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À ANNECY

*****

PERORAL DELIVERY OF THERAPEUTIC PROTEINS: FORMULATION IN THE FORM OF INTESTINAL MICROPATCHES

*****

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LIST OF ABBREVIATIONS

- API: Active Pharmaceutical Ingredient
- ANOVA: Analysis of Variance
- ATR-FTIR: Attenuated Total Reflection – Fourier Transformation InfraRed
- BA: Bioavailability
- BCS: Biopharmaceutics Classification System
- BSA: Bovine Serum Albumin
- Caco-2: Human epithelial colorectal adenocarcinoma cells
- CAGR: Compound Annual Growth rate
- C-CPE: C-terminal fragment of *Clostridium perfringens* enterotoxin
- CD: cyclodextrins
- CM-TMC: O-carboxymethyl N,N,N-TriMethylChitosan
- CMC: Critical Micelle Concentration
- DLS: Dynamic Light Scattering
- DSC: Differential Scanning Calorimetry
- DMEM: Dulbecco's Modified Eagle Medium
- FDA: Food and Drug Administration
- GI: Gastrointestinal
- GRAS: Generally Recognized As Safe
- HBSS: Hank's Balanced Salt Solution
- HNEC: Human Nasal Epithelial Cells
- HPLC-UV: High-Performance Liquid Chromatography – UltraViolet
- HPMC: HydroxyPropylMethylCellulose
- LMW: Low Molecular Weight
- MLC-pS^{19}: Myosin Light Chain at position S^{19}
- MP: Microparticles
- MW: Molecular Weight
- MMW: Medium Molecular Weight
- NBE: New Biological Entity
- NCE: New Chemical Entity
- NME: New Molecular Entity
- NP: Nanoparticles
- PBS: Phosphate Buffered Saline
- PE(s): Permeability (or Permeation) Enhancer(s)
- PEC: PolyElectrolyte Complexes
- P-gp: P-glycoprotein
- pI: Isoelectric point
- PPS: dimethyl Palmitoyl ammonium Propane Sulfate
- (N-)TMC: N,N,N-TriMethylChitosan
- R&D: Research & Development
- S-CMC: Sodium CarboxyMethylCellulose
- SCG: Sodium Glycolate
- SMEDDS: Self MicroEmulsifying Drug Delivery System
- TEER: TransEpithelial Electric Resistance
- TJ(s): Tight Junction(s)
- U(H)PLC: Ultra (High) Performant Liquid Chromatography
- WST-1: Water Soluble Tetrazolium (salts) 1

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INTRODUCTION

Innovation in the pharmaceutical industry is facing troubles: even though drug discovery activities are increasing, yet the outcome, i.e. number of new drugs on the market, is on decline.

The drug research and development pipeline keeps rising for decades and number of therapeutic molecules in development has never been so high (1), as illustrated in figure 1 below.

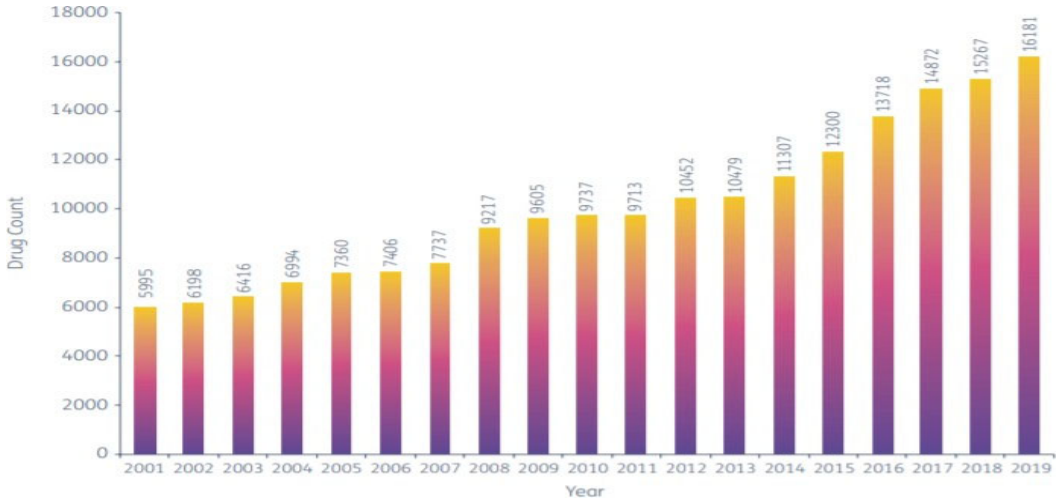

Figure 1: Total R&D pipeline size by year between 2001 and 2019 (1)
Pipeline considers all drugs in development, from preclinical stage, through various stages of clinical testing, regulatory approval, and launch. Launched drugs are also counted only if still in development for additional indications or markets.

However in 2018, only 59 new pharmaceuticals were approved by the US Food and Drug administration (FDA) out of more than 16 000 compounds in development (2,3). For the last two decades, the number of FDA-approved drugs has very slightly increased, mainly due to the development of New Biological Entities (NBE), as the number of New Chemical Entities (NCE) has remained steadily constant, as detailed in figure 2.

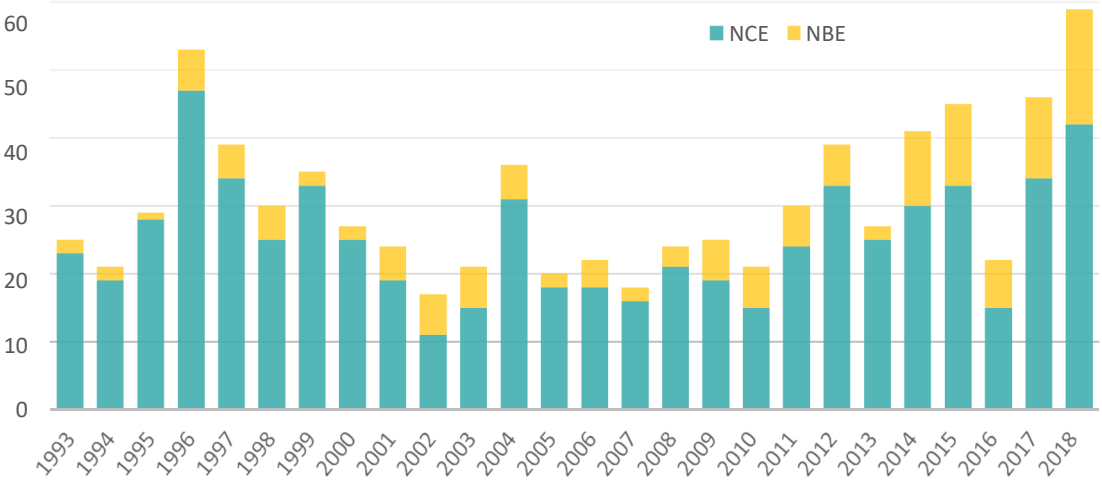

Figure 2: Number of New Chemical and Biological Entities approved by the US FDA, 1993- 2018 (3)

At the same time, R&D costs have hugely increased. The data in figure 3 show that the number of new FDA-approved drugs per billion US dollars of R&D spending in the drug industry has halved approximately every 9 years since 1950 (4).

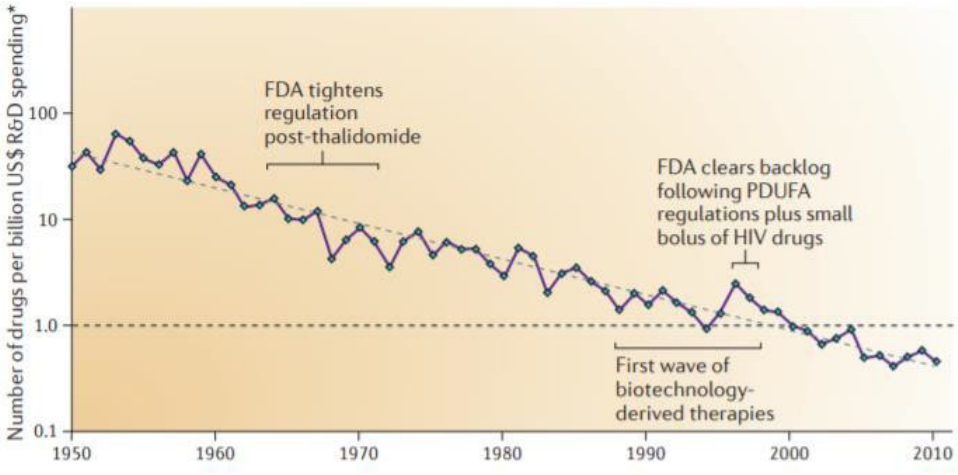

Figure 3: Overall trend in R&D efficiency (inflation-adjusted)(4)

This actual trendline, defined by Eroom's law (4), shows that New Molecular Entities (NME) output is essentially linear, and likely to remain so, while their costs of development are skyrocketing. Investment in R&D has dramatically increased during this period, up to $50 billion per year at present and for years to come, as plans show that worldwide R&D spending by pharmaceutical & biotech companies will keep growing from 178.9$bn in 2018 to 213$bn in 2024 (5). Despite all these massive investments, yet the number of new drugs annually approved is no greater now than it was 60 years ago (6). This increase in cost per approved molecule highlights the importance given to R&D but nonetheless its overall efficiency decline.

To counter this, the pharmaceutical industry requires alternative approaches to increase R&D productivity and one of the solutions seems to be therapeutic biomolecules. Over the past couple of decades, a clear move occurred in the laboratories from small chemical drugs towards biotech-centered strategies demonstrating potential to address many chronic diseases. Whereas biotech accounted for just 15% of the R&D pipeline back in 1995, by 2019 it has reached 39.7%, now meaning that out of every ten drugs under development 4 are biotech-derived. The proportion rose again in 2019, up from 37.9% in 2018, implying that this trend is not over yet (1). This evolution is even visible on the market: in 2018, 17 out of the 59 FDA-approved drugs were biotech products, setting a new record, and clearly surpassing the 12 approved in 2015 and 2017. As for market shares, biomolecules were worth $93 billion in 2010, valued at $140 million in 2016, and are forecast to reach $217 million by 2023, representing a compound annual growth rate of 6.5% from 2017 to 2023 (7).

All these figures confirm the increasing importance of biomolecules as drugs, which in the last five years (2014–2018) account for more than 25% of all drugs approved (59 out of 213). The potential for such therapies is still huge: biomolecules constitute a major type of active substances, for which the market potential is limitless. This augurs a promising future for innovative therapeutic proteins in the coming years (8).

However, to achieve the highest efficacy possible in patients, therapeutic proteins and peptides are currently administered by injection and this constitutes a real problem especially for compounds that are used chronically and require frequent dose administrations. As there is a clear need for non-injection alternatives (9) and ideally for oral administration, a main approach is to enhance the absorption of drugs that have already proven their efficacy through various methods: modification of the drug itself, of the environment or yet improving the system administered orally. Amongst those, focus is put on the use of absorption (or permeability) enhancers. Decline in the pharmaceutical R&D efficiency highlights the need to adopt new models of innovation and the concept of permeability enhancers could give a second lease of life to marketed biological drugs whose efficacy has already been proven. Their development has been on the rise for the last couple of decades and application in turning actual marketed injectable drugs into orally delivered systems would be a major revolution for pharmaceutics. Sticking to life cycle management, pharmaceutical companies consider this strategy to open up the field of innovative drug delivery systems with modified release dosage forms, for new routes of administration, with a better global efficacy, and allowing to maximize R&D investments.

The aim of this work is to tackle the development of a technology promoting the delivery of effective therapeutic biomolecules in the body *via* the convenient and patient-friendly oral route, in order to lift constraints of the actual injectable route of administration.

In a first part, this work particularly focused on the challenges of the oral route and on gastrointestinal absorption enhancement, which if successful, could have the greatest impact on drug therapy. In the second part, light will be shed on drug delivery systems specially designed for the oral route, and in particular on intestinal micropatches that constitute a rising strategy. Finally, the last and experimental part of this thesis will deal with the formulation and evaluation of intestinal micropatches.

THEORICAL PART: PERORAL PROTEIN DRUG DELIVERY

I- Therapeutic proteins

1. History

Emergence of protein therapy over the past few decades has been a major revolution in medicine and pharmaceutics, and covers peptides, proteins and macromolecule drugs such as DNA or RNA for example. Starting in the early 1920's with insulin and then in 1982 with the FDA approval of the first recombinant insulin, injection was back then the easiest way to deliver a precise dosage of drug but causes repeated pain and risks of infections. Ever since, research was conducted to find a way to administrate biomolecules *via* different routes besides parenteral one and many options were tested to achieve efficient drug delivery by the oral route. Even though this objective is not attained yet, over 200 different protein-based drugs have already been marketed worldwide and much more are under preclinical investigation or in clinical trials. So far, protein-based drugs have provided serious options for addressing new therapeutic challenges in multiple pathologies: diabetes, oncology, cardiovascular diseases, allergies, gastrointestinal dysfunctions, hematology, immunity diseases, infectious diseases, hormonal diseases, metabolic disorders, obesity, vaccines and so on (8,10–12).

Considering that protein therapy was discovered less than a century ago and constituted a niche for the following decades, it now constitutes a therapeutic class on its own, considered as the most rapidly expanding class of new therapeutics (13). Recent significant progress in manufacturing confirms that biomolecules have a huge potential for years to come to turn into highly effective protein drug candidates, with a more and more important place on the worldwide drug marketplace.

2. Therapeutic interest

A peptide is usually defined as a succession of amino acids linked by amide bonds and turns into a protein once this chain folds into its three-dimensional configuration, as illustrated in the following figure 4. Under the framework "therapeutic proteins" are considered peptides, proteins and macromolecules (such as antibodies, enzymes, interferons, hormones …(14)) that offer several advantages compared to traditional drugs. These biomolecules are complex structures, acting *via* specific mechanisms of action, whose main characteristics include high activity, high selectivity/affinity, high specificity, high potency to target complex functions, low

toxicity and minimal nonspecific and drug-drug interactions (15).

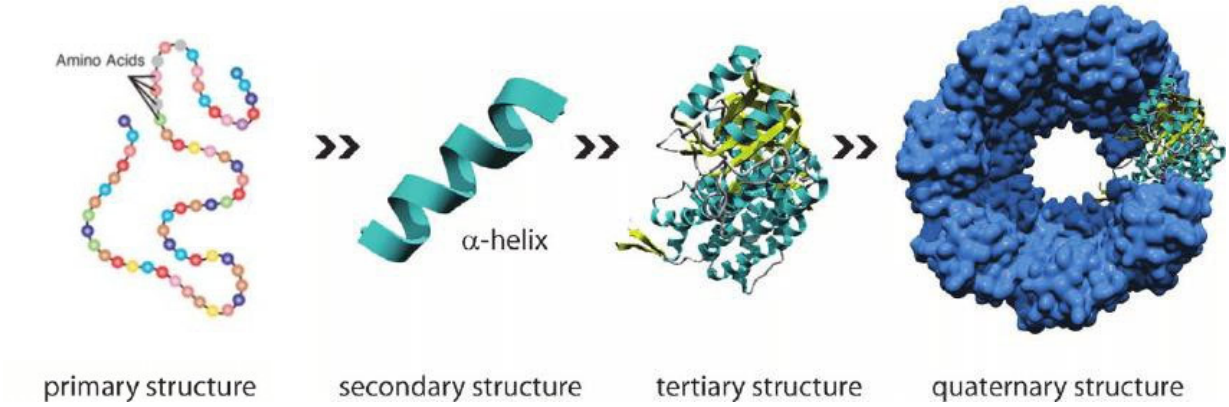

Figure 4: Hierarchy from peptide to protein structure (16)

Most therapeutic peptides act as receptor agonists (17) and in combination with the previous properties (in particular high specificity), low quantities are sufficient to activate the targeted receptors. This leads to reduction of both, toxicity risks and production costs. Furthermore, because of the short half-life of proteins, only few of those molecules would accumulate in tissues and their degradation products being amino acids, they would be metabolized as endogenous products thus reducing again the risk of toxicity usually existent with chemical drugs (8). Peptides even present some more advantages compared to proteins. Due to their smaller size, they are able to penetrate further into tissues, are less able to activate the immune system, are more effective and come with lower manufacturing costs. From an industrial point of view, the potential of therapeutic proteins is even growing as new technologies now allow manufacturing through transgenic, recombinant or synthetic methods such as solid phase protein synthesis (18).

The objective of the development of therapeutic proteins is to combine the right peptide with matching delivery technique so that the drug maintains its stability and activity once inside the body and up to its site of action (18). This would result in more effective medicines, with fewer off-target side effects and reduced toxicity, so those high value-added drugs would keep providing effective and innovative solutions for unmet medical needs.

3. Characteristics and administration

According to BCS classification, biomolecules such as proteins, peptides, DNA, etc., are considered class III compounds, meaning highly soluble yet poorly permeable and this is a major impediment for their absorption (19).

Bioavailability of biomolecules, no matter which route of administration is considered, depends

on one hand on their physicochemical properties such as high molecular weight, pH stability, hydro/lipophilicity, conformational structure, instability (enzyme- & pH-sensitivity), and on the other hand on metabolic properties such as immunogenicity and enzymatic degradation. They also present biochemical and structural complexities compared to low molecular weight pharmaceuticals (20). Consequently, administration of these therapeutic biomolecules must take into account all these characteristics to guarantee an optimum safety and efficacy for patients.

Since the beginning of biotherapies, the most common route for administration has always been parenteral injection via intravenous route, offering the advantage of 100% bioavailability while protecting both the structure and the activity of the proteins. However, this invasive drug administration does not permit specific targeting, and most of therapeutic proteins have short half-lives in serum (usually <30 min) because of rapid clearance effected by the mononuclear phagocytic system (MPS), combined with hepatic and renal clearance (21). Consequently, multiple injections a day or administration of higher doses are necessary for the drugs to be effective, with possibilities of toxicity issues, side effects and patient compliance concerns. Besides intravenous injection, other parenteral routes of administration such as subcutaneous and intramuscular were developed, but still are not solving the remaining issues of pain at the injection, infection risks, no self-injection, and expensive related costs.

Even though administration of therapeutic peptides/proteins is mainly limited to parenteral approach, several studies are ongoing to develop less constraining methods of administration. Several strategies have been evaluated amongst which oral drug delivery is on the rise. The development of oral administration for biomolecules as a non-invasive therapeutic method has emerged as an attractive alternative considering long-term dosing, potential for solid formulations, safety, convenience, less pain and fewer burdens of medical costs (22).

Hence, development of viable biopharmaceutical products for oral delivery should consider the physicochemical, biochemical, and physiological characteristics of both proteins and route of administration at the same time. Main objectives are to overcome absorption barriers, maintain therapeutic drug levels while limiting side effects to provide a better quality of life for patients. This challenge may be achieved *via* the development of efficient drug delivery systems specially designed for oral administration of therapeutic proteins.

II- Oral administration of drugs

1. Oral drug delivery

The peroral route, also called oral administration or PO (*per os*), is the most common route of administration. It is the favorite and most convenient route for drug administration according to patients (23,24), because this attractive approach has advantages that often outweigh its disadvantages, as shown in Table 1.

Table 1: Advantages and disadvantages of the oral route

ADVANTAGES	DISADVANTAGES
- Ease of administration (self) - Convenient, patient-friendly - Safe - Non-invasive, painless (needle-free delivery) - Accurate dosing - Cost-effective - Various galenic forms (feasibility for solid formulations) - Convenient for repeated and prolonged use - Possible protection against physiologic mechanisms (controlled / modified / sustained release, …) - No need for sterilization - Intensified immune response	- Slow absorption and onset of action, not usable for emergency - Unpredictable absorption - Pre-systemic degradation (acidic and enzymatic environment) - Hepatic first-past effect - Interactions (cytochromes P450, food, …) - Not suitable for all patients (uncooperative, vomiting, problems of deglutition, malabsorption syndrome, too young or too old patients) - Not suitable for irritant or unpalatable drugs

The oral pathway as a route of drug administration is of great interest. It can be used for local treatment but is mainly intended for systemic absorption, resulting from drug absorption through the intestine's large surface area and rich blood supply, which provide a quick access to systemic circulation. Moreover, another advantage is the possibility to control the release of the drug in order to improve its absorption, permeability and overall efficacy.

The main objective of any dosage form is to deliver an optimum amount of active pharmaceutical ingredient to the proper site in the body and to reach a desired drug concentration to ensure therapeutic efficacy. More specially for oral delivery systems, they

should ideally be easy to use, painless to the patient, cost-effective, non-toxic, non-irritating to the mucus membrane, and safe for prolonged administration (25–27). Currently, oral drug delivery systems include a large variety of pharmaceutical dosage forms such as tablets, capsules, powders, solutions, suspensions, or gels. Challenges in oral drug delivery now lie in the development of more innovative dosage forms, combining functional excipients with new technologies, in order to overcome the physiological barriers, to protect both structure and activity of the drugs up to the action site, and finally to induce efficient drug absorption across the intestinal epithelium.

2. Physiology of the digestive system

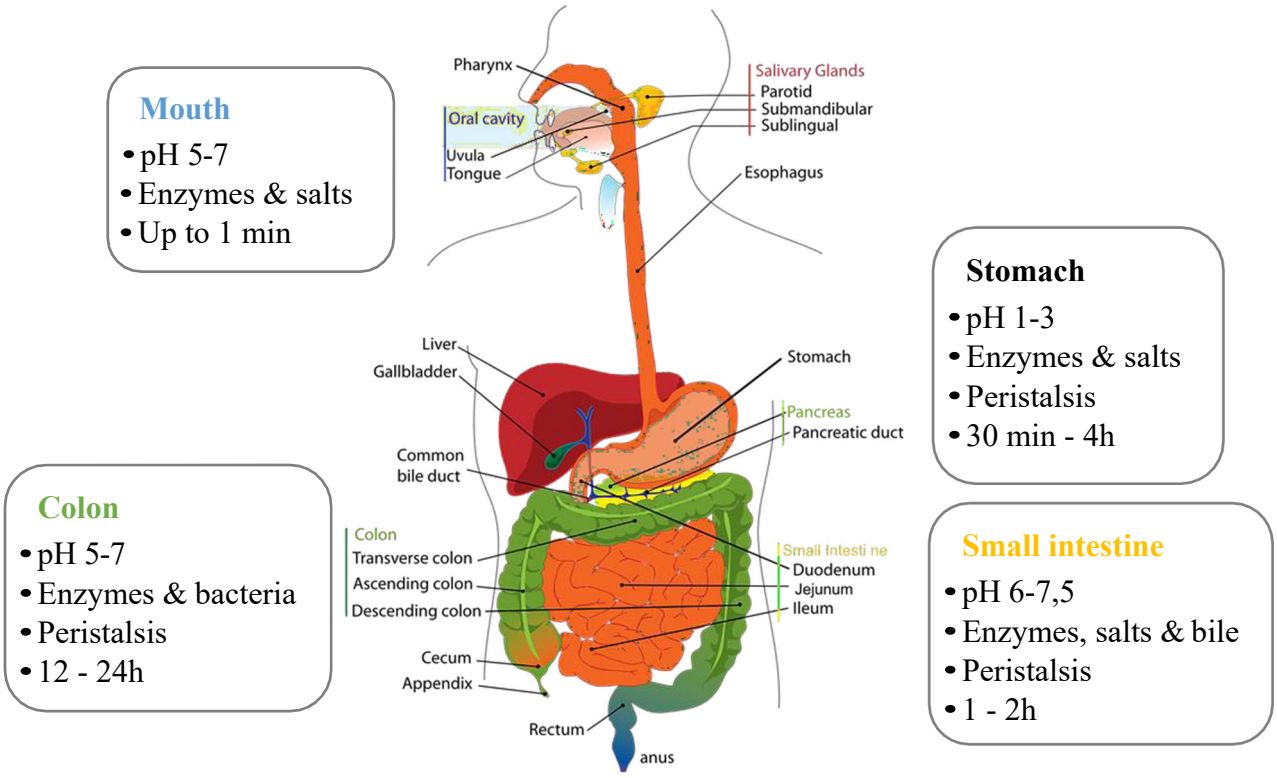

Figure 5: Structure of the digestive system (28)

As illustrated in figure 5, drug administration is made by the mouth and then the system goes into the digestive tract through the esophagus, followed by the stomach, small intestine and eventually the colon if not absorbed before.

Table 2: Major properties of the oral route for the delivery of macromolecule drugs (23,29–31)

	Physiological properties
Oral cavity	- Neutral pH (+) - Surface area: 200 cm² (-) - High permeability (+) - Amylase and lipase (-) - Salivary wash limiting buccal and sublingual administration (-)
Stomach	- Acidic pH (-) - Surface area: 1 m² (±) - Mucus thickness: 274±41 µm (+) - Low permeability (-) - Protease (-)
Small intestine	- pH 6-7,4 (+) - Surface area (microvilli): 200-250 m² (+) - Mucus thickness: 170±38 µm on duodenum, 123±4 µm on jejunum, 480±47 µm on ileum - High permeability (+) - High enzyme activity (-) - Tight junctions gap: 7-9 Å (ileum), 3-4 Å (jejunum) - Specialized immune system: Peyer's patches (+)
Colon	- pH 5,5-6,7 (+) - Surface area: 2-4 m² (+) - Mucus thickness: 830±110 µm (-) - Low permeability (±) - Low enzyme activity (+) - Tight junctions gap: 8-9 Å
(+): usually in favor of stability or absorption of macromolecule drugs (±): whether in favor or not in favor of stability/absorption of macromolecule drugs, depends on individual cases (-): usually not in favor of stability or absorption of macromolecule drugs	

3. Barriers to absorption

Oral delivery faces challenges as drugs encounter barriers during their journey in the digestive tract, as detailed in figure 6. Major barriers are (15,29–31):

- Biochemical barrier: Inside the digestive tract, biomolecules are submitted to extreme variations of pH from gastric acidic environment (pH 1,2 to 3) to intestinal alkaline environment (pH 6,5 to 8). These extreme pH variations might cause pH-induced oxidation, deamidation or even hydrolytic degradation of drugs. In addition, presence of bile salts and various enzymes considerably affects the integrity and efficacy of orally administered drugs. For example, main proteolytic enzymes are pepsin, trypsin, chymotrypsin, elastase, and peptidases at the brush border specifically degrading proteins and peptides, consequently leading to major loss of protein activities.

- Mucus barrier: Mucus consists of a hydrogel composed of more than 90-98% of water, 0,5 -5% mucins, 1% inorganic salts, 0,5-1% proteins and mucopolysaccharides. The main structural component is mucin, a high molecular weight glycoprotein produced by Goblet cells of the epithelium that is negatively charged. Auto-association of these molecules by means of non-covalent interactions forms the gel matrix. Mucus forms a dynamic semipermeable barrier, whose role is to lubricate the underlying epithelium and protect it from damages by foreign or noxious substances (32). Parameters such as mucus viscosity, porosity, turnover frequency, and pH also affect its activity. Also, mucus is the place where a range of interactions between physiological barrier and proteins take place, with various kinds of interactions: van der Waals, electrostatic, hydrogen bonds, … possibly resulting in drug structural changes and unfavorable absorption of proteins. As such, mucus can restrict drugs access to the epithelial surface and slows down drugs diffusion.

- Epithelial/cellular barrier: Mainly constituted of enterocytes, the intestinal epithelium surface is composed of phospholipidic bilayer membranes and cholesterol. Lipophilic molecules can access the blood circulation by transcellular passage (passive diffusion), but the majority of proteins being hydrophilic molecules with high molecular weight, intestinal cells constitute a main barrier. For those ones, paracellular delivery is an easier way to access systemic circulation.

Combination of those three barriers might affect the access of drugs to the systemic circulation, reduce their bioavailability and their overall efficacy.

