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Chitosan based hydrogels for transmucosal drug delivery

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List of the papers discussed

This thesis includes the papers listed below:

1. Abruzzo A., Bigucci F., Cerchiara T., Cruciani F., Vitali B., Luppi B. Mucoadhesive chitosan/gelatin films for buccal delivery of propranolol hydrochloride. Carbohydrate Polymers, 2012, 87, 581-588.
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2. Abruzzo A., Bigucci F., Cerchiara T., Saladini B., Cruciani F., Vitali B., Luppi B. Chitosan/alginate complexes for vaginal delivery of chlorhexidine digluconate. Original Research Article. 2013, 91(2), 651-658. DOI information: 10.1016j.carbopol.2012.08.074.
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3. Chitosan-cyclodextrin nanoparticles containing excipients with the capacity to modify the bioavailability of for insulin delivery. In preparation

My contribution:

I have contributed to all part of above papers except for the SEM analysis reported in paper 1 and 2 that were performed by Dr. M.C. Gallucci (Department of Chemistry, University of Calabria, Arcavacata di Rende, Cosenza), the thermal analyses (TGA and DSC) reported in paper 2, that were performed by Dr. Bruno Saladini (PolyCrystalLine s.r.l., Medicina, Bologna) and the antimicrobial analysis reported in paper 1 and 2 that were performed by Dr. Federica Cruciani and Dr. Beatrice Vitali (Department of Pharmaceutical Sciences, Bologna University, Bologna).

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BACKGROUND

Several mucosal routes, including nasal, buccal, rectal, ocular, pulmonary and vaginal routes, have been investigated over the last decades as alternatives to oral and parenteral drug administration (Banga and Chien, 1988; Zhou and Li Wan Po, 1991b). With respect to the oral route, their major advantagesis the circumvention of the hepatic first pass metabolism and of chemical and enzymatic degradations that generally occur in stomach. Moreover, transmucosal drug administration can allow to avoid pain or discomfort caused by injections, when drugs are administered through parenteral routes and especially if multiple daily injections are required, thus increasing patient compliance. On the other side, the major disadvantage of transmucosal drug administration is represented by the presence of biological fluids and mucus that can remove drug systems from the application site, thus reducing the contact time between drug and mucosa and consequently, decreasing drug bioavailability. For this reason, in the recent years new muycoadhesive polymers (Peppas and Sahlin, 1996) were employed for the formulation of drug delivery systems able to increase their residence time and to improve drug bioavailability. Among these polymers, chitosancan adhere to mucosal surface thanks to its positive charge that can interact with negatively charged mucus. Moreover, chitosan can promote drug permeation through opening the thigh junction of mucosal membrane. These properties, in addition to other characteristics like biocompatibility, biodegradability and non- toxicity, allow to use this material for the formulation of several transmucosal drug formulations (Luppi, et al., 2010b).

SUMMARY

In this project thesis, chitosan based mucoadhesive systems for buccal, vaginal and nasal drug administration were formulated and characterized in order to study their chemical-physical properties and their ability to release drugs.

In the paper 1, buccal films based on chitosan-gelatin complexes were prepared and loaded with propranolol hydrochloride. The complexes preapared with different amount of chitosan and gelatin, were characterized and studied in order to evaluate their physical-chemical properties and their ability to release the drugand to allow its permeation through buccal mucosa.

In the paper 2, vaginal inserts based on chitosan/alginate complexes were formulated for local delivery of chlorhexidine digluconate. Tests to evaluate the interaction between the polymers and to study drug release properties were performed, as well as the determination of antimicrobial activity against the patogens responsible of vaginitis and candidosis.

In the project 3, chitosan based nanoparticles containing cyclodextrin and other excipients, with the capacity to modify insulin bioavailabity were formulated for insulin nasal delivery. Nanoparticles were characterized in terms of size, stability and drug release. Moreover,*in vivo* tests were performed in order to study the hypoglycemic reduction in rats blood samples.

THEORETICAL SECTION

1. ADMINISTRATION ROUTES

Drugs can be introduced into the body by several routes, according to the physical-chemical properties of drugs, the existence of preparations appropriate for their uses and the patient state (Fig. 1.1). In particular, they may be:

- taken by mouth (orally);

- given by injection into a vein (intravenously), into a muscle (intramuscularly), into the space around the spinal cord (intrathecally), or beneath the skin (subcutaneously);

- placed under the tongue (sublingually);

- inserted in the rectum (rectally) or in the vagina (vaginally);

- placed in the eye (by the ocular route);

- sprayed into the nose and absorbed through the nasal membrane (nasally);

- breathed into the lungs, usually through the mouth (by inhalation);

- applied to the skin (cutaneously) for a local (topical) or bodywide (systemic) effect;

- delivered through the skin by a patch (transdermally) for a systemic effect.
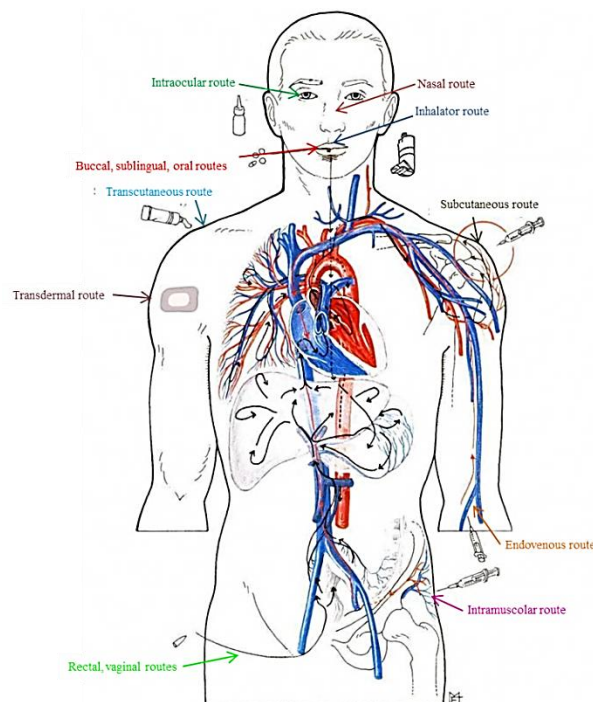
Fig 1.1 Schematic representation of several administration routes.

THEORETICAL SECTION-ADMINISTRATION ROUTES

Each route has specific purposes, advantages, and disadvantages.

1.1 Oral Route.The oral route is the most often used route, because it is convenient, safe and not expensive. Drugs can be administered orally as liquids, capsules, tablets, or chewable tablets and can be absorbed from the mouth until the intestine. Drugs, administered through oral route, pass through the intestinal wall and travels to the liver before they are transported via the bloodstream to its target site. The transit of drugs through the stomach and the liver can alter their physical-chemical structure, due to the presence of acidic medium in the stomach and of enzymatic process in the gastrointestinal tract and in the liver (first pass effect) that can metabolize or destroy many drugs. Consequently, a decreased amount of drugs reaches the bloodstream and an increase in daily administration is often necessary to obtain the pharmacological effect, with a consequent decrease in patient compliance. Moreover, in oral administration, food and other drugs in the digestive tract may affect the drug amount that is absorbed and the absorption velocity. For this reason, many drugs should be taken on an empty stomach, others should be taken with food, others should not be taken with certain other drugs. Some orally administered drugs, such as aspirin or non steroidal anti-inflammatory drugs, irritate the digestive tract and cause or aggravate preexisting ulcers. Finally, the oral route is not usable if the patient refuses to take it or vomits.

The oral route can be used for a local or general treatment:
- Local treatment: for the treatment of intestinal infection or a parasitosis. In this case, drugs will not be absorbed or only poorly absorbed.
- General treatment: it is the usual route of administration of drugs and in this case, drugs are absorbed and reach the bloodstream.

At the stomach level, drugs absorption is favored by the great absorption area (about $1 m^2$), but the pH, approximately around 1-2, can destroy drugs, especially peptide and proteins or drugs that are unstable under acidic conditions. Generally, only neutral molecules and not ionized acids in an acid pH are absorbed from the stomach.
The majority of drugs are absorbed through intestinal mucosa, that is characterized by an high area surface (from 200 to $300 m^2$), alkaline pH (from 6 to 8) and an important blood irrigation (1 L/minute). In the portion of digestive tract, including the rectum, drug absorption is very variable.

Generally, neutral and liposoluble molecules, but not those completely insoluble in water, can be adsorbed by passive diffusion through the lipid bilayer; amino acids, sugars and certain peptides by secondary active transport; elements in the form of ions, cations and anions by complex mechanisms.

Other routes of administration are required when the oral route cannot be used: for example, when a person cannot take anything by mouth, when a drug must be administered rapidly or in a precise or very high dose, or when a drug is poorly or erratically absorbed from the digestive tract(G.D. Anderson and Russell P. Saneto 2012).

1.2 Injection Routes: Administration by injection (parenteral administration) includes the subcutaneous, intramuscular, intravenous, and intrathecal routes. These routes are characterized by a low patient compliance due to the use of needles and the consequent discomfort caused by the injections.

The *subcutaneous route* is used for many protein and peptidic drugs, for example insulin and heparin,that can be destroyed in the digestive tract if they aretaken orally. In this case, drug is administered through a needle, inserted into the fatty tissue beneath the skin. After the injection, drug moves directly into small blood vessels (capillaries) and carried away by the bloodstream or reaches the bloodstream through the lymphatic vessels. Certain drugs may be given by inserting plastic capsules under the skin (implantation), thus providing a long-term therapeutic effect (for example, etonogestrel that is implanted for contraception may last up to 3 years).

For the *intramuscular route*drugs are usually injected into the muscle of the upper arm, thigh, or buttock. This route is preferred to the subcutaneous route when larger volumes of a drug product are needed or when it is necessary to inject aqueous or oily solutions. Generally,a longer needle is used because the muscles lie below the skin and fatty tissues. Drug absorption depends primarily on the tissue vascularization. Recently, delayed preparations that gradually release forever one or many weeks the drugs from the anatomic site of injection into the circulation,have beenproduced. The intramuscular injection should not be made in a vessel, or in contact with a nerve. It is contra-indicated if the patient is undergoing anticoagulant therapy.

For the *intravenous route*, a needle is inserted directly into a vein. A solution containing the drug may be given in a single dose or by continuous infusion, that is moved by gravity (from a collapsible plastic bag) or, more commonly, by an infusion pump through

thin flexible tubing to a tube (catheter) inserted in a vein, usually in the forearm. With an intravenous administration it is possible to deliver a precise dose quickly and in a well-controlled manner throughout the body. It is also used for irritating solutions, which would cause pain and damage tissues if given by subcutaneous or intramuscular injection. It is very important to control the speed of administration to avoid the risks of severe reactions or to ensure the effective therapeutic concentration. Moreover, the intravenous injection can be more difficult in obese person and in patient that refuses the needle. When given intravenously, a drug, delivered immediately to the bloodstream, tends to take effect more quickly than when given by any other route.

For the *intrathecal route*, a needle is inserted between two vertebrae in the lower spine and into the space around the spinal cord. The drug is then injected into the spinal canal to produce rapid or local effects on the brain, spinal cord, or the layers of tissue covering them (meninges)-for example, to treat infections of these structures. A small amount of local anesthetic is often used to numb the injection site. Anesthetics and analgesics (such as morphine) are sometimes given by this way.

1.3 Cutaneous Route. This route is often used to treat some superficial skin disorders, such as psoriasis, eczema, skin infections (viral, bacterial, and fungal), itching and dry skin.

1.4 Transdermal Route: Some drugs are delivered bodywide through a patch on the skin without any injection. Through a patch, the drug can be delivered slowly and continuously for many hours or days or even longer, thus maintaining the levels of a drug in the blood relatively constant. Patches are particularly useful for drugs that are quickly eliminated from the body. However, patches may irritate the skin of some people and are limited by how quickly the drug can penetrate the skin.

2. TRANSMUCOSAL ROUTES

Transmucosal routes involve the delivery of the drug through the mucosal linings of the nasal, rectal, vaginal, ocular and oral sites.

Traditionally drugs are administered by oral and by parenteral routes. Although generally convenient, both routes have a number of disadvantages. In fact, in the case of oral administration drugs are exposed to the harsh environment of the gastrointestinal tract and they are subject to the chemical and enzymatic degradation. Moreover, after gastrointestinal absorption the drug has to pass the liver, where, dependent on the nature of the drug, extensive first pass metabolism can take place with subsequent rapid clearance from the blood stream (Lalka et al., 1993; Taki et al., 1998). For macromolecular drugs a low permeability across the gastrointestinal mucosa is also often encountered (Yamamoto et al., 2001; Pauletti et al., 1997). Parenteral administration avoids drug degradation in the gastrointestinal tract and hepatic first pass clearance but, due to pain or discomfort during injection, patient compliance is poor, particularly if multiple daily injections are required (Hinchcliffe and Illum, 1999). The injections are responsible of several side effects like tissue necrosis and thrombophlebitis,leading to low patient acceptability (Zhou, 1994).

For these reasons, several mucosal routes,including nasal, buccal, rectal, ocular, pulmonary and vaginal,have been investigated over the last decades as alternatives to oral and parenteral drug administration (Banga and Chien, 1988; Zhou and Li Wan Po, 1991b). Their major advantage is the easy accessibility and circumvention of the hepatic first pass metabolism. In the following, a short overview over the different alternative mucosal drug delivery routes is given.

2.1 Buccal route. The oral cavity is lined by a stratified squamous epithelium that is non-keratinized in the buccal, lingual, and sublingual mucosa regions (Chien, 1995; Hoogstraate and Wertz, 1998). Although non-keratinized, the buccal mucosa contains intercellular lipids which are responsible for its physical barrier properties (Hoogstraate and Wertz, 1998; Shojaei, 1998), resulting in poor permeability for larger drugs, especially for peptides and proteins (Junginger et al., 1999; Veuillez et al., 2001). A more extended discussion on buccal mucosa as administration route will be madein the following chapters.

The sublingual epithelium is more permeable than the buccal one but the presence of saliva and the region motility can remove formulations from the absorption site (Shojaei, 1998). The sublingual route is especially used to obtain a fast and immediate effect because drugs can be adsorbed directly into the small blood vessels that lie beneath the tongue, without first passing through the intestinal wall and liver.

Dosage forms for buccal drug delivery include tablets, patches, films, lozenges, sprays, hydrogels, lollypops, chewing gums, powders, solutions (Hoogstraate and Wertz, 1998) and liposomal formulations (Veuillez et al., 2001). In order to enhance their permeation or to protect drugs from enzymatic degradations, absorption enhancer (Merkle et al., 1992) and proteases (Bird et al., 2001; Veuillez et al., 2001; Walzer et al., 2002) can be used, while the use of bioadhesive formulations can improve the residence time inside the oral cavity (Shojaei, 1998; Veuillez et al., 2001; Langoth et al., 2003).

2.2 Rectal Route. This route is preferable when people cannot swallow or have nausea or restrictions on eating. It is safe and convenient, especially for children, but the acceptance is low particularly among adults. The drugs are readily absorbed thanks the high vascularization of rectum tract and the thin wall. Moreover, the presence of a considerable protease activity and of bacterial flora (Lewin et al., 1986; Hacker et al., 1991; Zhouand Li Wan Po, 1991b) can alter drug activity and additionally, the circumvention of the hepatic first pass metabolismby rectal administration is only partial and depends on the positioning and/or spreading ofthe drug formulation (de Boer and Breimer, 1997; Kurosawa et al., 1998).Generally, drugs are administered with a substances that dissolve or liquefy after it is inserted into the rectum (Lejus et al., 1997; Jensen and Matsson, 2002). Traditional rectal dosage forms are suppositories, unguents and creams, as well as enemas.More recent studies have evaluated thermogelling dosage forms (Miyazaki et al., 1998), gels(de Leede et al., 1986), osmotic mini pumps (Teunissen et al., 1985) and hard gelatincapsules (Eerikainen et al., 1996) as rectal drug delivery systems.

2.3 Vaginal Route. This route can be used for local delivery of some drugs, such as antibacterial, antifungal, antiprotozoal, antiviral, labor-inducing, spermicidal agents, prostaglandins and steroids (Vermani and Garg, 2000) or to obtain a systemic drug absorption. In fact, vagina is characterized by a large surface area with high permeability and a high vascularization, so that can be used for administration of steroids (Ho et al.,

1976; Alvarez et al., 1983). The vaginal route has also the potential for uterine targeting of active agents such as progesterone and danazol (Bulletti et al., 1997; Cicinelli et al., 1998). However, changes of vaginal characteristics with age, stage of menstrual cycle, infections, and sexual arousal (Vermani and Garg, 2000) can influence the drug absorption. Moreover, vaginal flora can potentially contribute to enzymatic drug degradation in addition to the membrane-bound enzymes of the vaginal mucosa (Chien, 1995; Vermani and Garg, 2000). Limitations of systemic vaginal drug delivery next to the physiological barriers are also the gender specificity and the relatively low convenience.Drugs may be administered vaginally to women as creams, gels, tablets, capsules, pessaries, foams, films, tampons, vaginal rings and douches (Vermani and Garg, 2000). A more extended discussion on vaginal mucosa as administration route will be madein the following chapters.

2.4 Ocular Route. This route is typically used for a local drug delivery, especially for the treatment of glaucoma or ocular infection and inflammation. Drugs are mixed with excipients in order to obtain several formulations, such as liquid, gel or ointment. Liquid eye drops are relatively easy to use but may run off the eye too quickly to be absorbed well. Gels and ointment formulations keep the drug in contact with the eye surface longer but they may blur vision. Solid inserts, which release the drug continuously and slowly, are also available, but they may be hard to put in and keep in place. Fluids, introduced into the eye, arerapidly drained from the precorneal area to the nasal cavity and throat; a consequence of this mechanism is the low bioavailability. For this reason, in order to increase drug absorption, inactive substance that increase fluid viscosity or mucoadhesive polymers can be used. Novel formulations include drops, suspensions, oily drops, unguents and mucoadhesive ocular delivery systems such as solutions and microparticle suspensions, in-situ gelling systems triggered by pH, temperature or ions, colloidal delivery systems such as liposomes and nanoparticles and ocular inserts (Le Bourlais et al., 1995). Ocular inserts can be divided into non-erodible (Chetoni et al., 1998; Kawakami et al., 2001) and erodible inserts. Erodible ocular inserts, which do not need to be removed mechanically from the eye, have been prepared by powder compression from poly(ethylene oxide) (Di Colo et al., 2001), from bioadhesive mixtures of poly(ethylene oxide) with chitosan hydrochloride (Di Colo et al., 2002), and from mixtures of Carbopol® 974P with drum dried waxy maize starch (Ceulemans et al., 2001; Weyenberg et al., 2003). Finally, ocular inserts have also been prepared by freeze-drying

aqueous solutions of water soluble polymers such as HPMC resulting in a sponge-like structure (Diestelhorst et al., 1999; Lux et al., 2003).

Recently, this route has been investigated for the systemic delivery of peptides and proteins. Already in 1931, ocular administration of insulin produced sustainedlowering of the blood glucose level in proportion to the dose instilled (Christie and Hanzal,1931). However, systemic drug absorption afterocular instillation takes place across the nasal mucosa after drainage via the nasolachrymalduct (Lee et al., 2002). In addition, drugabsorption via the cornea is relatively low due to the lipophilicity of the corneal epithelium,dilution of the drug in the tear fluid (reflex tearing and reflex blinking) and drug binding toproteins in tear fluid and the presence of enzymes can alter drug activity (Zhou and Li Wan Po,1991b).

2.5 Nasal Route. The nasal route of administration has received a great deal of attention in recent years as a convenient and reliable method not only for local but also for systemic administration of drugs (Schipper et al., 1991; Sakar, 1992; Merkus and Verhoef, 1994; Kublik and Vidgren, 1998; Marttin et al., 1998; Davis, 1999; Hinchcliffe and Illum, 1999; Martini et al., 2000; Chow et al., 2001; Illum, 2003). Drug is absorbed through nasal mucosa, that show a large surface area and a great vascularization, and directly enters the blood stream.The nasal cavity offers a number of unique advantages such as easy accessibility, good permeability especially for lipophilic, low molecular weight drugs, avoidance of harsh environmental conditions and hepatic first pass metabolism, potential direct delivery to the brain. The ciliary movement and the considerable enzyme activity can remove the formulation from the site absorption and can destroy the drug. Nevertheless, a number of approaches have been used to overcomethese limitations such as the use of bioadhesive formulations to increase the nasal residencetime of dosage forms (Morimoto et al., 1991; Soane et al., 2001), addition of absorptionenhancers to increase the membrane permeability (De Ponti, 1991; Merkus et al., 1993; Illum,1999, Natsume et al., 1999), and the use of protease/peptidase inhibitors to avoid enzymaticdegradation of peptide and protein drugs in the nasal cavity (Morimoto et al., 1995; Dondetiet al., 1996). Several nasal dosage forms are under investigation including solutions (drops or sprays), gels, suspensions and emulsions, liposomal preparations, powders and microspheres,as well as inserts. A more extended discussion on nasal mucosa as administration route will be madein the following chapters.

2.6 Inhalation. Usually, this route is used to administer drugs that act specifically on the lungs, such as aerosolized antiasthmatic drugs in metered-dose containers, and to administer gases used for general anesthesia. The interesting properties of the lung, such as the large surface area of about 150 m^2 and an extremely well vascularized thin epithelium, lead many authors to study this route for the systemic drug delivery lung (Qiu et al., 1997; Adjei and Gupta,1998; Edwards et al., 1998).Drugs administered by inhalation through the mouth must be atomized into smaller particles than those administered by the nasal route, so that the drug can pass through the windpipe (trachea) and into the lungs. How deeply into the lungs they go depends on the size of the droplets and smaller droplets go deeper. Moreover, the particle size should be in the aerodynamic diameter window of 0.5 - 5 μm, ideally 2 - 3 μm, for deep lung delivery to avoid loss of delivered particles by impaction onto the mucus lined epithelia. The aerodynamic diameter relates the geometric particle diameter and the particle mass density. Thus, large porous particles are effective means for drug delivery to the alveolar region (Edwards et al., 1997; Vanbever et al., 1999; Crowder et al., 2002). In addition, the high humidity in the airways furthers particle agglomeration, thus decreasing the delivery efficiency due to hygroscopic growth (Malcolmson and Embleton, 1998; Crowder et al., 2002).

Drug administration through this route must be carefully monitored to ensure that a person receives the right amount of drug within a specified time. In addition, specialized equipment may be needed to give the drug by this route.

A number of technologies for the delivery of drug formulationshave been developed (Martini et al., 2000): (i) pressurized metered dose inhalers usingpropellants to deliver micronized drug suspensions (Autohaler®, Spacehaler®), (ii) dry powderinhalers which dispense micronized drug particles with/without carrier (lactose) byinhalation activation (Spinhaler®, Rotohaler®, Diskhaler®), and (iii) nebulizers and aqueousmist inhalers which aerosolize drug solutions using compressed air or ultrasound (AERx®,Respimat®).

A prolonged drug delivery can be obtained using polymeric particle formulations(Kawashima et al., 1999; Zhang et al., 2001) and mucoadhesive formulations (Takeuchi et al.,2001) but the accumulation of polymeric material in the alveoli has to be taken into consideration as well asthe possible delivery related development of fibrosis. Finally, the lungs contain high levels ofhydrolytic and other enzymes, which can become significant absorption barriers to drugs,although the metabolic activity of the lung is much lower than in the gastrointestinal tract (Adjei, 1997).

3. BUCCAL ROUTE

Delivery of drugs to the oral cavityhas attracted particular attention due to its potential for high patientcompliance and unique physiological features.

Within the oral mucosalcavity, the delivery of drugs is classified into two categories: (i) localdelivery,which is drug delivery into the oral cavityand (ii) systemic delivery either via the buccal mucosa,through the mucosal membranes lining the cheeks or sublingualmucosa, through the mucosal membranes lining the floor of the mouth. The sublingual mucosa is more permeable because more thin than the buccal mucosa and because it shows a considerable surface are and a high blood flow. It is used when a rapid onset is desired; in particular, for the treatment of acute disorders. It is not always useful, because the constant washing effect of saliva, the tongue activity and the lack of an expanse immobile mucosa limit the permanence of the dosage form at the administration site.

The buccal mucosa offers many advantages:

    a) a smooth and relatively immobile surface,

    b) suitability for the placement and removal of controlled-release systems,

    c) high patient compliance,

    d) relatively permeable in comparison to the other mucosal tissues,

    e) drugs can be administered to unconscious and trauma patients,

    f) avoidance of first-pass effect that leads to significant reduction in dose.

However, buccal drug delivery shows the following limitations:

    a) drug chemical modification due to saliva enzymes activity,

    b) involuntary swallowing and constant salivary scavenging canresult in drug loss fromthe site ofabsorption,

    c) relatively small absorption area and the barrier property of the buccalmucosa contribute to the inherent limitations of this delivery route,

    d) drug selection for oral transmucosal delivery is limited by thephysico-chemical properties of the drugs themselves and only few milligrams of drug can cross the oral mucosa (Harris and Robinson, 1992; Junginger et al., 1999; N. Salamat-Miller et al., 2005; Shojaei A.H, 1998, Viralkumar F. et al., 2011 and Ghandi et al., 1994).

3.1 Anatomy and nature of oral cavity

The oral cavity may be divided in two regions: the outer oral vestibule, boundedby the lips and cheeks; the oral cavity itself, the border being formed by the hard andsoft palates, the floor of the mouth and tonsils (Shojaei A.H, 1998; Satheesh Madhav et al., 2009; Salamat-Miller et al., 2005 and Ghandi et al., 1994).
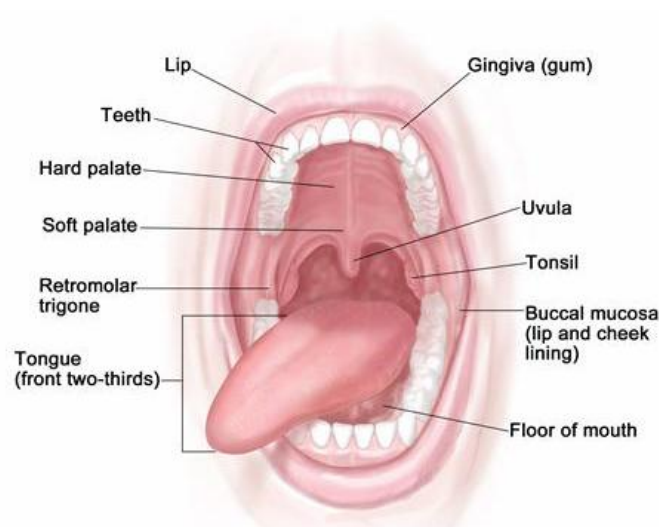
Fig. 3.1 Anatomy of oral cavity.

(http://www.cancer.gov/cancertopics/pdq/treatment/lip-and-oral-cavity/Patient/page1/AllPages)

The oral cavity comprises lips, cheek, tongue, hard palate, soft palate and floor of the mouth (Fig. 3.1) and its lining, referred to as the oral mucosa(including buccal, sublingual, gingival, palatal and labial mucosa).
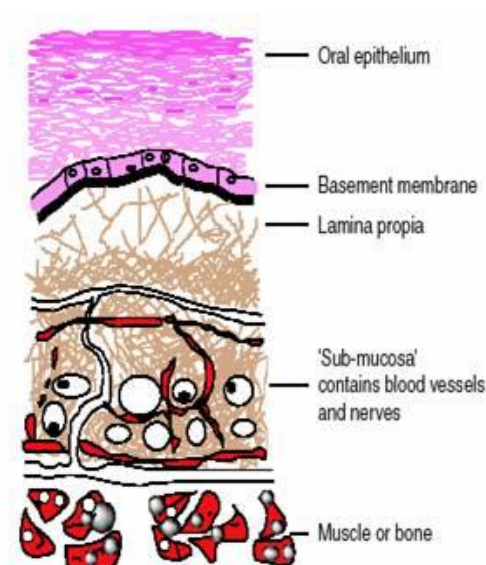
Fig. 3.2 Oral mucosa (Viralkumar F. et al., 2011)

The oral mucosa is made up of a layer of stratified squamous epithelium with closely compacted epithelial cells; their function is to protect the underlying tissue against potential harmful agents in the oral environment and from fluid loss (Fig. 3.2). Beneath the epithelium are the basement membrane, lamina propria and submucosa.

Three types of oral mucosa can be found in the oral cavity: the lining mucosa, the masticatory mucosa and the specialized mucosa approximately 60%, 25% and 15% of the total oral mucosal surface, respectively. The lining mucosa is found in the outer oral vestibule (buccalmucosa) and the sublingual region (floor of the mouth) and it has a non-keratinized epithelium, which sits on a thin and elastic lamina propria and a submucosa. The masticatory mucosa is found on the hard palate (the upper surface of the mouth) and the gingiva (gums) that are particularly susceptible to the stress and it consist of an epithelium with keratinized cells, a thick lamina propria and the underlying periosteum. In the dorsum of the tongue there is a specialized gustatorymucosa, with both keratinized and some non-keratinized cells. The keratinized epithelia contain neutral lipids like ceramides and acylceramides which have been associated with the barrier function. These epithelia are relatively impermeable to water. In contrast, non-keratinized epithelia, such as the floor of the mouth and the buccal epithelia do not contain acylceramides and only have small amounts of ceramide.

The most important sites for drug delivery are the buccal and the sublingual membranes that show a total area of 5.02cm^2 and 26.5cm^2, respectively.

The oral mucosal thickness varies depending on the site: thebuccalmucosameasures at 500-800 μm,while themucosal thickness ofthe hard and soft palates, the floor of the mouth, the ventral tongue andthe gingivae measure at about 100-200 μm.

3.1.1 Composition of Mucus Layer :

The epithelial surface is covered by a thin, continuous layer gel, called mucus that shows a mean thickness from about 50-450 μm(Satheesh Madhav et al., 2009). It is secreted by the goblet cells lining the epithelia or by special excessive glands with mucus cell acni. Mucus is composed of water (95%),glycoproteins,known as mucins,lipids (0.5-3.00%), mineral salts (1%) and free proteins (0.5-1.0%). Mucus protects and hydrates the oral cavity and plays an important role in bioadhesion of mucoadhesive drug delivery systems. At physiological pH, the mucus network carries a negative charge (due to the sialic acid and sulfate residues) and can form a strongly cohesive gel structure with the bioadhesive system.

3.1.2 Salivary secretion :

Saliva protects all tissues of oral cavity fromabrasion by rough materialsand from chemicals,aids the digestion of foods, lubricates the food for mastication and swallowing. Saliva is an aqueous fluid with 1% organic and inorganic materials and is secreted by *perotid, sublingual* and *sub-mandibular*glands. The salivary pH rangesfrom 5.5 to 7 depending on the flow rate. The amount of saliva in the oral cavity is around 1.1 ml, although the daily salivary production is between 0.5 and 2 l .

Saliva provides a water rich environment of the oral cavity where the pharmaceutical formulations must be dissolved to provide drug release. However, the presence of saliva can dilute the drug and remove the formulation form the site absorption before effective absorption occurs through the oral mucosa, thus also reducing the amount of permeated drug.

3.1.3 Blood Supply to Oral Mucosa:

The blood supply to the oral cavity tissue is delivered via the external carotid artery which branches into the maxillary, lingual and facial arteries. Blood from the capillary beds is collected by three main veins that finally flow into the internal jugular vein. Thus delivery of drugs via the oral mucosa drains directly to the *systemic circulation* and the hepatic first passmetabolism is avoided.

3.2 Oral drug delivery

Buccal cavity can be used for local and systemic drug delivery.

Local drug delivery. Buccal local administration is used for the treatment of topical pathologies such oral infections, dental caries, mouth ulcers andstomatitis.

Systemic delivery. Buccal administration is an effective way to systemically delivery of drugs as an alternative to oral and intravascular routes, thanks to interesting buccal mucosa properties.

3.2.1 Mechanism of drug absorption

Drugs can cross the oral mucosa through different routes.One route involves the paracellular pathway(also called extracellular route) and consist in drug passage through the inter-cellular space; while the other route, the transcellular (also called intracellular route), involvesits passage into and across the cell.

The hydrophilic nature of the paracellular spaces and cytoplasmprovides a permeability barrier to lipophilic drugs but can be favorable for hydrophilic drugs. In contrast, the lipophilic cell membrane offers a preferable route for lipophilic drugs compared to hydrophilic compounds (Fig. 3.3).
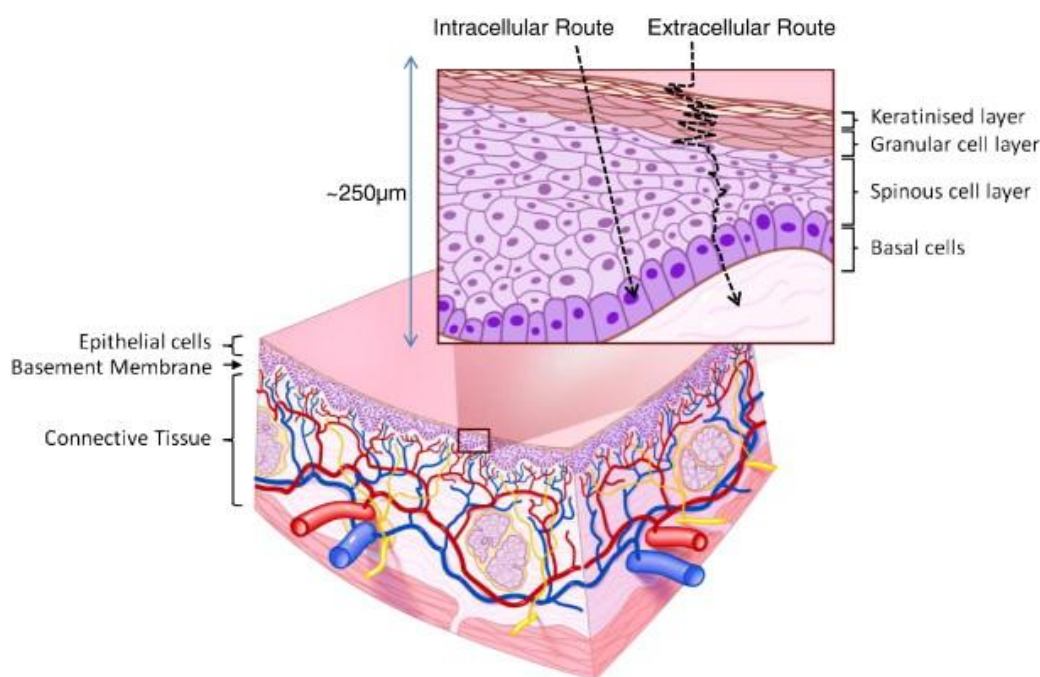
Fig. 3.3 Buccal mucosa and mechanism of drug absorption

3.2.2 Barriers to drug absorption

Drug permeability is influenced by the structure and the function of the several oral cavity regions. In particular, the permeability of the oral mucosa decreases in this order: sublingual greater than buccal and buccal greater than palatal (Satheesh Madhav et al., 2009); this behavior is due to the different mucosa thickness and degree of keratinization. Generally, the permeability decreases with the increase of thickness and keratinization degree. Moreover, the most important barrier to drug absorption is represented by the

intercellular lipid materials derived from the so-called membrane coating granules (MCGs), spherical or oval organelles, 100–300 nm in diameter, that migrate to the apical surface of the cell where their membranes fuse with the cell membranes and the lipid content is extruded in the extracellular space.

