sacrifice they found a statistically significant lower value of new bone formation in the condition without membrane (26%) compare to that with collagen membrane (46%)[55].

The injectable membrane demonstrated handling properties and to facilitate the control over the shape of the membrane, preventing flowing of the material during the time between application and gelation. After application of DexMA and Si-HPMC/DexMA, the viscous solutions were photocrosslink for 120 s forming solid membrane *in situ.* Through the entire healing period, none of animals exhibited adverse reactions or complications to the presence of the implants. Micro-CT analysis confirmed the presence an equal distribution of BCP in the defects treated with Si-HPMC, DexMA and IPN compare to collagen membrane. Quantitative assessment of mineral deposition showed in Si-HPMC, DexMA and IPN, a mineral deposition comparable to collagen membrane condition of about 33.43% after 8 weeks of healing. Further analysis and quantification are still on going to better understand the healing process and the implication of the membranes. Collagen and hydrogel membranes are not visible by micro-CT analysis, but histological staining (on going) will give further data about the use of the innovative IPN injectable hydrogel membrane for GBR.

5 CONCLUSION

In this work we designed a novel IPN hydrogel membrane composed of a self-setting Si-HPMC and photo-crosslinkable DexMA. The Si-HPMC/DexMA solution can be injected directly *in situ* and upon visible light irradiation, a IPN hydrogel membrane can be quickly obtained. *In situ* IPN hydrogel was compared to a commercial collagen membrane, in a relevant GBR animal model. IPN hydrogel membranes have been demonstrated to facilitate the handling and the application of membrane to cover the defect with BCP and results on bone tissue regeneration are comparable to the conventional product used on clinical practice. The analysis of *in vivo* experiments in calvaria defects in rabbits are still on going. The results will bring further information about the safety, bone regeneration and barrier effect against soft tissue for GBR.

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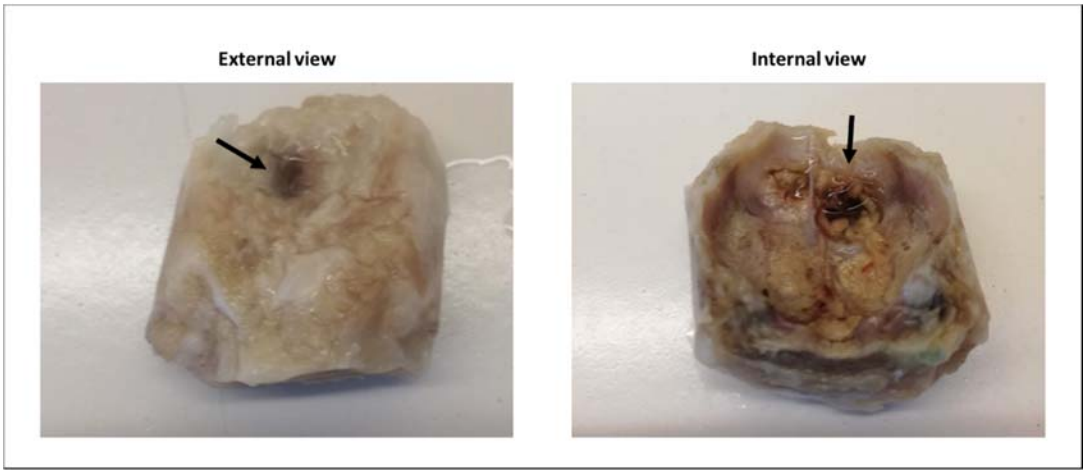
Appendix A. Supplementary Data



S. 1 – Picture of calvaria sample after 8 weeks from surgery. Left: external view of calvaria, right: internal view of calvaria. From internal picture (right) clockwise from top left: IPN, empty defect, control collagen membrane, DexMA. Arrows indicate the clot formation in the empty defect. DexMA: dextran methacrylate, IPN: interpenetrating polymer network.

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Discussion

In the framework of POsTURE project, an innovative periodontal regeneration will be developed based on: (i) a photocrosslinked interpenetrating polymer network as a membrane and (ii) a self-setting injectable bone grafting material containing Sr, Mg or Si substituted CaP nanoparticles with enhanced bioactivity. Hence, the second research axe conduct experiment in parallel to design a bone substitute material loaded with CaP. In particular, cross-linked Si-HPMC at low concentration was used as a 3D matrix to promotes the cohesion between the granules, ensuring homogeneity of the suspension and acting as a barrier to the outside of the bone defect. CaP nanoparticles containing Sr, Mg or Si were prepared in Riga Technical University. After *in vitro* experimentation and preliminary studies *in vivo*, the selected bone substitute formulation could be coupled with the photocrosslinkable membrane to validate the proof of concept of the novel medical device described in the project. At this moment, the *in vivo* experiments on CaP nanoparticles are on going. The described work corresponds to the second point of the project.

The objective of this work was the development of a IPN composed of Si-HPMC and DexMA. In addition, the suitability as hydrogel membrane for GBR was studied.

In the first part of the article, we synthetized the polymer and we focused on selected and characterized the Si-HPMC/DexMA formulations. We synthetized dextran methacrylate with 40, 100 and 100 kDa of molecular mass and we used vitamin B2 as a photoinitiators and a standard dentist's polymerization lamp to cure the hydrogel membrane. We selected an appropriate IPN formulation of Si-HPMC/DexMA. Rheological study showed that Si-HPMC and DexMA was able to crosslink in presence of the other polymer and the crosslinking time was reduced to 120 s of lamp irradiation compare to 15 min necessary to reach the gel point of Si-HPMC. The Si-HPMC/DexMA solution was demonstrated to be injectable through a syringe equipped with an 18 G needle. The *in vitro* experiments with neutral red uptake confirmed the cytocompatibility of the materials used.

In the second part of the article, we described the in vivo experiment. We compared the *in situ* obtained IPN hydrogel to a commercial collagen membrane, in a relevant GBR animal model. Calvaria critical size

defects (⌀ = 8 mm) in rabbits were filled with synthetic biphasic calcium phosphates bone graft in conjunction with experimental membranes to evaluate the biocompatibility and the efficacy for GBR technique. The synergistic effect obtained by the interpenetration of the two polymer networks could improve the physicochemical properties and the dense network of IPN ensures the barrier effect against soft tissue invasion and delayed the hydrogel resorbability. The injectable membrane demonstrated handling properties and to facilitate the control over the shape of the membrane, preventing flowing of the material during the time between application and gelation.

Quantitative analysis of newly formed bone were performed using micro-computed tomography system. Quantitative assessment of mineral deposition showed in the IPN test group a new bone formation comparable to the collagen membrane condition. Histological analysis and quantification are still on going to determine the morphology and quantification of mineral and soft tissue into the defects.

According with the results *in vivo* of IPN membrane, different parameters on polymer formulation could be modify to tune the IPN hydrogel membrane degradation. For example, a decrease in the degradation time, could be obtained by incorporating the dextranase enzyme into the system: by regulating the concentration of this enzyme within the hydrogel, the rate of degradation can be modulated. Another method to modify the degradation time is grafting on dextran backbone hydroxyethyl-methacrylate, instead of methacrylated groups. In fact, containing hydrolysable esters increase the biodegradability of the hydrogel network.

Also the degree of methacrylation (%MA) could have an impact on membrane degradation. In preliminary study, we analyzed the DexMA 40kDa with %MA of 10 and 20%. The hydrogel at 5% (w/v) was irradiated by UV lamp for 15 minutes. After reaction the hydrogel were treated with a solution of dextranase (Chaetomium erraticum, Sigma). DexMA with %MA of 10% was degraded after 2.5 h, instead of DexMA 20% appeared stable during the observation time, demonstrating a different accessibility of enzyme within the 3D network.

In conclusion, we designed a novel IPN hydrogel membrane composed of a self-setting Si-HPMC and photocrosslinkable dextran. The Si-HPMC/DexMA solution can be injected directly *in situ* and upon visible light irradiation, a hydrogel membrane can be quickly obtained to use as a GBR membrane.

The analysis of *in vivo* experiments in calvaria defects in rabbits are still on going. The results will bring further information about the safety, bone regeneration and barrier effect against soft tissue for GBR.

Chapter 3 : Interpenetrating polymer network hydrogel membrane of silanized hydroxypropylmethyl cellulose/methacrylated carboxymethyl chitosan

Rational of the study

In the first part of our research we developed a novel *in situ* IPN hydrogel membrane for the treatment GBR. The *in vivo* studies about this promising material are still on going. In this research, modified chitosan, namely carboxymethyl chitosan (CMCS), was used as polymer precursor for the synthesis of hydrogels. Indeed, this biocompatible and biodegradable polysaccharide, derivate from natural chitin, is water soluble at neutral pH[157]. Furthermore, to use chitosan instead of dextran could produce some interesting properties such as antibacterial activity and fast degradation. In addition, chitosan due to its ionic moieties can be involved in ionic links with biological apatite of cement or dentine for adhesive properties. For these reasons, we decide to study in parallel a second IPN formulation composed of Si-HPMC for its barrier effect and manageability, but with a methacrylated CMCS.

3.1 Chitosan

Chitosan is a biocompatible, biodegradable, linear polysaccharide composed of N-acetyl glucosamine and glucosamine units. Chitosan is a derivative of natural chitin, the second most abundant polysaccharide in nature after cellulose. Typically, chitosan is obtained by deacetylation of the N-acetyl glucosamine units of chitin by hydrolysis under alkali conditions at high temperature (degree of acetylation>60 mol%). In nature, chitin is present particularly in insects and crustaceans where it represents the major component of their exoskeleton or in cell wall of some mushrooms. Despite the fact that chitosan is a unique and versatile compound, there are hardly any available pharmaceutical products based on chitosan (only hemostatic dressings, preparations for wound-healing and nutraceutical products exist). This might be a result of the strong fact that chitosan material extracted from various sources differs significantly in terms of its purity level. This limitations, could be overcome using chitosan produced from mushrooms, as a non-animal source, is considered to be safer for biomedical and healthcare uses[78,79].

Chitosan has been presented in literature for a number of biomedical and pharmaceutical applications, including drug delivery systems [158–160], wound dressings [161], blood anticoagulants [75],

cartilage and bone tissue engineering scaffolds [162,163], and space filling implants [164]. The polymer is also considered as a promising candidate in obesity and hypercholesterolemia treatment as it is able to combine bile acids in the digestive tract and in consequence increase their excretion [165]. Numerous data have drawn attention to the use of chitosan as an antifungal and antibacterial agent [16,166]. Chitosan possesses good mucoadhesive properties resulting from the cationic behavior and the presence of free hydroxyl and amino groups allowing the polymer to interact with mucin by hydrogen and electrostatic bonding[167,168].

Chitosan undergoes enzymatic degradation to non-toxic components. Chitosan is degraded *in vivo* by several enzymes, mainly by lysozyme, which is a non-specific enzyme present in all mammalian tissues, producing non-toxic oligosaccharides which can be then excreted or incorporated to glycosoaminoglycans and glycoproteins. The molecular weight, polydispersity, deacetylation degree, purity level and moisture content play a crucial role in determining the mechanism and the speed of polymer degradation. Regardless of the mode of degradation, the process usually begins with random splitting of β-1,4-glycosidic bonds (depolymerization) followed by N-acetyl linkage (deacetylation). As a consequence, a decrease in average molecular weight and an increase in deacetylation degree are observed. Simultaneously with chitosan chain scission, cleavage and/or destruction of its functional groups (amino, carbonyl, amido, and hydroxyl) occur[75].

The main drawback of chitosan for the use as injectable ready-to-use products, is their poor solubility in water, which is a major disadvantage for biomedical formulations. Indeed, chitosan is only soluble in acidic solutions of pH below 6.5, required to insure the protonation of the primary amine. For that, many products with chemical modification of chitosan backbone are available today. Chitosan chains possess three attractive reactive sites for chemical modification: two hydroxyl groups (primary or secondary) and one primary amine. The site of modification is dictated by the desired application of the final chitosan derivative[78,79].