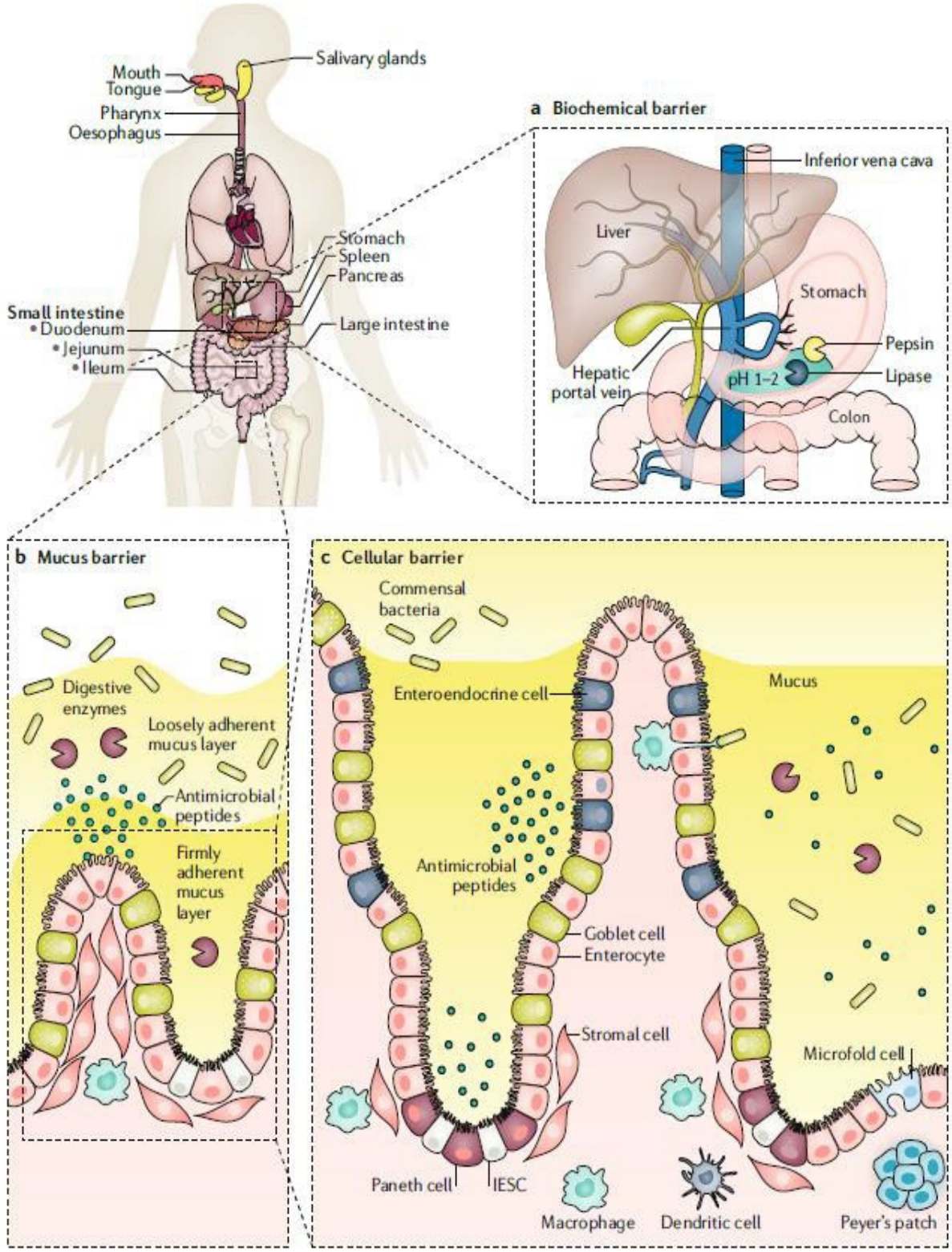

Figure 6: Physiological barriers to oral protein and peptide delivery (33)

In the end and as said previously, absorption is also limited by the proper physicochemical properties of the drugs. Especially for the macromolecules, high molecular weight, hydro/lipophilicity ratio and poor aqueous solubility, pH stability, differential surface charge &

fragile structure of the proteins are limiting factors of absorption (31,34).

To conclude, the GI tract has formidable physiological and chemical barriers that pose several challenges: oral delivery is hampered by the harsh environment and it might be restricted by pre-systemic degradation of drugs and their poor permeability across the intestinal epithelium. All these factors make an efficient administration of therapeutic proteins very limited in peroral approach, to some extent that unmodified or unprotected drugs usually display oral bioavailability that do not exceed 0,5-2%, failing to induce clinical response. Furthermore, low bioavailability has been associated with larger inter- and intra-subject variability in systemic exposure (20,21,35–37). Hence, overcoming those challenges is crucial to create effective oral proteic drug delivery systems. Yet, significant progresses have been made with each of these obstacles, as will be seen in the next part. By improving drug bioavailability, drugs action and side effects would be more closely controlled, reduction of doses to the minimum by oral route would reduce the waste of drugs that are not systemically exposed and would reduce costs *in fine* (9). At the present time, the potential of this emerging field is promising.

III- Mechanisms of absorption enhancement

Absorption enhancement is the technology aimed at enabling non-injection delivery of poorly membrane-permeable compounds (9). As barriers to absorption have now been defined, various strategies have been considered to overcome those limitations and to improve absorption of therapeutic peptides and proteins by the oral route.

Main key approaches to maximize oral absorption of biomolecules and their bioavailability consider either a change in the drug itself, modulations of the environment or a change in the formulation. Respective examples are chemical modification of proteins, use of enzyme inhibitors & permeability enhancers, use of mucoadhesive polymers and are developed right after (15,20).

1. Chemical modifications of drugs (15,38)

One idea to improve permeability and absorption is to chemically modify the structure of peptides and proteins. Derivatizations are usually used to optimize, to some extent, their therapeutic activities and to limit their presystemic metabolism *via* enhanced enzymatic stability, enhanced intestinal permeability and reduced immunogenicity.

For example, possible chemical modifications may include (20,39):

- PEGylation or covalent attachment of polyethylene glycol and PEG-derivatives. Addition of these moieties to a therapeutic agent ensure an overall enhancement of stability by limiting enzymatic degradation while improving solubility, increasing half-life and therapeutic utility of molecules. This technique has already been extensively discussed in the literature (38,40).
- Hyperglycosylation or covalent attachment of glycolic acid. Addition of these carbohydrates to therapeutic proteins serves to reduce interactions with clearance mechanisms and antigen presenting cells (APCs) in an effort to prolong circulation and to reduce immunogenicity (38).
- Lipidization or conjugation of medium chain fatty acids. Addition of these hydrophobic moieties improves their lipophilicity, metabolic stability, membrane permeability, bioavailability and implies changes in PK / PD profiles. Lipidization leads to dilatation of tight junctions and increase cell membrane penetration, with possible cytotoxicity (41,42).
- Palmitoylation or covalent attachment of palmitoyl group. It has been used on insulin not only to enhance its transport across the mucosal membrane of the large intestine but also to improve stability against enzymatic degradation in intestines (41).
- Substitution of amino acids. Analogue formation can concern a simple substitution of existing amino acids in favor of a new one or substitution of L/D enantiomers of the same amino acid. Application of this chemical modification turned vasopressin into desmopressin, which is twice more active at 1/75 fraction of dose (15).
- Transporters or covalent conjugation of carrier molecules to proteins. Presence of a transport-carrier molecule (or a compound that utilizes transporters for active drug absorption) enables recognition of these newly designed substrates by a specific endogenous membrane transporter or a receptor (9).
- Prodrug derivatization. Last but not least, this technique consists in the addition of protective groups (esters for example) or in the formation of new structures (via bioreversible cyclization for example) to turn active therapeutic molecules into pharmacologically inactive entities, that would need enzymatic or non-enzymatic metabolization crossing barriers to be active again. Those prodrugs structures are designed to improve solubility, stability, permeability, absorption and targeting capacities, but usually enhance cytotoxicity (20,43).

2. Reduction of metabolism

Drug absorption can be improved by reducing the physiological metabolism. For example, P-gp is an efflux membrane transporter responsible for limiting drug cellular uptake and for jeopardizing the success of drug delivery. Strategies to inhibit this transporter would improve enhance drug absorption and bioavailability (44).

Another example is enzyme degradation. As seen previously, many physiological mechanisms act on macromolecules to divide them into smaller molecules more easily absorbed. In fact, proteins or peptides are often cleaved into amino acids and sugars by various endo and exopeptidases at different levels of the digestive tract. At the contrary, reduction of this active metabolism would protect biomolecules until deeper barriers of the organism and possibly up to blood circulation. In order to reduce the metabolism, main approach consists in the addition of enzyme inhibitors in the formulation (9,45). Those molecules, pharmacologically inactive and consequently considered as excipients, are mainly protease inhibitors such as serine protease inhibitor (serpin), soybean trypsin inhibitor or aprotinin, this latter being widely used in oral protein delivery. They typically bind the enzymes in a reversible or irreversible way and complexation between an enzyme and its inhibitor let biomolecules free from cleavage and metabolism. As enzyme inhibitors alters the physiology of GI tract by reducing enzymatic degradation of proteins and thereby enhancing their bioavailability, co-administration of enzyme inhibitors with biomolecules facilitate their intestinal absorption. In particular, many studies revealed the interest of trypsin and chymotrypsin inhibitors such as apoprotein for the oral delivery of insulin (15,46).

Concerning drawbacks, first one is the variability of the enzyme presence, quantity and activity, which may greatly impact dosing and efficiency of this approach, leading to non-predictable inter-subject variability between patients. Second, non-site-specific activity of those compounds would alter the physiological metabolic pattern of the GI, affecting more globally food digestion and nutrients absorption. Third, being themselves sensitive to enzymatic degradation, inhibitors are often co-administered excessively in large amounts inducing possible toxicity. Finally, long-term consummation may cause enzyme deficiencies with metabolic issues leading to aforementioned problems concerning intestinal absorption and toxicity (15).

3. Development of interesting galenic forms

Development of a galenic form is a mandatory step in drug development and formulations are another means to help overcome the limitations of protein delivery that are not solved by the

previous options. They can be used to protect the drug's structure and activity or to target more precisely the specific site of action.

Here are some examples of recent drug formulation systems that are studied for oral protein delivery (15,20,29,38,45–47):

- Particulate carriers: Those oral delivery systems, shown in figure 7, consist in the encapsulation of proteins in microparticulate and nanoparticulate systems (e.g., liposomes, micelles, polymeric microspheres, polymeric nanoparticles) in the form of spherical carriers. They are protecting fragile molecules from the harsh environment of the GI tract (chemical and enzymatic unfavorable medium).
    - Micelles are colloidal carriers (5-100 nm) composed of amphiphilic molecules forming a hydrophobic core, that help to improve the aqueous solubility of hydrophobic therapeutic drugs. Their small size facilitates spontaneous penetration through tissues with enhancement of permeation efficiency and retention time in tissues. Latest generation, known as SMEDDS (self-microemulsifying DDS), is a combined formulation with microemulsions that seems promising to increase the oral absorption of poorly water-soluble drugs (48) .
    - Liposomes are lipid-based vesicles (20nm-10µm), composed of phospholipids and/or derivatives, with an aqueous inner core presenting the ability to encapsulate both hydrophilic and hydrophobic drugs, respectively, in the core or in the bilayer of the structure. Focus is now oriented on their derivatives called archaeosomes, made of archaeobacterial membrane showing better stability results.
    - Nanoparticles (10-1000nm) constitute the most promising approach for protein oral delivery. Made of various polymers (PLGA, PMMA, chitosan...), they can be classified into two types: nanospheres and nanocapsules. Nanospheres are matrix systems where the drug is dispersed, while nanocapsules are vesicles in which the drug is encapsulated in polymeric membrane. Though, it remains a challenge because of low loading capacity, stability issues through time, and because of the necessity to improve the controlled release profile.
    - Microparticles (1-1000µm) are comparable to nanoparticles with only difference in size. They are also composed of polymers, are easily degraded in the intestinal epithelium, and generally provide a better stability for labile compounds. Indeed, microencapsulation showed better results for numerous peptides, while nanoencapsulation is more complex owing to complexity and incompatibility during process with the physicochemical conditions required for peptide stability. NPs seem

more suitable for controlled delivery of biomolecules while MPs seem more suitable for immediate release.

The efficacy of those particulate-based delivery strategies depends on parameters such as composition, encapsulation efficiency, rate of drug release, stability, size, or yet surface charge. Lot of work is now oriented on release of these nano/microparticulate systems in order to control them and limit a burst effect. Those systems can also be functionalized at their surface to improve their efficacy. For example, fixation of targeting moieties (such as antibodies) can ensure a site-specific delivery of proteins and peptidic molecules thereby reducing toxicity, and groups (such as PEG) may limit opsonization and metabolism. Functionalization constitutes a major strategy to preserve the integrity of the carriers and the activity of contained bioactive molecules until the blood circulation.

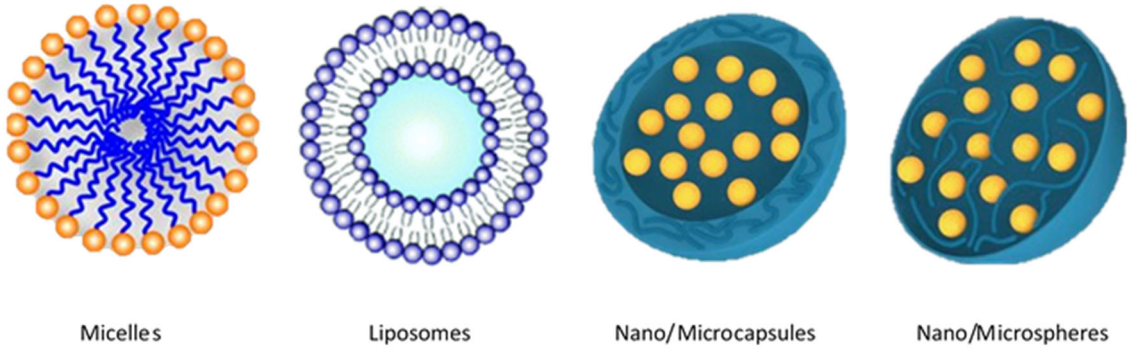

Figure 7: Representations of particulate delivery systems (49–51)

- Hydrogels (15,52): Composed of biodegradable, biocompatible and highly absorbent hydrophilic polymers, whose physical or chemical 3D networks constitute a gelled matrix in water that can capture therapeutic molecules. Chemical modifications of polymers allow tunability of gel properties (neutral or ionic nature, degree of water absorption, capacity for stimuli- responsive hydrogels). Either preformed or *in situ* forming gels, those systems can be sensitive to variations in surrounding stimuli such as temperature, ionic strength variation or pH alteration, turning them into physiological-responsive hydrogels. Main parameters to be considered are porosity and correlated mesh size (which is the distance between cross-links in the hydrogel network), that all together define the diffusion pattern of a therapeutic protein. Major interest for hydrogels relies on their potential for high encapsulation efficiency and controllable release. Actual development of this system focuses on optimization of porosity suitable for large biomolecules and even cells into hydrogels by forming microporous hydrogels.

- Mucoadhesive systems (33,38,53): Constituted of natural, semi-natural or synthetic polymers, those systems are made to bind and adhere to biological substrates such as mucosal membranes. Mucoadhesive polymeric drug systems present great qualities that enhance oral bioavailability of therapeutic proteins or peptides such as:
    - prolongation of the residence time of the system at the target site
    - presence of concentrated formulation at the target site increasing the drug concentration gradient
    - reduction of clearance of drug molecules at the absorption site
    - reduction of frequency of administration
    - controlled release
    - encapsulation of peptide and protein drugs within polymeric systems that ensure a protection towards enzymatic degradation.

  Main polymers considered for this approach are chitosan, PLGA or alginates, and chemical modifications can affect their swelling behaviors as they are depending on environmental conditions (ionic strength, electric charge, temperature, pH…). Greatest interest is now focused on thiolated polymers that present the advantage of a covalent bonding with the cysteine of mucus glycoprotein for stronger mucoadhesive properties.

- Cyclodextrins (54): Those cyclic molecules made of oligosaccharides constitute a cone shaped structure with a hydrophobic inner cavity and hydrophilic outer surfaces, making them able to interact with large biomolecules to form non-covalent inclusion complexes.

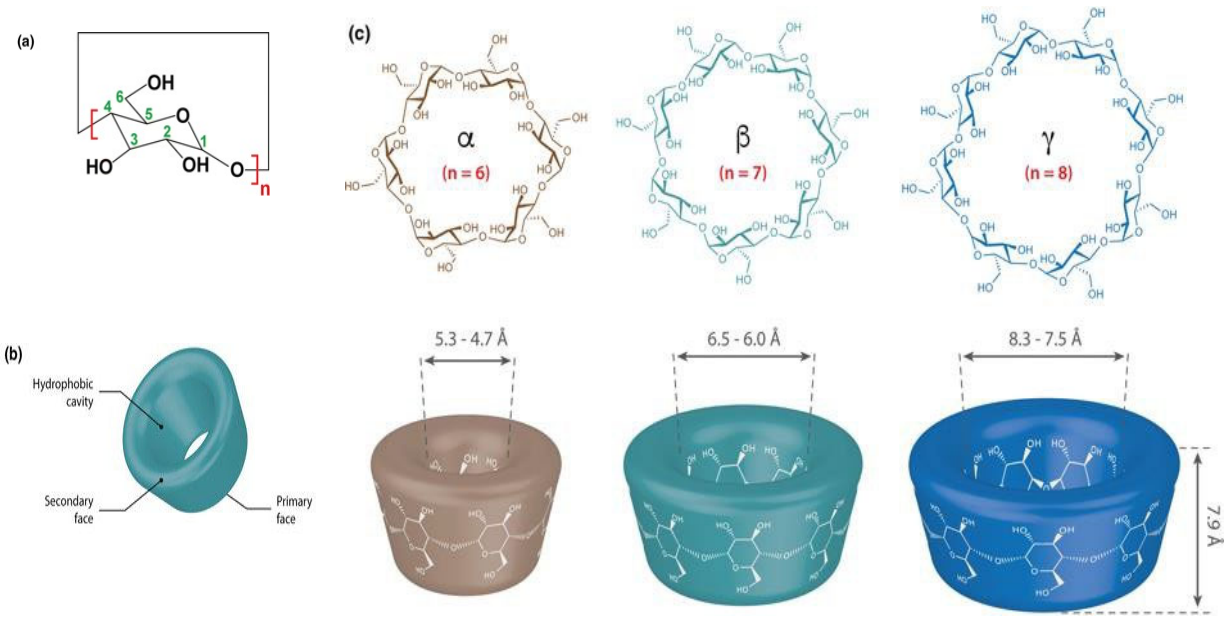

Figure 8: Concept of cyclodextrins - (a) monomeric structure, (b) global representation of CD, (c) structures of α, β, γ-cyclodextrins (54)

CD can be used as chemical- and enzymatic-protective carriers and are able to interact with biological membranes while inhibiting the P-gp efflux pump therefore improving drug absorption (15,30). However, complexity of CD-use relies on assuring the drug release.

- Colon-targeted delivery (38,55): As absorption in the GI tract is far from being uniform and as many molecules present absorption site specificity, colonic delivery appears to be an interesting approach for some proteins. To access this part of the GI tract, existing systems consist of particulate systems, pressure-induced systems, or yet pH- / time- / microbiota-dependent delivery systems. Advantages are prolonged transit time, lower proteolytic activity and a more basic pH than in upper part of the tract. However, variability in transit times, possible disease conditions and consideration of the microbiota leading to possible formation of biofilms barriers on the orally administered device really impact its efficacy.

- Oral delivery devices: Recently, new devices were developed for oral delivery of biomolecules such as a very innovative system of ingestible self-orienting vehicles (56) with the ability to release insulin directly in the gastric mucosa *via* drug-made needles. Other developments concern devices such as two-pulse spherical delivery systems (57), intestinal microneedles (58), Janus device (59), microfluidics and microdevices (60) or intestinal patches (61,62) that showed good results regarding drug release. They will be developed in the second and third parts of this report.

4. Permeability enhancers (15,63,64)

Permeability or permeation enhancers (PE) are defined as functional excipients capable of modulating and improving permeability, absorption and bioavailability of pharmacologically active biomolecules that are poorly absorbed if not directly injected in the systemic circulation (9). Studied since the 1990s, these substances could enhance absorption through epithelia of different routes: oral, buccal, nasal, ocular, vaginal, rectal and may permit reformulation of some injectable macromolecules to favor non-injection alternatives.

PEs are commonly classified according to their mechanism of action, which can be either transcellular, paracellular or a combination of both, as visible on figure 9. On one hand, transcellular PEs alter the epithelial permeability by a physical perturbation of the plasma membrane which increases the membrane fluidity and leads to an enhanced transcellular permeation. Most of them are surfactants or chelating agents. On the other hand, paracellular

PEs are tight junctions (TJ) selective compounds. Tight junctions are complex protein networks, localized between adjacent or endothelial cells at the apical pole, which regulate the passage of ions or molecules through the paracellular space. Physiologically, TJ sizes are location-dependent: for the GI tract, TJs are about 7-9 Å in the jejunum, 3-4 Å for ileum and 8-9 Å in the colon. They are composed of various transmembrane proteins such as claudins, occludin, junctional adhesion molecules and proteins of cytoplasmic scaffolding linked to cytoskeletal proteins (actin and microtubules) such as ZO-1, cingulin, or afadin (65,66).

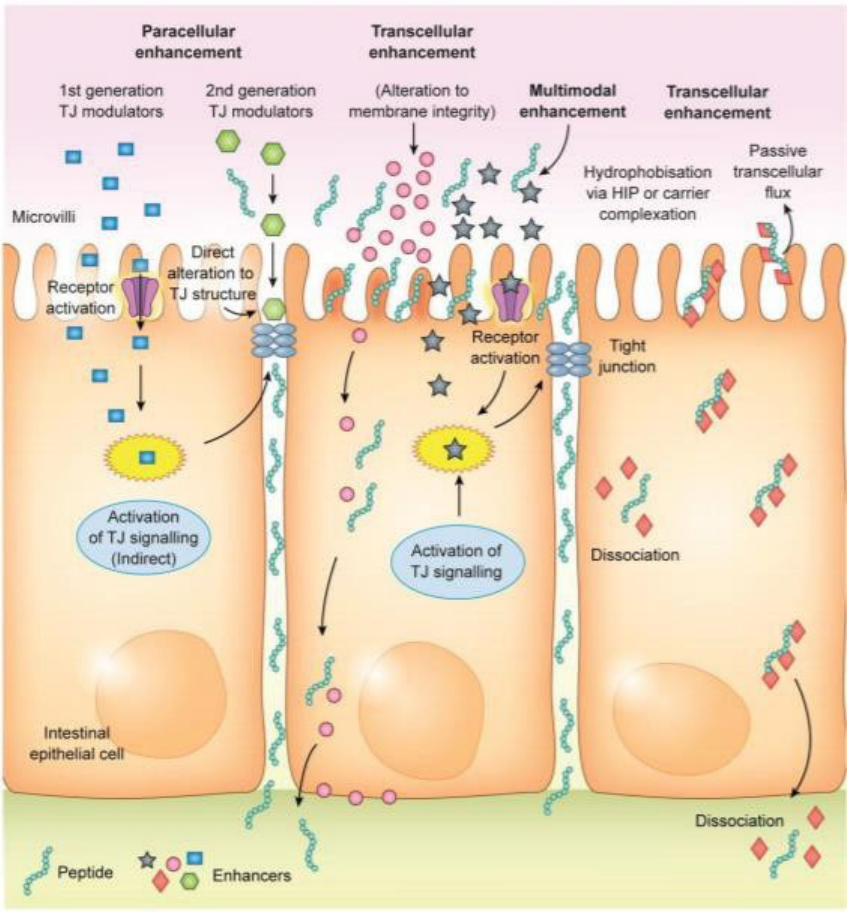

Figure 9: Mechanisms of action of Permeability Enhancers (PEs) on intestinal epithelium (63)

Going back to paracellular PEs, they are able to target various proteins composing the TJs and to modify their functional properties, including keeping the TJs open. The disruption of the structural integrity of the intestinal barrier leads to an increase in paracellular permeability, of which drug delivery could take advantage. Representatives of those TJ modulators are for example bacterial toxins and EDTA.

Peptides and protein drugs being hydrophilic in nature with large molecular weight, their absorption through epithelium is severely limited for both transcellular and paracellular routes. Under physiological conditions, movement of water and low-molecular weight solutes are regulated through the distension and constriction of the TJs of GI epithelial membranes, which

modify the paracellular porosity. Since TJs are opened and closed in response to physiological stimuli, this mechanism might afford a relatively safe and reversible means of permeation enhancement that, if combined with PEs, could potentially be extended to bigger compounds such as biotherapeutic molecules (9). Thereby, PEs constitute a technology that could allow therapeutic agents to permeate across biological membranes (such as the intestinal barrier) to access the systemic circulation and reach their site of action to exert their pharmacological actions without any chemical modification. As a matter of fact, PEs should be administered in combination with drugs within a specific formulation able to control their release. Ideally, PEs should be discharged prior to macromolecules from a two-pulse delivery system in order to establish a more favorable environment and improve mucosal permeation for the drugs.

One of the main concerns with PEs is an arguably direct correlation between potency and toxicity. Permeation enhancers should be safe and non-toxic, pharmacologically and chemically inert, non-irritant, and non-allergenic. However, because of their mechanism of action – by alteration of the epithelium or by alteration of cell membrane permeability and integrity – they can consequently cause disruptions in transmembrane ionic gradients, disfunctions in cellular activities, entry of impurities / toxins / allergens into cells and blood circulation. Moreover, the nature of a PE and its mechanism of action will determine whether the permeabilization is transient and whether tissues can regenerate in affected areas in case of cytotoxicity. Those are critical factors for an eventual long-term utilization as the most common drawback of PEs is that they may damage or even dissolve the biomembranes, leading to various gastrointestinal toxicity issues: local inflammation, ulceration, diminution of immunoprotective function of the intestinal epithelium in preventing pathogen entry (9). For example, amongst all the PEs developed during past decades (surfactants, cell-penetrating peptides (CPP), silicates, chelating agents bile salts, fatty acids…), many happened to be cytotoxic (67). Surfactants were shown to disrupt and partition the lipid packages composing cell membranes thus destroying their integrity, while other PEs can even extract important proteins from the cellular membranes. Chelating agents form complexes with calcium ions that break TJs in order to facilitate paracellular transport. As for CPP, they usually transport their cargo into the cytoplasm via perturbation of the lipid bilayer of the cell membrane or by endocytosis (68).

Various PEs have been investigated for the enhancement of protein and peptide absorption through the intestinal membrane. Although PEs are an effective and attractive approach to increase oral bioavailability of these biomolecules, safety and toxicity of these adjuvants needs to be assessed.

IV- Examples of applications

1. Therapeutic protein: insulin

Insulin is the most important regulatory hormone in the control of glucose homeostasis. It constitutes a very interesting biomolecule to represent peptide and protein drugs that could benefit from the development of non-injectable delivery systems and more particularly from oral alternatives. It is one of the main drugs on the market, with multiple daily administrations by the parenteral route for millions of diabetic patients requiring insulin therapy. Hence, development of an oral insulin-delivery system could really improve patient compliance to treatment. However, ever since the discovery of insulin, scientists started their quest for non-injection dosage form but did not succeed completely up to date (21,25,69,70).

From a pharmacological point of view, oral delivery would also be interesting as it would more closely simulate endogenous insulin than the subcutaneous administrated one. Insulin is normally secreted by the pancreas and accesses insulin receptors in the liver through the portal vein, avoiding the hepatic first-pass effect. Orally delivered insulin would mimic the pulsatile secretion pattern and also be delivered to the liver through portal circulation (69–72). The physiological metabolism of the protein would reduce the known side effects of parenteral administration: hyperinsulinemia, hypoglycemia, and troubles with weight control (9,71).

2. Permeability enhancer: 5-LR2 & proves of efficacy (73)

Protein kinase C (PKC) is a subfamily of serine/threonine kinases, implicated in several cellular functions and its isoforms are classified as conventional (α, β1, β2, γ), novel (δ, ε, η, μ, θ), and atypical (ζ, ι/λ). Peptidic inhibitors of protein kinase C isotype zeta (ζ) have been developed and already described as being effective against a wide spectrum of tumors, hyperproliferative disorders such as psoriasis and viral infections such as HIV (74). From a cellular point of view, they can also be used as permeability enhancers because PKC ζ inhibitors induce a transient redistribution of transmembrane proteins (occludin and ZO-1 for example) inside the intracellular compartment and a transient opening of the tight junctions. This property could be advantageously used to induce transient tissue permeabilization, and in particular to enhance penetration of large therapeutic molecules (antibodies, anti-opioid macromolecules, cancer treatment, induction of immune response for mucosal vaccination). When it comes to epithelial cells, PKC ζ inhibitors could be used to improve transmucosal delivery of high molecular weight drugs, e.g., peptides and protein drugs such as insulin.
This property can be assessed through measures of P_{app} (apparent permeability) and TEER

(Trans-Epithelial Electric Resistance) that reflect the barrier integrity of cell monolayers *via* tightness of the intercellular junctions.

