Faculdade de Engenharia da Universidade do Porto

Stomach Patch

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Resumo

Os problemas de estômago como úlceras, e perfurações estomacais são problemas patológicos comuns no mundo quotidiano. Por ano, cerca de 30000 pessoas padecem desta doença, que consiste num desfasamento na homeostasia do tecido epitelial do estômago, causado por um desequilíbrio na ação protetora e danosa do estômago.

Atualmente, o tratamento é inespecífico e consiste na administração de fármacos que diminuem o pH do ambiente do estômago (sob pena de mais tarde gerarem problemas secundários a nível nutricional) e/ou administração de antibióticos, que levam ao desenvolvimento de resistências bacterianas. Em casos mais adversos e avançados, no caso de a lesão não ter sido especificamente tratada, os pacientes desta patologia poderão ter que ser submetidos a cirurgia para remoção parcial do tecido da mucosa gástrica.

O atual projeto visa desenvolver um dispositivo biomédico de utilização no epitélio gástrico, utilizando uma tecnologia state of the art em regeneração de tecidos. Trata -se de um hidrogel feito de uma membrana polimérica de quitosano-Rosa de Bengal. Visa-se a introdução deste dispositivo por via endoscópica, por um profissional patologista, num procedimento rápido, confortável e não-invasivo.

Mostramos que este biomaterial tem capacidades mucoadesivas de adesão ao tecido gástrico, podendo criar uma barreira física ao pH ácido do estômago, permitindo assim a regeneração do tecido epitelial de uma forma mais eficaz. O filme também mostrou características mecânicas de elasticidade viáveis à sua utilização num órgão com elevada motilidade, como o estomago Este filme mostrou resistir a ambientes ácidos semelhantes ao existente no estomago. Da mesma forma, mostrou biocompatibilidade com linha celular MKN74, demonstrando o seu potencial no uso em meio clínico.

O seu desenvolvimento visa a sua aplicação futura na Medicina e na Medicina Veterinária.

Abstract

Pathologies associated with the gastrointestinal tract like Peptic ulcer disease (PUD) are very common nowadays. Per year, around 3000 people perish from these diseases that consists of an imbalance in the tissue epithelia's homeostasis, caused by a disparity between the protective and damaging forces in the stomach.

In present days, the treatment is unspecific and consists in the administration of pH lowering drugs (that give rise to problems on a nutricional level) and/or the administration of antibiotics/antimicrobials, which bring about the development of bacterial resistances. In many serious cases, patients must be submitted to surgery and/or removal of the injured tissue in the gastric mucosa.

This work seeks to develop a state of the art Chitosan-Rose Bengal (RB) film, a hydrogel film that will protect the gastric mucosa from the aggressions of the gastric acid environment, using state of the art tissue regeneration technologies. This hydrogel, protects the lesion directly creating a physical barrier to the acid juices of the stomach, allowing the mucosa to heal. The film is introduced by endoscopy, by an experienced professional medical practitioner, in a non-invasive and fast procedure.

In this work we show that the formulation has potential to be used in a clinical environment for the treatment of PUD, since it shows, firstly, to have the mechanical characteristics of elasticity to accompany the many movements of the stomach tissue. Also, in a gastric-like environment, the formulation can adhere to the mucosal tissue and resist its low pH medium. The film shows also high biocompatibility with MKN74 cells, that s heds some light on its biocompatilibility in a clinical mean.

In the future, this film will be used in both common medicine, but in veterinarian medicine as well.

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Abbreviations and symbols

List of abbreviations (alphabetical order):

COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
ECM	Extra-cellular matrix
GI	Gastrointestinal
H. pylori	*Helicobacter pylori*
IM	Intestinal Metaplasia
MM	Muscularis Mucosae
MSP	Medium Stomach Patch
NSAIDS	Nonsteroidal anti-inflammatory drugs
PUD	Peptic Ulcer Disease
RB	Rose Bengal
SSP	Small Stomach Patch
TCPS	Tissue Culture Polystyrene
WHO	World Health Organization

List of symbols:

tan δ	Damping, viscous-elastic modulus, mechanical loss of heat

Chapter 1

Introduction

1.1 - Stomach

The gastrointestinal tract (GI) is composed of the oesophagus, the stomach, the intestine (small and long) and the annexed organs reviewed by Robbins. Its main function is to breakdown food into its constituent nutrients and minerals for organism absorption and consumption [1]. The nutriment that is not absorbed is excreted, these processed are described and reviewed thoroughly by Robbins, Abbas and Kumar.

In the stomach, the digestion, in which all the enzymatic and chemical machinery act in the first break of nutrients such as proteins, is one of the main episodes in this gradual event [1,2]. Solid food is decomposed by the strong muscular peristaltic movements that starts in the oesophagus and heads afterwards to the stomach, where the acidic juices and enzymes (like pepsin and gastric lipase) secreted by the cells from the mucosa break down its proteins and lipids .

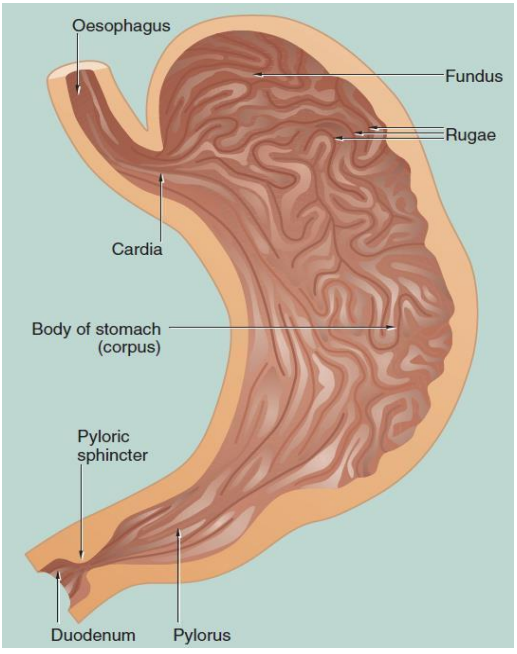


Figure 1.1 the anatomic regions of the stomach [1].

Though little components are absorbed in the stomach, except for water, alcohol and some drugs This has also been thoroughly described, and reviewed, namely by Robbins, Abbas and Kumar.

The stomach is anatomically composed of four regions: the *cardia*, the *fundus*, the *corpus* and the *pylori* (or antrum)[1] *(Figure 1.1)*, and generally has a pH close to 1 [1-3]. An alkaline protective mucous layer is produced in all regions of the stomach, but mainly in the *cardia* and *pylorus* regions. In the latter, two additional types of alkaline mucous constituents are produced, one of the reasons for the preference of *Helicobacter pylori* and other *Helicobacter pylori* like *Helicobacter* bacteria-colonization that cannot cope with the acidity of the other regions [6, 7].

Scattered throughout the gastric mucosa are gastric pits, tubular glands displaying a diverse array of different gastric cells [8]. Surface mucous cells cover the luminal surface of the stomach and partly line the gastric pits. These cells are usually referred to as gastric mucosa epithelia. Eosinophilic parietal cells, in the isthmus are responsible for the production of the concentrated HCl, in charge of the stomach's low pH, while the zymogen cells, or chief cells , in the neck of the gland, produce pepsin. On the depth most of the gastric gland, neck mucous cells and neuroendocrine cells can be found (Figure 1.2) [1].

Once the food has been processed into the *chime*, the peristaltic movements conduct it through the pyloric sphincter to the duodenum, where its processing will continue in the intestine [8].

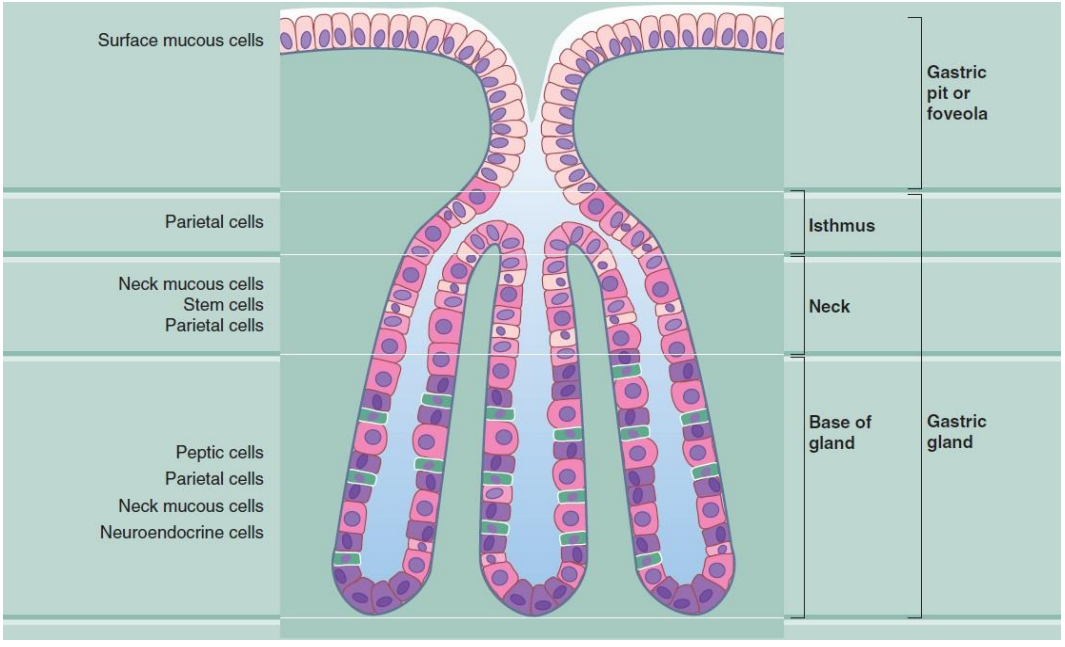


Figure 1.2 diagram of a generic tubular gland gastric pit found on the surface of the gastric mucosa [1].

1.2 - Peptic Ulcer Disease (PUD) in the Stomach

PUD's lesion can occur in throughout the whole mucosa of the gastrointestinal tract, but these disorders differ according to th location. In the stomach, these disorders are a frequent cause of clinical disease, with inflammatory and neoplastic lesions being particularly common, affecting 4,5 million people annually in the US [9-11].

Lesions less than 0.3 cm in diameter tend to be shallow, whereas those over 0.6 cm are likely to be deeper, as it deepens there is the possibility to reach a blood vessel or capillary in the mucosa and cause a stomach hemorrhage. Peptic ulcers are solitary in more than 80% of patients [1]. The classic peptic ulcer is a round to oval, sharply punched-out defect.

A healthy gastric mucosa matches the damaging forces of the gastric acidity with various defensive protective strategies. The stomach's mucosa has a high regenerative capacity, meaning that the gastric epithelial cell's turnover in this tissue is very high. This enables the gastric mucosa to regenerate itself between diet cycles hindering the development of lesions and gastric abrasions. Another hampering defensive force is the protective mucous layer mainly produced by the neck mucous cells displayed at the bottom of the gastric pits.