The membrane coating granules of keratinized epithelium are composed of lamellar lipid stacks, whereas the non-keratinized epithelium contains membrane coating granules that are non-lamellar. The membranecoating granule lipids of keratinized epithelia include sphingomyelin,glucosylceramides, ceramides and other non-polar lipids. However, fornon-keratinized epithelia, the major membrane coating granule lipidcomponents are cholesterol esters, cholesterol and glycosphingolipids.

3.2.3 Crucial factors for buccal drug delivery

For buccal administration, the design of a dosage form is influenced by several aspects, such as the physical-chemical and organoleptic drug properties, formulation characteristics and physiological conditions(Shojaei A.H, 1998; Satheesh Madhav et al., 2009).

Drug and system characteristics:

Molecular size and weight influence the diffusivity of the drug through theepithelial layer and generally, the larger the molecule the more difficult it is to moveabout, and the lower will be the diffusivity. Hydrophilic drugs andsmall molecules (<75-100 Da) appear to cross oral mucosa rapidly, althoughpermeability falls off rapidly as molecular size increases. The pKa of the drug plays also an important role in its absorption across the lipid membranes of the oral mucosa; in fact, drugs ionized at the oral cavity pH (around 6.8) can difficulty cross the oral mucosa.

The organoleptic properties of a drug and the type of delivery system can influence the patient compliance or the acceptance of the product. The design of a drug delivery system is also influenced by the area surface in contact with the formulation (about 2cm^2); for this reason a drug delivery system for buccal administration, that is easily applied, can deliver only small amount of drug (10-20 mg).Therefore, buccal drug delivery is suitable only for drugs whose daily dose is in the orderof a few mg.

Finally, idealformulation and its degradation products should be non-toxic, nonirritant and free from leachable impurities.

*Physiological conditions.*The formulation of a drug delivery system must take into account that the presence of saliva and the swallowing can remove the formulation from the absorption site; moreover, the buccal mucosa is relatively permeable due to the

presence of MCGs. For this reason, generally mucoadhesive polymers and absorption enhancers can be used in order to increase residence time formulation and drug absorption (Mizrahi and Domb, 2008). Thebioadhesion performance should not be impacted by surroundingenvironmental pH(Sudhakar et al., 2009).

Another barrier to the drug permeability across buccal epithelium is enzymatic degradation due to esterases, carbohydrases and phosphatases contained in saliva.

3.3 Oral transmucosal dosage forms

Several buccal drug delivery devices have been developed by many researchers either for local or systemic actions(Satheesh Madhav et al., 2009 and Viralkumar F. et al., 2011). They are broadly classified into (i) Solid dosage forms (ii) Semi-solid dosage forms (iii) Liquid dosage forms.

3.3.1 Solid buccal adhesive dosage forms

They are dry formulations which achieve bioadhesion via dehydration of the local mucosal surface.

Buccal Tablets and lozenges

Tablets and lozengeshave been the most commonly investigated dosage forms for buccal drug delivery (Llablot at., 2002); these dosage forms, when exposed to saliva rapidly dissolve and the total amount of the drug that can be delivered is limited. For this reason, mucoadhesive polymers can be used in order to increase the residence time of the formulation inside the oral cavity.Buccal mucoadhesive dosage forms can also be categorized into three types on the basis of geometry. Type I is a single layer device with multidirectional drug release that is characterized by a great loss of drug in the oral cavity due to the saliva washing effect and swallowing (Fig. 3.4 a,d). In the type II devices, an impermeable backing layer is superimposed on top of the drug-loaded bioadhesive layer, creating a double-layered device, preventingdrug loss from the top surface of the dosage form into the oral cavity (Fig. 3.4b, c). Type III is a unidirectional release device, from which drug loss is minimal, since the drug is released from the side adjacent to the buccal mucosa(Fig. 3.4e and f). This can be achieved by coating every face of the dosage form, except the one that is in contact with the buccal mucosa(Rossi et al., 2005).The bioadhesive polymers can be incorporated into a matrix containing the active agent and excipients and perhaps a second impermeable layer to allow unidirectional drug delivery.

The most important disadvantage of solid dosage forms is the poor patient compliance especially for children.
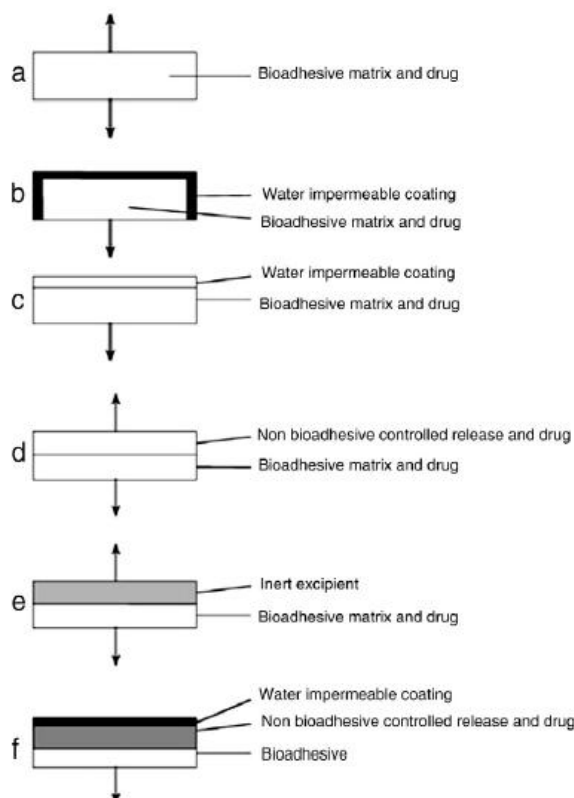
Fig. 3.4 Schematic representation representation of different types of matrix tablets designed for buccaldrug delivery system (Rossi et al., 2005)

Bioadhesive micro/nanoparticles

Recently, different studies have demonstrated the interesting properties of micro and nanoparticulate systems in buccal drug delivery (Kockisch et al., 2004 and 2003; Holpuch et al., 2010).Bioadhesive micro/nanoparticles have the advantage of being relatively small and more likely to be acceptable by the patients but their physical properties enable them to make intimate contact with a lager mucosal surface area. These are typically delivered as an aqueous suspension or are incorporated into a paste or ointment or applied in the form of aerosols

3.3.2 Semi-solid dosage forms

Medicated chewing gums

Chewing gum is one of the modern approaches to oral transmucosal drug delivery and is a useful means for systemic drug delivery. These formulation are convenient and can deliver the drug for an extended period of time, increasing drug bioavailability. Some commercial products are available in the market, such as the caffeine chewing gum (Stay

Alert®) that was developed for alleviation of sleepiness and nicotine chewing gums (e.g., Nicorette® and Nicotinelle®) for smoking cessation.

Adhesive Gels

Various adhesive gels may be used to deliver drugs *via* the buccal mucosa and allow sustained release. They may deliver variable amount of drug in comparison with a unit dosage formand as a result have limited uses for drugs with narrow therapeutic window; another limitation of semisolid dosage forms is the low retention time into the oral cavity. Gel forming bioadhesive polymers has been used to adhere to the mucosal surfaces for extended periods of time and provide controlled release of drug at the site of absorption (Perioli et al., 2008).

3.3.3 Buccal patches/films

Patches are laminates, 1-3 cm^2 in size, consisting of an impermeable backing layer, a drug-containing reservoir layer from which the drug is released in a controlled manner, and a bioadhesive surface for mucosal attachment. Flexible films/patches have been prepared either by solvent casting or hot melt extrusion technique to deliver drugs directly to a mucosal membrane. Compared to semisolid dosage forms, they offer advantages in delivering a measured dose of drug to the site.In general, oral mucosal patches can be classifiedinto three categories: patches with a dissolvable matrix, patches with anon-dissolvable backing and patches with a dissolvable backing.Patches with a dissolvable matrix are designed to release drug into theoral cavity. They work similarly to, and share many of the limitationsof, the solid dose form. The mucoadhesive layer, either in the drugmatrix or attached to drug matrix as an additional layer, prolongs theduration of drug matrix in the oral cavity. Therefore, compared withother open dosage forms, these types of patches are longer acting andcan potentially deliver more drug. Patches with non-dissolvablebacking are usually designed for systemic delivery. Since they areclosed systems and the formulations are protected from saliva, thedrug concentration is controlled and drug is continuously deliveredfor 10 to 15 h (Viralkumar F. et al., 2011). Recently, different authors have investigated buccal patch/film properties for drug delivery and a great attention has been focused on the possibility to deliver drugs through buccal route (Burgalassi et al., 1996; Cheng, et al., 1997 and 1993; Reinhold & Hans, 1989; Peh, et al., 1999; Kohda et al., 1997; Remuñán-et al., 1998and 1996).

3.3.5 Liquid formulations

Liquids have been investigated primarily to coat the mucosaand act as a protectant or a vehicle for drug delivery in the treatment oflocal disorders, including motility dysfunction and fungal infections. The major limitation of these preparations is thelow residence time in buccal cavity due to washing effect of saliva.

4. VAGINAL ROUTE

Vagina represents an interesting site of drug delivery thanks its unique features that can be exploited in order toachieve desirable therapeutic effect (Woolfson, et al., 2000).Traditionally,vaginal route is used mainlyfor the local pharmacological effect and for the treatment of infections pathologies and inflammations. Recently, vagina was also considered tobe an organ capable to ensure a systemic drug absorption thanks to mucous permeability anddense network of blood vessels. Moreover, systemic drug administration can allow to avoid the first pass metabolism, gastrointestinal irritation and side effect at gastrointestinal tract and is characterized by an easily administrationand the possibility to rapidly remove the drug delivery system.

However drug delivery through this route having several disadvantages like

    a) low bioavailability,

    b) gender specificity,

    c) culture sensitivity,

    d) personal hygiene,

    e) local irritation and influence of sexual inter course,

    f) changes of physiological conditionand estrogen concentration .

4.1 Anatomy and physiology of vagina

Vagina is an important organ of the reproductive tract with a major role in reproduction.

As reported in literature(Alamdar H. and Fakhrul A, 2005;Richardson et al., 1992;J. Paavonen, 1983), vagina is a slightly S-shaped fibromuscular collapsible tubes between 6 and 10 cm long extending from cervix of the uterus to thevestibule.It presents two distinctpositions; a lower convex portion and a wider upperportion that lies in an almost horizontal plane atstanding position of subject. The angle betweenupper and lower axes is about 130 degree. As the vagina enters the pelvis, it passes through two diaphragms: the urogenital and the pelvic diaphragms. The women ofreproductive age having numerous folds in vagina,named "rugae", which provide distensibility, supportas well as increase surface area of vaginal wall (Fig. 4.1).
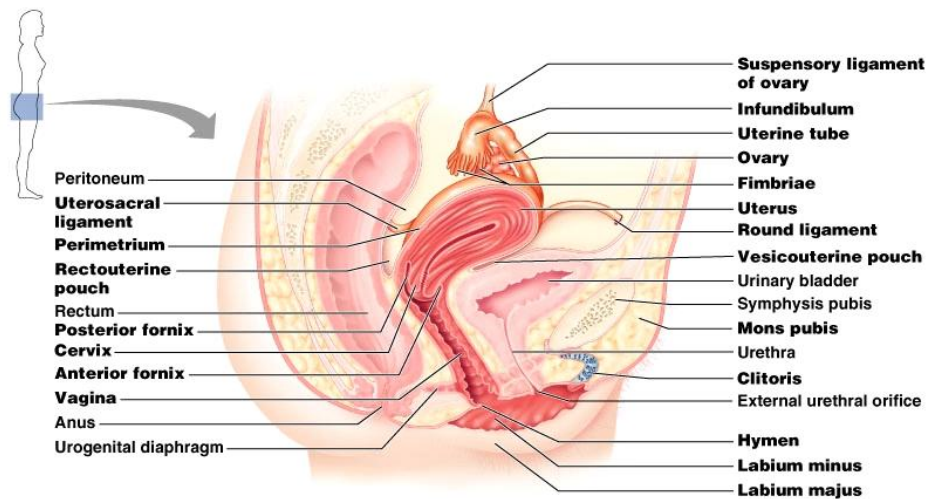
Fig. 4.1 Vagina anatomy

The vaginal histology is mainly consisting of four distinct layers. The superficial layer consists of anon-secretory stratified squamous epithelium and protects vaginal cavity from organisms that can invade or access the basement membrane/capillary bed; its thickness varies with age, menstrual cycles and several hormonal activities. The next is lamina propria or tunica, made of collagen and elastin, which contains a rich supply of vascular and lymphatic channels. The muscular layer is based on smooth muscle fibers running in both circular and longitudinal directions. The final layer consists of areolar connective tissue and a large plexus of blood vessels. An estimated cell turnover of vagina is about 10-15 layer in order of 7 days.

Vaginal tissue does not contain fat cells, glands or hair follicles, but it secrets a large amount of fluid containing transudates through the epithelium, cervical mucus, exfoliating epithelial cells, secretions of the Bartholin's and Skene's glands, leukocytes, endometrial and tubal fluids. The cervical mucus contains inorganic and organic salts, mucins, proteins, carbohydrates, urea and fatty acids (lactic and acetic acids). Vaginal secretions are influenced by the estrogens amount, menstrual cycle and the sexual stimulation; generally, they increase with the increase of hormones and sexual activity. Women of reproductive age produce fluid at a rate of 3-4 g/4 h, while the discharge produced by postmenopausal women is reduced by 50%. Lactic acid, present in the cervical mucus, is produced from glycogen by the *Lactobacillus acidophilus*and is responsible component able to maintain the vaginal pH between 3.8 and 4.8. Moreover, the vaginal pH is influenced by menstruation and acts of coitus, so that menstrual cycle

and sexual activity, as well as the high activity of enzymes, can potentially affect short- and long-term stability of intravaginal delivery systems and devices.

4.1.1 Innervation and blood supply.

Vagina is mainly consisting of two type of nervesupply. The peripheral nerve is the most important and supply to the lower quarter of the vagina making it ahighly sensible area. The other one is an autonomic fiber that isnot very sensitive topain or temperature. In addition, there are few sensory fibers in the upper vagina, making it a relatively insensitive area. For this reason, women rarely feel localized sensations or any discomfort when using vaginal products such as tampons, suppositories or vaginal rings. Moreover, the presence of smooth elastic fibers in the muscular coat vagina makes it a site with an excellent elasticity.

As regard the vascular supply of vagina, the uterine artery, the pudendal artery and the middle and inferior hemorrhoidal arteries cover the vagina. The vaginal, uterine, vescical, and rectosigmoid veins from the middle and upper vagina provide drainage to the inferior vena cava, which bypasses the hepatic portal system. In this way, drugs absorbed from the vagina do not undergo first-pass metabolism because blood leaving the vagina enters the peripheral circulation via a rich venous plexus, which empties primarily into the internal iliac veins. Moreover, the presence of an extensive vascular connections between the vagina and uterus allows to hypotize a "first uterine pass effect" when hormones are administered vaginally (Bulletti et al., 1997; Cicinelli et al, 1998).

4.1.2 Vaginal pH

The vaginal pH of healthy women of reproductiveage is acidic (pH 3.8-4.8); this value is maintainedby lactobacilli that convert glycogen ofexfoliated epithelial cells into lactic acid(Richardson et al., 1992). The pHchanges with age, stages of menstrual cycle, infectionsand sexual arousal. Menstrual, cervicaland uterine secretions, and semen act as alkalizingagents and increase pHto levels, where protective lactobacilli cannot survive (Sjorberg, et al., 1988).Female hygiene products and douches wash away a variety of the vaginal defenses and can promote colonization of bacteria or alter vaginal pH, allowing pathogenic bacteria and yeast to proliferate. Tampons or any absorbent material become media for bacterial colonization and growth.

4.1.3 Microflora

The vaginal flora is a dynamic system and is influencedby factors such as the glycogen contentof epithelial cells, glucose, pH, hormonal levels,trauma during sexual intercourse, birth-controlmethod, age and antimicrobial treatment.Lactobacillus is the mostprevalent organism in the vaginal environmenttogether with many other facultative and obligateaerobes and anaerobes; they are beneficial for vaginal health becausethey compete with exogenous microbes for nutrients. Moreover, they produce lactic acid and hydrogen peroxide from glycogen thus maintaining pH to acidic values thus protecting vagina from bacteria and yeast proliferation. In fact, hydrogen peroxide is toxic to othermicroorganisms that produce little or no hydrogen peroxide and its production make the environment less hospitable toother microbes such as *Escherichia coli* (*E. coli*), Group B*Streptococcus* and even human immunodeficiency virus(HIV).

The vaginal microflora alteration leads to a decrease of hydrogen peroxide and to an increase of bacterial vaginosis or candidosis onset. Generally, the alteration of microflora depends on the estrogen production, sexual activity and hygiene products use. For example, high levels of estrogen during pregnancy result in athick epithelium, high levels of lactobacilli and a low pH.

4.2 Vaginal drug delivery

The vagina has been studied as a favorable site for the local andsystemic delivery of drugs, specifically for female-related conditions.

4.2.1 Local delivery. Traditionally, the vaginal cavity has been used for the delivery of locally acting drugs such as antibacterial, antifungal, antiprotozoal, antiviral, labor-inducing and spermicidal agents, prostaglandins and steroids. In particular, local drug delivery was used for the treatment of vaginal candidiasis that is the most common gynaecological infections, caused by the presence of*Candida albicans* (Nyririesy et al.,2001; Sobel, 1988; Das Neves et al, 2008) and for the treatment of aerobic vaginitis that is accompanied by the presence of enteric commensals or pathogens,especially *Escherichia coli* and *Streptococcus galactiae* (Donders et al., 2002; Donders etal., 2011). Several drug delivery systems were studied for local drug delivery (Dobaria et al.m 2009, Kast et al., 2002; Shaaban et al., 2011; Garg et al., 2010; Furneri et al., 2008).In this thesis project, a pharmaceutical formulation for local drug delivery was formulated and more details will be discuss in the experimental section.

4.2.2 Systemic drug delivery. Becauseof its large surface area, rich blood supply and permeabilityto a wide range of compounds including peptides andproteins, the vagina has great potential alsofor systemic delivery (Benziger and Edelson, 1983). It offers a favorable alternative to the parenteralroute for some drugs such as bromocriptine (Varmesh et al., 1988), propranolol (Patel et al., 1984), oxytocin, calcitonin (Sayani and Chien, 1996), human growth hormone (Dezarnaulds and Fraser, 2003),insulin (Ning et al., 2005).However, despite all these advantages,the vagina has not been extensively explored for systemicdelivery because of gender specificity and cyclic variations.

The vaginal route has also potential for the uterine targeting ofactive agents such as progesterone and danazol (Cicinellli et al., 1998).

4.2.3 Mechanism of drug absorption

The drug transport across vaginal membrane mainly takes place by three major ways: transcellularly, via concentration dependent diffusion through the cells; paracellularly, mediated via tight junctions; vesicular or receptor mediated transport(Alamdar H. and Fakhrul A, 2005).

Drug absorption from vaginaldelivery systemtakes place in two mainsteps: drug dissolution in vaginal lumen and membranepenetration (in the case of systemic absorption). Any factors related to physiology orformulation that affects the above mentioned steps canpotentially alter the release and the absorption profile. Some of the factors which influence the drugabsorption are discuss in the following portion.

4.2.4 Factors Affecting Vaginal Absorption of Drugs

Physico-chemical Factors

The physico-chemical properties of drugs and polymers like lipophilicity, ionization, molecular weight, surface charge and chemical nature can influence the vaginal drug absorption.

Physiological Factors

Physiological factors like changes in thethickness of epithelium layer, cyclic changes, hormones, volume of vaginalfluid, alteration of vaginal pH and sexual arousal, asdescribe earlier, can potentially affect drug release fromany intravaginal delivery system and also alter its rateof absorption(Alamdar H. and Fakhrul A, 2005). The high volume of vaginalfluid may remove drug from the vaginal cavitywithsubsequent reduction of drug absorption. Changes in the pH ofvagina canalter degree of ionization of weakelectrolytic drugs and affect the release profile of pHsensitive drugs.

4.3 Vaginal dosage forms

For local action, a vaginal drug delivery system should distribute uniformly throughout the vaginal cavity. Several delivery systems can be used for local treatment; in particular, dosage forms can be classified into solid, semisolid and liquid dosage forms. Recently, new formulations such as vaginal rings have been studied especially for a systemic drug absorption.

4.3.1Tablets and Suppositories

A large number of intravaginal delivery systems are also available in the form of tablets and suppositories. Vaginal tablets contain components as like conventional oral tablets and are easy to manufacture and insert. Generally, mucoadhesive polymers are sometimes used in order to increase the vaginal residence time. These formulations can be used for local delivery of drug such as miconazole for vaginal candiasis and progesterone for hormonal replacementtherapy (Vukovic et al., 1977; Cicinelli et al, 1988). Inserts can be also used for vaginal drug delivery, thanks to their interesting properties such as the unique dosage form and the accurate dosage (Luppi et al., 2010a; Rabl et al., 2002).

4.3.2 Creams and Gels

They aremainly used for topical delivery of contraceptives andantibacterial drugs. These delivery systems are messyto use, uncomfortable, may not provide an exact dosebecause of non-uniformity and leakage. The desirable properties of vaginally administered creams or gels are acceptability and feasibility. They must be non-toxic and non irritating to the mucus membrane.

Several studies investigated creams and gels for local drug delivery (DuBouchel et al., 1998; Cruz and Uckun, 2001; Shettly et al., 2001; Rabl et al., 2002). Most of these conventional vaginal formulationsare associated with several disadvantages such as the low retention to the vaginal epithelium, leakage and messiness and poor patient compliance.

4.3.3 Vaginal Ring

Vaginal rings are circular ring type drug deliverydevices designed to release drug in a controlled releasefashion after insertion in the vagina(Alamdar H. and Fakhrul A, 2005). This type ofdosage forms can becontrolled by the user, does not interfere with coitusand allows continuous delivery of microbicidalcompounds. They are 5.5 cm in diameter with acircular cross section diameter of 4-9 mm. Drugs can be homogeneously dispersed, only on the surface ring or in the total area of the system in order to obtain a faster and

immediate release or a controlled drug release over time. Moreover, a vaginal ring sandwich or reservoir types of system have been developed in order to obtain a constant release of drug. Sandwich type devices consistof a narrow drug containing layer located below thesurface of the ring and positioned between a nonmedicatedcentral core and a non-medicated outerband. In reservoir type of rings, drugs are dispersed ina central core, which is than encapsulated by a drugfree layer. In a single ring, it is possible to have several cores of different drugs and thereby allowing administration of several drugs from the same device. Rate of drug release can be modified by changing the core diameter or thickness of the non-medicated coating. Several rings havebeen studied in recent years with different polymers such as ethylene vinyl acetate that provides increased flexibility, improved optical properties, greater adhesion and increased impact and puncture resistance (Roumen and Dieben, 1999; Laarhoven et al., 2002; Harwood and Mishell, 2001;Ballagh,2001).

Vaginal rings are mainly used forcontraceptive and hormonal replacement therapy. Formost contraceptive application the ring is placed invagina for 21 days followed by a week of ring freeperiod. Nuvaring is one of the example ofcontraceptive ring available in US market(Nova ́k et al., 2003).

5.NASAL ROUTE

5.1 Introduction

The nose as drug delivery site has a number of unique features related to its anatomy andphysiology. Conventionally, the nasal route has been used for local delivery of drugs for treating nasal allergy, nasal congestion or nasal infections. However, the interesting properties of nasal mucosa, such as the large surface area, the avoidance of the first pass effect, the high permeability and vascularization lead to take into account the nasal route as a potential route for the systemic drug delivery(Hinchcliffe and Illum, 1999). Moreover, the concentration-time profiles achieved after nasal administration are often similar to those after intravenous administration, resulting in a rapid onset of pharmacological activity.

The advantages of nasal drug delivery can be summarized as follow:

a) large nasal mucosal surface area for drug absorption,

b) rapid drug absorption via highly vascularized mucosa,

c) rapid onset of action,

d) ease of administration,

e) avoidance of the gastrointestinal tract and first pass metabolism,

f) possible transport directly into systemic circulation and CNS,

g) lower dose/reduced side effects,

h) improved convenience and compliance,

i) self-administration.

Moreover, nasal drug delivery shows the following limitations:

a) volume that can be delivered into nasal cavity is restricted to 25-200 μl,

b) not feasible for high molecular weight more than 1kDa,

c) adversely affected by pathological conditions,

d) ciliary movement and enzymatic inhibition can limit drug permeability,

e) nasal irritants drugs cannot be administered through this route.

The following sections will therefore give an introduction to the anatomyand physiology of the human nose.

5.2 Anatomy and physiology of nose

Human nasal cavity fills the space between the base of the skull and the roof of the mouth; above, it is supported by the ethmoid bones and, laterally, by the ethmoid, maxillary and inferior conchae bones. The lateral walls of the nasal cavity include a folded structure which enlarges the surface area in the nose to about 150 cm^2. This folded structure includes three turbinates, the superior, the median and the inferior (Gizurarson, 1990; Illum, 1996). The human nasal cavity is divided by middle (or nasal) septum into two symmetrical halves, each one opening at the face through nostrils and extending posterior to the nasopharynx (Fig. 5.1). Both symmetrical halves consist of four areas (nasal vestibule, atrium, respiratory region and olfactory region) that are distinguished according to their anatomic and histological characteristics (Merkus andVerhoef, 1994Mygind and Dahl, 1998 andMerkus andVerhoef, 1998).
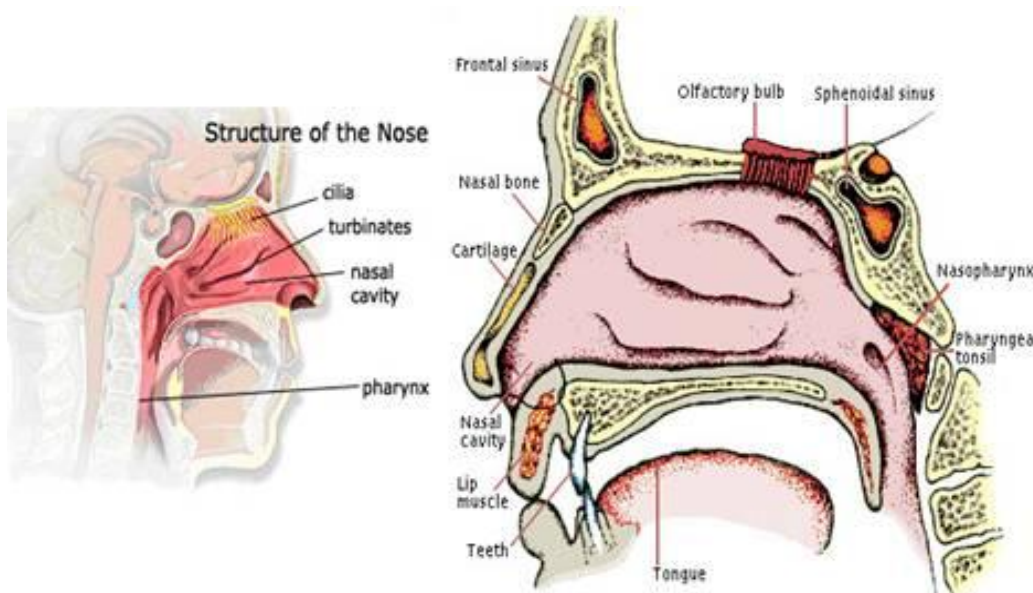
Fig. 5.1 Structure of nose(http://www.pharmainfo.net/pharma-student-magazine/drug-delivery-nasal-route)

Nasal vestibule is the most anterior part of the nasal cavity, just inside the nostrils, covered by a stratified squamous and keratinized epithelium with sebaceous glands. Here, there are nasal hairs, also called vibrissae, which filter the inhaled particles.

Atrium is the intermediate area between nasal vestibule and respiratory region. Its anteriorsection is constituted by a stratified squamous epithelium and the posterior area by pseudostratified columnar cells presenting microvilli.

The Respiratory Region

THEORETICAL SECTION-NASAL ROUTE

The nasal respiratory region, also called conchae, is the largest part of the nasal cavity and it isdivided in superior, middle and inferior turbinates which are projected from the lateral wall. These specialized structures are responsible for humidification and temperature regulation ofinhaled air.

The nasal respiratory mucosa (Merkus and Verhoef, 1994, Mygind and Dahl, 1998), considered the most important section for delivering drugssystemically, is constituted by the epithelium, basement membrane and lamina propria (Fig. 5.2).
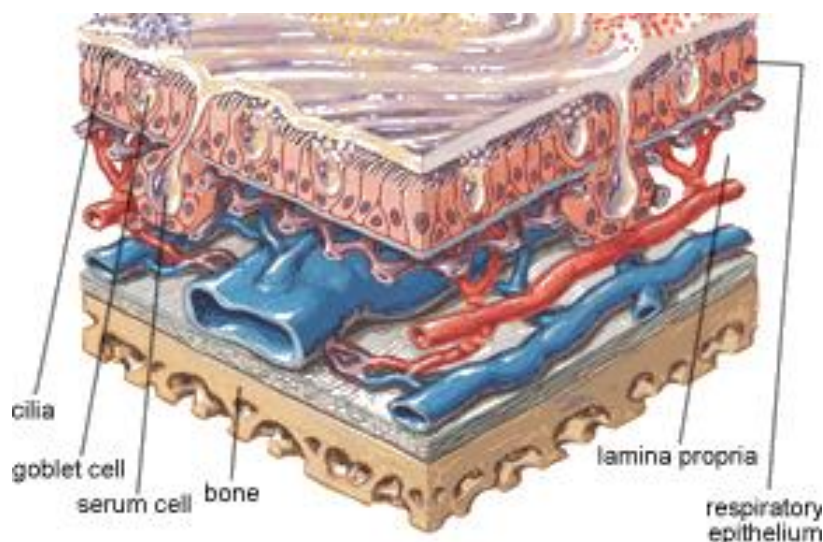
Fig. 5.2 Representation of nasal mucosa. (http://www.nozami.com/what.php)

The respiratory epithelium, 100μm in thickness, is composed of four types of cells, namely basal cells, non-ciliated and ciliated columnar cells and goblet cells.

Basal cells, which are progenitors of the other cell types, lie on the basement membrane and do not reach the airway lumen.

All columnar cells are covered on their apical surface with 300 microvilli, expansions that enhance the respiratory surface area, thus promoting exchange processes across the epithelium and that prevent drying surface, retaining moisture. Columnar cells are divided intociliated and non-ciliated cells. The ciliated cells are covered by fine projections, called cilia, 0.2 - 0.3 μm wide and 5 μm in length (100-300 cilia for every ciliated cells). Below the respiratory epithelium is a thick lamina propria, composed of a loose mesh of fibroelastic tissue with many blood vessels, nerves and glands. These submucosal glands possess both serous and mucous secretory cells and release directly onto the surface of the epithelium.

The globet cells secrete granules filled with mucin, a glycoprotein that determine the viscosity of the mucus. Mucus (or nasal secretion) is a complex mixture of materials, consisting of approximately 95% water, 2% mucin, 1% salts, 1% of other proteins such as albumin, immunoglobulins, lysozymes, lactoferrin and <1% lipids (Kaliner et al., 1984). Mucus is present in two layers (5 µm in thickness) on the epithelium, a viscous and dense gel layer, the 'mucus blanket' (gel layer, 2-4 mm thickness) that floats on the serous fluid layer ('sol layer', 3-5 mm thickness). The viscous gel layer is moved along by the hook shaped cilia termini during the energy dependent 'effective stroke' phase of the ciliary motion. Cilia are up to 7mm in length when fully extended but can fold to half this length during the recovery stroke where the hook terminus detaches from the gel layer and moves immersed in the sol layer in the opposite direction to the gel layer movement. The cilia of the columnar cells move with regular and symmetric beats at a frequency of about 10 Hz in the lower sol phase (Sleigh et al., 1988, Duchateau et al., 1985).Hence the mucus moves only in one direction from the anterior to the posterior part of the nasal cavity to the nasopharynx (Fig. 5.3).

The velocity of mucous transport is approximately 5- 8 mm/min (Procter et al., 1973; Andersen and Procter, 1983), thus renewing the nasal mucus layer every 10 - 20 min. The combined action of mucus layer and cilia is called mucociliary clearance, that is an important nonspecific physiological defense mechanism of the respiratory tract to protect the body against noxious inhaled materials. On the other hand, the mucociliary clearance is responsible for the generally observed rapid clearance of nasally administered drugs from the nasal cavity to the nasopharynx. To overcome the rapid removal of nasally administered drugs, the concept of bioadhesion can be applied. Thebaseline pH in the human nasal cavity is approximately 6.3, ranging from 5.2 - 8.1(Washington et al., 2000a).
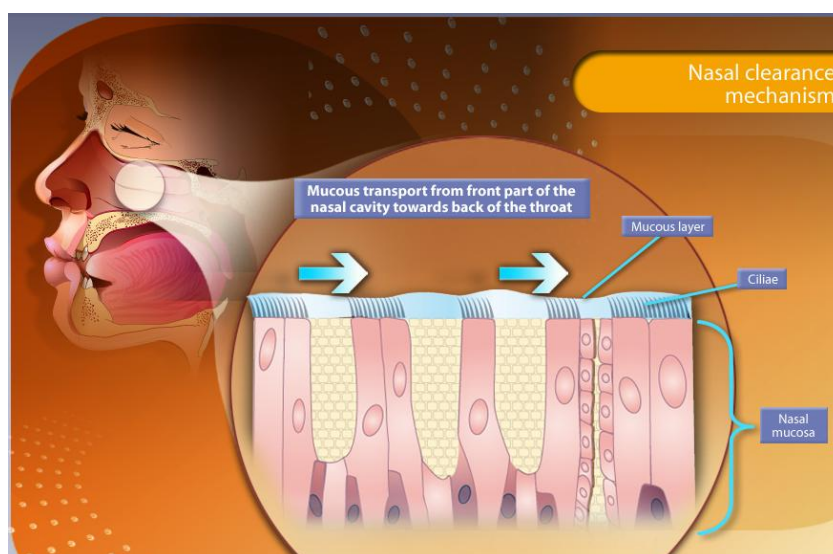
Fig. 5.3 Nasal clearance mechanism

(http://wwwtest.valois.com/pharma/en/publication/illustration.php)

*5.2.1 Vasculature and innervation***.** The lamina propria under the nasal epithelium and the basement membrane are rich in blood vessels and have an extensive blood supply (about 40 ml/min/100g, Bende et al., 1983) as well as a large lymph drainage system, particularly inthe respiratory region of the nasal cavity (Hinchcliffe and Illum, 1999). Three types of blood vesselscan be distinguished(Mygind and Dahl,1998):

(i)   Cavernous venous sinusoids are specialized vessels adapted to the functional demandsof the nose with respect to heating and humidification of inhaledair.