The permeability enhancer of interest here is L-5R2 which is a PKC ζ inhibitor, chemical analog of the pseudosubstrate, composed of 5 amino acids conjugated to a myristoyl acid, for a total molecular weight of 954 Da (73). As for the nomenclature, L- corresponds to the L enantiomer, 5 for the number of amino acids, and R2 stands for arginine in position 2. Its structure was optimized from a 13 amino acids structure and reduced to five, with various *in silico* and *in vitro* studies to determine which ones were crucial for the permeability enhancing activity (choice between L-5R2 and L-5K2). Peptide L was preferred to its enantiomer D as it showed a better activity on both cell models Caco-2 and Mucilair®, respectively standing for intestinal and respiratory epithelia (see Figure 9 – A, B and C).

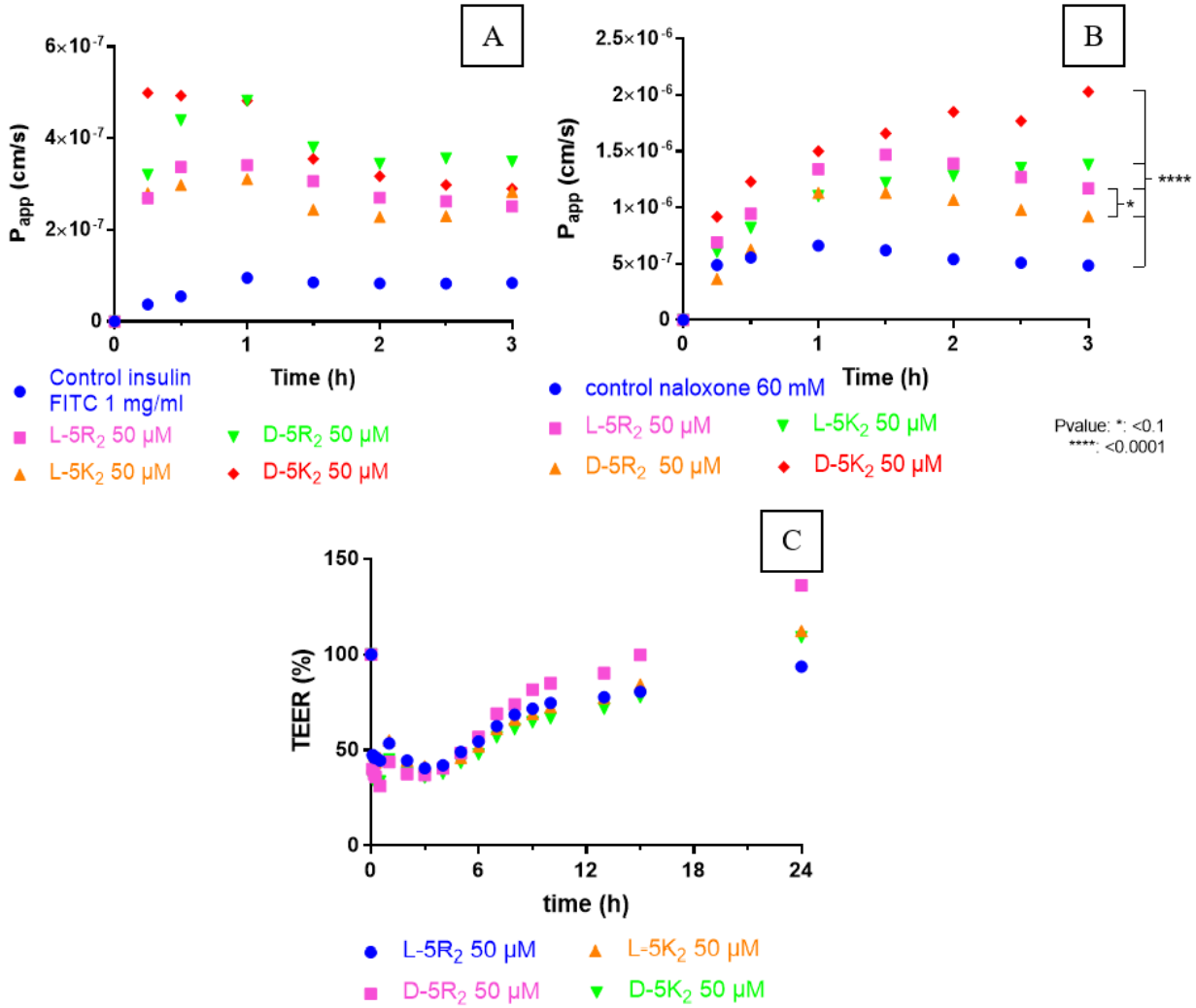

Figure 10: Impact of the peptide composition on its efficacy on Caco-2 cells
A and B: Permeability of insulin and naloxone co-administered with peptides,
C: Impact of peptides on TEER.

L-5R2 and D-5R2 correspond respectively to peptides L or D, R2 and K2 correspond respectively to amino acids Arginine or Lysin in position 2

Due to its amphiphilic composition, the peptide is supposed to arrange itself into a supramolecular structure known as micelles. It was shown that the peptide has a critic micelle concentration (CMC) of around 35µM, as shown in figure 11 below.

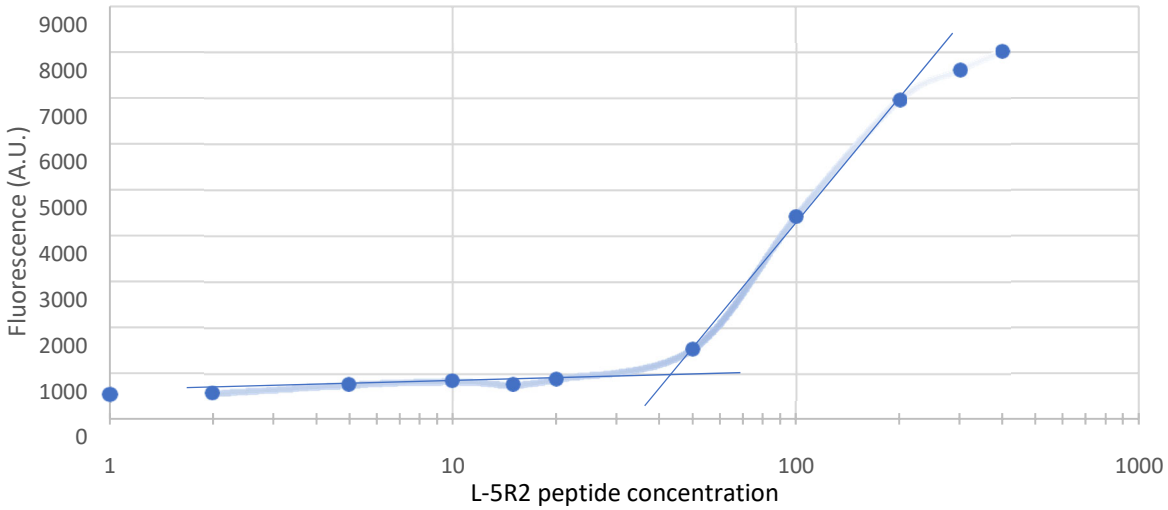

Figure 11: Determination of CMC based on the evolution of fluorescence versus L-5R2 concentration

Experiments showed that efficacy of the L-5R2 peptide is concentration-dependent, starting around 20µM (Fig. 12 below).

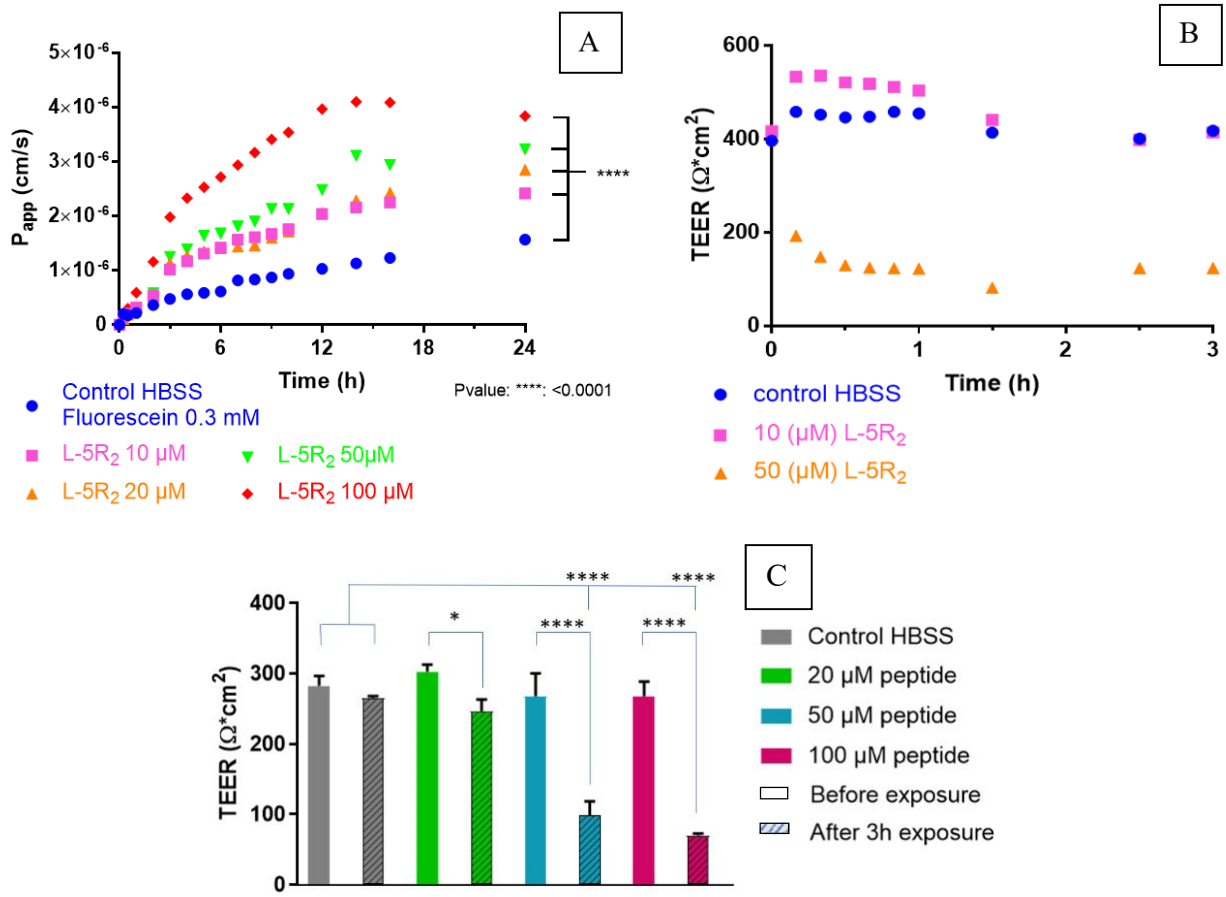

Figure 12: Efficacy of the peptide on Caco-2 cells permeabilization (A) and TEER (B and C) versus L-5R2 concentration

As for toxicity, a WST-1 assay revealed the absence of acute toxicity of the peptide in Caco-2 cell-line, within a large range of concentration from 10 µM to 30 mM (Fig. 13 below), and the reversibility of TJ opening (Fig. 12C).

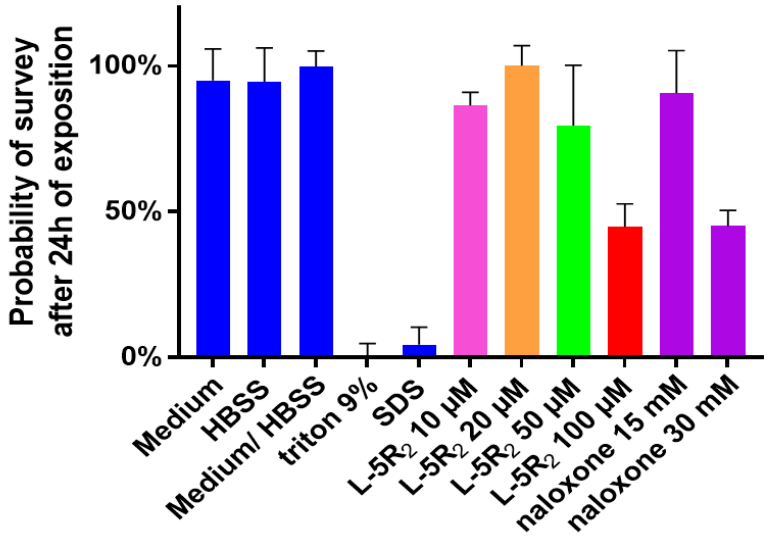

Figure 13: Evaluation of L-5R2 toxicity in Caco-2 cell line at various concentrations

Experiments showed that L-5R2 significantly reduces TEER and enhances paracellular Caco-2 permeability starting at a concentration of 20µM. However, with a CMC around 35µM, this implies that the peptide can act either as a single structure or as a micelle structure, without any direct interaction with the co-administered drug as shown in DLS (Fig. 14).

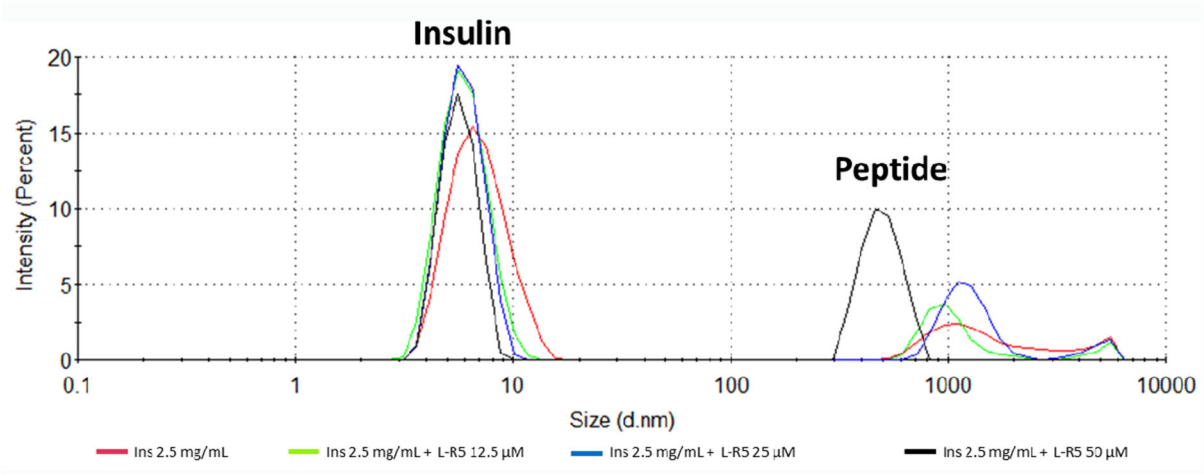

Figure 14: Proof of non-interaction between the L-5R2 peptide (single and micelles) and insulin by dynamic light scattering (DLS - Nanosizer®)

Even though specific mechanisms of action are still to be defined, L-5R2 optimum activity *in vitro* was measured for a concentration of 50 µM with a 7-hour duration (Fig. 10C), improving paracellular transport for macromolecules up to 150 kDa (Fig 10A and 10B), including insulin. Still more promising, initial *in vivo* results showed that nasal co-administration of L-5R2 with insulin resulted in a significant reduction in blood sugar level in mouse at 50 µM.

V- Conclusion

To conclude on this part, many strategies were studied to effectively address application of peptide and protein therapeutics by the oral route (Fig. 15). Overcoming biological barriers constitute the main challenge but technologies previously described succeeded in improving oral bioavailability of drugs.

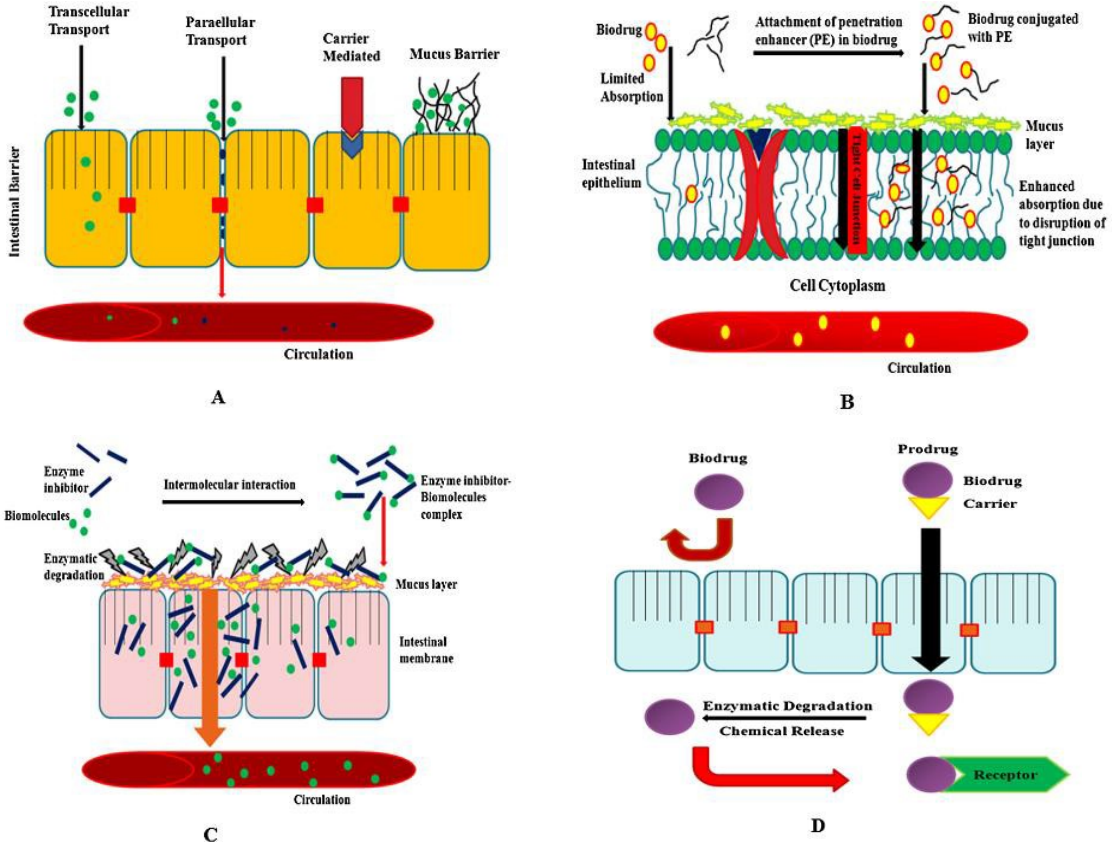

Figure 15: (A) Transport mechanism of biodrugs through the intestinal epithelium membrane and summary of various options for enhancing biomolecules permeability through epitheliums, (B) Permeability enhancers, (C) enzyme inhibitors, (D) prodrug derivatization (23)

Although those strategies are necessary steps for the development of oral delivery systems and present interesting capacities when used individually, they are still limited to ensure a sufficient bioavailability on their own. However, combinations of those concepts could result in synergistic effects that would constitute a compelling approach to develop the next generation of protein therapeutics.

As an example, focus will be put in the second part of this work on the development of intestinal micropatches that would combine the use of PEs and enzyme inhibitors within an innovative oral delivery system with sustained release, to successfully deliver peptide and protein therapeutics through the oral route.

THEORICAL PART: ORAL DRUG DELIVERY SYSTEMS

I- Ideal characteristics of drug delivery systems for the oral route

An ideal drug delivery system should be capable of:

- targeting the site of absorption,
- maintaining the integrity and stability of the carried drug molecules until there,
- releasing drugs at the target absorption site and maintaining position long enough for the system to deliver its whole drug load
- delivering a measurable and reproducible amount of drug to the target site
- and eventually promoting permeation to achieve systemic drug uptake.

DDS can be characterized by their flux of drug absorption, which in the case of passive transport are controlled by three main parameters: A the area of absorption, C_0 the drug concentration at the exchange surface (barrier) and P_{app} the apparent permeability of the intestinal tissue to the therapeutic drug (75). Consequently, the flux of drug absorption F can be defined by equation 1:

$$F = \frac{dQ}{dt} = A \text{ x } C_0 \text{ x } P_{app} \qquad \text{Eqn (1).}$$

Designing an efficient drug delivery system that facilitates oral administration of therapeutic proteins has then many parameters to consider to be optimum, amongst which:

- Combination of smart excipients: The strategy of employing enzyme inhibitors to protect the DDS contents from various enzymatic actions, combined with permeability enhancers that promote absorption, is a popular approach for oral protein drug delivery. Those latter reduce the mucosal barrier function and thereby directly enhance P_{app} from Eqn (1). Combination of these strategies were shown to improve drug uptake and prevent enzymatic breakdown of loaded therapeutic proteins (61).
- Co-localization: Co-localization of drug(s), PEs and enzyme inhibitors inside a unique solid DDS is a main asset to improve absorption. It ensures a synchronous release of those molecules and controlling the release of PE(s) and/or enzyme inhibitors prior to the release of biomolecules might enhance the effectiveness of these drugs by establishing a more favorable environment (15). Co-localization also ensures a synchronous release of these molecules within the same period of time, same environment and same volume of gastro-intestinal fluids. This would reduce the loss of drug molecules in the surrounding medium

(76) and would favor the set-up of a diffusion gradient that forces molecules to act on or to permeate through the intestinal barrier. Thereby, a small quantity of PE(s) delivered at the right localization will lead to greater enhancement of drug absorption than if dissolved in a larger volume of fluids (63).

- Size: Size of solid DDS is a critical parameter as it influences at the same time the quantity of API loadable and its release. For solid dosage forms, the bigger, the higher drug load is reachable but the smaller, the better for release and absorption. Multi-particular systems are characterized by the fact that the administered dosage form is constituted of several units with a size from 150µm to 2-3 mm of diameter, each one of them containing a small dose of API. Administration of several solid sub-units compared to a bigger one with an equal surface area, would deliver a higher quantity of drug as size reduction improves drug release. In fact, size of DDS influences the flux of drug absorption on two aspects: on one hand multi-units would be delivered over a bigger intestinal surface, directly improving the area of absorption A from Eqn (1), and on the other hand , reduction of size would concentrate drug molecules in a smaller surface so C_0 would also be enhanced too according to Eqn (1). Multi-units also offer flexibility in formulation and limit risks of therapeutics burst effect and of local irritation in the GI tract thanks to wider surfaces of dispersion and larger exchange surface areas. Finally, multiplicity of units composing the dosage form provide biopharmaceutical advantages compared to single dosages like various release profiles and more predictable transit times through the GI tract that reduces inter- and intra-batch variability.
- Composition & protection: Considering a frequent administration of oral DDS, choice of biocompatible and biodegradable excipients is recommended. At the same time, they should be able to overcome barriers and provide protection from harsh environmental conditions that may inactivate incorporated therapeutics. In particular for the oral route, ideal systems should be able to maintain themselves inside the gastrointestinal tract despite the transitory constraints (food / beverage passage, peristalsis) and this is why the choice of specific mucoadhesive polymers is crucial (15).
- Process: Ease of production with adequate industrial equipment is required. Tablets are classic galenic forms and plenty of mini-tablets already exist on the market. Multiplicity of units constitutes an advantage as it is industrially possible to commercialize a wide range of precise dosages from one unique tablet unit.

- Kinetics & release: Depending on the pathology, therapeutic proteins delivery should ideally match a specific drug-release profile. Either as controlled-release formulations (for drug delivery at predetermined rate, following zero-order kinetics) or sustained-release formulations (continuous release of drug over a prolonged period of time, following first-order kinetics), those DDS are designed to achieve therapeutic effects by releasing drug over an extended period of time after administration of a single dose, so plasma concentrations are maintained within therapeutic range as illustrated in figure 16. Among other advantages, there are reduced minimized side effects, reduced frequency of administration, fluctuations in steady-state drug level, increased safety margin & reduced toxicity, or reduction of costs.

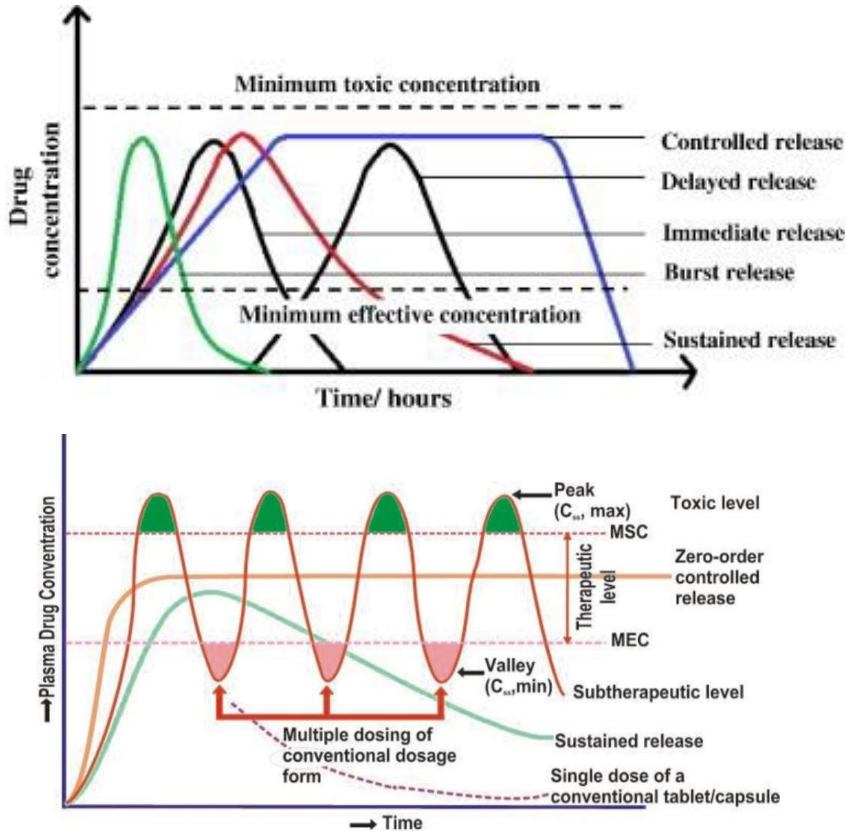

Figure 16: Classification of drug release profiles. Drug concentration in plasma versus time profile. MSC: maximum safe concentration, MEC: minimum effective concentration (77,78)

Oral delivery is considered to be the most convenient administration route due to its specific advantages, but it faces substantial challenges that need to be addressed. This is even more true in the context of biopharmaceuticals delivery. Formulation technology is the key to the effectiveness of an absorption-enhancing approach and initial data suggest that inclusion of PEs in oral formulations can safely assist absorption of selected potent peptides with a large therapeutic index (63). Work now relies on designing an optimum drug delivery system, adaptable for lots of therapeutic proteins and later on extending it to proteins with narrow therapeutic index.

II- Focus on intestinal micropatches: concept

Intestinal micropatches are multi-layered solid dosage-forms, with a size from 500 µm to 5 mm diameter, adhering to the intestinal mucosa by bioadhesion when orally administered. Initially inspired by transdermal patch technology, those millimeter-sized mucoadhesive devices aim to overcome the disadvantages of traditional oral delivery of protein drugs. The concept is based on a drug release depot, improving the bioavailability of the drug by providing a unidirectional diffusion regime towards the intestinal tissue after the attachment of the micropatches to the inner walls of the intestinal tract.

Intestinal micropatches are composed of several layers as illustrated in figure 17, each one of them able to perform specific tasks (61,75,79–81):

- First one is a mucoadhesive layer, ideally containing permeability enhancers to promote the access of drugs to the blood vessels of the mucosa. Its role is to have the device adhere to the intestinal wall and is usually composed of mucoadhesive polymers such as poly(acrylic)- acids (Carbopol®), derivatives of cellulose, polycarbophil cysteine, chitosan & derivatives, alginates or pectin.
- Second one is a layer where therapeutic proteins of interest are dispersed into a mixed matrix-diluent phase. It constitutes the drug reservoir and a strategic choice of excipients could help design a controlled delivery. For example, classic choices for diluents are microcrystalline cellulose, lactose, starch, calcium salts … and for matrix are HPMC, ethylcellulose, … Bibliography also highlighted the possibility to combine the first and second layer as a unique one (79,82), but this would expose the therapeutic proteins to partial degradation before adhesion of the patches to the mucosa.
- Third and final one is a water-impermeable backing layer. It is a solution that is sprayed over the entire micropatch except on one side (mucoadhesive layer - to allow adhesion to the GI tract) and forms a solid layer by solvent evaporation. It has a protective function towards the therapeutic proteins against GI fluids, minimizes the dilution effect and ensures the unidirectional flux through the unsprayed side. This layer is usually composed of inert and water-insoluble excipients such as ethylcellulose or cellulose acetate.

