GHENT UNIVERSITY
FACULTY OF PHARMACEUTICAL SCIENCES

MULTIFUNCTIONAL POLYMERS FOR ORAL PEPTIDE AND BIOADHESIVE DRUG DELIVERY

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Thesis submitted to obtain the degree of
Doctor in Pharmaceutical Sciences

2004

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Voorwoord

Bij het voltooien van dit doctoraatsonderzoek zou ik allen die een bijdrage leverden aan het tot stand komen van dit werk willen bedanken.

Ik zou Prof. Dr. J.P. Remon willen bedanken voor de kans die hij mij gaf om deze doctoraatsstudie te beginnen, voor zijn uitstekende begeleiding tijdens het onderzoek en voor het kritisch herlezen van dit proefschrift.

I am very thankful to Dr. P. Foreman (National Starch & Chemical Company, USA) for the perfect cooperation and the preparation of the polymers.

I also want to thank Prof. Geresh (Ben-Gurion University of the Negev, Israel) for the grafted starches.

Daniël Tensy zou ik willen bedanken voor zijn deskundige inzet bij het uitvoeren van de dierproeven.

Ik wil de volgende personen bedanken voor de goede samenwerking: Dr. P. Adriaensens (LUC) voor de NMR-opnames, B. De Pauw (Faculteit Diergeneeskunde) voor de SEM-opnames, Prof. Dr. M. Temmerman & Prof. Dr. Van Bortel (Faculteit Geneeskunde) voor de metronidazole-studie, en Prof. Dr. H. Nelis & Apr. K. Honraet (Labo voor Farmaceutische Microbiologie), Prof. Dr. H. Vermeersch & Dr. D. Loose (Faculteit Geneeskunde) voor de nystatine-studie.

Ik dank Dr. E. Adriaens voor de irritatietesten en haar hulp bij de statistische verwerking van de resultaten.

Alle collega's en ex-collega's van het Laboratorium voor de aangename werksfeer en de leerrijke discussies.

Ouders, familie en vrienden voor hun interesse.

Tenslotte wil ik vooral Sophie bedanken voor haar steun tijdens dit doctoraatsonderzoek en voor het aangename dagelijkse leven die we samen hebben.

Dieter Ameye, 10 januari 2004.

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Introduction & Aims of the Thesis

During the last decade multifunctional polymers have been used in developing controlled release systems, peroral peptide delivery and bioadhesive platforms for buccal, nasal, vaginal and oral drug delivery. Multifunctional polymers should have have good bio- or mucoadhesive properties. They can prolong the residence time of the drug delivery system at the site of drug absorption. They can increase the contact between delivery system and absorbing mucosa, resulting in a concentration gradient which can favour drug absorption. Drug delivery systems based on multifunctional polymers can be applied on specified mucosal tissues, such as the nasal, buccal and vaginal mucosa, to improve and enhance the bioavailability of the drug. Multifunctional polymers are supposed to improve the absorption of peptides across mucosal surfaces by increasing the permeability of epithelial tissues and inhibiting proteolytic enzymes. In principle, multifunctional polymers are hydrophilic macromolecules with good swelling properties. Because of these physical properties, they have matrix forming properties and can be used in sustained drug release systems ([1]Leuβen, 1996).

In this doctoral thesis multifunctional polymers were prepared by grafting starches with poly(acrylic acid). Two grafting methods to obtain the starch-*g*-poly(acrylic acid) copolymers were evaluated: ^{60}Co irradiation and chemical modification. A second series of multifunctional polymers was prepared by freeze-drying or spray-drying starch/carboxylated polymer mixtures.

The starch-*g*-poly(acrylic acid) copolymers, the freeze-dried and spray-dried starch/carboxylated polymer mixtures were evaluated as potential excipients for oral peptide delivery. The in vitro inhibition potency of the polymers towards the proteolytic enzyme trypsin was investigated. As most proteolytic enzymes have Ca^{2+} or Zn^{2+} at their active sites and as a reduction of extracellular Ca^{2+} concentration results in an opening of the tight junctions the calcium and zinc ion binding capacity of the multifunctional polymers was determined. (Insulin was used as model peptide in in vivo studies.)

In the second part of the thesis, the starch-*g*-poly(acrylic acid) copolymers, the freeze-dried and spray-dried starch/carboxylated polymer mixtures were evaluated as

potential buccal and vaginal bioadhesive drug carriers. The ex vivo bioadhesion properties and the mucosal irritation potency of the polymers were evaluated. Testosterone (buccal-systemic absorption), nystatin (buccal-local treatment) and metronidazole (vaginal) were used as model drugs in in vivo studies.

[1]Leuβen, Multifunctional polymers for peroral peptide absorption. Doctoral thesis, Leiden University, 1996.

Chapter 1 Multifunctional Polymers

CONTENTS:

Chapter 1.1 Synthesis and Process Details

1.1.1 Starch-*g*-ploy(acrylic acid) copolymers

The starch-*g*-poly(acrylic acid) copolymers, also named grafted starches, were synthesised by two different methods: ^{60}Co irradiation and chemical modification. By irradiation or chemical modification poly(acrylic acid) chains were grafted onto the starch molecules.

1.1.1.1 Grafted starches synthesised by ^{60}Co irradiation (IR)

These series of starch-*g*-poly(acrylic acid) copolymers were synthesised at the Institutes for Applied Research, Ben-Gurion University of the Negev, Beer-Sheva, Israel by Professor Geresh' group.