Carboxymethyl chitosan represents a water soluble chitosan derivatives. The carboxymethylation can occur at N, or O or both the atoms. Compared with other water-soluble chitosan derivatives, carboxymethyl chitosan (CMCS) has been widely studied because of its ease of synthesis, ampholytic character and possibilities of ample of applications[168–171]. It is described in literature for drug delivery and tissue engineering. In addition, can be further modified to get derivatives as amphiphilic, quaternized, or grafted by acylation, alkylation or grafting[169].

Second article: *in situ* photochemical crosslinking of hydrogel membrane for Guided Tissue Regeneration

In this project we used carboxymethyl chitosan as a started product to randomly insert methacrylate moieties to form an IPN hydrogel membrane with Si-HPMC for GTR in periodontal defects.

The results obtained will be described in the article **In situ photochemical crosslinking of hydrogel membrane for Guided Tissue Regeneration.** *Pauline Marie Chichiricco', Raphael Riva, Jean-Michel Thomassin, Julie Lesoeur, Xavier Struillou, Catherine Le Visage, Christine Jérôme and Pierre Weiss.* This article is in press in Dental Materials.

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In situ photochemical crosslinking of hydrogel membrane for Guided Tissue Regeneration

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ABSTRACT

Objective. Periodontitis is an inflammatory disease that destroys the tooth-supporting attachment apparatus. Guided tissue regeneration (GTR) is a technique based on a barrier membrane designed to prevent wound space colonization by gingival cells. This study examined a new formulation composed of two polymers that could be photochemically cross-linked *in situ* into an interpenetrated polymer network (IPN) forming a hydrogel membrane.

Methods. We synthetized and characterized silanized hydroxypropyl methylcellulose (Si-HPMC) for its cell barrier properties and methacrylated carboxymethyl chitosan (MA-CMCS) for its degradable backbone to use in IPN. Hydrogel membranes were cross-linked using riboflavin photoinitiator and a dentistry visible light lamp. The biomaterial's physicochemical and mechanical properties were determined. Hydrogel membrane degradation was evaluated in lysozyme. Cytocompatibility was estimated by neutral red uptake. The cell barrier property was studied culturing human primary gingival fibroblasts or human gingival explants on membrane and analyzed with confocal microscopy and histological staining.

Results. The IPN hydrogel membrane was obtained after 120 s of irradiation. The IPN showed a synergistic increase in Young moduli compared with the single networks. The CMCS addition in IPN allows a progressive weight loss compared to each polymer network. Cytocompatibility was confirmed by neutral red assay. Human cell invasion was prevented by hydrogel membranes and histological sections revealed that the biomaterial exhibited a barrier effect in contact with soft gingival tissue.

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Significance. We demonstrated the ability of an innovative polymer formulation to form *in situ*, using a dentist's lamp, an IPN hydrogel membrane, which could be an easy-to-use biomaterial for GTR therapy.

1. Introduction

Oral diseases, including dental caries and periodontitis, are among the most important global health burdens, affecting the majority of school-aged children and adults worldwide. The prevalence of periodontitis is reported to be between 20 and 50% of the worldwide population [1], with reported relationships between periodontitis and systemic diseases such as cardiovascular disease [2,3]. In Europe, the more severe forms of periodontitis affect 10% of the population [4].

Periodontitis is an inflammatory disease resulting from the presence of oral bacteria biofilm in the periodontal tissue, which leads to an immune-inflammatory response and destroys the tooth-supporting attachment apparatus [5]. The inflammation, if untreated, can spread to the whole gum and all periodontal tissues leading to the destruction of periodontal ligament and loss of the supporting tooth bone with ultimately the risk of spontaneous avulsion of the tooth [6]. For more severe cases, the dentist or periodontist generally selects a surgical approach that allows the elimination of periodontal pockets and the partial regeneration of the lost tissues with the use of various biomaterials. Several regenerative procedures have already been introduced to clinical practice to overcome these problems, including bone grafts, guided tissue regeneration (GTR), enamel matrix derivative and combined techniques [7].

GTR is a dental surgical procedure to regenerate lost components of periodontium. In this technique a biocompatible membrane is implanted around the periodontal lesion in order to prevent its colonization by soft tissues presenting a faster proliferation rate compared to the bone and ligament cells. In fact, during normal healing, it appears that the soft tissue migrates rapidly into the wound, avoiding tissue regeneration. The barrier membrane plays a key role in preventing undesirable tissue migration into the defective area, and consequently, it allows sufficient time for bone, cementum, and periodontal ligament regeneration.

For this purpose, the membrane, whether or not it is resorbable, must be biocompatible to prevent inflammatory processes and must present a selective permeability allowing the diffusion of nutrients without the passage of cells with an appropriated flexibility, compatible with the anatomical implantation. Among the nonresorbable membranes, polytetrafluoroethylene (e.g., Cytoplast® TXT-200, Osteogenics Biomedical) –based membranes are widely used, although a second surgery for their removal after use is mandatory. These nonresorbable membranes present high postoperative morbidity. To overcome this limitation, resorbable membranes, made from biodegradable materials, are largely studied to obviate the need for a second surgery and thus reduce complications (e.g., Bio-Gide®, Osteohealth) [7]. Nevertheless, the main drawback of resorbable membranes is their poor predictability in terms of resorption time largely influenced by the patient's characteristics. Generally, these membranes are made of polylactic acid, polyglycolic, polyurethane, collagen type I, etc. [8,9]. Most resorbable membranes used to date are characterized by rapid absorption kinetics after implantation. In fact, these membranes do not ensure, for a period of 8 weeks or more, the regenerating process below the barrier. To improve their properties, commercially available membranes are generally made of a cross-linked polymer given that the presence of physical or chemical crosslinking nodes delays the loss of barrier properties [8]. In addition, it should be taken into account that all the membranes now available are in a solid form, requiring skill and experience to be perfectly applied in narrow defects [10].

For complicated shapes or for a defect that is difficult to reach, liquid formulations, able to form solid membrane *in situ*, are easy-to-use materials that save time and money. To our knowledge, two kinds of free-flow membranes have been commercialized: Membragel® (Straumann, Austria) and Atrisorb® (Tolmar, USA), but they are no longer available according to the manufacturers [7]. The first one is composed of multi-arm PEG with thiol end-groups and acrylate end-groups that react forming a hydrogel membrane [11]. The second one is composed of poly(DL-lactide) (PLA) dissolved in *N*-methyl-2-pyrrolidone (NMP) [12].

Si-HPMC is a self-setting hydrogel that has been reported in the literature for many biological applications. It has the advantage of being injected as a viscous solution and then, due to the condensation reaction, it builds a 3D network *in situ*. This material was demonstrated to be biocompatible and slowly degraded in a rabbit model [13–16]. In addition, Struillou et al. demonstrated the capability of the cross-linked biomaterial to act as a physical barrier against cell invasion [17]. The main drawback of this self-setting hydrogel is an excessively slow crosslinking process for clinical needs.

We have developed an original mixture of biomaterials that could be used as a liquid formulation, a precursor of a resorbable interpenetrated polymer network hydrogel membrane formed by *in situ* curing under irradiation with a dentist's lamp. Indeed, photo-curing appears to be the most appropriate technique for this application given both its shape and the control of the curing time, and it has been reported in the literature for tissue engineering, cell encapsulation and drug delivery [18–21].

For several decades, interpenetrating polymer networks have been widely used because of the synergistic combination of each of the polymer networks [20,22,23]. Our membrane is composed of a Si-HPMC network, selected for its appropriate barrier effect against soft tissue invasion, which is interpenetrated in a methacrylated carboxymethyl chitosan network

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Table 1 – Macromolecular parameters of the polysaccharide precursors.

Carboxymethyl chitosan (HMC + Heppe Medical Chitosan GmbH)	Degree of deacetylation 94.2% Viscosity (1% water 20 °C) 22 mPas Molecular weight (from GPC) 30–500 kDa
Hydroxypropyl methylcellulose Methocel E4M (Colorcon)	Viscosity (2% water 20 °C) 3.023 mPas Methoxyl groups 28.2% Hydroxypropyl groups 9.3%

(MA-CMCS). Carboxymethyl chitosan allows the modification of membrane degradation due to its degradable backbone. This biocompatible and biodegradable polysaccharide, derived from natural chitin, is water soluble at neutral pH and presents antibacterial properties [24,25]. To allow photocrosslinking, methacrylate functions were randomly introduced along the CMCS backbone to provide the gelation under visible light irradiation using a photoinitiator solution (PIS) based on vitamin B2 [26–28]. Vitamin B2, or riboflavin, is a water-soluble vitamin widely present in both animal- and plant-derived foods [20]. In particular, we used its highly water-soluble riboflavin 5′-phosphate sodium salt hydrate derivative (RP); since it is a type II photoinitiator, it requires the addition of triethanolamine as a coinitiator.

The aim of this study was to associate the Si-HPMC and MA-CMCS polymers in an injectable viscous solution able to quickly gel under light irradiation into a biocompatible and resorbable hydrogel membrane for GTR.

This new formulation was analyzed from a chemical and rheological point of view. The cell viability and barrier membrane effect were evaluated with soft tissue cells and *ex vivo* gingiva cultures.

2. Materials and methods

2.1. Materials

Carboxymethyl chitosan (CMCS) was purchased from Heppe Medical Chitosan GmbH (Halle, Germany). Riboflavin 5′-phosphate sodium salt hydrate (RP), triethanolamine (TEOHA), glycidyl methacrylate, dimethylsulfoxide (DMSO), HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid), lysozyme (from chicken egg whites), RNAse, paraformaldehyde and neutral red were all obtained from Sigma Aldrich (Saint Louis, MO, USA). Hydroxypropyl methylcellulose (HPMC) was provided by Colorcon-Dow Chemical (Harleysville, PA, USA). Fetal bovine serum (FBS) was acquired from PAN biotech GmbH (Aidenbach, Germany). Penicillin, streptomycin, trypsin-EDTA, DMEM and phosphate buffered saline (PBS) were obtained from Gibco (Invitrogen, Carlsbad, CA, USA) and L929 cells from ATCC (Manassas, VA, USA). YOYO® -1 iodide (491/509) and Alexa Fluor 568 phalloidin were purchased from Thermo Fisher (Waltham, MA, USA). Harris Hematoxylin and Eosin Y were obtained from Surgipath (Richmond, IL, USA) and Safranin from VWR (Radnor, PA, USA) The macromolecule parameters of CMCS and HPMC are listed in the Table 1.

2.2. Methods

2.2.1. Si-HPMC synthesis

Si-HPMC was synthesized by grafting 3-glycidoxypropyltrimethoxysilane to HPMC as described elsewhere [30,31]. Then Si-HPMC powder was dissolved in 0.1 M NaOH overnight and sterilized by autoclave (121 °C for 20 min) as previously described [32].

2.2.2. MA-CMCS synthesis

Ten grams of CMCS was dissolved in 500 mL of Milli-Q water under magnetic stirring and the pH was adjusted to 9 with a 0.1 M NaOH solution. A total of 2.72 mL of glycidyl methacrylate was then added to target a 40 mol% percentage of methacrylation. After 48 h of stirring at room temperature under nitrogen atmosphere, the reaction mixture was dialyzed against distilled water (porosity of the dialysis membrane MWCO, 1 kDa, Spectrapor®) until the conductivity was less than 2 μS/cm before being freeze-dried for 3 days.

2.2.3. FT-IR

Infrared spectroscopy spectra were recorded with a Thermo Fisher Scientific Nicolet IS5 with module ATR ID5 in Germanium (700 cm^{-1}–4000 cm^{-1}). CMCS and MA-CMCS were analyzed in their lyophilized form.