This thromboxane and prostaglandin-induced alkaline layer decreases the acidity of most regions of the stomach mucosa protecting it from the HCl chemical aggression. The apical surface transport-derived secretion of bicarbonate onto the mucous layer also allows for an appropriate biochemical and chemical protection since it inhibits the peptidase effect of the gastric enzymes against the proteins of the extracellular region of the epithelial cells and it lowers the acidity of the gastric environment itself.

Ulcerogenesis arises when the damaging forces unbalance the defensive ones, and there are various factors that tip this meticulous homeostasis. PUD, in the stomach, is most often associated with *Helicobacter pylori* colonization [12], generally in the antral regions [12, 13] or non-steroidal anti-inflammatory drugs (NSAIDS), such as Aspirin, Ibuprofen or Nimesulide. These latter drugs, usually used in the treatment of acute pain and inflammation distresses, inhibit the activity of both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). These hinder the production of prostaglandins and thromboxane which, in turn, inhibit the production

of the protective alkaline mucin layer that prevents ulceration. [14-16]. PUD can also be stress-related or caused by the ingestion of specific foods [1].

The pathogenic role of *H. pylori* in chronic active gastritis and the association between *H. pylori* and PUD in 95-99% of patients is also well established, mainly due to the information perceived after *H. pylori* eradication, which proved to reduce the pathogenicity of PUD [10, 12]. Patients with PUD related gastric ulceration and *H. pylori* infection produce more acid

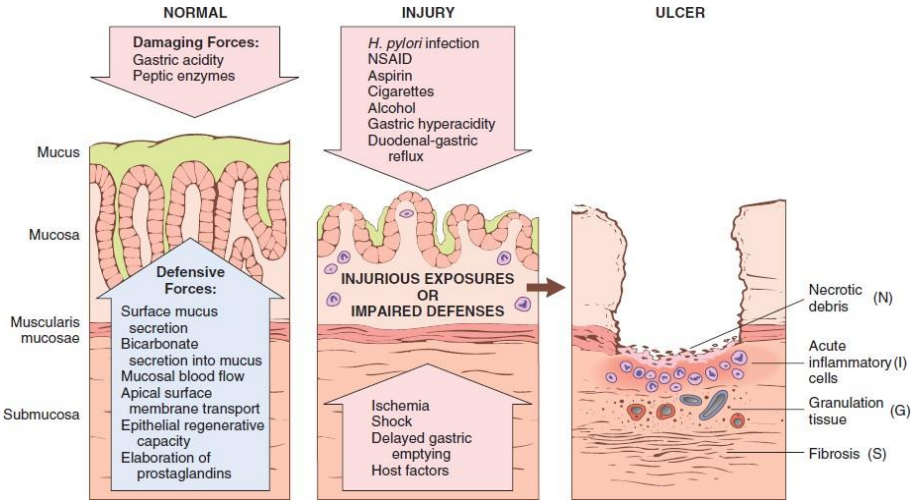


Figure 1.3 Mechanisms of gastric injury and protection, the progression from more mild forms of injury to ulceration that may occur with acute or chronic gastritis [1].

than infected people without ulcers in response to the same stimulation with gastrin. *H. pylori* has very high urease activity producing ammonia to protect the microorganism from its acidic gastric environment [10, 12].

Production of alkaline ammonia by bacteria on the surface epithelium and in the glands of the antrum prevents the gastric gland cells from sensing the true level of acidity. This lead to inappropriate release of somatostatin and an increase in gastrin that enables the excess production of gastric acid. This excess production causes the pH to drop significantly in each of the regions of the stomach and ulceration arises. *H pylori* also causes an inflammatory response in the gastric mucosa, with induction cytokines, that lead to an influx of neutrophils and macrophages into the gastric mucosa with release of lysosomal enzymes, leukotrienes, and reactive oxygen species. This enviroment is not favorable for mucosal regeneration and will start the process of ulcerogenesis [1].

1.3 - Gastric adenocarcinoma

PUD and gastric adenocarcinoma are generally associated in cases in which the protective and damaging forces are taken to a greater dimension [1]. Following this model once the damaging forces overcome the protective forces, and the ulcer is formed and not thoroughly treated this continuous aggression on the gastric mucosa can give rise to infection and chronic inflammation processes. These processes require a constant remodelling and

reshaping of the tissue surrounding the ulcer, known as metaplasia [17, 18]. Metaplasia involves genetic remodelling from an adult phenotype into a phenotypic younger cell, and back. In the gastric epithelia, the epithelial gastric cells develop into a phenotype that is more robust and better adapted to resist the low pH of the gastric juices, generally switching into intestinal epithelial cells, known as Intestinal Metaplasia (IM) [17, 18].

IM is known for being a pre-dysplasic state for gastric adenocarcinoma, starting with the display of small intestine specific goblet cells [17, 18]. This switch-on and switch-off of genes in the gastric epithelial cells can give rise to point mutations in important oncogenes (like the K-raps) and lead to the development of adenocarcinoma of the stomach.

1.4 - Epidemiological data

Table 1.1 Death rate of gastric ulcers per million per year [4].

Country	*Gastric ulcer*	
	Men	*Women*
Australia	29	21
Austria	49	29
Belgium	48	26
Canada	24	14
Denmark	41	34
England	32	25
Finland	35	23
France	42	18
West Germany	56	26
Greece	30	17
Iceland	17	25
Ireland	26	21
Italy	40	16
Japan	95	60
Netherlands	31	23
New Zealand	28	21
Northern Ireland	25	18
Norway	25	18
Poland	65	25
Portugal	84	31
Scotland	25	24
Spain	68	24
Sweden	60	44
Switzerland	32	26
U.S.A.	19	12

The epidemiology of PUD is very scattered around the globe predominantly facing dietary stigmas. Gastric and duodenal ulceration is more common in Mediterranean countries like Portugal, Spain, Greece and Italy with an incidence of approximately 9%, but also very high incidence in Japan with 19% of the population suffering from this pathology [19]. Other studies show a 3,4% incidence in the Iranian population [20] and 5% in the Danish population [21]. Although it is continuously decreasing, the death rates are still high. Japan leads the list (Table1.1) followed by Portugal [4].

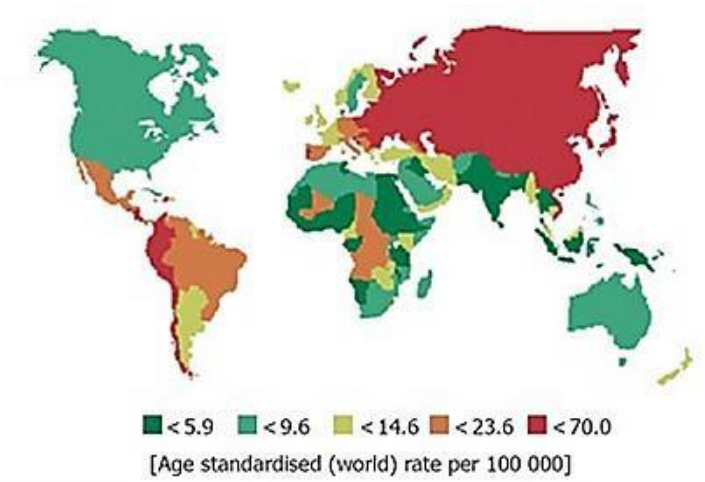


Figure 1.4 the incidence of intestinal-type gastric adenocarcinoma, as of 2004 [4].

At the bottom we can find the USA and Iceland. Men usually suffer and die more from PUD than women.

Gastric adenocarcinoma follows the same patterns as PUD, mostly very high in countries like Portugal, Russia and Japan and very scarce in countries like the USA (Figure 1.4).

The diagnosis of gastric adenocarcinoma and PUD is usually carried out by a gastroenterologist or pathologist, through an Upper Gastrointestinal Endoscopy (UGE) [22]. This procedure uses an endoscope, a small flexible tube-like structure with a light and a camera on its tip that is inserted in the patient's mouth and driven from the oesophagus until the stomach and used for searching for ulcers and other lesions of the upper gastrointestinal tract [22].

Table1.2 Classes of drugs with proven effec t on healing of PUD [1].

	Mechanisms	Use
H2-receptor antagonists (cimetidine, ranitidine, famotidine, nizatidine, roxatidine)	Acid inhibition	*H pylori*-negative peptic ulcer; replaced by PPI because of inferiority in acid suppression
PPI (omeprazole, pantoprazole, lansoprazole, rabeprazole, esomeprazole)	Most potent acid inhibition	Standard treatment for all *H pylori*-negative peptic ulcers; prevention of NSAID or aspirin ulcers; essential component in eradication regimen; given intravenously in bleeding ulcers
Prostaglandin analogues* (misoprostol)	Increase mucosal resistance; weak acid inhibition	*H pylori*-negative gastric ulcer; prevention of NSAID ulcers
H pylori eradication regimens (PPI plus two antibiotics)	Cure of *H pylori* infection	Standard therapy in all *H pylori*-positive ulcers
Bismuth salts (subcitrate, subsalicylate)	Weak antibacterial effect; increase of mucosal prostaglandin synthesis	In quadruple therapy for *H pylori* eradication

Several mucosal protectives used in some countries (ie, sucralfate, rebamipide, and others) do not have sufficient trial documentation to be included in the efficacy comparison with the listed standard therapies. PPI=proton-pump inhibitor. NSAID=non-steroidal anti-inflammatory drug. *Contraindicated in pregnancy.

Table 1.3 Helicobacter pylori eradication regimens [1].

First-line options (7–14 days)

- In populations with less than 15–20% clarithromycin resistance and greater than 40% metronidazole resistance: proton-pump inhibitor (PPI) standard dose, clarithromycin 2×500 mg, and amoxicillin 2×1000 mg, all given twice a day
- Less than 15–20% clarithromycin resistance and less than 40% metronidazole resistance: PPI standard dose, clarithromycin 500 mg, and metronidazole 400 mg or tinidazole 500 mg, all given twice a day
- In areas with high clarithromycin and metronidazole resistance: bismuth-containing quadruple therapy

Second-line option (10–14 days)

- Bismuth-containing quadruple therapy
- PPI plus metronidazole and amoxicillin, if clarithromycin was used in first-line treatment (in Latin America and China, furazolidone 2–4×100 mg is often preferred over metronidazole)

Rescue therapies (10–14 days)

- PPI twice a day plus amoxicillin 2×1000 mg with either levofloxacin 2×250 (500) mg, or with rifabutin 2×150 mg

1.5 - Treatment

Having in consideration the cause of the ulceration lesion, *H. pylori* and/or NSAIDS-related, treatment can be different (Table 1.2).