(ii)   Arterio-venous anastomoses allow the blood to bypass the capillaries. Their role is probably related to the temperature and water control. At least 50% of the blood flow is normally shunted through arterio-venous anastomoses (Anggard, 1974).

(iii)   Nasal vasculature shows cyclic changes of congestion (nasal cycle, every 3- 7 h). Different to the gastrointestinal tract, the venous blood draining from the nose passes directlyinto the systemic circulation, thereby circumventing hepatic first pass elimination. The lamina propria of the nasal mucosa embeds also nerves. Afferent nerve fibers run in thetrigeminal nerve. Stimulation of the trigeminus in the nasal mucosa results in the sneezing reflex (Faller, 1988). There is a rich parasympathetic innervation of the glands. Nervous stimulation of the glandular cholinoceptors causes marked hypersecretion and is often part ofthe reflex arc. Nasal blood vessels are both sympathetically and parasympathetically innervated, but are mainly controlled by sympathetic fibers (Mygind and Dahl, 1998).

*5.2.2 The Olfactory Region***.** It islocated on the roof of the nasal cavities, just below the cribriform plate of the ethmoid bone, which separates the nasal cavities from the cranial cavity. The olfactory epithelial layer predominantly contains three cell types: the olfactory neural cells, the subtentacular (also known as supporting) cells and the basal cellsthat are progenitor cells (of supporting cells) and also provide mechanical support via anchorage to other cells. The olfactory epithelium is a gateway for the non-invasive delivery of therapeutic agents to CNS thanks to a neuronal connection between the nasal mucosa and brain, thus providing a provide a unique pathway. The intraneuronal pathway involves axonal transport and require to hours or days for drugs to reach different brain regions, while the extraneuronal pathway probably relies on the bulk flow transport through perineural channelsand allows therapeutic agents to reach the CNS within minutes.

In this area there are also small serous glands (glands of Bowman) producers of secretions acting as a solvent for odorous substances (Fig. 5.4).
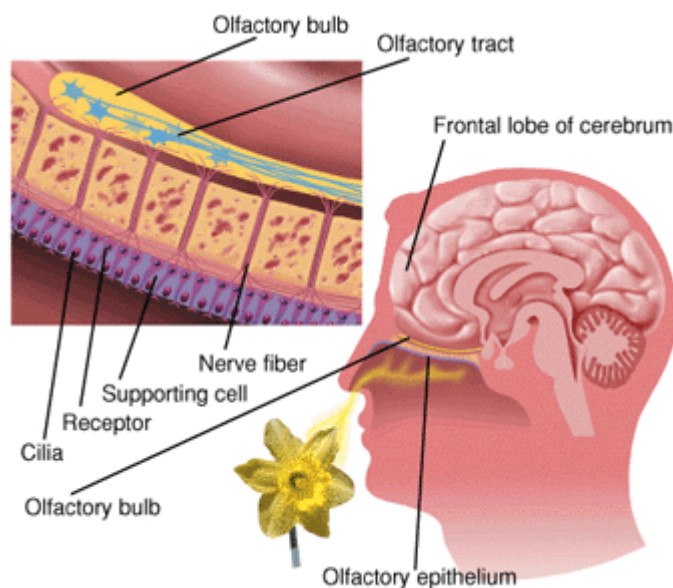
Fig. 5.4 Nose to brain delivery

(http://www.medicalook.com/human_anatomy/organs/Olfactory_sense.html)

5.3 Intranasal drug delivery

In the nasal drug delivery, several factors, such as the nature of pathologic condition (acute or chronic) and intended effects of drug treatment (local, systemic or at CNS), can be taken into account.

5.3.1 Local delivery

Intranasal local administration of medicines is thenatural choice for the treatment of some topical nasal disorders, such as rhinosinusitis. In these cases, intranasalroute is the primary option for drug deliverybecause it allows a rapid symptom relief with amore favorable adverse-event profile than oral or parenteral routes (Salib et al 2003).

5.3.2 Systemic delivery

The intranasal administration is an effective wayto systemically delivery of drugs as an alternativeto oral and intravascular routes. Actually, it seemsto present fast and extended drug absorption and it has been supported by many studiesplanned to compare intranasal drug deliveryagainst oral and parenteral administration. Consequently, the number of drugsadministered as nasal formulations intended toachieve systemic effects has widely increased (Illum 2002; Leonard et al., 2007; Illum 2004; Heidari et al., 2006; Ugwoke et al.,2000; Wang et al., 2006; Yu et al., 2004).

5.3.3 Mechanism of drug absorption through nose

The first step in the absorption of drug from the nasal cavity is passage through the mucus; generally, small unchanged particles easily pass through this layer. Subsequently, there are several mechanisms for absorption through the mucosa (Fig. 5.5). These include transcellular or simple diffusion across the membrane, responsible for the transport of lipophilic drugs, paracellular transport *via* movement between cell and transcytosis by vesicle carriers. Drugs also cross cell membranes by an active transport route *via* carrier-mediated means or transport through the opening of tight junctions (Illum, 2003; Arora et al., 2002).

Fig. 5.5 Drug absorption through nasal mucosa.

(http://wwwtest.valois.com/pharma/en/publication/illustration.php)
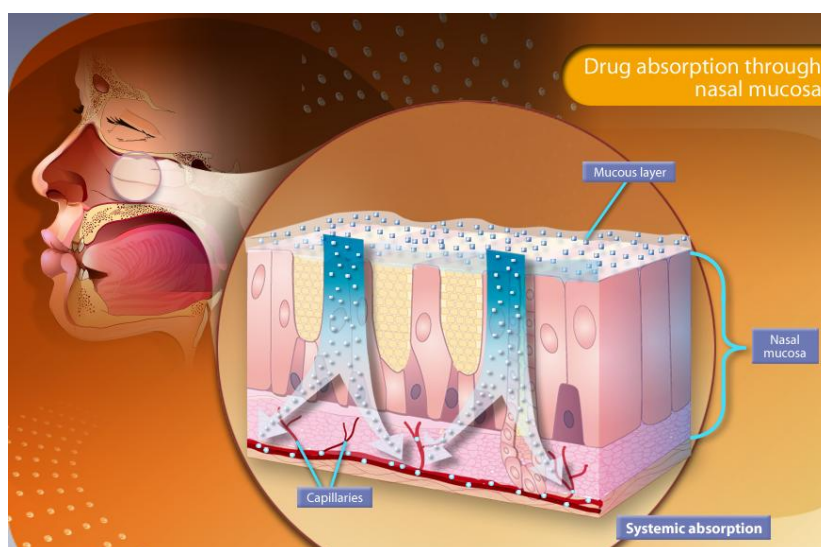
5.3.4Barriers for nasal delivery

*Low bioavailability***.** Bioavailability of polar drugs is generally low, about 10% for low molecular weight drugs and not above 1% for peptides such as calcitonin and insulin due to the low membrane permeability. Polar drugs with molecular weights below 1000 Da generally pass the membrane using the paracellular route. Although tight junctions are dynamic structures and can open and close to a certain degree when needed, the mean size of these channels is of the order of less than 10 Å and the transport of larger molecules is considerably more limited. Larger peptides and proteins are able to pass the nasal membrane using an endocytotic transport process but only in low amounts (Illum, 2003). Nasal absorption of such polar drugs can be greatly improved by co administration of absorption enhancing agents (see chapter 7).

*Mucociliary clearance*The mucociliary clearance can remove formulation form the absorption site, thus decreasing drug bioavailability, especially in the case of liquid formulations or formulations without mucoadhesive polymers. In fact,it has been shown that for both liquids and powder formulations, which are not bioadhesive, the half life for clearance is of the order of 15- 30 min (Pires et al., 2009). For this reason, the use of bioadhesive excipients in the formulations can be an approach to overcome the rapid mucociliary clearance.

*Enzymatic Degradation***.** The enzymatic degradation due to the presencein the lumen of the nasal cavity ofexo-peptidases, such as mono and diaminopeptidases and endo-

peptidases,can degrade drugs leading to their low bioavailability The use of enzyme inhibitors may be an approach to overcome this barrier (Illum, 2033).

5.4 Crucial factors for nasal formulations

Drug absorption through nasal mucosa must take into account the deposition of drug and, consequently, the deposition area, that is mainly dependent on the delivery system and the delivery device. The selection of delivery system depends upon the drug used, therapeutic indication, patient population and, last but not least, marketing preferences. Moreover, some of the physico-chemical characteristics of the drug formulation and physiological factors must be considered prior to designing an intranasal delivery system (Pires et al., 2009).

5.4.1 Physicochemical properties of drugs and delivery systems

Physicochemical properties are one of the important aspects in design of nasal formulation. In particular, increasing drug lipophilicity, the nasal absoption of the compound normally increases. In fact, lipophilic compounds tend to readily cross biological membranes via the transcellular route and diffuse into and traverse the cell in the cell cytoplasm.Unionized forms of drug are well absorbed compared with ionized forms of drug. Moreover, absorption decreases significantly if the molecular weight is greater than 1,000 Daltons except with the use of absorption enhancers (Illum, 2003).

It has been reported that particles greater than 10 μm in size are deposited in the nasal cavity. Particles that are 2 to 10 μm can be retained in the lungs, and particles of less than 1 μm are exhaled.

Additionally, drug delivery systems deposited in the nasal cavity need to dissolve in order to release the drug and to increase drug bioavailability (Pires et al., 2009). Generally,solvents or co-solvents such as glycols or small quantities of alcohol can be used in order to increase drug solubility.

The delivery volume is limited by the size of the nasal cavity. An upper limit of 25 mg/dose and a volume of 0.1-0.2 ml/nostril have been suggested.

Drug formulation should be isotonic in order to avoid cilliary activity inhibition.

Deposition of the formulation in the anterior portion of the nose provides a longer nasal residence time and better absorption, while a rapid elimination of the formulation occurs if the dosage form is deposited in posterior chamber of nasal cavity. The site of deposition is dependent on the delivery device mode of administration andphysico-

chemical properties of drug molecule. Drug absorption depends upon the vasoconstriction and vasodilatation of these blood vessels.

5.4.2 Physiological factors

The absorption of drugs is influenced by the residence time of drug formulation inside the nasal cavity. The mucociliary clearance removes the formulation from the absorption site and generally bioadhesive polymers can be used in the formulation in order to increase the residence time in the nasal cavity.

Several enzymes that are present in the nasal mucosa might affect the stability of drugs. For example, proteins and peptides are subjected to degradation by proteases and amino-peptidase at the mucosal membrane.

Finally,intranasal pathologies such as allergic rhinitis, infections or previous nasal surgery may affect the nasal mucociliary transport process and/or capacity for nasal absorption. During the common cold, the efficiency of an intranasal medication is often compromised. Nasal pathology can also alter mucosal pH and thus affect absorption of drugs.

5.5 Nasal formulations

5.5.1 Liquid dosage forms. Liquid dosage forms either in form of soluble, suspended or colloidal systems are normally used for formulating nasal delivery systems and included drops, sprays, nano and microemulsions and nanoparticles. They are rapidly removed from the nasal cavity due to the clearance mucociliary and are characterized by a lack of dose precision, even if innovative and precise dispositive for the systems deposition are used. Liquid formulation show also a low stability and generally, preservatives must be used.

5.5.2 Semisolid dosage forms. Semi-solid systems, including ointments and liquids containing polymers able to gel at particular pH changes, are usually employed for designing the nasal drug delivery systems. These systemsshow a greater viscosity with respect to liquid formulations and can remain inside the nasal cavity for a greater time.

5.5.3Solid dosage forms

Nasal powders

The advantages of a nasal powder dosage form are the absence of preservative and superior stability of the drug in the formulation. However, the suitability of the powder

formulation is dependent on the solubility, particle size, aerodynamic properties and nasal irritancy of the active drug and/or excipients. An additional advantage of this system is local application of drug, but nasal mucosa irritancy and metered dose delivery are some of the aspects that must be taken into account.

5.5.4 Novel drug formulations

Novel drug formulation for intranasal drug delivery include liposomes, microspheres and nanoparticles. These systems canbe formulated incorporating inside the system enzymatic inhibitors,nasal absorption enhancers or/and mucoadhesivepolymers in order to improve the stability,membrane penetration and retention time in nasalcavity.

Liposomes

Liposomes are phospholipids vesicles composedby lipid bilayers enclosing one or more aqueouscompartments and wherein drugs and othersubstances can be included. Liposomal drugdelivery systems present various advantages suchas the effective encapsulation of small and largemolecules with a wide range of hydrophilicity and pKa values (Law et al., 2001; Wyas et al., 1995).

Microspheres

Microsphere technology has been widely appliedin designing formulations for nasal drug delivery. Microspheres are usuallybased on mucoadhesive polymers (chitosan,alginate), which present advantages for intranasaldrug delivery. Furthermore, microspheres mayalso protect the drug from enzymatic metabolismand sustain drug release, prolonging its effect (Gavini et al., 2006).

Nanoparticles

Recently, much attention has been given tonanotechnology in many areas. Nanoparticle systems are being investigated to improve drugdelivery and intranasal drug administration.Nanoparticles are solid colloidal particles withdiameters ranging from 1-1000 nm. Theyconsist of macromolecular materials in which the active substance isdissolved, entrapped, encapsulated, adsorbed orchemically attach. Nanoparticles may offer several advantages due to their small size,but only the smallest nanoparticles penetrate themucosal membrane by paracellular route and in alimited quantity because the tight junctions are inthe order of 3.9-8.4 Å (Fernandez- Urrusuno et al., 1999a and 1999b).

5.6 Delivery of Peptide and Non-Peptide Drugs for Systemic Effect through Nasal Route

Most peptides and proteins, being hydrophilic polar molecules of relatively high molecular weight, are poorly absorbed across biological membranes with bio-availability obtained in the region of 1–2% when administered as simple solutions. But for certain peptide drugs such as insulin which does not have the luxury of wide therapeutic index it is essential to develop the novel formulation strategies. Different studies have been performed to evaluate the possibility to deliver insulin through nasal route. In particular, several drug delivery systemwere formulated and their delivery properties were evaluated from different point of view (Pillion et al., 1994; Bechgaard et al., 1996; Leary et al., 2005; Leary et al., 2006; Khafagy et al., 2009; Callens et al., 2003).

6. CHITOSAN

6.1 Chitosan and chitin

Chitosan is currently receiving a great deal of attention for medical and pharmaceutical applications due to itsinteresting properties. Indeed, chitosan is known for its biocompatibility allowing its use in various medicalapplications such as topical ocular application (Felt et al., 1999), implantation (Patashnik et al., 1997) or injection (Song et al., 2001). Moreover, chitosan can be considered as biodegradable because it is metabolized by certain human enzymes, e.g. lysozyme (Muzzarelli, 1997; Koga and Chen, 1998) and it can act as a penetration enhancer by opening epithelial tight-junctions (Junginger and Verhoef,1998; Kotze et al., 1998). Due to its positive charges at physiological pH, chitosan is also bioadhesive, thus increasing retention time at the site of application (He et al., 1998; Calvo et al., 1997) and has bacteriostatic effects (Liu et al., 2001). Finally, chitosan is abundantin nature and its production is of low cost and is ecologically interesting (Peter, 1995). In medical and pharmaceutical applications, chitosan is used as a component for the preparation of several drug delivery systems; in particular, our research group have investigated chitosan properties when it is formulated as hydrogels and in different dosage forms (Cerchiara et al., 2003a; 2003b; 2008; Bigucci et al., 2008a; 2008b; Luppi et al.,2009; 2010a; 2010b).

Chitosan (Fig. 6.1) is a polysaccharide formed primarily of repeating units of β-(1→4)-2-amino-2-deoxy-D-glucose (or D-glucosamine).
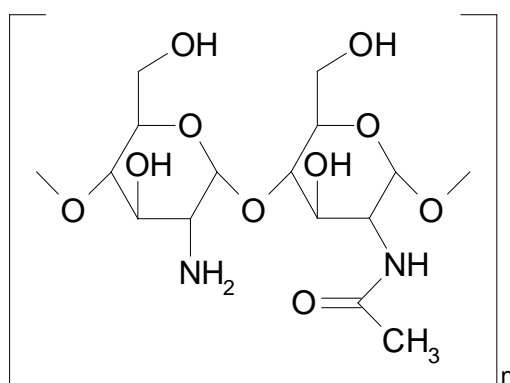
Fig. 6.1 Chitosan structure

Chitosan is the *N*-deacetylated derivative of chitin (Fig. 6.2), the most ubiquitous natural polysaccharide after cellulose,which is the main component of the exoskeleton of crustaceans, such as shrimps (Muzzarelli, 1973).
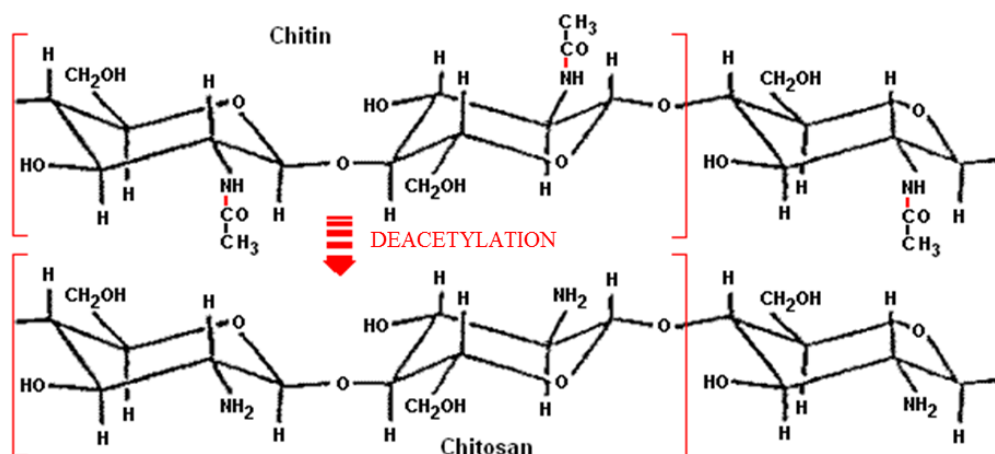
Fig. 6.2 Deacetylation process of chitin to obtain chitosan

Chitin is composed of 2-acetamido-2-deoxy-β-d-glucose through a β (1→4) linkage and can be degraded by chitinase (Dutta et al., 2002). It may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamido group and like cellulose, it functions naturally as a structural polysaccharide. Chitin is a white, hard, inelastic, nitrogenous polysaccharide. Chitin is present as ordered crystalline microfibrils forming a complex structure with proteins, minerals, and lipids and possesses three polymorphic forms (R, β, λ), where R is the most common structure, corresponding to a tightly compacted orthorhombic cell of alternate sheets of parallel and antiparallel chains. Commercial chitin is effectively isolated from crustacean shells after chemical treatments that lead to reduction in chitin molecular weights; but these methodologies do not allow the recovery of products such as protein hydrolyzates and pigments and can generate undesirable corrosive side products. Alternatively, biological approaches for chitin recovery have been proposed and among them, lactic acid fermentations (LAFs) are promising because minerals (calcium carbonate) are solubilized in situ and endogenous proteases are adequately activated for deproteinization.

Generally, alkali are used to simultaneously remove the protein and deacetylates chitin and allow to dissolve calcium carbonate which is present in crab shells in high concentrations. Then, chitin is deacetylated in order to obtain chitosan. Chitosan parameters such as molecular weight (MW) and degree of deacetylation (DD), representing the proportion of deacetylated units, are determined by theconditions selected during its preparation and are responsible of chitosan properties.The conditions employed for deacetylation of chitin, such as temperature, alkali concentration, time and the acidic depolymerisation can decrease the DD (Sorlier et al., 2001) and theMW (Dong

et al., 2001). For example, chitin is deacetylated in 40% sodium hydroxide at 120°C for 1–3 h,leading to 70% deacetylated chitosan.Moreover, chemical deacetylation conducted under heterogeneous conditions at high temperature during a short period of time is faster in amorphous regions, whereas homogeneous deacetylation, at relatively low temperatures and extended time, results in random distribution of deacetylated residues in the polymer backbone. In both cases, high deacetylation can be successfully achieved but with remarkable reduction in molecular weight. It has been suggested that the initial crystalline structure of the chitin is an important parameter during deacetylation and affects final chitosan structure as well as molecular mass and the DA. Therefore, this will influence its solubility, reactivity for chemical modification as well as biological, mechanical and rheological properties.

6.1.1 Chitosan solubilization

The solubilization occurs by protonation of the -NH$_2$ function on the C-2 position of the d-glucosamine repeat unit, whereby the polysaccharide is converted to a polyelectrolyte in acidic media (chitosan pK_b= 6.3; Lee et al., 1999).This characteristic can allow to use chitosan in different applications; in particular, it can be used as flocculants for protein recovery, as components to obtain solutions, gels, or films and fibers.

Chitosan solubility dependson its average DA, molecular weight, distribution of the acetyl groups and on pH and ionic concentration of solution used to chitosan dissolution. Generally, chitosan is characterized by typical degrees of deacetylation between 70 and 95% and molecular weights between 10 and 1,000 kDa and a low molecular weight can allow to obtain a fast chitosan dissolution. Moreover, chitosan can be dissolved in acetic acid or hydrochloric acid, pH and the pK of the acid influencing chitosan solubility. Finally, a salting-out effect was observed in excess of HCl (1 M HCl), making it possible to prepare the chlorhydrate form of chitosan.

6.2 Chitosan hydrogels

Peppas (Peppas, 1986) defined hydrogels as "macromolecular networks swollen in water or biological fluids". Hydrogels are often divided intothree classes depending on the nature of their network,namely entangled networks, covalently crosslinked networksand networks formed by physical interactions. Thelatter class contains all the intermediary cases situatedbetween the two other classes representing the extremes(Ross-Murphy, 1994). However, with respect to chitosan hydrogels, thisclassification is not entirely suitable and it can be suggested the following modified classification for

chitosanhydrogels; i.e. a separation of chemical and physicalhydrogels. Chemical hydrogels are formed by irreversiblecovalent links, as in covalently crosslinked chitosanhydrogels. Hydrogels formed by the addition of acrosslinker, namely covalently and ionically crosslinkedhydrogels are characterized by apotential toxicity of free unreacted covalent crosslinkers thatrequired a purification step during the manufacturing ofhydrogels. For this reason the development of alternative types of hydrogelswas desirable (Berger et al., 2004); in particular,hydrogels formed by directinteraction between polymeric chains without the additionof crosslinkers is advantageous. In physical hydrogels, chitosan can form reversible links such as ionic interactions, as inionically crosslinked hydrogels and polyelectrolyte complexes(PEC), or secondary interactions as in chitosan/polyvinyl alcohol (PVA) complexed hydrogels, graftedchitosan hydrogels and entangled hydrogels. The entangledchitosan hydrogels show a limited use due to their lack ofmechanical strength and their tendency to dissolve.Moreover, they do not exhibit characteristics that allow anefficient control of drug delivery or the modification ofproperties in response to changes in their physico-chemicalenvironment, such as pH or temperature.

6.3 Polyelectrolyte complexed hydrogels

As described before, polyelectrolyte complex networks are formed mixing oppositely charged polyelectrolytesthat can interact in solution thanks to the formation of ionic interactionsand without the use of covalent cross-linkers. The reaction isgenerally performed in aqueous solution, thus favoring biocompatibility and avoiding purificationbefore administration. For this reason, in general, these polymeric networks or hydrogels are well tolerated, biocompatible and are more sensitive to changes in environmental conditions (Long et al., 1996; Wang et al., 1997; Takahashi et al., 1990; Berger et al., 2004). They are characterized by a hydrophilic microenvironment with a high water content and electrical charge density and exhibit interesting swelling characteristics.

The formation and stability of these polyelectrolyte complexes depend on many factors such as the degree of ionization, the density of the charges, the charge distribution of the oppositely charged polyelectrolytes, the concentration of the polyelectrolytes, their mixing ratio, the mixing order, the duration of the interaction, the nature of the ionic groups, the position of the ionic groups on the polymeric chains, the molecular weight of the polyelectrolytes, the polymer chain flexibility as well as the temperature, ionic

strength and pH of the reaction medium. When the polyelectrolyte complex contains equal amounts of each opposite charge, it is usually insoluble and precipitate out of solution upon formation (Berger et al., 2004), due to its zero net charge. On the other hand, when in the mixed solution there is an excess of one charge (either positive or negative), a non-stoichiometric complex is formed that are usually soluble.

Chitosan can easily form polyelectrolyte complexes through the electrostatic attraction between the cationic amino groups on the C2 position of the repeating glucopyranose units of chitosan and the anionic groups of the other polyelectrolyte, even if other secondary binding interactions (Lee et al., 1999) can be occurred. In this way, chitosan that normally is dissolved in the stomach can be used for formulation of new drug delivery systems and it is also possible to maintain its interesting properties of biocompatibility and biodegradability. Generally, the formation of PEC can induce a conformational change of chitosan that has a rigid, stereo-regular structure containing (Berger et al., 2004).

The most commonly used polyanions are polysaccharides bearing carboxylic groups such as alginate (Kim et al., 1999), pectin (Bigucci et al, 2008b; Luppi et al., 2010a; Yao et al., 1997)or xanthan (Dumitriu and Chournet, 2000). Proteins, such as collagen (Taravel and Domard, 1996) and synthetic polymers, such as PAA (Wang et al., 1997) or even DNA (Borchard, 2001)have also been investigated.

In order to form a PEC, the pH solution values must be in the vicinity of the pKa interval ofthe two polymers, thus obtaining a great ionization of the two polymers. During complexation, polyelectrolytes can either coacervate or form a more or less compact hydrogel or precipitate. Precipitation can beavoided if electrostatic attraction is weakened by the addition of salts, such as NaCl, that reducestheattraction between the oppositely charged polyelectrolytes.

6.3.1 Properties and medical applications

As PEC hydrogels are formed by ionic interactions, theyexhibit pH- and, to a minor extent, ion-sensitive swelling. Inaddition, they have a high water content and electricalcharge density and allow the diffusion of water and/or drugmolecules (Berger et al., 2004). Therefore,chitosan hydrogels formed by PEC are well toleratedsystems and can be used in various applicationssuch as drug delivery systems, in cell culture and enzymeimmobilization or for tissue reconstruction and woundhealingmanagement.

PEC exhibit pH-sensitive swelling not onlyin acidic but also in basic conditions. pH of physiological fluids can modify the charge balance inside the gel andtherefore the degree

of interaction between the two polymers and the swelling properties. In particular, in acidic medium, the polyacid is neutralized and due to the free ammonium groups of chitosan, freepositive charges appear inside the gel and the consequent entry of water causes swelling(Fig. 6.3). On the other hand, in basic medium themechanism is the same but swelling is induced by the freenegative charges of the polyacid. If swelling becomestoo important, dissolution of the complex can occur at certainpH values if the global charge density of one of the polymersis no longer sufficiently high to ensure complexation (Berger et al., 2004).
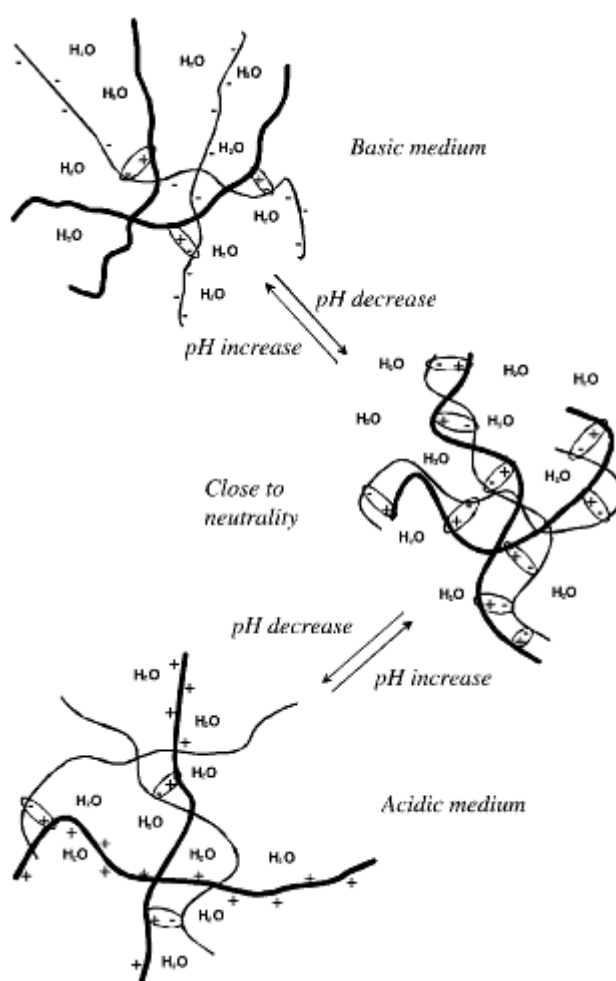
Fig. 6.3 Swelling properties of PEC in mediawith different pH (Berger et al., 2004).

6.4 Polyelectrolyte Complexes Chitosan/gelatin and Chitosan/alginate

As described before, chitosan can ionically interact with different polymers, natural and synthetic. In particular, in this thesis project chitosan/gelatin and chitosan/alginate were taken into account in order to obtain drug delivery system. These complexes were studied in terms of chemical-physical properties and of their ability to control drug release.

6.4.1 Chitosan-gelatin polyelectrolyte complex

Gelatin is a heterogeneous mixture of protein fractions consisting of single or multi-stranded polypeptides (Fig. 6.4), obtained by partial hydrolysis of animal collagen derived from skin, white connective tissues and bones. In nature there are two types of gelatin: type A gelatin, derived from pig skin by means of acid hydrolysis and type B gelatin, obtained from alkaline hydrolysis of cattle hides and bones [49]. The isoelectric point of gelatin is around a pH value of 4.7 and the formation of polyelectrolyte complexes is obtained in pH interval between this value and 6.3 that correspond to chitosan pKb (Yin et al., 2005). Chitosan-gelatin polyelectrolyte complex was used to obtain sponges containing tramadol hydrochloride; these formulations allow to obtain a control of drug release which followed Higuchi's diffusion mechanism over a 12 h periodand showed improved mechanical properties compared to sponges containing chitosan alone (Foda et al., 2007).
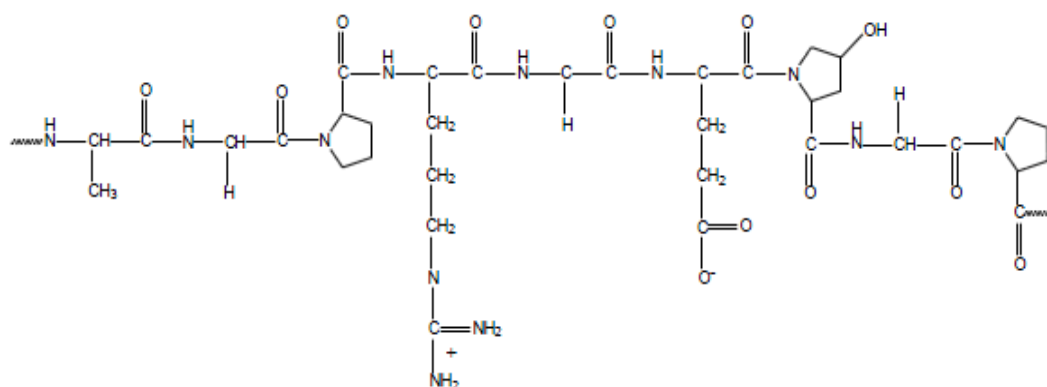
Fig. 6.4 Gelatin structure.