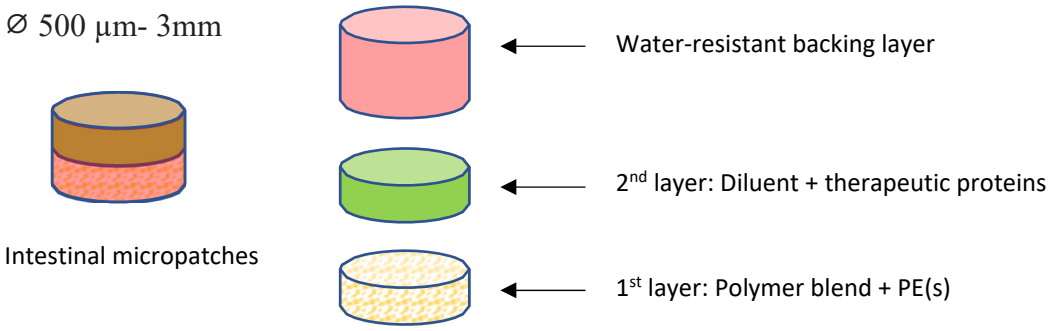

Figure 17: Schematic representation of the intestinal micropatches

Finally, for better protection, intestinal micropatches are then placed, with free protease inhibitors and acidity modifiers, into a gelatin capsule covered with a gastro-resistant film as illustrated in figures 18 and 19. The adjuvants often used are aprotinin and soybean trypsin inhibitors as protease inhibitors and citric acid as for acidity modifiers. The addition of citric acid in a capsule would lower pH in the intestinal medium and favor an acidic environment once the capsule would have disintegrated. The enteric coating is used to protect the capsule from gastric degradation so the patches would only be released in the first parts of intestines (duodenum / jejunum). The enteric coatings usually used are pH-sensitive poly(methyl methacrylate-co-methacrylic acid) copolymer derivates (Eudragit® L100 or S100 or other derivatives) or HPMC phtalate (HP-55).

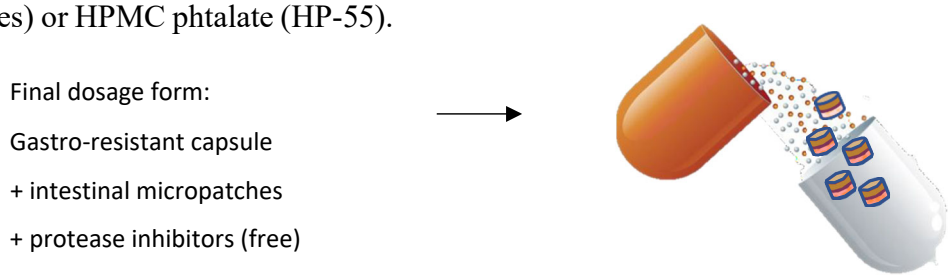

Figure 18: Schematic representation of the drug delivery system

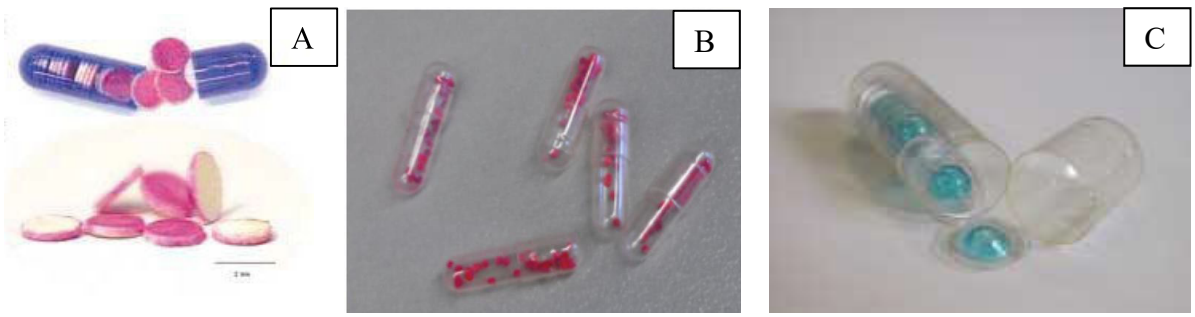

Figure 19: Examples of capsules containing gastrointestinal mucoadhesive micropatches: (A) insulin-loaded patches stained with sulforhodamine B (83), (B)rhodamine-loaded micropatches (59), (C) film-layered micropatches (75).

If desired, additional protection can be added to the intestinal patches. For example:

- The mucoadhesive bottom layer can also be protected *via* the addition of a pH-sensitive layer that enhances protection of the drug reservoir as it would react only in a specific intestinal segment with a specific pH thereby unmasking the second adhesive layer (61). In this case, the gastro-resistant coating of the capsule is useless.
- Because of their small size, micropatches are submitted to van der Waals forces, which make them susceptible to self-aggregation. This phenomenon can lead to non-attachment of tablets to the mucosa and consequently lead to significant drug loss and reduction of the overall efficacy of the device. As a solution, an additional coating can be used to prevent micropatches from self-aggregation such as Eudragit L-100. It is an anionic copolymer commonly used for its negative charges to electrostatically repulse themselves thereby preventing self-aggregation and ensuring an individual release of micropatches, without disturbing drug release (61,62).

Main parameters of this system to be considered are mucosal adhesion properties, loading capacity, controlled or sustained drug release. Moreover, attention should be paid to the thickness of the micropatches: as they adhere to the intestinal lining, the thinner the better. As a matter of fact, too thick patches could be detached from the lumen wall by food passage after the gastric area or by solid boluses of digested food formed along the GI tract (ileum), thereby decreasing their efficacy. This is why ideal release location of the micropatches is in the upper parts of the intestines (duodenum / jejunum), where they do still need strong bonding with the mucus to avoid being washed away by gastric juice (29).

Concerning the *in vivo* fate of the devices, one capsule is absorbed by the oral route, and successfully goes through the stomach, protected by its gastro-resistant coating. Once pH is high enough in the beginning of the duodenum, the gelatin capsule disintegrates to release on one hand the free protease inhibitors and acidity modifiers, and on the other hand the intestinal micropatches (Fig. 20A, B). Thanks to the more favorable environment, the microdevices can adhere to the duodenal mucosa *via* their mucoadhesive surface (Fig 20 C, D). Water from the mucus and the environment allows local dissolution of the first layer so permeability enhancers can act on tight junctions and open them. Then swelling achieve the second layer, releasing the therapeutic molecules that can directly access the blood circulation via paracellular transport. Nature of the excipients (biocompatible, biodegradable), food & beverage passages and mucus turnover are the main factors explaining the detachment and further disintegration of the patches, except for the water-resistant backing layer that would be eliminated intact in the feces.

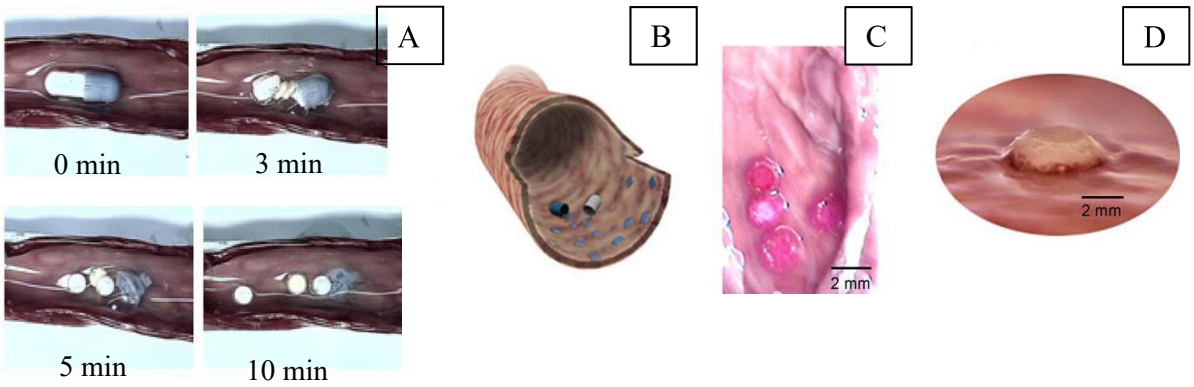

Figure 20: In vivo becoming of capsules containing micropatches (82)
(A) Time-lapse photographies of patch release and mucoadhesion on porcine intestines,
(B) Schematic representation of the drug delivery system,
(C) Adhesion of patches on porcine intestines in vitro,
(D) Close-up look at patch adhesion on rat intestine in vitro – early stage of swelling.

Considering manufacturing of such devices, it might seem a critical point but various strategies are possible (75):

- conventional techniques, usually consisting of classic tablet-manufacturing process and then in size reduction,
- microfabrication techniques, that can include photolithography or micromolding for minitablets like in figure 21A,
- rapid prototyping technology such as 3D-printing visible on figure 21B.

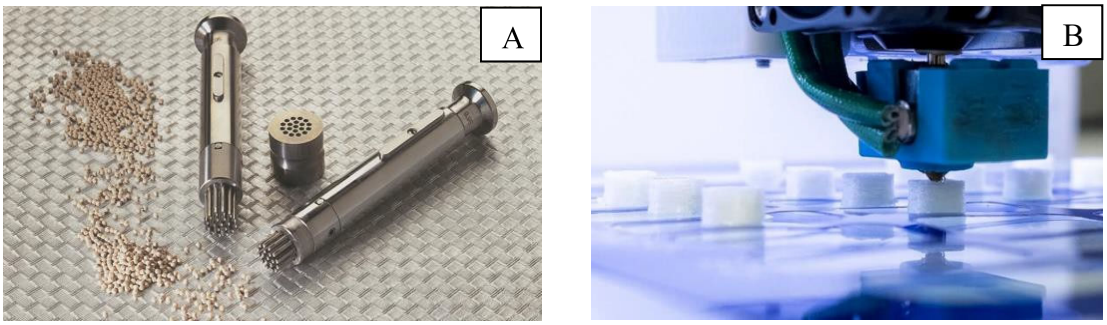

Figure 21: Examples of micropatches manufacturing processes
(A) minimolds for tableting (84), (B)3D-printing of minitablets (85)

At this time, manufacturing processes would need further considerations to find the most suitable processes, adapted to specific geometries of the products, but further developments should get these techniques more accessible and lower high manufacturing costs.

To conclude on the concept, intestinal micropatches have been especially attractive for improving the oral bioavailability of bioactive drugs. They are a combined strategy that includes encapsulation of the drug for its protection, mucoadhesion to increase the contact-time with the mucosal absorbing-barrier & the transit time in the GI tract, the co-localization of their activity

and the co-administration of adjuvants (enzymatic inhibitors to preserve drugs from degradation and permeability enhancers to enhance drug absorption) (75). Association of these strategies in a single device, such as these drug reservoirs, is an innovative approach: it isolates the drug molecules from the environment, forms an important drug concentration gradient in front of the intestinal mucosa, ensures a unidirectional release, avoids dilution effect & loss of proteins into GI fluids and reduces the used amounts of each active molecules and toxicity.

III- Proofs of efficacy

Intestinal micropatches have been tested for oral delivery of various proteins with very low (0-2%) bioavailability such as salmon calcitonin, exenatide, interferon-α, erythropoietin granulocyte-colony-stimulating factor (G-CSF) and insulin (29,33,82).

Comparison between administration of drugs in solution and as solid formulations (micropatches) demonstrated the interest of those latter:

- Micropatches significantly enhanced transport of sulforhodamine by 30% and of phenol red by 45% compared to 10% as solutions across rat intestines during 1-hour *in vitro* experiments (81).
- Experiments across Caco-2 cell lines showed a two-fold enhancement of transport of insulin and exenatide if administered as micropatches instead of protein solutions, without adverse effect on cells integrity (*via* TEER measures). To push forward, *in vivo* experiments were carried out to validate those results while taking into account the impact of mucus (not existent with Caco-2 cells) and both patches of insulin and exenatide increased respectively by 13 and 80-fold the relative bioavailability compared to intrajejunal injections of solutions. Same for salmon calcitonin that increased by 52-fold the relative bioavailability compared to intrajejunal injections in rats (80,86).
- Patches of insulin with dimethyl palmitoyl ammonium propane sulfate (PPS – acting as a PE that transiently opens TJs to improve paracellular transport), resulted in a 30% drop from baseline in blood glucose levels in 8 hours in diabetic rats compared to non-significant drop (<0,5%) of insulin -PPS solution (62).
- Takada et al developed gastro-intestinal mucoadhesive patch systems for the oral delivery, filled with gels containing PEs and EPO. Administration of the patches resulted in a 5-fold enhancement of C_{max} and a 6-fold enhancement of bioavailability compared to intrajejunal administration of EPO solution. Adaptation of the patches in favor of G-CSF as therapeutic

drug turned into a 23% bioavailability compared to intravenous injection (measured *via* the increase in total white blood cell count), which represents an important evaluation compared to 0-2% of protein oral bioavailability classically achieved (79,87).

- Finally, regardless of their composition, micropatches globally proved their ability to achieve strong mucoadhesion through various *ex vivo* studies and to withstand shear forces during intestinal peristalsis and water or food movements. In particular, prototypes of Mitragotri and his group usually released their drug load between 3 and 5 hours and adhesion to the intestinal mucosa did not induce any adverse effect: no toxicity observed on histological sections and no effect on membrane integrity by TEER evaluation (62,79,80,86).

Concerning the interest of permeability enhancers in co-administration with micropatches:

- Co-administration of PPS with salmon calcitonin inside intestinal micropatches showed a 56% decrease of plasma calcium levels, compared to no significant reduction with sCT alone (79,88).
- Addition of surfactants as PEs significantly improved EPO absorption through the oral route, with an enhancement of absolute bioavailability from 0.6 to 1.9. Moreover, in this example, switching of dosage form from solution into GI patches enhanced EPO absolute bioavailability from 1.9 to 12.1, completed with a significant difference ($p<0.001$) on AUC between patches and solution (with and without Labrasol) (87).

Table 3: Pharmacokinetics parameters of EPO (Dose of 100 IU/kg) following small intestinal administration of EPO formulations (87)

Dosage form	Permeability enhancer	C_{max} (mIU/ml)	T_{max} (h)	AUC_{0-6h} (mIU h/ml)	Absolute bioavailability
Solution	/	3.1	1.4	13.9	0.6
Solution	Labrasol® 94mg/kg	16.2	0.5	43.8	1.9
GastroIntestinal Patch System	Labrasol® 94mg/kg	84.1	3.0	271.6	12.1

Finally, combination of those strategies as intestinal micropatches containing permeability enhancers resulted in interesting results:

- Banerjee et al. (62) designed 500 µm-diameter insulin-PPS micropatches that were encapsulated inside a gastro-resistant capsule with free citric acid. During *in vitro* experiments, microdevices were able to adhere to the intestinal mucosa within 30 minutes and to release their entire content load, regardless of molecular weights: BSA and lysozyme as model proteins for release study, PPS as permeability enhancer and insulin (-FITC grafted for fluorescence measures) as therapeutic molecule. Results reported in figure 22 (A,B,C) confirmed that the specific composition of the micropatches constitute an interesting matrix for the delivery of any molecule, without major entrapment risk and that devices do not need an extra load of molecules to achieve a required delivery threshold.

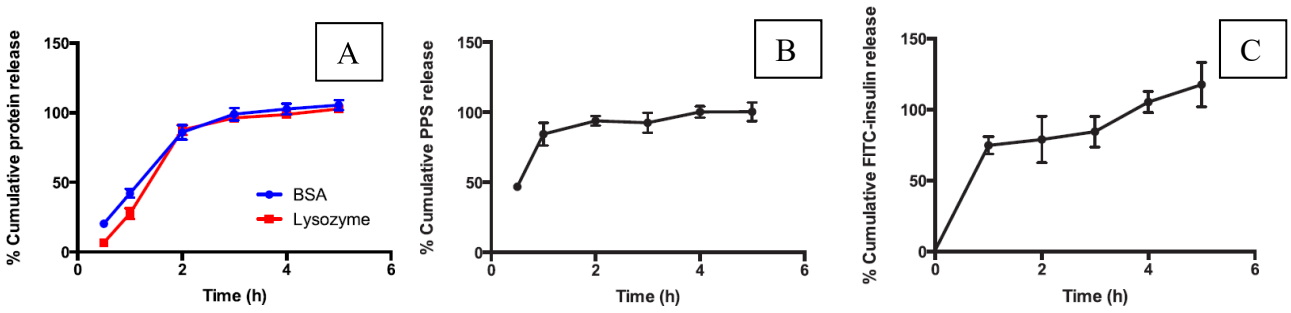

Figure 22: Protein release profiles from mucoadhesive devices: (A) BSA and lysozyme, (B) PPS, (C) FITC-insulin. Percent cumulative release of molecules tested in pH 7.4 PBS at 37°C. Error bars correspond to standard deviations (62).

In vivo experiments showed the ability of these micropatches to lower blood glucose levels, and its relative effectiveness compared to standard subcutaneous insulin injection. First in nondiabetic rats (Fig. 23 A), a significant difference is already seen 1 hour after administration of the insulin patches compared to the empty ones, with a 13% drop in blood glucose levels and down to 27% after 8h. As for PPS, co-administration with insulin in the capsule resulted in a significant - but moderate - effect on blood glucose level (90% after 6h, 77% after 8h), but experiments demonstrated that the best efficacy is achieved with micropatches containing insulin and 10% PPS wt/wt, among all the other orally administered formulations : a 18% drop after 1 hour and a decrease to 67% after 8 hours. The devices thereby showed a "long-term" effect compared to subcutaneous injection(drop to 60% by 1h but increased at 81% by 8h).

As for diabetic rats (Fig. 22 B), the groups "no treatment" and "empty patches" demonstrated no significant impact on blood glucose levels at 8 hours. The one with administration of

insulin patches resulted in a significant drop to 86% after 1h and 74% after 8h, while addition of PPS in the capsule did not achieve such an efficient drop (89% at 8h). As expected, the orally administered solution containing insulin and PPS resulted in a non-significant drop (99.8%). Once again, group with insulin + PPS in patches revealed the best efficacy in lowering blood glucose levels, with a 20% drop at 1h decreasing to 69% by 8h, with a sustained effect compared to the insulin injection that resulted in a significant drop down to 76% at 1h, 53% at 2h but ended increasing up to 86% at 8h. .

The study concludes that insulin and insulin-PPS devices showed statistically significant difference in blood glucose lowering efficacy compared to all other groups ($p<0.5$) in both nondiabetic and diabetic rats, with a global 30% drop from baseline (62).

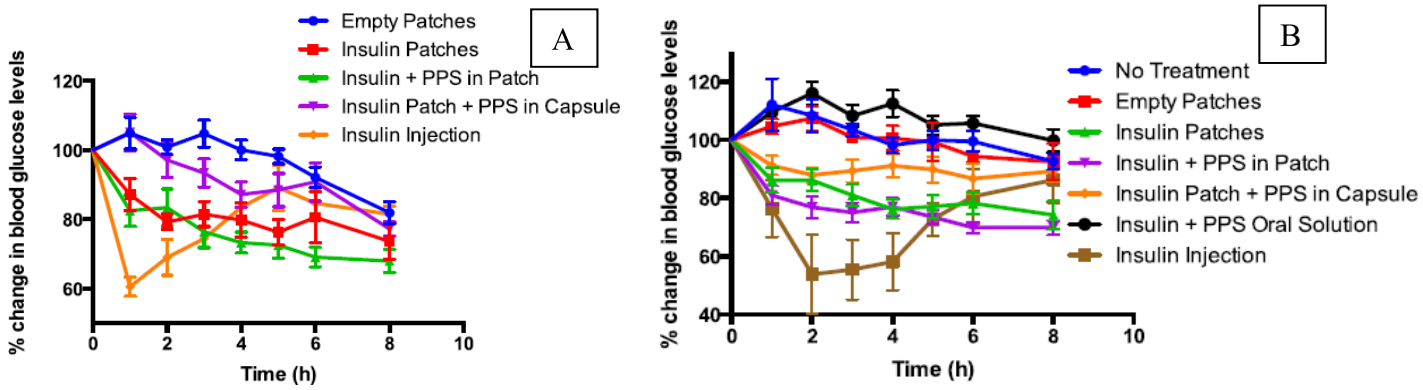

Figure 23: Efficacy of oral insulin formulations in lowering blood glucose levels in nondiabetic rats and diabetic rats (62).
Percent reduction in blood glucose levels with time after administration of various formulations in (A) nondiabetic and (B) diabetic rats. Blood glucose content was analyzed for 8 hours, in comparison to blood glucose levels at baseline (0hr). Animals were fasted prior to and during the 8-hour period of study. Micropatches contained 50 U/kg of insulin for nondiabetic rats and 100 U/kg of insulin for diabetic rats. PPS dosage was 0.6 mg/animal when administered in patches or 5 mg/animal when externally placed in capsules. Oral solution contained 100 U/kg insulin and 5mg PPS, while subcutaneous insulin injections were at the dosage of 1 U/kg. Error bars correspond to standard deviations.

- Finally, another article by Banerjee et al., with the same design of experiments showed that administration of a capsule with micropatches containing insulin and PPS in non-diabetic rats led to a 34% decrease within 3h compared to baseline, and even reached 41% decrease of blood glucose levels in 8 hours after 3 successive oral administrations separated of 30 minutes, thereby confirming that multi-administration could be an interesting pattern for sustainable release (79).

- Whitehead et al. (82) developed intestinal micropatches that revealed their efficacy in various aspects (adhesion, drug release, induced response) during both *in vitro* and *in vivo* experiments. First, *in vitro* tests showed that micropatches administered in a capsule were delivered within 10 min and 90% of them adhered to the mucosa on the mucoadhesive side. According to figure 24, more than 99% of the total insulin load was released over a 4-hour period, and only through the mucoadhesive side thereby confirming the unidirectional release.

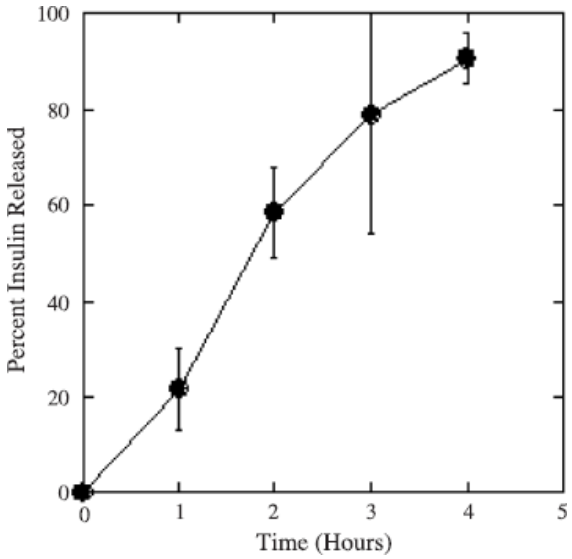

Figure 24: Insulin release from the patches, measured in vitro (82). Error bars correspond to standard deviations.

Then, *in vivo* experiments showed that intrajejunal administration in non-diabetic rats resulted in hypoglycemia with a maximum of 60% and 75% drops in blood glucose levels, respectively, at 5 and 10U/kg. Tests run with doses from 1 to 10 IU/kg in micropatches revealed a concentration-dependent effect on reduction of blood glucose level, as expected. Results were confirmed with negative controls (patches without insulin, or insulin solution) that did not induce hypoglycemia in rats. Moreover, intestinal patches offered significant hypoglycemia (>50%) at doses in the range of 5 to 10 IU/kg (Fig. 25 A). 10 U/kg patches-induced hypoglycemia was comparable to the one obtained with 1-5 U/kg subcutaneous injections, suggesting a higher insulin uptake from micropatches and meaning that doses only 2 to 10-fold higher for oral administration can induce the same clinical response than parenteral administration (Fig. 25 B).

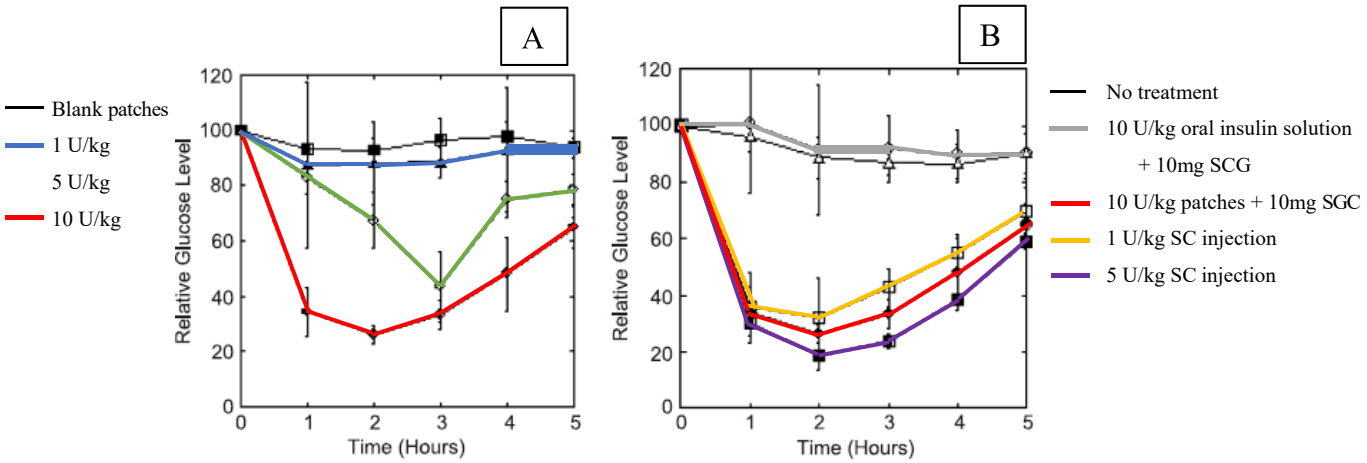

Figure 25: Relative blood glucose levels after intestinal administration of insulin formulations (82). (A) Relative blood glucose levels after intestinal administration of insulin micropatches patches at three doses. A total of 10 mg of sodium glycolate (SGC) was additionally co-administered in all experiments as permeability enhancer. Error bars correspond to standard deviations. (B) Comparison of hypoglycemia induced by various dosage forms: no treatment, oral solution, micropatches and SC injections. Error bars correspond to standard deviations.

Administration of insulin micropatches resulted in a significantly more important and sustained decrease of blood glucose level in contrast to a quick reversible drop observed with subcutaneous administration. Moreover, those experiments also confirmed that the solid form is a crucial parameter, regardless of the composition, and that patch design can constitute a solution by itself as dissolved patches did not induce any significant hypoglycemia (Fig. 26 A). Finally, the evolution of serum insulin concentrations were similar for 10 U/kg oral patches and 1 U/kg subcutaneous injection, suggesting that the insulin uptake through the mucosal barrier does not delay the absorption nor impact the global profile (Fig. 26 B) (82).

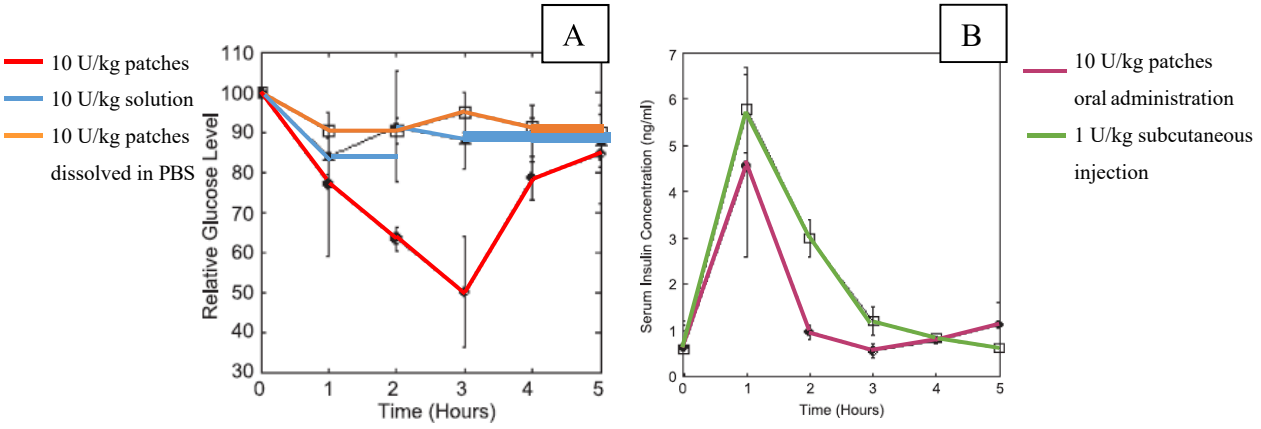

Figure 26: Impact of various dosage forms on induced hypoglycemia and serum insulin concentration (82) (A) Hypoglycemia induced by 10 U/kg insulin under various dosage forms: micropatches, solution and patches dissolved in PBS. All experiments were run without permeability enhancer. Error bars correspond to standard deviations. (B) Serum insulin concentration after insulin administration under two dosage forms: oral administration of 10 U/kg patches and subcutaneous injection of 1 U/kg. Error bars correspond to standard deviations.