Usually, *H. pylori*-related lesions are treated to eradicate the colonization. The therapy is done using antibiotics and acid inhibiting Proton-pump inhibitors (PPI). Two antibiotics often used in eradication regimens are clarithromycin and metronidazole but antibacterial action by itself is not sufficient (Table 1.3) [1]. For more complex matters usually a more thorough regime must be followed.

In patients with NSAID-related ulcers treatment starts by discontinuing the use of the drug, which sometimes can cause great discomfort to the patient and interrupt other treatments. Following this, the administration of PPI drugs and H2-receptor antagonists should heal the gastric lesion in 8 to 10 weeks [1].

Since the connection of the bacteria *H. pylori* to most gastrointestinal tract pathologies, many have sought to eradicate it. The use of antibiotics like amoxicillin, clarithromycin, tetracycline and metronidazole increased leading to an increase in resistances, which lead to stricter antibiotic regimens. (Reviewed in [23, 24]).

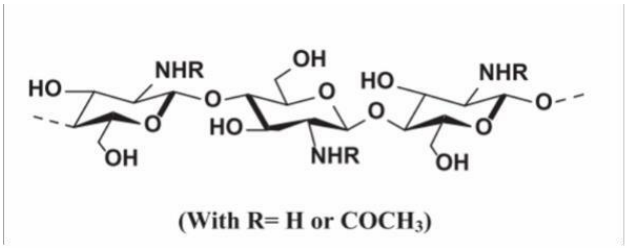
Antibiotic/antimicrobial resistance is important because it leads to treatment failure and overall development of extremely pathological bacteria. Antibiotic/ antimicrobi al resistance has been classified by the World Health Organization (WHO) as an emergent global problem, and that the use of alternatives to these drugs should be developed [25-27]. As of recent years, in PUD and other internal bleeding gastrointestinal (GI) lesions, Hemospray® by Cook Medical USA (patented in 1990) has been able to secure most of the urgent medical cases. It consists of an inorganic powder that achieves hemostasis by fixing on the bleeding site, which leads to mechanical tamponade and, by concentrating and activating platelets and coagulation factors, promotes thrombus formation [28]. It has been a remarkable success, with only 5% of failure, due to aneurism and excess thrombus formation in some patients [28]. However, in most European hospitals, the use of antibiotics and PPIs is still a first choice due to the elevated cost of the Hemospray. As another approach, as Guerrero, Oyarzun-Ampuero and his team thoroughly reviewed in 2016, technologies have been developed for the hydrogel nanoparticle -mediated release of drugs to improve he efficacy of the treatment of PUD and PUD -related gastric adenocarcinoma [29].

1.6 - Chitosan hydrogels: properties and applications

Chitin is a natural mucopolysacharide that can be found in the shells of s everal crustaceans, like shrimp or crabs. It is a supporting material like cellulose in structure, abundance and function. Chitin undergoes N-deacetylation in which the resulting substance is Chitosan [30].

Chitosan is a linear polysaccharide composed of randomly distributed ß-(1→4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (Figure 1.5A) is widely used in biomedical research due to its abundance and function but also its characteristics, mainly its muco-adhesive properties to the mucosal tissues [30]. This results from the strong electrostati c adhesive force between the cationic positively charged Chitosan N-H amines (Figure 1.5B) and the anionic negatively charged mucosal surface. The electrostatic interactions between Chitosan and the mucin layer of the stomach is stronger in acidic conditions, due to the protonation of the amines of the Chitosan molecule [5].

A



B

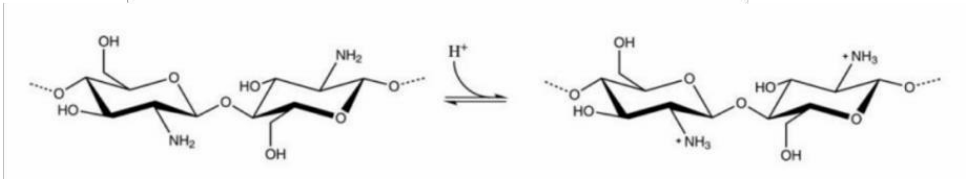


Figure 1.5 - A The c hemic al structure of Chitosan [2] while in **B** the figure shows the Chitosan balance when in an acid environment, exposing the cationic amines that bind to the negatively charged mucins [5].

There are several factors that can affect Chitosan stability, namely temperature [5], humidity [5, 31, 32] and pH [31], being the environmental pH the most relevant [2]. Because of the amine side groups, with increasing acidity, though it becomes more adhesive, Chitosan also becomes less stable and more soluble, meaning that in the gastric mucosa due to the acid juices the hydrogel could be degraded via hydrolysis [5]. To solve this issue of endurance and stability of Chitosan there are different methods to stabilize and to modulate its properties, such as crosslinking what with what and conjugation with what. Crosslinking is a method that is accomplished by creating chemical bonds using the reactive amine side groups that exist in the polymer chains of the Chitosan molecule [2]. It has been previously proved that crosslinked Chitosan increases its mechanical strength, decreases aqueous swelling and permits control of solubility in low pH enviroments because these reactive groups are capped from the acidic environment [2, 32]. Since Chitosan muco-adhesive properties depend on the number of primary amines available in the Chitosan conjugation functionalizes these amino groups with any functional group, used for various applications [33-36]. Some studies have been published regarding the use of Rose Bengal (RB) for this type of Chitosan functionalization. Rose Bengal is a xanthene dye, commonly used in corneal histological colouring. These latter studies have shown that Chitosan-RB conjugates maintain the mucoadhesion properties of the Chitosan hydrogel, but avoid the acid environment hydrolysis, possibly by restricting the reactive amino groups [37-39]. Lauto *et al*, in 2010 also showed a correlation between the use of irradiated light to enhance the muco-adhesive properties of the RB-Chitosan hydrogel's properties for a sutureless treatment for nerve traumatic truncation [38] and calf intestine perforation [40]. The mechanism for this conjugation was never fully disclosed by Lauto and his team, though some papers have shed some light on this, considering xanthene dyes' photobonding chemistry [3]. Encinas, and his

team, refer to a radicalization mechanism between any xanthene dye (like RB) with reactive amines, usually from peptides, proteins or monosaccharides when the compounds are placed under visible green light λ= 532nm (Figure 1.6A-B).

A

$$Dye^{2-} + h\nu \rightarrow {}^{1}(Dye^{2-})^{*}$$

$${}^{1}(Dye^{2-})^{*} \rightarrow \text{deactivation}$$

$${}^{1}(Dye^{2-})^{*} + Am \rightarrow \text{quenching}$$

$${}^{1}(Dye^{2-})^{*} \rightarrow {}^{3}(Dye^{2-})^{*}$$

$${}^{3}(Dye^{2-})^{*} + Am \rightarrow [(Dye^{2-})^{\bullet -} Am^{\bullet +}]$$

$${}^{3}(Dye^{2-})^{*} \rightarrow \text{deactivation}$$

$$[(Dye^{2-})^{\bullet -} + AmH^{\bullet +}] \rightarrow Dye + Am$$

$$[(Dye^{2-})^{\bullet -} + AmH^{\bullet +}] \rightarrow (DyeH^{2-})^{\bullet} + (Am(-H)^{\bullet}$$

B

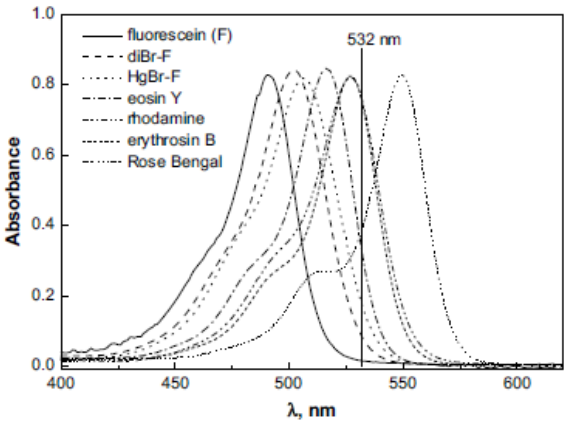


Figure 1.6 - A mec hanism for xanthene dye (like RB) radic alization with reac tive amine in Chitosan, adapted from [3] . In B, Encinas and his team show the absorbance spectrum for the conjugation reaction for different xanthene dyes, depicting the maximum absorbanc e wavelength for RB [3]

According to Encinas and his team, the bond between RB and Chitosan would be a -CONHR amide group between the excited triplet C=O of the RB and the Chitosan's reactive amine. In our work, we hypothesized that the RB-Chitosan conjugation will allow for a resistant and flexible film that can adhere to the gastric mucosa without dissolving under acidic environment.

With these characteristics, this RB-Chitosan film would be viable as a direct treatment for PUD, allowing the mucosa to regenerate without the arresting of the gastric acid.

Chapter 2

Materials and Methods

2.1 - RB-Chitosan films casting optimization assays

Different concentrations of purified-Mahlani medical grade Chitosan (90% deacetylation) were weighted. Under stirring 50 mL of MiliQ water were poured and left with the Chitosan to hydrate for 30 minutes. Afterwards,144 µL of Glacial acetic acid (Fluka) were added to the previous under stirring inside the Hotte. The Chitosan solutions were left, under horizontal stirring (Certomat by BRAUN), at 50°C until fully dissolution. The Chitosan solutions were centrifuged (Sigma 3-12 by BRAUN) for thirty minutes, at 5000rpm, for degasification and debris separation. RB (Sigma) was then added according to the respective concentrations shown below.

Afterwards each 50mL solution was poured, into a 100mmx20mm Pyrex Petri Dish and left to dry for 48h under the Hotte. All these procedures were performed either under the Hotte. Thickness of the patches was then measured using a stereo microscope (Magnifier Olympus SZX10).

Table 2.1 - Different concentration of Chitosan and RB used in the Chitosan -RB films throughout this work * the concentration Lauto and team used in 2010 for a sutureless treatment for nerve traumatic truncation.