2.2.4. ^1H NMR

The substitution degree was determined by ^1H NMR 400 MHz at 80 °C (Bruker). The polysaccharide samples were solubilized in deuterium oxide at a concentration of 14 mg/mL. To calculate the grafting percentage, formula 1 was used:

$$\%MA = \frac{(DA/100) \times \int CH3MA}{\int CH3DA} \times 100 \quad (1)$$

where DA is the degree of residual acetylation on the chitosan backbone, CH_{3MA} is the integral of the methyl group of the methacrylate moiety (δ = 2.5 ppm) and CH_{3DA} is the integral of the methyl group of the acetyl group in carboxymethyl chitosan (δ = 2.6 ppm).

2.2.5. Liquid formulation preparation

IPN precursor solution was prepared in a four-step procedure: (1) Si-HPMC solution preparation, (2) acid buffer preparation, (3) MA-CMCS solution preparation and (4) a mixing step to obtain the Si-HPMC/MA-CMCS solution. The main steps are described as follows:

(1) Si-HPMC was dissolved in 0.1 M NaOH at 4% (w/v).
(2) Acid buffer was prepared with 0.06 M HCl, 1.8% (w/v) NaCl and 6.2% (w/v) HEPES.
(3) MA-CMCS was dissolved in distilled water at 5% (w/v). To allow the crosslinking under irradiation, 5 μL of PIS was added to the MA-CMCS per each final milliliter of total solution (stock solution of photoinitiator composed of RP 4.2 mM and 4.2 M of TEOHA [27]).
(4) MA-CMCS, Si-HPMC and acid buffer were successively mixed in a 4:2:1 ratio in volume to obtain a 4% (w/v) solution.

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To allow the correct comparison within different samples, Si-HPMC and MA-CMCS solutions were prepared at the same final concentration present in Si-HPMC/MA-CMCS formulation (1.14% (w/v) and 2.86% (w/v)).

2.2.6. *Hydrogel preparation*

The Si-HPMC/MA-CMCS and MA-CMCS solutions were poured into a Teflon mold and irradiated for 120 s from one side by means of a BA-Optima 10 dentistry lamp curing light 1200 mw/cm^2, 420–480 nm (B.A. International, Northampton, UK). Si-HPMC was mixed with acid buffer, previously described, and transferred to a mold. We used a Teflon mold measuring 25 mm in diameter and 4 mm high for rheological/mechanical analysis and 5 mm in diameter and 2 mm high for the *in vitro* test. The ratio chosen between the two polymers to compose the interpenetrating polymer network was previously assessed testing different ratios of polymer and photoinitiator. The formulation selected was the only one that provided the correct crosslink of MA-CMCS in a short time, even in presence of Si-HPMC. Si-HPMC is a self-setting hydrogel, which, due to the condensation reaction, builds a 3D network. Struillou et al. demonstrated the capability of this biomaterial to act as a physical barrier against cell invasion when it is fully cross-linked [17]. The main drawback of this self-setting hydrogel, for this application, is an overly slow crosslinking time. For the *in vitro* study, so that Si-HPMC could be compared with the other hydrogels, we used Si-HPMC cross-linked for 4 days in a mold. To maintain the same conditions for all samples, MA-CMCS and Si-HPMC/MA-CMCS were prepared as described previously and preserved 4 days before the *in vitro* tests.

2.2.7. *Rheology*

Rheological measurements were taken on an ARES G2 rheometer (TA Instruments). The data were collected using the TRIOS software. Time-sweep experiments were conducted (1% strain, 6.28 rad/s) using a parallel plate geometry (20 mm in diameter) with an upper transparent quartz plate, allowing the material to be irradiated with a lamp to follow the gelation (λ = 450 nm, 430 mW, 350 mA, royal blue).

2.2.8. *Compression test*

The hydrogel stiffness was obtained from uniaxial static compression. Mechanical compression was performed using Texture Analyzer (TA HD plus) with a 25 mm cylindrical aluminum probe run at 0.1 mm/s. The stress-strain curves were recorded and the compression modulus was derived as the slope divided by the corresponding cross-section of the hydrogel sample.

2.2.9. *Enzyme degradation*

Hydrogel membrane degradation was investigated by incubating the hydrogels in 5 mL of PBS buffer containing lysozyme from chicken white eggs (10 mg/mL) at 37 °C. The solvent was replaced twice a week. The samples were weighed at different time points, eliminating excess solvent. The weight loss, reported as a ratio of final over initial weight percent (W/W_0)%, was followed over a period of 24 days. Hydrogels pictures were taken at every time point. All the experiments were conducted in triplicate.

2.2.10. *In vitro cell viability*

To evaluate cytocompatibility, a neutral red uptake assay was carried out with the exposure of murine fibroblasts (L929, ATCC, VA, USA) to (1) PIS, (2) MA-CMCS extract and (3) Si-HPMC, Si-HPMC/MA-CMCS and MA-CMCS hydrogel.

The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 mg/mL glucose, 10% fetal bovine serum and 1% penicillin–streptomycin. Cells were detached by 0.2% trypsin and seeded 16,000 cells/mm^2 in a 96-well Plate 24 h before every test.

To perform the neutral red uptake assay, a solution was prepared 24 h before the test with 0.04 mg/mL of neutral red in PBS and incubated at 37 °C. Before being added to cells, the solution was centrifuged to remove any crystals. One hundred microliters of neutral red solution was added to cells and incubated for 3 h. After incubation, the solution was removed and a destain solution (50% ethanol 96%, 49% deionized water, 1% glacial acetic acid) was used to solubilize the dye trapped in living cells. The optical density was read at 550 nm using a microplate reader (Victor3 V, PerkinElmer, Wellesley, MA, USA). The averages of optical density units were calculated after blank subtraction.

All the tests were performed in triplicate. The results in neutral red uptake were expressed as the percentage of the ratio between the optical density of the experimental condition and the optical density of the control well, named untreated cells.

2.2.10.1. Photoinitiator solution. A PIS of 4.2 mM RP and 4.2 M of TEOHA [27] was prepared and HCl was added to neutralize the pH solution as in polymer liquid formulation [33]. In fact, although we analyzed only the impact of triethanolamine, as expected the unbalanced pH strongly impacted cell viability (data not shown). The PIS was added to culture medium at the same concentration as required for the polymer photocrosslinking (5 μL/mL). To examine the effect of irradiation, visible light curing was used to expose cells containing or not containing the PIS for 120 s. Any plates not exposed at a given time were carefully covered to prevent any unregulated light exposure. After cell exposure to visible light, all the plates were returned to the 37 °C incubator for 24 h. The control well consisted of cells without PIS and not irradiated. After 24 h, the medium was removed, the cells rinsed with sterile PBS and neutral red solution added to each well plate to perform the assay.

2.2.10.2. MA-CMCS extract. MA-CMCS at 2% (w/v) was inserted in the dialysis tube (porosity of the dialysis membrane MWCO = 1 kDA, Spectrapor®). The tube was introduced in a Falcon tube with 10 mL of distilled water under stirring at room temperature for 72 h. The extract, containing the polymer leach-out product, was sterilized on a 0.22-μm filter and used for testing the effect of leach-out products on cell viability. The 96-well plates, incubated 24 h before with L929 cells, were recovered from the incubator. The culture medium was discarded and replaced with medium containing 10% (v/v) polymer extract. The control well consisted in cells with culture medium without polymer extract. To examine the effect of irradiation, visible light curing was used to expose cells containing or not containing polymer extract. After 1 and 3 days

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of incubation, the medium was removed, the cells rinsed with sterile PBS and neutral red solution added to each well plate to perform the assay.

2.2.10.3. Si-HPMC, MA-CMCS, Si-HPMC/MA-CMCS. Si-HPMC, Si-HPMC/MA-CMCS and MA-CMCS hydrogels were prepared as described in the Section 2.2.6. The 96-well plates, incubated 24 h before with L929 cells, were recovered from the incubator. The cell medium was removed and hydrogels were transferred in each well and successively covered with 100 μL culture medium. The control well consisted in cells with 100 μL culture medium without hydrogel. After 1 and 3 days, the hydrogels and the culture medium were removed, the cells rinsed with sterile PBS and neutral red solution added to each well plate to perform the assay.

2.2.11. *In vitro barrier effect*

Primary cultures of human gingival fibroblasts (HGFs) were used to prove the physical barrier role of this hydrogel membrane. In collaboration with Nantes University Hospital (CHU Nantes), with written informed consent, healthy human gingival tissue was obtained from patients and HGFs were isolated as described in Dreno et al. [34].

Si-HPMC, MA-CMCS and Si-HPMC/MA-CMCS were prepared according to Section 2.2.6 in a 5-mm-diameter Teflon mold 2 mm in height. The hydrogels were preincubated in DMEM culture medium in a 24-well plate for 24 h. After incubation the medium was removed and the hydrogels were ready for cell seeding.

HGFs were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 mg/mL glucose, 10% fetal bovine serum and 1% penicillin–streptomycin. The cells were used between passage 2 and 8. They were seeded on the top of the Si-HPMC, MA-CMCS and Si-HPMC/MA-CMCS hydrogel with 14 μL of cell suspension (40,000 cells/hydrogel) [35]. After seeded hydrogels, cell medium was gently added at the bottom of the hydrogels and the samples were maintained in culture for 4 days [4]. As a control, a porous pullulan/dextran hydrogel was used [36]. After that the samples were fixed using PFA 4%, permeabilized with a 0.1% triton X-100 solution and treated with an RNAse solution prior to staining with the label nuclear Yoyo-1 and actin staining phalloidin-Alexa 568, to visualize cell presence and morphology. The samples were then observed using confocal microscopy using 488-nm and 543-nm laser (Eclipse TE2000-E-Nikon). Samples were carefully transferred from the well plate to a glass slide for observation of the hydrogel surface. Z-stacks from the top of the hydrogel surface to a 300-μm thickness were obtained with × 40 magnification; 2D images at the hydrogel surface were taken with a × 60 zoom.

2.2.12. *Ex vivo model*

Human gingival explant was obtained from a healthy patient undergoing dental surgery. The explant was rinsed three times in PBS with penicillin–streptomycin (2%) and divided into four samples. The explants were placed at the top of a porous pullulan/dextran hydrogel, Si-HMPC hydrogel, MA-CMCS hydrogel and Si-HMPC/MA-CMCS hydrogel membrane using a Teflon cylinder to maintain the contact for 1 week in 12-well plates and incubated with DMEM with 4.5 mg/mL glucose, 20% fetal bovine serum, 1% penicillin–streptomycin and 1% Fungizone. After culture, the explant/hydrogel samples were fixed with

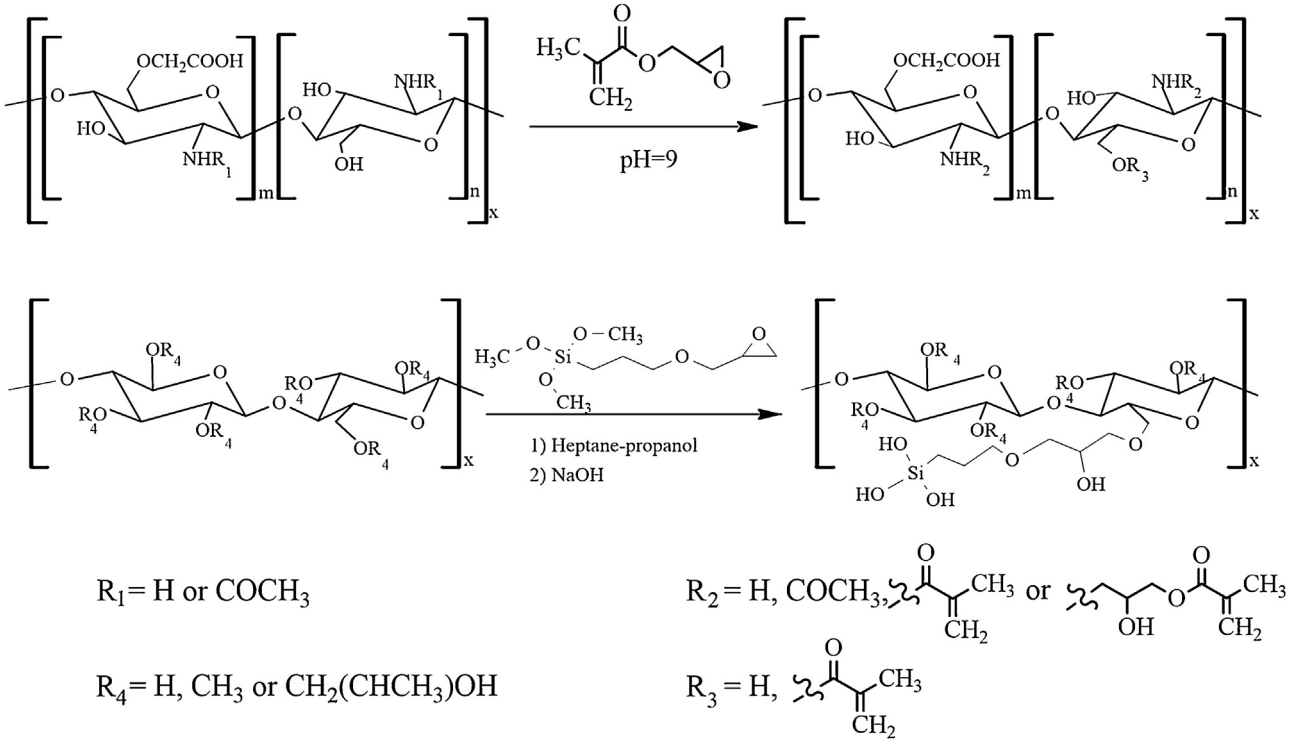

Fig. 1 – Synthesis diagram of methacrylated carboxymethyl chitosan (MA-CMCS) and silanized hydroxypropyl methylcellulose (Si-HPMC).