The starch was first pregelatinised by heating a stirred starch slurry in water (5 % w/v) at 85°C for 30 min. By pregelatinisation the starch granules are gelatinised, disrupted and solubilised, leading to a clear starch dispersion in water. The dispersion was cooled to room temperature and the (partially neutralised) acrylic acid monomer (AA) was added in varying amounts to the stirred starch dispersion. The graft polymerisation reaction was initiated by γ-rays from a ^{60}Co source (1300 rad/min). By ^{60}Co irradiation free radicals are formed on the starch backbone and the acrylic acid monomers are allowed to polymerise and to react with the free radicals, resulting in starch-*g*-poly(acrylic acid) copolymers (Figure 1). The starch/acrylic acid mixtures were irradiated for 24h at room temperature. The gels so formed were cut into small pieces and dried in air at ambient conditions. Finally, the dried gels were milled to obtain a powder with an IKA Labortechnic Staufen Mill Type A 10 (IKA, Germany).

Table 1 shows the synthesis details of the ^{60}Co irradiated starch-*g*-poly(acrylic acid) copolymers. The irradiated grafted starches received the code IR.

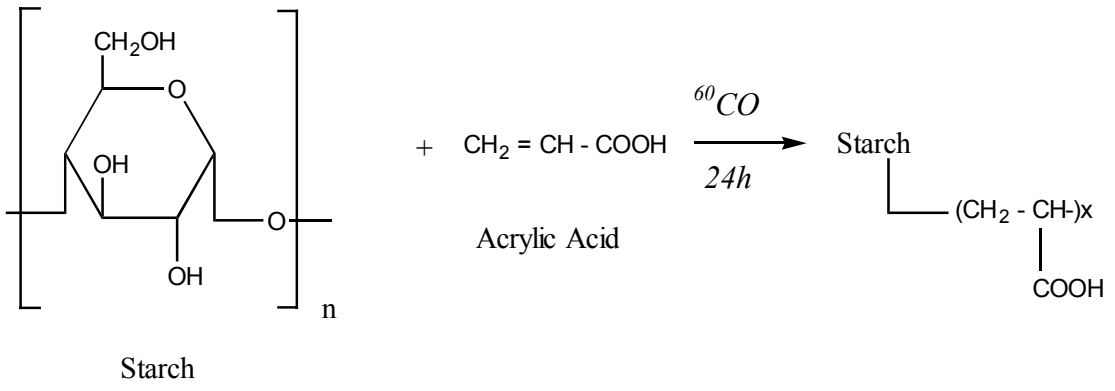

Figure 1: Grafting reaction between starch and acrylic acid initiated by ^{60}Co irradiation.

Polymer code	Starch	Starch/AA ratio	Degree of neutralisation
IR 1	Rice	1:5	1/5
IR 2	Rice	1:5	not neutralised

Table 1: Synthesis parameters of the ^{60}Co irradiated grafted starches (IR).

Rice starch was used as former studies reported better bioadhesive properties with rice starch based grafted starches than with potato or corn starch as starch source. The ratio starch/acrylic acid was 1/5. One sample was partially neutralised (1/5), while the second sample was not neutralised. The acrylic acid solution was neutralised to 1/5 with NaOH before grafting.

Most starches such as rice, corn and potato are composed of two types of polysaccharides: amylose and amylopectin.

Amylose is a linear polymer in which anhydroglucose units are linked by α-D-(1,4) glucosidic bonds to form linear chains. The level of amylose and its molecular weight vary between different starch types. Amylose molecules are typically made from 200-2000 anhydroglucose units. Aqueous solutions of amylose are very unstable due to intermolecular attraction and association of neighbouring amylose molecules. This leads

to viscosity increase, retrogradation and, under specific conditions, precipitation of amylose particles.

Amylopectin has a branched structure. In addition to α-D-(1,4) glucosidic bonds, which are present in amylose and the linear segments of amylopectin, the amylopectin molecule has α-D-(1,6) glucosidic bonds which occur every 20-30 anhydroglucose units. The level of amylopectin varies between different starch types. Waxy starches contain almost 100% amylopectin. Aqueous solutions of amylopectin are characterised by high viscosity, clarity, stability, and resistance to gelling.

Starches from different sources contain different amounts of amylose. Corn starch contains 28 % amylose, potato starch 20 % and rice starch 18.5%. (Young, 1984)

1.1.1.2 Grafted starches synthesised by chemical modification (CM)

The chemical modified starch-*g*-poly(acrylic acid) copolymers were synthesised by National Starch and Chemical Company, Bridgewater, New Jersey, USA.

The chemical modified grafted starches were synthesised in two steps. First, the starch was modified. The starch was reacted with allyl glycidyl ether (AGE) to introduce olefinic functionality onto the starch polymer backbone (Figure 2). The used AGE concentration was 0.2 or 0.5%. (Shih et al., 1987)