Chitosan-RB films	%(w \v)	Chitosan (g) per 50mL	RB (m g) per 50mL
$C_{0,75}R_{0,01}$	0,75 % w /v Chitosan + 0.01% w/v RB	0,0375	5,0
$C_{1,5}R_{0,01}$ *	1,5% w /v Chitosan + 0.01% w/v RB	0,75	5,0
$C_2R_{0,01}$	2,0 % w /v Chitosan + 0.01% w /v RB	1,00	5,0
$C_{1,5}R_{0,005}$	1,5% w /v Chitosan + 0.005% w /v RB	0,75	2,5
$C_{1,5}R_{0,2}$	1,5% w /v Chitosan + 0.2% w /v RB	0,75	10,0
$C_{0,75}$	0,75% w /v Chitosan	0,75	-
$C_{1,5}$	1,5% w /v Chitosan	1,50	-
C_2	2,0% w /v Chitosan	1,00	-

2.2 - Acid dissolution assay

Chitosan-RB films were cut to 15mm x 15mm squares, weighed and put into 12 well plates. These wells were after filled with 5mL Simulated Gastric Fluid solution(Sigma) pH=1,1 to 1,3 (at 25˚C) each. Each Chitosan-RB film was kept in gastric acid, at 37˚C, for a different time. After, the film was taken from the well, dried for 24h, in a 37 ˚C incubator, as previously described [41], and weighed again to allow compare with the same film, before Simulated Gastric Fluid incubation. The time points used were 1h, 2h, 12h, 24h, 2 days, 3 days , 1, 2 e and 4 weeks. For time points longer than 24h, the Simulated Gastric Fluid was replaced every 24h. As a negative control the respective Chitosan-RB film was incubated in PBS, instead of Simulated Gastric Fluid, and as a positive control, the C1,5 film, containing only Chitosan, was used. This assay was done parallel to stereo Microscope imaging (Magnifier Olympus SZX10).

2.3 - In vitro biocompatibility assay – according to ISO 10993-12:2016

MKN74 gastric adenocarcinoma cell line, kindly provided by Dra Carla Oliveira, were used throughout this assay.

These cells were kept in culture under well-established conditions of O2, CO2 and humidity [42], in an incubator (ESCO) . The cells were continuously maintained in RPMI media (GIBCO) supplemented with 10% FBS (GIBCO), and 1% Penicillin/Streptomycin, in T75 flasks. The cells were cultured every other day, using trypsin to release cells anchorage, and a split ratio of 10:1. The passage number while working the biocompatibility assay was 61 -64.

MKN74 cells were then cultured in 12 wells plates - previously placed with the Chitosan-RB films, as stated previously. Visual cell morphology and proliferation was assayed through Inverted Fluorescence Microscopy.

The protocol that was followed for the biocompatibility assay was the Resazurin metabolical activity assay. 100k cells were seeded into each well in a 12 well plate. These were left to adhere for 24 hours. After the cells were either placed in direct contact with the Chitosan-RB film, or Chitosan-Rb conditioned medium, except controls. For positive control cells were cultured normally, and for negative control 0.02% of H 2O2 was added to the culture medium. For the conditioned medium, following the rulings of the ISO 10993 -6:2016, 5 cm^2 of film were cut and left for 24h incubating in 50mL of normal culture medium, at 37˚C. Afterincubation, the conditioned medium was used as culture medium for the MKN74 cells. 24h later the cells were incubated with 20% Resazurin (in RPMI 20%FBS, 1%P/S) for 3 hours. After

incubation, the samples' fluorescence was measured using a 560 nm excitation/590 nm emission filter set, in a Multimode microplate reader (SynergyTM 2).

2.4 - Ex vivo mucoadhesion texturometer assay

The mucoadhesion assay was performed using the Texturometer TA - XT2 Texture Analyzer (Stable Microsystems, Surrey, England), using a previously described mucoadhesion protocol [43, 44], also described in Figure2.1.

Male Winstar rats, kindly provided by Doutora Joana Alves, Doutora Filipa Sousa and Mestre Sofia Macedo the I3S Animal Facility, were used throughout this assay. These animals were left under fasting 24 hours prior to euthanasia. The animals were anesthetized the Animal facility's CO2 chamber or by cervical dislocation, by their holders, in ICBAS (BALB\c mice), the Animal House of the Faculty of Medicine (Winstar rat) and the I3s' Animal Facility.

MUCOAHESION ASSAY:
Pre-Test Speed: 0,10 mm/s
Test Speed: 0,10 mm/s
Post-Test Speed: 0,50 mm/s
Applied Force: 30g
Return Distance: until breaking point
Initial time of contact: 20 s
Probe 5mm Cyl . Stainless Steel

Figure 2.1 - Mucoadhesion assay as described by Piccelle *et al* and optimized by Muldoon *et al*.

Right after, in a specialized flow hood (Sanyo), the stomach of the animals was removed, and the cleaning of the food remains was performed using heated PBS medium injected through a 5mL syringe. After cleaning, the animals' stomachs were cut through the longer curvature, opened and cleaned again with PBS. The organs were frozen with 20%FBS RPMI medium for future use.

Circular cut-outs, with 1,50 cm of diagram, of rat's stomach mucosa were placed on the device's platform, a cylinder probe with a 5mm diameter with an attached Chitosan-RB film punches the mucosa and measures delay and opposite force done by the adhesion of the film to the mucosa.

2.5 - Dynamic Mechanical Analysis:

Mechanical properties of the were assayed by using a DMA TRITEC2000B (Triton Technology). Chitosan-RB films were cut to a specific size (15x15mm) and then loaded onto the tension mode clamp assembly. Measurements were performed under RT conditions (humidity 44%, 20°C). A user defined small tension load was applied to guarantee an

adequate contact between the samples and the device. A temperature scan (1 Hz, within the linear viscoelastic region (LVR) 25◦C to 50°C, 4°C/min), and the tension storage modulus (E', elastic component), loss modulus (E'', viscous component) and damping (E''/E', tan δ) were calculated. At least five replicas were tested for each condition.

2.6 - Fourier Transform Infra-Red (FTIR) Spectroscopy

Spectroscopic measurements were performed using the Frontier FTIR spectrometer (Perkin -Elmer, USA) coupled with the attenuation accessory ATR (Perkin- Elmer, USA). All measurements were acquired with a spectral resolution of 8 cm^{-1} and a spectral range of 4000–400 cm^{-1} and a mirror speed of 0,2 cm/s. Spectra were analysed without further data retreatment except those where spectra were baseline corrected based on wavenumber using the Spectrum 10 software (Perkin Elmer). The free- ware image spectrometer image analysis software Spectrographs was used for the spectrum image and analysis. The films were cut into a 1,5 x 1,5mm square and placed under the reader with an applied sensor force. The 2 mg of RB powder was directly placed onto the FTIR plate for reading. All samples were repeated three times, for data significance.

Chapter 3

Results and discussion

3.1 - RB-Chitosan films casting optimization assays

Lauto and his team in 2010, for the first time, showed a possible treatment for the traumatic truncation of nerves using a Chitosan-RB film. It adheres to the mucosa around each loose end of each nerve, allowing thus the regeneration of the tissue itself and its components. According to the author, this 1,5% w/v Chitosan + 0.01% w/v RB film (which we will name $C_{1,5}R_{0,01}$) displays a muco-adhesive functionality due to the RB that chemically bonds to the mucosa's epithelial components, as described in Figure 1.5A. We hypothesized that this mechanism could be like a Chitosan crosslink mechanism, capping the amine ends of the Chitosan chains, preventing its dissolution in low pH solutions, like the gastric acid. Taking up this hypothesis, this film could be used as a treatment for PUD. The Chitosan-RB film would adhere to the gastric mucosa and protect it against the low pH of the gastric acid, allowing the tissue to regenerate without changing the gastric environment.

To test our hypothesis, we decided to try different formulations beginning with the formulation by Laouto and his team, $C_{1,5}R_{0,01}$. To best assess the function of each of its components we decided change the concentration in each, that can be seen in Table 2.1. The main visual property of the films is its colour. All films with RB displayed a strong rose colour, that became stronger with the higher the concentration of RB ($C_{1,5}R_{0,2}$) and less strong in lower concentration ($C_{1,5}R_{0,005}$). Another characteristic was the Thickness of the film itself, that increases more significantly when the concentration of Chitosan increases ($C_2R_{0,01}$) and decreases when the concentration is lower (Table 3.1). This also happens when there is no RB in the formulation ($C_{0,75}$, $C_{1,5}$, C_2), with increasing concentration of Chitosan the thickness of the films also increases, and since no RB was added, no rose colour is displayed.

With the increase in thickness, the film became less malleable and elastic, and more rigid, showing that some mechanical characteristics changed.

Table 3.1 - Different concentration of Chitosan and RB used in the Chitosan -RB films throughout this work. * Thickness of the patches was then measured using a stereo microscope and image processing software ImageJ with a sample of n=6 each concentration, ** this is the concentration Lauto and team used in 2010 for a sutureless treatment for nerve traumatic truncation.

Chitosan-RB films	%(w \v)	Thic kness (mm)*
$C_{0,75}R_{0,01}$	0,75 % w/v Chitosan + 0.01% w/v RB	0,059 ±0,010
$C_{1,5}R_{0,01}$ **	1,5% w/v Chitosan + 0.01% w/v RB	0,065 ±0,008
$C_2R_{0,01}$	2,0 % w/v Chitosan + 0.01% w/v RB	0,102 ±0,009
$C_{1,5}R_{0,005}$	1,5% w/v Chitosan + 0.005% w/v RB	0,067 ±0,009
$C_{1,5}R_{0,02}$	1,5% w/v Chitosan + 0.02% w/v RB	0,066 ±0,010
$C_{0,75}$	0,75% w/v Chitosan	0,052 ±0,013
$C_{1,5}$	1,5% w/v Chitosan	0,062 ±0,012
C_2	2,0% w/v Chitosan	0,110 ±0,006

3.2 - Dynamic Mechanical Analysis:

According to ISO 13485:2018, on the implantation of Medical Devices on the Surface of Mucosal Tissues, a film or hydrogel or patch to be implanted with direct contact on the surface of a mucosa should have a viscoelasticity ratio that is equal or lower than that of the tissue where it shall be implanted. That means that a film that is to be implanted in the gastric mucosa should be as elastic as the stomach tissue, or more. The stomach is one of the most elastic tissues in the organism, mainly due to components of the extra-cellular matrix (ECM) laminin and collagen type IV, that allow the extension and distension of the tissue during peristalsis [45]. We thus used the DMA to measure the viscoelasticity modulus for the different Chitosan-RB formulations. All hydrogel or film melts are viscoelastic materials, that have a viscous component and an elastic component [46]. Through a series of precise vertical tension movements, the DMA assesses the elastic and viscous modulus of the film, putting it to a ratio called tan delta or viscoelastic ratio. The tan delta also changes with temperature, because some reaction can take place within some temperature frame changing its properties [46]. This ratio allows to compare different materials, tissues or hydrogels to assess their elastic and viscous properties. According to Sellers and his team, the viscoelastic ratio of the gastric tissue

mucosa and its glycocomponents is 0,25 for physiological temperatures until 50˚C [47]. This mechanical ratio has been previously confirmed [45, 48, 49]. Having in consideration the mechanical property prerequisites of the ISO 13485:2018, all the formulations are within the permitted values to be used as a possible implantation hydrogel in the stomach mucosa, since their tan delta (viscoelastic ratio) is lower than that of the tissue itself. This means that all the Chitosan-RB films produced are more elastic than the gastric mucosa tissue.

