OBTAINING CHITOSAN MICROSPHERES ON SUPERHYDROPHOBIC SURFACES FOR CONTROLLED RELEASE OF 5-FLUOROURACIL

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Hydrogels are three-dimensional networks consisting of hydrophilic polymers that are crosslinked. They are able to absorb large amounts of water or biological fluids, but they are prevented from dissolving due to the presence of crosslinks. Because of their high water content, they resemble biological tissue (Bhattarai et al., 2010; Peppas et al., 2000). Hydrogels are suitable as drug delivery systems because they are able to deliver the drug at the desired site and rate. Generally, they release the drug when swollen and when they shrink, release slows down or even stops (Alvarez-Lorenzo and Concheiro, 2008).

The main aim of this work was to obtain microspheres for the controlled release of 5-fluorouracil (5-FU). Microspheres were obtained by a biomimetic method that involved polystyrene superhydrophobic surfaces, where hydrogel spheres are formed at the solid-air interface (Song et al., 2010). Chitosan (QT) was used as the main component of the microspheres. It is a natural polysaccharide that has unique properties like a cationic nature, low toxicity and high biocompatibility (Bhattarai et al., 2010). The microspheres were loaded with 5-FU, which is an anti-cancer drug that is widely used, but that can cause severe side effects. Controlled release systems could be useful to keep the concentrations of 5-FU at a low level, so that side effects can be reduced (Rokhade et al., 2007).

In an attempt to obtain controlled release, QT was successfully combined with EUDRAGIT® E PO, EUDRAGIT® NE 30D or EUDRAGIT® RS 30D. Release studies with these formulations indicated that the latter two were not useful to obtain controlled delivery due to the high burst release in all three media (MilliQ® water, HCl 1% and phosphate buffer pH 7.4). Formulations with EUDRAGIT® E PO seemed to show a lower burst release, but when cytotoxicity assays were performed, it was seen that these formulations were highly toxic, and therefore could not be used for oral delivery of 5-FU. Release studies showed that there were no significant differences between QT particles that were crosslinked 1 hour and particles that were crosslinked 4 hours. Cytotoxicity assays indicated that QT particles showed acceptable toxicity and that 5-FU is efficient as an anti-cancer drug as it killed around 50% more cancer cells than formulations without drug. Further investigations are necessary to try to decrease the toxicity of the particles that contain EUDRAGIT® E PO and to obtain further controlled release, as these particles only decreased the burst release after 15min in HCl 1% and phosphate buffer pH 7.4.
SAMENVATTING

Hydrogels zijn driedimensionale netwerken die bestaan uit gecrosslinkte hydrofiele polymeren. Ze kunnen grote hoeveelheden water of biologische vloeistoffen opnemen, maar lossen niet op door de aanwezigheid van crosslinks. Door hun hoge watergehalte lijken ze op biologisch weefsel (Bhattarai et al., 2010; Peppas et al., 2000). Hydrogels zijn geschikt als drug delivery systemen omdat ze de drug met de gewenste snelheid op de gewenste plaats kunnen afleveren. In het algemeen stellen ze het geneesmiddel vrij als ze gezwollen zijn en verminderd of stoppen de vrijlating wanneer ze krimpen (Alvarez-Lorenzo and Concheiro, 2008).

Het voornaamste doel van dit werk was de productie van microsferen op basis van chitosan (QT) voor gecontroleerde vrijstelling van 5-fluorouracil (5-FU). Dit werd nagestreefd met behulp van een biomimetische methode, die gebruik maakt van polystyreen superhydrofobische oppervlakken waar sferen gevormd werden aan het scheidingsvlak tussen lucht en vaste stof (Song et al., 2010). QT is een natuurlijk polysaccharide met unieke eigenschappen, zoals een kationische natuur, lage toxiciteit en hoge biocompatibiliteit (Bhattarai et al., 2010). De microsferen werden geladen met 5-FU, dat wereldwijd wordt gebruikt tegen kanker maar ernstige neveneffecten kan veroorzaken. Systemen die zorgen voor vertraagde vrijstelling zouden nuttig kunnen zijn om de concentratie laag te houden, zodat neveneffecten herleid kunnen worden tot een minimum (Rokhade et al., 2007).

Vertraagde vrijstelling werd nagestreefd door partikels, die QT combineren met EUDRAGIT® E PO, EUDRAGIT® NE 30D of EUDRAGIT® RS 30D, te bereiden. Release studies gaven aan dat de laatste twee variëteiten niet gebruikt konden worden, vanwege een hoge burst release in zowel MilliQ® water, HCl 1% als fosfaatbuffer pH 7.4. Formulaties met EUDRAGIT® E PO toonden een lagere burst release, maar tijdens cytotoxiciteit studies werd hoge toxiciteit waargenomen, waardoor deze partikels niet gebruikt kunnen worden voor toediening van 5-FU. Release studies toonden geen verschil in vrijstellingsprofielen tussen partikels die 1 uur en 4 uur gecrosslinkt zijn. Cytotoxiciteit studies toonden dat de toxiciteit van QT partikels aanvaardbaar was en dat 5-FU efficiënt was als anti-kanker geneesmiddel omdat geladen QT partikels ongeveer 50% meer cellen dooden dan partikels zonder drug. Verder onderzoek is nodig om de toxiciteit van de partikels die EUDRAGIT® E PO bevatten te verminderen en om meer gecontroleerde vrijstelling te krijgen omdat de partikels enkel een verlaagde burst release vertonen na 15min in HCl 1% en fosfaatbuffer pH 7.4.
First of all, I would like to thank Prof. Carmen Alvarez-Lorenzo and Prof. Angel Concheiro Nine for welcoming me in their laboratory for 3.5 months. Thank you for revising my work so many times and for giving me great advice when I had troubles or doubts.

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Lotte
USED ABBREVIATIONS

5-FU: 5-Fluorouracil
CA: Contact Angle
DNA: Deoxyribonucleic acid
DMEM: Dulbecco’s modified eagle medium
dATP: Deoxyadenosine triphosphate
dCTP: Deoxycytidine triphosphate
dGTP: Deoxyguanosine triphosphate
dTMP: Deoxythymidine monophosphate
dTTP: Deoxythymidine triphosphate
dUMP: Deoxyuridine monophosphate
dUTP: Deoxyuridine triphosphate
FBS: Fetal Bovine Serum
FdUMP: Fluorodeoxyuridine monophosphate
FdUTP: Fluorodeoxyuridine triphosphate
FUTP: Fluorouridine triphosphate
GI tract: Gastro-intestinal tract
IC50: Half maximum inhibitory concentration
IPN: Interpenetrating polymeric network
MES: 2-(4-morpholine)ethanesulfonic acid
NEAA: Non-essential amino-acids
PB7.4: Phosphate buffer pH 7.4
PS: Polystyrene
RNA: Ribonucleic acid
SEM: Scanning Electron Microscope
TS: Thymidylate synthase
UV/VIS: Ultraviolet/Visible
VCRF: Variation coefficient of the response factors
QT: Chitosan
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1. INTRODUCTION

1.1. HYDROGELS

1.1.1. General information

Hydrogels are three-dimensional networks consisting of crosslinked polymers that have a high number of hydrophilic groups or domains (Hoare and Kohane, 2008; Bhattarai et al., 2010). They are able to absorb large amounts of water or biological fluids but they are prevented from dissolving due to the presence of crosslinks (Peppas et al., 2000). When water penetrates the network, swelling occurs (Bhattarai et al., 2010). Because fully swollen hydrogels resemble biological tissue, they have a wide range of applications in clinical practice and experimental medicine. Their biocompatibility makes them suitable as contact lenses, membranes for biosensors or drug delivery devices (Peppas et al., 2000; Hoare and Kohane, 2008).

To characterize the network structure of hydrogels, three important parameters are used: molecular weight of the polymer chain between two neighboring crosslinking points, the corresponding mesh size and the polymer volume fraction in the swollen state (Peppas et al., 2000).

One of the most important factors to regulate the swelling of hydrogels is the crosslinking ratio, which is the ratio of moles of crosslinking agent to the moles of polymer repeating unit. The more hydrogels are crosslinked, the tighter their structure becomes and the less they will swell compared to hydrogels with lower crosslinking ratios. Swelling also depends on the chemical structure of the polymer. Hydrophobic groups collapse in the presence of water. As a result, these hydrogels swell less than hydrogels containing hydrophilic groups (Peppas et al., 2000). In addition to the hydrophilic/hydrophobic balance and the degree of crosslinking, the degree of ionization and interaction with counterions are important parameters which control swelling, dimensional change and the release pattern of drugs out of the hydrogels (Ganji et al., 2010).

Hydrogels can act as a depot formulation where drug loading and drug release occur at a rate dependent on the diffusion coefficient (Hoare et al., 2008). Some hydrogels have extra
features that make them excellent drug delivery vehicles, for example muco- and bioadhesive characteristics that enhance residence time (Bhattarai et al., 2010).

1.1.2. Crosslinking

Stabilizing linkages are formed between the functional moieties of hydrogel polymers in order to prevent the dissolution of the matrix and to maintain the mechanical integrity (Bhattarai et al., 2010; Lima et al., 2012). Polymer binding is either accomplished by non-covalent physical associations or by covalent chemical bonds. The main difference is that physical associations are reversible and covalent crosslinks between polymer chains are not (Bhattarai et al., 2010).

1.1.2.1. Physical crosslinking

In physically crosslinked gels, dissolution is prevented by physical interactions between different polymer chains. An increased interest in physical or reversible hydrogels has been seen because of the ease of production and because the use of crosslinkers is avoided (Hennink and van Nostrum, 2001; Gulrez et al., 2011).

A first method to obtain physically crosslinked hydrogels is by heating/cooling a polymer solution. For example in the case of polyethylene oxide-polypropylene oxide block copolymers, simply warming the polymer solutions causes aggregation of the polymer chains and thus hydrogel formation (Gulrez et al., 2011).

Ionic polymers can undergo ionic interactions when di- or tri-valent counterions are added, leading to the formation of the hydrogel (FIGURE 1.1). An example is the reaction of the anionic groups of alginate (COO−) with Ca2+ (Gulrez et al., 2011).

Another method of physical crosslinking is complex coacervation. In this case, hydrogels are formed by mixing a polyanion with a polycation. Because these polymers have opposite charges, they stick together and form soluble and insoluble complexes depending on the pH and the concentration of the polymers (FIGURE 1.1) (Gulrez et al., 2011).
FIGURE 1.1 Hydrogel formation by ionic interactions with di- or trivalent counterions and by complex coacervation (Hoffman, A.S., 2002).

Another case are polymers containing carboxyl groups in their structure such as carboxymethylcellulose. A decrease in the pH of an aqueous solution of these polymers leads to the replacement of sodium with hydrogen. This way, hydrogen bonds are promoted and a hydrogel is formed. (FIGURE 1.2) (Gulrez et al., 2011).