To conclude on this part, the efficacy of intestinal micropatches can be assessed by the achieved bioavailability of therapeutic molecules. Benefits of patches over other dosage forms, in particular solutions, are the very limited dilution effect leading to the reduction of proteolytic degradation, increased permeability and increased local concentration. This latter is thought to be the primary reason for relatively high bioavailability, because even if animal doses are quite moderate, the small size of patches still lead to quite high concentrations (400U/cm²), that is to say up to 20 times higher than in controls. In the case of insulin, in comparison with the typical dose of 1 U/kg for SC injections and in comparison with the most promising techniques of oral delivery currently studied - where classic doses administered to reduce by 50% blood glucose levels are around 75 to 100 U/kg - micropatches showed their ability to drop these concentrations at doses of around 10 U/Kg, suggesting their tremendous potential. Experiments also showed that the efficacy of the system originates more from the engineering design than its composition. Efficacy can also be pushed forward *via* the use of selected permeability enhancers that work synergistically with the patches, in regard of the significant interest of PPS but not SGC. Finally, safety studies showed the non-toxicity of the devices on intestinal tissues, but this is actually more dependent on the nature of the permeability enhancer if existent in the patch.

IV- Areas of improvement for existing micropatches

Intestinal patch-based devicesaddress all aforementioned issues by releasing drug at a specific intestinal location and unidirectionally, in a controlled fashion, with a role of protection and delivery. However, those systems could still be optimized to achieve better efficacy, to broaden new opportunities for the development of oral protein delivery systems.

First, patch design and geometry can be optimized. Choice of patch diameter is influenced by the amounts of excipients to load and the dose of drug to administer but impacts absorption and concentration gradient per unit. Same for thickness of the patches, as devices might interfere with food or beverage flow with the risk of being dislodged. Ideally, the flatter, the better as it also reduces the distance between proteins and the mucosa, but this is obviously process challenging.

Second, composition of the patches is critical as it impacts on adhesion and dissolution time. More globally, composition impacts on drug release and absorption and consequently defines the kinetic profile of the device. By optimizing the patch composition, the oral drug delivery system can be improved and be controlled over a wide range (82). As for the lower layer, many

studies among those quoted before suggested to further investigate the concept of mucoadhesion of the devices and possibly develop stronger mucoadhesive properties with "recently-discovered" polymers, in order to improve the residence time in the intestines. As for the second layer containing the therapeutic proteins, formulations should be optimized by choosing tunable materials to exhibit the desired drug-release profile from this matrix (immediate / controlled / sustained release). Possibilities are specific coatings, modifying cross-linking density, gel inclusion or the encapsulation of drugs in multiparticulate systems and their incorporation inside the micropatches. For example, Kalavadia et al. proved the superiority of patches containing FITC-dextran in chitosan nanoparticles over simple FITC-dextran patches (89). Same for Shen et al., who showed that incorporation of drug-loaded microspheres into the patches, instead of direct loading of the drugs, can provide significantly enhanced control over the release behavior of the drug, while Toorisaka et al. made a lipophilic formulation impregnated on the mucoadhesive matrix of bilayered intestinal patches (29). Applied to diabetes and insulin, it should then be possible to design various intestinal patches including delivery of basal insulin doses over long periods of time, delivery of bolus doses over short periods of time and potentially a combination approach that delivers basal as well as boluses by using different types of patches (82). In addition, delivery of two different molecules inside a unique multi-reservoir / multi-layered type of micropatches could be considered in order to design new profiles of blood glucose levels (combination of metformin and insulin for example).

Third, even though initial studies showed no side effects, no histological issues on the intestines and no toxicity of the micropatches, thorough studies of toxicity would be necessary to establish their safety for chronic use.

EXPERIMENTAL PART: FORMULATION OF INTESTINAL MICROPATCHES

I- Formulation of the first layer: Impact on mucoadhesion

1. Concept, theories, and factors affecting mucoadhesion

Mucoadhesion may be defined as a state in which a material binds to a mucosal tissue upon intimate contact and is held together for extended periods of time by interfacial forces (90–93). The phenomenon of bioadhesion can also be described as the process by which a drug delivery device is designed to stick to a biological tissue (in particular mucosa) for an extended period of time.

Mucoadhesion in drug delivery presents a major interest because of the ability of mucoadhesive polymers to adhere to mucins on top of the mucosal epithelium that can prolong residence time at the targeted administration site. This can be asset for both local and systemic treatments. Combined with other induced advantages like reduced clearance rate of drug molecules from the site of absorption, reduced administration frequency, possibility of targeting, and possibility of controlled release, this property helps improving oral bioavailability of drugs, protein and peptide therapeutics.

Mucoadhesion is a complex phenomenon, for which involved mechanisms are not yet totally defined. Yet, the six main generalized theories are (90,91,93–98):

- Wetting theory: mainly applies to liquid mucoadhesive forms. It correlates the surface tension of mucus and of the mucoadhesive formulation into the ability of the liquid to spread over the mucosal surface. The affinity of the liquid to spread spontaneously over one mucosal surface is defined by the contact angle (the lower, the greater the affinity), the surface energies and the work of adhesion that represents the energy required to separate the two phases.
- Electronic (or electrostatic) theory: adhesion as result of electron transfer between mucus and the mucoadhesive polymers (given that they have different electronic characteristics). This results in the formation of an electrical double layer at the interface with electrostatic attraction to maintain contact between the two oppositely charged surfaces.
- Diffusion theory: interpenetration of mucoadhesive molecules inside the mucus gel layer and of mucins into the dosage form. It is a concentration gradient-driven process and is much dependent on the characteristics of the mucoadhesive polymers involved. Depth of

interpenetration depends on the diffusion coefficient and the time of contact and a sufficient depth can lead to a semi-permanent adhesive bond.

- Adsorption theory: adhesion to mucosa due to hydrogen bonds, van der Waals' forces or hydrophobic interaction. Also considers the possibility of chemisorption, which is a strong covalent interaction between polymers and mucins.
- Fracture theory: describes the strength required for the detachment of two surfaces after adhesion is established. It is regarded as being equal to the strength of adhesive bonds.
- Mechanical theory: considers the effect of irregularities on a rough surface and its porosity, both thought to favor the contact area.

However, each of the previous theories can only explain a limited number of interactions that constitute the bioadhesive bond. Mucoadhesion is probably achieved through a combination of several of those mechanisms.

The mucoadhesion process can be divided into sequential phases as visible in figure 27, each of which is associated with a different mechanism. First, the drug dosage form wets and swells (wetting theory), after which noncovalent (physical) bonds are created within the mucus/polymer(s) interface (electronic and adsorption theories). This is the so-called contact stage where an intimate contact occurs between the membrane & the materials. Then, polymers and mucus chains interpenetrate (diffusion theory) and entangle together to form further non-covalent and covalent (chemical) bonds (electronic and adsorption theories). This last step is known as the consolidation stage where various physiochemical interactions occurs to strengthen the adhesion (90,91,97). The relative importance of each mechanism would depend on the nature of the mucus membrane at the site of attachment, the local environment, the mucoadhesive polymers and the type of formulation (91).

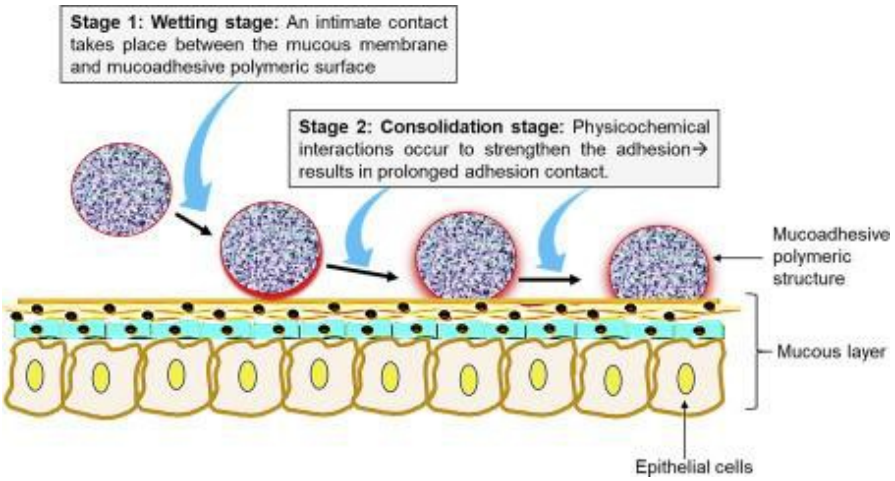

Figure 27: A two-stage model describing the mucoadhesion phenomenon (99)

Moreover, the adhesiveness of a bioadhesive polymer to a mucosal surface is influenced by various factors (90,91,93–98) that can be polymer-related properties:

- Molecular weight: Interpenetration of polymeric molecules is favored by low-molecular weight molecules (chains small enough to allow easy interpenetration) whereas entanglements are favored at higher molecular weight (chains large enough). Theoretically, bioadhesive forces increase with the molecular weight of the polymer, and an optimum range was assessed between 10^4 to $4x10^4$ Da (still depending on the type of polymer).
- Solubility: Adhesiveness and time of retention depends on whether the polymer is soluble or insoluble in water.
- Chain flexibility: a critical parameter for polymeric chains to achieve the required interpenetration & entanglement with mucus and is reflected by their hydrodynamic size. Mobility and flexibility of polymers can also be related to their viscosities and diffusion coefficients, as higher flexibility of a polymer causes greater diffusion into the mucus network. Cross-linked polymers show less mobility, reduced desired entanglement with mucus, thus decreased mucoadhesion. This parameter can be improved thanks to chemical modification such as PEGylation, which offers more structural flexibility.
- Hydrogen bonding capacity: Mucoadhesive polymers should present functional groups able to form hydrogen bonds and chain flexibility can improve their hydrogen bonding potential.
- Concentration: Theoretically, when concentration of polymers is too low, the number of penetrating polymer chains per unit volume of mucus is small and interaction between polymer & mucus is unstable. Consequently, more concentrated polymers would result in a longer penetrating chain length and better adhesion. However, each polymer seems to present an optimum concentration for the best mucoadhesion, beyond which mucoadhesion seems to drop significantly.

or environment-related properties:

- pH: can influence the formal charge on the surface of mucus because of the dissociation of functional groups of the amino-acid backbone. It can also influence the charge of certain ionizable bioadhesive polymers and affect their degree of hydration and their global bioadhesive capacity. For example, protonated carboxyl groups allow the formation of hydrogen bonds with negatively charged mucin molecules for a better mucoadhesion than in a pH where carboxyl groups are not ionized.

- Contact time: Initial contact time between the mucoadhesive excipients and mucus layer defines the degree of swelling and of interpenetration of chains. The strength of adhesion usually increases with the duration of initial contact time.
- Swelling: This parameter is related to both bioadhesive polymers and the environment. Swelling depends on polymers concentrations, ionic strength, and on the presence of water as this later causes polymeric chains' expansion and mobility. Optimum swelling (and subsequent mucoadhesion) are obtained with a critical water content (too few and the chains will not have enough flexibility / interpenetration, overhydration and turns out in the formation of a wet slippery mucilage without adhesion).
- Physiological variables: such as mucin properties (viscosity), pathological states and mucus turnover. This latter parameter is very widely estimated, depending on location and method of measurement. Measures between a few hours (50 and 270 min in the intestinal mucosa according to (100)) to a day have been reported. However, experiments showed that residence time of mucoadhesive devices are typically longer than the reported mucin turnover, suggesting that the contact of bioadhesive polymer(s) with mucus may alter its turnover rate (98).

The role and potential of mucoadhesion in drug delivery has been of interest in pharmaceutical technology since the early 1980's and got developed for many polymeric dosage forms in buccal, oral, nasal, ocular and vaginal drug delivery (91). As for intestinal micropatches, most of their potential relies on the efficacy of mucoadhesion. The formulation of an efficient mucoadhesive layer is a critical step to ensure the adhesion of the reservoirs, to prolong their time of residence and time of contact with the intestinal membrane for drug release. *In fine*, mucoadhesion is the key to success and a possible limiting factor for drug absorption.

2. Choice of mucoadhesive polymers

Materials constituting mucoadhesive matrix of intestinal micropatches usually are mucoadhesive polymers. That is to say synthetic or natural hydrophilic macromolecules able to interact with mucosal surfaces. They can be water-soluble or insoluble polymers, and possibly charged to design an optimal polarity with mucus in order to form swellable networks.
Ideal mucoadhesive polymers (and their degradation products) should be non-absorbable, non-toxic, and non-irritant for the GI tract and mucosal tissues in particular. Polymers should form a quick and strong bond with the mucin at the epithelial cell surfaces, and eventually with some

site specificity. Most importantly, the polymeric network should allow drug molecules to go through it without hindrance. Finally, as for any excipient, polymers should not decompose during storage or shelf-life of the dosage form and remain economically interesting for a large-scale production (101).

Different classifications exist concerning polymers used in mucoadhesive drug delivery. One of them divides polymers into three broad classes:

- Hydration-mediated adhesion: polymers that become sticky when placed in aqueous media and owe their mucoadhesion to stickiness.
- Bonding-mediated adhesion: polymers that adhere through covalent or noncovalent interactions (mainly electrostatic such as ionic bonds or van der Waals forces, but also hydrogen and hydrophobic bonds).
- Receptor-mediated adhesion: polymers that bind to specific receptor site on the cell surface.

Another one is based according to their surface charge: anionic, cationic, non-ionic or amphiphilic. Chemical composition and surface charges influence the mechanisms of action of mucoadhesive polymers: they usually contain hydrogen bond forming groups (hydroxyl -OH, carboxyl -COOH or amine -N- groups) to favor the consolidation stage and to establish strong adhesive bonds with mucosal surfaces.

Based on experiments, better mucoadhesive performance is typically observed for polymers possessing charged groups or non-ionic functional groups over neutral polymers. It was also proven that degree of binding is proportional to the charge density on the polymer surface, that polyanions are better than polycations in terms of binding, that anionic polymers with sulfate groups bind more effectively than those with carboxylic groups and that water-insoluble polymers offer greater flexibility in design than water-soluble polymers (101,102).

Although materials used in previous studies have shown good bioadhesion *in vitro* and *in vivo*, results in humans were disappointing. First, this can be linked to the fact that intestinal micropatches must attach to the surface of the mucus layer, which is itself a continuously eroding surface. Second is that the delivery system must withstand motility of the GI tract, which is highly efficient at transiting ingested food or beverages and usually prevents any form of adhesion on the tract. Consequently, overcoming those obstacles requires to enhance the strength of mucoadhesion of intestinal micropatches, which could be achieved by the addition of stronger mucoadhesive polymers (62,98).

Typical examples of highly binding polymers include carbomers (Carbopol), cellulose derivatives (carboxymethylcellulose (CMC)), chitosan, gelatin, sodium alginate, hyaluronic acid, … and those planned to be used in the following experiments are developed below:

- Pectin (Fig. 28): It is a natural polymer, originally found as a cell wall structural carbohydrate in many plants and are now extracted from citrus fruits or in apple pomace for commercialization. As for its structure, it is a complex polysaccharide, which consists primarily of D-galacturonic acid monomers in a α(1-4) chain, that can partially be esterified with methoxy groups and sometimes with amine groups. The degree of esterification (DE) and degree of amidation (DA), which are both expressed as a percentage of carboxyl groups (esterified or amidated), allows for the classification of pectins in two groups: high-methoxy (HM, >50%) and low-methoxy (LM, <50%) gelation.

  These anionic, hydrophilic and water-soluble polymers present mucoadhesive performances due to interactions between the numerous carboxylic groups of pectins and the functional groups of mucus. However, mucoadhesive properties are largely dependent on pectin characteristics (i.e. DE, molecular weight, global electrical charges) such as decrease of DE implies the increase of the wetting behavior of pectin surfaces, and high net value of electrical charges shows a better mucoadhesion in porcine tissues than low charged ones (103–105). In practice, gelation mechanism for LM pectins is made by an 'egg-box' binding process governed by specific non-covalent ionic interaction between blocks of galacturonic acid residues of the pectin backbone and with divalent ions such as calcium. As for HM pectins, the gelation mechanism relies more on the formation of junction zones in which there are chain associations stabilized by hydrogen bonding and by hydrophobic interaction between functional groups.

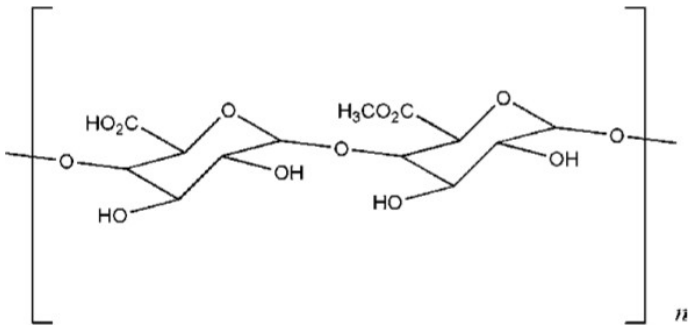

Figure 28: Structural formula of pectin (103)

- Cellulose & derivatives (Fig. 29): Cellulose is the most abundant naturally occurring biopolymer as present in every plant cell wall. It is a polysaccharide whose structure is composed of a linear chain of β(1-4)D-glucose units. Cellulose is insoluble in water and in most common solvents, its poor solubility being primarily attributed to the strong intramolecular and intermolecular hydrogen bonding between the individual chains. As for cellulose derivatives, they are semi-synthetic ether and ester derivatives and the range covers compounds with different physico-chemical characteristics including both charged and uncharged material, soluble and insoluble material, … Most used ones in the pharmaceutical industry are methylcellulose (MC), hydroxypropylcellulose (HPC), hydroxypropylmethylcellulose (HPMC) and carboxymethylcellulose (CMC). In particular, this carboxymethylcellulose – and more precisely sodium salt of CMC (Na-CMC) - has been explored for its mucoadhesive properties and tested in various mucoadhesive formulations for mucosal delivery. Its mechanism of adhesion involves formation of strong hydrogen bonds between carboxylic acid group of cellulosic polymers and glycoprotein of mucin, and also takes the pH into consideration. For instance, as CMC is an anionic polymer, better absorption is observed when pH<pKa to avoid repulsion with glycoproteins of the mucus. Even though non-biodegradable, CMC is commercialized at different viscosity grades and mucoadhesion potential, depending on their molecular weight, pKa and polymer hydration status (106,107).

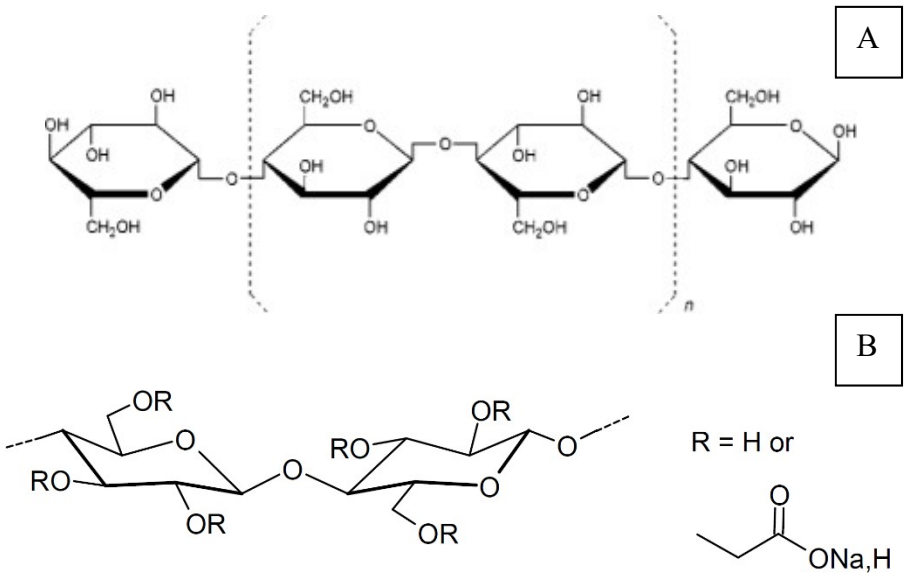

Figure 29: Structural formula of (A) cellulose and (B) sodium-carboxymethylcellulose (103,108)

- Carbomers (Fig. 30): They are synthetic carboxyvinyl polymers of very high molecular weight, made of cross-linked acrylic acid (also known as PAA, poly(acrylic acid)). They are anionic, hydrophilic, water-swellable but essentially water-insoluble polymers, which makes them suitable for use in controlled drug delivery systems. These polymers mainly express their mucoadhesive properties through four mechanisms: hydrogen bonds and hydrophobic interactions with the functional groups of mucus components, interpenetration & entanglement and finally, electrostatic interactions. In fact, for carbomers, swelling and viscosity increase with pH, as neutralization of environment causes the electrostatic repulsions of negatively charged carboxyl groups and the expansion of polymer chains. Commercially known as Carbopol® and presented as dry fluffy powders, the range of carbomers include various grades depending on their molecular weight, their architecture and the ether molecules used to reticulate acrylic acid. Carbomer resins are being extensively used in the formulation development of controlled drug delivery systems, and in particular for oral and mucosal applications for which the "P" (Pharmaceutical) line is recommended (Carbopol® 934P and 974P as highly cross-linked polymer, Carbopol® 971P as lightly cross-linked polymer). Carbomer formulations proved their efficacy, even at low resin concentration, for controlled release, thickening and finally appeared to be on top of classification of hydrogels for mucoadhesion (103,109,110).

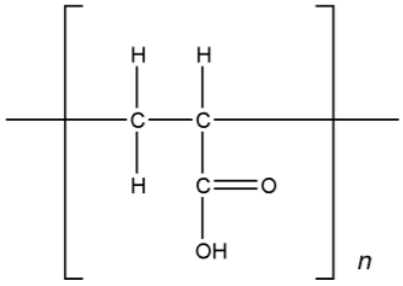

Figure 30: Structural formula of poly(acrylic acid), also called carbomer (103)

- (Poly)methacrylates (Fig. 31): They are fully polymerized copolymers made of dimethylaminoethyl methacrylates, methacrylic acid and methacrylic acid esters in varying ratios. Those synthetic cationic and anionic polymers offer a large range of products, with variation in their content and solution viscosities, widening their potential applications (film-coating agents, enteric coatings, binders for wet granulation, matrix for controlled release).

  In the following study and based on literature articles, focus is done on Eudragit® E PO for the formulation of intestinal micropatches as a major component of the mucoadhesive layer

and as a controlled-release agent. It is a linear cationic copolymer based on dimethylaminoethyl methacrylate, butyl methacrylate, and methyl methacrylate with a ratio of 2:1:1. The combination of these repeating units within this polymer ensures its solubility in water only under acidic conditions. As for its mechanism of action, main interactions include ionic interactions, van der Waals forces and hydrogen bonding. Even though these are weak bonds, numerous interaction sites (carboxyl and amino groups in here for hydrogen bonds) lead to strong mucoadhesion. Moreover, the ability of cationic Eudragit E PO to form interpolyelectrolyte complexes with various anionic polymers (such as Na-CMC and Carbopol) was also previously used in the preparation of solid dosage forms for gastrointestinal delivery (103,111–114).

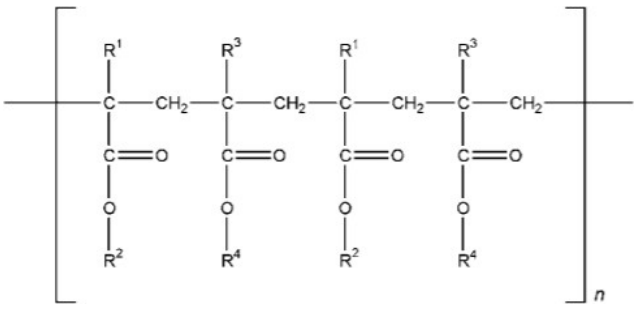

Figure 31: Structural formula of polymethacrylates (103)

For Eudragit E PO, $R^1 = R^3 = CH_3$, $R^2 = CH_2CH_2N(CH_3)_2$ and $R^4 = CH_3, C4H9$

- Chitosan & derivatives (Fig. 32): It is a linear polysaccharidic polymer, composed of randomly distributed D-glucosamine and N-acetyl-D-glucosamine units. This hemisynthetic molecule is usually chemically produced by deacetylation of chitin derived from the exoskeleton of crustaceans (shells of crabs or shrimps) or from mushrooms. Various chitosan products can be obtained, by variations in molecular weight and degree of deacetylation (DD, percentage of primary amino groups in the polymer backbone). Biocompatible and biodegradable, chitosan is considered one of the most suitable polymers in regards of safety. Its cationic character - even though of relatively low charge density - and its functional groups (hydroxyl (-OH) and amine ($-NH_2$) groups) make it an attractive biopolymer for many mucoadhesive applications. Its main mechanism explaining its gel-forming ability involves electrostatic interactions between negatively charged mucin chains and cationic chitosan and is completed with an important contribution of hydrogen bonds and hydrophobic interactions globally resulting in remarkable mucoadhesive properties. Moreover, chitosan also shows antimicrobial property and an important ability to reversibly disrupt intercellular tight junctions, which makes it an absorption enhancer for

transepithelial drug delivery. Due to all those properties and versatility, chitosan is an ideal biomaterial for controlled drug delivery formulations. It has been already much investigated for use in powders, tablets, capsules, films, gels and offers much potential for the future (107,115–123).

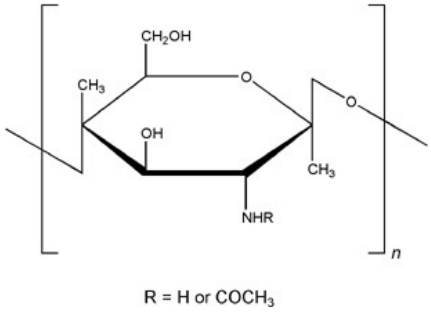

Figure 32: Structural formula of chitosan (103)

However, because chitosan is water-insoluble at neutral and basic pH (chitosan's pKa = 6,5), it shows its unique properties only in acidic medium. Its limited solubility is a major obstacle for its applicability, in particular for intestinal micropatches released in the small intestine where pH stands between 6 and 7,4. Another limitation in the use of chitosan for the preparation of sustained release systems arises from its rapid water adsorption capacity and its high swelling degree in aqueous environments, both leading to fast drug release. To improve its properties such as charge density, mucoadhesion, release profile and aqueous solubility, many derivatives of chitosan have been synthesized: carboxymethyl chitosan (CMC), trimethyl chitosan (TMC), thiolated chitosan, chitosan- EDTA, acrylated chitosan … Two of them are detailed here:

- TMC (Fig. 33): N,N,N-trimethyl chitosan is a cationic polymer, quaternized methylated derivative of chitosan and soluble over a wider pH range than chitosan (pH 1 to 9). Being a derivative of a cationic polymer enriched with protonated groups ($–N^+(CH_3)_3$), TMC presents a much denser positive charge and consequently better mucoadhesive, permeation enhancement and drug delivery properties. The degree of quaternization (DQ) is, once again, an important characteristic to consider. TMC polymers with high DQ present a high number of positive charges available for electrostatic interactions with mucus for mucoadhesion, and available for interactions with cell membrane for absorption enhancement process. Even at low DQ, TMC proved to have superior solubility and basicity compared to chitosan and salts (122,124–129). However, a contrary conclusion was withdrawn from a study due to the steric hindrance. Indeed, addition of methyl groups

may force the polymer to change its conformation with a reduction in polymer-chain flexibility and may also hide the positive charges of the amino groups. Steric effects would then negatively affect the exchanges between the negatively charged sialic groups of the mucus and the positive charge of the TMC polymers and would badly affect interpenetration into the mucus layer with a subsequent lower mucoadhesion (127).