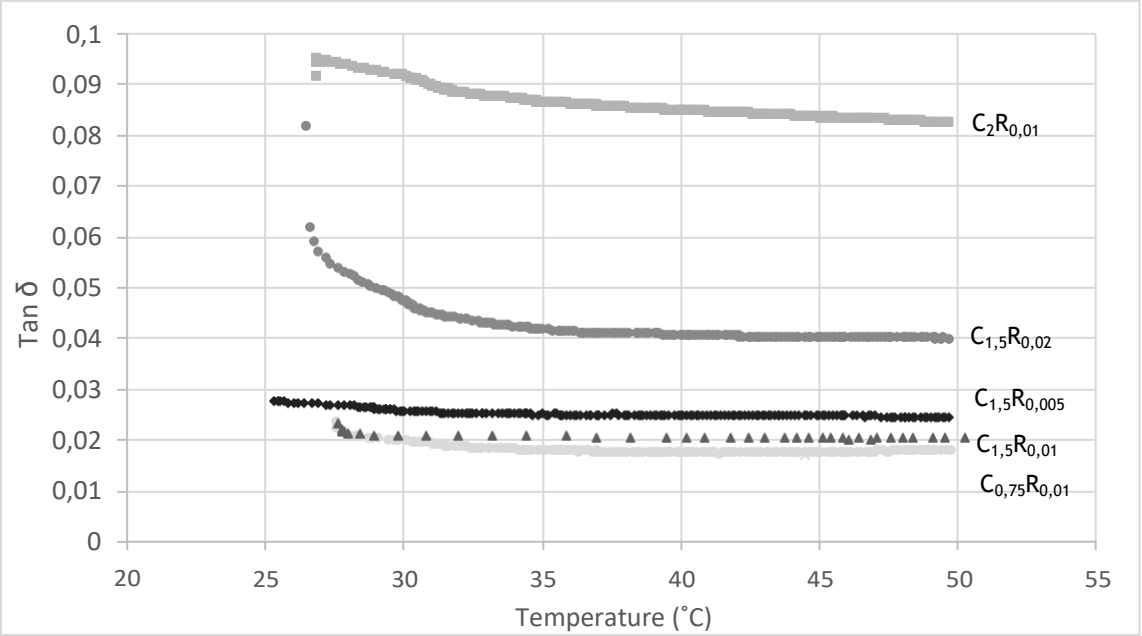


Figure 3.1 - Average tan δ values for the five different Chitosan -RB formulations n=3, DM A mechanical temperature assay in tension mode, 1mm of displacement and 1Hz frequency of vertical movement, temperature range from 25˚C to 50˚C with a rate of 3˚C/min. For each formulation, three signific ant samples were used (n=3).

Within the different formulation, we can also notice some significant changes that correlate with the thickness of the film. As expected, films with higher Chitosan concentration, thus thicker hydrogels, have less elasticity than films with less concentration of Chitosan. This can be seen if we compare $C_2R_{0,01}$ (the less elastic of the formulations and thickest) with $C_{0,75}R_{0,01}$ (the most elastic of the formulations and thinnest). The RB doesn't have a significant effect on the film's mechanical properties when Chitosan concentration is maintained. If we compare $C_{1,5}R_{0,01}$ and $C_{1,5}R_{0,005}$, when the RB concentration is halved the tan delta does not change significantly, nor when we compare $C_{1,5}R_{0,01}$ and $C_{1,5}R_{0,02}$. Within the temperature range in which the experiments were done, we also did not see any statistically significant effect of the temperature on the mechanical properties of the films.

3.3 - Fourier Transform Infra-Red (FTIR) Spectroscopy

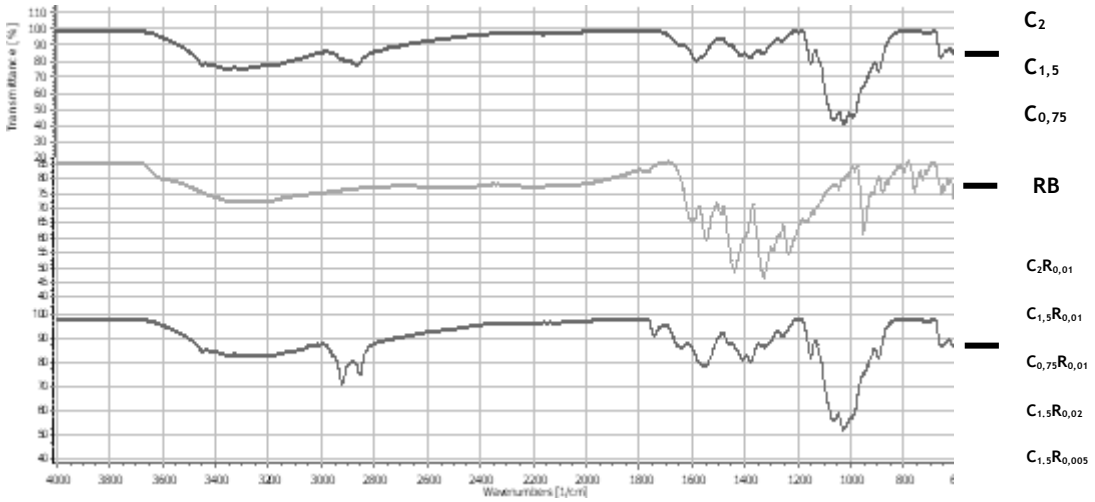


Figure 3.2 - FTIR spectra of RB powder and Chitosan-RB formulations. Spectra were analysed without further data retreatment except those where spectra were baseline c orrected based on wavenumber using the Spectrum 10 software (Perkin Elmer). The free -ware image spectrometer image analysis software SpectraGryph was used for the spectrum image and analysis.

Table 3.2 - FTIR spectra band peak table for highest peaks oh the RB powder and chitosan formulations. The free-ware image spectrometer image analysis software SpectraGryph was used for the spectrum analysis and table. a) RB peak wavenumbers ref (Jiao, Wu et al. 2016) b) Chitosan film peak wavenumbers ref (Krishna Rao, Vijaya Kumar Naidu et al. 2006).

Peak wavenumber (cm^{-1})	**RB**	**ref**	**Chitosan film**	**ref**	**Chitosan-RB film**
759	-C-Cl	a)	not present	-	not present
880	aromatic ring vibration	a)	not present	-	aromatic ring vibration
1020	-Na-O	a)	not present	-	-Na-O
1089	not present	-	-C-O	b)	-C-O
1235	-C-O-C-	a)	-C-O-C-	b)	-C-O-C-
1332	not present	-	Amide Band II	b)	Amide Band II (lower)
1339	-C-O-O	a)	not present	-	not present
1405	not present	-	Amide Band III	b)	Amide Band III
1448	aromatic ring vibration	a)	not present	-	not present
1492	aromatic ring vibration	a)	not present	-	not present
1547	aromatic ring vibration	a)	not present	-	not present
1613	-C-O-O	a)	not present	-	not present
1650	not present	-	Amide Band I	b)	Amide Band I
1747	not present	-	not present	-	Possible -CONHR
3446	-OH	a)	-OH / -NH	b)	-OH / -NH

What we hypothesized was that the chemical mechanism that Lauto and his team described in the reaction of Chitosan and RB, is also a crosslink of the Chitosan hydrogel that caps the amine groups in the Chitosan chain, allowing it to resist dissolution in low pH environments. To collect some information on the bonds that form the Chitosan-RB film, or if it isn't just a mesh of its components, we performed FTIR spectroscopy.

One first observation from the analysis of Figure 3.1, in these formulations, the different concentrations don't have a significant importance. So, in all the Chitosan-RB formulation that had in its composition Chitosan and RB ($C_{1,5}R_{0,01}$, $C_2R_{0,01}$, $C_{0,75}R_{0,01}$, $C_{1,5}R_{0,005}$, $C_{1,5}R_{0,02}$) the spectra overlay perfectly. The same happened with the films in which the only component was Chitosan (C_2, $C_{1,5}$, $C_{0,75}$). Thus, the different concentrations had no significant effect on the transmittance of the peaks in the assay and no different band showed.

We can also observe that in fact, the Chitosan-RB formulations are not a mesh of the two components in the film (Figure 3.1, Table 3.1). If there had been no reaction, the spectrum of the Chitosan-RB mesh would be an addition of the RB powder spectrum and the Chitosan spectrum, yet we see that other bands appear, more significantly one band arises that is not in the RB spectra nor in the Chitosan's . This band positioned at 1747cm^{-1}, in the Chitosan-RB formulations, could possibly be the -CONHR that Encinas and his team, in 2009, described as the possible photo-mediated bond for conjugation of RB with a reactive amine. Some papers have already described the appearance of this band, related to the duality of the bond C=O and C-N in this -CONHR amide, in proteins, at the same wavenumber [50, 51].

Also, in the Chitosan-RB formulations, the Amide II band shows a lower transmittance than the Chitosan film spectra alone. Since the Amide II band is due to the stretching vibration of the N-H bending, we could hypothesize that the lack of transmittance in the Chitosan-RB formulations means that this N-H bond is taking part in the RB-Chitosan bond. For more information, we would still need to perform other types of analysis on these formulations to have more insight on the nature of these bonds.