FIGURE 1.2 Hydrogel network formation due to intermolecular H-bonding in carboxymethylcellulose at low pH (Gulrez et al., 2011).

1.1.2.2. Chemical crosslinking

In chemically crosslinked gels, dissolution is prevented by the formation of covalent, irreversible bonds between different polymer chains (Hennink and van Nostrum, 2001). Covalently bonded networks can be synthesized based on two approaches: simultaneous crosslinking and polymerization or crosslinking after synthesis of polymer chains. Crosslinking during polymerization can occur through two different mechanisms: condensation and addition (Osada, 2001).
Condensation involves multifunctional monomers that are able to form crosslinked networks. Links, such as amide or ester bonds, are formed between the functional groups of monomers. During the formation of these links, small molecules, such as water, are eliminated. (Mishra, 2000; Osada, 2001).

\[
A+B \rightarrow C+H_2O
\]

In the addition mechanism, double bonds are necessary because they give rise to free radicals that are required to produce the crosslinked network. Addition occurs between monomers that are the same (Mishra, 2000).

\[
R_1=CH_2 + R_1=CH_2 \rightarrow R_1-CH_2-R_1=CH_2
\]

Three stages can be distinguished: initiation, propagation and termination. During the initiation phase, double bonds are converted into free radicals (FIGURE 1.3). Based on the way the free radicals are obtained, different polymerization methods, like thermal polymerization or radiation polymerization, are distinguished. In the propagation stage, these free radicals interact with new monomer units (FIGURE 1.4). Propagation progresses into termination when an inactive covalent bond is formed. The most important way in which this happens, is the one where two active chain ends interact. This can happen in two different ways: combination or disproportionation (FIGURE 1.5) (Mishra, 2000; Sellergren, 2001).

![Initiation stage of polymerization of methylmethacrylate (Sellergren, 2001).](image)
When the same mechanism occurs between two different monomers, the reaction is referred to as copolymerization (Mishra, 2000).

\[ R_1=CH_2 + R_2=CH_2 \rightarrow R_1-CH_2-R_2=CH_2 \]

Crosslinking can also occur after polymerization, using condensation or high energy crosslinking. In the condensation reaction, functional groups of the polymers react with each other, thus forming a crosslinked network. In high energy crosslinking, \( \gamma \)-rays are used to create polymer free radicals and couple the polymers (Osada, 2001).

### 1.1.3. Classification

Hydrogels can be classified in many different ways. First of all they can be classified according to the nature of the side chain into neutral or ionic. As mentioned above, they can also be classified in chemical and physical gels. Based on the method of preparation they can be classified into homopolymer or copolymer networks. Furthermore they can be classified according to their mechanical and structural characteristics or based on the physical structure of the network (amorphous, semicrystalline, ...). When release out of the hydrogel is dependent on the environment, the polymers are called stimuli-responsive hydrogels (Peppas et al., 2000). Based on drug release, hydrogels can be classified as swelling-controlled, diffusion-controlled or chemically-controlled release systems (Bhattarai et al., 2010). The different kinds of release systems are explained in section 1.1.7.
1.1.4. Different shapes of hydrogels

Hydrogels can be divided in macroscopic networks or confined to smaller dimensions such as microgels, which are crosslinked polymeric particles, or nanogels, which are microgels in the submicron range (FIGURE 1.6) (Oh et al., 2008).

![FIGURE 1.6 Structure of hydrogels (Alvarez-Lorenzo and Concheiro, 2008).](image)

Microgels/nanogels offer unique advantages for the development of drug delivery systems using polymers, such as tunable size from nanometers to several micrometers and a large surface area (Oh et al., 2008). Due to their high specific area, mass transfer limitations are avoided during the loading of bioactive substances. It also enables a fast response to environmental stimuli and thus allows a fine regulation of the drug release rate (Lima et al., 2011).

Several techniques have been proposed to produce polymeric particles that contain bioactive substances. The applicability of these techniques is limited by the need of using organic/toxic solvents, high temperature and low encapsulation efficiency. To avoid these complications, superhydrophobic surfaces can be used (Lima et al., 2011).

1.1.5. Stimuli-responsive hydrogels

Hydrogels that respond to stimuli, internal or external to the body, can exhibit dramatic changes in their swelling behavior, network structure, permeability or mechanical strength in response to those stimuli (FIGURE 1.7). Stimuli can be physical stimuli (e.g. ion strength and pH), chemical stimuli (e.g. glucose or morphine) or external stimuli (e.g. magnetic or electric field) (Gupta et al., 2002). When working with stimuli-responsive networks, the crosslinking degree should be low enough to enable the network to undergo remarkable conformational changes in response to stimuli, but it should be high enough to provide mechanical stability to the network so that it can maintain its functionality after several cycles of changes in the environment (Alvarez-Lorenzo and Concheiro, 2008).
1.1.6. Drug loading

Diffusion, entrapment and tethering are the three major approaches to drug loading (FIGURE 1.8). The easiest method is to place the fully formed hydrogel into a medium that is saturated with the drug. The drug will diffuse slowly into the gel with a rate dependent on the porosity of the hydrogel, the size of the drug and the chemical interactions of drug and hydrogel. This method works well on small molecules but larger therapeutics are not able to migrate through the small pores of the hydrogel. In that case it is better to entrap the drug during the gelation process. Therefore, the drug is mixed with the polymer solution and the crosslinker is added. Because diffusion and entrapment allow free movement of the drug out of the hydrogel network, an initial burst release is often observed. The loss of therapeutic reserve and the risk of toxic exposure can be reduced by using the tethering method. Drugs can be covalently or physically linked to the polymer chains prior to gelation. In this way tissue only gets exposed to the therapeutic when the hydrogel breaks down or when the molecular tether is broken (Bhattarai et al., 2010).

1.1.7. Drug release

Drugs can be released from the hydrogel due to diffusion, swelling or through chemical reactions. Diffusion is the most common release method for hydrogels. It refers to the movement of molecules according to the concentration gradient. Swelling-controlled release occurs when diffusion of the drug is faster than hydrogel swelling. Solvent molecules
penetrate in between polymer chains, thereby expanding the meshes, causing the hydrogel to swell. The dissolved drug has to wait until the mesh size is big enough to be able to move out of the hydrogel. Chemically-controlled release involves chemical reactions like dissolution (where the crosslinks are broken) or degradation (destruction of the polymer chains themselves) (Peppas et al., 2006; Bhattarai et al., 2010; Ganji et al., 2010).

1.1.8. Hydrogels and controlled drug delivery

Controlled drug delivery is used to make the required amount of drug available at the desired time and site of action in the body (Gupta et al., 2002). Generally, hydrogels release the drug when swollen and when they shrink, the release slows down or even stops (FIGURE 1.9) (Alvarez-Lorenzo and Concheiro, 2008). By controlling the swelling properties or bioadhesive characteristics, hydrogels can be used to control drug release through oral, rectal, ocular, epidermal and subcutaneous applications (Peppas et al., 2000).

![Swelling](image.png)

**FIGURE 1.9 Control of drug delivery through volume phase transition of the hydrogel. The swelling promotes the release by diffusion (Alvarez-Lorenzo and Concheiro, 2008).**

The use of intelligent hydrogels reduces the dose needed and the side effects that originate from exposure to healthy, non-targeted tissue. Targeted drug delivery to stomach and colon is very important for the treatment of local maladies such as Crohn’s disease, ulcerative colitis and carcinomas. The GI tract offers a great opportunity to pH sensitive release systems to perform controlled delivery due to the wide ranging pH microenvironments (Bhattarai et al., 2010).

1.2. CHITOSAN NETWORKS

1.2.1. Chitosan

Bhattarai et al. (2010) stated that: “Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine units.” Chitosan has primary aliphatic amines that can be protonated under acidic conditions (amine pKa is 6.3). For this reason, chitosan has a cationic nature under acidic conditions.
Characteristics of chitosan are influenced by two important parameters: molecular weight and degree of deacetylation (FIGURE 1.10) (Bhattarai et al., 2010).

![Chemical structure of chitosan](image)

FIGURE 1.10 Chemical structure of chitosan (Bhattarai et al., 2010).

Chitosan is produced by partial deacetylation under alkaline conditions of chitin (>60%), which is found in crustaceans and insects (Rinaudo, 2006; Bhattarai et al., 2010). After cellulose, chitin is the most abundant natural biopolymer. In addition to the benefits of other natural polymers, which are biocompatibility, low toxicity and susceptibility to enzymatic degradation, chitosan does not show immunogenicity and there is no risk of transmitting animal-originated pathogens (Bhattarai et al., 2010).

Due to its polymeric cationic character and its gel and film forming properties, chitosan allows the development of controlled release drug delivery systems. Because chitosan is the only pseudonatural cationic polysaccharide, it has many applications (Illum, 1998; Rinaudo, 2006). Chitosan hydrogels have been prepared with a variety of different shapes and geometrics in different formulations (e.g. beads, films, tablets, microspheres). In each preparation chitosan is physically or chemically crosslinked to form a hydrogel (Bhattarai et al., 2010).

### 1.2.2. Stimuli-responsive chitosan networks

The three most important stimuli-responsive release mechanisms concerning chitosan are pH-responsive release, enzyme-responsive release and electro-sensitive release. Hydrogels that are formed to provide pH-responsive release, react to changes in pH and ionic strength, which cause a volume phase transition that leads to drug release. In this case the chemical properties of the polymer are critical. Neutral polymers do not change their degree of swelling under acidic conditions, while anionic polymers will shrink, and chitosan, which is cationic, will swell more. Chitosan is also an excellent target for colon-specific drug delivery. Enzymes secreted by intestinal bacteria cleave the chitosan polymers, leading to
release of the loaded drug. Electric stimuli can cause swelling/shrinking of the hydrogel. In this way drug release can be controlled externally (Bhattarai et al., 2010).

1.2.3. Preparation of chitosan hydrogels

1.2.3.1. Physical crosslinking

Two conditions must be satisfied to gain the required features of a physical chitosan hydrogel. First, inter-chain interactions must be strong enough to form semi-permanent junction points in the molecular network. Second, the network should promote the access and residence of water molecules inside the polymer network. The four major physical interactions are ionic complexation, polyelectrolyte complexation, interpolymer complexation and hydrophobic associations (FIGURE 1.11) (Bhattarai et al., 2010).

![FIGURE 1.11 Networks of chitosan formed with ionic molecules, polyelectrolyte polymers and neutral polymers (Bhattarai et al., 2010).](image)

A chitosan-based physical gel can be obtained by mixing the components of the hydrogel under the appropriate conditions. Because these gels have a short life time in physiologic media, they are mainly used for short-term drug delivery. Physical association does not require any potentially toxic covalent linker molecules, which means it is usually safe for clinical application. The reason for their limited application is their weak mechanical strength and their inconsistent performance in vivo (Bhattarai et al., 2010).