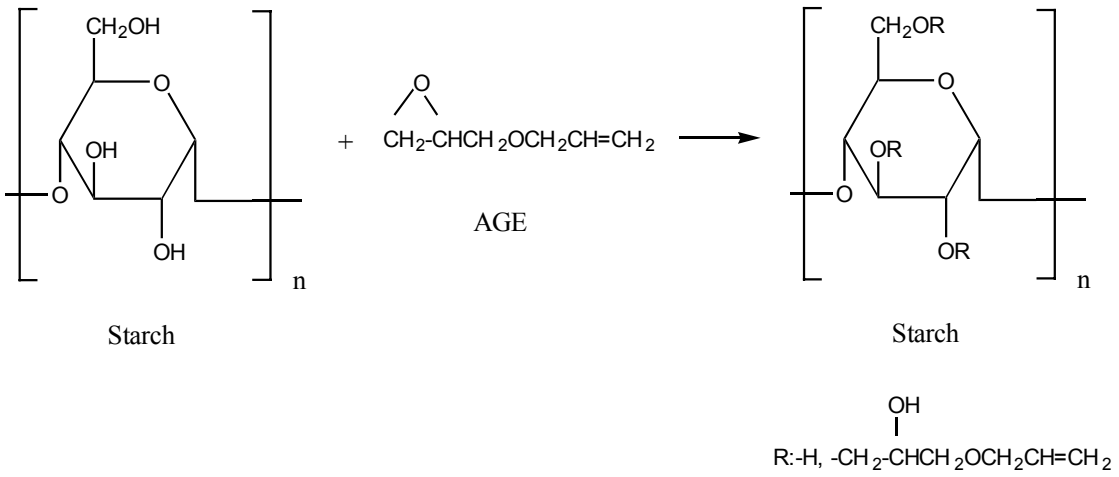

Figure 2: Introduction of olefinic functionality onto the starch by chemical reaction with allyl glycidyl ether (AGE).

In the second phase of the graft polymerisation process the unsaturated olefinic groups will polymerise with acrylic acid. The AGE modified starch was cooked in degassed water to obtain a starch solution. If required, acrylic acid was partially neutralised in a separate beaker while cooled in an ice water bath. The cooked starch solution was then mixed with the (partially neutralised) acrylic acid (AA) solution in the required ratios. The polymerisation process was chemically initiated with potassium persulfate ($K_2S_2O_8$) and was performed under nitrogen at 75-80°C. By adding potassium persulfate free radicals are formed and during the polymerisation process the unsaturated olefinic groups will first react with the acrylic acid, while acrylic acid monomers will polymerise into poly(acrylic acid) polymers, so forming starch-*g*-poly(acrylic acid)

copolymers (Figure 3). The polymerisation reaction was stopped by adding some drops of a 1% hydroquinone/ethanol solution. The viscous polymer product was precipitated in ethanol. After filtering off the ethanol/water fraction, drying and grinding (Cemontec sample mill, MTI, Selters, Germany – room temperature) a white powder was obtained.

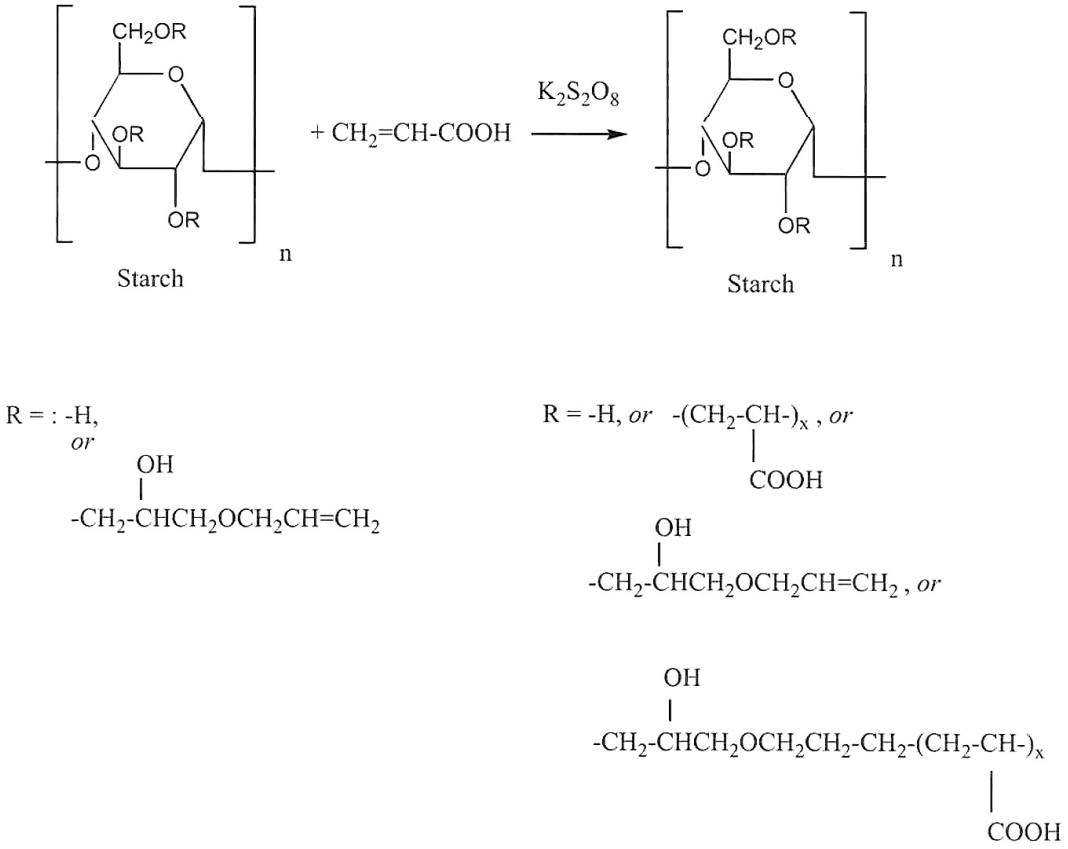

Figure 3: Graft polymerisation process initiated by potassium persulfate.