6.4.2 Chitosan-alginate polyelectrolyte complex

Alginates are natural polysaccharide polymers isolated from brown seaweed such as *Laminaria hyperborea*, *Ascophyllum nodosum* and *Macrocystis pyrifera*(Oliveira et al., 2009; Sankalia et al., 2007). The seaweed is extracted with a dilute alkalinesolution which solubilizes the alginic acid.Free alginic acid is obtained on treatment ofthe resulting thick and viscous mass with mineral acids and then converted to asalt, such as sodium alginate, that is the major form currentlyused.
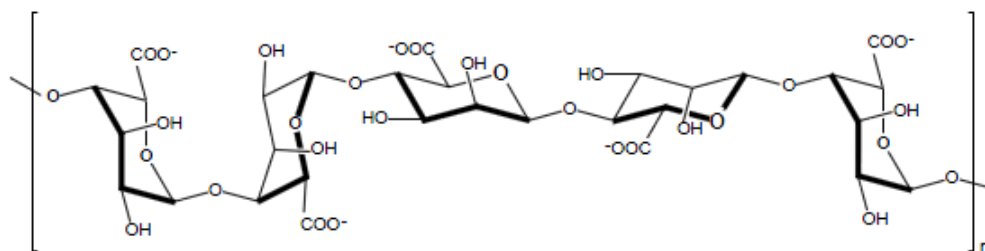
Fig. 6.5 Alginate structure

Alginic acid (Fig. 6.5) is a linear polymer consistingof D-mannuronicacid (M) and L-guluronic acid (G) residues that are arranged in the polymer chain in blocks, M and G blocks. In particular, M and G residues in alginatesare joined together in a blockwise fashion-homopolymeric M blocks (MMMMM) and Gblocks (GGGGG) or heteropolymeric blocks of altering M and G (MGMGMG).In the polymerchain the monomers tends to find their most energetically favorable structure. For G-G itis the 1C4 chair form linked together with an α-(1→4) glycosidic bond. For M-M it is the 4C1chair form linked together with a β-(1→4) glycosidic bond. The rather bulky carboxylic groupis responsible for an equatorial/equatorial glycosidic bond in M-M and an axial/axial glycosidicbond in G-G and an equatorial/axial glycosidic bond in M-G. The result of this is a buckledand stiff polymer in the G-block regions and a flexible, ribbon-like polymer in the M-block andMG-block regions (Fig. 6.6).
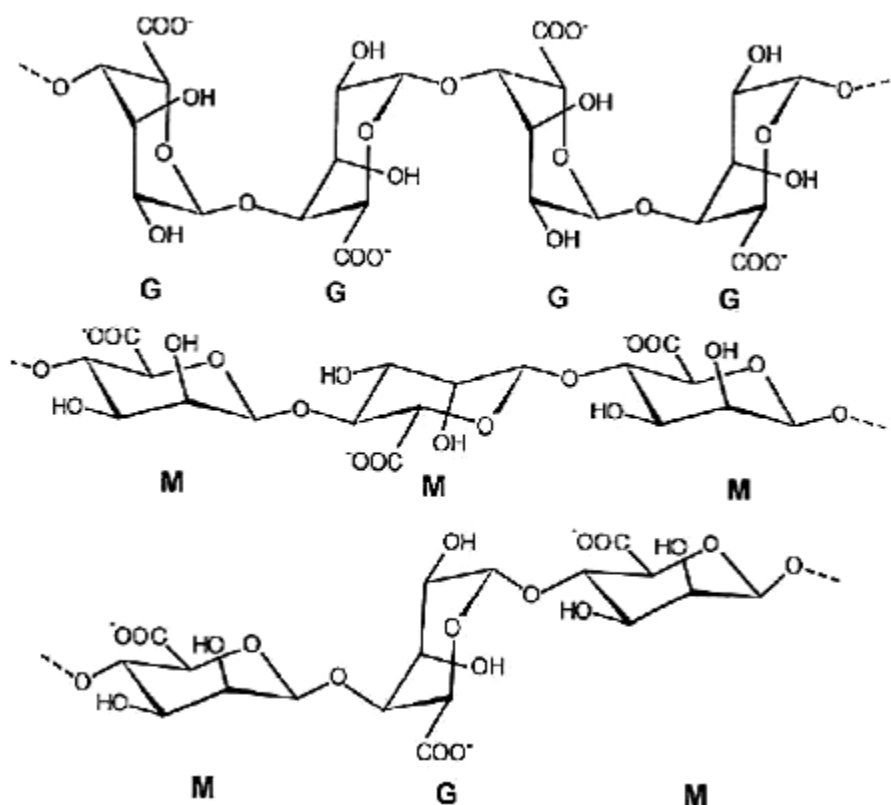
Fig. 6.6 Alginate block types

The proportion and distribution of these blocks (Fig. 6.6) determine the chemicaland physical properties of the alginate molecules.

The chemical composition of alginates varies accordingto seaweed species and even within different parts of the same plant, even if a selection of manufacture process allows to obtain alginates with constant properties in a wide range of grades.The block structure distribution of an alginate extracted from brown algae is determinedby alginate biosynthesis in the brown algae and its genetical and environmental control. Thepathway of alginate biosynthesis in brown algae ends up with poly(mannuronate), the homopolymerof M, as an obligate intermediate. An epimerase is then acting on the polymer level andworks along the polymer chain in controlling the epimerization from M to G in certain regionsof the polymer. As a rule of the thumb, one can say that the transformation from M to G willbe more and more complete as the plant tissue grows older.

Hydration of alginic acid leadsto the formation of a high-viscosity ''acid gel'' due to intermolecular binding; water molecules are physically entrapped inside thealginate matrix, but are still free to migrate. Moreover, monovalent metal ions form soluble salts

withalginate whereas divalent and multivalent cations(except Mg^{2+}) form gels or precipitates(Tønnesen and Karlsen, 2002). The variouscations show different affinity for alginate andselective ion binding is the basis for the ability ofalginate to form ionotropic hydrogels (Fig. 6.7). Alginateswith a high content of guluronic acid blocks givegels of considerably higher strength compared toalginates rich in mannuronate, as the G residuesexhibit a stronger affinity for divalent ions than the M residues.
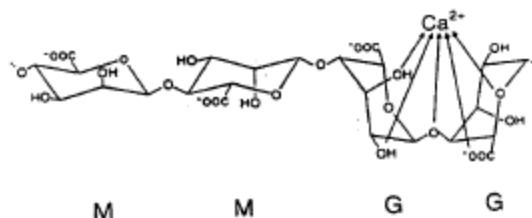
Fig. 6.7Binding mode between Ca^{2+} ions and alginate

Calcium and sodium alginate are the most extensively studied due to their properties such as the non-toxicity and biocompatibility (Tønnesen and Karlsen, 2002). In particular, sodium alginate can be used as a bindingand disintegrating agent in tablets, as asuspending and thickening agent in water-misciblegels, lotions and creams, and as a stabilizer foremulsions.

An important characteristic of alginate is represented by the presence of carboxylic acid groups of manuronic and guluronic acid units that can be present under ionized form and can interact electrostatically with the positively charged amino groups of chitosan to form a polyelectrolyte complex (Hamman, 2010). Alginate is one of the most studied anionic polyelectrolytes in complexation with chitosan. The polyelectrolyte complex formed between these two polymers is still biodegradable and biocompatible, but mechanically stronger at lower pH values where chitosan dissolves (Hein et al., 2008). Moreover, chitosan-alginate polyelectrolyte complexes are less degraded by lysozymes with respect chitosan alone (Li et al., 2009) and they can be used in tissue engineering for scaffolds and support materials (Li et al., 2009). It has been shown that charge ratio, molecular weight, ionic strength, pH, mixing order, as well as speed and diameter of the dispersing element, influence the particle size, particle surface charge (zeta potential) and stability of alginate-chitosan polyelectrolyte complexes (Sætheret al., 2008).

6.5 Cyclodextrins

CDs are useful pharmaceutically because they can interact with drug molecules to form inclusion complexes (1:1), thus improving their physicochemical and biological properties. In particular, the complex formation can enhance drug solubility, physical and chemical stability, thus resulting in greater biological performance (Uekama et al., 1994; Loftsson, 1995; Albers and Muller, 1995; Loftsson and Brewster, 1996; Challa et al., 2005). The complex formation is already widely used in many industrial products, technologies and analytical methods as well as in pharmaceutical fields. Cyclodextrin complexation with drugs can protect them against oxidation, light induced reactions, decomposition and thermal decomposition; it can also improve their solubility and stability. Moreover, it was demonstrated that they can increase dramatically the loading capacity of carriers such as liposomes, nanospheres and microspheres (Dunchene and Ponchel, 1999; Duchene et al., 1999). Cyclodextrins are used in food formulations for flavour protection and delivery. Artificial flavours are volatile oils or liquids and complexation with cyclodextrins provide a promising alternative to the conventional encapsulation technologies used for flavour protection. In cosmetic field, the major benefits of cyclodextrins in this sector are stabilization, odour control and improvement upon conversion of a liquid ingredient to a solid form. It is also possible to eliminate or reduce undesired tastes or odours and microbiological contaminations.

The CDs of biomedical and pharmaceutical interest are cyclic oligosaccharides made up of six to eight dextrose units joined through one to four bonds. They are crystalline, homogeneous, non-hygroscopic substances, which are torus-like macro-rings built up from glucopyranose units.The α-cyclodextrin comprises 6 glucopyranose units, β-CD comprises 7 such units and γ-CD comprises 8 such units(Fig. 7.1).
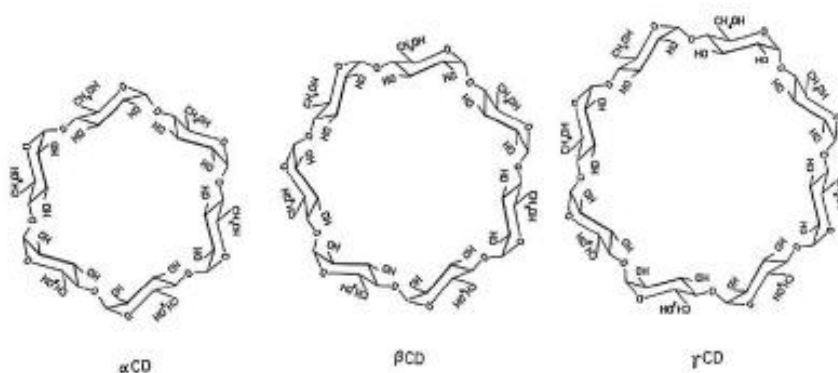
Fig. 6.8Cyclodextrin structure

As a consequence of the 4C1 conformation of the glucopyranose units, all secondary hydroxyl groups are situated on one of the two edges of the ring, whereas all the primary ones are placed on the other edge. Cyclodextrin rings are amphipathic with the wider rim displaying the 2- and 3-OH groups and the narrower rim displaying 6-OH group on its flexible arm. These hydrophilic groups are on the outside of the molecular cavity whereas the inner surface is hydrophobic lined with the ether-like anomeric oxygen atoms and the C3-H and C5-H hydrogen atoms.

The C-2-OH group of one glucopyranoside unit can form a hydrogen bond with the C-3-OH group of the adjacent glucopyranose unit. In the βCD molecule, a complete secondary belt is formed by these H-bonds, therefore the βCD is a rather rigid structure. This intramolecular hydrogen bond formation is probably the explanation for the observation that βCD has the lowest water solubility of all CDs.

6.5.1 Cyclodextrin derivates

In the cyclodextrins every glucopyranose unit has three free hydroxyl groups which all differ in their functions and reactivity. The relative reactivities of C(2) and C(3) secondary, and the C(6) primary hydroxyls depend on the reaction conditions (pH, temperature, reagents). In β-CD 21 hydroxyl groups can be modified substituting the hydrogen atom or the hydroxyl group with a large variety of substituting groups like alkyl-, hydroxyalkyl-, carboxyalkyl-, amino-, thio-, tosyl-, glucosyl-, maltosyl-, etc. groups, thousands of ethers, esters, anhydro- deoxy-, acidic, basic, etc. derivatives can be prepared by chemical or enzymatic reactions. The aim of such derivatizations may be:

• to improve the solubilityof the CD derivative (and itscomplexes)

• to improve the fitting, and/or the association between the CD and its guest, with concomitant stabilization of the guest.

6.5.2 Cyclodextrin inclusion complexes

In an aqueous solution, the slightly apolar cyclodextrin cavity is occupied by water molecules which are energetically unfavored (polar-apolar interaction), and therefore can be readily substituted by appropriate "guest molecules" which are less polar than water. The dissolved cyclodextrin is the "host" molecule, and part of the "driving force" of the complex formation is the substitution of the high-enthalpy water molecules by an

appropriate "guest" molecule. Most frequently the host:guest ratio is 1:1, however 2:1, 1:2, 2:2, or even more complicated associations and higher order equilibria exist, almost always simultaneously. The inclusion complexes formed can be isolated as stable amorphous or microcrystalline substances. The association of the CD and guest (D) molecules and the dissociation of the CD/guest complex formed, is governed by a thermodynamic equilibrium. Upon dissolving these complexes, an equilibrium is established very rapidly between dissociated and associated species, and this is expressed by the complex stability constant Ka. The most important primary consequences of stirring a poorly soluble guest with an aqueous CD solution are as follows:

a) The concentration of the guest in the dissolved phase increases significantly, while the concentration of the dissolved free CD-decreases. This latter point is not always true, because ionized guests or hydrogen-bond establishing (e.g. phenolic) compounds may enhance the solubility of the CD.

b) The spectral properties of the guest are modified. For example, the chemical shifts of the anisotropically shielded atoms are modified in the NMR spectra. Also when achiral guests are inserted into the chiral CD cavity, they become optically active, and show strong induced Cotton effects on the circular dichroism spectra. Sometimes the maximum of the UV spectra are shifted by several nm.

c) The reactivity of the included molecule is modified. In most cases the reactivity decreases, i.e. the guest is stabilized, but in many cases the CD behaves as an artificial enzyme, accelerating various reactions and modifying the reaction pathway.

d) The diffusion and volatility (in case of volatile substances) of the included guest decrease strongly.

e) The complexed substance is effectively protected against any type of reaction, except that with the CD-hydroxyls, or reactions catalyzed by them.

f) Sublimation and volatility are reduced to a very low level.

g) The complex is hydrophilic, easily wettable and rapidly soluble.

6.6 Kinetics of Hydrogel Swelling

The favorable property of these hydrogels is their ability to swell when put in contact with an aqueous solution. The water attacks the hydrogel surface and penetrates into the polymeric network. Regularly, the meshes of the network in the rubbery phase start expanding, allowing other solvent molecules to penetrate within the hydrogel network. Therefore, the unsolvated glassy phase is separated from rubbery hydrogel region with a moving front.

One of the very important features of hydrogel swelling is the rate of swelling or swelling kinetics, that aredetermined by the sample/particle size, porosity extent and the type of the porous structure. In particular, hydrogels may be divided into four main classes: a) non-porous that have molecular size pores equal to the macromolecular correlation length (10-100 Å); micro-porous (100-1000 Å); macro-porous (0.1-1 μm) and super-porous hydrogels (SPHs) that is usually in the range of several hundred micrometers, characterized by a rapid uptake of water into the porous structure.

The most important parameters that define the structure and properties of swollen hydrogels are the polymer volume fraction in the swollen state, $v_{2,s}$, the effective molecular weight of the polymer chain between cross-linking points, Mc, and the correlation distance between two adjacent cross-links, ξ. The polymer volume fraction in the swollen state ($v_{2,s}$) describes the amount of liquid that can be imbibed in hydrogels and is defined as a ratio of the polymer volume (V_p) to the swollen gel volume (V_g).

$$v_{2,s} = V_p / V_g$$

Swelling is a continuous process of transition from unsolvated glassy or partially rubbery state to a relaxed rubbery region. Although penetrant sorption by rubbery polymers may be described by Fickian transport with a concentration dependent diffusion coefficient, this description usually is not successful for glassy polymers that are influenced by the glass transition temperature (Tg) in the delivery process. In particular, it is possible to distinguish the Fickian or Case I transport, which appears when the Tg of polymer is well below the medium temperature and a non-Fickian diffusion, which appears when the Tg of polymer is well above the experimental temperature.

In the first case, the polymer chains have a high mobility and the water penetrates easily in the rubbery network. Therefore, the solvent diffusion rate, Rdiff, is clearly slower than

the polymer chain relaxation rate, Rrelax, (Rdiff << Rrelax). In the second situation, the polymer chains are not adequately mobile to permit urgent penetration of water into the polymer core. Depending on the relative rates of chain relaxation and diffusion, they commonly classified the non- Fickian diffusion to two subsections: "Case II transport" and "anomalous transport". Case II transport is dominated when the diffusion is very rapid compared to relaxation (Rdiff >> Rrelax), with relaxation occurring at an observable rate. The anomalous transport is observed when the diffusion and relaxation rates are comparable (Rdiff ≈ Rrelax). Since most polymers swell when they are in contact with certain solvents, Fick's laws can be used.

A simple and useful empirical equation is commonly used to determine the mechanism of diffusion in polymeric networks:

$(M_t/M_\infty = k*t^n)$

The constants k and n are characteristics of the solvent-polymer system. The diffusional exponent (n) is dependent on the geometry of the device as well as the physical mechanism of solute uptake or drug release.

By determining the diffusional exponent, information about the physical mechanism controlling solute uptake or about drug release from a particular device can be obtained (Table 1). For a film, n = 0.5 indicates Fickian diffusion, n > 0.5 indicates anomalous transport and n = 1 implies case II (relaxation-controlled) transport.

Type of transport	Diffusional exponent (n)	Time dependence
Fickian diffusion	n= 0.5	$t^{1/2}$
Anomalous transport	0.5 < n < 1	t^{n-1}
Case II transport	n= 1	Time dependent

Table 1 Drug transport mechanisms and diffusional exponents for hydrogel slabs

The Fickian diffusion, actually, refers to a situation where water penetration rate in the gels is less than the polymer chain relaxation rate. Therefore, n = 0.5 indicates a perfect drug transport mechanisms and diffusional exponents for hydrogel slabs. Nevertheless, when the water penetration rate is much below the polymer chain relaxation rate, it is

possible to record the n values below 0.5. This situation, which is still regarded as Fickian diffusion, is named as "Less Fickian" behaviour.

The previously discussed power law equation , even though effectively describes the major portion of the swelling behaviour , fails to give a precise analysis above $M_t/M_\infty = 0.60$. To obtain a better model beyond 60%, the Berens-Hopfenberg proposed the following differential equation:

$$dM_t/dt = k_2 (M_\infty - M_t)$$

where k_2 (min-1) is the relaxation rate constant.

For the case of anomalous transport, Peppas et al. developed the following model to describe the release behavior of dynamically swelling hydrogels

$$M_t/M_\infty = k_1t + k_2t^{1/2}$$

This expression describes the release rates in terms of relaxation-controlled transport process (k1t) and the diffusion-controlled process ($k_2 t^{1/2}$).

The exponent (n) is also influenced by the matrix geometry of the delivery systems (Table 2).

Matrix Geometry	Diffusion-controlled delivery system (Case I)	Swelling-controlled delivery system (Case II)
Slab	n= 0.5	n= 1
Cylinder	n= 0.45	n= 0.89
Spere	n= 0.43	n= 85

Table 2 Matrix geometry influence on diffusional exponent

The diffusional Deborah number (De), which relates water motion to the rate of polymer relaxation, and the swelling interface number (Sw), which measures water penetration into a network relative to diffusion of a dispersed drug out of the polymer, are the two dimensionless parameters that describe the dominant behavior of swelling-controlled systems.

Deborah number, De(δr), is used to relate the relaxation time of polymeric chains and the drug diffusion time. δr is the gel layer thickness which is defined as $\delta(t)$- $\delta g(t)$, where $\delta(t)$ is the distance from the gel/water edge to the centre of the hydrogel, and $\delta g(t)$ is the distance from the centre of the hydrogel to the interface between glassy and rubbery regions of the material

$De = \lambda/t = \lambda D/ \delta(t)^2$

The swelling interface number

$Sw = V \delta(t) /D$

where V is the velocity of swelling process and D is the drug diffusion.

If the swelling process is subjected by water diffusion (De<<1 or Sw>>1) the Fickian diffusion dominates the drug release process. If the swelling process is controlled by the relaxation time (De>>1 or Sw<<1), the Case II transport is dominated and results in zero-order release kinetics.

During the swelling process, it is possible to distinguish three front: the diffusion front, that is characterized by the movement of drug inside the gel system; the swelling front that is the limit between the polymers in the glass state and rubbery state; the erosion front that is represented by the limit where polymer, after reaching the critic water concentration (disentanglement concentration), start to disaggregate (Fig. 6.8)
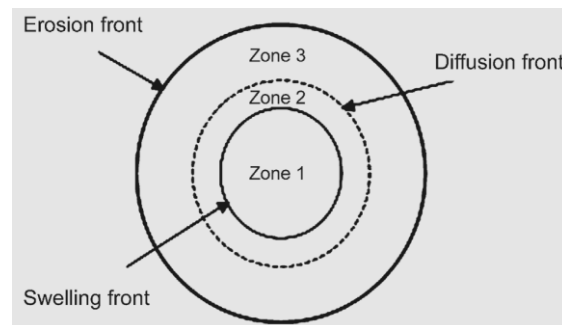
Fig. 6.9Schematic illustration of a swellable tablet during radial drug release.

8. BIOADHESION

Conventional formulations are characterized by a low retention time at the absorption site due to the biological fluids that rapidly remove the systems or reduce their contact time with biological surface, with the consequent decrease of drug bioavailability. For this reason, the recent research is increasinglyfocused on the use of bioadhesive/ mucoadhesive polymers that can increase the residence time of the formulations, thus allowing a greater contact with the mucous membrane and improving drug bioavailability. This feature can allow to decrease the drug administration frequency and to increase patient compliance.

Bioadhesion can be defined as the state in which two materials, at least one of which is biological in nature, are maintained together for a prolonged time period by means of interfacial forces (Smart, 2005). It is also defined as the ability of a material (synthetic or biological) to adhere to a biological tissue for an extended period of time, thereby increasing drug bioavailability and promoting local or systemic effects (Woodley, 2001).

Bioadhesive systems applied to mucous membrane are frequently defined as mucoadhesive, but the terms are interchangeable (Leung, Robinson, 1990). Mucosal membranes of human organism are characterized by an epithelial layer whose surface is covered by mucus. The mucus contains glycoproteins, lipids, inorganic salts and 95% water by mass, making it a highly hydrated system. Mucin is the most important glycoprotein of mucus and its main functions are protecting and lubricating the epithelium. Mucus thickness can vary from 50-450 μm in the stomach to less than 1 μm in the oral cavity (Smart, 2005). Different dosage forms can interact with mucin and the mucus component, for example with ionic interaction, or they can be hydrated by mucus thus allowing an entanglement process between the formulation and mucin chains.

8.1 Mechanism of mucoadhesion

When a drug delivery system comes into contact the mucosal surface, attraction and repulsion force are established, but for a mucoadhesive to be successful, the attraction forces must dominate.

It was possible to consider the mucoadhesion as a process based on different stages:

Stage-1 Involves an intimate contact between a bioadhesive system and a mucosal membrane; this depend by the bioadhesive and membrane wetting and by the swelling of bioadhesive.

Stage-2: After contact is established, penetration of the bio-adhesive into the surface of the tissue takes place. These two stage represent the contact step. In some cases, such as for ocular or vaginal formulations, the delivery system is mechanically attached over the membrane. In other cases, the deposition is promoted by the aerodynamics of the organ to which the system is administered, such as for the nasal route.

Stage-3: Inter penetration of the chains of the bioadhesive with those of the mucous takes place. In this step there was a consolidation of linkage between drug delivery system and mucosal surface(Fig. 8.1).
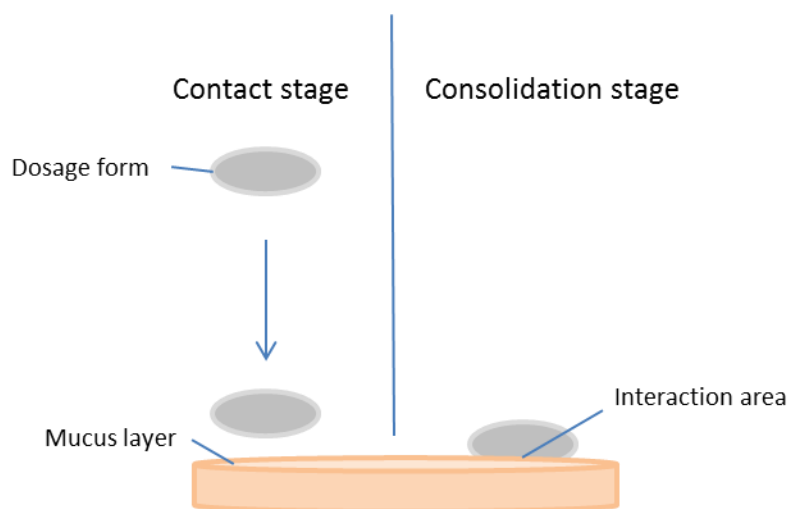
Fig. 8.1 Steps of mucoadhesion process (Carvalho et al., 2010)

Essentially, there are two theories explaining the consolidation step: the diffusion theory and the dehydration theory. According to diffusion theory, the mucoadhesive molecules and the glycoproteins of the mucus mutually interact by means of interpenetration of their chains and the building of secondary bonds (Smart, 2005). In particular, polymers with hydrogen bonds building groups (–OH, –COOH), with an anionic surface charge, high molecular weight, flexible chains and surface-active properties can establish a chemical and mechanical contact with mucus thus increasing mucoadhesion process.

According to dehydration theory, materials that are able to readily gelify in an aqueous environment, when placed in contact with the mucus can cause its dehydration due to the difference of osmotic pressure. In fact the difference in concentration gradient can favor the entry of water into the formulation until the osmotic balance is reached, promoting the

mixture of formulation and mucus and consequently ensuring the consolidation step. However, the dehydration theory is not applicable for solid formulations or highly hydrated forms (Smart, 2005).

8.2 Mucoadhesion theories

There are six classical theories adapted from studies on the performance of several materials and polymer-polymer adhesion which explain the phenomenon (Hägerström, 2003; Huang *et al.*, 2000; Smart, 2005).

8.2.1 Electronic theory

This theory is used when mucoadhesive material possesses electrical charges that are opposed to those of biological membrane; in this case, both materials come into contact and transfer electrons leading to the building of a double electronic layer at the interface, where the attractive forces within this electronic double layer determines the mucoadhesive strength.

8.2.2 Adsorption theory

According to this theory, after an initial contact between two surfaces, the materials adhere because of two types of chemical bonds. In particular, it is possible to distinguish primary and secondary chemical bonds. The first are covalent in nature and undesirable in bioadhesion because their high strength may result in permanent bonds, while the secondary chemical bonds include electrostatic forces, Vander Waals forces and hydrogen and hydrophobic bonds.

8.2.3 Wetting theory

Wetting theory is predominantly applicable to liquid bioadhesive systems and analyses adhesive and contact behavior in terms of a liquid or a paste to spread over a biological system (Smart, 2005).

8.2.4 Diffusion theory

According to this theory, the polymer chains and the mucus mix to a sufficient depth, to create a semipermanent adhesive bond(Fig. 8.2). In particular, the polymer chains penetrate the mucous; the exact depth of penetration depends on the diffusion co-efficient, time of contact and flexibility and nature of the mucoadhesive chains, mobility and contact time (Huang *et al.*, 2000; Lee, Park, Robinson, 2000; Smart, 2005).
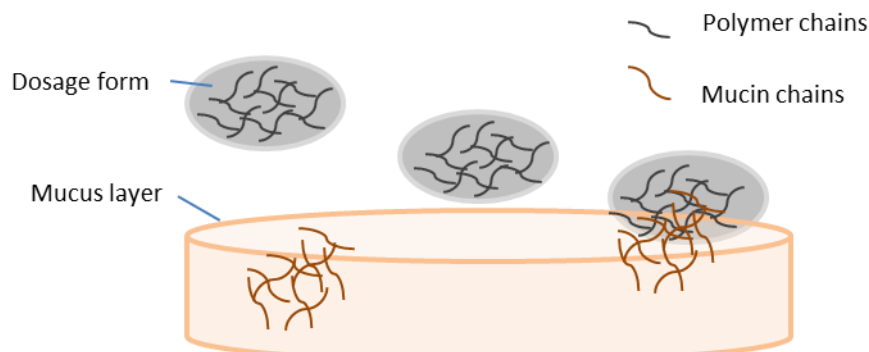
Fig. 8.2 Diffusion theory

According to the literature, the depth of interpenetration required to produce an efficient bioadhesive bond lies in the range 0.2-0.5 μm. This interpenetration depth of polymer and mucin chains can be estimated by equation

$L = (t* \, D_b)^{1/2}$

where t is the contact time, and D_b is the diffusion coefficient of the mucoadhesive material in the mucus. The adhesion strength for a polymer is reached when the depth of penetration is approximately equivalent to the polymer chain size.

8.2.5 Fracture theory

This theory is the most-used theory in studies on the mechanical measurement of mucoadhesion. It analyses the force, S_m, required to separate two surfaces after adhesion is established (Smart, 2005) that is calculated by the ratio of the maximal detachment force, F_m, and the total surface area, $A0$, involved in the adhesive interaction.

$S_m = Fm/A0$.

It has been demonstrated that the rupture rarely occurs at the surface, but near it or at the weakest point, which can be the interface itself, the mucus layer or the hydrated region of the mucus, as illustrated in Figure (Smart, 2005).

Since the fracture theory is concerned only with the force required to separate the parts, it does not take into account the interpenetration or diffusion of polymer chains. Consequently, it is appropriate for use in the calculations for rigid or semi-rigid bioadhesive materials, in which the polymer chains do not penetrate into the mucus layer.

8.2.6 Mechanical theory

Mechanical theory considers adhesion to be due to the filling of the irregularities on a rough surface by a mucoadhesive liquid. Moreover, such roughness increases the interfacial area available to interactions thereby aiding dissipating energy and can be

considered the most important phenomenon of the process (Peppas, Sahlin, 1996; Smart, 2005).

It is unlikely that the mucoadhesion process is the same for all cases and therefore it cannot be described by a single theory. In fact, all theories are relevant to identify the important process variables (Lee, Park, Robinson, 2000).

8.3 Factors affecting muco/ bioadhesion

Mucoadhesion can be influenced by several factors affecting the polymers or the environment or the physiological properties.

In particular, polymers related factors regard the molecular weight, its flexibility and spatial conformation. High molecular weight polymers are generally used for mucoadhesion and it seems that the bioadhesive force increases with molecular weight of the bioadhesive polymer, up to 10,000 and that beyond this level there is no much effect. Hydrogen bonding due to presence of hydrophilic groups such as -COOH or -OH, plays a significant role in mucoadhesion.

The polymer nature influences the degree of swelling in water which in turn determines interpenetration of polymeric molecules within the mucus. To allow chain interpenetration, the polymeric molecule must have an adequate length. Size and configuration of the polymer molecules are also important factors.

Flexibility is an important factor for interpenetration and entanglement. As water soluble polymers become cross-linked, the mobility of individual polymer chain decreases. As the cross-linking density increases, the effective length of the chain which can penetrate into the mucous layer decreases and mucoadhesive strength is reduced.

The pH of the environment influences the charge on the surface of both mucus and the polymers. In particular, mucus contains sialic acid and sulphate residues of the glycoprotein that are negatively charged under physiological conditions; these negatively charges can interact with polymers through several strengths.

The physiological factors are principally related to the natural turnover of mucin molecules from the mucus layer. The mucin turnover limit the residence time of the mucoadhesives on the mucus layer, but mucin turnover also results in substantial amounts of soluble mucin molecules. These molecules interact with mucoadhesives before they have chance to interact with the mucus layer.

Moreover, the physicochemical properties of mucus are known to change during diseased states such as common cold, gastric ulcers, ulcerative colitis, cystic fibrosis, bacterial and fungal infections of the female reproductive tract and inflammatory conditions of the eye. If the mucoadhesives are to be used in the diseased states, the mucoadhesive property needs to be evaluated under the same conditions.

8.4 Mucoadhesive materials

Mucoadhesive materials interact with the mucus layer covering the mucosal epithelial surface and the main molecules constituting a major part of mucus. They can be synthetic or natural polymers and can be water-soluble and water insoluble polymers. Water insoluble polymers are swellable networks, jointed by cross-linking agents, or highly polar to ensure a good hydration and consequently an optimal fluidity that permits the mutual adsorption and interpenetration of polymer and mucus to take place.

An ideal mucoadhesive polymer has the following characteristics:

1. The polymer and its degradation products should be nontoxic
2. It should be non-irritant to the mucous membrane.
3. It should preferably form a strong non-covalent bond with the mucin-epithelial cell surfaces.
4. It should adhere quickly to most tissue and should possess some site-specificity.
5. The polymer must not decompose on storage or during the shelf life of the dosage form.
6. The cost of polymer should not be high so that the prepared dosage form remains competitive.

Generally, mucoadhesive materials can be divided into first and second generation materials.

The first generation mucoadhesive materialsare natural or synthetic hydrophilic molecules containing numerous organic functions that generate hydrogen bonds such as carboxyl, hydroxyl and amino groups, which do not adhere specifically onto several surfaces and the most known examples are carbomers, chitosans, alginates and cellulose derivatives. These polymers can be subdivided into three classes: cationic, anionic and nonionic. Cationic molecules, such as chitosan, are positively charged at physiological pH and can interact with the mucus surface. In particular, an electrostatic interactions of

the amino groups (or positive charge) with the sialic groups of mucin in the mucus layer can occur. Chitosan has been extensively investigated as a drug delivery mucoadhesive systems (Woodley, 2001; Bravo-Osuna *et al*., 2007; Luppi et al., 2010b).

In the case of anionic molecules, mucoadhesion results from physical-chemical processes, such as hydrophobic interactions, hydrogen and van der Waals bonds, which are controlled by pH and ionic composition (Woodley, 2001) and from interpenetration of polymers chains (Luppi et al., 2010a).

Nonionic polymers, including hydroxypropylmethylcellulose, hydroxyethylcellulose and methylcellulose, present weaker mucoadhesion force compared to anionic polymers (Mortazavi, Moghimi, 2003).

Studies on novel mucoadhesive systems involve the use of multifunctional materials.

These novel multifunctional mucoadhesive systems are classified as second generation polymers (Lee, Park, Robinson, 2000). They are an alternative to non-specific bioadhesives (Smart, 2005) because they bind or adhere to specific chemical structures on the cell or mucus surface. Good examples of these molecules are lectins, invasins, fimbrial proteins (Woodley, 2001), and those obtained by the addition of thiol groups to known molecules (Bravo-Osuna *et al*., 2007).

8.5 Methods of analyzing mucoadhesion

No technology has still been developed specifically to analyze mucoadhesion. Most of the tests available were adapted from other preexisting techniques but are useful and necessary for selecting the promising candidates as mucoadhesives as well as in elucidating their mechanisms of action.

8.5.1 Tests measuring mucoadhesive strength

Most *in vitro/ex vivo* methodologies found in the literature are based on the evaluation of mucoadhesive strength, that is, the force required to break the binding between the model membrane and the mucoadhesive. Depending on the direction in which the mucoadhesive is separated from the substrate, is it possible to obtain the detachment, shear and rupture tensile strengths (Carvalho et al., 2010).

The force most frequently evaluated in such tests is rupture tensile strength (Bromberg *et al.*, 2004; Bruschi *et al*., 2007; Luppi et al., 2010 a). Generally, the equipment used is a texture analyzer or a universal testing machine that measure the force required to remove

the formulation from a model membrane. The model membrane can be a disc composed of mucin (Bruschi *et al*., 2007), a piece of animal mucous membrane, generally porcine nasal mucus (Luppi et al., 2010a) or intestinal mucus from rats (Bromberg *et al*., 2004).

8.5.2 Rheological methods

This category of methods are all carried out *in vitro* and were first proposed by Hassan and Gallo (1990), who used viscosimetric assays to macroscopically analyze the formulation-mucin interaction. From this test, it is possible to obtain the mucoadhesion force by monitoring the viscosimetric changes of the system constituted by the mixture of the polymer chosen and mucin. The energy of the physical and chemical bonds of the mucin-polymer interaction can be transformed into mechanical energy or work. This work, which causes the rearrangements of the macromolecules, is the basis of the change in viscosity.