1.2.3.2. Chemical crosslinking

Robust chitosan hydrogels are produced using irreversible bonds. Covalently crosslinked chitosan networks are prepared using the available –OH and –NH₂ functional groups. Condensation can be carried out using small molecules, polymer-polymer reactions, photosensitive agents or enzyme-catalyzed reactions (Bhattarai et al., 2010).
When small molecules are used, a chemical reaction occurs (e.g. Schiff base formation where the C=O double bond is replaced by a C=N double bond) to form the crosslinks. Glutaraldehyde and genipin, for example, are used as crosslinkers. The main drawback of these types of hydrogels is that the biocompatibility of many crosslinkers is unknown, while others have been found to be quite toxic (Bhattarai et al., 2010).

Polymer-polymer crosslinking can be used to avoid the use of crosslinking molecules during gelation. In order to achieve covalently bonded hydrogels, the use of pre-functionalized polymer chains is necessary so that they can participate in chemical reactions like Schiff base formation, Michael addition or disulfide bonding. A problem is that chitosan, functionalized with a reactive group, might be cytotoxic, even if the parent chitosan polymer is highly biocompatible (Bhattarai et al., 2010).

Crosslinkers that are photosensitive or sensitive to enzymes can be used too. These type of crosslinkers become reactive after exposure to environmental factors. For example, when chitosan is functionalized with azide groups, irradiation with UV-light causes the azide to convert into a reactive nitrene group that binds chitosan’s free amino groups. This way, hydrogels can be formed in situ. Advantages of this technique over chemical methods are the safety, low cost and speed. There are still some drawbacks concerning the use of photosensitive polymers like the prolonged irradiation time that is sometimes required to crosslink the polymers. (Bhattarai et al., 2010).

1.2.4. Interpenetrating polymeric networks (IPNs)

To further strengthen entangled polymer networks, secondary polymers can be interlaced within the crosslinked networks. A full-IPN is formed when both polymers are crosslinked and a semi-IPN is formed when only one of the polymer networks is crosslinked (Bhattarai et al., 2010). Figure 1.12 gives a schematic presentation of a full and a semi-IPN. IPNs offer drug delivery systems for both small and large drug molecules and are usually formed to combine the individual properties of 2 (or more) polymers (Suresh et al., 2011). In this work, chitosan is crosslinked in the presence of EUDRAGIT® varieties to form a semi-IPN.
FIGURE 1.12 a) Full IPN b) Semi-IPN (Sperling, 1994).

1.3. EUDRAGIT®

Different varieties of EUDRAGIT® are used in this work to form a semi-IPN with chitosan. EUDRAGIT® is a trade name that is used to describe poly(meth)acrylates for pharmaceutical applications. The EUDRAGIT® polymers are mainly used for the production of enteric formulations, to obtain moisture/light protection and odor/taste masking and to achieve sustained-release formulations (http://eudragit.evonik.com/product/eudragit/en/products-services/eudragit-products/pages/default.aspx 19/5/12).

1.3.1. EUDRAGIT® E PO


The variety E PO of EUDRAGIT® is based on dimethylaminoethyl methacrylate, butyl methacrylate and methyl methacrylate, resulting in a positively charged copolymer in acidic conditions (FIGURE 1.13). The polymer is soluble in gastric fluid up to pH 5.0 and is used to obtain targeted drug delivery to the stomach (http://eudragit.evonik.com/product/eudragit/en/products-services/eudragit-products/pages/default.aspx 19/5/12).

1.3.2. Other varieties of EUDRAGIT®

In this work, EUDRAGIT® NE 30D and EUDRAGIT® RS 30D are also used. These varieties of EUDRAGIT® provide time-controlled release and are pH independent. EUDRAGIT® NE 30D is
a neutral copolymer based on ethyl acrylate and methyl methacrylate and is delivered as a 30% aqueous dispersion. EUDRAGIT® RS 30D is a copolymer that is based on ethyl acrylate, methyl methacrylate and a low content of methacrylic ester with quaternary ammonium groups which make the polymer permeable. EUDRAGIT® RS 30D is also delivered as a 30% aqueous dispersion (http://eudragit.evonik.com/product/eudragit/en/products-services/eudragit-products/pages/default.aspx 19/5/12).

1.4. SUPERHYDROPHOBIC SURFACES

1.4.1. General information

Hydrogel spheres are usually produced in a liquid environment starting from the initial components in solution. During the hardening of these spheres, a portion of the drug molecules can diffuse from the spheres to the liquid environment. This is why the encapsulation of drug in these wet methods is not totally efficient. Therefore, scientists developed a biomimetic approach, inspired on the Lotus leaf, where hydrogel spheres are formed at the solid-air interface (FIGURE 1.14) (Song et al., 2010).

FIGURE 1.14 Preparing hydrogel spheres using superhydrophobic surfaces (Song et al., 2010).

Another advantage concerning the encapsulation efficiency is that the contact surface with the solid substrate covers a negligible area. Other advantages of this method are the ease of preparing hydrogel spheres, low production costs and scale-up possibilities. In addition, it is possible to use mild processing conditions without the need of mechanical forces during particle formation (Song et al., 2010).

1.4.2. The Lotus effect

Because it shows good self-cleaning properties, the Lotus leaf is considered a symbol of purity for thousands of years (Guo et al., 2011). The Lotus effect describes the self-cleaning action of some leaf surfaces. If the hydrophobicity of the surface is high enough, water droplets will roll off the surface instead of sliding off, picking up dirt particles as they move (FIGURE 1.15) (Crick and Parkin, 2010).
1.4.3. Surface properties

1.4.3.1. Contact angle

The water contact angle (CA) is the angle subtended by the droplet and the surface (FIGURE 1.16). The surface is said to be hydrophilic when the CA is lower than 90°, with a minimum of 0°. If the CA is higher than 90° (and maximum 180°, giving perfectly spherical particles), the surface is said to be hydrophobic. A surface is called superhydrophobic when the CA is higher than 150° (Crick and Parkin, 2010).

1.4.3.2. Surface energy and surface roughness

Surfaces with low surface energy tend to be hydrophobic because water will not be attracted strongly and the CA will be higher. Surface roughness can be defined by looking at the difference between the planar area and the actual surface area. The planar area assumes a smooth surface whereas the actual surface area takes into account the peaks and valleys of the surface. The CA increases with surface roughness. As implied in section 1.4.3.1., the higher the CA is, the more hydrophobic the surface is (Crick and Parkin, 2010).

1.4.3.3. Structure of superhydrophobic surfaces

Two approaches are used to create artificial superhydrophobic surfaces: creating rough structures on hydrophobic surfaces (CA > 90°) or modifying a rough surface using materials
with low surface free energy like materials with alkyl or fluorinated alkyl groups. The combination of hierarchical roughness and low surface free energy material leads to the fabrication of surfaces with an apparent water CA > 150° and a low sliding angle (<10°), showing self-cleaning properties (FIGURE 1.17) (Feng et al., 2002; Crick and Parkin, 2011; Guo et al., 2011).

FIGURE 1.17 a) and b) are the SEM images of Lotus leaf with low and high magnifications respectively. The inset of b) is a water CA with a value or about 162° (Guo et al., 2011).

1.4.4. Applications of superhydrophobic surfaces

In this work superhydrophobic surfaces are used to create microspheres because liquid droplets acquire spherical shape when deposited on a superhydrophobic surface. The droplets harden as hydrogel structures under mild conditions without being in contact with another liquid medium (Song et al., 2010). Superhydrophobic surfaces are not only found to be useful in the pharmaceutical industry. One example to demonstrate this are their anti-icing properties. They have the capability to reduce accumulation of snow and ice and even to completely prevent formation of ice on solid surfaces (Guo et al., 2011).

1.5. 5-FLUOROURACIL (5-FU)

1.5.1. General information

Zhang et al. (2008) stated that: “5-FU is a heterocyclic aromatic organic compound with a structure similar to that of the pyrimidine molecules of DNA and RNA; it is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen” (FIGURE 1.18).

5-FU, developed in the 1950s, is widely used to treat a variety of cancers, including colon cancer. The antitumor mechanism follows from its ability to inhibit thymidylate synthase, misincorporate nucleotides in RNA and deplete dTTP. These actions lead to cell apoptosis. 5-
FU, an antimetabolic agent, has found to cause serious side effects. To decrease these side effects and to increase the therapeutic index, scientists are trying to formulate controlled release systems that keep the concentration of 5-FU at a low level (Rokhade et al., 2007).

After entering the cell, 5-FU is converted into the active metabolites fluorodeoxyuridine monophosphate (FdUMP) and fluorouridine triphosphate (FUTP). These metabolites interfere with RNA synthesis and with the action of thymidylate synthase (TS) (Longley et al., 2003).

### 1.5.2. Thymidylate synthase inhibition

TS catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). This reaction is a reductive methylation that uses reduced folate (CH$_2$THF) as a methyl donor and is the only de novo source of thymidylate, which is needed for DNA-replication and -repair. Both subunits of the dimeric TS protein contain a nucleotide-binding site and a binding site for CH$_2$THF. The 5-FU metabolite FdUMP binds to the nucleotide binding site of TS where it forms a stable complex with the enzyme and CH$_2$THF. This way, it blocks the binding of the normal substrate (dUMP), thereby blocking the synthesis of dTMP and subsequently the formation of deoxythymidine triphosphate (dTTP). Through various feedback mechanisms this disturbs the levels of other deoxynucleotides (dATP, dGTP and dCTP). The disturbed deoxynucleotide levels (especially the dATP/dTTP ratio) disrupt DNA synthesis and repair, resulting in lethal DNA damage (FIGURE 1.19) (Longley et al., 2003).

Because dUMP is no longer converted into dTMP, dUMP accumulates, which might lead to increased levels of deoxyuridine triphosphate (dUTP). dUTP and FdUTP (metabolite of 5-FU) can both be misincorporated into DNA. Uracil-DNA-glycosylase works as a nucleotide excision repair enzyme for uracil- and 5-FU-containing DNA, but it is futile in the presence of high (F)dUTP/dTTP ratios and only results in further false-nucleotide incorporation. These
cycles of misincorporation, excision and repair eventually lead to the breaking of DNA strands, causing cell death. The salvage pathway, where thymidylate is formed out of thymidine through action of thymidine kinase, alleviates the effects of TS-inhibition and presents a possible mechanism of resistance to 5-FU (Longley et al., 2003).

![FIGURE 1.19 Thymidylate synthase inhibition by 5-fluorouracil leading to DNA damage (Longley et al., 2003).]