The chemical modified grafted starches were coded CM. Table 2 gives an overview of the synthesis parameters of the CM starch-*g*-poly(acrylic acid) copolymers, used.

Amioca® (National Starch and Chemical Company) is a corn starch consisting primarily of amylopectin (waxy corn). It is frequently used as a native thickener and texturizing agent in the food industry. The rice starch used was a native waxy rice starch (Remyline®) and was obtained from Orafti, Tienen, Belgium.

Polymer code	Starch	Starch/AA ratio	[AGE] %	Degree of neutralisation
CM 1	Amioca®	1:3	0.2	½
CM 2*	Amioca®	1:3	0.2	½
CM 3	Amioca®	1:3	0.5	½
CM 4	Amioca®	1:3	0.5	not neutralised
CM 5	Amioca®	1:5	0.5	½
CM 6	Amioca®	1:3	0.5	not neutralised**
CM 7	Amioca®	1:3	0.5	not neutralised***
CM 8	waxy rice	1:3	0.5	½
CM 9	waxy rice	1:3	0.5	not neutralised***
CM 10	waxy rice	1:3	0.5	¼
CM 11	waxy potato	1:3	0.5	½
CM 12	tapioca	1:3	0.5	½

Table 2: Synthesis parameters of the chemically modified grafted straches (CM).
(*polymerised to a higher viscosity compared to CM 1)
(**Sodium carboxylate functions were converted to carboxylic acid with diluted HCl)
(***Ion exchange resin used to convert carboxylate groups to carboxylic acid)

1.1.2 Freeze-dried starch/poly(acrylic acid) mixtures (FD)

The freeze-dried starch/poly(acrylic acid) mixtures were prepared by National Starch and Chemical Company, Bridgewater, New Jersey, USA.

First, the starch was pregelatinised by jet cooking in a custom made jet cooker. Temperature was set at 138°C, at a pressure of 3.1-3.2 bar and a flow rate of 1.2-1.5 liter/min. Steam jet cooking is an effective means of rapidly forming an aqueous starch solution. A slurry of granular starch is brought into contact with high-pressure steam, leading to the gelatinisation, disruption and solubilisation of the granules (Byars, 2003). After jet cooking the obtained aqueous starch dispersion was mixed with an aqueous solution (35% w/w) of a linear poly(acrylic acid) (PAA) (average Mw 250.000) (Sigma-Aldrich, USA). The aqueous starch/PAA mixture was freeze-dried using a Flexi Dry™ MP freeze-dryer (FTS Systems, Stone Ridge, NY, USA) to obtain a powder. After freeze-drying some powders were additionally heat treated at 120°C during 15 min., intending to effect cross-linking. (Foreman et al., 2000)

The poly(acrylic acid) used was a linear polymer of acrylic acid monomers with an average molecular weight of 250.000 (PAA) (Figure 4).

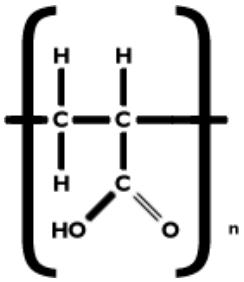

Figure 4: Chemical structure of a linear poly(acrylic acid) (PAA).

The freeze-dried powder mixtures were coded FD. Table 3 gives the process details of the used freeze-dried starch/PAA mixtures.

Polymer code	Starch	PAA (Mw)	Starch/PAA ratio	Heat Treated
FD 50/50	Amioca®	Linear (250.000)	1:1	No
FD 25/75	Amioca®	Linear (250.000)	1:3	No
FD 50/50 HT	Amioca®	Linear (250.000)	1:1	Yes
FD 25/75 HT	Amioca®	Linear (250.000)	1:3	Yes

Table 3: Details of the freeze-dried starch/PAA mixtures (FD).

1.1.3 Spray-dried starch/carboxylated polymer mixtures (SD)

The spray-dried starch/carboxylated polymer mixtures were prepared by National Starch and Chemical Company, Bridgewater, New Jersey, USA.

As for the freeze-dried starch/PAA mixtures, the starch was first pregelatinised by jet cooking. The obtained aqueous starch dispersion was then mixed with an aqueous dispersion of a carboxylated polymer. The aqueous starch/carboxylated polymer mixture was spray-dried using a Bowen spray-dryer model BE-1393 (Arnold Equipment Company, Cleveland, OH, USA) to obtain a powder. After spray-drying some powders were additionally heat treated at 120°C for 15 min. to induce cross-linking. (Ameye et al., 2003)

As carboxylated polymer a linear poly(acrylic acid) (average Mw 250.000) (PAA) (Sigma-Aldrich, USA), Carbopol® 974P (a cross-linked poly(acrylic acid)) (C 974P) and sodium carboxymethylcellulose (a cellulose derivate) (CMC) were used.