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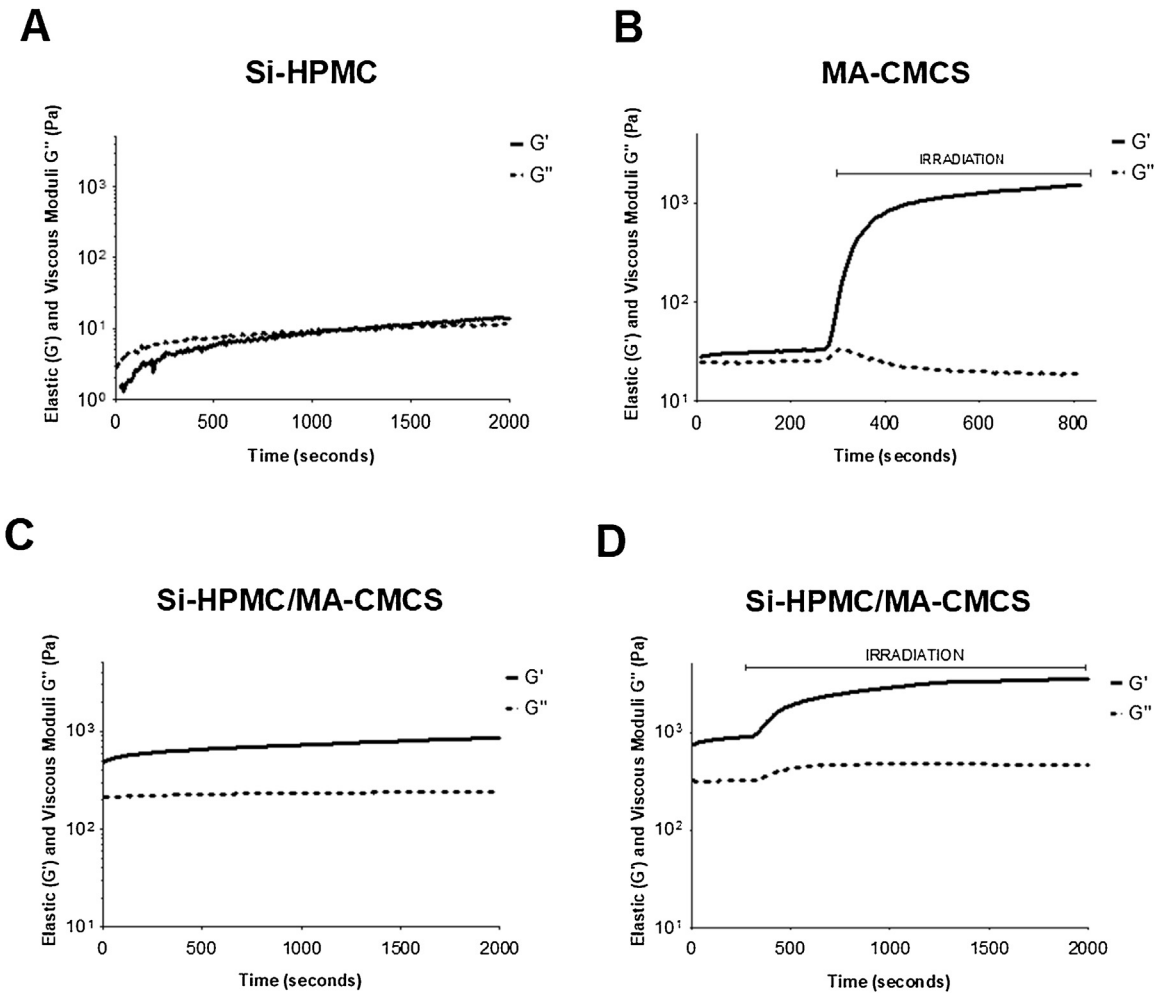

Fig. 2 – Variation of elastic and viscous moduli of polymer solutions as a function of time (seconds) of (A) Si-HPMC without irradiation, (B) MA-CMCS with irradiation, (C) Si-HPMC/MA-CMCS without irradiation and (D) Si-HPMC/MA-CMCS with irradiation. Data recorded at 6.8 rad/s frequency, 1% strain, room temperature; the lamp used for irradiation with λ = 450 nm, 430 mW, 350 mA. Si-HPMC: silanized hydroxypropyl methylcellulose, MA-CMCS: methacrylated carboxymethyl chitosan, Si-HPMC/MA-CMCS: silanized hydroxypropyl methylcellulose/methacrylated carboxymethyl chitosan.

4% PFA for 1 h, embedded in 30% sucrose solution and in OCT (optimal cutting temperature compound). Every sample was then frozen in isopentane with liquid nitrogen. After cryosectioning, the frozen slices were stained using hematoxylin, eosin Y and safranin.

2.2.13. *Statistical analysis*

GraphPad 6 was used to perform statistical analysis on cytocompatibility tests using the two-way ANOVA Bonferroni test and on rheological-mechanical tests with a one-way ANOVA post Tukey test.

3. Results

3.1. *Chemical characterization*

In this study we developed an interpenetrating polymer network to make an *in situ* photocrosslinkable hydrogel membrane. Previous work demonstrated the good cell barrier effect of Si-HPMC; however, certain characteristics limit the use for this application. The addition of MA-CMCS combines its favorable properties with the Si-HPMC properties, such as photosensitivity and a degradable backbone.

The CMCS was modified as described in Fig. 1 to introduce photocrosslinkable moieties, i.e., methacrylic groups, providing phototriggered crosslinking ability to the material. FTIR and ^{1}H NMR confirmed the grafting of methacrylic pendant groups onto CMCS backbone. On the infrared spectrum, the peak of methacrylate carbonyl group appears at 1723 cm^{-1}. After reaction with glycidyl methacrylate, three new signals also appear on the ^{1}H NMR spectra, compared to the spectrum of the starting CMCS, corresponding to the CH3 at 2.5 ppm and 6.3 ppm, and the 6.75 ppm peaks of double-bond protons of the grafted methacrylate groups. The integration of peaks compared to the 2.6 ppm peak of the methyl group of the acetyl group in carboxymethyl chitosan confirms a

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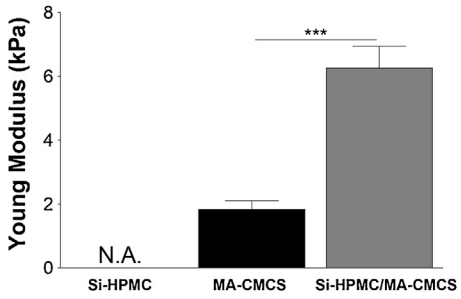

Fig. 3 – Mechanical characterization of photochemically cross-linked hydrogel (120 s with a visible light lamp). Young modulus obtained in compression experiment for Si-HPMC, MA-CMCS and Si-HPMC/MA-CMCS after photocrosslinking (mean value ±SEM, n = 3). Statistical analysis was performed with one-way ANOVA with post-Tukey test (*p < 0.001). Si-HPMC: silanized hydroxypropyl methylcellulose, MA-CMCS: methacrylated carboxymethyl chitosan, Si-HPMC/MA-CMCS: silanized hydroxypropyl methylcellulose/methacrylated carboxymethyl chitosan.**

grafting density around 35% (Fig. S1 in the online version, at DOI:10.1016/j.dental.2018.09.017).

3.2. *Rheological and mechanical properties*

Fig. 2 shows the elastic and viscous moduli as a function of time (seconds) of four different systems: Si-HPMC (1.14% (w/v) without irradiation), MA-CMCS (2.86% (w/v) with irradiation after 300 s), Si-HPMC/MA-CMCS (1.14%/2.86% (w/v) without irradiation) and Si-HPMC/MA-CMCS (1.14%/2.86% (w/v) with irradiation after 300 s). As expected, the Si-HPMC sample (Fig. 2A) shows a gradual increase of both moduli with time as a consequence of the crosslinking by silanol condensation. This increase of moduli is relatively slow with a crossover of the moduli only achieved after 15 min. In the MA-CMCS sample (Fig. 2B) the elastic modulus is already higher than the viscous modulus at the beginning of the measurement, with a small difference between them, suggesting the existence of physical interactions between the MA-CMCS chains. The moduli remain constant without irradiation (0–300 s) showing that no reaction occurs in these conditions. Interestingly, a sharp and immediate increase of the elastic modulus is observed with stabilization around 10^3 Pa after less than 100–200 s. These results confirm the potential of MA-CMCS to quickly cross-link upon irradiation. To impart this potential to the Si-HMPC membranes, MA-CMCS has been added to reach a Si-HMPC/MA-CMCS 1.14%/2.86% (w/v) ratio. This ratio was chosen after preliminary results that showed that the smaller amount of MA-CMCS does not efficiently cross-link the membrane upon irradiation. At the beginning of the measurement, the moduli were significantly higher than those observed with the individual component demonstrating that interactions occur between both components. Without irradiation (Fig. 2C), a small increase of the moduli is observed due to the silanol condensation of Si-HMPC. When the irradiation starts (after 300 s in Fig. 2D), a sharp and immediate increase of the moduli is observed to reach a plateau around 10^3–10^4 Pa. The stability of the MA-CMCS and Si-HPMC/MA-CMCS hydrogels was confirmed by frequency sweep measurement after 120 s of irradiation (Fig. S2 in the online version, at DOI:10.1016/j.dental.2018.09.017). A plateau-like behavior is observed in both cases with no decrease of the storage modulus at low frequencies.

Mechanical compression tests were performed on the different samples after 120 s upon irradiation. As expected Si-HPMC was not sufficiently cross-linked after 120 s to perform this analysis, confirming that the second polymer network was necessary to obtain a liquid-to-solid material with a fast gelification time. The Si-HPMC/MA-CMCS (1.14%/2.86% (w/v)) samples show a significantly higher compression modulus than the MA-CMCS (2.86% (w/v)) material, suggesting that the double network improves the mechanical properties of the hydrogel (Fig. 3).