Rheological tests are performed totally *in vitro* and consequently are conducted in combination with the rupture tensile strength test, most frequently used in studies on mucoadhesion. Moreover, it is applicable to semi-solids and liquids.

8.5.3 Tests analyzing molecular interactions involved in mucoadhesion

In order to study mucoadhesion process form a microscopical point of view, several tests analyzing the molecular interactions involved in mucoadesion are used.

The use of low frequency dielectric spectroscopy represents an attempt to study gel-mucus interactions near the molecular level. It evaluates the possible physic-chemical interactions between molecules and glycoproteins of the mucus at the interface, which is considered the step preceding the formation of bonds during the mucoadhesion process. This technique involves the study of material response to the application of an electrical field. A sinusoidal voltage is applied throughout the sample and the response is measured in function of the frequency. From the responses, the impedance or permittivity of the sample is obtained and the property of charges changing in the system can be determined (carvalho et al., 2010). This technique can provide information about the compatibility between mucus and mucoadhesive system by means of the evaluation of the movement of the charged particles.

Another test was applied for the analysis of mucoadhesion by Takeuchi *et al.* (2005). This test is based on the passage of a mucin suspension through a sensor containing the immobilized polymer. When a mucin particle binds to the polymer at the sensor, both the solute concentration and the refraction index on this surface undergo changes, where the

interaction is quantitatively evaluated and reproduced on a diagram. The sensor is a chip with a glass surface covered in a fine gold layer, where functional groups are introduced and the polymer is attached (Takeuchi *et al.*, 2005).

8.5.4 In vivo tests

Säkkinen *et al.* (2006) applied gamma scintigraphy to analyze mucoadhesion *in vivo* of chitosan within the gastrointestinal tract. Gamma scintigraphy allows the immediate visualization of all the formulation transit, with low exposure of the subjects to radiation. The study emphasized the importance of *in vivo* studies, because although chitosan exhibits an outstanding mucoadhesion capacity *in vitro*, the retention time at the absorption site in the human gastrointestinal tract was relatively short and not sufficiently reproducible (Säkkinen *et al.*, 2006). The gastrointestinal transit time in animals can also be evaluated in a non-invasive way, in which the release systems can be formulated with opaque radioisotopes and signals can be followed by X-rays, without affecting normal gastrointestinal motility (Chowdary, Rao, 2004).

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AIM OF THE THESIS

The aim of this thesis was the formulation of new chitosan based delivery systems for transmucosal drug administration. With respect to the oral route, the transmucosal route, such as buccal, vaginal and nasal routes, allowsthe circumvention of the hepatic first pass metabolism and of chemical and enzymatic degradations that generally occur in the stomach. Moreover, transmucosal drug administration can allow to avoid pain or discomfort caused by injections, when drugs are administered through parenteral routes and especially if multiple daily injections are required, thus increasing patient compliance. On the other side, the major disadvantage of transmucosal drug administration is represented by the presence of biological fluids and mucus that can remove drug systems from the application site, thus reducing the contact time between drug and mucosa and consequently, decreasing drug bioavailability. For this reason, in this study, the investigation of chitosan delivery systems as mucoadhesive formulations able to increase drugs residence time and to improve their bioavailability, was taken into account.

Polyelectrolyte complexes were prepared with chitosan and polyanions such as gelatin and alginate. Several parameters of production process were evaluated in order to obtain a final drug delivery system (buccal films and vaginal inserts) able to interact with water,to adhere with mucosal surface and to deliver the selected drugsover the time. Results related to these aims are shown in the following papers (N°1 and 2):

Mucoadhesive chitosan/gelatin films for buccal delivery of propranolol hydrochloride. Carbohydrate Polymers, 2012, 87, 581-588.
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Chitosan/alginate complexes for vaginal delivery of chlorhexidine digluconate. Original Research Article. 2013, 91(2), 651-658. DOI information: 10.1016j. carbopol.2012.08.074.
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Finally, chitosan was employed for the preparation of nanoparticles intended for insulin nasal delivery. This research was conducted in Santiago di Compostela under the supervision ofProf. Marcos-García Fuentes and Prof. Maria José Alonso. In particular, nanoparticles were prepared with chitosan and cyclodextrin including different amount of two excipients with the capacity to modify insulin bioavailabity. I will disclose the names of these formulation components due to IP issues and I will call them with the codenames C1 and C2. Nanoparticles were characterized in terms of size, PDI, zeta potential and stability and the influence of these excipients on drug release and permeation were evaluated. These results are shown in the paper N°3:

4. Chitosan-cyclodextrin nanoparticles containing two excipients with the capacity to modify insulin bioavailabity. In preparation

EXPERIMENTAL SECTION

MUCOADHESIVE CHITOSAN/GELATIN FILMS FOR BUCCAL DELIVERY OF PROPRANOLOL HYDROCHLORIDE

Abstract

The aim of this work was to develop and characterize chitosan/gelatin films as innovative mucoadhesive system for buccal delivery of propranolol hydrochloride. FT-IR and TGA analysis confirmed the interaction between chitosan and gelatin. The presence of higher chitosan amounts in chitosan/gelatin films allowed the lowest percent water-uptake ability (235.1±5.3%) and the highest *in vivo* residence time in the buccal cavity (240±13 minutes). Moreover, the presence of mannitol in the formulation allowed 80% drug permeation through porcine buccal mucosa in 5 hours. This behaviour suggests that the application of four and two films containing 5 mg of propranolol hydrochloride could be suitable for achieving the proposed daily dose for hypertension and atrial fibrillation treatment, respectively. Another interesting aspect of chitosan/gelatin films was their compatibility with buccal microflora in the absence of drug and their ability to determine growth inhibition for pathogen bacteria, but not for probiotic species, when loaded with drug.

Keywords: Chitosan/gelatin complexes; Buccal delivery; Mucoadhesive films; Propranolol hydrochloride.

1. Introduction

Buccal route offers several advantages than oral route (Harris and Robinson, 1992) due tothe high total blood flow which ensures systemic bioavailability, avoiding first-pass hepatic metabolism and gastrointestinal drug degradation (Junginger et al., 1999; Salamat-Miller et al., 2005). Moreover, it is easily accessible for self-medication and suitable for dosage forms administration and removal. However, the accidental swallowing of delivery systems and the continuous dilution of the released drug by saliva could determine a low residence time of formulation in buccal cavity and, consequently, a low drug bioavailability (Shojaei, 1998).For this reason, various bioadhesive buccal formulations (Sudhakar et al., 2006), such as tablets(Llabot et al., 2002), gels (Pelin et al., 2004; Mortazavi, 2002), patches (Burgalassi et al., 1996; Reinhold and Hans, 1989; Cheng et al., 1997; Wong et al., 1999), and films (Kohda et al., 1997;Remuñán-López, 1998), have been developed using mucoadhesive polymers which can establish a strong adhesive contact with the buccal mucosa, allowing to increase residence time of delivery systems and to optimize drug bioavailability. In particular, mucoadhesive buccal films can ensure an accurate drug dosing with respect to liquid formulations and gels, which can be easily washed away by saliva, and can be more comfortable with respect to conventional solid formulations. In fact, films are flexible and elastic, so that patient compliance is increased and also adequately strong to withstand breakage, caused from mouth movements (Peh and Wong, 1999).

In this study the properties of films based on chitosan/gelatin polyelectrolyte complexes were investigated. Chitosan, a N-deacetylated product of the polysaccharide chitin, shows interesting biological properties, including biocompatibility, non-toxicity, biodegradability and mucoadhesivity (Koga, 1998; He et al., 1998; Muzzarelli, 1997; Luppi et al., 2010a). Chitosan is also a promising matrix carrier for sustained drug release and it possesses excellent film-forming properties (Remuñán-López and Bodmeier**,** 1996). At pH below its pKa, chitosan is a polycation and has been used extensively to prepare ionically crosslinked hydrogels with anionic polymers (Hamman, 2010, Berger et al., 2004; Meshali and Gabr, 1993). In this study, type B gelatin was used as anionic polymer. Type B gelatin is a heterogeneous mixture of protein fractions consisting of single or multi-stranded polypeptides and it is derived from alkaline hydrolysis of cattle hides and bones (Hamman, 2010).

Propranolol hydrochloride is a β-blocker almost completely absorbed although it shows a low bioavailability due to extensive first-pass metabolism, so that only 25% approximately reaches systemic circulation (Reiter, 2004). It is used clinically for hypertension, angina, postinfarction, sinus tachycardia, arrhythmias, and obstructive cardiomyopathy. Because of differences in clearance and variation in drug binding there is a wide range of effective oral dosage. In particular, for hypertension treatment, the initial average daily dose of propranolol hydrochloride is 40 mg twice daily, while for atrial fibrillation, the initial usual dose is 10 mg three or four times daily. Considering drug oral bioavailability of approximately 25%, for hypertension treatment and for atrial fibrillation, the anticipated buccal doses of drug are 10 mg twice daily and 7.5-10 mg daily, respectively.

The aim of this work was to develop mucoadhesive chitosan/gelatin films able to easily administer propranolol hydrochloride by buccal route, allowing suitable drug permeation. In particular, their use for chronic treatment can be suggested due to their tolerability and compatibility with buccal mucosa.

2. Materials and methods

2.1 Materials

Type B gelatin from bovine skin (~225 Bloom, isoelectric point in the range of pH 4.5-5.5) was obtained commercially from Sigma-Aldrich (USA); chitosan (Mr. 150,000; deacetylation degree 84%; pKa 6.3) and propranolol hydrochloride were obtained commercially from Fluka (Milan, Italy). All other chemicals and solvents were of analytical grade and purchased from Carlo Erba (Milan, Italy). Water-uptake, mucoadhesion, release and permeation studies were carried out in aqueous buffers with the following compositions (g) per liter of distilled water: 2.38 $Na_2HPO_4 \cdot 10H_2O$, 0.19 KH_2PO_4, 8.0 NaCl for buffer solution pH 7.4; 4.609 KH_2PO_4, 16.748 $Na_2HPO_4 \cdot 12H_2O$ adjusted with hydrochloric acid to pH 6.8.

2.2 Preparation of chitosan/gelatin complex buccal films

As described in Cheng M. et al., (2003), known amounts of chitosan and gelatin were dissolved separately in 1% w/v acetic acid and water, respectively. Then chitosan

solution and gelatin solution were mixed obtaining two final polymeric concentrations, F1 (1% w/v) and F2 (2% w/v) and different weight mixing ratios. The mixing ratio r (i.e. the percentage of gelatin in the mixture) was defined as:

$r = W_g/(W_c+W_g)$;

where W_c and W_g were the weights of chitosan and gelatin, respectively.

50 mL of the final mixture were cast into a petri dish (11 cm in diameter) and dried at 50 °C for 24 h through casting-solvent evaporation method. Loaded films were prepared by the same procedure, adding a known amount of propranolol hydrochloride into the polymeric solutions, in order to obtain films containing 1.67 mg/cm^2.

Mannitol, a hydrophilic absorbing material, was added to F1 polymeric solutions obtaining Fm films (1.55 mg/cm^2 of mannitol).

Films were washed with 80% ethanol until neutrality (pH=7), cut into appropriate sizes, packed in aluminium foil and stored at 4°C for further studies.

2.3 FT-IR spectroscopy, Thermogravimetric analysis (TGA) and Differential Scanning Calorimetry (DSC)

To verify interactions between chitosan and gelatin, FT-IR spectroscopy (FT-IR-4100 spectrophotometer recorded with a Jasco, 650-4000 cm^{-1}) and TGA (Mettler TA 4000 apparatus equipped with a TG 50 cell on 8-10 mg samples; β=10 K min^{-1}, static air atmosphere, 30-400°C temperature range) of unloaded films, chitosan and gelatin powders and their physical mixture were performed. Measurements were carried out at least in triplicate (relative standard deviation ± 5%). To verify the absence of crystal drug in films, thermal analysis were performed using a thermocryostat (Mettler 821e/800/847) connected to the thermal analyzer (Mettler- Toledo S.p.a., Novate Milanese, Italy).Samples of loaded films and propranolol hydrochloride powder (about 5 mg) were sealed in a 30 μL aluminium pan and were scanned between 30°C and 340°C at a heating rate of 10°C/min.

2.4 Characterization of buccal films

In order to determine film thickness, three circles of 3 cm^2 were cut from each film. The average thickness of the buccal films was determined using a Mitutoyo pocket thickness gauge; Mitutoyo Mfc. Co. Ltd, Tokyo, Japan.

For determination of weight uniformity, circles of 3 cm^2 of each film were randomly selected and accurately weighted using an electronic balance. The results are expressed as the mean values of three determinations.

Drug content was calculated as follows: three circles of 3 cm^2 were dissolved in 10 mL of phosphate buffer (pH 7.4) containing 2 mL of HCl 0.1M solution, in order to determine the amount of propranolol hydrochloride in the films. The amount of drug was determined with chromatographic system, composed of a Shimadzu (Milan, Italy) LC-10ATVP chromatographic pump and a Shimadzu SPD-10AVP UV-Vis detector set at 254 nm. Separation was obtained on a Phenomenex (Torrance, CA, USA) Sinergy Fusion-RP 80A (150 x 4.6 mm I.D., 5 µm) coupled to a Phenomenex (Torrance, CA, USA) SecurityGuard C18 guard cartridge (4 x 3.0 mm I.D., 5 µm). The mobile phase was composed of a mixture of acetonitrile-pH 3.0 solution of triethylamine (0.5%) 30:70 (v/v). The flow rate was 0.4 mL/min and manual injections were made using a Rheodyne 7125 injector with a 50 µL sample loop. Data processing was handled by means of a CromatoPlus computerised integration system (Shimadzu Italia, Milan, Italy). Calibration curve of concentration versus peak area ratio was plotted at concentration range of 0.1µg/mL-10µg/mL; good linearity was found ($r^2 = 0.9998$). Repeatability assays were carried out on propranolol hydrochloride standard solutions, at concentrations corresponding to the lower and upper limit and the middle point of the calibration curve. Method precision was satisfactory: RSD% values of 3.1, 3.0 and 1.3 were obtained for propanolol hydrochloride concentrations of 0.1, 1.0 and 10.0 $\mu g\ mL^{-1}$, respectively.

The results were expressed as milligrams of drug for square centimetre (mg/cm^2). All determinations were carried out in triplicate.

2.5 *Scanning electron microscopy (SEM)*

The morphological structure of buccal films was studied by SEM analysis. Buccal films were fixed on supports and coated with gold-palladium under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. Samples were then observed with LEO 420 (LEO Electron Microscopy Ltd, England) using secondary electron imaging at 15 kV in order to examine the structure of the films.

2.6 In vitro water-uptake studies

In vitro water-uptake studies were performed in phosphate buffer at pH 6.8 that simulated human saliva and measuring the increase of weight for predetermined periods of time. Circles of 3 cm^2 of each films were weighted (W_1) and dipped in simulated saliva fluid for predetermined periods of time. Then, the circles were wiped off from the excess surface water using filter paper and weighted (W_2). Water-uptake (WU) ability was determined as a weight increase of the films after 5 h, according to the follow equation:

WU (%) = [(W_2-W_1) x 100/W_1],

where W_1 was the initial weight of dry film and W_2 is the weight of hydrated films.

2.7 In vitro and in vivo mucoadhesion properties

For these studies porcine buccal mucosa was used as biological membrane due to the similarity to the human buccal tissue (Shojaei, 1998; Hoogstraate et al., 1992).Porcine buccal mucosa was removed from a freshly killed male pig obtained from a local slaughter house. The buccal cavity was placed in phosphate buffer at pH 7.4; then buccal mucosa was surgically removed from the oral cavity using fine-point forceps and surgical scissors to turn away the connective tissue. Finally, buccal mucosa was cleaned in phosphate buffer at pH 7.4 and immediately used for tests.

The *in vitro* mucoadhesion was measured in terms of the force needed to pull out a freshly excised buccal mucosa (surface area 1 mm^2) from a film with an adapted tensiometer (Krüss 132869; Hamburg, Germany). As reported in Luppi et al. (2010b), the mucosa was fixed to a support with cyanoacrylate adhesive and then suspended from the tensiometer spring. The mucosa was lowered until it just contacted the surface of the film, previously hydrated with phosphate buffer at pH 6.8 for 5 minutes. A 20 dyne force, measured by the torsion balance of the instrument as a negative force, was applied to the films for 60 seconds. Then the mucosa was raised until it was separated from the film. The assay was performed for three different circles from each film and it was calculated the average.

The *in vivo* mucoadhesion properties of buccal unloaded films were tested in five healthy volunteers aged 25–40 years. The volunteers were instructed to press the films against the gingival mucosa above the canine tooth for 60 seconds (Perioli et al., 2004; Yehia et al., 2008).The films were observed for 5 h. The volunteers were refrained from food and drinks during the test and were asked to monitor for irritation and to record the residence time which was taken as the time for the film to dislodge completely.

2.8 In vitro release of propranolol hydrochloride from buccal films

The release studies of propranolol hydrochloride were performed in 10 mL of phosphate buffer at pH 7.4 at 37±0.5°C under magnetic stirring. Aliquots of 0.2 mL were withdrawn at different time intervals, filtered through cellulose acetate membrane (0.45 μm), and replaced by fresh medium. The studies were carried on for 5 h. The release studies were conducted in triplicates and the mean values were plotted versus time.

2.9 In vitro permeation studies

In vitro permeation studies through buccal porcine mucosa were conducted in a Franz-type permeation cell with a diffusional area of 1.76 cm^2. At time zero, films were placed in the donor compartment and 20 μL of phosphate buffer at pH 6.8 simulating human saliva were placed on mucosa. The receiver phase (6.0 mL of a phosphate buffer solution, pH 7.4, maintained at 37 °C by means of a surrounding jacket) was stirred constantly and, at predetermined time intervals, samples of 100 μL were taken and replaced by fresh medium, in order to assess the amount of drug permeated. As control formulation, the permeation study of propranolol hydrochloride from 50 μL of solution containing 5 mg of drug was performed. The studies were carried on for 6 h. The permeability coefficient (*P*) was calculated using the following equation: $P = (dM/dt)/(M_0 A)$, where dM/dt represents the permeability rate and M_0 stands for the initial concentration in the donor chamber, while *A* is the effective surface area of the mucosa.

2.10 Antimicrobial activity assay

The antimicrobial activity was evaluated against Gram-positive bacteria (*Lactobacillus acidophilus* LA14, *Bifidobacterium infantis* BI07, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 29213), Gram-negative bacteria (*Escherichia coli* ATCC 11105, *Pseudomonas aeruginosa* ATCC 9027) and yeasts (*Candida albicans* ATCC 10231). *L. acidophilus* LA14 and *B. infantis* BI07 are probiotic strains purchased by Danisco Inc. (Madison, WI). *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* were grown aerobically in LB medium (Difco, Detroit, MI) at 37°C for 24 h. *L. acidophilus* and *B. infantis* were cultured in MRS medium (Difco) supplemented with 0.05% L-cysteine at 37°C for 18-36 h under an anaerobic atmosphere by using Anaerocult A (Merck, Darmstadt, Germany). *C. albicans* was grown aerobically in SD medium (Difco) at 30°C

for 48 h. The disc-agar diffusion method was used to test the antimicrobial activities of unloaded and loaded films (6 mm diameter) containing different concentrations of chitosan and gelatin (r=0.2, r=0.4, r=0.6). In parallel, the antimicrobial activity was evaluated for: (i) chitosan solutions (1%, w/v), without and with propranolol hydrochloride (25 mg/mL) in order to simulate polymer and drug amounts in the films; (ii) chitosan/gelatin (1%, w/v) solutions, without and with drug. Suspensions of the test microorganisms (10^8 colony-forming units [CFU]/mL) were spread on the agar plates containing the appropriate culture media (LB, MRS or SD). Sterile paper discs of 6 mm diameter (Schleicher and Schuell, Dassel, Germany) were impregnated with 20 μL of each solution. These paper discs and the circular films with the same diameter were placed on the surface of the agar plates. Plates were incubated at the appropriate conditions and the diameter of the inhibition zone around the paper discs and films were measured. The experiments were performed in triplicate.

The minimal inhibitory concentration (MIC) of propranolol hydrochloride was determined by the agar dilution method. A stock solution of 20 mg/mL of drug in water was used to prepare agar plates containing scalar concentrations of the drug (3.75–120 μg/mL). Pure propranolol hydrochloride was used to obtain agar plates containing 25 mg/mL of the drug. Microbial suspensions of 10^5–10^6 CFU/mL, prepared from broth cultures in log phase growth, were used to inoculate plates containing propranolol hydrochloride. Plates were made in triplicate and incubated at the appropriate conditions.

2.11 Statistical analysis

All the experiments were done in triplicate. Results are expressed as mean ± SD. Kruskal–Wallis and Anova tests were used to determine statistical significance of permeation studies and of all other studies, respectively. Differences were considered to be significant for values of $P < 0.05$.

3. Results and discussion

3.1FT-IR spectroscopy, Thermogravimetric analysis (TGA) and Differential Scanning Calorimetry (DSC)
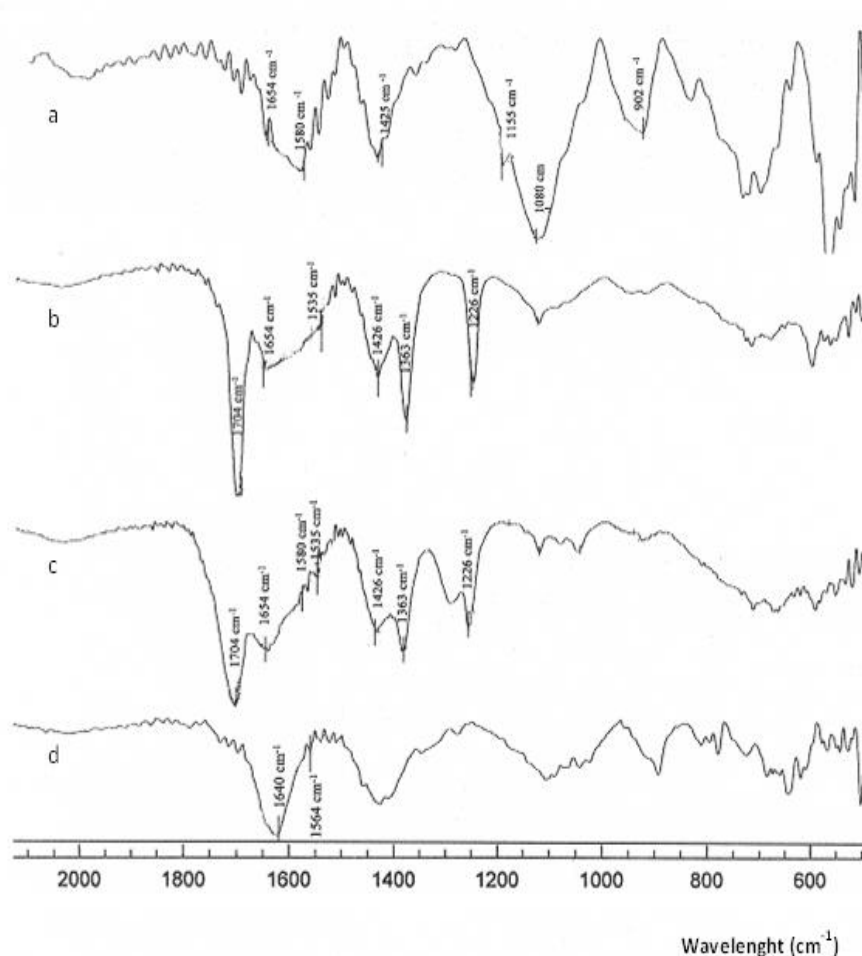
Fig. 1. FT-IR of chitosan (a), gelatin (b), chitosan/gelatin physical mixture (c), F1 film r=0.6 (d).

Fig. 1 shows the FT-IR spectra of chitosan (a), gelatin (b), chitosan/gelatin physical mixture (c) and F1 film, r=0.6 (d).

The FT-IR spectra of chitosan showed bands at 1654 cm^{-1} relative to the vibration of the carbonyl group of acetylated amide and at 1580 cm^{-1} relative to stretching of the free amino group. Gelatin showed the bands at 1654 cm^{-1} and 1535 cm^{-1} relative to the vibration of the amide carbonyl and stretching of the free amino groups, respectively; it

also showed band at 1704 cm^{-1} relative to undissociated carboxyl group. These characteristics bands were also in the FT-IR spectra of physical mixture of chitosan and gelatin. The FT-IR of film showed the shift in amide carbonyl group to 1640 cm^{-1} and the shift in amino group of chitosan to 1564 cm^{-1}, confirming the interaction between chitosan and gelatin, also reported in Yin et al. (1999).
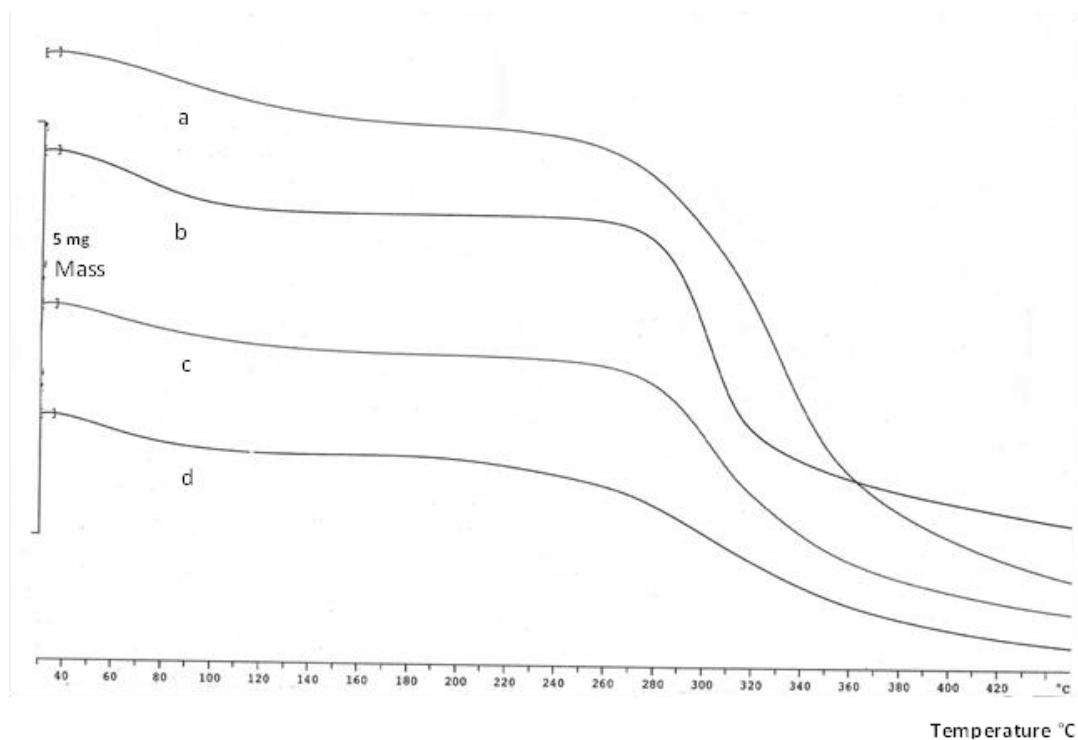
Fig. 2. Thermogravimetric analysis of gelatin (a), chitosan (b), chitosan/gelatin physical mixture (c), F1 film r=0.6 (d).

Fig. 2 shows the thermograms of chitosan (a), gelatin (b), chitosan/gelatin physical mixture (c) and F1 film, r=0.6 (d).

Chitosan and gelatin degraded at about 285 °C (inflection point temperatures). The degradation of polyelectrolyte complex film showed one event at 250 °C that can be considered as a proof of chitosan and gelatin complexation. The shift to a lower temperature in the thermal degradation of the complex indicated a loss of organization, probably due to the formation of ionic bonds between chitosan and gelatin.

As can be seen from the Fig. 3, propranolol hydrochloride showed a melting point at 165.6 ± 0.2 °C. DSC analysis of all films showed the absence of exothermic melting peak of propranolol hydrochloride and consequently the absence of crystal drug in the films.
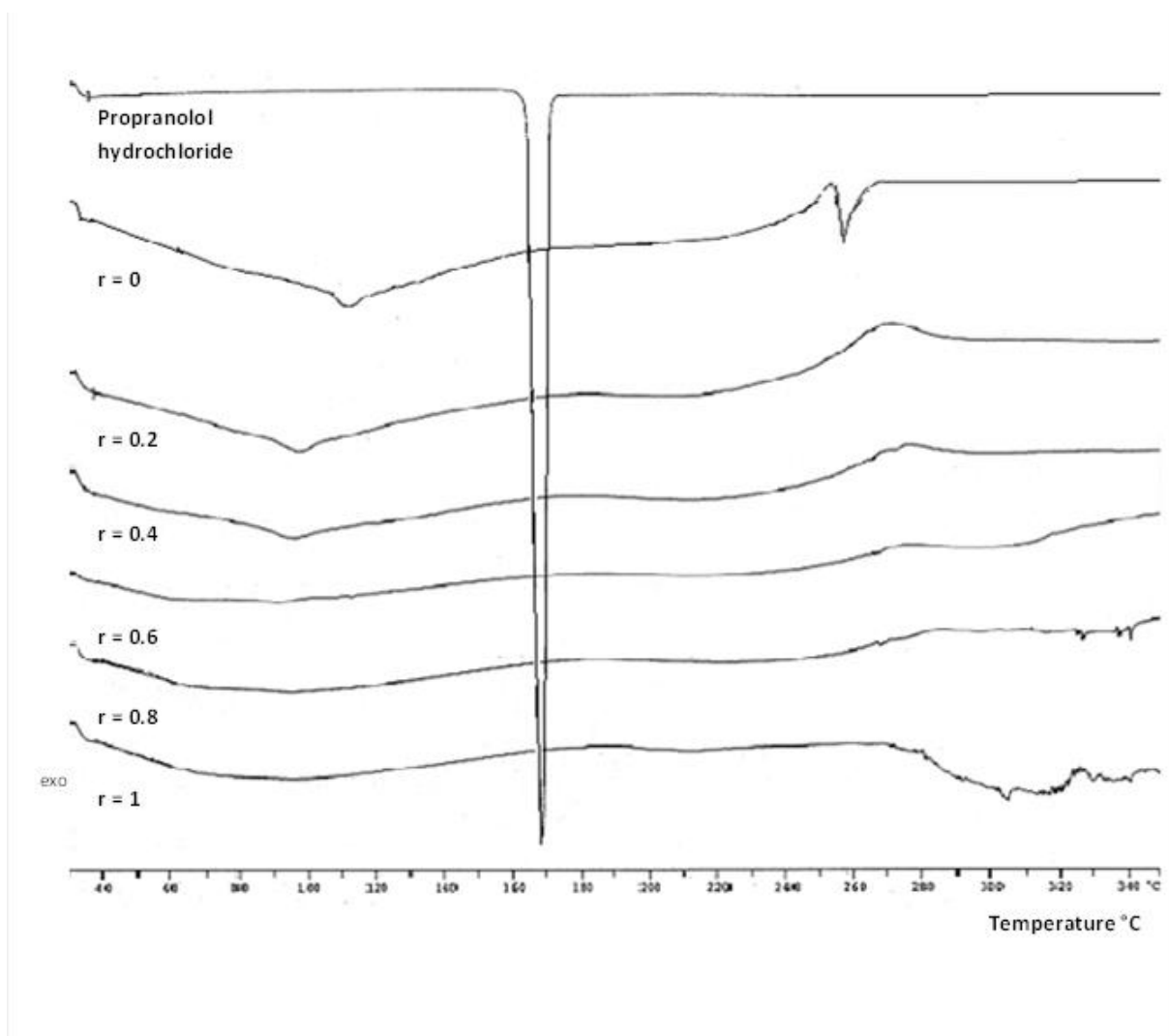
Fig. 3. DSC of propanolol hydrochloride and F1 films.

3.2 *Characterization of buccal films*

Table 1 reports drug content, thickness and weight of loaded and unloaded films.

Formulation type	Drug content (mg/cm^2)	Film thickness (μm)	Weight films (mg/cm^2)
F_1 unloaded	-	50 ± 3	7.08 ± 0.06
F_2 unloaded	-	70 ± 5	10.12 ± 0.13
F_m unloaded	-	62 ± 3	10.01 ± 0.02
F_1 loaded	1.67 ± 0.05	70 ± 8	8.53 ± 0.32
F_2 loaded	1.68 ± 0.03	100 ± 9	11.73 ± 0.36
F_m loaded	1.70 ± 0.04	82 ± 7	11.48 ± 0.33

Table 1. Characteristics of the different films (mean $\pm$ S.D., n = 3).

All loaded films consisting exclusively of chitosan did not show uniformity of drug content, weight and thickness (data not reported) and they were not considered for the subsequent tests. The others films showed weight uniformity and different thickness that can be related to the different polymeric concentration (for F1 and F2) and to the presence of mannitol in the Fm formulations.

Moreover, the experimental drug content of loaded films was close to the theoretical one (1.67 mg/cm^2 for all films) with low standard deviation, suggesting that the method employed for their preparation was capable of giving an uniform drug distribution.

3.3 Scanning electron microscopy (SEM)

Fig. 4 show the morphology of unloaded F1 film (r=0.6, a) and all loaded F1 films (r= 0.2, b; r=0.4, c; r= 0.6, d; r=0.8, e; r=1, f) which showed a homogeneous structure and devoid of crystals.