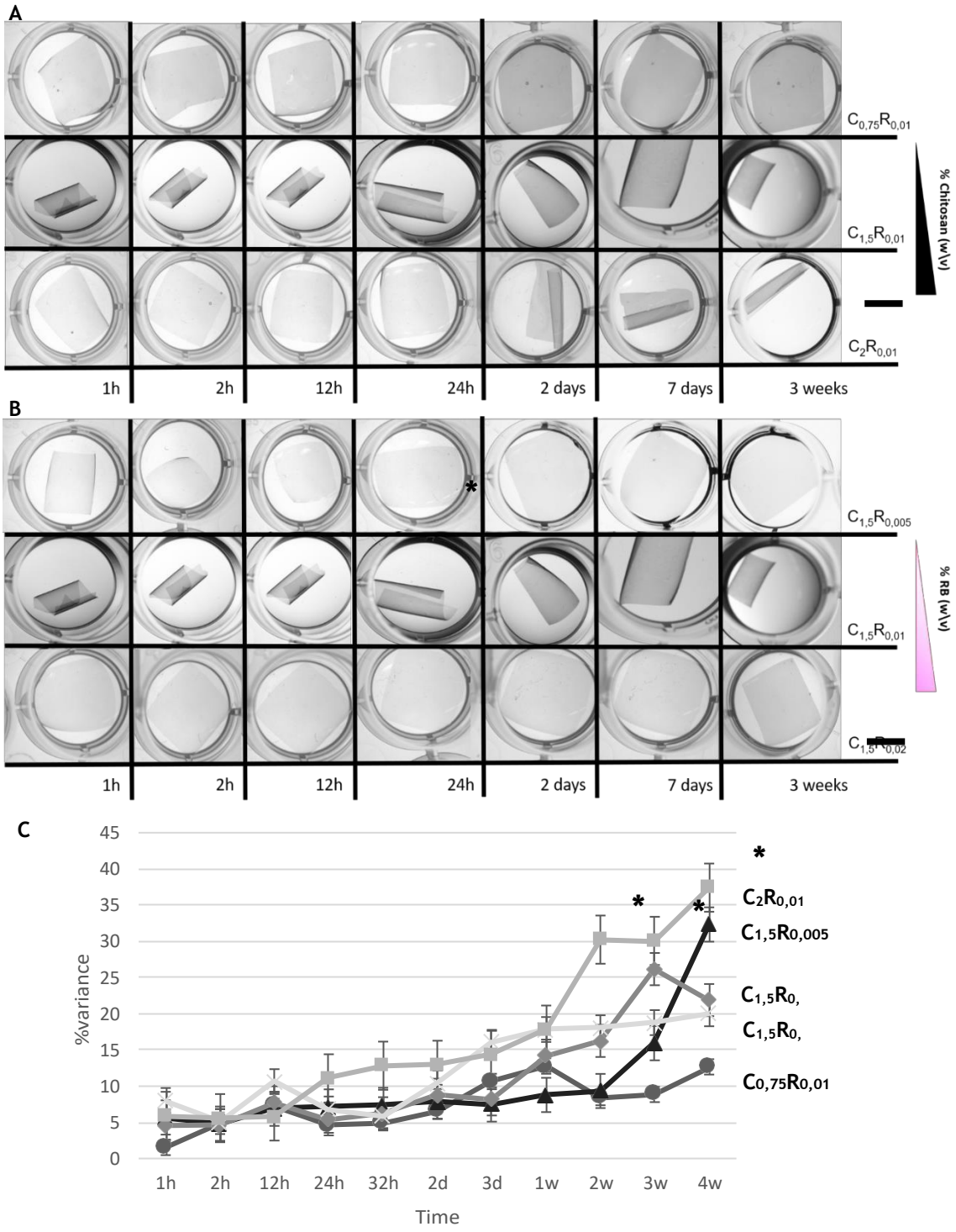


Figure 3.3 - A Stereo microscope (Magnifier Olympus SZX10) 5 x 1.5mm lense, 40mm base images of the Chitosan-RB formulations with increasing Chitosan % (w/v) during incubation in Simulated Gastric Fluid, and respective time point. Positive control not shown due to instantaneous degradation, scale = 1 cm; **B** Stereo microscope (Magnifier Olympus SZX10) 5 x 1.5mm lense, 40mm base images of the Chitosan-RB formulations with increasing RB % (w/v) during incubation in Simulated Gastric Fluid, and respective time point. Positive control not shown due to instantaneous degradation, scale = 1 cm; **C** % of weight variance between the Chitosan-RB film prior to Simulated Gastric Fluid incubation, and after incubation. Each formulation and timepoint was repeated three time (n=3). Positive control not shown due to instantaneous degradation. * statistical significance following mathematical analysis $p<0.001$.

3.4 - Acid dissolution assay

One important step in the development of a possible treatment for PUD is the gastric environment's low pH [52]. Though the Chitosan film on its own would be a proper barrier to adhere to the mucosa allowing the tissue to heal, the hydrogel instantaneously dissolves in low pH solutions, due to the acid-base characteristics of the N-H bonds in Chitosan [53]. If the Chitosan-RB reaction, like we hypothesized, functionalizes the N-H bond in Chitosan, the Chitosan-RB formulations will not dissolve in an acid solution. We, thus, performed a dissolution assay, using Sigma's Simulated Gastric Fluid, that besides the HCl, (accountable for the low pH), also has in its composition digestive enzymes , like gastric lipase and pepsin, that breakdown lipids and peptides, respectively.

As we can see from Figure 3.2 A and B, none of the Chitosan-RB formulations fully dissolved in the Simulated Gastric Fluid, for three weeks. These results show that there is in fact a reaction occurring between the Chitosan's N-H Bond and the RB, though no information is shed on its nature. This also shows that our formulations are not a Chitosan-RB mesh, because without any new bond substituting the N-H bond in Chitosan, with or without the RB, the formulation would dissolve. The results also show us that the Chitosan-RB ratio is to be considered. From Figure 3.2C, we can see that $C_2R_{0,01}$, the formulation with the highest concentration (w\v) of Chitosan was the one that got the most degraded with time (approx 37% over 4 weeks), during incubation, while $C_{0,75}R_{0,01}$ with the same RB concentration (w/v) was the least degraded over time (approx 12% over 4 weeks). For the same concentration of Chitosan ($C_{1,5}R_{0,02}$, $C_{1,5}R_{0,01}$, $C_{1,5}R_{0,005}$), with increasing RB concentration(w/v), the degradation decreases (approx 32%, 30% and 20%, respectively, over 4 weeks). The same happens when the Chitosan concentration (w/v) decreases. For $C_2R_{0,01}$, $C_{1,5}R_{0,01}$ and $C_{0.75}R_{0,01}$, with decreasing concentration (w\v) of Chitosan, the degradation decreases meaning that the Chitosan-to-RB ratio is more important than the sole increase of RB. We can also see that with $C_{1,5}R_{0,02}$ and $C_{0,75}R_{0,01}$, where even though the RB concentration(w\v) is higher the film over time has a higher degradation than the formulation with less RB concentration (w\v), yet with less Chitosan concentration(w/v). Even so, all formulations, over the 4 weeks timespan, could be used in a clinical environment due to their low degradability in acid medium.

3.5 - In vitro biocompatibility assay – according to ISO 10993-12:2016

According to ISO 10993-12:2016, on the implantation of Medical Devices on the Surface of Mucosal Tissues, in order to be EU approved for clinical use, a film or hydrogel or patch to be implanted with direct contact on the surface of a mucosa, for a prolonged period, over 30 days, should have a high (over 75%) of biocompatibility, during both Direct Contact assays and Conditioned medium (Eluition) assays. As we can see from Figure 3.3, all formulations were within the boundaries set by the ISO. In each formulation, the direct contact showed less compatibility then conditioned medium, possibly due to cell death where the film was placed on the well, after 24h incubation, as the ISO states. The RB concentration (w\v) is important for the films biocompatibility, C1,5R0,005 with less RB content shows higher compatibility while C1,5R0,02 with more shows less compatibility, both in a direct contact assay and in a conditioned medium assay. Unlike dissulotion in acid medium, Chitosan-to-RB does not have any significancy because, when the Chitosan content is changed, for C2R0,01, C1,5R0,01 and C0.75R0,01, we see no significant changes in cell biocompatibility with MKN74 cell line.

3.6 - Ex vivo mucoadhesion texturometer assay

To test mucoadhesion, *Ex vivo* texturometer assay was used. From Figure 3.4 A, we can see the Work of Adhesion, meaning the Force per Area, needed to detach the film from the mucosa [54]. From the results, we see that the Chitosan content has a more significant role in adhesion than RB. From the Figure, we see that both C2R0,01 and C2 have the equally highest adhesion values and C0,75R0,01 and C0,75 have equally lowest, for the same RB concentration (w\v). If we compare the Chitosan-RB film with its Chitosan film concentration counter part we also see there is never a significant icrease or decrease in Work of Adhesion. For this conclusion, we can also see that for C1,5R0,02, C1,5R0,01 and C1,5R0,005, with decreasing RB concentration(w\v) , show no significant change in the Work of Adhesion of the Chitosan-RB film. With the information we have thus far, we can show that the RB component of the Chitosan-RB film is more important for the acid resistance in gastric environment than for the mucoadhesive properties of the hydrogel.

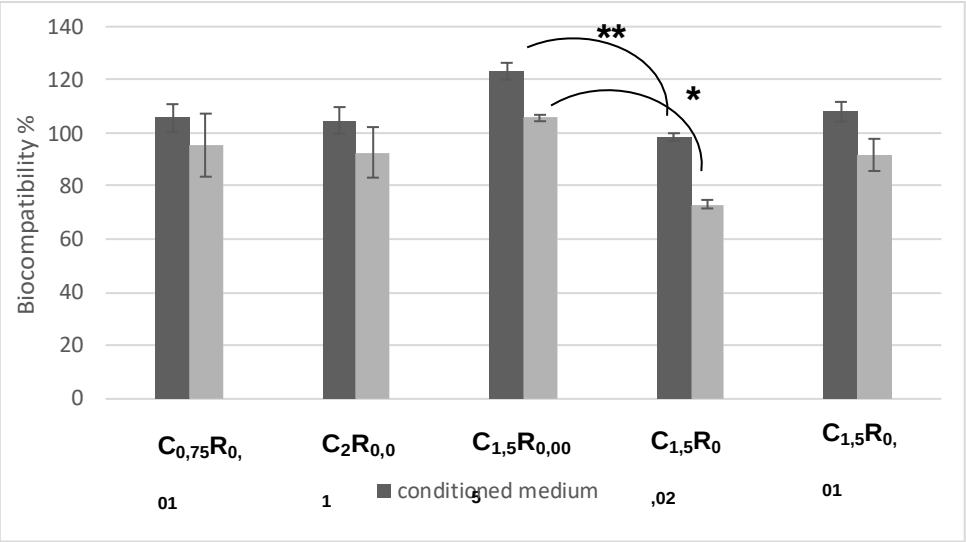


Figure 3.4 - Biocompatibility comparison between the different formulations of Chitosan -RB formulation in MKN74, using Resazurin metabolic activity assay and fluorescence reader Multimode microplate reader (SynergyTM ^{2}). The assay was repeated three times (n=3) * statistical significance following mathematical analysis $p<0.05$ ** statistical significance following mathematical analysis $p\leq0.001$

We can also assess that information in Figure 3.4 C, where we can see the Bonding Distance. This variable tells us of the specificity of the bond between the two materials adhering [54]. If there is specificity in bond between the two materials, the bonding distance is greater than two materials that are bonding without specificity [54]. The biggest statistical significance we can see is between C1,5R0,02, C1,5R0,01 and C1,5R0,005, with decreasing RB concentration(w\v), the bonding distance increases. As previously mentioned, the mucoadhesion between Chitosan and mucosal tissues is due to the electrostatic bonds between the positively charged amines of the chitosan chain and the negatively charged glycoprotei ns from the mucosa's mucous layer. In the Chitosan-RB film, as we discussed previously, our hypothesis is that the Chitosan-RB light- dependent reaction is capping the amines so there is no low pH dissolution, in the gastric environment. It is, thus, understandable that when there's less concentration(w/v) of RB, so more amines, the Chitosan-RB film adheres more specifically to the gastric tissue. Even so, the presence of RB in the film is important, as we can see from Figure 3.4C, because if we decrease the RB concentration(w\v) (like in $C_{1,5}R_{0,02}$, $C_{1,5}R_{0,01}$ and $C_{1,5}R_{0,005}$) the bonding distance increases, but if we take the RB from the film the bonding distance significantly decreases (like $C_{1,5}R_{0,01}$ and $C_{1,5}$ or $C_2R_{0,01}$ and C_2). To know the reason for this decrease we would need to have more information on the nature of this Chitosan-RB bond.