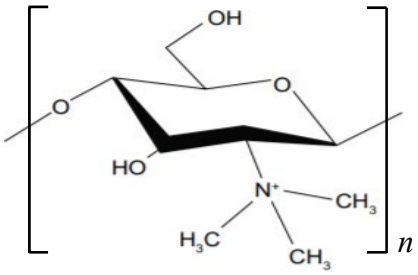

Figure 33: Structural formula of N,N,N-trimethyl chitosan (TMC) (130)

- CM-TMC (Fig. 34): TMC can be further derivatized for modulating properties to keep enhancing solubility, antibacterial activity, biocompatibility and most of all, mucoadhesion. Reaction of carboxymethylation on hydroxyl functional groups of TMC allows the formation of O-carboxymethyl-N,N,N-trimethyl-chitosan (CM-TMC), a water-soluble amphiphilic polymer formulated as chloride salt. The amphoteric properties of CM-TMC originate from the presence of both amino (basic) and carboxylic (acidic) groups in important quantities in its chemical structure. Consequently, it has a bigger charge density than the above chitosan and TMC molecules, which have an important impact (positive or negative) on every mechanism. In fact, quaternization and carboxymethylation of chitosan leading to CM-TMC implies the enhancement of hydrophilicity and a major increase of charges available for electrostatic interactions. However, it also implies a massive reduction of free amino & hydroxyl bonds available for the formation of inter / intramolecular hydrogen bonds and an important steric hindrance, illustrated by the fact that an increase in the degree of carboxymethylation reduces the activity of the molecule (128,131).

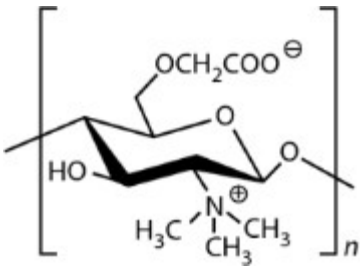

Figure 34: Structural formula of N,N,N-trimethyl-O-carboxymethyl chitosan (CM-TMC) (128)

In the following part, experiments were realized to compare formulations of intestinal micropatches using classic polymers and which proved their efficacy in the literature. They are considered as “formulas of reference”. To be noticed, all polymers and excipients used in the following formulations have already been widely used in oral pharmaceutical applications and are classified as GRAS (Generally Recognized As Safe) by FDA.

The aim of the experimental part is also to improve their properties while assessing the impact of the integration of chitosan & derivatives molecules inside. In particular, comparison of formulas containing chitosan, TMC or CM-TMC interacting with mucus would be interesting to analyze in regards to their molecular particularities as chitosan is slightly positively charged when solubilized and rather neutral when dispersed, TMC is very positively charged and the amphiphilic CM-TMC presents both positive and negative charges at intestinal pH.

3. Mucoadhesion studies

Materials:

Pectin from apple, citric acid and chitosan low molecular weight (LMW) were purchased from Sigma Aldrich (St. Louis, MO, USA). Carboxymethylcellulose sodium salt, ethylcellulose, sodium phosphate dibasic dihydrate and sodium dihydrogen phosphate monohydrate were purchased from Fluka Chemie (Buchs, Switzerland). Carbopol 934 was bought from BF Goodrich Chemicals (Cleveland, Ohio), microcrystalline cellulose Avicel® from FMC Biopolymer (Philadelphia, PA, USA) and finally Eudragit E PO® and Eudragit L100® came from Röhm GmbH/ Evonik (Darmstadt, Germany). Finally, pig intestines were fetched from Loëx slaughterhouse (Bernex, Switzerland), with the authorization of the Swiss Veterinary Services (SCAV). As for the equipment, a Stable Micro Systems TA-XT Plus Texture Analyser equipped with 50g load cell and connected to the Exponent Connect software, was used to measure the bioadhesion bond strength and other adhesive properties.

Methods:

Various bases of polymeric matrices were obtained from polymer blends at a specific dry weight ratio, as shown in table 4. Mix A et B were inspired from articles (62) and (82) , as references. 15mm-large and about 400 µm-thick tablets of polymers were obtained by compression of 100mg mixtures under a 3-ton pressure using a powered hydraulic bench press (Atlas™ Power Press 8T, Specac Inc., Fort Washington, PA).

Table 4: Composition of polymeric matrices for mucoadhesion tests

MIX	COMPONENTS				WEIGHT RATIOS
A	Pectin	S-CMC	Eudragit E PO	/	(1:1:2)
B	Pectin	S-CMC	Carbopol 934	/	(1:1:2)
C	/	S-CMC	/	Chitosan (MMW shell)	(1:1)
D	Pectin	S-CMC	Carbopol 934	Chitosan (MMW shell)	(1:1:1)
E	Pectin	S-CMC	Eudragit E PO	Chitosan (MMW shell)	(1:1:1:1)
F	Pectin	S-CMC	Carbopol 934	Chitosan (MMW shell)	(1:1:1:1)
G	Pectin	S-CMC	Eudragit E PO	Chitosan (LMW powder)	(1:1:1:1)
H	Pectin	S-CMC	Carbopol 934	Chitosan (LMW powder)	(1:1:1:1)
I	Pectin	S-CMC	Eudragit E PO	N-TMC	(1:1:1:1)
J	Pectin	S-CMC	Carbopol 934	N-TMC	(1:1:1:1)
K	Pectin	S-CMC	Eudragit E PO	CM-TMC	(1:1:1:1)
L	Pectin	S-CMC	Carbopol 934	CM-TMC	(1:1:1:1)

Mucoadhesion of those polymeric matrices was assessed *ex vivo* by tensile testing, measuring the adhesion strength of the tablets on porcine intestinal mucosa as it was reported to have characteristics similar to human intestinal mucosa. It has been widely used in literature as a model membrane in various types of mucoadhesion testing. Jejunum was specially chosen as intestinal micropatches would be encapsulated inside gastro-resistant gelatin capsules to form the final device and some time is needed for them to be disintegrated through duodenum, before micropatches release their drug load inside the jejunum. Mucosa was chosen from freshly sacrificed healthy adult pigs, quickly rinsed with phosphate buffer saline solution (PBS) to remove stomach contents, degraded mucus & mucus degrading enzymes. Tissues were then cut longitudinally to expose the inner mucosa and cut in 3.5x3.5 cm portions before finally being placed in Petri boxes in which a fixed volume of pH 7.4 PBS was pipetted onto the mucosa. Standardization of the hydration prior to testing is a crucial step because samples' state of hydration is a key element affecting its bioadhesiveness. Polymeric tablets were then applied on the irrigated inner mucosa for 15 min, kept together in a heat chamber at 37°C with a shaking movement (80rpm/min) to simulate peristaltic motion.

Set-up parameters were inspired by Tobyn et al. experiments (132–134). Tissues (with tablet on top) were disposed on a device called mucoadhesion test rig, designed as a clamp with the purpose of firmly holding down the samples in order to perform the test successfully. Then, a

10mm-diameter probe descends to stick to the tablet with double-sided tape for a 5min-dwell time applying a downward 2N force to begin the binding process. After attachment, the probe was withdrawn from the mucosal tissue at the constant speed of 0.1mm/s as visible in figure 35 below and the parameters were measured.

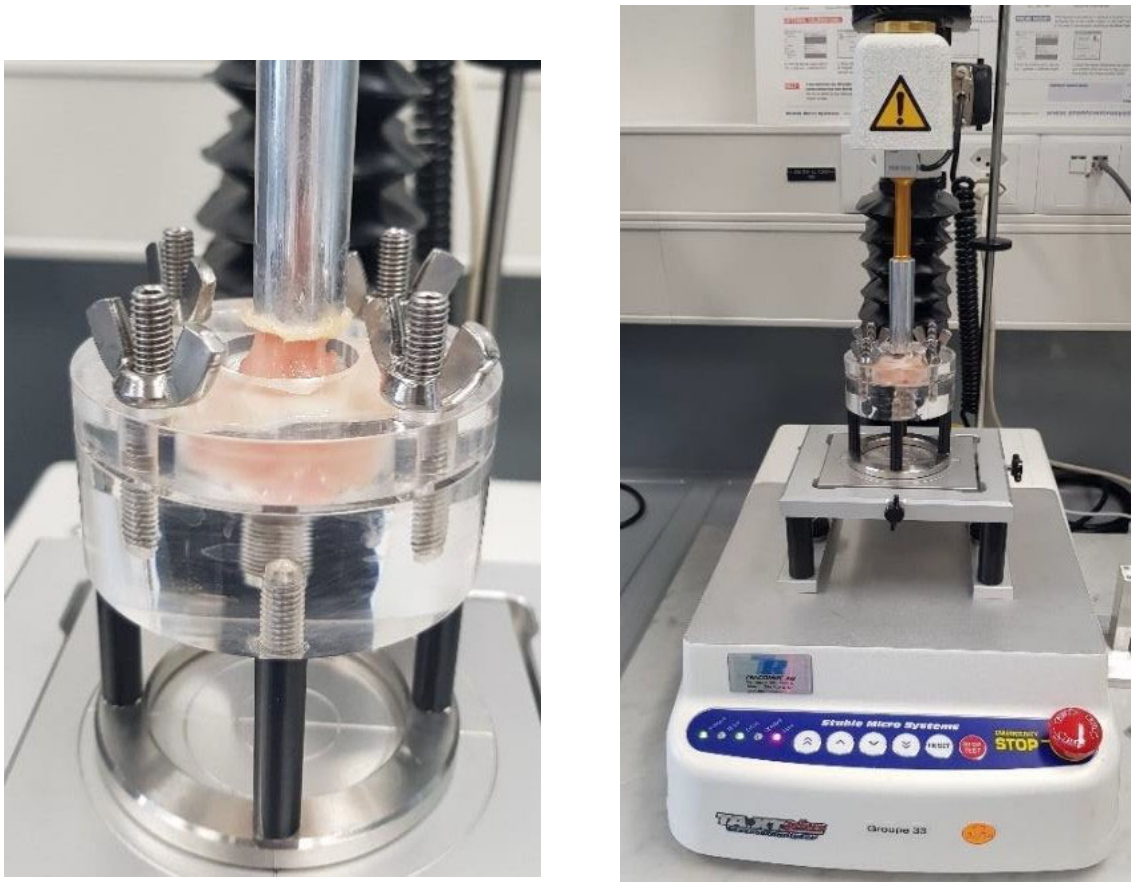

Figure 35: Texture analyser TA.XT and mucoadhesion rig during test

It should be noticed that in order to assess mucoadhesion in the closest possible conditions to normal environment, the rig provides the ability to set-up the tissue samples in vessel of temperature regulated gastric fluid (or similar). However, this possibility was not used in these experiments because immersion of tissues and tablets in PBS interfered with the correct binding of the tape with both probe and tablets. As a matter of fact, solid materials (such as tablets) were usually attached to the underside of the upper testing probe using cyanoacrylate adhesive but double-sided tape is now privileged as it is more reliable and reproductible for quantity and contact surface created between samples (135). Because the adhesion test would last less than 5 minutes, decision was taken to realize the detachment test with double-sided tape and without liquid environment.

The following adhesive properties were measured (132,136–140):

- separation distance – also called debonding distance and referring to stringiness property, it corresponds to the distance completed by the withdrawing probe at constant speed before the tablet detach from the mucosa
- detachment force – sometimes related to mucoadhesive force / adhesiveness / stickiness. It is taken as the peak force that is to say the maximum force necessary to overcome the attractive forces between the surface of a product and a tissue in contact, thereby corresponding to the force needed to remove the tablets from mucosal substrate
- work of adhesion, the area under the force-displacement curve corresponding to the total work during the withdrawal of the probe.
- positive area under curve, which is taken as a measurement of toughness and tackiness (property of being cohesive and sticky).

The higher the values, the stronger the mucoadhesion of the sample.

In a general way concerning (muco)adhesion tests, areas provide a better measure of adhesive capability because they are more sensitive parameters to analyze than peak forces. Whilst peak forces for different products can be similar, the debonding behavior shown by the calculation of parameters from other regions of the curve, such as work of adhesion and debonding distance, can be very different. Knowing that a mucoadhesive device should hold against mucosal linings where food, liquids and peristalsis are able to dislodge it, it must resist very aggressive conditions. And because few real forces in physiological situations are sharp enough to generate momentarily high peak forces, area of work is a much better measure of the adhesive capability to withstand the dislodging forces than peak forces.

Ex vivo studies carried out in triplicates have their data represented as mean ± standard deviation (SD). Statistical analyses and graphs were obtained using GraphPad Prism 7 (GraphPad Software, LaJolla, CA). Two-way analysis of variance (ANOVA) tests were conducted using Tukey's multiple comparisons test. A p-value <0.05 was considered statistically significant.

Results:

Visual aspect and texture of the obtained polymeric matrices were studied and led to the exclusion of formulations C, D, E and F depicted in figure 36 B. Those latter were too fragile to be manipulated and initial tests of micropunching were not successful due to the presence of chitosan pieces from shrimp shell which lead to the heterogeneity of these powder blends. Further mucoadhesion tests on the texture analyzer were run for formulations A, B, G, H, I, J,

K and L which showed good visual aspect, homogeneity and solidity as visible in Figure 36 A.

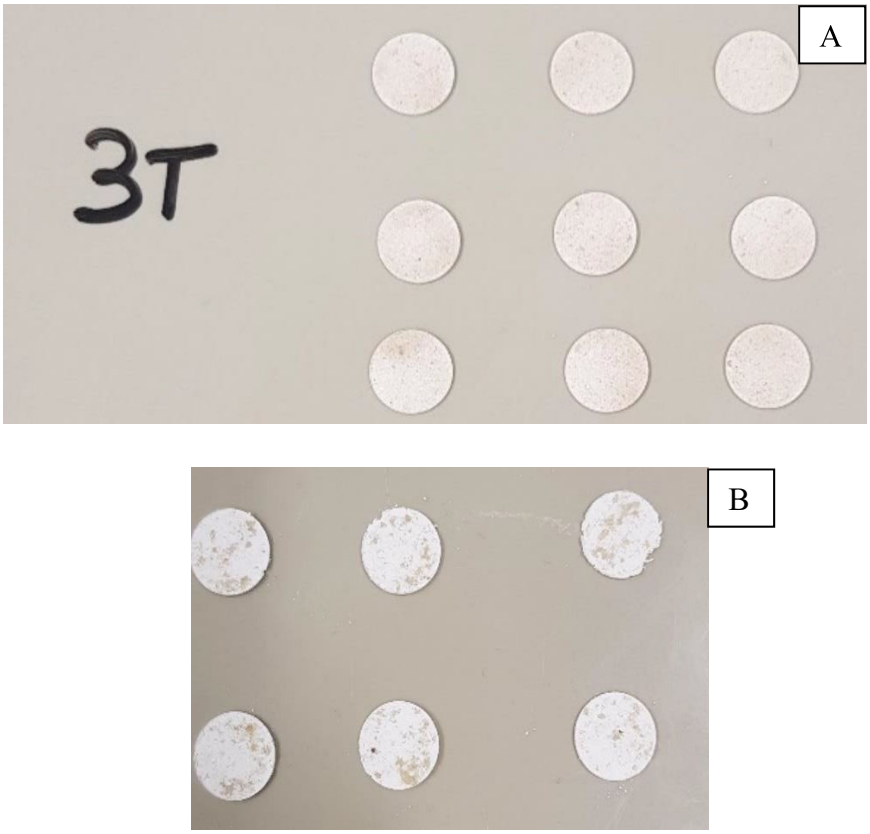

Figure 36: Visual aspects of polymeric matrices after a 3-ton compression
A : homogeneous tablets such as formulations A, B, G, H, I, J, K and L
B: heterogeneous and fragile tablets such as formulations C,D, E, F

Results of the mucoadhesion tests, illustrated in figure 37, showed similarity between the referent formulations A and B, with the same composition except that Eudragit E PO® was changed for Carbopol 934®. Even though separation distance is short (around 1mm), those formulations presented valuable force of detachment and positive area. Concerning formulations I and J containing N-TMC, they showed medium mucoadhesive properties in all adhesive properties measured. Formulations K and L containing CM-TMC presented similar properties to referent formulations A/B, and a slight superiority to formulations I/J. Finally, the only formulation which seems to have a clear improvement in all mucoadhesive properties is G (with Eudragit E PO and chitosan LMW), even if no significant difference got highlighted during statistical analysis. However, the addition of chitosan did not enhance the mucoadhesive properties of the formulation H with Carbopol 934 and shows the lowest values in separation distance and work of adhesion.

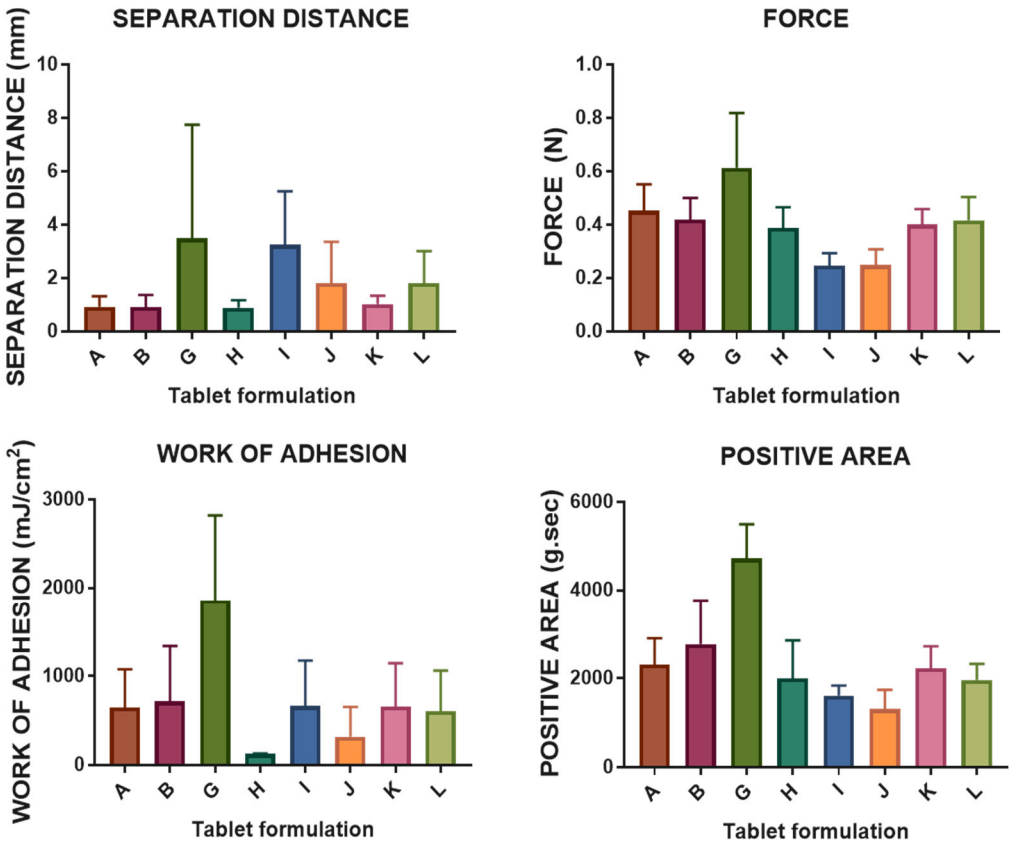

Figure 37: Graphic representations of mucoadhesion tests results

4. Rheology studies

Materials:

Same excipients were used for the rheology studies as in the mucoadhesion ones. In addition, type II mucin from porcine was purchased from Sigma Aldrich (St. Louis, MO, USA). Mechanical properties of gel formulations were determined using a software-controlled rheometer, HAAKE Mars 40 (Thermo Fischer Scientific, Karlsruhe, Germany), equipped with a cone plate (C35, angle of 2°TI) and analyzed via Rheowin Data Manager.

Methods:

To assess the interaction of polymeric matrices with intestinal mucins, rheology studies were initiated considering both viscosity and viscoelastic measurements. Polymeric gels were formulated based on previous formulas (A, B, G, H, I, J, K, and L). They were prepared dispersing the required amounts of polymers in PBS at pH 6.8 as dissolution media. This latter was stated equivalent to Simulated Intestinal Fluid USP (141) due to its equal characteristics concerning pH at mucus surface, ionic strength, buffer capacity and osmolarity. For all

formulations, pH was adjusted to 6.8 with HCl 0,1 and 1M or NaOH 1M and left for stirring overnight in sealed vials to ensure a total hydration of the polymers. Finally, 4% (w/w) of type II mucin was added last minute to mimic the type of interaction that could take place at the intestinal mucosa between polymeric tablets and the mucus and the mix were placed under stirring for 15 minutes prior to analysis. Quantity of mucin added in the formulations was found in the literature (142–144), and precise composition of the gels is given in the following table 5.

Table 5: Composition of gel formulations for rheology studies

MIX	QUANTITIES				
A	1% Pectin	+ 2% SCMC	+1% Eudragit E PO		+4% mucin
B	1% Pectin	+ 2% SCMC	+1% Carbopol 934		+4% mucin
G	1% Pectin	+ 2% SCMC	+1% Eudragit E PO	+1% chitosan	+4% mucin
H	1% Pectin	+ 2% SCMC	+1% Carbopol 934	+1% chitosan	+4% mucin
I	1% Pectin	+ 2% SCMC	+1% Eudragit E PO	+1% N-TMC	+4% mucin
J	1% Pectin	+ 2% SCMC	+1% Carbopol 934	+1% N-TMC	+4% mucin
K	1% Pectin	+ 2% SCMC	+1% Eudragit E PO	+1% CM-TMC	+4% mucin
L	1% Pectin	+ 2% SCMC	+1% Carbopol 934	+1% CM-TMC	+4% mucin

As for the equipment, a cone plate was used as measuring system for a constant shear rate all over the surface of contact, completed with a sample hood to avoid evaporation and dry-out effects. In order to get reproducible initial states, all measurements of 400 µL of samples were allowed to equilibrate for 1 minute at 37° ± 1◦C prior to testing to mimic reaction in intestinal medium. Finally, before recording experiments. samples were sheared for 40s of a $10s^{-1}$ shear rate if liquid preparations, 80s of $100s^{-1}$ shear rate if viscous preparation.

Stability tests were first realized at 37°C for 10 min to ensure a low variation in viscosity during variation of shear stress. Second, steady shear studies were conducted to determine viscosity. Finally, dynamic oscillatory tests were performed within the linear viscoelasticity range previously determined. Storage modulus (G') and loss modulus (G'') were measured at frequencies ranging between 0.5 and 5Hz, and value obtained at the intermediate value of 1.0Hz have been chosen to compare the results.

Results:

Visual inspection of the samples led to the exclusion of formulations K and L, which presented a biphasic and heterogeneous aspect with presence of particulate material, closer to a solid dispersion than a gel. This was confirmed by the low values of viscosities measured by the rheometer (low curves on the graphs). Formulations I and J also presented an intermediate visual aspect between gel and particulate dispersions but were kept for further experiments.

For all formulations, graphs of figure 38 show that polymeric dispersions see their apparent viscosity decrease while shear rate increases: they are classically called shear-thinning fluids. This is an expected result for polymeric solutions as the decreasing viscosity whilst the shear rate increase is linked to two phenomena: the separation and progressive alignment of the entangled polymeric macromolecules with structuration in layers, and the rupture of polymer clumps, releasing the liquid phase.

Formulation G seems to have the highest viscosity of all the formulations, and this behavior is constant through increasing shear rate, compared to other formulations. The referent formulations A and B showed good rheological properties for a gel, with slightly lower values.

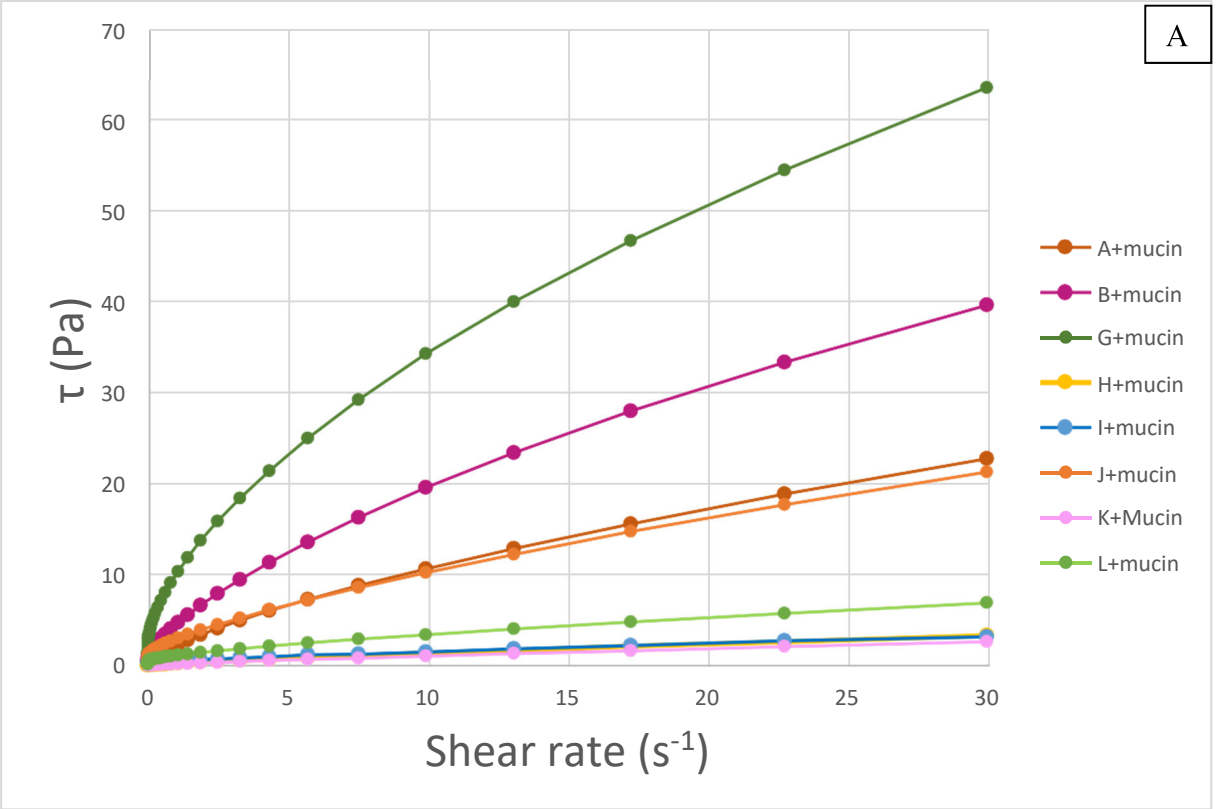

Figure 38 (A): Shear strain versus shear rate plots of mucin dispersed in polymeric solutions (pH 6.8)

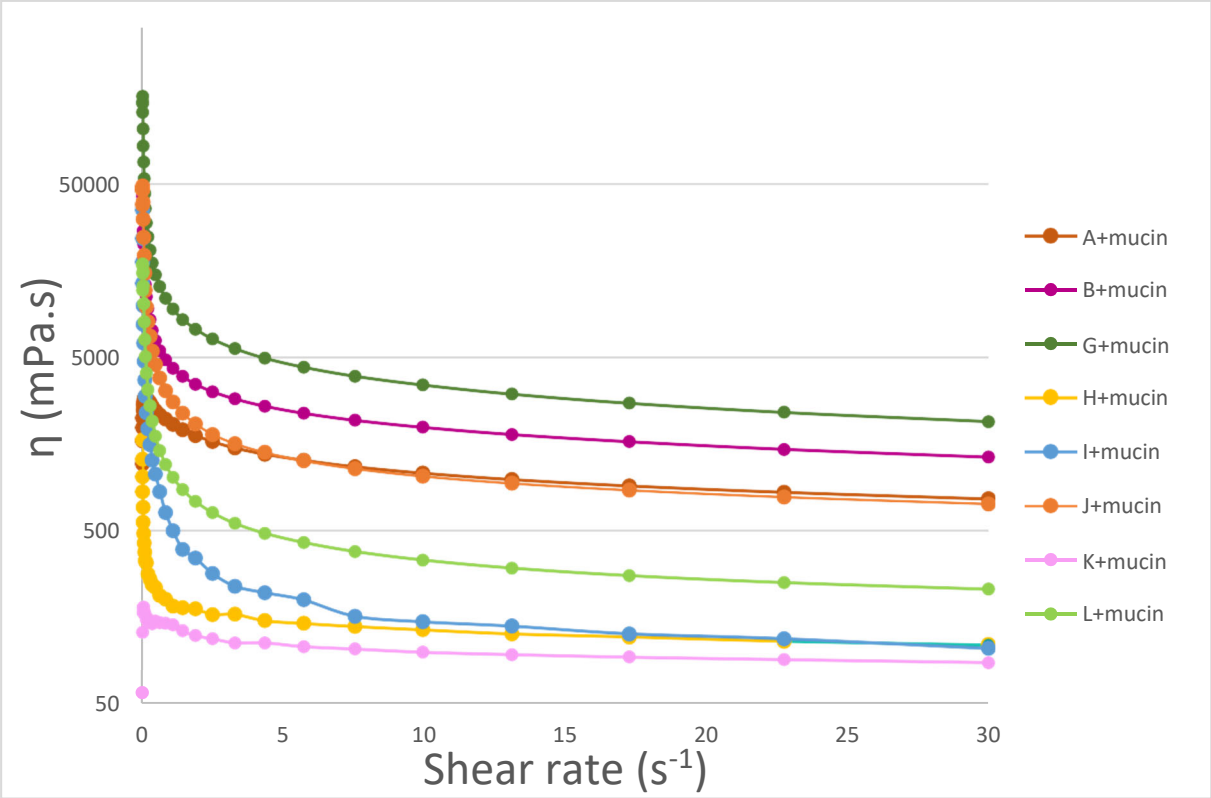

Figure 38(B): Shear viscosity versus shear rate plots of mucin dispersed in polymeric solutions (pH 6.8)

5. Bioadhesion study

Materials:

Formulations elaborated for rheology studies were used again in this part and analytical data were measured at the same time on the Datawin software-controlled HAAKE Mars 40 rheometer.