Moreover, loaded films containing an excess of gelatin (d, e, f) did not show a continue structure but an increasingly evident convex pattern on the top surface.
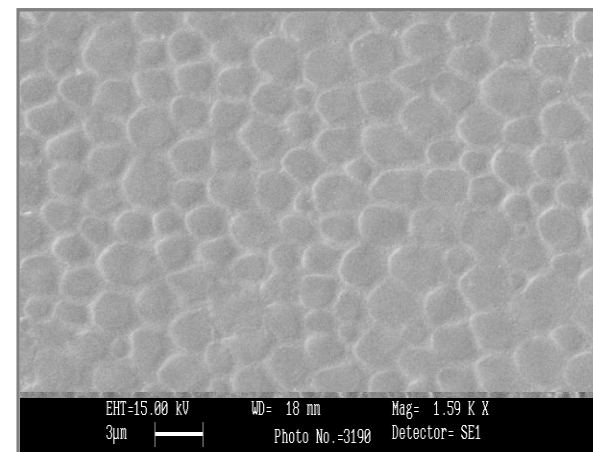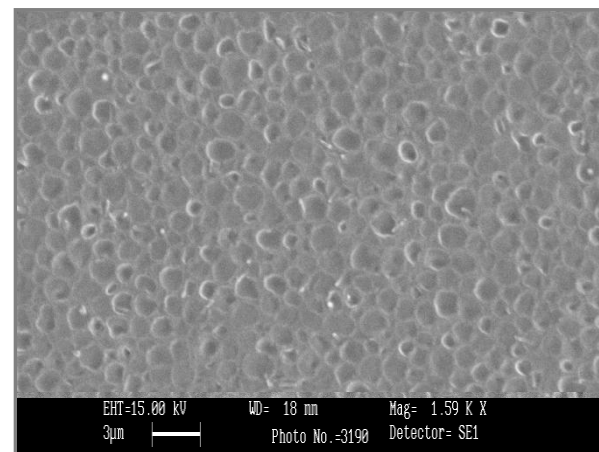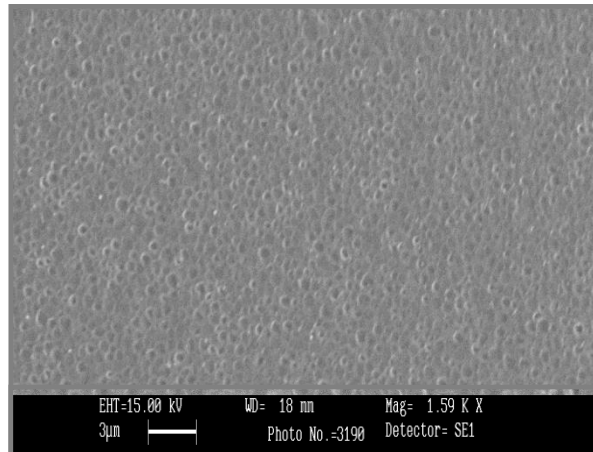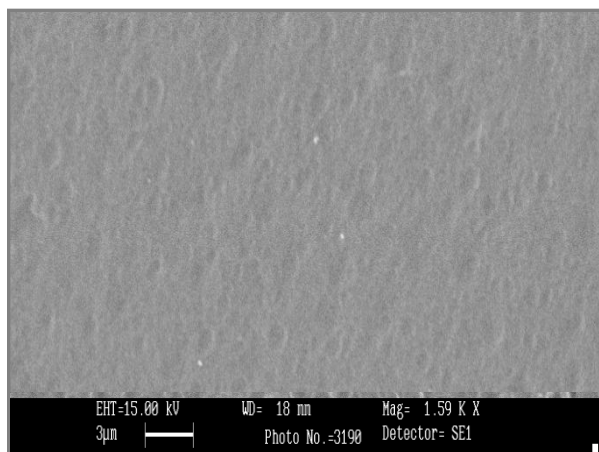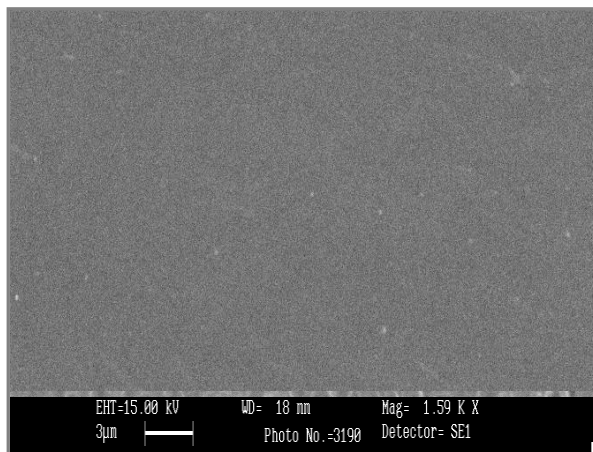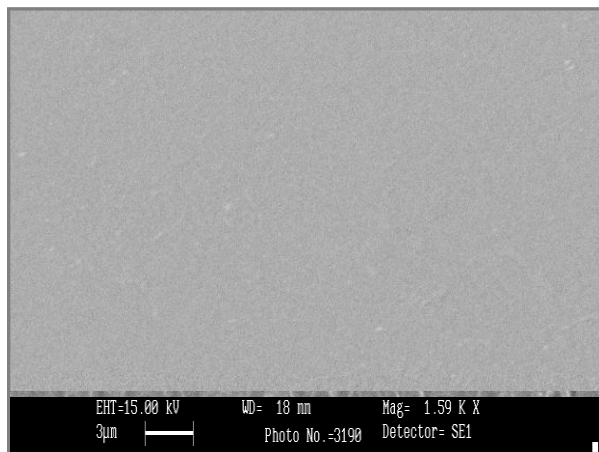
Fig. 4 Scanning electron micrographs of unloaded F1 film r=0.6 (a) and of loaded F1 films: r= 0.2 (b), r=0.4 (c), r=0.6 (d), r=0.8 (e), r=1 (f).

3.4 In vitro water-uptake studies

In vitro water-uptake studies, performed at pH 6.8 on unloaded F1 films, showed the highest water-uptake ability % at 30 minutes.

In particular, film consisting exclusively of gelatin completely solubilised in the medium in 10 minutes, while in the complex based films, the presence of a greater amount of gelatin provided a higher water-uptake ability % than films with an excess of chitosan (P <0.05). In fact, for films with r=0, r=0.2, r=0.4, r=0.6 and r=0.8 the water-uptake ability % values were 190.0±7.9, 235.1±5.3, 254.1±4.3, 286.5±6.4, 352.7±8.7, respectively. This behaviour can be correlated to the presence of a great number of ionized amino acids in gelatin structure and consequently to the presence of free charges favouring the entry of water.

This trend was also observed for loaded F1 films. However, the presence of propranolol hydrochloride provided the formation of loaded films characterized by a lower water-uptake ability % than unloaded films (P<0.05). In particular, water-uptake ability % was as follows: 193.8± 8.4 for film r=0.2; 209.3± 7.0 for film r=0.4; 237.5±8.3 for film r=0.6; 295.3±4.5 for film r=0.8. We must consider that at pH 6.8 propranolol hydrochloride is present in its ionized form (pKa 9.5) and its positive charge can interact with free negative charges of acidic amino acids of gelatin. These possible drug/protein ionic interactions can reduce free charges on gelatin structure thus determining the lower tendency of film hydration.

Fig. 5 showed the water-uptake ability after 60 minutes for all loaded films (F1, F2 and Fm).
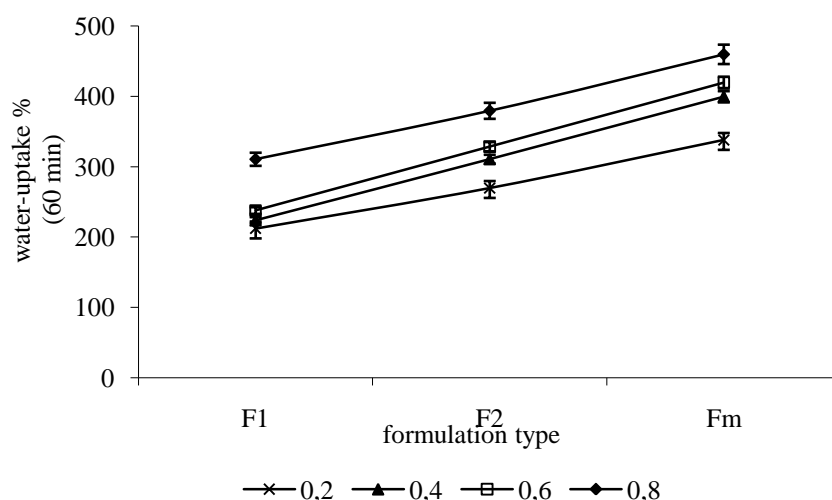
Fig. 5 Water-uptake ability after 60 minutes of different film formulation (F1, F2 and Fm) at pH 6.8 (n = 5, the SD did not exceed the 5%).

As can be seen, the presence of a higher amount of polymer (F2 films) increased water-uptake ability % respect to F1 films. As previously described the presence of free charges in the formulations is extremely important for film hydration and greater amount of polymers in the films can improve water-uptake ability % ($P < 0.05$).

As can be expected, films with the addition of mannitol (Fm) showed a greater water-uptake ability % than F1 and F2 films ($P < 0.05$), due to the presence of a hydrophilic molecule able to favour a major entry of water in the system.

3.5 In vitro and in vivo mucoadhesion properties

Table 2 reports the results of *in vitro* and *in vivo* mucoadhesion testsfor unloaded and loaded F1 films.

r	0	0.2	0.4	0.6	0.8	1
In vitro force detachment (dyne)	18.7 ± 0.4	15.8 ± 0.3	14.6 ± 0.5	13.8 ± 0.3	11.4 ± 0.5	10.3 ± 0.2
In vivo residence time (min)	270 ± 15	240 ± 13	230 ± 12	220 ± 11	150 ± 7	50 ± 4

Table.2 *In vitro* mucoadhesive capacity (expressed as detachment force, mean ± SD, n = 3) and *in vivo* residence time in buccal cavity (mean ± SD, n = 3) of unloaded films (F1).

As can be seen, films with an excess amount of chitosan showed the best *in vitro* mucoadhesive properties among all films. In fact, amino groups of chitosan chains were positively charged and could interact with sialic acid (pKa 2.6) and sulphate residues of mucin glycoprotein, that, at pH 6.8, were negatively charged (Peppas and Sahlin, 1996).

F2 films showed higher mucoadhesion values than F1 films; in fact, for film with r=0, for example, the detachment force increased until a value of 22.8±0.5 (other data were not reported). This behaviour can be explained with the presence of a greater amount of polymer respect F1 film and consequently, with a greater presence of positively charged amino groups.

Moreover, Fm films, containing mannitol, showed the best mucoadhesion properties (30.7±0.9 for film with r=0); in fact, in addiction to ionic interaction, mannitol promoted the entry of water, a more efficient chain mobility and physical entanglement with mucus. Despite loaded films showed a lower significant water-uptake ability than unloaded films, as a consequence of interaction between drug and gelatin (see section 3.5), the ionic interaction between chitosan and mucus provided not significantly different mucoadhesion properties respect mucoadhesion of unloaded films.

In vivo mucoadhesive tests were performed to assess the ability of films, without drug, to adhere to the gingivae and to study the potential irritant effect. Films did not have irritating effects on the buccal mucosa; in fact, after the removal of the film, buccal tissue revealed no signs of damage to the mucosa. Volunteers reported no irritation during or after the study.

Moreover, films with an excess of chitosan showed the best *in vivo* mucoadhesive properties, confirming *in vitro* mucoadhesion studies, while films with an excess of gelatin showed a lower residence time respect to films with an excess of chitosan (p<0.05). For F1 and Fm films (r=0) the residence times were 300±12 minutes and 320±5 minutes, respectively (other data were not reported).

3.6 In vitro release of propranolol hydrochloride from buccal films

In vitro release studies showed that propranolol hydrochloride release stopped in the first 30 minutes for all films analysed.

In particular, only film based on gelatin alone (r=1) provided complete drug release due to its dissolution. Films with an excess of chitosan (r=0.2 and r=0.4) showed a higher release of drug with respect to films with a greater amount of gelatin (P<0.05) allowing

83% and 66% of drug released in 30 minutes, respectively. On the contrary, films containing an excess of gelatin, r=0.6 and r=0.8, provided a percentage drug release of 54% and 48% respectively in 30 min.

Propranolol hydrochloride was not completely released from all the formulations containing chitosan/gelatin complexes; this behaviour can be related to the presence of possible interactions between drug and gelatin, which, proportionally to gelatin increase in the films, limit drug diffusion through the chitosan/gelatin polymeric network.

Differently, all films containing mannitol provided a complete drug release (100%) in 30 minutes, due to a greater entry of water favouring polymeric chain mobility and thus drug diffusion through the hydrated films.

3.7 In vitro permeation studies

Fig. 6 showed the permeation profiles and permeability coefficients P (cm/min) of propranolol hydrochloride across porcine buccal mucosa, after application of loaded F1 films.
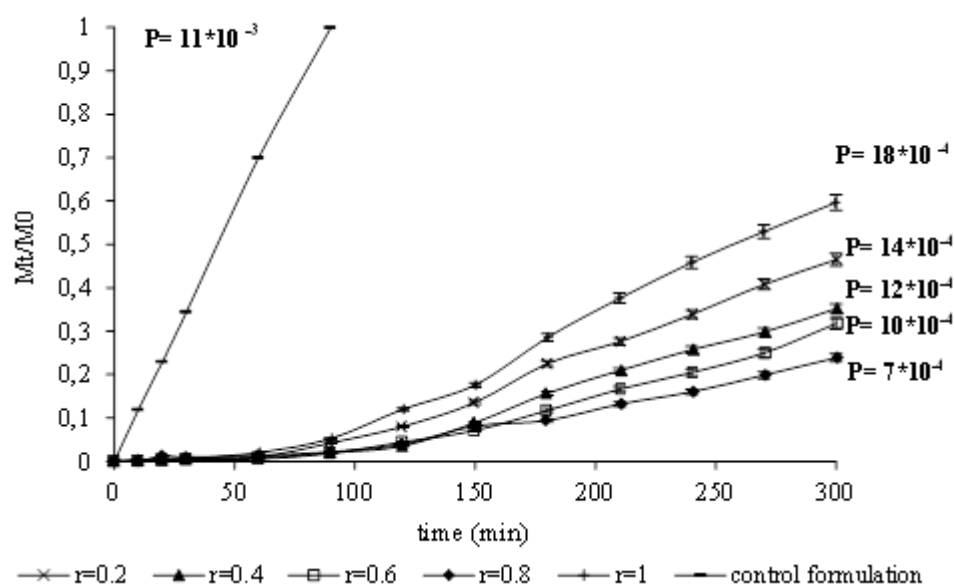
Fig. 6. Permeation profiles (mean ± SD, n = 3) and permeability coefficients P (cm/min) of propranolol hydrochloride across the porcine buccal mucosa from F1 films.

As can be seen from the figure, control formulation provided the complete permeation of the drug in 90 minutes. Films consisting exclusively of gelatin provided the greater amount of permeated drug, due to its rapid dissolution. Instead, films containing chitosan/gelatin complexes provided a lower amount of permeated drug; in particular, films with a higher content of gelatin provided the lower amount of permeated drug.

This behaviour can be correlated with drug release profiles from buccal films, which influenced drug availability at the absorption site.

While control formulation was rapidly removed from buccal cavity by saliva and swallowing, mucoadhesive films, in particular films containing an excess of chitosan (r=0.2 and r=0.4), provided a higher buccal residence times, optimizing drug bioavailability.

As can be seen from the Fig. 6, these films provided the permeation of only 46% and 35% ($P= 14*10^{-4}$ cm/minand $P= 12*10^{-4}$ cm/min) of drug, respectively. Differently, permeation studies relative to films (r=0.2 and r=0.4) containing mannitol showed an amount of permeated drug around 80% and 68% ($P= 28*10^{-4}$ cm/min), respectively.

As underlined in section 1, considering drug oral bioavailability of approximately 25%, for hypertension treatment and for atrial fibrillation the anticipated buccal doses of propranolol hydrochloride are 10 mg twice daily and 7.5-10 mg daily, respectively. The application of four and two films containing mannitol and an excess of chitosan could allow achieving the proposed daily dose for hypertension treatment and for atrial fibrillation, respectively.

3.8 Antimicrobial activity assay

Buccal preparations intended for chronic treatment should guarantee the adequate dosing regimen avoiding any potential undesirable side effects related to their prolonged residence at the administration site. An interesting characteristic of chitosan/gelatin films was their compatibility with buccal microflora.

The disc-agar diffusion test showed an antibacterial activity associated with propranolol hydrochloride. Loaded films (r=0.2, r=0.4, r=0.6), chitosan/drug and chitosan/gelatin/drug solutions determined growth inhibition zones for the pathogen bacteria *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* with diameters ranging from 8 to 28 mm. No growth inhibitory effect was observed against the probiotic species *L. acidophilus* and *B. infantis* and against the yeast *C. albicans*.

Differently, unloaded films, chitosan and chitosan/gelatin solutions did not show any noticeable inhibition zone for the microorganisms tested, indicating the absence of

antimicrobial activity for chitosan and gelatin at the concentrations used in the preparation of the films.

The MIC of propranolol hydrochloride was evaluated by the agar dilution method. MIC resulted > 120 µg/mL, indicating that propranolol hydrochloride can not be considered a strong antimicrobial agent. Since drug concentration in the films was 25 mg/mL, the antimicrobial activity of this concentration was also evaluated. The antimicrobial effects of propranolol hydrochloride at 25 mg/mL was demonstrated for all the microorganisms tested, confirming the data related to the inhibitory zones. Notably, the formulation of propranolol hydrochloride in the polymeric films counteracted the antibacterial effects of this drug against the probiotic species *L. acidophilus* and *B. infantis,* suggesting a selectiveaction against pathogen bacteria.

4. Conclusion

Polyelectrolyte complexes based on chitosan and gelatin can be successfully employed for the formulation of buccal films.

The selection of the appropriate chitosan/gelatin ratio and polymer concentration in the film, as well as the addition of mannitol, supports the goalof ensuring the necessary dosefor treatment of hypertension and atrial fibrillation.

Moreover, along with adequate drug release and permeation, desirable film characteristics such as suitable hydration and mucoadhesion, were obtained. Finally, film tolerability and compatibility with buccal mucosa suggests their possible use as formulations intended for treatment of chronic diseases.

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CHITOSAN/ ALGINATE COMPLEXES FOR VAGINAL DELIVERY OF CHLORHEXIDINE DIGLUCONATE

Abstract

Chitosan/alginate complexes were prepared at different polycation/polyanion molar ratios and freeze-dried vaginal inserts were obtained for chlorhexidine digluconate local delivery in genital infections. Complex yield, FT-IR spectra and TGA thermograms were studied to confirm the interaction between the two polyions. The influence of different complexes on physical handling, morphology and drug distribution in the samples were evaluated by friability test, scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS), respectively. *In vitro* water-uptake, mucoadhesion and release tests were performed as well as microbiological tests towards pathogenic vaginal microorganisms. The results showed that the selection of suitable chitosan/alginate molar ratio and drug loading allowed modulate insert ability to hydrate, adhere to the mucosa and release chlorhexidine digluconate. The insert containing an excess of alginate was found to be the best performing formulation and showed good antimicrobial activity towards the pathogens *Candida albicans* and *Escherichia coli*.

Keywords: chitosan/alginate complex, chlorhexidine digluconate, vaginal delivery, mucoadhesive inserts.

1. Introduction

Disturbances in the vaginal environment due to abnormal vaginal flora and vaginal infections are highly prevalent among reproductive-aged women. Vaginal candidiasis is ranked as one of the most common gynaecological infections, and it has been estimated that about 75% of women experience an acute episode once in their lifetime. It has been reported that 30-35% of vaginitis episodes are due to *Candida albicans* (Nyririesy et al., 2001; Sobel, 1988; Nitin et al., 2009; Das Neves et al, 2008). Aerobic vaginitis is another frequent form of abnormal vaginal flora which has been considered an important cause of pregnancy complications, such as ascending chorioamnionitis, preterm rupture of the membranes and preterm delivery. Aerobic vaginitis is defined as a disruption of the lactobacillary flora, accompanied by signs of inflammation and the presence of a predominantly aerobic microflora, composed of enteric commensals or pathogens, especially *Escherichia coli* and *Streptococcus agalactiae* (Donders et al., 2002; Donders et al., 2011).

Topical imidazoles are considered standard treatments of candidiasis, while kanamycin or quinolones are a good choice for the therapy of aerobic vaginitis (Tempera and Furneri, 2010). In the case of mixed vaginitis, the use of a monotherapy becomes ineffective, whereas treatment with a wide-spectrum antibacterial and antifungal substance, such as chlorhexidine digluconate, may be promising for a more rapid healing (Molteni et al., 2004).

Several drug delivery systems are used for treatment of vaginal infections (Alamdar et al., 2005). Indeed, conventional vaginal formulations (suspensions, pessaries, cream and solutions) are characterized by short residence time at the site of administration, due to washing action of physiological secretions of vaginal fluids. Bioadhesive vaginal drug delivery systems, such as tablet, inserts and gels, may adhere to vaginal mucosa in order to bring drug in contact with target tissues for sufficient period of time and prevent expulsion of formulation (Ceschel et al., 2001; Dobaria et al., 2007; Kast et al., 2002; Valenta, 2005, Woodley, 2001). Tablets and some gel-based vaginal delivery systems are associated with problems like messiness and leakage of formulations causing inconvenience to users and leading to poor patient compliance (Dobaria et al., 2007). For this reason, in this study we focused the attention on the possibility to formulate a new suitable delivery system, able to overcome these limitations and characterized by a convenient application and easy handling. To achieve this goal, the vaginal insert was

chosen as final dosage form, easily applicable and able to deliver a unique dose of drug in the vaginal cavity, while chitosan and sodium alginate were selected in order to obtain good insert mucoadhesion ability. Furthermore, different chitosan/alginate molar ratios were tested in order to obtain a system releasing the suitable chlorhexidine digluconate amount, accordingly to the therapeutic needs and providing the complete inhibition of pathogens, such as *Candida albicans* and *Escherichia coli.*

Chitosan, a N-deacetylated product of the polysaccharide chitin, shows interesting biological properties, including biocompatibility, non-toxicity, biodegradability and mucoadhesivity (Koga, D., 1998; Kumar Ravi, M., 2000; Dutta et al., 2004; Muzzarelli R.A.A, 1997 and 2010). It was also widely used for different type drug delivery systems (Dodane et al., 1998, Luppi et al., 2010a) and largely employed to prepare vaginal mucoadhesive dosage forms (Bonferoni et al., 2008, Perioli et al., 2008, Valenta, 2005, Rossi et al., 2003). Chitosan can also interact with anionic polymers in order to prepare ionically crosslinked hydrogels (Remuñán-López and Bodmeier, 1996; Hamman, 2010; Berger et al., 2004; Meshali and Gabr, 1993). Sodium alginate, an anionic, biocompatible, hydrophilic and biodegradable polymer, derived primarily from brown seaweed and bacteria, is a linear polysaccharide that consists of β-D-mannuronic acid and α-L-guluronic acid repeating units in various ratios (Hanne and Jan, 2002).

Chitosan/alginate complexes were obtained mixing polymeric solutions with different molar ratios of chitosan and alginate and then freeze-drying the precipitates. Complex yield, FT-IR analysis, TGA thermograms were studied to investigate the interaction between the two polyions. The complexes were used to prepare vaginal inserts loaded with chlorhexidine digluconate.Physical handling, morphology and drug distribution in the samples were studied by friability test, scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) analysis. *In vitro* water-uptake, mucoadhesion, release and microbiological tests were performed in order to investigate the polyelectrolyte complexes ability to adhere to mucosa, to release chlorhexidine digluconate and to study the antimicrobial activity towards *Candida albicans* and *Escherichia coli.*

2. Materials and methods

2.1 Materials

Sodium alginate low viscosity (Mw ≈140000 Da, viscosity 100-300 cP, 2 %),chitosan low molecular weight (Mw ≈ 150000 Da, viscosity 20-300 cP, T=20°C, 1% in 1% acetic acid; deacetylation degree 97%) and chlorhexidine digluconate used for this study were obtained commercially from Sigma-Aldrich (Milan, Italy). All other chemicals and solvents were of analytical grade and purchased from Carlo Erba (Milan, Italy). Complex preparation, water-uptake, mucoadhesion and release studies were carried out in aqueous buffers with the following compositions per liter of distilled water: 8.99 mL CH_3COOH 2N and 2.62g CH_3COONa for acetate buffer at pH 5.0; 13.61g KH_2PO_4, adjusted with hydrochloric acid to pH 4.5, for buffer simulating vaginal secretions.

2.2 Preparation of chitosan/alginate complex and solid complex weight measurement

Chitosan/alginate was prepared according to a method reported in a previous work (Bigucci et al., 2008) with some modifications. Briefly, chitosan(1.50 mmol of monomer in 200 ml) and alginate (1.50 mmol of monomer in 200 ml)were separately dissolved in acetate buffers at pH 5.0 at the same ionic strength (50 mM). Different volumes of chitosan solutions were added to alginate solutions and stirred at room temperature for 24 h, in order to obtain different chitosan/alginate molar ratios (1:9, 3:7, 1:1, 7:3 and 9:1).

The precipitate was separated by ultracentrifugation at 10,000 rpm for 10 min (ALC 4239R centrifuge; Milan, Italy). Then it was washed with deionized water and homogenized at 17,500 rev min^{-1} for 5 min (Ultra-Turrax, T 25 basic homogenizer; IKA, Dresden, Germany) for three times in order to eliminate sodium acetate. Finally, the precipitate was suspended again in deionized water and freeze-dried (Christ Freeze Dryer ALPHA 1-2, Milan, Italy), obtaining five different chitosan/alginate complexes:

CH/ALG(1:9), CH/ALG(3:7), CH/ALG(1:1), CH/ALG(7:3) and CH/ALG(9:1).

Each precipitate was weighted for the determination of solid complex weight.

2.3 FT-IR spectroscopy and thermogravimetric analysis (TGA)

To verify interactions between chitosan and alginate, FT-IR spectroscopy (FT-IR-4100 spectrophotometer recorded with a Jasco, 650-4000 cm^{-1}) and TGA (STA 409 PC Luxx® Netzsch, temperature range: 5-1700 °C, heating and cooling rates: 0.01 K/min-50 K/min,

inert atmospheres) of unloaded complex, chitosan and alginate powders and their physical mixture were performed. The IR spectra for the test samples were obtained using KBr disk method. Measurements were carried out at least in triplicate (relative standard deviation ± 5%).

2.4 Preparation of chitosan/alginate complex vaginal inserts

The freeze-dried chitosan/alginate complexes were used to prepare vaginal inserts. For unloaded inserts (average diameter 0.6 cm, height 1.0 cm) 200 µl of phosphate buffer at pH 4.5 were added to 20 mg of different complex/mannitol mixtures (9:1; w/w). Mannitol was added, as a bulking agent in order to improve mechanical strength of lyophilized vaginal inserts when handled (Luppi at al., 2009; McInnes et al., 2005). Loaded insertswere prepared in the same way adding 200 µl of chlorhexidine digluconate solutions (in phosphate buffer at pH 4.5) at different concentration in order to obtain three different complex/drug weight ratios (2:0.5, 2:1 and 2:2) for every type of complex. The resultant suspensions, filled into polypropylene microcentrifuge tubes, were allowed to settle to swell and remove air and finally lyophilized, obtaining cone-like shaped solid inserts. The inserts were stored in a desiccator until use (Luppi et al., 2010b).

Moreover, control formulations were prepared, without chitosan/alginate complexes, using 20 mg of mannitol and 200 µl of chlorhexidine digluconate solutions at different concentration (mannitol/drug weight ratio 2:0.5, 2:1 and 2:2).

2.5 Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS)

The morphology of vaginal inserts was studied by SEM analysis. Inserts were cut with a razor blade to expose the inner structure, fixed on supports and coated with gold–palladium under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. Samples were then observed with LEO 420 (LEO Electron Microscopy Ltd., England) using secondary electron imaging at 15 kV in order to examine the surface morphology and structure of the inserts.

Moreover, drug distribution in the samples was evaluated by energy dispersive X-ray spectroscopy (EDS).

2.6 Friability studies

Friability tests were conducted by subjecting at least 10 inserts to repeat revolutions using a friability tester. Inserts were weighted before and after the testing and % friability was measured as a percentage of weight lost during a standardized abrasion.

2.7 Water-uptake ability

Water-uptake studies were performed in phosphate buffer at pH 4.5 that simulate vaginal fluids and with the procedure reported in our previous work (Luppi et al., 2010b).

The water-uptake behavior of loaded inserts with different complex/drug weight ratios: 2:0.5, 2:1 and 2:2 was also investigated.

2.8 Insert mucoadhesion properties

For these studies, vaginal mucosa obtained from freshly slaughtered pig was used. In fact, porcine vaginal mucosa was found to be very similar to human one in many characteristics, such as lipid compositions and histological properties (Kremer et al., 2001 and Van Eyk AD, 2005).The *in-vitro* mucoadhesion was measured in terms of the force needed to pull out a freshly excised porcine vaginal mucosa (surface area 1 mm^2) from the inserts with an adapted tensiometer (Krüss 132869; Hamburg, Germany) as reported in a previous work (Luppi et al., 2010c).

The mucosa, suspended from the tensiometer spring, was lowered until it just contacted the surface of the insert, previously immersed in phosphate buffers at pH 4.5 for 15 min.A 500 µN force, measured by the torsion balance of the instrument as a negative force, was applied to the inserts for 30 s. Then, the vaginal mucosa was raised until it was separated from the formulations. This point represents the adhesive bond strength between these elements and is expressed as a positive force in dyne.

2.9 In-vitro release studies

In-vitro release studies were performed as reported in Luppi et al., 2010b. Briefly, loaded inserts were placed on the sintered-glass filter plate of a Borosil glass filter crucible and the whole system was closed with Parafilm to avoid evaporation of release medium (filled with 10 ml of pH 4.5 phosphate buffer) and adjusted exactly to the height of the release medium surface so that the porous glass membrane was wetted but not submersed. The experiments were performed at 37 °C under magnetic stirring. Samples

of 200 µl were taken at predetermined time points and replaced by fresh medium and analyzed using UV- spectrophotometer set at 254 nm.

2.10 Microbiological assays

The antimicrobial activity was evaluated against *Escherichia coli* ATCC 11105 and *Candida albicans* ATCC 10231. *E. coli* was grown aerobically in LB medium (Difco, Detroit, MI) at 37°C for 24 h. *C. albicans* was grown aerobically in SD medium (Difco) at 30°C for 48 h.

Viability of *E. coli* and *C. albicans* in phosphate buffer (pH 4.5) was compared with viability of the respective bacterium and yeast cultured in the presence of vaginal insert based on CH/ALG (1:9) complex and containing chlorhexidine digluconate (complex/drug weight ratio 2:1). A microbial suspension, prepared from a broth culture in log phase growth of *E. coli* or *C. albicans*, was used to inoculate the Erlenmeyer flasks containing 120 ml of phosphate buffer. The initial concentration of *E. coli* and *C. albicans* was about 6 log of colony forming unit (CFU) per ml of experimental medium, corresponding to the physiological amounts in cases of infection. Counts of viable *E. coli* and *C. albicans* were carried on LB and SD agar plates, respectively, at the inoculum time (T0) and after 6 h (T6), and 24 h (T24) of incubation at 37°C (physiological temperature). LB plates were incubated aerobically at 37°C for 24 h. SD plates were incubated aerobically at 30°C for 48 h. All plates were made in triplicate. Microbial concentration was expressed as a mean of log CFU/ml ± standard deviation (SD).

2.11 Statistical analysis

All the experiments were done in triplicate. Results are expressed as mean ± SD. Anova tests were used to determine statistical significance of studies, respectively. Differences were considered to be significant for values of $P < 0.05$.

3. Results and discussion

3.1 Chitosan/alginate polyelectrolyte complex weight measurement

Fig. 1shows the effect of chitosan/alginate molar ratio on complex formation at pH 5.0.
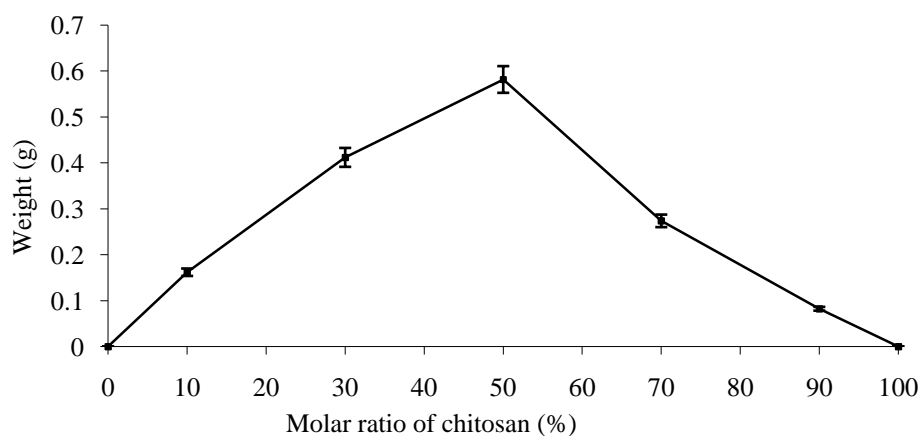
Fig. 1. Solid complex weights as a function of chitosan/alginate molar ratio at pH 5.0 obtained from 400 mlof final polymeric solutions.

The molar ratio for maximum insoluble complex formation at pH 5.0 was 1:1. In fact, at pH 5.0 most of the chitosan amino groups and alginate carboxylic groups were charged (pKb value of chitosan= 6.3 and pKa value of alginate= 3.5), thus providing the greater interaction between the polymers. Moreover, the amount of precipitated complexes CH/ALG(7:3) and CH/ALG(9:1) was lower with respect to that of CH/ALG(3:7) and CH/ALG(1:9), respectively (P<0.05); this suggest that greater moles of alginate were charged with respect to chitosan and that the presence of greater amount of alginate provided the formation of major amount of complex. For this reason, the amount of positively and negatively charges was evaluated. In particular, NH_3^+ and COO^- theoretical concentration (mM) was calculated considering a complexation reaction between chitosan (5mM) and alginate (5mM) as a function of pKa values of the two polysaccharides and molar ratio chitosan/alginate at pH 5.0. NH_3^+ and COO^- theoretical concentration (mM) was 4.28 and 0.49 for CH/ALG(9:1), 3.33 and 1.45 CH/ALG(7:3), 2.38 and 2.42 for CH/ALG(1:1), 1.43 and 3.39 CH/ALG(3:7), 0.48 and 3.36 for CH/ALG(1:9), respectively, thus demonstrating the greater ionization of alginate with respect to chitosan.