3.3. *Enzyme degradation*

Hydrogel membrane degradation was evaluated following the weight loss in presence of lysozyme solution (10 mg/mL) over a period of 24 days. Fig. 4A shows that cross-linked Si-HPMC

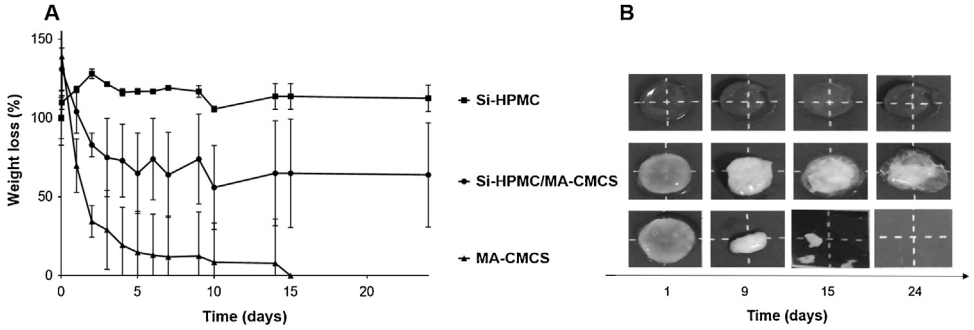

Fig. 4 – Degradation profile of Si-HPMC, MA-CMCS and Si-HPMC/MA-CMCS hydrogels in presence of lysozyme. (A) Weight loss of hydrogels incubated in lysozyme solution (10 mg/mL in PBS), at 37 °C, was assessed as a function of time. Results are expressed as a percentage of the initial weight (mean value ±SEM, n = 3). (B) Representative pictures of hydrogels taken after 1, 9, 15 and 24 days of incubation. Si-HPMC: silanized hydroxypropyl methylcellulose, MA-CMCS: methacrylated carboxymethyl chitosan, Si-HPMC/MA-CMCS: silanized hydroxypropyl methylcellulose/methacrylated carboxymethyl chitosan.

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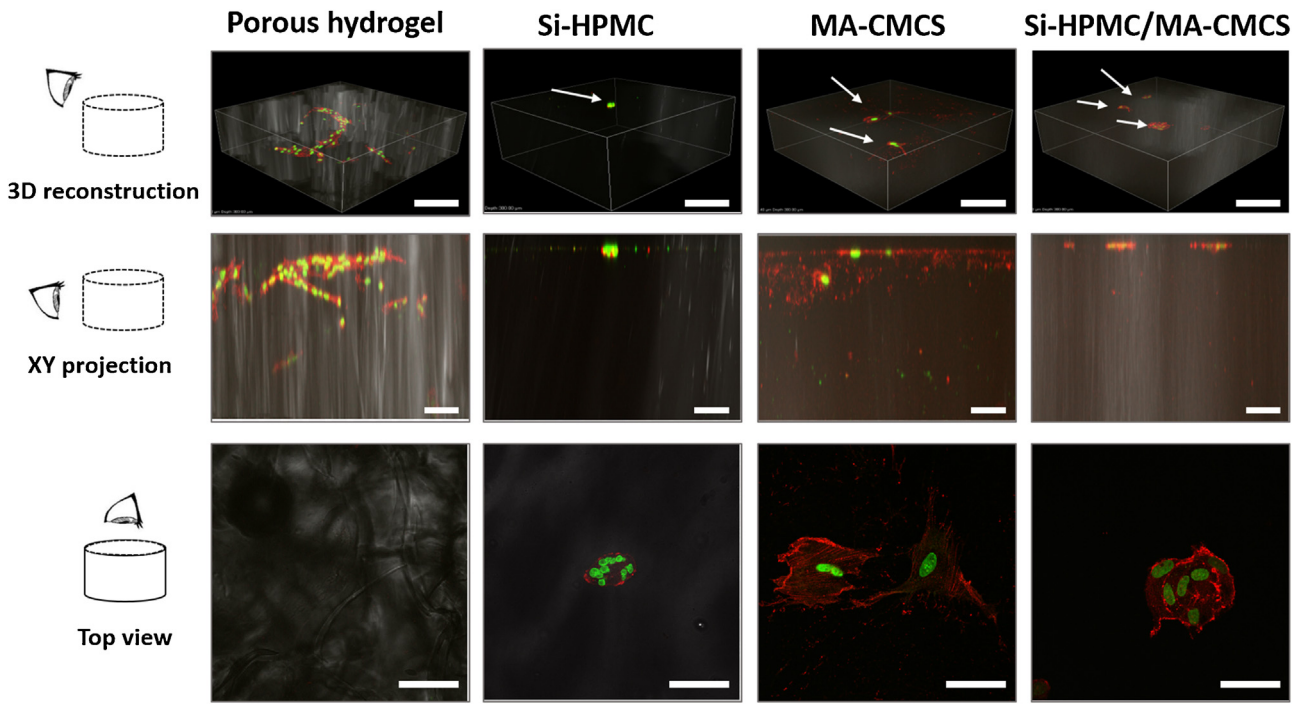

Fig. 5 – Hydrogel barrier effect against primary human gingival fibroblasts. Confocal microscopy images of porous hydrogel, Si-HPMC hydrogel, MA-CMCS hydrogel and Si-HPMC/MA-CMCS hydrogel after 4 days in culture with cells seeded on the top surface (nuclear staining: Yoyo-1; actin staining: phalloidin-Alexa Fluor 568). Top: 3D reconstruction of volume observed from hydrogel top surface to a 300 μm thickness. Cells on the surface are indicated by arrows. Scale bar = 300 μm. Middle: XY projection of the volume observed. Scale bar = 150 μm. Bottom: hydrogel on the top surface of biomaterials. Scale bar = 50 μm. Si-HPMC: silanized hydroxypropyl methylcellulose, MA-CMCS: methacrylated carboxymethyl chitosan, Si-HPMC/MA-CMCS: silanized hydroxypropyl methylcellulose/methacrylated carboxymethyl chitosan.

hydrogel maintained a stable weight throughout the observation time. MA-CMCS started to lose weight after 1 day of incubation in lysozyme. The weight reduced progressively until insoluble fragments were formed at day 15. When we combined the two polymers, forming IPN, the hydrogel membrane showed a reduction in weight compared to Si-HPMC hydrogel; however, the reduction proceeded more slowly than with the MA-CMCS hydrogel.

Comparing the pictures taken during degradation experiments (Fig. 4B), the Si-HPMC results were stable for all the observations in its shape and its transparent appearance. MA-CMCS, on the other hand, appeared whiter compared to Si-HPMC. The addition of MA-CMCS to Si-HPMC biomaterial increased, at first, the whiter aspect of hydrogel and successively a transparent part appeared in the border area. Observing the weight loss and the pictures, the degradation seemed to proceed with a surface erosion mechanism. The presence of a CMCS network in the hydrogel membrane, in the lysozyme solution, made it possible to obtain a progressive decrease in weight loss compared to Si-HPMC hydrogel.

3.4. *In vitro cell viability*

In this study we prepared a multicomponent hydrogel membrane for GTR. The cytocompatibility of the components and hydrogel were analyzed.

Firstly, we measured the influence on cell viability of the PIS under visible light curing irradiation for 120 s. Untreated cells were used as a positive control of cytocompatibility. The results (Fig. S3A in the online version, at DOI:10.1016/j.dental.2018.09.017) show that, after 24 h from contact with PIS, whether or not it was irradiated, the neutral red uptake of untreated cells was comparable with the cells in the experimental conditions.

MA-CMCS was synthetized to bring a particular synergistic combination of properties to Si-HPMC. After synthesis an extract of MA-CMCS polymer was made to assess the cytocompatibility. The extract was added to cell medium at 10% (v/v). Neutral red uptake of cells was evaluated after 24 and 72 h of contact between cells and polymer extract and 120 s of lamp exposure. All the results of the experimental conditions were compared to the untreated cells. No variation in neutral red uptake was evidenced for extract presence or for irradiation (Fig. S3B in the online version, at DOI:10.1016/j.dental.2018.09.017).

Lastly, cytocompatibility of cells in contact 24 and 72 h with Si-HPMC, MA-CMCS and Si-HPMC/MA-CMCS hydrogels was evaluated. Neutral red uptake results were compared to cell cultured without hydrogel. No significant differences were found between the viability of cells alone and the cultured cells in contact with Si-HPMC, MA-CMCS and Si-HPMC/MA-CMCS hydrogel membrane (Fig. S3C in the online version, at DOI:10.1016/j.dental.2018.09.017).

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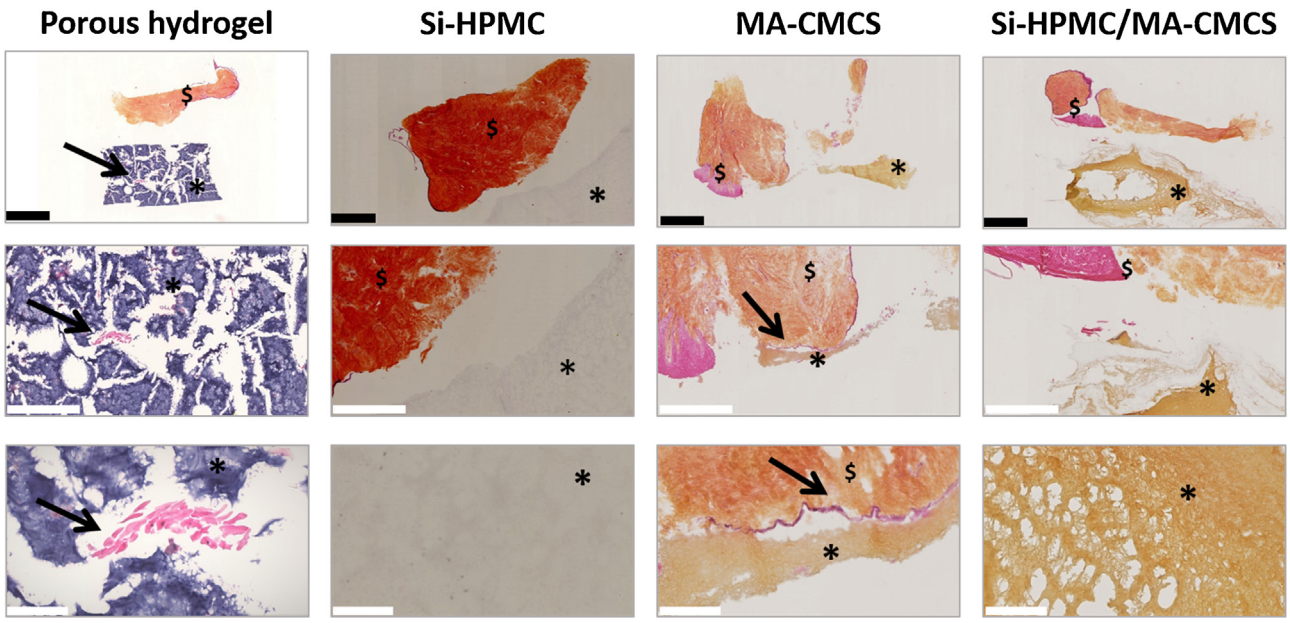

Fig. 6 – Hydrogel barrier effect against human gingival tissue. Histological sections of gingiva explant were cultured with porous hydrogel, Si-HPMC hydrogel, MA-CMCS hydrogel and Si-HPMC/MA-CMCS hydrogel. The hydrogels were maintained in contact with the explants in culture for 1 week. (Histological staining: hematoxylin, eosin Y and safranin). Asterisk = hydrogel, dollar sign = explant, arrow = cell infiltrate/in contact with hydrogel. Scale bar: top = 1 mm, middle = 250 μm, bottom = 50 μm. Si-HPMC: silanized hydroxypropyl methylcellulose, MA-CMCS: methacrylated carboxymethyl chitosan, Si-HPMC/MA-CMCS: silanized hydroxypropyl methylcellulose/methacrylated carboxymethyl chitosan.