**1.5.3. RNA misincorporation**

FUTP is regularly misincorporated into RNA, causing disruptions in normal RNA processing and function. The misincorporation can result in toxicity to RNA in several ways (Longley et al., 2003).
2. OBJECTIVES

5-Fluorouracil (5-FU) is an anti-cancer drug that is widely used for the treatment of various kinds of cancer, including colon cancer. It is found to cause severe side effects when the concentration is too high. Controlled release systems could be useful to decrease these side effects by keeping the concentration of 5-FU at a low level and by targeting the drug delivery so that healthy tissue will not be harmed (Rokhade et al., 2007).

The main goal of this work is to produce microspheres for the controlled release of 5-FU, using chitosan as the main component of the hydrogel. In addition to the biocompatibility, low toxicity and susceptibility to enzymatic degradation, chitosan does not show immunogenicity and there is no risk of transmitting animal-originated pathogens (Bhattarai et al., 2010). Microspheres are obtained by a biomimetic approach, using superhydrophobic surfaces. Liquid droplets are placed on superhydrophobic surfaces to create spherical particles. In this work, polystyrene surfaces are used, which are treated to obtain both hierarchical roughness and low surface energy (Song et al., 2010). Particles are crosslinked by putting the particles under glutaraldehyde atmosphere in a desiccator.

Controlled release is pursued by creating a semi-interpenetrated polymeric network by crosslinking the chitosan in the presence of different EUDRAGIT® varieties. Release profiles in water, HCl 1% and phosphate buffer pH 7.4 are obtained by performing in vitro release studies. It is investigated whether the EUDRAGIT® varieties influence 5-FU release and if the time of crosslinking has an effect on the release profiles.

Cytotoxicity assays, using HeLa cells, are performed to evaluate the toxicity of the chitosan particles and of the particles that contain EUDRAGIT® E PO. The efficiency of 5-FU against cancer cells is also investigated.
3. MATERIALS AND METHODS

3.1. CHEMICALS

5-Fluorouracil (5-FU) was purchased from Fagron Iberica S.A.U. (Terrassa, Spain). All the varieties of EUDRAGIT® (E PO, L100-55, FS 30D, L30 D-55, RL 30D, NE 30D and RS 30D) were obtained from Evonik (Darmstadt, Germany). Chitosan (QT, molecular weight 359150Da, 76.2% deacetylation degree and an intrinsic viscosity of 9.55 dl g⁻¹ (Barreiro-Iglesias et al., 2005) was purchased from Georges S. Daras SA (Marseille, France). Glutaraldehyde solution (25%), ortho-phosphoric acid (85%) and potassium dihydrogen phosphate (KH₂PO₄) were bought from Merck-Schuchardt OHG (Hohenbrunn, Germany). Hydrochloric acid (HCl, 37%) and dipotassium hydrogen phosphate (K₂HPO₄) were obtained from Panreac Quimica SA (Barcelona, Spain). Acetic acid was sourced from VWR international (Fontenay sous Bois, France) and sodium hydroxide (NaOH) was obtained from VWR international (Leuven, Belgium). Di-sodium hydrogen phosphate anhydrous (Na₂HPO₄) was obtained from Scharlau (Sentmenat, Spain). Crystal violet, formic acid, fetal bovine serum (FBS), glutamine, non-essential amino-acids (NEAA) and penicillin-streptomycin were obtained from Sigma Aldrich (St. Louis, USA). Dulbecco’s modified eagle medium (DMEM) high glucose was purchased from Gibco (Invitrogen, Spain). 2-(4-morpholine)ethanesulfonic acid (MES) was obtained from Fischer Bioreagents (Leicestershire, UK). MilliQ® water, obtained by reverse osmosis (with a resistivity of 18.2 MQ•cm at 25°C) was used. All chemicals that are used are of analytical quality and are used without further purification.

3.2. PREPARATION OF PHOSPHATE BUFFER pH 7.4

To prepare 2000ml of phosphate buffer pH 7.4, 1000ml of a potassium dihydrogen phosphate (KH₂PO₄) 0.2M solution and 500ml of sodium hydroxide (NaOH) 0.2M solution have to be made. For the first solution, 27.22g of KH₂PO₄ is weighted and diluted to 1000ml with MilliQ® water. For the NaOH solution, 4g NaOH pellets are weighted and diluted to 500ml with MilliQ® water. Both solutions are shaken at least three times and are placed under magnetic stirring until all the reactive material is dissolved. Next, 500ml of the KH₂PO₄ 0.2M solution is transferred to a volumetric flask of 2000ml together with 380ml of the NaOH 0.2M solution. MilliQ® water is added until a little below the mark of the volumetric flask. A magnetic stirrer is then introduced and the solution is mixed. When the mixture is homogeneous, 50ml is transferred to a beaker to measure the pH using a pH meter (Crison
GLP 22). If necessary, pH is adjusted by adding NaOH or KH₂PO₄ solutions, until reaching pH 7.4 (Universidad de Santiago de Compostela, 2008).

3.3. SUPERHYDROPHOBIC SURFACES

The polystyrene (PS) superhydrophobic surfaces used in this work are produced in Porto, Portugal using a phase-inversion method. The first step involves the preparation of a 60mgml⁻¹ PS solution in tetrahydrofuran and then this solution is mixed with ethanol (2:1.3 v/v). This mixture is then dispensed onto a smooth PS substrate. Next, the PS substrate is immersed in ethanol for 1min and dried under nitrogen flow. To further increase the hydrophobicity, the rough PS surface is modified with Argon plasma (Plasma Prep5, Gala instruments, Germany) at 30W for 20s and with 1H,1H,2H,2H-perfluorodecyltrimethoxysilane (Song et al., 2010).

![Schematic showing of the construction of the superhydrophobic surface](Crick and Parkin, 2010).

3.4. UV/VIS-SPECTROPHOTOMETRY

3.4.1. General information

The electromagnetic spectrum includes gamma rays, X-rays, ultraviolet radiation, visible light, infrared radiation, microwaves and radio waves (http://imagine.gsfc.nasa.gov/docs/science/know_l1/emspectrum.html 11/4/12). The different kinds of radiations can be characterized by their wavelengths or frequencies. Formula 3.1 says that when wavelength decreases (and thus frequency increases), the energy of the wave increases.

\[ E = \frac{hc}{\lambda} \]  

(3.1)

Where: \( E \) = energy (J)
\( h \) = Planck constant = 6.624x10⁻³⁴ J s
\( c \) = velocity of light in vacuum (=3x10⁸ ms⁻¹)
\( \lambda \) = wavelength (m)
The region that covers the visible and the accessible part of the UV/VIS region, stretches out from 200 until 800nm. The measurement of the absorption of UV and visible radiation by components in solution is one of the most widely used methods of quantitative analysis (Thomas, 1996).

### 3.4.2. Beer-Lambert Law

![FIGURE 3.2 Schematic representation of light absorption by a solution with pathlength l](http://5e.plantphys.net/article.php?ch=7&id=66 15/4/12)

The intensity of the incident beam is reduced when light is absorbed by a solution to the transmitted light beam (FIGURE 3.2). The terms transmittance and absorbance are defined in formula 3.2 and 3.3 respectively (Thomas, 1996):

\[
T = \frac{I}{I_0} \quad (3.2)
\]

Where:

- \(T\) = transmittance
- \(I\) = transmitted beam
- \(I_0\) = incident beam

\[
A = \log(I_0/I) = \log (1/T) \quad (3.3)
\]

Where:

- \(A\) = absorbance
- \(I_0\) = incident beam
- \(I\) = transmitted beam
- \(T\) = transmittance

The Beer-Lambert law (formula 3.4), proposed in 1852, shows that absorbance depends on the total amount of absorbing species in the radiation path through the cell. This means that absorption is affected by both concentration and path length (Thomas, 1996).
\[ A = \varepsilon c l \]  

(3.4)

Where:

- \( A \) = absorbance
- \( \varepsilon \) = molar absorption coefficient (Lcm\(^{-1}\)mol\(^{-1}\))
- \( c \) = concentration (mol/L)
- \( l \) = path length (cm)

3.4.3. Calibration curve of 5-FU

Three calibration curves are used in this work to determine the concentration of 5-FU: one in water, one in HCl 1% and one in phosphate buffer pH 7.4. The calibration curve in water was set up in the work of Van Poucke (2011). The calibration curve in HCl 1% and the one in phosphate buffer pH 7.4 are constructed during the time this work is written. All three curves are constructed in the same way, described here: 0.005g 5-FU is weighted and dissolved under magnetic stirring in 250ml HCl 1% to achieve a concentration of 0.020mg/ml. Starting from this stock solution, 9 dissolutions are prepared with a concentration of 0.018mg/ml, 0.016mg/ml, 0.014mg/ml, 0.012mg/ml, 0.010mg/ml, 0.008mg/ml, 0.006mg/ml, 0.004mg/ml and 0.002mg/ml. All the solutions are made and measured in triplicate, using the FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany) at 280nm, and the data are corrected with a blank. Measurement is carried out using a 96-well plate (Costar, Corning Incorporated). Different volumes were tested before and samples of 200μl turned out to be the most appropriate (Van Poucke, 2011).

3.5. PREPARATION OF CHITOSAN PARTICLES

3.5.1. Prepared solutions

Chitosan 2% is prepared by weighing 2g of solid chitosan and dissolving it in 100ml acetic acid 1% under magnetic stirring. Acetic acid 1% is prepared by diluting 1ml of acetic acid 100% to 100ml using MilliQ® water. The following solutions are prepared with the aim to produce particles:
1. Chitosan 1.5% 
2. Chitosan 1.5% 
   EUDRAGIT® E PO 2.5% 
3. Chitosan 1.5% 
   5-FU 0.5% 
4. Chitosan 1.5% 
   EUDRAGIT® E PO 2.5% 
   5-FU 0.5% 

Solutions 1 to 4 are used to produce particles for the cytotoxicity assay. Solutions 3 to 6 are used to produce particles for the in vitro release study.

### 3.5.2. Use of superhydrophobic surfaces

Polystyrene (PS) superhydrophobic surfaces are used to create spherical particles of the previously prepared solutions (FIGURE 3.3). Liquid droplets of 2.5μl are dropped onto the PS-plates using a micropipette, which controls the size of the droplets. For small volumes, it has been shown that the shape of the droplets on superhydrophobic surfaces is almost spherical. A more deformed geometry is obtained when bigger droplets are made due to the effect of gravity (Song et al., 2010).