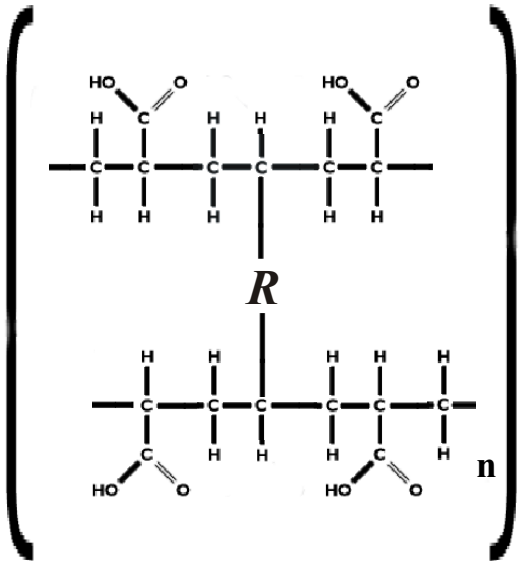

Figure 5. Structure of Carbopol® 974P. *R* = cross-linking agent (allyl pentaerythritol)

Carbopol® 974P (C 974P) (BF Goodrich, Cleveland, Ohio, USA) is a synthetic water-swellable, high molecular weight, cross-linked acrylic acid-based polymer (Figure 5). C 974P is cross-linked with allyl pentaerythritol and is polymerised in ethyl acetate, a

GRAS solvent. Carbopol® 974P is a flocculated powder of primary particles. Each primary particle can be viewed as a three-dimensional network structure of polymer chains interconnected by cross-links, which result in a molecular weight in the billions.

Carbopol® 974P is a pharmaceutical grade polymer and can be used as controlled release agent in tablets, as bioadhesive agent in buccal, ophthalmic, nasal, intestinal, vaginal, etc. applications, as a thickener, as suspending and emulsifying agent.

Sodium carboxymeyhylcellulose (CMC) (Sigma-Aldrich, USA) is a sodium salt of carboxymethylcellulose, a semi-synthetic water-soluble polymer in which -CH_2COOH groups are substituted on the glucose units of the cellulose chain through an ether linkage (Figure 6). The molecular weight (Mw) was 700.000 and the degree of substitution 0.65 – 0.85. It is used as a viscosity controller, thickener, suspending agent and emulsion stabilizer.

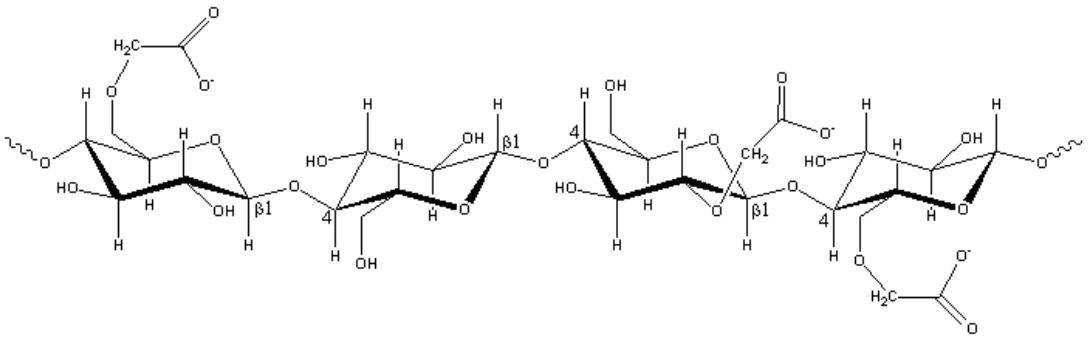

Figure 6. The carboxymethylcellulose structure is based on the β-(1-4)-D-glucopyranose polymer of cellulose.

National® 5730 (National Starch and Chemical Company) is a waxy corn starch pregelatinised by drum drying. During drum drying the starch is cooked and dried on heated rolls at the same time. After pregelatinisation a starch is obtained which will hydrate and swell in cold water.

Ultrasperse® A (National Starch and Chemical Company) is a waxy corn starch pregelatinised according a proprietary of National Starch and Chemical Company.

Tapioca (National Starch and Chemical Company) is a native starch which has application in powder and emulsion systems. It is also used in food products because of its flavour advantage. Tapioca contains 16.7 % amylose (Young, 1984).

The spray-dried starch/carboxylated polymer mixtures were coded SD. Table 4 gives the details of the spray-dried mixtures, used.

Polymer code	Starch	Carboxylated Polymer	Ratio	Heat Treated
SD PAA 50/50	Amioca®	PAA	50/50	No
SD PAA 50/50 HT	Amioca®	PAA	50/50	Yes
SD PAA 25/75	Amioca®	PAA	25/75	No
SD PAA 25/75 HT	Amioca®	PAA	25/75	Yes
SD 25/75 HT	Amioca®	Carbopol® 974P	25/75	Yes
SD 25/75	Amioca®	Carbopol® 974P	25/75	No
SD 50/50	Amioca®	Carbopol® 974P	50/50	No
SD 60/40	Amioca®	Carbopol® 974P	60/40	No
SD 70/30	Amioca®	Carbopol® 974P	70/30	No
SD 75/25	Amioca®	Carbopol® 974P	75/25	No
SD 80/20	Amioca®	Carbopol® 974P	80/20	No
SD 85/15	Amioca®	Carbopol® 974P	85/15	No
SD 90/10	Amioca®	Carbopol® 974P	90/10	No
SD 95/5	Amioca®	Carbopol® 974P	95/5	No
SD Nat 75/25	National® 5730	Carbopol® 974P	75/25	No
SD Ultra 75/25	Ultrasperse® A	Carbopol® 974P	75/25	No
SD CMC 50/50	Amioca®	CMC	50/50	No
SD CMC 50/50 HT	Amioca®	CMC	50/50	Yes
SD CMC 25/75	Amioca®	CMC	25/75	No
SD CMC 25/75 HT	Amioca®	CMC	25/75	Yes
SD Rice 50/50	waxy rice	PAA	50/50	No
SD Rice 50/50 HT	waxy rice	PAA	50/50	Yes
SD Tap 50/50	tapioca	PAA	50/50	No
SD Tap 50/50 HT	tapioca	PAA	50/50	Yes

Table 4: Details of the spray-dried starch/carboxylated polymer mixtures (SD).