3.5. *In vitro cell barrier effect*

The cell barrier effect is one of the most important characteristics of the material to be used as a membrane for GTR. To prove the cell obstruction, human gingival fibroblasts were seeded on the top of hydrogels for 4 days. The hydrogels were analyzed as the above-mentioned conditions. In Fig. 5 (top), 3D reconstructions of the Z-stack of the top surface in contact with cells up to 300 μm thick are reported; in the middle a 2D projection from a lateral view of volume is reported; and on the bottom pictures of hydrogels' top surface are reported. A porous pullulan/dextran hydrogel was used as a negative control in which cells are able to infiltrate the biomaterial. Fig. 5 shows that cells were found in the volume and no cells are observable at the material's surface. On the Si-HPMC sample, the cells are found on top of the biomaterial, round in shape and organized in a cluster. From the lateral projection view, the barrier effect of Si-HPMC is clearly confirmed. On MA-CMCS, cells are also found on top but are more elongated, suggesting a different interaction between cells and the hydrogel. From a later projection, the barrier effect is confirmed compared to porous hydrogel. Si-HPMC/MA-CMCS presented cells on the hydrogel surface organized in groups with a rounded appearance, but more extended than Si-HPMC. From the lateral view, the occlusive aspect of the biomaterials is confirmed and it is similar to the occlusive aspect of Si-HPMC.

3.6. *Ex vivo model*

Further investigation on the cell barrier effect analyzed *ex vivo* gingiva explant cultured in contact with hydrogels. In Fig. 6, histological sections after staining are reported. Gingival explants appear on a more yellow/orange layer due to the collagen composition, surmounted by a rich pink layer of cells. Porous pullulan/dextran hydrogel was used as a negative control of the material, in which cells are able to infiltrate the volume. Porous hydrogel presents cells inside the biomaterials shown by arrows. Si-HPMC hydrogel remains transparent after staining, however, a visual analysis was possible, confirming that no cells were inside the biomaterial. MA-CMCS appears yellow/orange, similar to the explant. The material was found partially in contact with the biological tissue (shown by arrow), suggesting the different interaction between tissue and hydrogel, as already observed with human gingival fibroblasts in contact with MA-CMCS hydrogel. The Si-HPMC/MA-CMCS hydrogel membrane also appears in yellow/brown and no cells were visible inside the biomaterial.

The Si-HPMC hydrogel was already proved *in vivo* in an animal model to possess a barrier effect against soft tissue cell invasion. In this experiment this property was confirmed and also MA-CMCS and IPN hydrogel showed cell occlusion against cell infiltration compared to hydrogel with macroporosity, which is not adapted for this kind of application.

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4. Discussion

In this study, we developed a liquid mixture able to cross-link under irradiation with a standard dentist's lamp into an interpenetrated polymer network hydrogel membrane composed of Si-HPMC and MA-CMCS for GTR in periodontal defects. Si-HPMC is a functional polymer able to undergo, at neutral pH, a condensation reaction that results in the formation of a three-dimensional network [30,37]. This viscoelastic polymer can be injected as a viscous solution and then build a 3D network *in situ*. Previous work in periodontal defects in an animal model demonstrated its ability to act as a physical barrier against cell invasion [38]; however, a fast crosslinking reaction is required for this application.

An interpenetrated polymer network was made because it presents a synergistic combination of the favorable properties of each of the polymer networks. Si-HPMC was selected to assess the barrier effect against gingival tissue invasion and CMCS allows the modification of membrane degradation due to its degradable backbone. In addition, the methacrylate grafted chains on CMCS can cure the biomaterial in a short period of time. MA-CMCS was synthesized to impart the photosensitivity to the formulation. FT-IR and NMR peaks confirm the functionalization reaction on CMCS backbone. No specifical grafting site was targeted, but a random distribution was sought. In the literature the reaction between glycidyl methacrylate (GMA) and many polysaccharides has been reported. Van Dijk-Wolthuis studied the reaction between GMA and Dextran. He demonstrated by NMR studies that two reactions are in competition the ring opening of epoxide and a transesterification reaction between the hydroxyl groups of polymer and the carbonyl of methacrylic groups [39]. Furthermore, Elisseeff, reported about the reaction of GMA and chondroitin-sulphate, that the transesterification reaction is a rapid reversible reaction, in contrast to the ring-opening product which slowly appears along the time [40,41]. In addition, Hutmacher described that in presence of free amino groups, such as gelatin polymer, GMA preferentially reacts with them in a percentage about 90% of all the grafted functionality [42]. In 2007, Poon successfully grafted methacrylate groups onto CMCS by reaction of CMCS with glycidyl methacrylate in water under basic conditions. The ^{1}H and ^{13}C NMR spectra of the collected MA-CMCS confirmed the grafting of methacrylate groups by transesterification or transamidation mechanisms and not by the ring-opening of the epoxide of glycidyl methacrylate. The ^{1}H NMR spectrum (Fig. S1 in the online version, at DOI:10.1016/j.dental.2018.09.017) recorded on our sample confirms the successful grafting of methacrylic bonds but does not allow to determine undoubtedly by which reaction (transamidation/transesterification) it occurs. Moreover, we cannot exclude that a part of the grafted methacrylic groups is coming from the ring-opening of the epoxide by the amino groups. However, independently from mechanisms, the content of methacrylate groups randomly grafted on CMCS backbone required for the targeted mechanical and gelification properties was reached.

To photocrosslink our biomaterials, we chose to use a dentistry visible light lamp (λ 420–480 nm). This lamp is already used by dentists to cure composites for dental restoration. Photocrosslinking uses light to dissociate the photoinitiator into radicals, which can propagate to macromolecules. The ratio between the polymers and the photoinitiator concentration is a key point for successful crosslinking. In addition, the concentration of the photoinitiator is not excessive, thus preventing phototoxicity consequences. At the beginning of the project, camphorquinone was tested to initiate the radical reaction; however, overly long irradiation was required with this photoinitiator. We then attempted the riboflavin molecule but rapidly switched to its phosphate derivatives. Riboflavin 5′-phosphate sodium salt hydrate, given its higher water solubility, avoids the use of organic solvent or ethanol, often used to solubilize the photoinitiator as reported in the literature [43]. When the use of RP as photoactivator was considered, triethanolamine was used as a coinitiator. This molecule is described in the literature by Previtali et al. as being more efficient as the alkyl-substituted amine [44].

The *in situ* formation of hydrogel membrane remarkably differs from what is available today on the market. To our knowledge, all the membranes used for GTR are in solid form and they need to be adapted by the dentist in their shape before implantation. The liquid formulation, able to form a solid membrane *in situ*, is an easy-to-use material, especially for complicated shapes or for a defect that is difficult to reach. In addition, a better shape adaption to the wound could increase the intimate contact with the defect and consequently improve the cell barrier effect compared to solid membranes. Rheological analysis of Si-HPMC/MA-CMCS showed a strong decrease in gelling time compared to Si-HPMC alone with the advantage of being able to control the start of the crosslinking with the start of irradiation. The combination between the two polymers and the PIS results in an IPN hydrogel membrane after 2 min of irradiation, compared to Si-HPMC in which the gel point appears after 15 min. Gelation seems coherent with the gel time already found in the literature with riboflavin and a visible light lamp [26,28,29,45,46].

IPN materials, which can be obtained by either chemical or physical crosslinking, in most cases show physicochemical properties that can differ remarkably from those of the macromolecular constituents [47,48]. MA-CMCS and Si-HPMC/MA-CMCS were analyzed under uniaxial compression after 120 s of irradiation. As expected at this time, Si-HPMC alone was not sufficiently cross-linked to perform this analysis, confirming a second polymer network is required to obtain a liquid-to-solid material that is adapted to clinical conditions in a few minutes. The compression test showed similar values to the study reported in the literature and the mechanical increase in stiffness seems coherent with the presence of second polymer network formation [26,49,50]. The formation of the IPN enhances the mechanical strength of the composite hydrogel.

An ideal membrane is described as a biocompatible material, to avoid tissue response, preferably biodegradable to avoid a second surgical intervention, acting as a soft tissue physical barrier, but also clinically manageable. Phototoxicity (photoirritation) is an acute light-induced tissue response to a photoreactive chemical. Excitation of molecules by light can lead to generating reactive species (ROS). We therefore assessed the photo safety of the new material by neutral red uptake [43,51–53]. We analyzed the cytocompatibility using

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murine fibroblasts and we measured the neutral red uptake on cells after incubation with the PIS, the MA-CMCS extract and hydrogels. A photoinitiator, under irradiation, gives radical species to react with the methacrylate unsaturated groups. The results showed that the PIS at this concentration, in combination with the time of irradiation chosen, did not demonstrate diminution of cytocompatibility compared with the untreated cells. Also, the contact between the cells and the polymer extract or in contact with Si-HPMC, MA-CMCS and Si-HPMC/MA-CMCS hydrogel membrane did not exhibit a decrease in cell viability compared to values found for cells cultured without biomaterials.

The degradation rate is a key point in membrane for GTR. Chitosan can be degraded with human enzymes such as lysozyme. The degradation could be impacted with factors that modify its water solubility, such as the degree of deacetylation or cross-linked chains [54]. To determine the influence of two-polymer materials, cross-linked hydrogels were placed in high concentrated lysozyme solution in physiological conditions. Lysozyme is the common enzyme used to study chitosan degradation [55,56], it is produced by cells and it is contained in saliva in different amounts depending on patients' habits and conditions. Glycol methacrylate chitosan was treated with lysozyme by Shapka [57]. The concentration was 4 mg/mL and the hydrogels with different degrees of substitution were incubated for 5 months, without complete degradation. Lee and co-workers analyzed the effect of glycol methacrylate chitosan mixed with hyaluronic acid. However, their hydrogels made of glycol methacrylate chitosan did not completely degrade in the 42-day observation time, and in the composite hydrogel they observed a slower gel mass loss. The comparison between experimental studies appears difficult due to the different chitosan types, degrees of modification, photoinitiators and irradiation conditions. They appear to have in common the difficulty in the enzyme diffusion in the volume in relationship with a more cross-linked network or a denser network for the addition of second polymer [26]. In the current study, we followed the weight variation of cross-linked Si-HPMC, MA-CMCS and Si-HMCP/MA-CMCS hydrogel membrane in lysozyme solutions. Si-HPMC was selected to assess the barrier effect against gingival tissue invasion, and CMCS allows the modification of membrane degradation due to its degradable backbone. We confirmed that the presence of the CMCS network in membrane can reach a progressive mass loss, compared with Si-HPMC, which is stable throughout the experiment. The critical time for soft tissue cell migration has been reported to be 14 days, the length of time the membrane has to be intact for wound healing. MA-CMCS hydrogel alone reduces its mass until the formation of fragments at day 15, making it poorly adapted to be used alone for this membrane application. In the current experiment, the stability of Si-HPMC and the degradability of MA-CMCS could be balanced in a favorable combination of the two polymer networks.

In GTR, the membrane has to have a resorption time compatible with tissue regeneration, but has to act as a physical barrier against soft tissue cells. To assess the barrier effect of IPN hydrogel membrane, we conducted two experiments. For the first experiment, isolated human gingival fibroblasts were seeded on top of hydrogel. After culture, cells were observed by confocal microscopy. In Si-HPMC and Si-HPMC/MA-CMCS cross-linked material, cells were found on the top surface of the biomaterial, within the cluster, but they were not able to enter the volume, confirming the barrier effect. In MA-CMCS cells appear more elongated and in the area of observation, from observation of lateral projection, a cell seems to start infiltrating the hydrogel volume. This could be related to a different interaction between cells and MA-CMCS hydrogel or to a degradation rate of chitosan backbone. In the second experiment, human gingiva explant was cultured in contact with biomaterials for 1 week and then analyzed using histological staining. The results confirmed that the cells could not infiltrate the cross-linked hydrogels. Porous hydrogels were used as a negative control in both experiments. Isolated cells and cells from soft tissue have been able to infiltrate the biomaterial. Cells were found in groups inside the volume, probably passing through the hydrogel macroporosity. The comparison between IPN and control hydrogel showed the strong correlation between the hydrogel structure and the cell's occlusive potential, demonstrating that IPN hydrogel could be an adapted membrane for GTR. In previous experiments, we used Si-HPMC as a self-setting hydrogel for cell encapsulation. The *in vitro* results confirm the ability of Si-HPMC to act as a physical barrier trapping cells inside the hydrogel. In addition, cell viability was demonstrated up to 21 days and diffusion experiments confirm the possibility of nutrient diffusion, essential for cell survival [14,58–60]. These previous experiments could be correlated with the *in vitro/ex vivo* results found in this study. In fact, due to the presence of Si-HPMC, the cells cannot cross the hydrogel barrier, maintaining cell viability. These physical barrier properties are also confirmed in several *in vivo* studies [60]. Moreover, the results obtained in periodontal lesions in dogs treated with cross-linked Si-HPMC membrane suggested that the hydrogel may act as an occlusive barrier to protect bone area from soft connective tissue invasion. However, the placement and adaptation of this membrane is described as being quite difficult during the surgical phase [61]. Hence, *in situ* curing hydrogel membrane may be the most appropriate strategy to overcome these limitations.