![FIGURE 3.3 A and B: 2.5μl particles (QT 1.5% + 5-FU 0.5%) on polystyrene superhydrophobic surfaces before crosslinking.](image)

Then, the surfaces are put in a desiccator under glutaraldehyde atmosphere for 1 or 4 hours, to crosslink the particles. Afterwards, the particles are detached from the surface using a spatula and they are kept in eppendorf tubes. Some of the particles are then put under vacuum for a predetermined period of time. The superhydrophobic surfaces are reusable because of their self-cleaning properties. The surfaces are rinsed with distilled water and dried in an oven at 40°C.
3.5.3. Chemical crosslinking

Glutaraldehyde is used to crosslink the chitosan polymers (FIGURE 3.4). The carbonyl functions of the glutaraldehyde molecule form covalent imine bonds with the amino groups of chitosan through a Schiff reaction (Gonçalves et al., 2005).

![Chemical structure of glutaraldehyde and chitosan crosslinking](image)

FIGURE 3.4 Crosslinking process of chitosan treated with glutaraldehyde (Gonçalves et al., 2005).

3.6. RELEASE STUDY IN VITRO

3.6.1. Release study with varieties of EUDRAGIT®

To carry out the release study, particles are made as described in section 3.5. with the particles in table 3.1 as result. 8 different kinds of particles, tested in 3 different media and all tests done in triplicate, gives a total of 72 eppendorf tubes, each filled with 5 particles.

The release profiles of these particles are obtained in water, HCl 1% and phosphate buffer pH 7.4. The tubes containing the particles are filled with 1ml of medium. Because of the contact with fluid, the particles swell and drug is being released. Samples of 200μl are gathered in 96-well plates (Costar, Corning Incorporated) at predetermined periods of time. This is every 15min for the first 3 hours and every 30min for the next 3 hours. When a sample of 200μl is taken out of the tube, 200μl of the corresponding medium is added to keep the volume constant. When the samples are gathered in the 96-well plate, absorption is measured using the FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany) at 280nm. Blanks consisting of only the corresponding medium are included and are used to correct the sample measurements.
Table 3.1 Particles prepared to use in the release study with unpurified materials.

<table>
<thead>
<tr>
<th>Crosslinked for 1 hour</th>
<th>Crosslinked for 4 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Chitosan 1.5%</td>
<td>Chitosan 1.5%</td>
</tr>
<tr>
<td>5-FU 0.5%</td>
<td>5-FU 0.5%</td>
</tr>
<tr>
<td>2. Chitosan 1.5%</td>
<td>Chitosan 1.5%</td>
</tr>
<tr>
<td>EUDRAGIT® E PO 2.5%</td>
<td>EUDRAGIT® E PO 2.5%</td>
</tr>
<tr>
<td>5-FU 0.5%</td>
<td>5-FU 0.5%</td>
</tr>
<tr>
<td>3. Chitosan 1.5%</td>
<td>Chitosan 1.5%</td>
</tr>
<tr>
<td>EUDRAGIT® NE 30D 1%</td>
<td>EUDRAGIT® NE 30D 1%</td>
</tr>
<tr>
<td>5-FU 0.5%</td>
<td>5-FU 0.5%</td>
</tr>
<tr>
<td>4. Chitosan 1.5%</td>
<td>Chitosan 1.5%</td>
</tr>
<tr>
<td>EUDRAGIT® RS 30D 1%</td>
<td>EUDRAGIT® RS 30D 1%</td>
</tr>
<tr>
<td>5-FU 0.5%</td>
<td>5-FU 0.5%</td>
</tr>
</tbody>
</table>

3.6.2. Release study with purified materials

The release study is done a second time using purified chitosan (see section 3.8.) and purified EUDRAGIT® E PO (see section 3.9.). The procedure used to carry out this study is the same as described in section 3.6.1. The difference is that purified materials are used and that the particles are placed under vacuum for 4 hours after crosslinking. Only the formulations with EUDRAGIT® E PO and the formulations that only consist of chitosan are tested.

3.7. CYTOTOXICITY ASSAY

3.7.1. Templates

The template used to carry out the first two cytotoxicity assays is shown in table 3.2. The particles for these assays are obtained as described in section 3.5. The difference between the first and the second assay is that in the first assay, unpurified chitosan is used and in the second one, purified chitosan.

The negative controls only include the growth medium. The positive controls consist of 5-FU in different concentrations (2.4μM, 2.4mM and 24mM) in acetic acid 10%. 2.4μM is chosen because that concentration is near the IC$_{50}$ of 5-FU (IC$_{50}$ of 5-FU is 2.9μM (Takara et al., 2002)). 2.4mM is chosen because this is the concentration the 200μl-wells should have when the 5 drug-containing particles have released their amount of drug completely. 24mM is chosen to make sure that there is a well where all the cells have died after 24 hours.
The template that is used for the third cytotoxicity assay is shown in Table 3.3. Like the second assay, this assay also uses purified chitosan. The same controls as in the first two assays were included (negative controls and 5-FU in concentrations of 2.4µM, 2.4mM and 24mM), supplemented with an extra positive control (5-FU 0.48mM, corresponding to the concentration obtained when 1 drug-containing particle releases its amount of drug completely). The particles used in the third assay are obtained as described in section 3.5., with the difference that after crosslinking, they are put under vacuum for 2 hours.

For the fourth cytotoxicity assay, the template in Table 3.3 is also used. In this assay, purified chitosan and purified EUDRAGIT® E PO are used and the particles are put under vacuum for 4 hours after crosslinking. The positive control of 1.45mM corresponds to the concentration obtained when 3 drug-containing particles release their amount of drug completely.

3.7.2. Preparation crystal violet solution 0.1%

1.35ml fosforic acid, 3.904g MES and 1.360g formic acid are dissolved in 100ml MilliQ® water. Then, the pH is adjusted to pH 6 using a 5M solution of NaOH. Then, 100mg of crystal violet is added. In this way, a 0.1% crystal violet solution with 200mM fosforic acid, 200mM MES and 200mM formic acid is prepared.

### Table 3.2: Template for cytotoxicity assay 1 and 2.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tbody>
<tr>
<td>A</td>
<td>QT 1.5%</td>
<td>QT 1.5%</td>
<td>QT 1.5%</td>
<td>Control (-)</td>
<td>Control (-)</td>
<td>Control (-)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>QT 1.5% + 5-FU 0.5%</td>
<td>QT 1.5% + 5-FU 0.5%</td>
<td>QT 1.5% + 5-FU 0.5%</td>
<td>Control (-)</td>
<td>Control (-)</td>
<td>Control (-)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>QT 1.5% + EU 1.2.5%</td>
<td>QT 1.5% + EU 1.2.5%</td>
<td>QT 1.5% + EU 1.2.5%</td>
<td>Control (-)</td>
<td>Control (-)</td>
<td>Control (-)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>QT 1.5% + EU 1.2.5% + 5-FU 0.5%</td>
<td>QT 1.5% + EU 1.2.5% + 5-FU 0.5%</td>
<td>QT 1.5% + EU 1.2.5% + 5-FU 0.5%</td>
<td>Control (-)</td>
<td>Control (-)</td>
<td>Control (-)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>QT 1.5% + EU 1.2.5% + 5-FU 0.5%</td>
<td>QT 1.5% + EU 1.2.5% + 5-FU 0.5%</td>
<td>QT 1.5% + EU 1.2.5% + 5-FU 0.5%</td>
<td>Control (-)</td>
<td>Control (-)</td>
<td>Control (-)</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>5-FU 2.4 mM</td>
<td>5-FU 2.4 mM</td>
<td>5-FU 2.4 mM</td>
<td>5-FU 24 mM</td>
<td>5-FU 24 mM</td>
<td>5-FU 24 mM</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>5-FU 2.4 µM</td>
<td>5-FU 2.4 µM</td>
<td>5-FU 2.4 µM</td>
<td>5-FU 24 µM</td>
<td>5-FU 24 µM</td>
<td>5-FU 24 µM</td>
<td></td>
</tr>
</tbody>
</table>

EU= EUDRAGIT® E PO
### TABLE 3.3 Template for cytotoxicity assay 3 and 4.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>QT 1.5% 3particles</td>
<td>QT 1.5% 3particles</td>
<td>QT 1.5% 3particles</td>
<td>CONTROL (-)</td>
<td>CONTROL (-)</td>
<td>CONTROL (-)</td>
<td>QT 1.5% + EU¹ 2.5% 3particles</td>
<td>QT 1.5% + EU¹ 2.5% 3particles</td>
<td>QT 1.5% + EU¹ 2.5% 3particles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>QT 1.5% 5particles</td>
<td>QT 1.5% 5particles</td>
<td>QT 1.5% 5particles</td>
<td>CONTROL (-)</td>
<td>CONTROL (-)</td>
<td>CONTROL (-)</td>
<td>QT 1.5% + EU¹ 2.5% 5particles</td>
<td>QT 1.5% + EU¹ 2.5% 5particles</td>
<td>QT 1.5% + EU¹ 2.5% 5particles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>QT 1.5% + 5-FU 0.5% 3particles</td>
<td>QT 1.5% + 5-FU 0.5% 3particles</td>
<td>QT 1.5% + 5-FU 0.5% 3particles</td>
<td>QT without crosslinking 7.5 µl</td>
<td>QT without crosslinking 7.5 µl</td>
<td>QT without crosslinking 7.5 µl</td>
<td>QT 1.5% + EU¹ 2.5% + 5-FU 0.5% 3particles</td>
<td>QT 1.5% + EU¹ 2.5% + 5-FU 0.5% 3particles</td>
<td>QT 1.5% + EU¹ 2.5% + 5-FU 0.5% 3particles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>QT 1.5% + 5-FU 0.5% 5particles</td>
<td>QT 1.5% + 5-FU 0.5% 5particles</td>
<td>QT 1.5% + 5-FU 0.5% 5particles</td>
<td>QT without crosslinking 12.5 µl</td>
<td>QT without crosslinking 12.5 µl</td>
<td>QT without crosslinking 12.5 µl</td>
<td>QT 1.5% + EU¹ 2.5% + 5-FU 0.5% 5particles</td>
<td>QT 1.5% + EU¹ 2.5% + 5-FU 0.5% 5particles</td>
<td>QT 1.5% + EU¹ 2.5% + 5-FU 0.5% 5particles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>QT 1.5% + 5-FU 0.5% 5particles</td>
<td>QT 1.5% + 5-FU 0.5% 5particles</td>
<td>QT 1.5% + 5-FU 0.5% 5particles</td>
<td>QT without crosslinking 12.5 µl</td>
<td>QT without crosslinking 12.5 µl</td>
<td>QT without crosslinking 12.5 µl</td>
<td>QT 1.5% + EU¹ 2.5% + 5-FU 0.5% 5particles</td>
<td>QT 1.5% + EU¹ 2.5% + 5-FU 0.5% 5particles</td>
<td>QT 1.5% + EU¹ 2.5% + 5-FU 0.5% 5particles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>QT 1.5% + 5-FU 0.5% 5particles</td>
<td>QT 1.5% + 5-FU 0.5% 5particles</td>
<td>QT 1.5% + 5-FU 0.5% 5particles</td>
<td>QT without crosslinking 12.5 µl</td>
<td>QT without crosslinking 12.5 µl</td>
<td>QT without crosslinking 12.5 µl</td>
<td>QT 1.5% + EU¹ 2.5% + 5-FU 0.5% 5particles</td>
<td>QT 1.5% + EU¹ 2.5% + 5-FU 0.5% 5particles</td>
<td>QT 1.5% + EU¹ 2.5% + 5-FU 0.5% 5particles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>5-FU 24 mM</td>
<td>5-FU 24 mM</td>
<td>5-FU 24 mM</td>
<td>5-FU 0.48² mM or 1.45³ mM</td>
<td>5-FU 0.48² mM or 1.45³ mM</td>
<td>5-FU 0.48² mM or 1.45³ mM</td>
<td>QT 1.5% + EU¹ 2.5% without crosslinking 7.5 µl</td>
<td>QT 1.5% + EU¹ 2.5% without crosslinking 7.5 µl</td>
<td>QT 1.5% + EU¹ 2.5% without crosslinking 7.5 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>5-FU 2.4 mM</td>
<td>5-FU 2.4 mM</td>
<td>5-FU 2.4 mM</td>
<td>5-FU 2.4 µM</td>
<td>5-FU 2.4 µM</td>
<td>5-FU 2.4 µM</td>
<td>QT 1.5% + EU¹ 2.5% without crosslinking 12.5 µl</td>
<td>QT 1.5% + EU¹ 2.5% without crosslinking 12.5 µl</td>
<td>QT 1.5% + EU¹ 2.5% without crosslinking 12.5 µl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹EU = EUDRAGIT* E PO  
²0.48mM = positive control used in the third cytotoxicity assay  
³1.45mM = positive control used in the fourth cytotoxicity assay
3.7.3. Realisation of the assay