3.2. FT-IR spectroscopy and thermogravimetric analysis (TGA)

Fig. 2 showed the FT-IR spectra of chitosan and alginate powders, physical mixture and CH/ALG(1:1) complex.
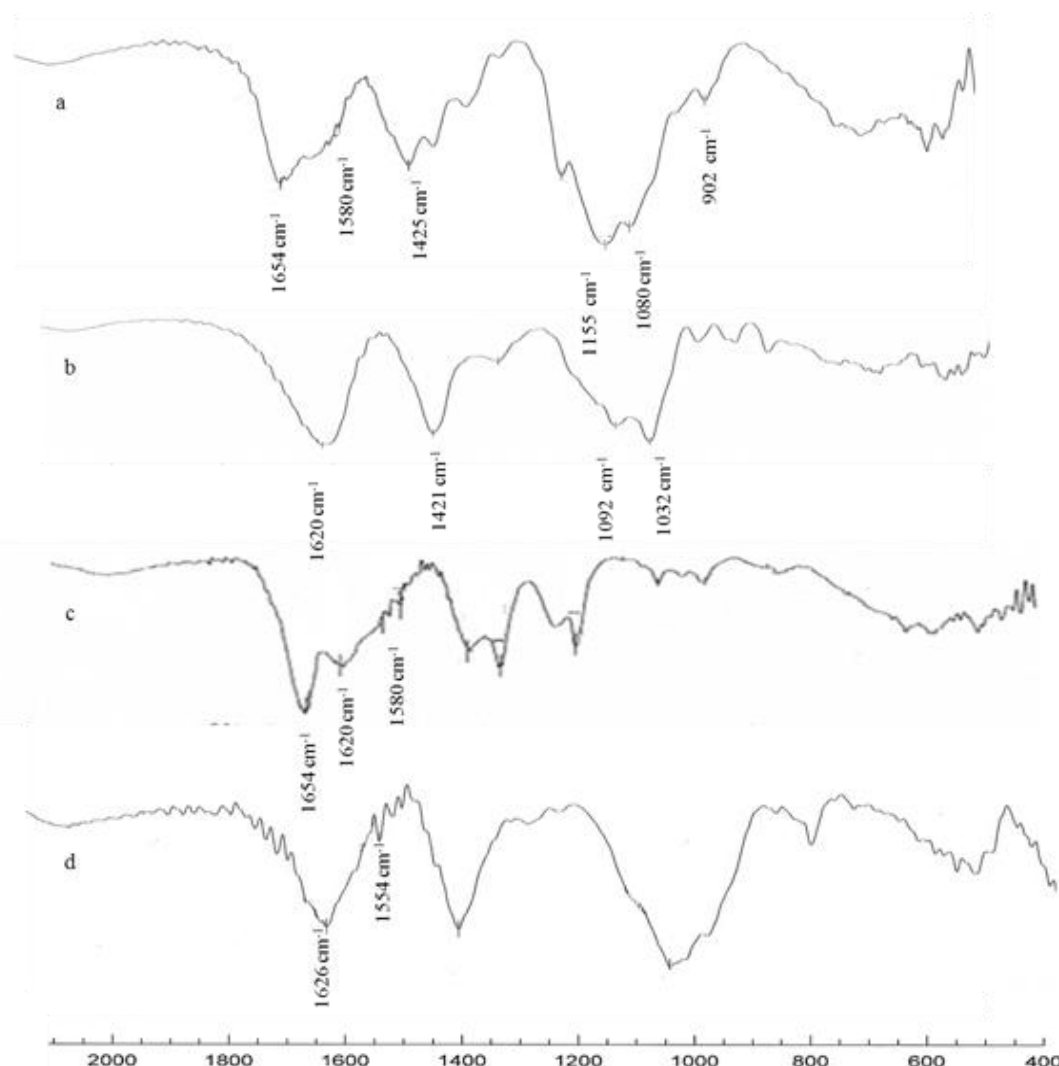
Fig. 2. FT-IR of chitosan (a), alginate (b), chitosan/alginate physical mixture (c) and complex CH/ALG (1:1) (d)

The FT-IR spectra of chitosan showed bands at 1654 cm^{-1} relative to the vibration of the carbonyl group of acetylated amide and at 1580 cm^{-1} relative to stretching of the free amino group. Alginate showed the typical band at 1620 cm^{-1}relative to the vibration of C=O group. These characteristics bands were also in the FT-IR spectra of physical mixture of chitosan and alginate.The FT-IR of complex CH/ALG(1:1) showed the shift in amide carbonyl group to 1626 cm^{-1} and the shift in amino group of chitosan to 1554cm^{-1},

confirming the interaction between chitosan and alginate, also reported in Muzzarelli C., 2003.

Fig. 3shows thermograms of chitosan, alginate, physical mixture and CH/ALG(1:1) complex.
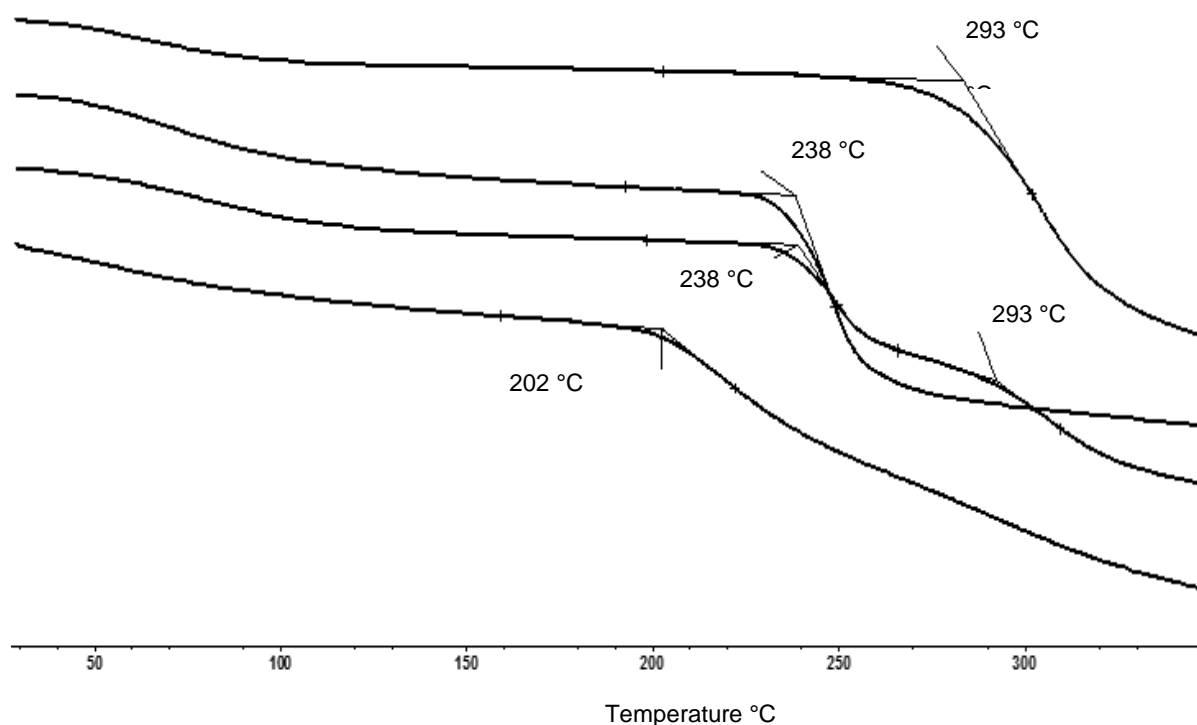
Fig. 3. Thermogravimetric analysis of chitosan (a), alginate (b), chitosan/alginate physical mixture (c), CH/ALG (1:1) (d).

Chitosan and alginate degraded at 293 and 238 °C, respectively. In the physical mixture there were two peaks at 238 and 293 °C that could be related to the weight loss of alginate and chitosan, respectively. The complex CH/ALG(1:1) showed one event at 202 °C that can be considered as a proof of chitosan and alginate complexation. The shift in a lower temperature in the thermal degradation of the complex indicates that there was a loss of organization, due to the formation of ionic bonds between chitosan and alginate.

3.3 Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS)

The structure of the inserts, observed by scanning electron microscopy (SEM), depends on the composition of chitosan/alginate complexes.
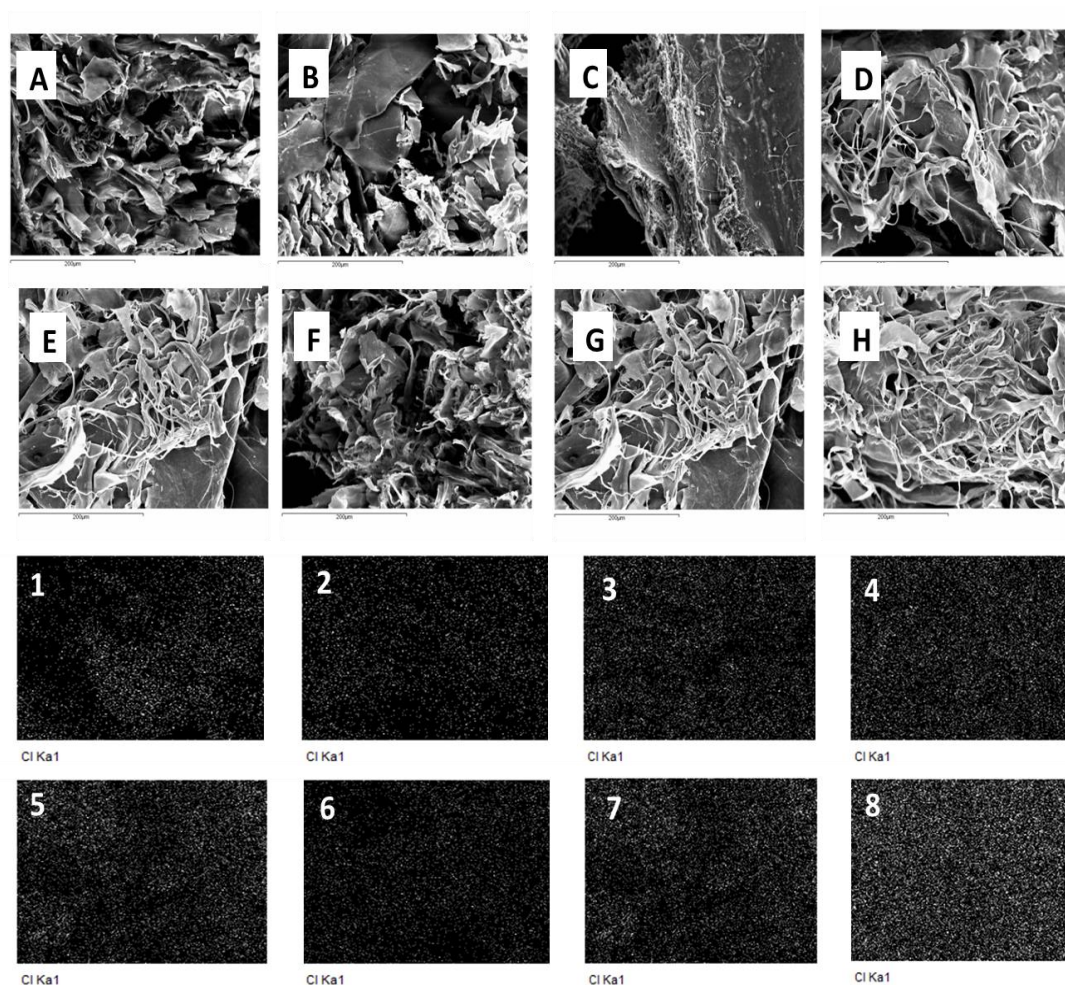
Fig. 4. Scanning electron micrographs/EDS of the different chitosan/alginate complexes: A/1: CH/ALG (9:1), B/2: CH/ALG (7:3), C/3: CH/ALG (1:1), D/4: CH/ALG (3:7), E/5: CH/ALG (1:9) with complex/drug molar ratio 2:1. F/6: CH/ALG (1:9) with complex/drug molar ratio 2:0.5, G/7: CH/ALG (1:9) with complex/drug molar ratio 2:1, H/8: CH/ALG (1:9) with complex/drug molar ratio 2:2.

Fig 4(A-E)show the morphology of vaginal inserts based on the different complexes with 10 mg of chlorhexidine digluconate (complex/drug weight ratio 2:1). The presence of drug in the complexes produced a rough and less porous surface rather than smooth as unloaded complexes (images of unloaded complexes are not reported).

Moreover, the complexes structure was more rough with the increase of the content of alginate in the complexes (Fig. 4D e 4E), probably due to the interaction between alginate and chlorhexidine digluconate. This interaction was studied measuring the turbidity (UV

spectrophotometer set at 500 nm) of alginate solutions (2% w/w) with increasing chorhexidine digluconate content (Bertram and Bodmeier, 2006). The drug concentration, at which precipitation started, was determined by extrapolating the linear correlation of the measured data points to an absorption of zero. Drug polymer interaction was observed as precipitation in polymeric solutions at concentration >0.06mg/ml.

Fig. 4 (F-H) show the influence of different amount of drug on CH/ALG(1:9) complex. As can be seen, inserts based on complex/drug weight ratio 2:0.5 provided a more smooth surface with respect to inserts based on complex/drug weight ratio 2:1 and 2:2.

Fig. 4 (1-8) shows the drug distribution in the inserts. As can be seen, the drug was homogeneously distributed in all the inserts based on the different complexes and also in the case of CH/ALG(1:9) inserts with complex/drug weight ratios 2:0.5, 2:1 and 2:2.

3.4 Friability Studies

Inserts should be hard enough to be easily removed from their packaging and to be placed intact into the vaginal cavity. Friability is a function of the hardness of a solid form and was measured in order to assess insert tendency to chip, crack or crumble due to friction and abrasion resulting from physical handling. The lower the friability, the more resistant the solid dosage form is to handling. In general, friability is affected by factors such as the size, shape and weight of the dosage form, as well as the formulation. In particular, friability values of loaded inserts based on CH/ALG(1:9), CH/ALG(3:7), CH/ALG(7:3) and CH/ALG(9:1) were 9.3±0.6, 10.1±0.3, 9.5±0.5, 9.8±0.4 %, while complex CH/ALG(1:1) provided a friability of 80±1.7%. All loaded inserts, except CH/ALG(1:1), were handled without damage and can be considered as promising formulations for vaginal application.

3.4 Water-uptake ability

Water-uptake was influenced by the medium (phosphate buffer pH 4.5) and by chitosan/alginate molar ratio. All the complexes showed the highest water-uptake ability at 120 minutes. In particular, the complex CH/ALG(1:1) showed the lower water-uptake ability among all the complexes (404.4 ± 11.2); while a large excess of chitosan and alginate allowed a greater water-uptake ability. In fact, water-uptake ability % of CH/ALG(9:1), CH/ALG(1:9), CH/ALG(7:3) and CH/ALG(3:7) were 686.2 ± 9.5, 767.9 ± 10.2, 484.9 ± 7.1, 548.7 ± 7.6 . This behavior is due to the presence of major charges in

the complexes CH/ALG(9:1) and CH/ALG(1:9) that provided the entry of major amount of water in the systems. Moreover, in the complex CH/ALG (1:1), the ionization of the same amounts of polymers provided a system with a major interaction between chitosan and alginate and a minor amount of charges, thus limiting water-uptake ability. Furthermore, the complexes CH/ALG(1:9) and CH/ALG(3:7) showed a major water-uptake ability ($P<0.05$) with respect to CH/ALG(9:1) and CH/ALG(7:3), due to the major ionization of alginate at pH 4.5 (see section 3.1).

The influence of chlorhexidine digluconate on the water-uptake ability of the insert was also investigated (Fig. 5).
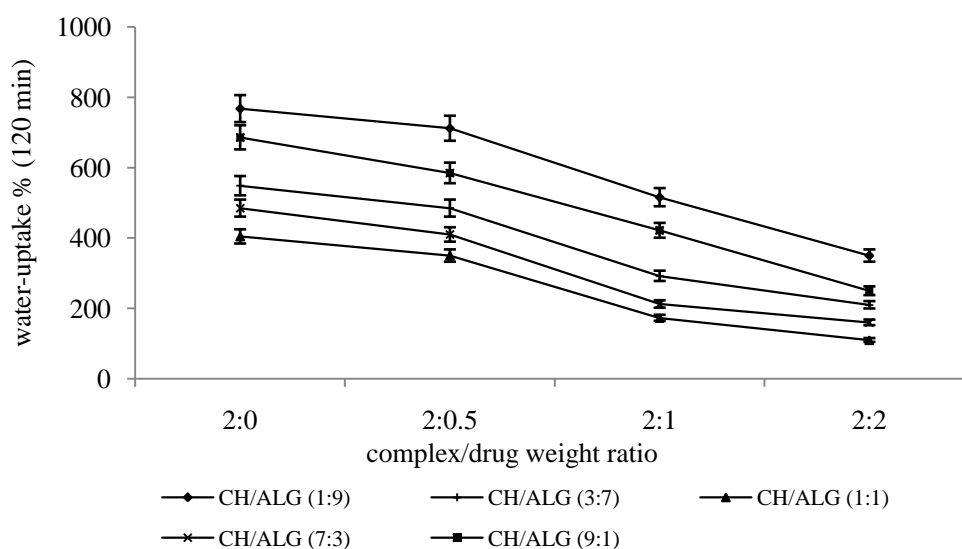
Fig. 5. Water uptake ability after 120 minutes of differently loaded (complex/drug weight ratios: 2:0.5, 2:1, 2:2 and 2:4) complexes at pH 4.5 (n = 3, the SD did not exceed the 5%).

As can be seen, the presence of the drug in the vaginal inserts gradually reduced water-uptake. In fact, for the preparation of loaded inserts, the drug was dissolved in phosphate buffer at pH 4.5 and the amino group of chlorhexidine (pKb 10.3) and the carboxylic groups of gluconic acid (pKa 3.6) were positively and negatively, charged, respectively. When drug solution was added to complex/mannitol mixture, these groups can interact with negative (alginate carboxylic groups) and positive (chitosan amino groups) charges, respectively, thus reducing the amount of free charges in the inserts.

3.5 Insert mucoadhesion properties

The presence of different amounts of chitosan or alginate in the formulations influenced significantly theinsertsmucoadhesion properties. In particular, chitosan hydrochloride insert showed a higher detachment force value with respect to that of alginate insert (150 ± 7 µN and 55±3 µN, respectively). This behaviour can be due to the presence of chitosan amino groups that at pH 4.5, were positively charged and could interact with the negatively charge of sialic acid (pKa 2.6) and sulphate residues of mucin glycoprotein (Peppas and Sahlin, 1996). For the same reason, a decrease in the mucoadhesion values was observed with reduction of chitosan amount in the inserts. In fact, the detachment force for CH/ALG(9:1) and CH/ALG(7:3) was higher with respect to that for CH/ALG(1:9) and CH/ALG(3:7) inserts (88±4, 70±6, 55±3, 57±4 µN, respectively). Moreover, in the complex CH/ALG(1:1) the high interaction between chitosan and alginate limited the presence of the positively charges, and, consequently, the detachment force (27±2 µN). There was no significant difference in mucoadhesion results between loaded and unloaded inserts (P>0.05).

3.6 In-vitro release studies

Release profiles from loaded vaginal inserts (complex/drug weight ratio 2:1) at pH 4.5 are shown in Fig. 6.

Fig. 6. Fractional amount of chlorhexidine digluconate released over time at pH 4.5 from the different chitosan/alginate complexes (Mt drug amount released over time, M0 drug

amount in the formulation at t=0). Each datum represents the average of three determinations ± SD.
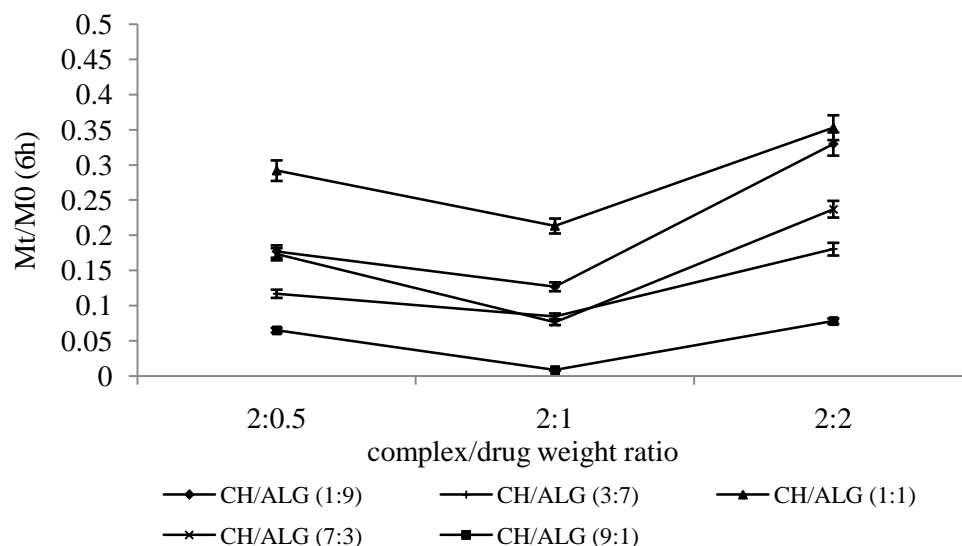
Fig. 7. Fractional amount of chlorhexidine digluconate released after 6 h from differently loaded (complex/drug weight ratios: 2:0.5, 2:1 and 2:2) chitosan/alginate complexes at pH 4.5. Each datum represents the average of three determinations ± SD.

In the case of the control formulation, the total amount of loaded drug was released after 30 minutes, due to the fast dissolution of the mannitol insert (data not reported in Fig. 6). On the other side, a sustained drug release can be observed for all the complex based formulations, due to the interaction of chlorhexidine digluconate with alginate and chitosan. Among all the inserts based on the different complexes, CH/ALG(1:1) showed the higher drug release due to the higher degree of interaction between chitosan and alginate in the complex and the lower free charges amount able to interact with chlorhexidine digluconate. Surprisingly, despite the comprovate interaction between alginate and chlorhexidine digluconate suggesting a major sustained release, CH/ALG(1:9) insert allowed higher drug release with respect to CH/ALG(9:1) insert.

As previously described, drug-complex interaction provided a decreased water-uptake. However the same interaction determined a decrease of density from unloaded to loaded inserts, indicating the capability of the hydrogel network to extend the polymeric chains in a greater way in presence of drug. In particular, the density values for unloaded and

loaded (complex/drug weight ratio 2:1) inserts based on CH/ALG(1:9) and CH/ALG(9:1) were 0.54 ± 0.02 g/cm^3, 0.65 ± 0.03 g/cm^3, 0.45 ± 0.02 g/cm^3 and 0.52 ± 0.03 g/cm^3, respectively.From these data we can also observed that the decrease in density was more evident for the insert with the excess of alginate, thus resulting in a major drug release from the polymer matrix, due to a greater drug diffusion ability. Finally in the case of the complex/drug ratio 2:2 the all inserts were unable to control drug release (Fig. 7).

3.7 Antimicrobial activity of the vaginal insert containing chlorhexidine digluconate

Viability of *E. coli* and *C. albicans* in phosphate buffer at pH 4.5 in the absence and presence of unloaded and loaded vaginal insert based on CH/ALG(1:9) complex (complex /drug weight ratio 2:1) is shown in Table 1.

[a] Counts of viable *E. coli* and *C. albicans* were carried at the inoculum time (T0) and after 6h (T6) and 24h (T24) of incubation at 37°C.

	Microbial concentration (log CFU/ml ± SD)[b]			
	Escherichia coli		*Candida albicans*	
Time point (h)[a]	Unloaded insert	Loaded insert	Unloaded insert	Loaded insert
T0	6.15 ± 0.24	6.18 ± 0.15	5.90 ± 0.19	5.90 ± 0.23
T6	4.38 ± 0.28	< 2	5.85 ± 0.20	5.00 ± 0.31
T24	3.15 ± 0.19	< 2	4.86 ± 0.16	< 2

[b] Microbial concentration was expressed as a mean of log CFU/ml ± SD.

Table 1. Viability of *Escherichia coli* and *Candida albicans* in phosphate buffer at pH 4.5 with and without the vaginal insert containing chlorhexidine digluconate.

Cell concentration of *E. coli* decreased after 6 h (T6: 4.38 log CFU/ml) and 24 h (T24: 3.15 log CFU/ml) of incubation at 37°C, compared to the baseline value (T0: 6.15 log CFU/ml), with a survival of 71.2% and 51.2% at T6 and T24, respectively. Loaded insert exerted a strong antibacterial activity against *E. coli* as evidenced by the bacterial concentrations that were below the detection limit (< 2 log CFU/ml) at the time points T6 and T24.

A good viability in phosphate buffer was demonstrated by *C. albicans*, as its concentration remained approximately constant after 6 h (T6: 5.85 log CFU/ml) and

decreased about one logarithmic unit after 24 h (T24: 4.86 log CFU/ml) of incubation, compared to the baseline value (5.90 log CFU/ml). The survival of the yeast was found to be 99.2% and 82.4% at T6 and T24, respectively. The addition of the loaded vaginal insert to the experimental medium caused the loss of almost one logarithmic unit in the viability of *C. albicans* at T6 (5.00 log CFU/ml; survival: 84.7%) and a strong reduction of the yeast count at T24 (< 2 log CFU/ml).

The present microbiological data demonstrated the inhibitory activity of the chlorhexidine digluconate formulated in vaginal insert against the principal pathogens which are responsible for aerobic vaginitis and candidiasis.

Conclusions

This investigation verified the formation of polyelectrolyte complexes between chitosan and sodium alginate in the vicinity of the pKa interval of the two polymers and confirmed the potential of these complexes, able to hydrate and adhere to vaginal mucosa. Moreover, these complexes can be used to prepare new suitable carrier system capable to overcome limits of the conventional delivery formulations, such as messiness and leakage of formulations, thus increasing patient compliance. The selection of the appropriate chitosan/sodium alginate molar ratio as well as the drug amount allowed the modulation of insert water-uptake behavior and chlorhexidine digluconate release. In particular, inserts based on the complex CH/ALG(1:9) provided the higher amount of released drug and microbiological data demonstrated that chlorhexidine digluconate released from this insert can inhibit the principal pathogens responsible of aerobic vaginitis and candidiasis.

Acknowledgements

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CHITOSAN/ CYCLODEXTRIN NANOPARTICLES FOR INSULIN BIOAVAILABITY

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Abstract

The purpose of this work was to investigate the potential of a new chitosan nanocarrier composed of cyclodextrins and two excipients (*C*) with the capacity to modify insulin bioavailabity. In particular, the selected excipients, *C1* and *C2*, were included in the chitosan/cyclodextrins nanocarriers in order to study their influence on nanoparticles physico-chemical properties and on insulin bioavailability upon nasal administration. Nanoparticles were obtained with the ionic gelation method, adding cyclodextrin/*C* solutions, with or without TPP, to the chitosan phase. The size of the resulting nanoparticles,ranging between 260 and 398 nm, was affected by the nature and the amount of the cyclodextrins and *C*whereas the zeta potential of the NPs was always positive.Nanoparticles *in vitro* stability was evaluated in different media and the empirical amount of *C* in nanoparticles was quantified using a kit. Finally, *in vitro* release and *in vivo* studies were performed in order to evaluate loaded nanoparticles ability to release insulin and to modify its bioavailability. Results showed good nanoparticle stability and capacity to decrease blood glucose levels in rats after nasal administration.

Keiwords: nanoparticles, chitosan, cyclodextrin, insulin nasal delivery.

1.Introduction

Peptides and proteins administration through oral route is characterized by a low drug absorption and bioavailability, due to the gastrointestinal environment and first-pass metabolism that lead to chemical and enzymatic drug degradations (Woodley, 1994; Saffran et al., 1997; Ram et al., 2003; Singh et al., 2008, Sood and Panchagnula, 2001; Chen et al., 2011). Other conventional administration routes, such as the intravenous route, are no convenient and show a poor patient compliance, due to the repetitive injections, generally necessary for a chronic treatment. In the recent years, nasal route has been studied as an alternative administration route thanks to the interesting properties of nasal mucosa. In fact, the large surface area, the porous endothelial membrane, the avoidance of first-pass metabolism and the ready accessibility of the nasal cavity can allow to obtain faster and higher level of drug absorption and a good patient compliance (Ugwoke et al., 2005; Costantino et al., 2007; Prego et al., 2005a). On the other hand, the mucociliary clearance mechanism removes rapidly the formulations from the nasal cavity, thus determining their low residence time and, consequently, low drug permeability and bioavailability (Merkus et al., 1998; Illum, 2003; Andersen and Proctor, 1983; Inagaki et al., 1985; Harris et al., 1986). In order to overcome this limitation, in the past years many mucoadhesive materials were analyzed to evaluate their ability to adhere to nasal surface and to increase the contact time of drug systems with the nasal mucosa (Duchene et al., 1988). Different studies have shown that chitosan nanocarriers can facilitate the interaction of the drug with mucosa (Csaba et al., 2006; Felt et al., 1998; Prego et al., 2005b; Luppi et al., 2010), thanks to the interactions between positively charged chitosan amino groups and the sialic acid and sulphate aminoacidic residues of the mucus layer, thus providing a longer contact time for drug absorption (Lehr et al., 1992). Moreover, another limitation with nasal route administration is the low permeability of polar and large molecular weight drugs, that generally cross the epithelial cell membrane by the paracellular route through the tight junctions between the cells. Although the tight junctions are dynamic structures, their mean size channels limit the transport of large molecules (Illum, 2003). Recent studies have demonstrated that chitosan can transiently open the tight junctions of the human barrier thus increasing drug permeability (Smith et al., 2004; Garcia- Fuentes and Alonso, 2012).

In this study, in order to improve insulin bioavailability through nasal route, two excipients were used (C1 and C2) for nanoparticles formulation. Nanoparticles (*NPs*)

were obtained with the nanoprecipitation technique and were characterized with regard to their size, surface charge, C1/C2 experimental amount and stability in different media. Finally, *in vitro* release and *in vivo* studies were performed in order to study nanoparticles ability to drug deliver and to modify insulin bioavailability.

2.Materials and methods

2.1. Materials

The following chemicals were obtained from commercial sources and used as received. Ultrapure chitosan (CS) hydrochloride salt (Protasan UP CL 113, *Mw* = 110 KDa and deacetylation degree = 86%) was purchased from FMC Biopolymers (Norway). 2-hydroxypropyl-*β*-cyclodextrin (HP-*β*-CD, *Mw* = 1540, substitution degree = 4.69), pentasodium tripolyphospate (TPP), C1 and C2 were all purchased from Sigma-Aldrich (Spain). Sulphobutyl ether-*β*-cyclodextrin sodium salt (SBE-*β*-CD, *Mw* = 2163, substitution degree = 6.40) was obtained from CyDex, Inc. (USA). Recombinant human free Zn insulin (27.4 units/mg, isoelectric point = 5.8) was kindly donated by Novo Nordisk A/S (Målov, Denmark). The kit for xcipients C1 and C2 amount determination was purchased from Cayman Chemical (Michigan, USA). All other chemicals were reagent grade or higher.

2.2 Preparation of cyclodextrin/C

The two selected excipients (*C*) and *CD*s (*CD/C* molar ratio of 1:1) were solubilized in an ethanol/water mixture (1:11 v/v) with the adding of an equimolar amount of NaOH with respect to *C*. We obtained HP-*β*-CD/*C1*, SBE-*β*-CD/*C1*, HP-*β*-CD/*C2* and SBE-*β*-CD/*C2* solutions with different *C* concentrations. In particular, for C1 and C2the concentrations ranges were 0.01-2mg/ml and0.01-1mg/ml, respectively. These solutions were evaporated with rotavapor until a volume reduction of tree times. In order to obtain control formulations, solutions without *C* were prepared with the same procedure.

2.3 Preparation of NPs

NPs were prepared according to the procedure previously described by our group (Calvo et al., 1997a; Calvo et al., 1997b). *CS/TPP* nanoparticleswere spontaneously formed upon

addition of 1 ml of TPP aqueous solution (0.15% w/v, polyanionic phase) to 3 ml of the CS solution (0.20% w/v, polycationic phase) under stirring. For *CS/CD/C NPs,* different volumes of *CD/C1* and*CD/C2* solutions, prepared as described above, were added to the polycationic phase, with or without TPP, obtaining NPs with different components weight ratio (table 1a, 1b and 2).In particular, in the case of nanoparticles based on HP-*β*-CD, TPP was added maintaining a CS/TPP weight ratio of 4/1; while in the case of SBE-*β*-CD NPs, the weight ratio CS/TPP was 4/0 or 4/0.5. Using the same procedure, *CS/CD* control *NPs* were obtained using solutions composed of only *CD*.

The resulting NPs were isolated by ultracentrifugation (16000 × *g*, 30 min, 15°C; Beckmann Avanti 30, Beckmann, USA) and resuspended in ultrapure water. For loaded NPs, human insulin was added to the anionic phase until a final concentration in nanoparticles suspension of 0.25 mg/mL.

2.4 Physicochemical and morphological characterization of NPs

The mean particle size and the size distribution (PDI) of the NPs were determined by photon correlation spectroscopy (PCS) using a Zetasizer 3000 HS (Malvern W Instruments, Malvern, UK). The ζ-potential determination was performed by laser Doppler anemometry (Zetasizer 3000 HS, Malvern Instruments, Malvern, UK) after dilution with KCl 1 mM.

The morphological examination of NPs was performed by transmission electron microscopy (TEM) (CM12 Philips, Eindhoven, Netherlands). All samples were stained with 2% (w/v) phosphotungstic acid and placed on copper grids with Formvar® film for TEM observation.

2.5 Stability in different media

Unloaded and insulin loaded nanoparticles were tested for their stability in different media: water, phosphate buffer at pH 6.8 and at pH 6.0, simulating nasal secretions. Nanoparticles stability was evaluated taking into account the change of nanoparticles size and their possible precipitation. Aliquots of fresh suspensions of nanoparticles were diluted in these media reaching a concentration of 1 mg/mL, and the evolution of size was assessed using photon correlation spectroscopy (Zetasizer 3000HS, Malvern Instruments, UK) for 2 h at 37°C (*n=3*). The size and PDI of the nanoparticles in the fluids were measured after 30, 60 and 120 min by photon correlation spectroscopy.

2.6 Determination of Process Yield

For the calculation of the nanoparticles production yields, the nanoparticles suspensions were centrifuged (30000g, 15 °C, 40 min), and the supernatant was discarded. The sediments were dried at 50°C until constant weight, and the difference of the theoretical solid weights and the actual dried nanoparticles weights were obtained ($n = 3$). The yield of the process was calculated as follows: process yield (%) = nanoparticles weight x 100/ total solids weight.