1.1.4 References

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Chapter 1.2 Scanning Electron Microscopy and Solid State NMR Analysis of Spray-Dried Amioca®/Carbopol® 974P Mixtures

1.2.1 Introduction

The spray-dried Amioca®/Carbopol® 974P mixtures were analysed using Scanning Electron Microscopy (SEM) and solid state NMR (Nuclear Magnetic Resonance) spectroscopy to investigate interactions and miscibility at the molecular level.

The Scanning Electron Microscope (SEM) is designed for direct studying of the surfaces of solid objects. By scanning with an electron beam that has been generated and focused by the operation of the microscope, an image is formed. The SEM allows a greater depth of focus than the optical microscope. For this reason the SEM can produce an image that is a good representation of the three-dimensional sample. Electromagnets are used to bend an electron beam. Once the electron beam hits the sample, other electrons are ejected from the sample and collected by detectors and converted to a signal that is sent to a viewing screen similar to the one in an ordinary television, producing an image. Since the SEM uses electrons to produce an image, most conventional SEM's require that the samples are electrically conductive. In order to view non-conductive samples, these must be covered with a thin layer of a conductive material (i.e. gold, platinum) using a sputter coater.

Solid state NMR spectroscopy and relaxometry is a powerful non-invasive and non-destructive technique to investigate the segmental chain dynamics and molecular miscibility of polymer blends and copolymers on the nanometer level (Komoroski, 1986; Schmidt-Rohr and Spiess, 1996; Fedotov and Schneider, 1989; Adriaensens et al., 2002). Below the glass transition temperatures (T_g), the proton relaxation decay times T_{1H} and $T_{1\rho H}$ provide information about the level of heterogeneity (phase morphology) of a polymer mixture on the nanometer scale due to the process of proton spin diffusion. Under the condition of spin diffusion, both proton decay times can be directly related to the dimensions of the molecular domains. The proton $T_{1\rho H}$ decay time (spin-lattice

relaxation time in the rotating frame), in the order of milliseconds, will be averaged out over a short distance (in the order of 1-2 nanometer), making it a local property. Since the $T_{1\rho H}$ decay time is sensitive to molecular frequency motions of several tens of kilohertz, it reflects the motion of short segments in the polymer chain. On the other hand, the T_{1H} decay time (spin-lattice relaxation time), in the order of seconds, is sensitive to the spectral density of Larmor frequency motions (here 200 MHz) and is averaged out over a larger distance (in the order of tens of nanometers), making it a more large-scale molecular property. The maximum path length L, over which proton-proton spin-diffusion can occur, is approximately given by

$$L \approx (6DT_{iH})^{1/2} \qquad (1)$$

in which D is the spin diffusion coefficient ($\approx 10^{-16} m^2/s$ for rigid solids) and T_{iH} the decay time T_{1H} or $T_{1\rho H}$ (McBrierty and Douglas, 1981). Measuring the proton decay times via the chemical shift selective carbon signals by means of the ^{13}C-CP/MAS (Cross Polarisation/Magic Angle Spinning) technique allows to obtain information about the degree of phase separation in polymer mixtures. The T_{iH} decay time, as measured via the carbon resonances of Amioca® will only be different from this measured via the carbon resonances of Carbopol® 974P if molecular domains larger than L appear in the mixtures.

1.2.2 Materials

Carbopol® 974P (C 974P) was supplied by BF Goodrich (Cleveland, Ohio, USA). Amioca® starch is a National Starch product (National Starch and Chemical Company, Bridgewater, New Jersey, USA). All other chemicals used were of analytical grade.

1.2.3 Methods

1.2.3.1 Amioca®/Carbopol® 974P physical mixtures

Amioca®/Carbopol® 974P physical mixtures were prepared by blending granular Amioca® starch with Carbopol® 974P in the required ratios.

1.2.3.2 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) pictures were made with a JEOL JSM 5600 LV (JEOL B.V. Europe, Zaventem, Belgium).

The SEM analyses were performed by Bart De Pauw at the *Department of Morphology (Prof. P. Simoens), Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium*.

1.2.3.3 Solid-state ^{13}C-CP/MAS NMR

Solid state NMR analyses were performed by Dr. Peter Adriaenssens, Dr. Liesbet Storme and Prof. Jan Gelan at the *Institute for Materials Research (IMO), Division Chemistry, Limburgs Universitair Centrum (LUC), Univeritaire Campus, Building D, B-3590 Diepenbeek, Belgium*.

Solid-state ^{13}C-CP/MAS NMR spectra were recorded at room temperature on an Inova 200 Varian spectrometer (Varian, Belgium) operating at a static magnetic field of 4.7 T. Magic angle spinning was performed at 3.1 kHz, making use of ceramic Si_3N_4 rotors.

1.2.4 Results and Discussion

Scanning Electron Microscopy

Figure 1 is a SEM picture of an Amioca®/Carbopol® 974P physical mixture (75/25 w/w) and shows a clear phase separation between the Amioca® starch granules and the Carbopol® particles. The dimension of most of the starch granules is in the order of 10 μm, while the Carbopol® particles have dimensions situated between 1 and 10 μm. This in contrast to a spray-dried Amioca®/Carbopol® 974P mixture with the same composition (SD 75/25), in which no phase separated Carbopol® particles are observed (Figure 2).