The cells used in this work are HeLa-cells (CCL2, American Type Culture Collection) which are cervix carcinoma cells. The cells are cultivated in growth medium in the oven (at 37°C, 90% relative humidity and 5% CO₂). Growth medium consists of DMEM high glucose supplemented with 10% FBS, 2% glutamine, 1% NEAA and 1% penicillin-streptomycin. When the cells are cultivated, they are counted using a coulter and 200µl is placed in each well, so that in every well there are approximately 15000 cells. The cells are left incubating in an oven at 37°C, 90% relative humidity, 5% CO₂ for 24 hours so that they can attach to the bottom of the wells.

Following the templates, 3 or 5 particles or solutions (7.5 or 12.5µl, corresponding to 3 or 5 particles) are added to the growth medium. Positive controls (24mM, 2.4mM, 0.48mM or 2.4µM) are made by removing the growth medium and replacing it by 200µl of drug solution. Samples were all measured in triplicate. Afterwards, the 96-well plates are put back into the oven to incubate for 6, 24 or 48 hours. After 6 hours, the first plate is taken out of the oven and the culture medium is removed. Then, 20µl glutaraldehyde (11%) is added and the well-plate is incubated for 15min under shaking. Then, glutaraldehyde is removed and the wells are washed 3 times with 100µl of MilliQ® water.

The cells are treated with 100µl of crystal violet solution 0.1% for 15min. When the color is retained, the cells are washed 4 times with MilliQ® water and the plates are dried. Next, 100µl acetic acid 10% is added to dissolve the amount of crystal violet, retained by the cells, to obtain a homogeneous color in the wells. The absorbance of the crystal violet solution is measured using the FLUOstar OPTIMA plate reader (Offenburg, Germany) at 595nm. After 24 and 48 hours the same procedure is performed on the other plates.

3.8. PURIFICATION CHITOSAN

10g solid chitosan is dissolved in 1000ml of acetic acid 2% (v/v) in order to obtain a 1% (w/v) chitosan solution. This chitosan solution is filtered twice: once over a filter with a diameter between 7 and 11µm (ALBET-Hahnemühle, Barcelona, Spain) and once over a nylon filter with a diameter of 0.45µm (Lida Manufacturing Corp., Kenosha, WI, US) to remove the sludges. After this, the solution is washed several times with distilled water and the pH is adjusted to 8 by slowly adding 2M NaOH in order to form a precipitate of the
polymer. The precipitated material is filtered over the ALBET-Hahnemühle filter again and washed with distilled water until pH reaches 7. The pH is measured using a pH meter (Crison GLP 22). After the pH reaches 7, the precipitate must be filtered again using the ALBET-Hahnemühle filter and 300ml of an ethanol-water solution (80:20) is added. The suspension is filtrated again with the nylon filter in each washing process in order to remove the maximum of ethanol solution from the purified chitosan. The purified chitosan is transferred to Petri dishes and is frozen at -80°C overnight. To completely remove the water, the frozen material is put in a freeze-dryer (VirTis Genesis, Gardiner, NY, US) for 4 days. After freeze-drying, the purified chitosan is grinded and stored in a plastic bottle.

3.9. PURIFICATION EUDRAGIT® E PO

3.9.1. Preparation of phosphate buffer pH 2

8.95g Na$_2$HPO$_4$ and 3.40g KH$_2$PO$_4$ are weighted and dissolved in a volumetric flask of 1000ml using MilliQ® water. The pH is adjusted to pH 2, using orthophosphoric acid (European Pharmacopoeia, 2007).

3.9.2. Pretreatment membranes

The membranes that are used are high retention seamless cellulose tubings with a molecular cut off of 12400Da (Sigma Aldrich, Steinheim, Germany). To remove the glycerol from the membranes, the tubings are washed in running water for 3-4 hours. Next, sulfur compounds are removed by treating the tubings with a 0.3% (w/v) solution of sodium sulfide for 1min at 80°C. Then, they are washed with water at 60°C for 2min and acidified with a 0.2% (v/v) solution of sulfuric acid. The acid is removed by rinsing the tubings with hot water (http://www.sigmaaldrich.com/catalog/product/SIGMA/D0530?lang=es&region=ES 15/5/12)

3.9.3. Purification

Purification is carried out by performing dialysis. First, 5g EUDRAGIT® E PO is weighted and dissolved in 50ml of phosphate buffer pH 2 to make a 10% (w/v) solution. On this solution a dialysis is carried out, using previously described membranes (Sigma Aldrich, Steinheim, Germany). The dialysis is first carried out in phosphate buffer pH 2 and then in MilliQ® water for a total period of 3 days. After dialysis is completed, the EUDRAGIT® E PO-solution is frozen with liquid nitrogen and freeze-dried for 3 days (VirTis Genesis, Gardiner, NY, USA).
4. RESULTS

4.1. CALIBRATION CURVE

The calibration curve for 5-fluorouracil (5-FU) in water (FIGURE 4.1) was set up in the work of Van Poucke (2011).

![Calibration curve 5-FU in water](image)

**FIGURE 4.1 Calibration curve of 5-FU in MilliQ® water, measured with the FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany) at 280nm (Van Poucke, 2011).**

The calibration curves in HCl 1% and phosphate buffer pH 7.4 were set up in this work. The absorbance of the stock solution and the 9 dilutions, measured using the FLUOstar OPTIMA plate reader (Offenburg, Germany) at 280nm are shown in table 4.1. All solutions were prepared and measured in triplicate.

**TABLE 4.1 Absorbance at 280nm in HCl 1% and phosphate buffer pH 7.4, measured with FLUOstar OPTIMA plate reader in triplicate.**

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>0.002</th>
<th>0.004</th>
<th>0.006</th>
<th>0.008</th>
<th>0.010</th>
<th>0.012</th>
<th>0.014</th>
<th>0.016</th>
<th>0.018</th>
<th>0.020</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absorbance in HCl 1%</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.038</td>
<td>0.066</td>
<td>0.098</td>
<td>0.126</td>
<td>0.153</td>
<td>0.181</td>
<td>0.207</td>
<td>0.237</td>
<td>0.268</td>
<td>0.290</td>
<td></td>
</tr>
<tr>
<td>0.037</td>
<td>0.067</td>
<td>0.096</td>
<td>0.123</td>
<td>0.149</td>
<td>0.179</td>
<td>0.206</td>
<td>0.235</td>
<td>0.266</td>
<td>0.294</td>
<td></td>
</tr>
<tr>
<td><strong>Absorbance in PB 7.4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.040</td>
<td>0.070</td>
<td>0.103</td>
<td>0.132</td>
<td>0.165</td>
<td>0.197</td>
<td>0.229</td>
<td>0.263</td>
<td>0.295</td>
<td>0.290</td>
<td></td>
</tr>
<tr>
<td>0.037</td>
<td>0.069</td>
<td>0.099</td>
<td>0.134</td>
<td>0.168</td>
<td>0.199</td>
<td>0.232</td>
<td>0.263</td>
<td>0.289</td>
<td>0.296</td>
<td></td>
</tr>
<tr>
<td>0.040</td>
<td>0.072</td>
<td>0.099</td>
<td>0.129</td>
<td>0.164</td>
<td>0.195</td>
<td>0.230</td>
<td>0.262</td>
<td>0.289</td>
<td>0.295</td>
<td></td>
</tr>
</tbody>
</table>

*PB 7.4 = phosphate buffer pH 7.4.

Using the results in table 4.1, two calibration curves were set up. These curves are shown in figure 4.2 and figure 4.3, where in figure 4.2 the calibration curve for 5-FU in HCl 1% is shown and in figure 4.3 the calibration curve for 5-FU in phosphate buffer pH 7.4. The
equations on the right side of the curves were used to determine the concentration of 5-FU in the corresponding medium, for example during the in vitro release study.

![Calibration curve 5-FU in 1% HCl](image)

**FIGURE 4.2** Calibration curve 5-FU in HCl 1% measured with the FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany) at 280nm.

![Calibration curve 5-FU in phosphate buffer pH 7.4](image)

**FIGURE 4.3** Calibration curve 5-FU in phosphate buffer pH 7.4 measured with the FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany) at 280nm.

4.2. RELEASE STUDY

4.2.1. Release study with varieties of EUDRAGIT®

The graphs that represent the cumulative amount of 5-FU released in function of time, in MilliQ® water, HCl 1% and phosphate buffer pH 7.4 are shown in figure 4.4, 4.5 and 4.6 respectively. The release of 5-FU (in percentages) for the different formulations after 15, 30 en 360min is given in table 4.3. The release after 15 and 30min represents the burst release
that occurs when the particles are put in a liquid environment. The release after 360min represents the total cumulative amount of 5-FU that is released from the particles.

**FIGURE 4.4** Release study with purified materials: percentage of 5-FU released as a function of time in MilliQ® water.

**FIGURE 4.5** Release study with purified materials: percentage 5-FU released as a function of time in HCl 1%.
FIGURE 4.6 Release study with unpurified materials: percentage 5-FU released as a function of time in phosphate buffer pH 7.4.