Methods:

From a molecular point of view, bioadhesion could be investigated by the changes in rheological properties that mucoadhesive polymers undergo when they are mixed with mucus. It is related as rheological synergism (Δη, also called $\eta_{bioadhesion}$), the strength of the interaction between polymeric systems and mucin (143–146). Calculation of this synergism was defined by Hassan and Gallo (143) as the following equation 2:

$$\eta_{(mix)} = \eta_{(mucin)} + \eta_{(polymer)} + \eta_{(bioadhesion)} \qquad \text{Eqn (2).}$$

which turns into

$$\Delta\eta = \eta_{(mix)} - (\eta_{(mucin)} + \eta_{(polymer)}).$$

Same equation can be applied to values of storage (G') and loss (G'') modulus to determine viscoelastic interaction parameters. Thereby, synergism is generally measured using rheological interaction parameters (146):

- Δη: the extent to which the viscosity of the polymer-mucin mixture differed from the value expected on the basis of addition of polymer and mucin contributions.
- ΔG': the difference between the storage (elastic) modulus (G') of the polymers-mucin mixture and that of the polymer solutions alone.
- ΔG'': the difference between the loss (viscous) modulus (G'') of the polymers-mucin mixture and that of the polymer solutions alone.

Results:

It is observed in figure 39 that formulation I developed a very slightly positive variation in viscosity in the presence of mucin, while formulations A and G showed a real increase in the viscoelastic parameters with positive synergism values when in contact with mucin. Once again, formulation G exhibited the most pronounced interaction in comparison. This positive rheological synergism is attributable to physical chain entanglements and to non-covalent bonds between the polymers and mucin chains (hydrogen, van der Waals, …).

At the contrary, formulations B, H and L presented negative values of synergism. This suggests a lack of rheological interaction, indicating that the mixture viscosity is lower than the viscosity of the polymeric solution alone (mucin contribution being negligible since its viscosity was about 11 mPa.s for a shear rate of $2{,}5 s^{-1}$).

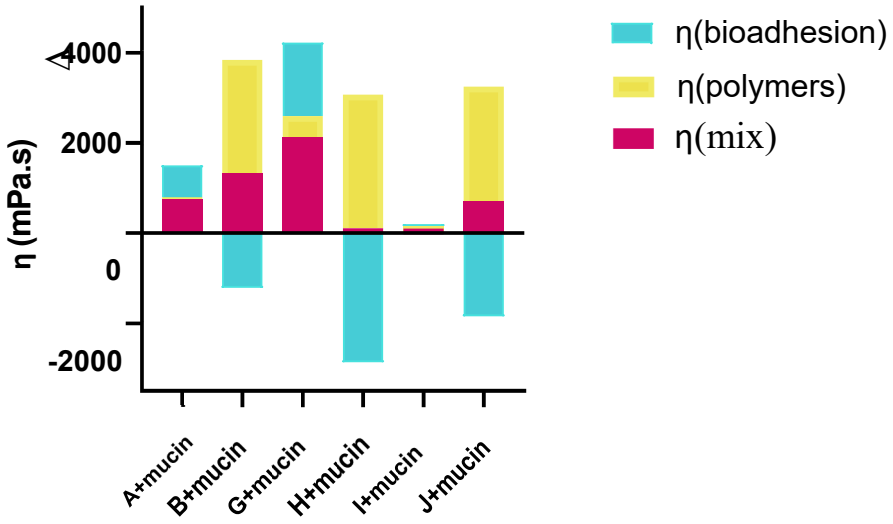

Figure 39: Results of the bioadhesion study: highlight of the rheological synergism between polymers and mucin

In conclusion, bioadhesion test revealed that formulations containing Eudragit E PO (A and G) presented positive values of rheological synergism when in contact with mucin while formulations containing Carbopol 934 presented negative synergism (B, H and J).

6. Conclusion on experiments

Experiments conducted on the mucoadhesive layer of intestinal micropatches were conclusive: all mucoadhesive, rheological and bioadhesive tests agree on the superiority of formulation G containing pectin, S-CMC, Eudragit E PO and chitosan LMW powder.

As a matter of fact, significant increase in work of adhesion (mucoadhesion), highest value of viscosity (rheology) and highest value of synergism in bioadhesion were observed specifically for the formulation G in presence of mucin. The interactions between mucin and the mix of polymers were visible at a macroscopic scale when both were put in physical contact (mucoadhesion of tablets) and at a microscopic scale during *in situ* interpenetration of polymers and mucin (rheology and bioadhesion).
The relationship elaborated between rheological and tensile parameters proves that for formulation G, the strengthening of the polymers-mucin interface is linked to the rheological changes, and more precisely mainly physical entanglements, occurring when the polymers are mixed with the glycoprotein (144). Amongst all tested formulations, G is the one developing the most of interactions with mucin from the intestinal membrane, leading to the best mucoadhesion possible for those micropatches.

II- Formulation of the matrix and backing layers: impact on release

1. Matrix of the second layer

Mucoadhesive formulations can, if properly formulated, control the release profile of the incorporated drug(s) (147). In the case of intestinal micropatches, ideal profile would be a sustained release. The second layer being the reservoir of therapeutic proteins, its formulation is a key point to achieve the desired release profile.

Formulation of controlled-release tablets can consist of the creation of an internal matrix, made of selected functional excipients. Those latter are defined as pharmacologically inactive ingredients with function to form a network or obstacles aiming to slow down the release of active molecules towards the media. In most case, those functional excipients being polymers.

Three types of matrices exist for sustained release oral forms and they can be classified as hydrophilic, hydrophobic, or inert. The most commonly used are hydrophilic matrices and are based on the ability of a functional polymer to form a hydrated and gelled layer when in contact with aqueous media.

As for the mechanisms of drug release out of these matrices, they are also three: diffusion, osmosis and erosion. Diffusion of water from GI fluids is supposed to favor the dissolution of active molecules inside the matrix before helping their exit into the media. Osmosis happens depending on conditions in the GI tract and on the nature of polymers: penetration of water into the tablets creates an osmotic pressure, itself generated by the soluble components composing the tablets thereby forcing the dissolved active substances to escape from the solid form. Finally, erosion is a mechanical effect much depending on conditions of motility and pH in the GI tract. Proportion of these mechanisms depends on drug's nature: for water-soluble molecules, dissolution is mainly controlled by the diffusion of the substance through the gelled matrix. If molecules present a limited water-solubility, the release is mostly controlled by the matrix erosion (21,148–150). In the particular case of intestinal micropatches, erosion is in fact a restrained phenomenon due to mucoadhesion and the much-limited distance travelled in the GI tract. Moreover, the only surface possibly submitted to erosion would be the backing layer, made out of a water-resistant film. Consequently, drug release mechanisms applying to intestinal micropatches are osmosis and diffusion, fluids only coming directly from the intestinal membranes.

To end up on release mechanisms, attention should be paid concerning burst effect in controlled-release formulations. The burst effect is characterized by a quick release of drugs from the dosage form at the beginning of dissolution due to a latency period before gelation or formation of the matrix network. Even though intestinal absorption is favored by a burst-release effect that creates a high local concentration generating a high flux of absorption (75), this phenomenon should be restricted to insure a sustained-release over a longer period of time.

As for the choice of matrix composition for controlled release dosage forms, the most popular one in literature is hydroxypropylmethylcellulose (HPMC, also called hypromellose). It is a hydrophilic polymer, non-toxic, easily compressible, with good swelling-ability and fast gel-forming characteristics to not only control the initial release but also exert sustained release effect through strong viscous gel formation (151). Literature suggests that high-viscosity grades have a better effect on sustained release (103,151). However, Lopes et al. showed that different grades of HPMC did not impact drug release profiles in the case of mini-tablets due to chemical

similarity in between the polymers and due to the small size of tablets leading to non-significant difference in the relaxation of polymeric chains and the swelling capacity (125). Other possibilities for polymeric matrix consist in xanthan gum (presenting the highest swelling index forming a dense matrix with a much developed chain network and leading to a close to zero-order kinetic release profile), carbopol, poly(ethylene) oxide or ethylcellulose (whose main mechanism is diffusion) for example.

As for proportions, many studies showed that drug release has inverse relation with polymer concentration and that a minimum threshold of matrix agent was necessary to achieve a sustained release. It was observed that formulations with polymer contents less than 40% remained ineffective in controlling drug release. Globally, matrix with about 50% polymer (HPMC or ethylcellulose) achieved 45-53% drug released at 4h, 82-85% at 8h and 94-99% in 24h (151,152). However, reduction of the proportion of functional excipient forming matrix improves flow mix (reduction of seizures and sticking) and compressibility. This is why matrix-based formulations need combination of a matrix agent with other materials to achieve an equilibrium between flow properties, ease of process and drug release control. Those excipients -without sustained-release ambition- can be diluents, binding agents, glidants, bulking / control or gelling agents to limit the burst effect.

Materials:

Taking the direct compression (DC) process into consideration, chosen excipients for the following experiments were: HPMC as matrix agent, microcrystalline cellulose and DC lactose as diluent / charge agents (the first one being water-soluble and the second water-insoluble, impact on release profile could present an interest) and a flow mix including talc and Aerosil® as glidants and magnesium stearate as lubricant. A hydrophile dye, indigo carmine, was selected in order to simulate drug release profile – this will be developed in the following paragraphs. Precise composition is given in the following table 6, with an extra 7,4mg of dye per tablet.

HPMC Methocel® E5 Premium LV came from Colorcon (Kent, UK), ethylcellulose Ethocel® NF premium STD 100 from DOW chemical (Michigan, USA), microcrystalline cellulose Avicel® type PH-101 was purchased from FMC Biopolymer Europe (Belgium), FlowLac 100 DC lactose from Meggle (USA), Aerosil® 200 from Evonik Degussa Operations GmbH (Germany) and Indigo carmine from Sigma-Aldrich chemie GmbH (Germany).

Table 6: Composition of micropatches second layer for sustained release

Formulation	*Matrix components*			
1	66,5% HPMC	+ 30% µC cellulose		+3,5% flow mix*
2	66,5% HPMC	+15% µC cellulose	+ 15% DC lactose	+3,5% flow mix*
3	66,5% HPMC		+30% DC lactose	+3,5% flow mix*
4	46,5% HPMC	+25% µC cellulose	+25% DC lactose	+3,5% flow mix*

*Flow mix such as 2% talc + 0,5% Aerosil® + 1% magnesium stearate

Methods:

Matrix blends were prepared by mixing HPMC, diluents and indigo carmine together, before addition of the flow mix only in the end to avoid segregation. Tablets were elaborated with 75mg of formulation G as mucoadhesive layer (composed of Eudragit E PO, chitosan LMW, pectin and S-CMC), on top of which were added 100 mg of the matrices aforementioned in table 6. 15mm-large tablets of polymers were obtained by compression of those layers under a 4,5-ton pressure for 1 minute, without precompression, using a powered hydraulic bench press (Atlas™ Power Press 8T, Specac Inc., PA, USA).

Results:

15mm-bilayered tablets were obtained without any process issues and showed good visual characteristics as visible in figure 40. White parts correspond to the mucoadhesive layer while blue parts correspond to the second layer supposed to contain therapeutic proteins.

Figure 40: 15mm-bilayered tablets

2. Backing layer

Backing layer has for aim to offer a water-impermeable protection for the active molecules against the intestinal environment and erosion, while designing them a unidirectional release pattern. Based on literature, the gold standard is ethylcellulose. It is an inert, non-toxic and hydrophobic polymer, widely used in sustained release formulations.

Materials:

Ethylcellulose Ethocel® NF premium STD 100 from was purchased from DOW chemical (Michigan, USA) and acetone from VWR chemicals.

Methods:

Coating solution was prepared by dissolution of the polymer in heated acetone under magnetic shear to achieve a clear solution of 5% ethylcellulose (w/v). Tablets were coated manually over the second layer surface and the edges with this solution, three times consecutively with allowance to dry in between.

Results:

The manual coating method was not very appropriate. Tablets being small and the solution being very viscous, deposit uniformity could not be assured. Moreover, some tablets presented a bit of coating on their mucoadhesive surface, which could have delayed their dissolution, creating some variability between tablets. Use of a coating spray gun would probably be more efficient and uniform.

Even though Wong et al. proved that the ideal coating is 5 layers concerning enteric coating (153), the 3 applied layers were enough to cover evenly the surface and edges of tablets. Even after an 8-hour dissolution assay that will be described below, backing layers were found intact, without any hole, cracking or erosion as observable in figure 41 below, proving that the backing layer plays its protective role in aqueous media efficiently.

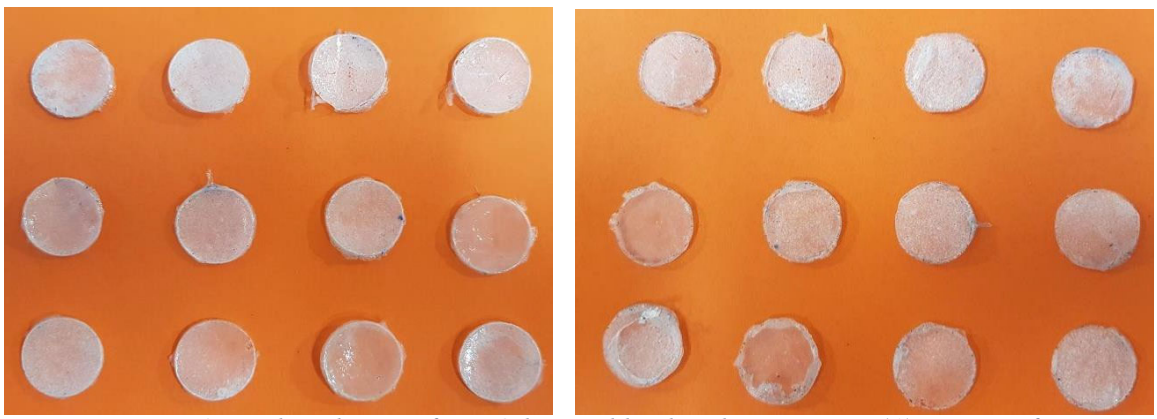

Figure 41: Backing layers after a 8-hour tablet dissolution assay: (A) outer surface, (B) inner surface in contact with the matrix layer.

3. Controlled release: dissolution profile

In order to evaluate the impact of the changes in formulation, tablets were loaded with indigo carmine to simulate therapeutic drugs. It was chosen because of its hydrophilicity (such as insulin).

Materials:

The dissolution tests were performed using two Sotax AT6 dissolution apparatuses (Sotax, Alschwil, Switzerland), equipped with paddles for each of the twelve bowls. Analysis was run thanks to a Synergy plate reader (Bio Tek, Switzerland) loaded with 96-well plates and interpreted with Graphpad Prism 7 (GraphPad Software, LaJolla, CA, USA).

Methods:

For practical reasons, dissolution tests were realized on 15-mm tablets instead of tablets of about 3mm that match the size of intestinal micropatches. As said in the first part of this work, size of tablets has a major influence on release and absorption. Literature showed that tablet dimensions, more precisely the surface area / volume ratio, had an important effect on release profile. In particular for HPMC matrix tablets with equivalent surface, tablets having the different SA/Vol values did not result in similar drug release, tablets with bigger ratio values had faster release profiles (154). However, in this precise case, edges and top surface of the tablets being waterproof coated, drug release is only possible through one surface of exchange. Consequently, at equivalent drug concentration / surface, the release profile would be notably identical to the one obtained with 15-mm tablets, the only difference being timings.

The dissolution tests were performed as follows: each bowl was filled with 900mL of distilled water, allowed to equilibrate at 37°C±1°C and paddle rotation speed was set at 50rpm. Timing started at T0 when 15-mm tablets were added to the media. Samples of 1mL of each bowl were collected at a defined point (in the center, upper third) and placed in Eppendorf® vials for future analysis. Samples were immediately replaced by 1mL of warm distilled water to keep sink conditions valid until the end of experiments. For a more precise follow-up in the beginning of the tests than requirements of the European Pharmacopoeia, samples were collected every 30 min for the 6 first hours and every hour for the last 2 hours. Each formulation was tested in triplicate.

As for analysis, the absorption spectrum of indigo carmine was studied to determine the wavelength resulting in the maximum of absorbance of the dye, and the value would be further used to establish the calibration curve.

For the elaboration of a calibration curve, standard solutions were prepared by dilution of a stock solution of indigo carmine with distilled water in a range from 0,5 to 15mg/L to be centered on the expected dose of indigo carmine in the tablets (being 7,4mg/tablet, expected 8,23mg/L). Standard solutions and samples of dissolution tests were submitted in triplicate for measures of absorbance in the UV spectrophotometer at the defined wavelength. Calibration curve was established based on the spectrophotometric determination and expresses the absorbance of standard indigo carmine solutions versus corresponding concentrations of standard solutions. For dissolution tests, average values were calculated for each tablet and reported on the calibration curve to define their concentrations at each sampling time. Average values were calculated for each formulation and represented as release profile graphs.

Results:

Tests resulted in full disintegration of the 12 tablets made out of 4 different formulations, and most of the objective was achieved after 5 hours. Indeed, the only exception to disintegration concerns the water-impermeable backing layers that were found intact after 8 hours of assay in every bowls. As for dissolution, powders from formulation 1 were totally dissolved while bowls of formulations 2,3 and 4 contained few white particles as visible in figure 42, corresponding to DC lactose that is water-insoluble.

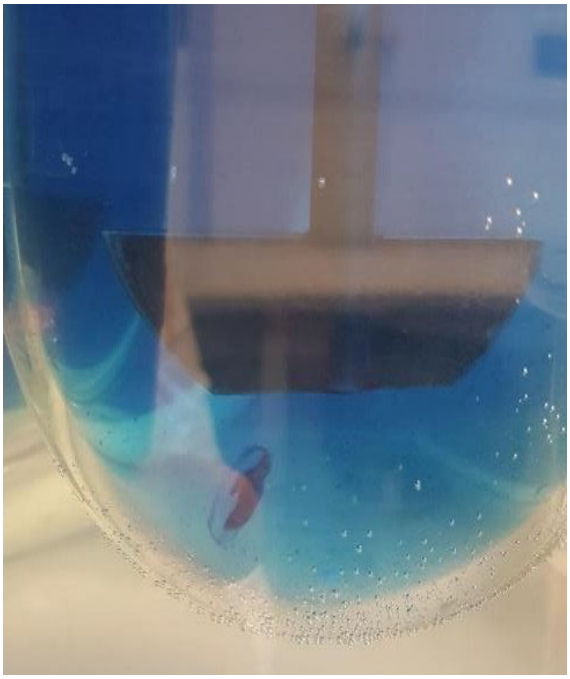

Figure 42: Tablet during the dissolution test: powder part is partially disintegrated, backing layer is intact

Spectrum of absorption of indigo carmine, illustrated in figure 43 below, was obtained by UV-spectroscopy. The maximum of absorbance is λ_{max}=608 nm which is accordance with literature (103,155).

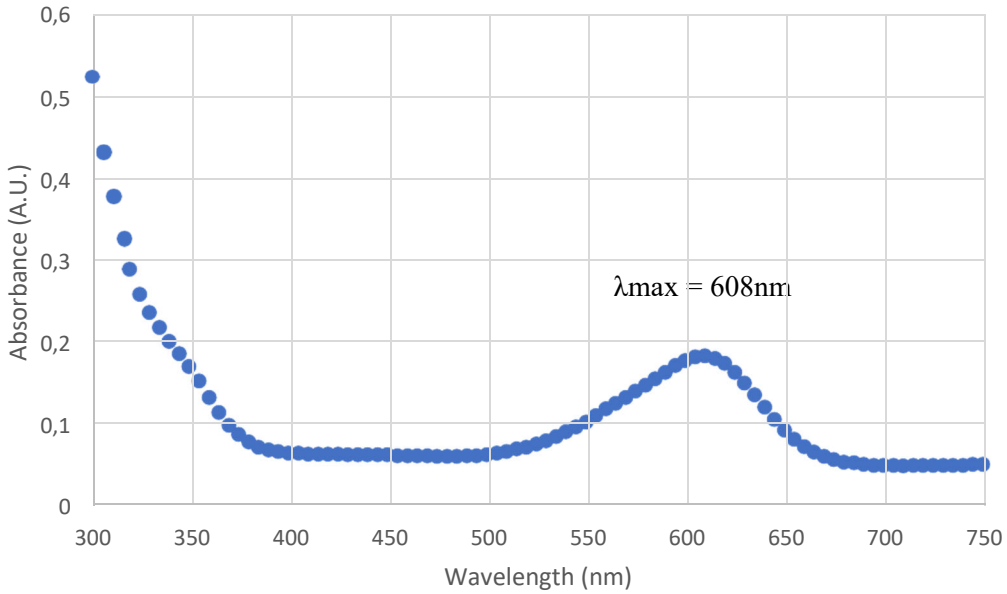

Figure 43: Spectra of absorption of indigo carmine dye

λ=608nm was used as wavelength of excitation to establish the calibration curve represented in the figure 44 below, and used to determine the concentration of indigo carmine in the samples collected during dissolution test. The concentration range of 0,5-15 mg/L was found to result in linear relationship between dye concentration and absorbance. The correlation coefficient R^2 was determined to be 0,9989 and the curve did not present any outlier.

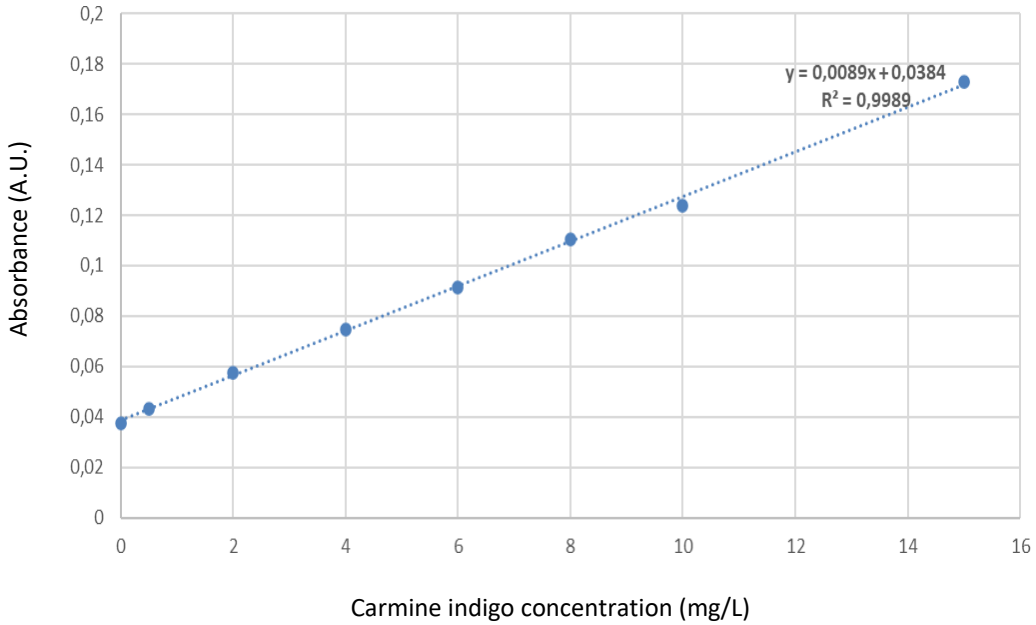

Figure 44: Calibration curve of indigo carmine (λ=608nm)

The following release profiles and fitting curves, respectively represented in figure 45 and 46, were obtained:

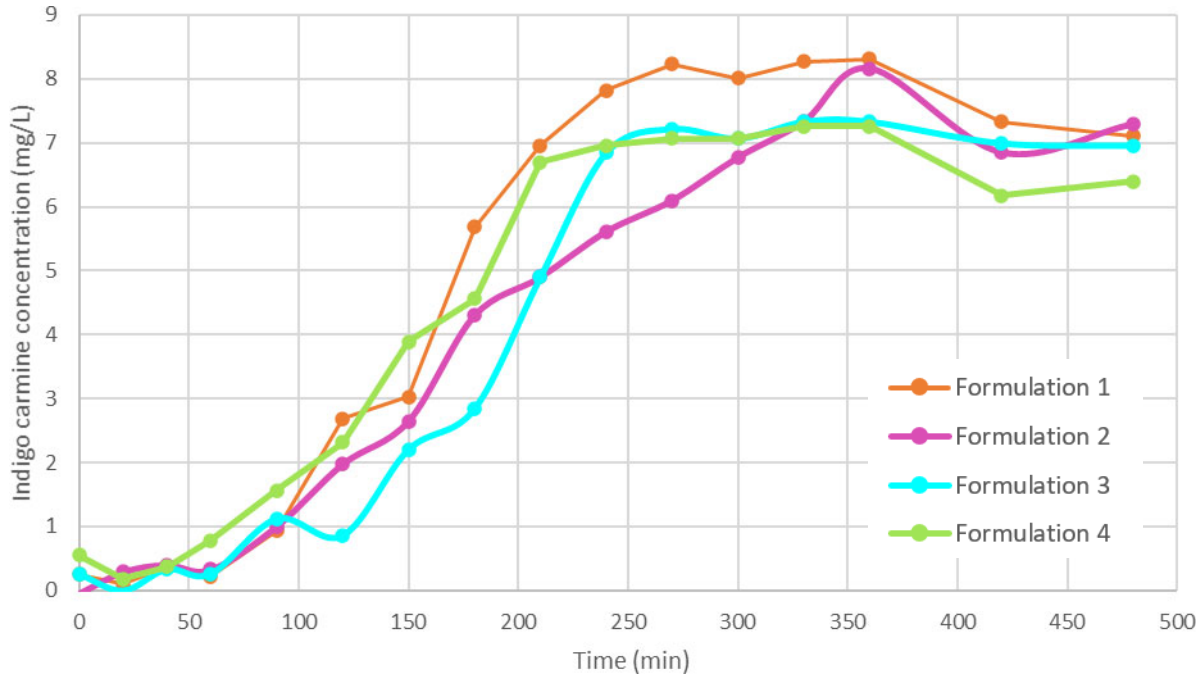

Figure 45: Indigo carmine release profiles

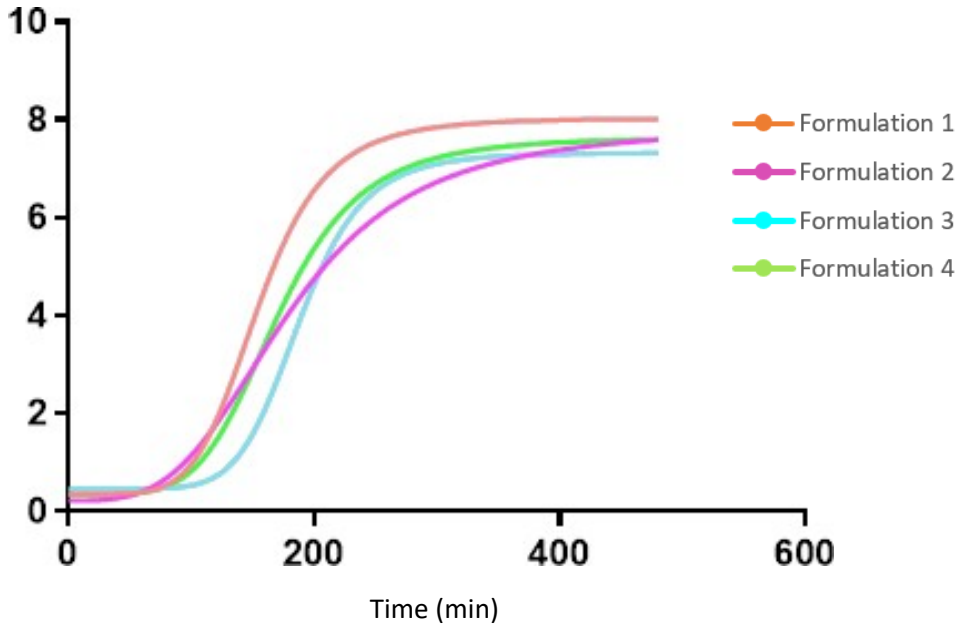

Figure 46: Fitting curves of indigo carmine release profiles

Tablets showed a delayed and prolonged release of indigo carmine. At least 1 hour of dissolution was necessary to detect the first significant concentrations of dye, this delay corresponding to the swelling of the polymeric first layer (60 to 90 minutes). After that, slopes of the fitting curves (figure 46) around 60-90 minutes of dissolution suggest a

brief burst effect that gets reduced around 120 minutes, once the HPMC is hydrated and the matrix plays its role to reduce the release rate. In the end, tablets achieved complete release after approximatively 5 hours.