In order to study the miscibility of the individual components, physical and spray-dried mixtures of Amioca® starch and Carbopol® 974P were studied by ^{13}C-CP/MAS relaxometry.

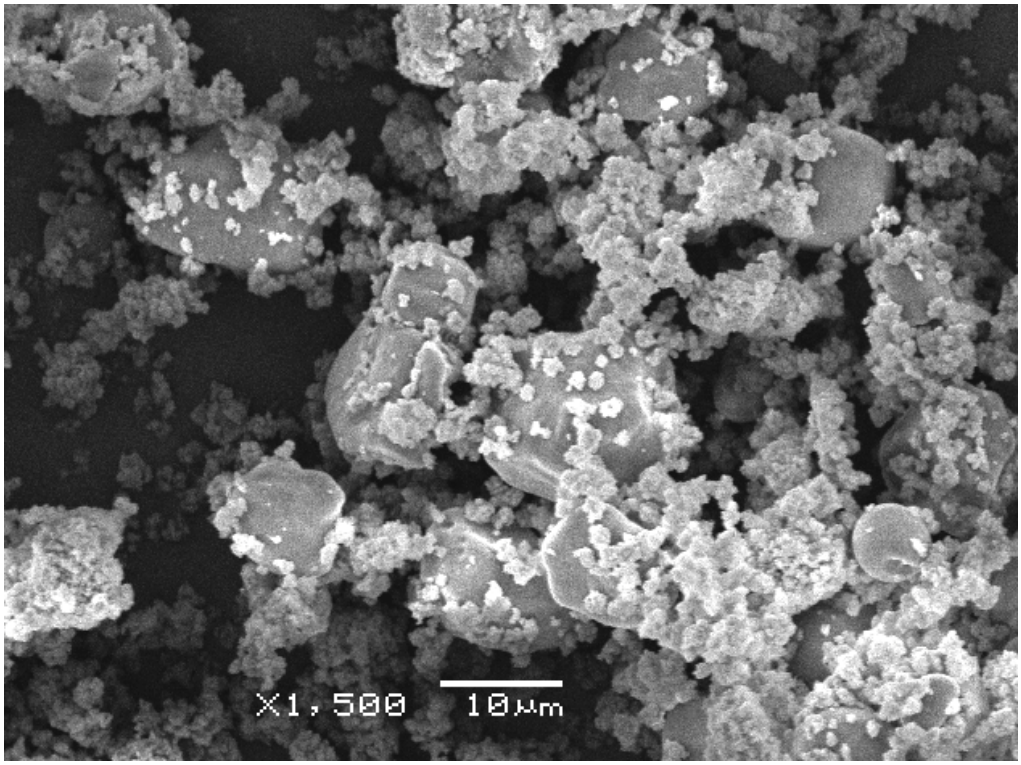

Figure 1. Scanning Electron Microscopy (SEM) picture of an Amioca®/Carbopol® 974P 75/25 (w/w) physical mixture.

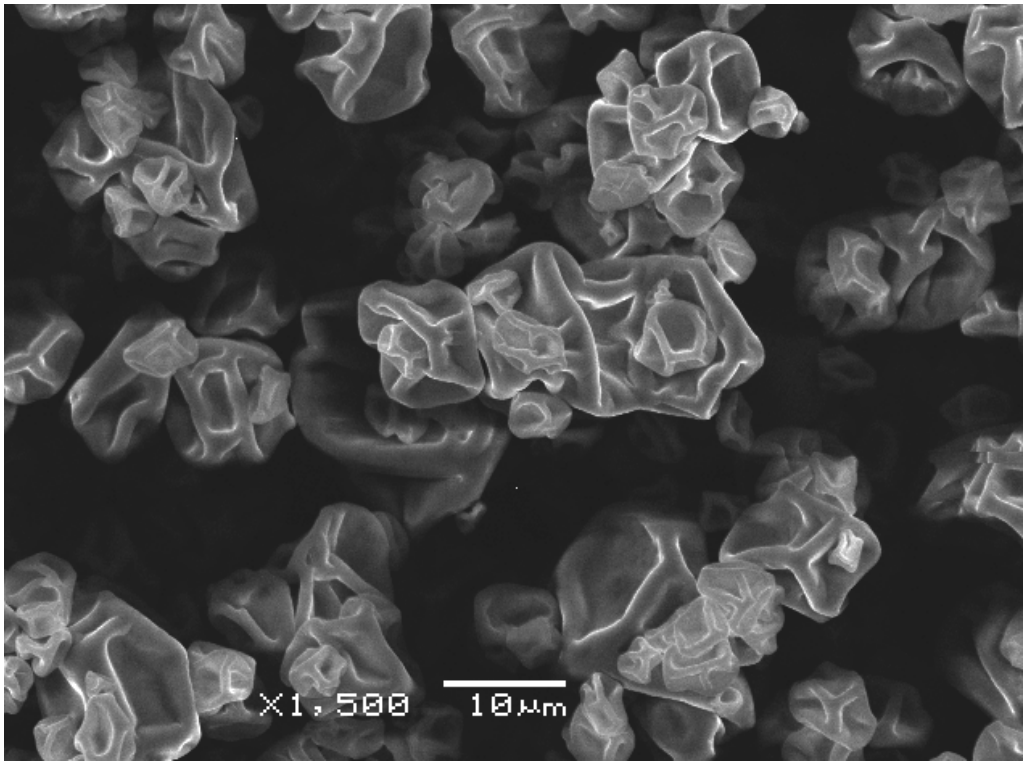

Figure 2. Scanning Electron Microscopy (SEM) picture of a spray-dried Amioca®/Carbopol® 974P 75/25 (w/w) mixture (SD 75/25).