TABLE 4.3 Release of 5-FU after 15, 30 and 360 min in MilliQ® water, HCl 1% and phosphate buffer pH 7.4 (in percentages) with unpurified materials.

<table>
<thead>
<tr>
<th></th>
<th>H₂O</th>
<th>HCl 1%</th>
<th>PB 7.4¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15min</td>
<td>30min</td>
<td>360min</td>
</tr>
<tr>
<td>QT 1.5% 4h</td>
<td>13.8 ± 1.88</td>
<td>81.7 ± 20.2</td>
<td>107 ± 9.63</td>
</tr>
<tr>
<td>QT 1.5% 1h</td>
<td>10.6 ± 2.02</td>
<td>83.0 ± 3.03</td>
<td>96.6 ± 8.03</td>
</tr>
<tr>
<td>QT 1.5% + EUDRAGIT® E PO 2.5% 4h</td>
<td>24.8 ± 7.81</td>
<td>63.7 ± 14.7</td>
<td>99.1 ± 23.1</td>
</tr>
<tr>
<td>QT 1.5% + EUDRAGIT® E PO 2.5% 1h</td>
<td>26.0 ± 3.67</td>
<td>59.6 ± 9.52</td>
<td>97.9 ± 23.2</td>
</tr>
<tr>
<td>QT 1% + EUDRAGIT® NE 30D 1% 4h</td>
<td>21.4 ± 14.3</td>
<td>82.2 ± 3.58</td>
<td>89.5 ± 4.38</td>
</tr>
<tr>
<td>QT 1% + EUDRAGIT® NE 30D 1% 1h</td>
<td>52.8 ± 18.9</td>
<td>86.9 ± 5.83</td>
<td>88.5 ± 5.15</td>
</tr>
<tr>
<td>QT 1% + EUDRAGIT® RS 30D 1% 4h</td>
<td>18.1 ± 4.32</td>
<td>82.5 ± 2.15</td>
<td>104 ± 0.767</td>
</tr>
<tr>
<td>QT 1% + EUDRAGIT® RS 30D 1% 1h</td>
<td>73.6 ± 18.5</td>
<td>93.6 ± 8.12</td>
<td>106 ± 2.75</td>
</tr>
</tbody>
</table>

¹PB 7.4 = phosphate buffer pH 7.4.
4.2.2. Release study with purified materials

The cumulative amount of 5-FU released in function of time in MilliQ® water, HCl 1% and phosphate buffer pH 7.4 are shown in figure 4.7, 4.8 and 4.9 respectively. The release of 5-FU (in percentages) after 15, 30 en 360min is given in table 4.4. As in section 4.2.1., release after 15 and 30min represents the burst release and the release after 360min represents the total cumulative amount of 5-FU that is released from the particles.

![Graph showing release study with purified materials: percentage 5-FU released as a function of time in MilliQ® water.](image)

**FIGURE 4.7** Release study with purified materials: percentage 5-FU released as a function of time in MilliQ® water.

![Graph showing release study with purified materials: percentage 5-FU released as a function of time in HCl 1%.](image)

**FIGURE 4.8** Release study with purified materials: percentage 5-FU released as a function of time in HCl 1%.
FIGURE 4.9 Release study with purified materials: percentage 5-FU released as a function of time in phosphate buffer pH 7.4.

TABLE 4.4 Release of 5-FU after 15, 30 and 360 min in MilliQ® water, HCl 1% and phosphate buffer pH 7.4 (mean values in percentages ± standard deviation) with purified materials.

<table>
<thead>
<tr>
<th></th>
<th>H2O</th>
<th>HCl 1%</th>
<th>PB7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15min</td>
<td>30min</td>
<td>360min</td>
</tr>
<tr>
<td>QT 1.5% 4h</td>
<td>41.5 ± 5.90</td>
<td>74.1 ± 6.01</td>
<td>99.7 ± 4.26</td>
</tr>
<tr>
<td>QT 1.5% 1h</td>
<td>46.9 ± 7.52</td>
<td>70.3 ± 8.05</td>
<td>84.8 ± 4.68</td>
</tr>
<tr>
<td>QT 1.5% + EUDRAGIT® E PO 2.5% 4h</td>
<td>47.7 ± 6.74</td>
<td>81.37 ± 3.88</td>
<td>90.01 ± 3.86</td>
</tr>
<tr>
<td>QT 1.5% + EUDRAGIT® E PO 2.5% 1h</td>
<td>40.7 ± 7.16</td>
<td>72.6 ± 5.37</td>
<td>90.3 ± 9.61</td>
</tr>
</tbody>
</table>

*PB7.4 = phosphate buffer pH 7.4.

4.3. CYTOTOXICITY ASSAY

Cytotoxicity assays were performed as explained in section 3.7. Figure 4.10 shows the percentage cell growth inhibition (after 6 or 24 hours of incubation) after exposure to the particles or 5-FU positive controls. 45.1 ± 15.6% of the cells died within 6h after exposure to the particles that were only made up out of chitosan and 82.0 ± 9.80% of the cells within 24 hours after exposure. For this reason, cytotoxicity assay was performed a second time, using purified chitosan. The results of this second assay are shown in figure 4.11.
FIGURE 4.10 Cytotoxicity assay with unpurified materials: percentage cell growth inhibition after 6h and 24h.

35.5 ± 14.4% of the cells died within 6h after exposure to the particles that were only made up out of chitosan and 67.5 ± 15.8 % of the cells within 24 hours after exposure.

Figure 4.12 shows the results of the third cytotoxicity assay. 8.12 ± 3.64% of the cells died within 6h after exposure to 3 particles that were only made up out of chitosan and 8.78 ± 2.98 % of the cells within 24 hours after exposure. 12.0 ± 8.11% of the cells died within 6h after exposure to 5 particles that were only made up out of chitosan and 25.8 ± 11.9 % of the cells within 24 hours after exposure.
70.7 ± 6.34% of the cells died within 6 hours after exposure to 7.5µl QT 1.5% + EUDRAGIT® E PO 2.5% without crosslinking and 90.8 ± 1.61% within 24 hours. 71.8 ± 4.43% of the cells died within 6 hours and 80.5 ± 6.97% within 24 hours after exposure to QT 1.5% + EUDRAGIT® E PO 2.5% without crosslinking. To reduce the toxicity of these formulations, another cytotoxicity assay was performed, using purified EUDRAGIT® E PO.

![Cytotoxicity assay with purified chitosan and 2h vacuum treatment: percentage cell growth inhibition after 6 and 24 hours.](image)

The results of the fourth cytotoxicity assay are shown in figure 4.13. Mean values and standard deviations are shown in table 4.5.
FIGURE 4.13 Cytotoxicity with purified chitosan, purified EUDRAGIT® E PO and 4h vacuum treatment: percentage cell growth inhibition after 6, 24 and 48 hours.

Table 4.5 Results of the fourth cytotoxicity assay: mean values of the percentage cell growth inhibition ± standard deviation after 6h, 24h and 48h.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>6h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>QT 1.5% 3 particles</td>
<td>3.07 ± 2.10</td>
<td>15.2 ± 2.74</td>
<td>28.4 ± 4.97</td>
</tr>
<tr>
<td>QT 1.5% 5 particles</td>
<td>8.01 ± 1.91</td>
<td>28.9 ± 3.50</td>
<td>40.4 ± 1.45</td>
</tr>
<tr>
<td>QT 1.5% + 5-FU 0.5% 3 particles</td>
<td>18.6 ± 3.84</td>
<td>48.0 ± 1.07</td>
<td>85.0 ± 1.43</td>
</tr>
<tr>
<td>QT 1.5% + 5-FU 0.5% 5 particles</td>
<td>26.4 ± 3.60</td>
<td>73.8 ± 5.14</td>
<td>90.2 ± 0.603</td>
</tr>
<tr>
<td>QT 1.5% + EUDRAGIT® E PO 2.5% 3 particles</td>
<td>40.2 ± 7.09</td>
<td>78.4 ± 2.81</td>
<td>91.2 ± 1.07</td>
</tr>
<tr>
<td>QT 1.5% + EUDRAGIT® E PO 2.5% 5 particles</td>
<td>56.7 ± 4.67</td>
<td>79.5 ± 3.33</td>
<td>90.0 ± 4.29</td>
</tr>
<tr>
<td>QT 1.5% + EUDRAGIT® E PO 2.5% + 5-FU 0.5% 3 particles</td>
<td>60.2 ± 6.14</td>
<td>81.1 ± 5.82</td>
<td>91.0 ± 1.87</td>
</tr>
<tr>
<td>QT 1.5% + EUDRAGIT® E PO 2.5% + 5-FU 0.5% 5 particles</td>
<td>60.2 ± 4.43</td>
<td>83.8 ± 4.46</td>
<td>93.7 ± 2.17</td>
</tr>
<tr>
<td>QT 1.5% without crosslinking 7.5µl</td>
<td>27.6 ± 1.92</td>
<td>42.8 ± 1.28</td>
<td>76.0 ± 1.19</td>
</tr>
<tr>
<td>QT 1.5% without crosslinking 12.5µl</td>
<td>31.3 ± 2.44</td>
<td>84.1 ± 2.26</td>
<td>92.1 ± 1.40</td>
</tr>
<tr>
<td>QT 1.5% + EUDRAGIT® E PO 2.5% without crosslinking 7.5µl</td>
<td>69.2 ± 4.19</td>
<td>82.9 ± 3.31</td>
<td>92.8 ± 0.690</td>
</tr>
<tr>
<td>QT 1.5% + EUDRAGIT® E PO 2.5% without crosslinking 12.5µl</td>
<td>55.4 ± 3.87</td>
<td>74.8 ± 6.63</td>
<td>87.0 ± 1.21</td>
</tr>
<tr>
<td>5-FU 24 mM</td>
<td>31.0 ± 2.45</td>
<td>65.0 ± 1.21</td>
<td>86.0 ± 1.24</td>
</tr>
<tr>
<td>5-FU 2.4 mM</td>
<td>30.3 ± 2.74</td>
<td>57.8 ± 3.61</td>
<td>83.3 ± 1.14</td>
</tr>
<tr>
<td>5-FU 1.45 mM</td>
<td>25.8 ± 3.89</td>
<td>54.2 ± 1.81</td>
<td>78.1 ± 1.42</td>
</tr>
<tr>
<td>5-FU 2.4 µM</td>
<td>27.2 ± 2.41</td>
<td>32.2 ± 3.09</td>
<td>53.6 ± 2.27</td>
</tr>
</tbody>
</table>
5. DISCUSSION

5.1. PREPARATION OF PARTICLES

To obtain particles that demonstrate controlled release of 5-fluorouracil (5-FU), a trial and error approach was adopted. Different varieties of EUDRAGIT® were mixed with chitosan to evaluate if the formation of particles was possible. Only the varieties included in section 3.5.1. (EUDRAGIT® E PO, NE 30D and RS 30D) led to the formation of spherical particles and were included in the release study. Attempts were made to prepare formulations that combined chitosan with varieties of EUDRAGIT® that provide targeted drug release to the duodenum (EUDRAGIT® L100-55 and L30 D-55) or to the colon (EUDRAGIT® FS 30D) (http://eudragit.evonik.com/product/eudragit/en/products-services/eudragit-products/pages/default.aspx 19/5/12). It was not possible to obtain these formulations because a precipitate was formed when these varieties of EUDRAGIT® were mixed with the chitosan solution. This is probably because these varieties are soluble at basic pH and chitosan is dissolved in acetic acid 1%. In another attempt it was tried to mix EUDRAGIT® RL 30D with chitosan, because this variety shows time-dependent release. The particles with EUDRAGIT® RL 30D were not spherical after crosslinking because the EUDRAGIT®-polymers sedimentated and made the particles spread.