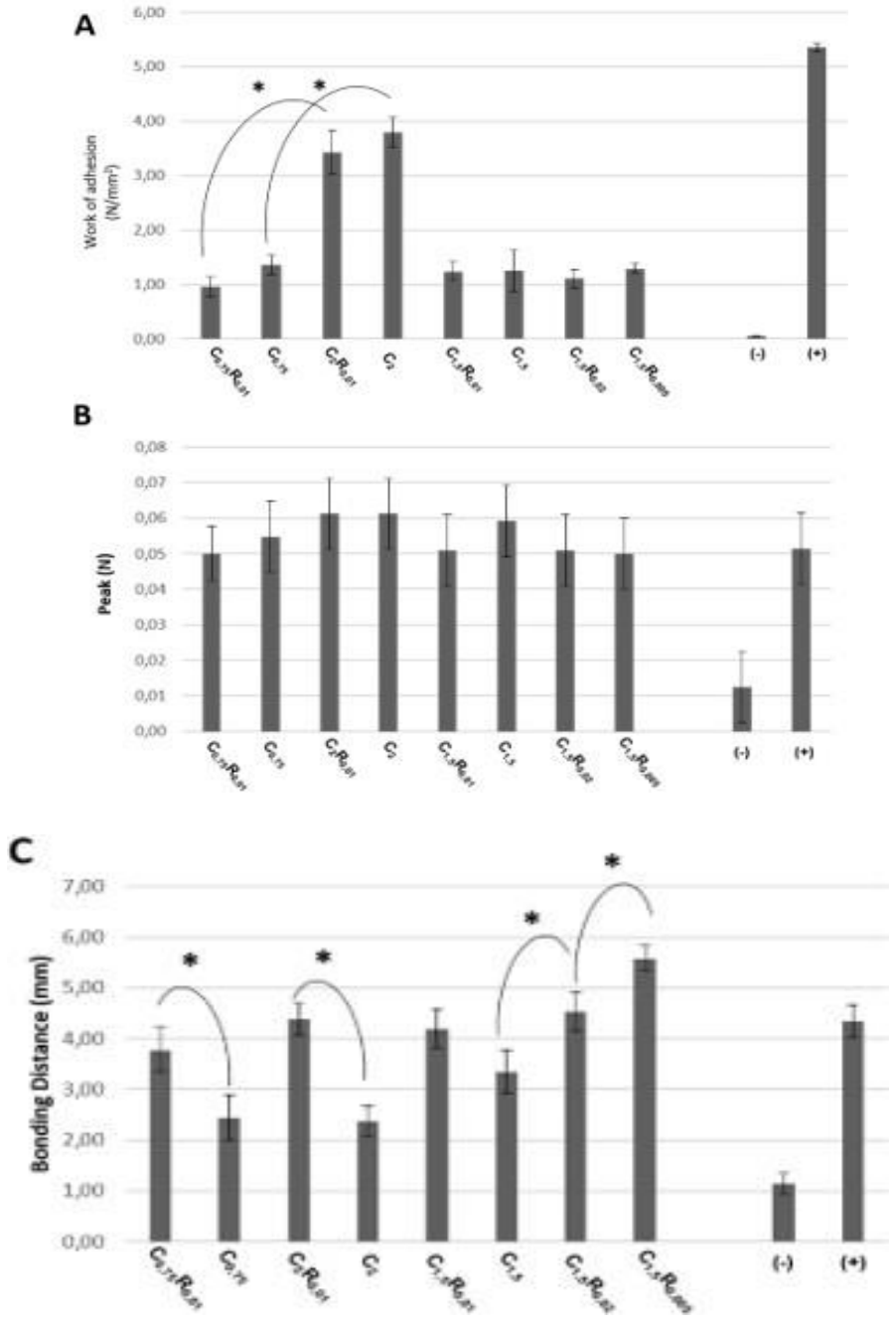


Figure 3.5 - A Work of adhesion, the force needed to detach the respective film per unit of area (mm^2). The positive control used for this assay was duct tape applied to the probe to adhere directly on the gastric tissue whilst the negative control h ad no film to adhere, only letting the probe press unspecifically to the mucosa. **B** Peak force, the highest force (in N) needed to detach the respective film. The positive control used for this assay was duct tape applied to the probe to adhere directly on the gastric tissue whilst the negative control had no film to adhere, only letting the probe press unspecifically to the mucosa. **C** Bonding distance, distance (in mm) needed to detach the respective film. The positive control used for this assay was duct tape applied to the probe to adhere directly on the gastric tissue whilst the negative control had no film to adhere, only letting the probe press unspecifically to the mucosa. * statistic al significance following mathematical analysis $p<0$.

Chapter 4

Conclusion and Future Work

The stomach is a versatile and complex organ that has been the target of an increasing number of pathologies and lesions. Is this work we start to scratch the surface of what we hope to one day be one of therapeutics for one of its antagonists. In this work, we have started to develop a Chitosan-RB film that can adhere to the mucous layer of the gastric mucosa and be used as therapeutics for Peptic Ulcer Disease. This formulation was previously proposed by Lauto and his team, in 2009, for treatment of traumatic truncation of nerve ends. During this work, we have shed some light on the nature of this Chitosan-RB reaction, and its usefulness in PUD treatment.

We showed that the RB allows the Chitosan-RB film to resist acid dissolution, in a mechanism we hypothesized would cap the amine ends of the Chitosan chain in the film. Yet in the future we would still need to perform other tests on the films, like H -NMR, to have more specific information on the film and on the nature of the bonds created. The low pH dissolution is dependent on a Chitosan-to-RB ratio so that the formulation cannot have a high concentrati on of Chitosan to a low concentration of RB. The consequence is that within 3 to 4 weeks the formulation will degrade when in contact with the low pH medium. We also showed that, mechanically, according to the ISO 10993-12:2016, all the formulation can be clinically used in the gastric mucosal tissue, since their elasticity is higher than that of the stomach. This is important because the gastric tissue is a very motile and elastic tissue. The formulation would have to be able to accompany that movement and elasticity during as the peristalsis of digestion, maintaining its physical integrity. Another important step is, according to ISO 10993-12:2016, the biocompatibility of the formulations shows potential to be used for a permanent treatment of the surface mucosa of the stomach. Even though that a higher RB concentration (w\v) shows more cytotoxicity and less biocompatibility with MKN74 cells, these are still within the allowed values of the ISO. This data show a lot of potential, yet acording to the standart, in order to be EU approved for clinical use the film would still have to undergo further testing. Sensibilization tests and intracutaneous reactivity tests should be done in the future, to acquire EU approval and accute systemic toxicity assay should also be done for the formulation to be FDA approved for clinical use. The formulations also adhere to the gastric mucosa. Using the texturometer, we assessed the adhesion of the formulations to the gastric tissue. The Chitosan is significantly more important for the adhesion fo the patch and C2R0,01 is the most adherent formulation. This mechanism could confirm our hypothesis that RB caps the amine groups in Chitosan, allowing

it to resist acid dissolution, but inhibiting a greater adherence to the mucosa. Yet, the adhesion all formulation show would allow their use in a clinical setting. In the future, we would like to use other mucoadhesion assays to find more information on this issue, using also other animal models, like piglets or bovine.

References:

[1] V. Kumar, A. K. Abbas, J. C. Aster, and S. L. Robbins, *Robbins basic pathology*. Philadelphia, PA: Elsevier/Saunders, 2013.

[2] N. Mati-Baouche, P.-H. Elchinger, H. de Baynast, G. Pierre, C. Delattre, and P. Michaud, "Chitosan as an adhesive," *European Polymer Journal,* vol. 60, pp. 198-212, 11// 2014.

[3] M. V. Encinas, A. M. Rufs, S. G. Bertolotti, and C. M. Previtali, "Xanthene dyes/amine as photoinitiators of radical polymerization: A comparative and photochemical study in aqueous medium," *Polymer,* vol. 50, no. 13, pp. 2762-2767, 2009/06/19/ 2009.

[4] K. D. Crew and A. I. Neugut, "Epidemiology of gastric cancer," (in eng), *World J Gastroenterol,* vol. 12, no. 3, pp. 354-62, Jan 21 2006.

[5] S. Dimida, C. Demitri, V. M. De Benedictis, F. Scalera, F. Gervaso, and A. Sannino, "Genipin-cross-linked chitosan-based hydrogels: Reaction kinetics and structure-related characteristics," *Journal of Applied Polymer Science,* vol. 132, no. 28, pp. n/a-n/a, 2015.

[6] A. Allen *et al.*, "Mucus and H. pylori," *J Physiol Pharmacol,* vol. 48, no. 3, pp. 297-305, Sep 1997.

[7] J. R. Clamp and D. Ene, "The gastric mucosal barrier," *Methods Find Exp Clin Pharmacol,* vol. 11 Suppl 1, pp. 19-25, 1989.

[8] W. L. Maxwell, "Wheater's functional histology–a text and colour atlas by H.G. Burkitt, B. Young, and J.W. Heath, 3rd edition, 416 pp. Edinburgh: Churchill livingston; 1993. (Distributed in the United States of America by churchill livingston, inc.). £27.50," *Clinical Anatomy,* vol. 7, no. 1, pp. 52-53, 1994.

[9] S. C. Fiedorek, R. J. Shulman, and W. J. Klish, "Endoscopic detection of peptic ulcer disease in cystic fibrosis," *Clin Pediatr (Phila),* vol. 25, no. 5, pp. 243-6, May 1986.

[10] W. Rubin, "Medical treatment of peptic ulcer disease," *Med Clin North Am,* vol. 75, no. 4, pp. 981-98, Jul 1991.

[11] V. Tolia and R. S. Dubois, "Peptic ulcer disease in children and adolescents. A ten-year experience," *Clin Pediatr (Phila),* vol. 22, no. 10, pp. 665-9, Oct 1983.

[12] A. Ateshkadi, N. P. Lam, and C. A. Johnson, "Helicobacter pylori and peptic ulcer disease," *Clin Pharm,* vol. 12, no. 1, pp. 34-48, Jan 1993.

[13] M. L. Partipilo and P. S. Woster, "The role of Helicobacter pylori in peptic ulcer disease," *Pharmacotherapy,* vol. 13, no. 4, pp. 330-9, Jul-Aug 1993.