The two experiments conducted on the barrier effect of IPN could be a preliminary alternative to study the biomaterial barrier effect *in vitro*. However, we intend to conduct detailed animal studies to confirm the characteristics discussed in this paper and explore the potential of *in situ* IPN hydrogel membrane in an *in vivo* animal model of periodontitis.

5. Conclusion

The aim of this study was to develop an innovative *in situ* hydrogel membrane for GTR of periodontal defects. A previous study demonstrated the capability of Si-HPMC to act as a physical barrier against cell invasion in periodontal defects. However, the crosslinking rate was not well adapted for this clinical application. Therefore, we developed an innovative mixture by adding a biodegradable polymer to Si-HPMC that can be cross-linked photochemically, namely MA-CMCS. We used a photoinitiator based on riboflavin phosphate and visible light lamp (λ 420–480 nm), already used in dentistry. With the presence of this second polymer in the injectable viscous

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solution, the system becomes phototriggered, reducing the gelation time drastically in agreement with clinical needs. The resulting IPN hydrogel presents reinforced mechanical properties with preserved biocompatibility. This mixture is easy to handle because it is possible to apply it and cross-link it *in situ* using a common dentistry lamp. In addition, the presence of a chitosan polymer increases the degradability of the resulting biomaterial, making it possible to find a favorable combination of different polymer networks and to tune the degradation time in the future in accordance with *in vivo* needs. The results of the *in vitro* test and histological analysis confirm the biocompatibility of the material and the barrier effect against soft tissue cell invasion.

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Discussion

The results of this work showed the potentiality of the IPN hydrogel membrane to be used in GTR technique.

In particular, the first part of the work was dedicated to the polymers preparation and hydrogel membrane preparation and characterization. First, we prepared a methacrylated carboxymethyl chitosan for our hydrogel formulation. The random grafting of methacrylate groups onto CMCS, was chosen for its one-pot reaction in water medium, representing an easy preparation and purification step by dialysis against distilled water. The synthesis showed a higher percentage of yield mass and a grafting percentage coherent with the amount of reactive molecules used.

The Si-HPMC/MA-CMCS solution showed, using photocrosslinking, a decrease in gel point according to our objective reducing the crosslinking from 15 min of Si-HPMC to 2 min. This results are in compliance with the clinical application suggested in the work, but they also open the biomaterial to some novel biomedical applications such as cell therapy or drug delivery. In fact, the rapid curing and the use of visible light lamp could increase the cell viability usually decreased from UV lamp or more toxic photoinitiator[172,173].

The carboxymethyl chitosan has degradable backbone also once reticulated. The degradation depends on different parameters such as the molecular mass, the degree of deacetylation and the crosslinking. In our work the hydrogel was degraded in presence of lysozyme in physiological medium. Also the IPN material showed a weight loss and picture suggested a surface erosion mechanism of the 3D network. Different ratio of the two biomaterial or different a substitution degree in CMCS could modified the range of resorption with the possibility to tune the degradation time in future according with the desired application.

The second part of the paper was focused on cell/biomaterial study. The results of neutral red uptake confirmed the cytocompatibility of hydrogel system. During the experimental study, also antibacterial test was performed using the IPN hydrogel membrane and the corresponding hydrogel systems. In chitosan based hydrogels the antibacterial effect is related to the presence along the chain of free amino group, which

could interact with pathogens exerting a powerful bacteriostatic. A number of studies have been demonstrated that chitosan and derivatives are able to inhibit growth of both Gram-negative and Gram-positive bacteria[167]. Periodontitis development is related to the bacteria biofilm presence. For that we analyzed the antibacterial properties of Si-HPMC, MA-CMCS and Si-HPMC/MA-CMCS hydrogels. The hydrogel was prepared as described in the previous paper. The antibacterial test was performed in collaboration with Prof. Gilles Amador Del Valle in the department of microbiology of Nantes Hospital. The hydrogels have been deposited in contact of petri with schaedler medium with Porphyromonas G ingivalis in an anaerobic condition. The test was repeated twice. No antibacterial properties were observed in the experiment due to the hydrogels presence. The negative results of the experiment could be related with the decrease of free amino amine during the grafting reaction. In the next experiments a selective introduction of methacrylated groups could be planned to maintained the amino groups free to maintain the antibacterial properties described in literature, instead of testing different pathogens that could be differently impacted by the presence of chitosan derived hydrogel.

Cell barrier effect was investigated cultivating the hydrogels in contact with human gingival fibroblasts and with human gingival explants. During this work also human gingival keratinocytes were isolated from human gingival explant to demonstrate the barrier effect. However, the low viability of cells in culture induces us to study directly the *ex vivo* explant instead of the isolated cells. Nevertheless, *ex vivo* gingiva culture and human gingival fibroblasts in contact with hydrogels showed the occlusive property of the investigated IPN, confirming the potentiality of this biomaterial to be used in GTR therapy as an *in situ* membrane.

Further investigations prior to preclinical study are still on going, however we believe that these IPNs hydrogels due to their high tuneable properties, like degradation or crosslinking time, offer various perspectives for GTR therapy.

Chater 4 : conclusions and perspectives

Summary

Hydrogels are three-dimensional networks well described in pharmacy and medicine for biomedical applications such as drug delivery, tissue engineering or for medical devices. Polysaccharides represent a class of macromolecules of particular interest for hydrogels design, as they are usually abundant and available from renewable sources. In addition, they have a large variety of compositions and properties. Moreover, they allow tailored chemical modifications to tune the biological/mechanical properties of the resulting network. In recent years, multicomponent hydrogels such as interpenetrated polymer networks (IPNs), which are systems composed of two or more networks, have emerged as innovative biomaterials. The success of this strategy is due to the synergistic combination of favourable properties of each of the polymer networks. [174–176].

The first part of **chapter 1** provides an introduction on polymer-composite hydrogels as a strategy to modify the properties of a hydrogel systems. An overview of such polymer-composite hydrogels is presented with particular attention for IPNs.

The second part of **chapter 1** presents an overview on periodontitis and guided regeneration techniques. We focused on reviewing the membranes commercially available for guided tissue/bone regeneration (GTR/GBR) with an overview on novel strategies and future perspectives for membranes design. A large panel of membranes are available today on the market. As evidenced by bibliographic review, many techniques are often used together to try to combine or achieve a synergism of properties. New materials and fabrication techniques appear from biomaterials and biological research. *In situ* forming hydrogels, able to fill complex defects and quickly form solid membranes upon injection, are particularly appealing owing to their ease of handling and cost- and time-saving features.

The main objective of the thesis is presented at the end of **chapter 1**, as the development and the characterization of two IPNs based on Si-HPMC and two methacrylated polysaccharides: dextran and

carboxymethyl chitosan. The two IPNs were designed and characterized. In addition, a possible application as a membrane for GTR/GBR were investigated.

In **chapter 2** we described an IPN hydrogel membrane composed of Si-HPMC and dextran methacrylate (DexMA). DexMA derivatives are well-known described in literature. Many biological applications have been described for this 3D grafted polymer network. This material was selected with Si-HPMC in the framework of POsTURE project to design an *in situ* IPN hydrogel membrane. Different DexMA derivatives were evaluated for final formulation. The Si-HPMC/DexMA solution was demonstrated to be injectable through a syringe equipped with a 18 G needle. The Si-HPMC/DexMA solution can be injected directly *in situ* and upon visible light irradiation, an IPN hydrogel membrane can be quickly obtained. The *in vitro* experiments with neutral red uptake confirmed the cytocompatibility of the materials used. In addition, we compared the *in situ* obtained IPN hydrogel to a commercial collagen membrane, in a relevant GBR animal model. Calvaria critical size defects (⌀ = 8 mm) in rabbits were filled with synthetic biphasic calcium phosphates bone graft in conjunction with experimental membranes to evaluate the biocompatibility and the efficacy for GBR technique. Quantitative analysis of newly formed bone were performed using micro-computed tomography system and histological stain was used to visualize soft tissue in presence of IPN hydrogel membrane. Quantitative assessment of mineral deposition showed in the IPN test group a new bone formation comparable to the collagen membrane condition. Histological analysis and quantification are still on going to determine the morphology and quantification of mineral and soft tissue into the defects. According with the results *in vivo* of the IPN hydrogel membrane, further detailed animal studies could be realized to confirm the characteristics obtained.

In **chapter 3** an IPN based on Si-HPMC and methacrylated carboxymethyl chitosan (MA-CMCS) was studied. In this study, modified chitosan, was used as polymer precursor for the synthesis of hydrogels. Indeed, this biocompatible and biodegradable polysaccharide, derivate from natural chitin, is water soluble at neutral pH[157]. Furthermore, to use chitosan instead of dextran could produce some interesting properties such as antibacterial activity and fast degradation. The modified chitosan was successfully

prepared and characterized. This new formulation was analysed in a chemical, rheological and mechanical point of view. The addition of methacrylate polymer reduced the gel point irradiating the polymers solutions with a standard dentist polymerization lamp. In addition, the presence of a MA-CMCS polymer increased the degradability of the resulting IPN. Cell barrier effect was investigated cultivating the hydrogels in contact with human gingival fibroblasts and with human gingival explants. The results showed the occlusive property of IPN hydrogel membrane, confirming the potentiality of this biomaterial to be used in GTR therapy as an *in situ* membrane.

Conclusions and perspectives

The main topic of this thesis was the development and the characterization of two IPNs composed of Si-HPMC and DexMA or MA-CMCS. In addition, we explored the suitability of these novel systems as a formulation for the development of injectable *in situ* forming IPN hydrogel membrane for GTR/GBR. Membranes act as a physical barrier to avoid gingival cell invasion. The barrier membranes play a key role in preventing undesirable tissue migration into the defective area, and consequently, they allow sufficient time for bone, cementum, and periodontal ligament regeneration. These materials have to present good integration with hosting tissue, avoiding inflammations reaction, but also good mechanical properties against compressive force of overlying soft tissue and maintain the space for tissue regeneration[43,61,62]. [63,64]. To this end, injectable formulations, able to cover complex defects and quickly form solid membranes upon injection, are particularly appealing owing to their ease of handling and cost- and time-saving features [43,124,125].

A large panel of membranes are available today on the market. According to our knowledge only two kind of liquid membranes have been commercialized: Atrisorb® (Tolmar, USA) and Membragel® (Straumann, Austria), but they are no longer available according to the manufacturers [43]. The first one is composed of poly(DL-lactide) (PLA) dissolved in *N*-methyl-2-pyrrolidone (NMP)[124]. The second one was composed of multiarms PEG with thiol end-groups and acrylate end-groups that react forming a hydrogel membrane via Michael-type addition[125].