After obtaining controlled release profiles with EUDRAGIT® E PO, attempts were made to further delay the release of 5-FU by increasing the amount of EUDRAGIT® E PO in the particles. Different compositions of chitosan and EUDRAGIT® E PO were tried, but formation of particles was not successful.

It is said that even if hydrogels are purified, the presence of unreacted glutaraldehyde can not be completely excluded and toxic effects may occur. To decrease the possibility of toxic side-effects, different steps can be undertaken. The use of genipin as a crosslinking agent is an interesting alternative. Genipin is shown not to be cytotoxic in vitro and to be biocompatible after injection in rats, but biocompatibility on humans has not been assessed yet (Berger et al., 2004).

5.2. CALIBRATION CURVES

To prove that the obtained equations can be used, a validation has been carried out. The results of the validation for the two calibration curves are shown in table 5.1.
TABLE 5.1 Results of the validation of the calibration curves.

<table>
<thead>
<tr>
<th></th>
<th>HCl 1%</th>
<th>PB 7.4(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R^2)</td>
<td>0.9994</td>
<td>0.9993</td>
</tr>
<tr>
<td>VCRF (%)</td>
<td>0.579</td>
<td>1.51</td>
</tr>
<tr>
<td>Mean recovery (%)</td>
<td>99.8</td>
<td>100</td>
</tr>
<tr>
<td>(t_{\text{exp}})</td>
<td>0.512</td>
<td>0.796</td>
</tr>
<tr>
<td>(t_{\text{theor}})</td>
<td>1.83</td>
<td>1.83</td>
</tr>
<tr>
<td>VC mean values (%)</td>
<td>1.27</td>
<td>1.60</td>
</tr>
</tbody>
</table>

\(^1\)PB 7.4 = phosphate buffer pH 7.4

The variation coefficient of the response factors (VCRF) must be lower than 2%. When this is combined with \(R^2\) higher than 0.999, this indicates that the requirements for the linearity are fulfilled. The mean recovery must be around 100%. To prove that the mean recovery percentages are not significantly different from 100%, a t-test is carried out with n-1 degrees of freedom. When the experimental value \((t_{\text{exp}})\) is lower than the theoretical value \((t_{\text{theor}})\), it can be concluded that there are no significant differences between the calibration curve and the theoretical values and thus that the curves are accurate. The variation coefficient of the mean values must be lower than 5.5% in order to obtain the required level of precision. As seen in table 5.1, both curves meet the requirements to pass the validation (Universidad de Santiago de Compostela, 2004).

5.3. IN VITRO RELEASE STUDIES

5.3.1. Release study with varieties of EUDRAGIT®

When comparing the mean values of burst release of 5-FU after 15min of the different formulations, lowest release of 5-FU was observed with the chitosan particles. The big difference was situated around 30min where it seemed that in the three different media, release of 5-FU out of the formulations with EUDRAGIT® E PO was lower than (or at least the same as) the release out of the formulations that only contained chitosan. The other varieties of EUDRAGIT® were excluded from further research because they seemed to show a high burst release in all three media. For these reasons, combined with the overall form of the curve, which was more slowly bended than the other curves, the formulation with EUDRAGIT® E PO was chosen to carry out the cytotoxicity assays.
5.3.2. Release study with purified materials

The release study was done a second time to make sure that deviations, resulting from purification processes or the removal of glutaraldehyde, are taken into account.

ANOVA tests, carried out on the obtained data after 15min in the second release study, pointed out that in all three media, no significant difference (at a 95% confidence level) was seen between particles that are crosslinked for 1 hour and particles that are crosslinked for 4 hours.

ANOVA tests (95% confidence level) showed that, for the particles that only consist of chitosan, the release profiles in phosphate buffer pH 7.4 were not significantly different from the release profiles in HCl 1%. It was also shown that the burst release after 15min in MilliQ® water was significantly lower than in phosphate buffer pH 7.4 or in HCl 1%. It could be expected that drug release would be higher at acidic pH than at basic pH because protonation of the chitosan amino groups led to faster swelling and thus faster release rates. To explain the obtained release profiles, it is thought that the chitosan is extensively crosslinked. This would mean that most of the amino groups of chitosan are linked to glutaraldehyde, so that protonation under acidic pH does not occur. This would explain why the release rate at acidic pH is similar to the release rate at basic pH. To confirm this theory, fourier transform infrared spectroscopy and a swelling assay should be carried out. The release profiles of the formulations that contained EUDRAGIT® E PO did not show significant differences on a 95% confidence level between the three media. Deviating observations in the first release study could be explained due to the presence of impurities.

Results of ANOVA tests (95% confidence level) also showed that the formulations that contained EUDRAGIT® E PO and were crosslinked for 4 hours, decreased the burst release after 15min in phosphate buffer pH 7.4 and in HCl 1%. The ideal release profile would be that the drug would not be released at acidic pH (in the stomach) and that a slow release rate is obtained at basic pH, which is present in the colon. Release in the stomach and the small intestine could be avoided by putting the particles in capsules that are coated. The coating makes sure that the capsule does not break down before it reaches the colon. This way, the particles are not able to release any of the loaded drug before they are in contact with the pH that is present in the colon.
It should be noted that the conditions under which the release study was conducted should be optimized to further mimic the biological environment of the GI-tract. For instance, it was not tested whether the presence of enzymes have an influence on the release profiles. To be able to make more definite conclusions, release studies should also be optimized so that smaller standard deviations are obtained.

5.4. CYTOTOXICITY ASSAYS

Results of the first assay indicated that there was not much difference between the toxicity of the chitosan particles that contain 5-FU and the ones that do not contain 5-FU. A possible explanation for this observation is that the chitosan that was used, caused lethal damage to the HeLa cells. For this reason, purification of chitosan was performed and the cytotoxicity assay was repeated, now using the purified chitosan.

In the results of the second assay, it seemed that the toxicity of the particles only consisting of chitosan was decreased due to the purification, but the percentage cell growth inhibition was still quite high. In a second attempt to decrease the toxicity, particles were put under vacuum for 2 hours in order to eliminate any residual glutaraldehyde, that was used to crosslink the particles and is known to be toxic.

As indicated by the results from the third assay, removing the residual glutaraldehyde seemed to make the toxicity of the chitosan particles lower. The results also seemed to indicate that the formulations using EUDRAGIT® E PO without 5-FU are highly toxic. To try to decrease this toxicity, purification of EUDRAGIT® E PO was carried out.

Results from the fourth cytotoxicity assay also showed that the particles that only contained chitosan had low toxicity. It is said that cytotoxicity under 30% is not considered cytotoxicity (Lim et al., 2010). Results also showed that toxicity of the particles containing EUDRAGIT® E PO did not decrease due to purification. Further investigation is necessary to investigate if it is possible to decrease the toxicity.

The results of the fourth assay also showed that chitosan particles containing 5-FU seemed to be considerably more toxic to the HeLa cells than the particles without drug after 24h exposure. The particles that consist of QT 1.5% + 5-FU 0.5% showed around 50% more
toxicity than the particles without 5-FU. This implicated that 5-FU is able to induce the death of cancer cells and is efficient as an anti-cancer drug.

It was also seen that after 48h, the 2.4µM solution of 5-FU (that was chosen to represent the IC₅₀ of 5-FU at 48h) reached 50% cell growth inhibition which indicates that the cytotoxicity assay was successfully performed.
6. CONCLUSIONS

The main aim of this work was to obtain microspheres, using a biomimetic methodology, for the controlled release of 5-fluorouracil (5-FU). In an attempt to achieve controlled release, chitosan (QT) was combined with different EUDRAGIT® varieties in order to obtain a semi-interpenetrated polymeric network. Mixtures of chitosan with EUDRAGIT® L100-55, L30 D-55, FS 30D and RL 30D were not successful because the formation of a precipitate occurred. Particles with chitosan 1.5% and particles combining chitosan 1.5% with EUDRAGIT® E PO 2.5%, chitosan 1% with EUDRAGIT® NE 30D 1% and chitosan 1% with EUDRAGIT® RS 30D were successfully obtained using polystyrene superhydrophobic surfaces. Particles were chemically crosslinked by placing them under glutaraldehyde atmosphere.

Because release studies indicated high burst release after 15min for the formulations with EUDRAGIT® NE 30D and EUDRAGIT® RS 30D, they were excluded from further research. Results of the release studies also indicated that burst release after 15min of the particles that contained 2.5% EUDRAGIT® E PO and were crosslinked for 4 hours was lower (in HCl 1% and phosphate buffer pH 7.4) than with particles that only contained chitosan. Despite the overall release profiles of the particles that contained EUDRAGIT® E PO, which were more slowly bended than the release profiles of particles that only consisted of chitosan, additional investigation is needed to further sustain the release of 5-FU.

Release studies also showed that there are no significant differences at a 95% confidence level between the release profiles of chitosan particles that were crosslinked for 1 hour and particles that were crosslinked for 4 hours.

Cytotoxicity assays showed that both chitosan and EUDRAGIT® E PO were highly toxic without purification. Purification of chitosan combined with a 4 hour vacuum treatment of the particles led to a significant decrease in toxicity. Mean values of toxicity indicated that toxicity was acceptable, as some sources indicate that toxicity lower than 30% can be due to apoptosis of the cells (Lim et al., 2010). Purification of EUDRAGIT® E PO on the other hand, did not lead to a decrease in toxicity. Because toxicity of these particles was so high, they can not be used to deliver 5-FU to the colon. Further investigation is necessary to decrease the toxicity of these particles.
Results of the cytotoxicity assay indicated that 5-FU is efficient as an anti-cancer drug, as chitosan particles containing 5-FU killed around 50% more of the HeLa cells after 48 hours than particles without 5-FU.
REFERENCES


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