[14] B. A. Dijkmans, M. Janssen, J. P. Vandenbroucke, and C. B. Lamers, "NSAID -associated upper gastrointestinal damage in patients with rheumatoid arthritis," *Scand J Gastroenterol Suppl,* vol. 212, pp. 105-8, 1995.

[15] S. Schneeweiss, R. J. Glynn, J. Avorn, M. Mamdani, H. Mogun, and D. H. Solomon, "NSAID switching and short-term gastrointestinal outcome rates after the withdrawal of rofecoxib," *Pharmacoepidemiol Drug Saf,* vol. 18, no. 12, pp. 1134-42, Dec 2009.

[16] J. Sung, "NSAID and PUD complications," *Rev Gastroenterol Mex,* vol. 68 Suppl 3, p. 60, Nov 2003.

[17] C. Arslan Pagnini and M. Rugge, "Gastric cancer: problems in histogenesis," *Histopathology,* vol. 7, no. 5, pp. 699-706, Sep 1983.

[18] P. J. Matley, D. M. Dent, M. V. Madden, and S. K. Price, "Gastric carcinoma in young adults," *Ann Surg,* vol. 208, no. 5, pp. 593-6, Nov 1988.

[19] J. H. Kurata and B. M. Haile, "Epidemiology of peptic ulcer disease," (in eng), *Clin Gastroenterol,* vol. 13, no. 2, pp. 289-307, May 1984.

[20] F. Barazandeh, A. Yazdanbod, F. Pourfarzi, S. G. Sepanlou, M. H. Derakhshan, and R. Malekzadeh, "Epidemiology of peptic ulcer disease: endoscopic results of a systematic investigation in iran," *Middle East J Dig Dis,* vol. 4, no. 2, pp. 90-6, Apr 2012.

[21] S. J. Rosenstock and T. Jorgensen, "Prevalence and incidence of peptic ulcer disease in a Danish County--a prospective cohort study," *Gut,* vol. 36, no. 6, pp. 819-24, Jun 1995.

[22] B. J. Dicken, D. L. Bigam, C. Cass, J. R. Mackey, A. A. Joy, and S. M. Hamilton, "Gastric Adenocarcinoma: Review and Considerations for Future Directions," *Annals of Surgery,* vol. 241, no. 1, pp. 27-39, 2005.

[23] A. Gallay *et al.*, "Campylobacter antimicrobial drug resistance among humans, broiler chickens, and pigs, France," *Emerg Infect Dis,* vol. 13, no. 2, pp. 259-66, Feb 2007.

[24] F. Megraud, "Helicobacter pylori and antibiotic resistance," *Gut,* vol. 56, no. 11, p. 1502, Nov 2007.

[25] P. M. Nischal, "First global report on antimicrobial resistance released by the WHO," *Natl Med J India,* vol. 27, no. 4, p. 241, Jul-Aug 2014.

[26] J. McConnell, "WHO announce strategy to combat antimicrobial resistance," *Lancet Infect Dis,* vol. 1, no. 3, p. 140, Oct 2001.

[27] A. Gulland, "Antimicrobial resistance is now widespread, warns WHO," *BMJ,* vol. 348, p. g3062, 2014.

[28] J. J. Sung *et al.*, "Early clinical experience of the safety and effectiveness of Hemospray in achieving hemostasis in patients with acute peptic ulcer bleeding," (in eng), *Endoscopy,* vol. 43, no. 4, pp. 291-5, Apr 2011.

[29] A. R. Guerrero, F. Oyarzun-Ampuero, N. Hassan, A. H. Corvalan, A. F. Quest, and M. J. Kogan, "Gastric Cancer: Nanoparticles as Tools to Improve Treatment Efficacy," *Curr Pharm Des,* vol. 22, no. 39, pp. 5988-5997, 2016.

[30] M. N. V. Ravi Kumar, "A review of chitin and chitosan applications," *Reactive and Functional Polymers,* vol. 46, no. 1, pp. 1-27, 11//2000.

[31] S. Maya, S. Bruno, N. Amrita, N. S. Rejinold, V. N. Shantikumar, and R. Jayakumar, "Smart Stimuli Sensitive Nanogels in Cancer Drug Delivery and Imaging: A Review," *Current Pharmaceutical Design,* vol. 19, no. 41, pp. 7203-7218, 2013.

[32] X. Z. Shu, K. J. Zhu, and W. Song, "Novel pH-sensitive citrate cross-linked chitosan film for drug controlled release," *International Journal of Pharmaceutics,* vol. 212, no. 1, pp. 19-28, 1/5/2001.

[33] P. Chanphai and H. A. Tajmir-Riahi, "tRNA conjugation with folic acid-chitosan conjugates," *Int J Biol Macromol,* vol. 105, no. Pt 1, pp. 810-815, Dec 2017.

[34] N. Jafary Omid, N. Bahari Javan, A. R. Dehpour, A. Partoazar, M. Rafiee Tehrani, and F. Dorkoosh, "In-vitro and in-vivo cytotoxicity and efficacy evaluation of novel glycyl-glycine and alanyl-alanine conjugates of chitosan and trimethyl chitosan nano-particles as carriers for oral insulin delivery," *Int J Pharm,* vol. 535, no. 1-2, pp. 293-307, Jan 15 2018.

[35] Y. Jing, J. Huang, and X. Yu, "Preparation, characterization, and functional evaluation of proanthocyanidin-chitosan conjugate," *Carbohydr Polym,* vol. 194, pp. 139-145, Aug 15 2018.

[36] A. M. Piras *et al.*, "A water-soluble, mucoadhesive quaternary ammonium chitosan-methyl-beta-cyclodextrin conjugate forming inclusion complexes with dexamethasone," *J Mater Sci Mater Med,* vol. 29, no. 4, p. 42, Mar 30 2018.

[37] L. DaSilva, Y. Finer, S. Friedman, B. Basrani, and A. Kishen, "Biofilm formation within the interface of bovine root dentin treated with conjugated chitosan and sealer containing chitosan nanoparticles," *J Endod,* vol. 39, no. 2, pp. 249-53, Feb 2013.

[38] A. Lauto, D. Mawad, M. Barton, A. Gupta, S. C. Piller, and J. Hook, "Photochemical tissue bonding with chitosan adhesive films," *Biomed Eng Online,* vol. 9, p. 47, Sep 8 2010.

[39] A. Shrestha, M. R. Hamblin, and A. Kishen, "Characterization of a conjugate between Rose Bengal and chitosan for targeted antibiofilm and tissue stabilization effects as a potential treatment of infected dentin," *Antimicrob Agents Chemother,* vol. 56, no. 9, pp. 4876-84, Sep 2012.

[40] M. Barton *et al.*, "In vitro cell compatibility study of rose bengal-chitosan adhesives," *Lasers Surg Med,* vol. 44, no. 9, pp. 762-8, Nov 2012.

[41] R. Lieder, M. Darai, G. Örlygsson, and O. E. Sigurjonsson, "Solution casting of chitosan membranes for in vitro evaluation of bioactivity," *Biological Procedures Online,* journal article vol. 15, no. 1, p. 11, November 06 2013.

[42] S. K. Linden, K. M. Driessen, and M. A. McGuckin, "Improved in vitro model systems for gastrointestinal infection by choice of cell line, pH, microaerobic conditions, and optimization of culture conditions," *Helicobacter,* vol. 12, no. 4, pp. 341-53, Aug 2007.

[43] C. Eouani, P. Piccerelle, P. Prinderre, E. Bourret, and J. Joachim, "In-vitro comparative study of buccal mucoadhesive performance of different polymeric films," *Eur J Pharm Biopharm,* vol. 52, no. 1, pp. 45-55, Jul 2001.

[44] D. S. Jones, B. C. O. Muldoon, A. D. Woolfson, and F. D. Sanderson, "An Examination of the Rheological and Mucoadhesive Properties of Poly(Acrylic Acid) Organogels Designed as Platforms for Local Drug Delivery to the Oral Cavity," *Journal of Pharmaceutical Sciences,* vol. 96, no. 10, pp. 2632-2646, 2007/10/01/ 2007.

[45] V. I. Egorov, I. V. Schastlivtsev, E. V. Prut, A. O. Baranov, and R. A. Turusov, "Mechanical properties of the human gastrointestinal tract," *J Biomech,* vol. 35, no. 10, pp. 1417-25, Oct 2002.

[46] G. Perfetti, K. M. Jansen, W. J. Wildeboer, P. van Hee, and G. M. Meesters, "Characterization of physical and viscoelastic properties of polymer films for coating applications under different temperature of drying and storage," *Int J Pharm,* vol. 384, no. 1-2, pp. 109-19, Jan 15 2010.

[47] L. A. Sellers, A. Allen, E. R. Morris, and S. B. Ross-Murphy, "Mechanical characterization and properties of gastrointestinal mucus gel," *Biorheology,* vol. 24, no. 6, pp. 615-23, 1987.

[48] F. Mearin, V. Fonollosa, M. Vilardell, and J. R. Malagelada, "Mechanical properties of the gastro-esophageal junction in health, achalasia, and scleroderma," *Scand J Gastroenterol,* vol. 35, no. 7, pp. 705-10, Jul 2000.

[49] H. Saraf, K. T. Ramesh, A. M. Lennon, A. C. Merkle, and J. C. Roberts, "Mechanical properties of soft human tissues under dynamic loading," *J Biomech,* vol. 40, no. 9, pp. 1960-7, 2007.

[50] H. Yang, S. Yang, J. Kong, A. Dong, and S. Yu, "Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy," (in eng), *Nat Protoc,* vol. 10, no. 3, pp. 382-96, Mar 2015.

[51] R. Pribic, I. H. M. Vanstokkum, D. Chapman, P. I. Haris, and M. Bloemendal, "Protein Secondary Structure from Fourier Transform Infrared and/or Circular Dichroism Spectra," *Analytical Biochemistry,* vol. 214, no. 2, pp. 366-378, 1993/11/01/ 1993.

[52] J. L. Achord, "Comparison of basal gastric acid and pepsin output in controls vs. active peptic ulcer disease (PUD)," *Am J Gastroenterol,* vol. 70, no. 1, pp. 46-9, Jul 1978.

[53] Q. Wang, X. Xie, X. Zhang, J. Zhang, and A. Wang, "Preparation and swelling properties of pH-sensitive composite hydrogel beads based on chitosan-g-poly (acrylic acid)/vermiculite and sodium alginate for diclofenac controlled release," *Int J Biol Macromol,* vol. 46, no. 3, pp. 356-62, Apr 1 2010.

[54] J. G. Brennan, R. Jowitt, and O. A. Mughsi, "Some Experiences with the General Foods Texturometer," *J Texture Stud,* vol. 1, no. 2, pp. 167-184, Mar 1970.

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