2.7. Determination of excipients (C)experimentalamount

The *C*experimental amount was investigated using a kit. First, nanoparticles composed of *CD/C* and of *CDs* (used as control formulations) were isolated as described in section 2.2 and resuspended in ultrapure water. Then, chitosanase-RD was added to NPs suspensions (1:2 chitosanase-RD/NPs w/w ratio, 1 h, 37°C) in order to promote chitosan degradation. Following this process, the experimental amount of *C* in the nanoparticles were calculated using a kit.

2.8 Loading and encapsulation efficiency of nanoparticles

For the calculation of loading % and encapsulation efficiency, loaded nanoparticles were isolated by centrifugation as described in Section 2.2. The amount of unbound insulin was determined in the supernatant by HPLC analyses (Agilent1100 Series, Santa Clara, CA, USA), with a method described by Marsüchtz, M.K. and Bernkop-Schnürch (2000) that was thereby modified. Briefly, 20 µL of the supernatant was injected into HPLC. Insulin and/or degradation products were separated on a protein and peptide C18 column (Grace Vydac, W.R. Grace & Co., Columbia, MD, USA) at room temperature. Gradient elution was performed as follows: flow rate 1.0 mL/min, 0-10 min; linear gradient from 70% A/30% B to 39% A/61% B (eluent A: 0.1% trifluoroacetic acid in water; eluent B: 80% of acetonitrile and 20% of eluent A). Insulin and/or degradation products were detected by absorbance at 220 nm with a diode array absorbance detector. Insulin concentration was quantified from integrated peak areas and calculated by interpolation from an according standard curve.

The loading % and the encapsulation efficiency of insulin, respectively, were calculated as follows:

Loading % = (Total amount of drug −Amount of unbound drug) x 100/ Nanoparticles weight

Encapsulation efficiency (EE) = (Total amount of drug −Amount of unbound drug)x 100/ Total amount of drug

2.9 In vitro release studies

Insulin release studies were performed by incubating 0.1 mg insulin-loaded nanoparticles in 1mL of phosphate buffer at pH 6.0, simulating nasal secretions, at 37 °C. At appropriate intervals, the samples were filtered and the amount of released insulin was evaluated by HPLC analysis. Insulin concentrations were quantified and calculated by interpolation from an according standard curve.

2.10 In Vivo Studies

Male Sprague-Dawley rats (160-190 g) from the Central Animals House of the University of Santiago de Compostela (Spain) were fasted for 12 h before experiments but allowed water ad libitum. Animals were kept conscious during the experiments. The following formulations were administered intranasally to rats: (1) insulin aqueous solution, (2) *CS/SBE-β-CD/C1/TPP* (4/2/0.17/0.5) and (3) *CS/SBE-β-CD/TPP* (4/2/0.5) NPs. In all cases the insulin dose administered was 5 UI/kg and the volumes of formulations used were 40 µl (20 μL/nostril). Formulations were intranasally administered using a polyethylene tubing inserted about 1 cm into the nostril. Blood samples were collected from the tail vein 30 min prior the nasal administration (t) -30 min, in order to establish the baseline glucose levels, and at different times after dosing (t) 0, 30, 60, 120, 180, 240 and 300 min. Glycaemia was determined in plasma samples by the glucose-oxidase method (Glucose-TR, Spinreact S.A, Spain). Results are shown as the mean values of plasma glucose levels % of initial level of six animals.

2.11 Statistics

Pairs of groups were compared by performing one-tailed Student's t-test and multiple group comparison was conducted by one-way analysis of variance (ANOVA). All data are presented as a mean value with its standard deviation (mean ± SD). p-Values less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1 Preparation of NPs

In previous works, we have demonstrated the interesting properties of nanoparticles based on chitosan and cyclodextrins for insulin delivery (Fernandez-Urrusuno et al., 1999; Maestrelli et al., 2006; Trapani et al., 2008; Teijeiro- Osorio et al., 2009). These nanocarriers combine the enhancement effect and the mucoadhesion properties of chitosan and the cyclodextrins capacity to load molecules with high efficiency, to protect drugs from enzymatic degradation and to promote their transport across mucosal surfaces. In the present work, in order to improve insulin bioavalability, we designed *CS/CDs* nanocarriers containing two excipients (*C*),C1 and C2, with the capacity to modify insulin bioavailabity. Moreover, for this study, we selected a neutral and a polyanionic cyclodextrin, HP-*β*-CD and SBE-*β*-CD.

3.1.1. Conditions for NPs formation.

First of all, we prepared *CDs/C* solutions with different *C* concentrations and then we formulated *CS/CD/C/TPP* nanoparticles with the ionic gelation technique, developed in our laboratory (Calvo et al., 1997a; Calvo et al., 1997b).

CDs/C solutions were prepared with 1:1 molar ratio between cyclodextrins and *C* and in order to increase *C* solubility, an equimolar amount of NaOH was added in the solutions. Transparent solutions with the higher amount of *C* were selected for the preparation of nanoparticles. In particular, in the case of C1, *C*concentrations were 1.0 and 0.25 mg/ml for HP-*β*–CD/C1 and SBE-*β*-CD/C1 solutions, respectively. For solutions based on C2, C2concentration was lower (0.1 mg/ml) due to its lower solubility. These concentrations allowed us to evaluate the optimal conditions, in terms of components amount, for nanoparticle formulations. In fact, as reported in previous studies (Trapani et al., 2008), the formation of the different types of *CS/CD*s NPs were clearly influenced not only by the TPP and CS concentrations, but also by the nature of *CDs* and their amount added during the preparation process.

3.2 Physicochemical and morphological characterization of NPs

Tables 1a, 1b and 2 show the physicochemical properties of the different NPs: *CS/TPP* NPs, *CS/CD/C/TPP* NPs and *CS/CD/TPP* NPs, for every cyclodextrin and C.

Ratio CS/CD/C/TPP (w/w/w)	Size (nm)	Polydispersity	ζ (mV)
CS/TPP 4/1	361 ± 20	0.36-0.44	+35.1 ± 0.3
CS/ HP-β-CD-C1/ TPP 4/ 3/ 0.33/ 1.0	340 ± 4	0.30-0.35	+27.8 ± 1.4
CS/ HP-β-CD / TPP 4/ 3/ 1.0	398 ± 2	0.28-0.31	+32.8 ± 0.4
CS/ HP-β-CD- C1/ TPP 4/ 6/ 0.67/ 1.0	291 ± 5	0.26-0.29	+27.4 ± 0.3
CS/ HP-β-CD / TPP 4/ 6/ 1.0	383 ± 5	0.30-0.33	+33.6 ± 1.2
CS/ HP-β-CD- C1/ TPP 4/ 12/ 1.34/ 1.0	260 ± 5	0.29-0.34	+35.5 ± 1.0
CS/ HP-β-CD/ TPP 4/ 12/ 1.0	360 ± 4	0.28-0.32	+31.0 ± 0.5

Table 1a. Physicochemical properties of *CS/HP-β-CD/C1/TPP* NPs and their respective control formulations (NPs without C1), means ± S.D., *n* = 3.

Ratio CS/CD/C/TPP (w/w/w)	Size (nm)	Polydispersity	ζ (mV)
CS/TPP 4/1	361 ± 20	0.36-0.44	+35.1 ± 0.3
CS/ HP-β-CD-C2/ TPP 4/ 0.76/ 0.13/ 1.0	322 ± 9	0.34-0.38	+34.3 ± 0.3
CS/ HP-β-CD / TPP 4/ 0.76/ 1.0	335 ± 7	0.27-0.39	+31.0 ± 1.8
CS/ SBE-β-CD-C2/ TPP 4/ 1/ 0.13/ 0.5	375 ± 19	0.23-0.28	+33.9 ± 2.9
CS/ SBE-β-CD/ TPP 4/ 1/ 0.5	b	-	-

b = transparent solution

Table 1b. Physicochemical properties of *CS/HP-β-CD/C2/TPP* and *CS/SBE-β-CD/C2/TPP* NPs and their respective control formulations (NPs without C2), means ± S.D., *n* = 3.

Ratio CS/CD/C/TPP (w/w/w)	Size (nm)	Polydispersity	ζ (mV)
CS/TPP 4/1	361 ± 20	0.36-0.44	+35.1 ± 0.3
CS/ SBE-β-CD- C1/ TPP 4/ 1/ 0.08/ 0.5	381 ± 5	0.23-0.30	+31.5 ± 1.7
CS/ SBE-β-CD / TPP 4/ 1/ 0.5	b	-	-
CS/ SBE-β-CD- C1/ TPP 4/ 2/ 0.17/ 0.5	290 ± 7	0.22-0.28	+33.6 ± 1.6
CS/ SBE-β-CD / TPP 4/ 2/ 0.5	337 ± 6	0.37-0.39	+31.4 ± 1.0

b = transparent solution

Table 2. Physicochemical properties of *CS/SBE-β-CD/C1/TPP* NPs and their respective control formulations (NPs without C1), means ± S.D., *n* = 3.

It is well known that the NPs size largely depends on the cyclodextrins nature (Maestrelli et al., 2006; Trapani et al., 2008).

In particular, data reported in the tables confirmed that the presence of HP-*β*-CD has no critical impact in the NP formation process and, therefore, no particular limit of CD amount needs to be respected for the preparation of these NPs, while SBE-*β*-CD can readily interact with CS, and an excess of cyclodextrin can lead to aggregates formation (data were not reported). In the case of nanoparticles based on HP-*β*-CD, the sizes decreased with the increase of the cylcodextrin amount, probably due to the formation of a more compact structure. For SBE-*β*-CD nanoparticles a reduction in size was also evident; this behavior can be explained with the formation of ionic interaction between chitosan and the anionic cyclodextrin that lead to smaller particles size, as demonstrated in our previous work (Trapani et al., 2008).

Tables 1a and 2 report the sizes, polydispersity and potential zeta of nanoparticles with C1, in which it was possible to relate *CS/CDs/C1/TPP* NPs with the respective control NPs. In particular, for nanoparticles based on HP-*β*-CD, a decrease in size (p < 0.05) from *CS/CDs/TPP* to *CS/CDs/C1/TPP* was observed. This behavior has been attributed mainly to the presence of C1 that in the nanoparticles suspensions, was negatively charged and could interact with the positively charged amino group of chitosan; this ionic interaction could lead to the formation of a more compact structure. For SBE-*β*-CD

nanoparticles, *CS/CDs/C1/TPP* also showed a lower size than the control nanoparticles (without C1). In this case, the reduction in size of *CS/CDs/C1/TPP* was mainly attributed to the presence of the anionic cyclodextrin that can interact with chitosan, thus reducing nanoparticle size in a greater way with respect to HP-*β*-CD that weakly interacts with chitosan.

In the case of NPs containing HP-*β*-CD and C2 (table 1b), the low amount of C2 contained in the formulation did not provide a significant reduction in nanoparticles size. On the other hand, nanoparticles based on SBE-*β*-CD and C2 showed size in the nanometer range, but control preparations lead to transparent solutions.

NPs composed of SBE-β-CD can be prepared also without TPP by direct incorporation of the *SBE-β-CD/C* solutions into the CS phase; in this case it was possible to incorporate a greater amount of cyclodextrin, because the cyclodextrin/TPP cooperative effect in the ionic interaction was absent. Moreover, we also observed a reduction of nanoparticles size with the increase of cyclodextrin and *C* amounts (data not showed).

With respect to the zeta potential of the four series of NPs, it was interesting to observe that all the series exhibited positive charge values (tables 1a, 1b and 2). The fact that all the NPs showed positive zeta potential suggests that CDs and also the excipients *C* are mainly entrapped inside the NP matrix and do not mask the inherent charge of CS on the surface. Moreover, for NPs containing HP-*β*-CD/C1 a reduction of zeta potential was observed with respect to control formulations, probably due to the presence of the negative charges of C1 that, interacting with chitosan, reduced the positive charge of the surface.

In all cases, the size, ranging between 260 and 398 nm, can allow nasal administration and promote an efficient drug transport through biological barriers, while the presence of a positive surface can improve the interaction between the formulations and the mucosal surface, ensuring a good mucoadhesion properties.

Loaded NPs (table 3) showed a higher size with respect to unloaded NPs probably due to the incorporation of insulin inside the NPs, while a zeta potential decrease was observed. This zeta potential reduction could be explained by the interaction between CS and insulin that leads to a positive charge reduction in the NPs surface.

Ratio CS/CD/C/TPP (w/w/w)	Size (nm)	Polydispersity	ζ (mV)	EE%	Loading %
CS/ HP-β-CD- C1/ TPP 4/ 12/ 1.34/ 1.0	308 ± 2	0.28-0.33	+27.6 ± 0.6	89.5 ± 1.1	25.3 ± 1.1
CS/ HP-β-CD-C2/ TPP 4/ 0.76/ 0.13/ 1.0	340 ± 3	0.29-0.32	+30.7 ± 0.6	71.5 ± 5.2	19.3 ± 0.7
CS/ SBE-β-CD-C1/ TPP 4/ 2/ 0.17/ 0.5	370 ± 4	0.19-0.27	+29.7 ± 0.9	46.7 ± 4.3	6.1 ± 0.4
CS/ SBE-β-CD-C2/ TPP 4/ 1/ 0.13/ 0.5	387 ± 7	0.23-0.29	+30.5 ± 1.0	21.0 ± 1.1	7.1 ± 0.3

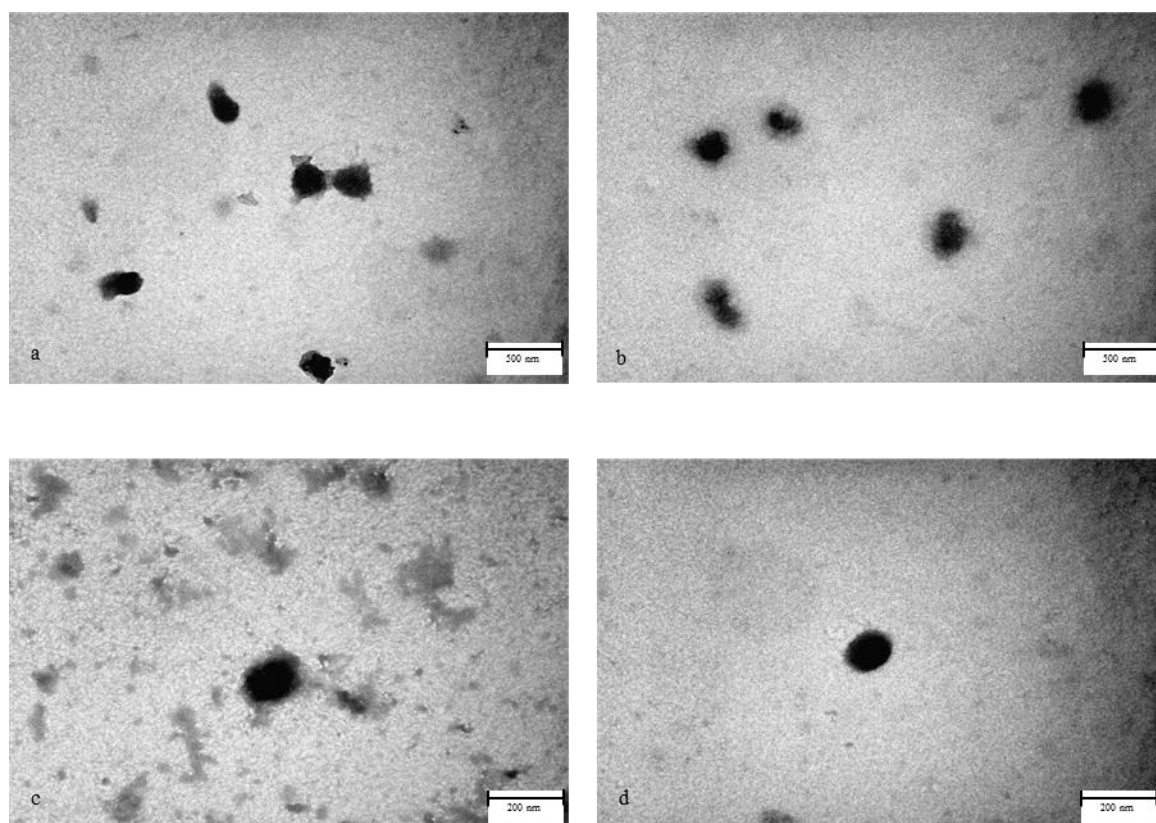Table 3. Physicochemical properties, EE% and Loading % of loaded nanoparticles (means±S.D., *n*=3).

Fig. 1. Morphological Characterization performed by transmission electron microscopy of blank NPs: *CS/ HP-β-CD/C1/TPP* (*4/12/1.34/1*) (a/c) and *CS/SBE- β-CD/C1/TPP* (*4/2/0.17/0.5*) (b/d).

Fig. 1 shows the morphological appearance of *CS/CD/C1/TPP* NPs.In general, particles size measured by TEM is smaller than that determined by photon correlation

spectroscopy, which is attributed to the loss of water during the drying step prior to electron microscopy. It can also be observed that NPs containing HP-β-CD showed an irregular morphology (figure 1(a/c)), whereas those containing SBE-β-CD exhibits a round shape (figure 1(b/d)).

3.3 Stability in different media

In order to evaluate whether nanoparticles size changed in the different tested media and to predict their suitability for administration, NPs stability was investigated. Fig. 2 shows the stability of the following nanoparticles composed of C1: *CS/HP-β-CD/C1/TPP 4/12/1.34/1.0*, *CS/SBE-β-CD/C1/TPP 4/2/0.17/0.5*, *CS/SBE-β-CD/C1/TPP 4/4.3/0.33/0.*
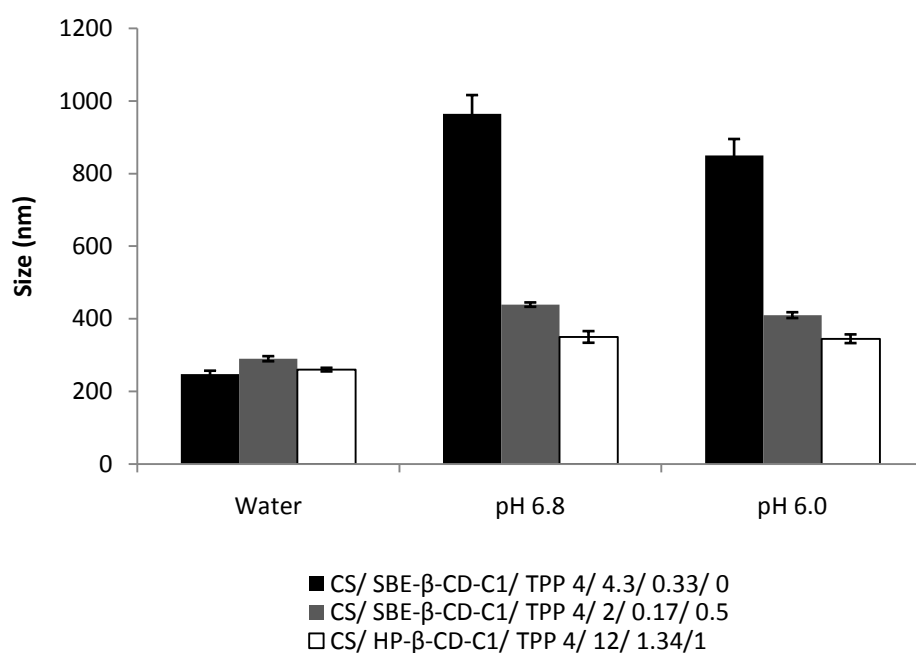

Fig. 2. Stability of nanoparticles composed of C1 in different media (mean ±SD., n=3)

As can be seen, all NPs were stable in water with small changes in size and DI values (PDI values are not reported). Furthermore, NPs with TPP were also stable in phosphate buffer at pH 6.8 and 6.0, while NPs without TPP aggregate and cannot be used for nasal administration. For this reason, for the next studies we selected NPs with the higher amount of *C* and with TPP.

3.4 Determination of Process Yield

In order to study the influence of *C* on drug absorption through nasal mucosa, we selected the formulations with the higher amount of *C*. In table 4 we report the selected nanoparticles and the results of the process yield determination.

Ratio CS/CD/C/TPP (w/w/w)	Process yield (%)	Theoretical C content(%)	Experimental C content (%)
CS/ HP-β-CD-C1/ TPP 4/ 12/ 1.34/ 1.0	11 ± 1	7.29	2.89 ± 0.14
CS/ HP-β-CD-C2/ TPP 4/ 0.76/ 0.13/ 1.0	26 ± 2	2.28	0.17 ± 0.02
CS/ SBE-β-CD-C1/ TPP 4/ 2/ 0.17/ 0.5	42 ± 2	2.56	3.53 ± 0.17
CS/ SBE-β-CD-C2/ TPP 4/ 1/ 0.13/ 0.5	24 ± 1	2.36	0.58 ± 0.05

Table 4.   Yield percentage for the prepared   nanoparticles and theoretical and experimental amount of C in nanoparticles (mean ± S.D., $n = 3$).

As can be seen, for NPs containing HP-*β*-CD the yield was lower with respect to nanoparticles containing SBE-*β*-CD. In fact, HP-*β*-CD weakly interact with chitosan and its incorporation efficiency within the NPs was very low (about the 3% as demonstrated in another study (Trapani et al., 2008). In this work, we can also suppose that *C* were included in the hydrophobic cavity of the cyclodextrinandthat their extremities, negatively charged, was located outside of the cyclodextrin andcan interact with chitosan. For this reason, we hypotized that also the incorporation efficiency of *C* was lower for NPs based on HP-*β*-CD with respect to SBE-*β*-CD nanoparticles. On the other side, the ionic interaction between chitosan and SBE-*β*-CD can lead to the formation of a great number of precipitation nuclei, increasing the incorporation efficiency of the cyclodextrin and consequently, of the excipients C, and leading to a greater process yield.

3.5 Determination of excipients C experimental amount

Table 4 reports the theoretical amount of *C* with respect to the total amount of nanoparticle components, while the *C* experimental amount was correlated to the weight of nanoparticles obtained after ultracentrifugation. As can be seen, in SBE-β-CD

nanoparticles, the amount of *C* was higher compared to that of HP-*β*-CD nanoparticles. As described in section 3.4, the incorporation efficiency of HP-*β*-CD was around the 3% and lead to a low process yield; for the same reason, the experimental amount of *C*, that was incorporated in the hydrophobic cavity of the cyclodextrin, was lower with respect to the theoretical amount. Moreover, the experimental content % of C1 was higher with respect to that of C2, although for Nps with HP-*β*-CD/C2 the process yield was higher than the yield of HP-*β*-CD/C1 NPs, thus demonstrating an higher incorporation capacity of C1. For the same reason, C1 was also mainly incorporated in nanoparticles with SBE-β-CD with respect to C2; moreover, SBE-β-CD incorporation efficiency was very high through its interaction with chitosan and this aspect can allow to incorporate a great amount of C1 into nanoparticles.

We concluded that the presence of the excipients in the formulations was influenced by the incorporation efficiency of cyclodextrin and by the capacity of the excipients to be included in the cyclodextrins and consequently, into the nanoparticles. In particular, data demonstrated that C1 can be incorporated in a higher amount inside the nanoparticles, with respect to C2. Moreover, in the case of SBE-β-CD/C1 nanoparticles, this aspect, together with the high incorporation efficiency of cyclodextrin, led to a greater C1 experimental amount with respect to that theoretical.

3.6 Loading and encapsulation efficiency of nanoparticles

In the table 3 the loading % and the encapsulation efficiency of loaded nanoparticles were reported. As described in our previous study (Fernandez-Urrusuno et al., 1999), the association of insulin to chitosan nanoparticles is primarily based on electrostatic interaction such as hydrophobic interactions. As can be seen, nanoparticles with HP-*β*-CD showed a very high encapsulation efficiency (>70%), leading to insulin loading values of 25%. Moreover, as discussed before, the incorporation efficiency of cyclodextrin and the *C* experimental amount were lower in the case of HP-*β*-CD based *NPs* than *SBE-β-CD* NPs; this aspect leads to a greater presence of chitosan in HP-*β*-CD *NPs*that can interact with insulin. On the other hand, the insulin encapsulation efficiency in NPs composed of *SBE-β-CD* was lower; in fact, the anionic cyclodextrin and *C*, included in cylcodextrin, can interact with chitosan leading to a reduction of positive charges, able to bind insulin.

3.7. In vitro release studies

In order to study the influence of the excipients *C* on drug release, we tested nanoparticles with and without *C*. In particular, fig. 3 shows the insulin amount released in phosphate buffer at pH 6.0 during 2 hours from *CS/SBE-β-CD/C1/TPP (4/2/0.17/0.5)* and*CS/SBE-β-CD/TPP (4/2/0.5)*.
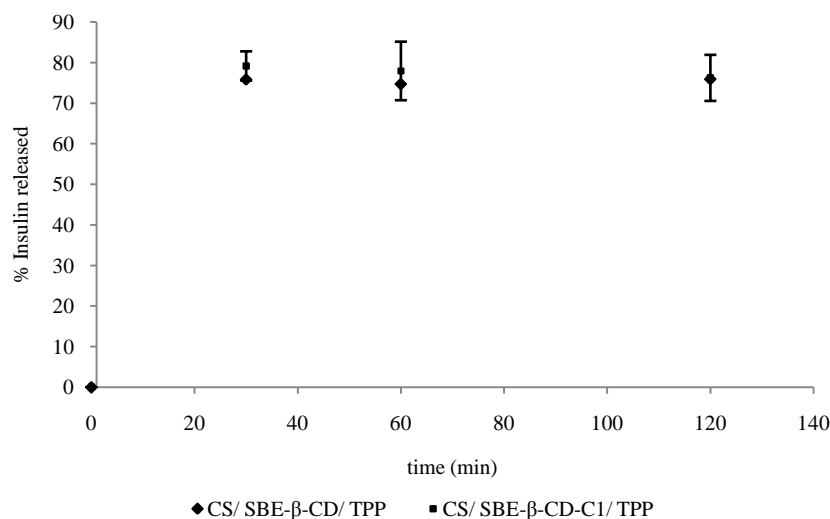

Fig. 3. Insulin release from loaded nanoparticles *CS/SBE-β-CD/CA/TPP* (*4/2/0.17/0.5*) and *CS/SBE- β-CD/TPP* (*4/2/0.5*) in phosphate buffer, pH 6.0, 37 ∘C (means ± S.D., *n* = 3).

As can be seen, the 80 % of loaded insulin was rapidly released from nanoparticles, confirming that the insulin release depends on a simple dissociation mechanism based on the ionic interaction between the chitosan amino group and insulin negative charges. Significant difference between formulations with C1 and without C1 can not be found.

3.8. In Vivo Studies

Fig. 4 shows the reduction of plasma glucose concentration after intranasal administration of different formulations. In particular, the figure reports the plasma glucose levels achieved following administration of loaded *CS/SBE-β-CD/C1/TPP* and*CS/SBE-β-CD/TPP* NPs, unloaded *CS/SBE-β-CD/C1/TPP* NPs as well as insulin solution instilled to conscious rats.
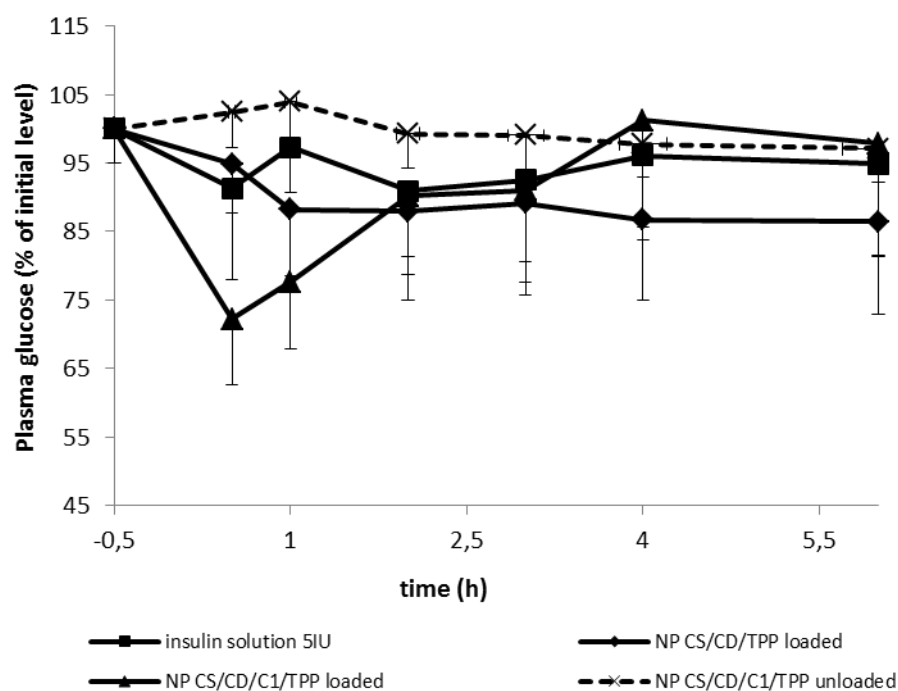
Fig. 4. Plasma glucose levels achieved in rats following nasal administration of: insulin solution, *CS/SBE-β-CD/C1/TPP* (*4/2/0.17/0.5*) unloaded nanoparticles and *CS/SBE-β-CD/C1/TPP* (*4/2/0.17/0.5*) and *CS/SBE- β-CD/TPP* (*4/2/0.5*) loaded nanoparticles (mean ± S.D, n=6).

As shown in the figure 4, the administration of unloaded nanoparticles to rats did not provide any modification in plasma glucose levels during the experimental period, thus indicating the absence of stress that could be caused by the administration procedure or the blood sampling. The same figure shows that the administration of insulin control solution (5IU/Kg) resulted in a decrease blood levels less than 10 % at 30 min post administration. The blood glucose level fell to 87 % basal levels when insulin was associated with *CS/SBE-β-CD/TPP* NPs, confirming CS/CD nanoparticles properties to promote the nasal insulin absorption. In particular, chitosan ability to adhere to the mucosa and to transiently open the tight junctions and cyclodextrins properties to protect drugs from enzymatic degradation and to disrupt the epithelial membrane by extraction of phospholipids and proteins (Marttin et al., 1998; Fernandez-Urrusuno et al., 1999; Teijeiro- Osorio et al., 2009) can improve insulin absorption. Moreover, these results indicate that the nanoparticles preparation method not influenced insulin structure and activity.

Interestingly, a greater response was achieved after the administration of insulin loaded *CS/SBE-β-CD/C1/TPP* NPs. In fact, the blood glucose concentration decreased to about 72% respect to the baseline levels at 30 minutes post-administration. This plasma glucose concentration reduction was the maximum and it was significantly different from that induced by the insulin control solution at least 1 hour and from that induced by nanoparticles without C1 at least 30 minutes post-administration. Indeed, the presence of C1 in nanoparticles could improve the pharmacological effect of the formulation, probably due to the bioavailability enhancement effect of C1.

It is also interesting to note that the decrease in plasma glucose level obtained in this study with the administration of *CS/SBE-β-CD/TPP* loaded nanoparticles was lower with respect to the decrease observed with a similar formulation (*CS/SBE-β-CD/TPP4/3/0.25*) in our previous study (Teijeiro- Osorio et al., 2009). These differences can be explained by the different experimental approach: different animal model (rabbits vs. rats) and different insulin (insulin from bovine pancreas vs. Zn-free human insulin).

According to our previous studies, we can summarize the interesting results obtained when insulin was associated with CS/CD and CS/CD/C/TPP nanoparticles. First, CS/CD nanoparticles can enhance the absorption of macromolecules, such as insulin, thanks to chitosan and cyclodextrin ability to adhere to the epithelium and to open the tight junctions, to disrupt the epithelial membrane, as well as to protect drug from enzymatic degradation. Second, as demonstrated in this study, the presence of C1 in the formulation can promote a systemic absorption of insulin, leading to a reduction in plasma glucose level that was significant different from that induced by the same formulation but without C1.

On the other hand, in our previous works, we hypothesized another mechanism that can influence drug nasal absorption. In fact, these nanocarriers can act as a true macromolecule carriers, internalizing in the nasal epithelium (as shown in the CLSM cross section of a previous work, Teijeiro- Osorio et al., 2009), thus operating simultaneously with the mucoadhesion and penetration enhancements mechanisms.

4.Conclusions

In this work, CS/CD nanoparticles containing two excipients, C1and C2, able to improve insulin bioavailabilitywere prepared. Nanoparticles showed size in the nano range and a positive charge due to the presence of chitosan, that allows the interaction with the nasal epithelium. Nanoparticles with *SBE-β-CD* and C1 showed the higher content of the excipient C1 and interesting properties in terms of encapsulation efficiency and drug release. *In vivo* studies showed that the presence of the C1 modify insulin bioavailability, as demonstrated by the significant glucose decrease in the plasma rats.

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Concluding remarks

Chitosan represents a multifunctional polymer, featuring both mucoadhesive and permeation-enhancing properties and therefore is a widely studied excipient for transmucosal drug delivery. Chitosan have been used for the preparation of gels, solid inserts, powders and nanoparticles in which ionic interactions with anionic molecules can be recognized. This thesis have contributed to a deeperinvestigation of the properties of some chitosan based formulations.

In particular, the paper 1 have demonstrated that chitosan and gelatin can be successfully employed for the formulation of buccal films that ensure the necessary dosefor treatment of hypertension and atrial fibrillation.Moreover, along with adequate drug release and permeation, desirable film characteristics such as suitable hydration, film tolerability and compatibility with buccal mucosaand mucoadhesion, were obtained.

In the paper 2, polyelectrolyte complexes between chitosan and sodium alginate were obtained in the vicinity of the pKa interval of the two polymers. The complexes were able to hydrate and adhere to vaginal mucosa and can be used to prepare vaginal inserts for local deliver of chlorhexidine digluconate. The selection of the appropriate chitosan/sodium alginate molar ratio as well as the drug amount allowed the modulation of insert water-uptake behavior and chlorhexidine digluconate release and provided the inhibition of the principal pathogens responsible of aerobic vaginitis and candidiasis.

In the last paper, CS/CD nanoparticles loaded with insulin and containing two excipinets with the capacity to modify the bioavailabity of drug, C1 and C2, were prepared and characterized. Results demonstrated that nanoparticles with *SBE-β-CD* and C1 showed interesting properties in terms of encapsulation efficiency and drug release. *In vivo* studies showed that the presence of C1 improve insulin bioavailability, as demonstrated by the significant glucose decrease in the plasma rats.

I hope that these works will stimulate further investigations in the fieldtransmucosal drug delivery using chitosan based formulations and thatresearchers will collect further data and concrete clinical perspectives for a real application in the pharmaceutical industry.