In this work we prepared two IPNs composed of Si-HPMC and DexMA or MA-CMCS. Si-HPMC is a self-setting hydrogel that has been reported in the literature for many biological applications. It has the advantage of being injected as a viscous solution and then, due to the condensation reaction, it builds a 3D network *in situ*. This material was demonstrated to be biocompatible and slowly degraded in a rabbit model[131–134]. In addition, it has been demonstrated the capability of the cross-linked biomaterial to act as a physical barrier against cell invasion[60]. The main drawback of this self-setting hydrogel is an excessively slow crosslinking process for clinical needs. The combination of DexMA or MA-CMCS with Si-HPMC reduced in both case the gel point and allows to have temporal control over the crosslinking process. We obtained the IPNs hydrogel membranes after 120 s of lamp irradiation compare to 15 min for Si-HPMC necessary to reach the gel point. Si-HPMC/MA-CMCS before photocrosslinking presented elastic moduli higher than viscous moduli, probably due to ionic interaction between the polysaccharides chains. This interaction could facilitate the *in vivo* product deposition, avoiding the flow away from complex/difficult to reach wound during clinical practice. On the other hand, DexMA derivatives are well-known polymers with an established characterization. In addition, the polymer solution stability was proved in the described conditions (4°C, avoiding contact from light).

The degradation rate is a key point in membrane for GTR/GBR. The synergistic effect obtained by our IPNs improved the physicochemical properties and the dense network might assure the barrier effect against soft tissue invasion and delayed the hydrogel resorbability. Most resorbable membranes, such as polylactic acid or collagen, used until now are characterized by a rapid absorption kinetics after implantation. In fact, these membranes do not ensure, for a period of 8 weeks or more, the regenerating process below the barrier. With the purpose to improve their properties, commercially available membranes are generally made of a cross-linked polymer. Indeed, the presence of physical or chemical crosslinking nodes delays the loss of barrier properties[70]. However, nondegradable based membranes are widely used, although a second surgery for their removal after use is mandatory with an increase of postoperative morbidity.

Si-HPMC/DexMA IPN was implanted in calvaria defect. According with the results *in vivo* of IPN membrane, different parameters on polymer formulation could be modify to tune the IPN hydrogel membrane degradation. For example, a decrease in the degradation time, could be obtained by incorporating the dextranase enzyme into the system: by regulating the concentration of this enzyme within the hydrogel, the rate of degradation can be modulated. Also the degree of derivatization (%MA) could have an impact on membrane degradation. Another method to modify the degradation time is grafting on dextran backbone hydroxyethyl-methacrylate, instead of methacrylated groups. In fact, containing hydrolysable esters increase the biodegradability of the hydrogel network.

We confirmed that the presence of the MA-CMCS network in Si-HPMC/MA-CMCS membrane can reach a progressive weight loss, compared to Si-HPMC, which was stable through the experiment. The critical time for soft tissue cell migration has been reported to be 14 days, the length of time the membrane has to be intact for wound healing. MA-CMCS hydrogel alone reduced its mass until the formation of fragments at day 15, making it poorly adapted to be used alone for this membrane application. According to future *in vivo* experiments in GTR/GBR animal model, the stability of Si-HPMC and the degradability of MA-CMCS could be balanced in a favorable combination of the two polymer networks.

To cure the IPNs hydrogels membranes, the photocrosslinking technology appeared the more appropriate technique for this application for its spatio-temporal control over the crosslinking process. In addition, this technique is already used in dentist in methacrylate resins for the treatment of dental decays. For this reason, we decided to use a standard dentist's lamp to irradiate our photosensible solutions. We selected vitamin B2 or riboflavin as a photoinitiator in its phosphate derivatives to increase the water solubility and avoid the use of organic solvent. Moreover, riboflavin phosphate is already commercialized as a photoinitiator for keratoconus treatment.

Phototoxicity (photoirritation) is an acute light-induced tissue response to a photoreactive chemical. Excitation of molecules by light can lead to generating reactive species (ROS). We therefore assessed the photo safety of the new material by neutral red uptake[177–180]. Nonsignificant variation in the results in

the two IPNs were observed compare to the untreated cells. The irradiation along 120 s, also in presence of photoinitiator, did not show any difference in neutral red uptake compare to the untreated cells.

The *in vivo* results about Si-HPMC/DexMA IPN hydrogel membrane will bring further information about the safety, bone regeneration and barrier effect against soft tissue for GTR/GBR.

For Si-HPMC/MA-CMCS IPN hydrogel membrane the occlusive barrier properties were proved *in vitro* against cell invasion using HGFs and gingiva explant, confirming the potentiality of this membrane. However, prior preclinical studies, further investigation are necessary to understand some issues such as antibacterial properties. Some studies are ongoing to modify the grafting synthesis of CMCS, increasing the free ammine, described in literature to have antibacterial properties.

Further investigations are still on going, however we believe that these IPNs hydrogels due to their high tuneable properties, like degradation or crosslinking time, offer various perspectives for biomedical applications and in particular in peri-implantitis diseases. Dental implants are prevailing treatment solution for tooth replacement in the current restorative dentistry. The most frequent complication of dental implant is peri-implantitis, referring to the chronic inflammatory disease of the supporting peri-implant tissues, due to an infection development caused by local bacteria. The treatment approach consists of anti-infective mechanical treatment stage to eliminate bacterial biofilms and inflammatory products and GBR technique to restore bone defects using different biomaterials. On the bottom of the treated site, (bone/gum interface) a liquid *in situ* IPN hydrogel membrane could be applied to prevent the ingrowth of the epithelial/connective soft tissues and stabilize the bone filling biomaterials. These two points are recognized as a main complication of peri-implant bone healing. Also in this treatment, clinical handling by the physicians when applying the membrane could be improved with the use of injectable formulations. Currently, it is accepted that the presence of threads on the implant surface together with surface micro-roughness compromises elimination of bacterial biofilms, hence the insufficient implant surface decontamination was suggested as the major cause of incomplete disease resolution and respective high disease recurrence. This was additionally supported by the findings that repeated use of local antimicrobials as an adjunct to anti-infective

mechanical treatment substantially improved treatment outcomes. Therefore, the design of liquid membrane-drug delivery system could be a new challenge for optimal bone regeneration. The possibility to produce antibacterial membranes were investigated in literature. Metronidazole and Amoxicillin were loaded in polymer solutions to produce electrospun fibers for membranes or directly integrated in a layer of the membranes inhibiting bacterial growth[43,95,96]. Also non antibiotics antimicrobial agents were investigated like zinc- or silver-based material, lauric acid or chlorhexidine[97–103]. These composite membranes revealed antimicrobial activity versus Porphyromonas Gingivalis and others oral major pathogens bacteria. In our knowledge, no antibiotics/antimicrobials liquid products membranes have been commercialized. Due to their high water content, the properties of hydrogels are similar to those of biological tissues, resulting in an excellent biocompatibility. These hydrogels facilitate the localized and sustained release of a drug, thereby decreasing the number of administrations, preventing damage to the drug and allowing for relatively low doses. IPNs could be used to extend the release time and to enhance the mechanical stability. Drug delivery or bioactive molecules release are governed from different parameters such as the swelling degree or the chemical nature of the polymer networks. For example, Si-HPMC/MA-CMCS IPN hydrogel could form an ionic interaction between the charged polymer with ionic molecules such as (e.g. Ag+) of interest modulating the release.

It can be concluded that the interpenetrating polymer networks described in this thesis are attractive candidates for *in situ* hydrogel membrane for GTR/GBR procedures. Moreover, to their high tuneable properties, we believe they are also a promising candidate in peri-implantitis treatment and in a broad range of pharmaceutical and biomedical applications.

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Titre : Réseaux interpénétrés d'hydrogels d'hydroxypropyl méthylcellulose silanisé/polysaccharides méthacrylés pour des applications biomédicales.

Mots clés : photo-réticulation, régénérations tissulaire et osseuse guides, membrane, biomatériaux dentaire, parodontite, dextrane, carboxymethyl chitosane, lampe dentaire pour la photopolymérisation

Résumé : La régénération tissulaire guidée et la régénération osseuse guidée sont des interventions chirurgicales visant la régénération des tissus perdus du parodonte et de l'os alveolaire. Dans ces techniques, une membrane biocompatible est implantée autour de la lésion intra osseuse parodontale, remplie ou non d'une greffe osseuse, afin de prévenir sa colonisation par les tissus mous. L'hydroxypropyl méthylcellulose silanisée (Si-HPMC) est un hydrogel auto-réticulant qui peut être injecté sous forme de solution visqueuse puis, grâce à la réaction de condensation, construire un réseau 3D. Il a été démontré que le Si-HPMC réticulé agissait comme barrière physique contre l'invasion des tissus mous dans les défauts parodontaux chez le chien. Le principal inconvénient de cet hydrogel auto-réticulant est une cinétique de réticulation trop lente pour des applications cliniques optimales. L'objectif principal de cette thèse était donc le développement et la caracterisation de deux resaux interpenetres d'hydrogels (IPNs) composé de Si-HPMC et d'un polysaccharide : soit le carboxyméthyl chitosane (CMCS), soit le dextrane Nous avons greffé sur le CMCS et le dextrane des groupes méthacrylate capables de réagir sous irradiation d'une lampe à photopolymériser standard utilisée en dentisterie en présence d'un photo-initiateur composé de vitamine B2.

Les polymères ont été synthétisés et caractérisés avec succès. Deux IPNs innovants ont été réalisés et les propriétés chimiques et physico-chimiques ont été étudiées. De plus, des études *in vitro* et *in vivo* ont été réalisées pour évaluer la cytocompatibilité et pour étudier l'aptitude de ces deux membranes innovantes hydrogel IPN à promouvoir la régénération tissulaire ou osseuse guidée.

Les résultats sont encourageants et nécessitent d'autres investigations *in vivo* pour caractériser les biomatériaux et confirmer leur potentiel en tant que biomatériaux dentaires et implantaire.

Title : Interpenetrating polymer networks hydrogels of silanized hydroxypropylmethyl cellulose/methacrylated polysaccharides for biomedical applications

Keywords : photo-crosslinking, guided tissue and bone regeneration, membrane, dental biomaterial, dental visible light lamp periodontitis, dextran, carboxymethyl chitosan, visible light lamp.

Abstract : Guided tissue regeneration and guided bone regeneration are surgical procedures aiming the regeneration of lost components of periodontium. In these techniques a periodontal intraosseous defect is filled or not with a bone graft material and covered with a biocompatible membrane in order to prevent its colonization by soft tissues. In fact, during a physiological healing, it appears that the soft tissue migrates rapidly into the wound, avoiding the regeneration. The barrier membrane plays a key role to prevent undesirable tissue migration into the defective area, and consequently, it allows sufficient time for bone, cementum, and periodontal ligament regeneration. Silanized hydroxypropylmethyl cellulose (Si-HPMC) is a self-setting hydrogel that can be injected *in vivo* as a viscous solution and then, due to the condensation reaction, build a 3D network. It has been demonstrated demonstrated that cross-linked Si-HPMC acted as a physical barrier against soft tissue invasion in periodontal defects in dogs. The main drawback of this self-setting hydrogel is the too slow crosslinking process, which is not suitable for clinical needs. Nevertheless, the results were quite promising due to the easy preparation and handling of polymer solution, which could lead to simplified periodontal treatment.

Therefore, the main objective of this thesis was the development and the characterization of two injectable interpenetrating polymer network hydrogels (IPNs) composed of Si-HPMC and two polysaccharides: carboxymethyl chitosan (CMCS) or dextran. We grafted on CMCS and dextran methacrylate groups able to react under irradiation of standard photopolymerization dentistry lamp. We selected vitamin B2 as a photoinitiator.

The polymers were successfully synthetized and characterized. Two innovative IPNs were realized and the chemical and physico-chemical properties were studied. In addition, *in vitro, in vivo* study were performed to assess the cytompatibility and to study the suitability of these two innovative IPNs hydrogel membrane for guided tissue or bone regeneration.

The encouraging results need *in vivo* further investigations to characterize the biomaterials and confirm the potentiality as dental biomaterials.