Formulations 1, 2 and 3 all contained 66,5% of HPMC as matrix agent and are different in the nature and quantities of diluents. Formulation 1 contained 30% of water water-soluble microcrystalline cellulose, formulation 3 30% of water-insoluble DC lactose and formulation 2 a mix of both. Release profiles confirmed that F1 has the quickest dissolution profile (steepest slope, first to achieve plateau and maximum release) as expected while formulation 3 is staggered over time because of lactose hydrophobia. Formulation 2 was expected to be in the middle of the previous release profiles but surprisingly slows the release of the dye (flattest slopes at both tangential points). Formulation 4 with lowest content in HPMC matrix and highest content of both diluents had an intermediate release profile, which exceeded expectations concerning speed of release. In the end, formulations 1,3 and 4 are rather identical in the shape of their curves, while formulation 2's curve suggests a slower release.

Even though statistical analysis revealed no significant differences between the four formulations ($p>\alpha=0,05$) based on Tukey's multiple comparisons, formulation 2 seems like the best equilibrium to achieve a sustained release.

According to the European Pharmacopoeia's definition of sustained release, 3 points need to be defined: 1st at 20-30% of dissolution to make sure dose dumping is avoided, 2nd at 50% dissolution to define the release profile and 3rd one at minimum 80% release to guarantee a maximum release. Considering all these four formulations equivalent, average timings are 120 minutes (2h) for 1st point at 25% release,180 minutes (3h) for 2nd point at 50% and 240 minutes (4h) for the 3rd point at 80% release.

III- Discussion

Worldwide, there is a serious health concern about chronic pathologies, such as diabetes, auto-immune pathologies or cancers, being on a rise. For these diseases, the proportion of biological treatments keeps growing and there is an enormous potential for therapeutic proteins and peptides in the coming decades (20). Actual treatments by parenteral injection are often source of anxiety, pain, infections, leading to poor patient compliance and poor adherence to treatment (24,156–158). Finding a way to deliver those drugs orally would help millions of patients.

Route of administration is a critical factor in any therapeutic intervention and governs both the pharmacokinetics and efficacy of the drug. For protein and peptide therapeutics, an interplay of poor permeability characteristics, harsh conditions of the GI tract and the epithelial mucosa, all together form a complex mechanical, biochemical, microbial, and immune barrier resulting in their poor absorption. Thereby, achieving sufficient bioavailability inducing clinical response makes oral delivery of therapeutic proteins extremely challenging.

Several techniques have been developed over the years to improve oral bioavailability such as chemical modifications, co-administration with enzyme inhibitors, absorption enhancers (PE) or formulation of innovative drug delivery systems like enteric-coated solid dosage forms (63,153,159) and intestinal micropatches (25,31,38,58,59,158–160). Even though those techniques are quite efficient at their scale, none of them is strong enough to develop a significant oral bioavailability compared to parenteral injection (161). However, combination of several of these approaches in a unique drug delivery system might be a synergic strategy to achieve sufficient bioavailability.

The ideal DDS should be able to protect drugs from harsh environment, to target site of action, to enhance drug absorption through prolonged contact with tissues and achieve sustained release. This should avoid, or at least reduce, adverse reactions (88). To reach this objective, the idea of this thesis was to design intestinal micropatches with mucoadhesive and sustained-release properties, containing permeability enhancers and therapeutic drugs, co-administered with protease inhibitors in enteric-coated capsules. Final objective was to assess the potential synergism of these interactions.

The rational design of this DDS formulation, made by a wise choice of excipients and process parameters, was promising concerning its ability to overcome physiological challenges.

About the mucoadhesive layer

Mucoadhesion is a critical step as it constitutes a limiting factor for absorption, bioavailability, and efficacy of the treatment. This is why the choice of polymers constituting the mucoadhesive layer was crucial. Carbopol, Eudragit and chitosan are among the most frequently studied in literature. Mucoadhesive experiments showed the significant superiority of formulation G above all others, composed of Eudragit E PO and chitosan in addition to the basis formulation of S-CMC and pectin. A significant difference with formulation A, that did not contained chitosan, proved the interest of its addition in the enhancement of mucoadhesion as expected. However, derivatives of chitosan (TMC or CM-TMC) showed no significant difference in mucoadhesion results, contrary to what was expected.

Concerning mucoadhesion experiments, even though intestinal tissues were irrigated right before tablet application, evaluation of mucoadhesive bond in aqueous medium would be a closer representation of reality in intestinal tract. In addition, those conditions would allow to realize tests in physiological conditions of pH and temperature. In fact, literature data demonstrated the importance of experimental set-up by revealing that significant differences in mucoadhesion have been observed between testing of formulations at body (37 °C) and room temperature (20 °C) on vaginal tissues (162). These conditions would also permit to evaluate capacity of pH-responsive polymers to interact with mucosa in realistic conditions. For example, mucoadhesive tests with Eudragit L100-55 would give the best results in specific pH conditions because this pH-sensitive methacrylic acid copolymer has a dissolution threshold at pH 5,5. This constitutes an important parameter to consider for site-specific delivery into the upper intestine as it is in between stomach (pH 2-3) and small intestines (pH 6,5 -7) (163). Moreover, for *ex-vivo* and *in-vivo* experiments, mucoadhesion tests should be extended to more than triplicates due to important variability in tissues (variable composition and quantity of mucus from one animal to another).

As for rheology studies of polymeric preparations in contact with mucin, results coincide with those of mucoadhesion as formulation G presented the highest values of viscosity, and reference formulations A and B presented valuable results. Once again, results of formulations containing N-TMC and CM-TMC were disappointing. Rheology and bioadhesion tests investigated the interactions between mucoadhesive polymers and homogenized mucus gel. Results proved that incorporating a mucoadhesive polymer into a mucus gel induces rheological synergism, either positive or negative, which is a reflection of inter-macromolecular interactions between polymers and mucus. In the cases of positive synergism, formulations lead to “weakly” cross-

linked gel network. In fact, due to polymers chemical structure, gel networks are thought to be caused by physical chain entanglements and non-covalent bonds, probably hydrogen (145).
As rheological experiments did not provide complete explanation of the mucoadhesion phenomenon, it should not be considered as a stand-alone method to characterize the mucus-polymer interactions. Further investigations with spectroscopic methods such as ATR-FTIR (Attenuated Total Reflection – Fourier Transformation InfraRed spectroscopies) would assess the extent of chain interpenetration at the polymer / mucin interface & hydrogen bonds (145). This should permit to study more precisely macromolecular interactions between polymers and mucus via comparison of spectrum of components alone vs mixture) or yet dielectric spectroscopy.

Results obtained in mucoadhesion, rheology and bioadhesion tests all agreed on the superiority of formulation G concerning adhesion properties. Moreover, bioadhesion tests proved that all formulations containing Carbopol 934 instead of Eudragit E PO had negative values of synergism when mixed with porcine intestinal mucin, meaning that mixture Carbopol + mucin has a lower viscosity than Carbopol alone. This supposes that the carbomer has a lack of rheological interaction when in contact with mucus.
As described in literature, sensitivity of polyanionic carbomers, which are PAA-derivatives, to pH and ionic strength of the hydration medium might be the cause of this negative synergism. Possible explanation is the presence of ions in the commercial mucin used during experiments. These ions, whose presence is probably linked to mucin extraction and purification processes, may interact with PAA-derivatives macromolecules. In fact, presence of cations to polyanionic carbomer solution causes a shielding of the polymer charges and a subsequent recoiling of the macromolecules themselves. In its coiled conformation, carbomers cannot easily diffuse to interact with another hydrogel like mucus and moreover, number of free groups capable of giving secondary chemical bonds with biological tissues significantly decreases. Furthermore, calcium ions can also cause mucin aggregation by reducing the electrostatic charge of the phosphate groups of glycoproteins and by inducing a simultaneous change in protein 3D structure. Both ionic phenomena lead to a breakdown in gel network, thereby explaining the lack of interactions highlighted during tests (143,146).
This could be proved by doing comparative rheology tests between the used mucin and ions-free mucin, obtained by dialysis of intestinal mucin samples.

Concerning comparisons of the effect of chitosan and its derivatives (N-TMC and CM-TMC), they have been used as polymer of choice to increase mucoadhesion of intestinal micropatches. Based on literature, and contrary to slightly charged chitosan, N-TMC and CM-TMC present strong positive charges supposed to interact with negative charges of the mucosal glycoproteins. The formation of hydrogen bonds between them ensures the stronger adhesion of derivatives to mucus, compared to chitosan. Normally, in conditions of acidic pH, chitosan is ionized with high charge density, molecules uncoil and swell to exhibit an elongated shape used to develop its mucoadhesive properties. In neutral or basic conditions, chitosan is no more soluble and only exists as coiled molecules. Consequently, its derivatives were synthetized to offer a wider range of solubility into neutral and basic pH, and thereby better mucoadhesion properties.

To mimic physiological conditions, experiments of mucoadhesion and rheology were run in pH 6,8 PBS. In these conditions, pH being slightly superior to the pKa of chitosan (6,5), chitosan is supposed to have limited mucoadhesion properties while its derivatives are in the good conditions for ionization, full chain elongation and mucoadhesion. However, results of previous experiments do not agree with that.

This suggests that at least another parameter interfered in the expected results. A possible explanation might be the molecular weight of the polymers we used. Chitosan being a linear molecule, it can easily interpenetrate with a mucin glycoprotein and form hydrogen bonds thanks to its numerous hydroxyl and amino groups. By contrast, derivatives of chitosan carry additional carboxymethyl and trimethyl groups compared to the structure of chitosan. This steric hindrance affects their dynamic volume (164) and reduces the mobility and flexibility of polymeric chains reacting with mucin, leading to limited mucoadhesion.

Therefore, although a critical molecular weight (and length) of the polymers is necessary to produce the interpenetrating layer, an excessive one may impair interpenetration (144). A possible compromise between chitosan used in these experiments (LMW) and its derivatives would be chitosan of medium or high molecular weight. In comparison with grafted chitosan derivatives that present voluminous and numerous ramifications (depending on the functionalization ratio), linear chitosans (MMW or HMW) present much less side ramifications able to perturbate interactions with mucus. Their dynamic volumes are reduced compared to derivatives, and their flexibility increased. This reminds that optimal gel formation not only depends on the nature and molecular weight of polymers, but mainly depends on environmental conditions (pH, electrolytes, …) that impact degree of ionization, charge density, conformation, mobility & flexibility of polymeric and mucus molecules (97).

Finally, the fact that gel formation of our micropatches is pH-dependent confirms the non-

covalent nature of the bond between polymers and mucus (145).

As for the comparison of formulation G with referents A and B, the addition of chitosan resulted in its superiority in all tests. A possible explanation could be the interaction between chitosan and a polymer present in both reference solutions: pectin. Polysaccharides of opposite charges such as chitosan and pectin can have a very strong intermolecular interaction. Due to its cationic nature (amino groups $-NH_3^+$), chitosan is able to form complexes with anions like pectin (ionized carboxylic groups $-COO^-$) by electrostatic interactions, giving rise to polyelectrolyte complexes (PEC). Because of their multiple advantages like high biocompatibility and pH-sensitivity, *in situ* formation of pectin/chitosan PEC was found to be potential carriers for sustained drug delivery and are already widely used as excipients of drug formulations (165–168). Nature of the interaction could be proven by differential scanning calorimetry (DSC).
Potential of chitosan is great in the type of formulation developed here due to its double mechanism of action: mucoadhesive polymer and permeability enhancer (115–118). Literature combined with the results of previous experiments suggest that chitosan micropatches can be an effective asset in oral delivery systems for therapeutic drugs, hence its addition in future formulations is recommended.

To open up about actual hot topics in pharmaceutical research, many scientific studies showed that thiolated chitosan possesses clearly improved mucoadhesion and permeation properties compared to chitosan (118,124,125,169). The presence of free thiol groups (-SH) in the polymeric skeleton helps in the formation of disulfide (S-S) covalent bonds with that of the cysteine-rich subdomains present in mucin, which can substantially improve the mucoadhesive properties of the polymers (170). Advantages of thiolated chitosan over chitosan include higher hydrophilicity, more efficient uptake process for macromolecules delivered, enzyme inhibitory activity, improved PE (171). Comparison between the formulation developed in this thesis, containing chitosan, and another one with thiolated chitosan could confirm its interest in the formulation of intestinal micropatches

Concerning the matrix layer of the devices

Controlling the rate, extent and time of drug delivery can optimize their performance in many ways, like increase their therapeutic benefits, minimize side effects and enhance the patient compliance.
Employing a polymer as an entrapment matrix is a common feature among the different types of systems currently being pursued for protein delivery. Development of sustained release tablets with a direct compression process is a real challenge due to low flow properties of

functional polymers. The equilibrium between matrix agent, diluent and flow agents is the key to achieve desired release profile without restriction on production process.
Dissolution test proved that the fourth formulations had a sustained release profile, without significant difference. Based on theory, formulation 2 containing high dosage of HPMC, an equal dosage of microcrystalline cellulose and DC lactose and a small quantity of flow mix represents the best potential to achieve the desired kinetics. To finish with formulation, no modification has been tested on the backing layer as tests proved certainly its efficacy as it stands, in accordance with literature that did not reveal any other interesting formulation.

Nonetheless, results of the dissolution tests should be taken with caution for several reasons. First, dissolution tests represent a very important hydrodynamic movement, with lot of water able to react with tablets. This phenomenon is much restricted with intestinal micropatches because the exchange is limited to content in water of the tissues (limited water inflow). Release would be delayed as it would happen over a longer period of time *in vivo*.
Second, those experiments were made in distilled water from calibration curve to dissolution tests, as recommended by the European Pharmacopoeia. To keep it aligned with physiological mechanisms, those tests could be realized in media such as simulated intestinal fluids. Finally, molecular size has a major influence when it comes to drug release, even more when the main mechanism is diffusion. Indigo carmine having a molecular weight of 466,36 $g.mol^{-1}$ and insulin 5808 $g.mol^{-1}$, release profile would be different. Matrix mesh size and dynamic volume of biomolecules would be important parameters to predict the release profile. For all these reasons, *in vivo* release profiles of intestinal micropatches will be different than the one obtained with those dye-loaded patches in aqueous dissolution tests but these experiments have given an important information concerning the ability of our formulations to design a sustained release profile. This truly suggests hope for a sustained release of intestinal micropatches containing therapeutic molecules such as insulin.

Perspectives

Next step for formulation experiments would be characterization of the obtained micropatches with *in vitro* drug release studies to confirm that biomolecules are able to cross the polymeric gel formed in contact with the mucosa. Franz cell diffusion system or flow-through set-up constitute the most appropriate *in vitro* methods for evaluating drug release properties and the enhancement of transport across the intestinal wall (81). Moreover, evaluation of the DDS stability in the presence of intestinal fluid simulant and its safety in both cell model and suitable

in vivo model would be necessary. Further experiments, after optimization of the drug delivery system, would be *in vivo* experiments in both diabetic and non-diabetic rats to evaluate controlled release of the insulin and efficacy on blood glucose level. Final *in vivo* evaluation would be the comparison of effects between the selected formulation of intestinal micropatches and commercially available dosage forms of insulin.

Caution will be required concerning extrapolation of *in vitro* to *in vivo* results. *In vivo* effects might be reduced compared to *in vitro* due to dilution effect (even though reduced due to the backing layer and mucoadhesive properties), and due to the resilience of living tissues to the action of permeability enhancers than excised membranes or cultured cells.
Furthermore, knowing that formulations of therapeutic drugs by the oral route rarely exceed 0,5-2% of bioavailability, reaching the double-digit range (>10%) would already be an achievement. In fact, it is important to assess whether the extent of drug absorption is acceptable, with regard to average bioavailability as well as inter-subject variability. In fact, low bioavailability has been shown to be associated with large inter-subject variability in systemic exposure. Thereby, a successful PE formulation may increase bioavailability for negligeable / very low levels to low / moderate levels and still be very efficient. Reduction of intra- and inter-variability is another advantage to enhancement of bioavailability, helping to improve the control of the drug's intended and unintended actions (9).

Once an absorption-enhanced delivery technology would prove to be successful for one particular drug, that technology might be readily adapted to improve more broadly the delivery of other poorly absorbed drugs. The developed technology may afford alternatives for proteins and peptides currently only administered by injection but would also create new opportunities for chemical entities which demonstrated good pharmacologic activities but poor biopharmaceutical properties and that otherwise were not developed into drugs. Moreover, new and more potent permeability enhancers that are designed to function through specific mechanisms than those currently in clinical trials may follow (9). Those technologies could later be developed for large variety of indications, starting with the delivery of cancer treatment, antibodies for passive vaccination or treatment of the opioid crisis.

CONCLUSION

There is no doubt that the oral route is the most favored because of patient compliance and acceptability, but probably the most complex route of administration of protein and peptide drug. Worldwide, novel therapeutics now are peptides and proteins for chronic pathologies, and there is a great need in oral delivery of these biomolecules. Delivering therapeutic proteins at efficient quantity via non-injectable route is challenging considering the physiology of this route. However, better understanding of the GI tract physiology, with harsh environment and physiological barriers, permits the development of strategies to overcome the hurdles encountered in oral drug delivery.

Several approaches like chemical modifications, use of functional excipients, or development of innovative delivery platforms were developed through decades. Recently, attention was focused on permeability enhancers that are small functional molecules with a great potential to enhance oral bioavailability by disturbing cellular tight junctions, and that could be co-administered with drugs to potentialize their effect. However, none of these approaches resulted in significant results in oral bioavailability, or at least without side-effects. To ensure protection of the active molecules and efficient delivery, the idea of this work was to combine several strategies including use of permeability enhancers, enzyme inhibitors, enteric coating and mucoadhesive polymers inside a unique drug delivery system designed for sustained drug release.

These drug delivery systems, called intestinal micropatches, proved their efficacy in literature but improvement of formulations could lead to even better drug absorption. This work focused on the preparation of intestinal micropatches and their characterization through various tests.

To select the best components for the mucoadhesive layer, various polymeric mixtures were submitted to mucoadhesion, rheological and bioadhesive tests. All agreed on the superiority of formulation G, composed of pectin, S-CMC, Eudragit E PO and chitosan. Comparison of formulations containing Eudragit E PO and Carbopol 934 resulted in respectively positive and negative values of rheological synergism when in contact with mucin. Results of Carbopol are thought to be due to pH and ionic conditions that did not favor its interactions with mucus. Interest of addition of chitosan and its derivatives in the mucoadhesive layer was shown for the native molecule only. As for derivatives, steric hindrance of N-TMC and CM-TMC in intestinal conditions did not allow those molecules to achieve correct mucoadhesion properties, contrary to literature expectations. For chitosan, the molecule enjoyed favorable pH conditions and the

formation of polyelectrolyte complexes with pectin to improve the mucoadhesive properties. Moreover, the permeability enhancer activity of chitosan makes it an important asset of the mucoadhesive layer. As for the second layer of the intestinal micropatches, all tested formulations resulted in the formation of matrices capable of sustained release, without significant difference. However, formulation 2 with 66,5% of HPMC, 15% microcrystalline, 15% DC lactose and 3% flow mix is thought to be the most suitable, considering process parameters.

Those experiments proved the ability of improving existing formulations of intestinal micropatches by addition of Eudragit E PO and chitosan in order to enhance the mucoadhesion properties. Selection of HPMC as a matrix agent with both water-soluble and insoluble diluents to form the second layer resulted in sustained release kinetic profile. Optimization of those drug delivery systems is not over yet and many other experiments need to be done to confirm their efficacy *in vitro* and *in vivo*, but improvements highlighted in this work should theoretically lead to an increase of bioavailability of therapeutic drugs administered by the oral route.

Intestinal patches are promising devices, with the ability to improve oral absorption of peptide and protein therapeutics. In this work and in literature, they have shown their tremendous potential on oral drug delivery. Thereby, it seems reasonable that once the delivery technology would have proven to be successful for one particular drug (insulin for example), that technology might be readily adapted to improving the delivery of other molecules demonstrating poor absorption.

To conclude, studies and experiments proved that the best approach seems to be the combination of multiple strategies acting in a synergic way to improve oral bioavailability. Special focus was given here on permeability enhancers and innovative drug delivery platforms. Only limited publications study the overall effectiveness of multiple approaches considering intestinal physiology, delivery concept and specific formulation, and this work could be considered as an addition to the list.

Le Président de la thèse,
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Vu et permis d'imprimer, Lyon, le 15 FEV. 2021
Vu, la Directrice de l'Institut des Sciences Pharmaceutiques et Biologiques, Faculté de Pharmacie

Signature :

Pour le Président de l'Université Claude Bernard Lyon 1,

Professeure C. VINCIGUERRA

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Au moment d'être reçu Docteur en Pharmacie,

En présence des Maitres de la Faculté, je fais le serment :

- D'honorer ceux qui m'ont instruit(e) dans les préceptes de mon art et de leur témoigner ma reconnaissance en restant fidèle aux principes qui m'ont été enseignés et d'actualiser mes connaissances

- D'exercer, dans l'intérêt de la santé publique, ma profession avec conscience et de respecter non seulement la législation en vigueur, mais aussi les règles de Déontologie, de l'honneur, de la probité et du désintéressement

- De ne jamais oublier ma responsabilité et mes devoirs envers la personne humaine et sa dignité

- En aucun cas, je ne consentirai à utiliser mes connaissances et mon état pour corrompre les mœurs et favoriser des actes criminels.

- De ne dévoiler à personne les secrets qui m'auraient été confiés ou dont j'aurais eu connaissance dans l'exercice de ma profession

- De faire preuve de loyauté et de solidarité envers mes collègues pharmaciens

- De coopérer avec les autres professionnels de santé

Que les Hommes m'accordent leur estime si je suis fidèle à mes promesses. Que je sois couvert(e) d'opprobre et méprisé(e) de mes confrères si j'y manque.

L’ISPB - Faculté de Pharmacie de Lyon et l’Université Claude Bernard Lyon 1 n’entendent donner aucune approbation ni improbation aux opinions émises dans les thèses ; ces opinions sont considérées comme propres à leurs auteurs.

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PRADAL Pauline

PERORAL DELIVERY OF THERAPEUTIC PROTEINS: FORMULATION IN THE FORM OF INTESTINAL MICROPATCHES

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SUMMARY

Innovation in pharmaceutical industry illustrates itself nowadays with a growing pool of biologic therapies launched on the market. They are mainly administered by parenteral route, which is a problem for patient compliance, efficacy, and side effects.

Formulation of therapeutic proteins for the oral route constitutes one of the biggest challenges in pharmaceutical sciences ever, as it is restricted by a harsh environment and biological barriers, resulting in very low bioavailability and absence of efficacy. Because co-administration of biodrugs with permeability enhancers inside a specific drug delivery system could overcome those physiological issues, the present study reports about formulation and efficacy of three-layered intestinal micropatches designed to provide prolonged residence time on the site of action and to favor a diffusion pathway to the intestinal mucosa.

Based on literature, experiments were conducted to optimize formulations of existing micropatches. Mucoadhesion tests were carried out, combined with rheology and bioadhesion tests, and all formulations containing Eudragit E PO presented better characteristics than those containing Carbopol 934, while the addition of chitosan and its derivatives (N-TMC and CM-TMC) gave surprising results with domination of simple chitosan. The best polymeric combination possible for the first layer, with overall superiority over other formulations, was found to be formulation G containing both Eudragit E PO and chitosan. For the second layer, dissolution tests assessed the release profiles of various matrix formulations, and even though all formulations lead to sustained release, formulation with HMPC + two types of diluents + flow mix is thought to be the most suitable. The third backing layer, already being efficient, was kept unchanged.

Experiments consequently proved that formulation of intestinal micropatches could be improved in regard to mucoadhesion and confirmed that combination of various strategy can be synergic for oral drug delivery. Further experiments are needed to confirm those results and to assess impact of this formulation on drug oral bioavailability. If successful, development of such drug delivery system could have the greatest impact on drug therapy.

RESUME

L'innovation dans l'industrie pharmaceutique s'illustre de nos jours par un nombre grandissant de biothérapies sur le marché. Elles sont principalement administrées par voie orale ce qui soulève un problème pour la compliance des patients et pour l'efficacité et les effets indésirables de ces traitements.

La formulation de protéines thérapeutiques pour la voie orale constitue un des enjeux majeurs de la recherche pharmaceutique, du fait qu'elle soit limitée par un environnement difficile et par des barrières biologiques, le tout engendrant une faible biodisponibilité et une absence d'efficacité. Parce que la co-administration de biomolécules avec des promoteurs d'absorption au sein d'un dispositif de délivrance de médicament spécifique pourrait permettre de franchir ces obstacles physiologiques, cette thèse s'intéresse à la formulation et à l'efficacité de micropatches intestinaux tri-couches, conçus pour prolonger le temps de résidence au site d'action et pour favoriser un sens de diffusion uniquement en direction de la muqueuse intestinale.

Des expériences basées sur la littérature ont été menées pour optimiser les formulations existantes de micropatchs. Des études de mucoadhésion, combinées à des tests de rhéologie et de bioadhésion, ont été conduites et toutes les formulations contenant de l'Eudragit E PO ont présenté de meilleurs résultats que celles contenant du Carbopol 934, alors que l'ajout de chitosan et ses dérivés (N-TMC and CM-TMC) ont donné des résultats surprenants en faveur du chitosan simple. La meilleure combinaison polymérique pour la première couche, avec une supériorité sur toutes les autres formulations, s'est révélée être la formulation G contenant à la fois l'Eudragit E PO et le chitosan. Pour la seconde couche, des tests de dissolution ont permis d'évaluer les profils de libération de différentes formulations de matrices et, bien que toutes les formulations présentent une libération prolongée, celle contenant de l'HPMC + 2 types de diluants + un mélange lubrifiant semble la plus adaptée. La troisième couche, déjà efficiente, a été conservée intacte.

Ces expériences ont prouvé que la formulation de micropatchs intestinaux peut être améliorée en regard de la mucoadhésion et ont confirmé que la combinaison de différentes stratégies peut être synergique quant à l'administration de biomolécules par voie orale. D'autres expériences seraient nécessaires pour confirmer ces résultats et évaluer l'impact de cette formulation sur la biodisponibilité des protéines thérapeutiques. Le succès du développement de telles plateformes de délivrance de biomolécules pourrait alors avoir un impact majeur sur les traitements pharmaceutiques.

MOTS CLES Oral drug delivery, intestinal micropatches, permeability enhancer, mucoadhesion, intestinal absorption

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