Solid-state ^{13}C-CP/MAS NMR spectroscopy and relaxometry

Figure 3 presents a typical ^{13}C-CP/MAS spectrum of a spray-dried and physical mixture of Amioca® starch and Carbopol® 974P (75/25 w/w). The signals of Amioca® can be assigned as follows: the resonance around 100 ppm to the glycosidic carbon C1, this around 60 ppm to C6, this around 79 ppm to C2 and those around 71 ppm to C3-C5 (Kalinowski et al., 1984). The signals of Carbopol® 974P appear around 22-50 ppm (backbone methine and methylene) and 178 ppm (carbonyl carbon). It has to be noticed that, independently of the mixture composition, the Amioca® C2 resonance (79 ppm) is more resolved in the spray-dried mixtures as compared to the physical mixtures. This is a first spectroscopic indication that points to a different interaction between Amioca® and Carbopol® in the spray-dried and physical mixtures.

Figure 4 shows a plot of the T_{1H} decay times as a function of the mixture composition for physical mixtures of Amioca® starch and Carbopol® 974P. Independently on the mixture composition, the T_{1H} decay time obtained via the Carbopol® carbon resonances is different as compared to the one observed via the Amioca® carbon resonances.

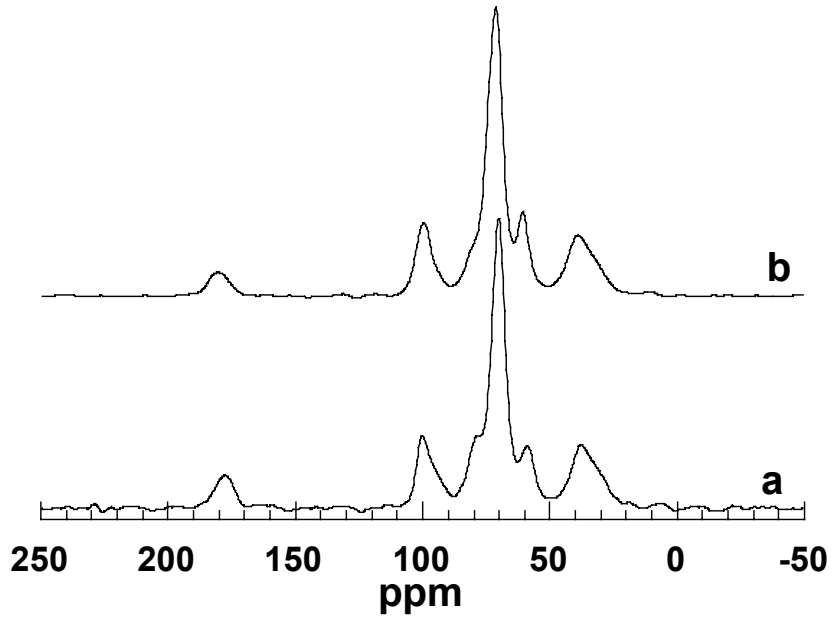

Figure 3. ^{13}C-CP/MAS spectrum of a 75/25 (w/w) mixture of Amioca® starch and Carbopol® 974P prepared by (a) spray-drying and (b) physical blending.

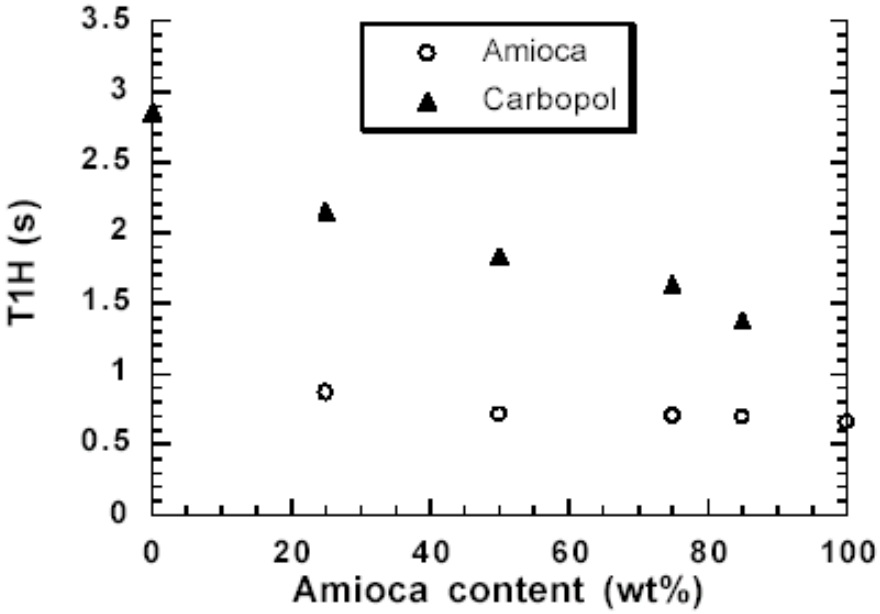

Figure 4. The T_{1H} relaxation decay time (s) as measured via the Amioca® carbon signals (❍) and Carbopol® 974P signals (▲) vs. the composition (% w/w) of the physical mixture.

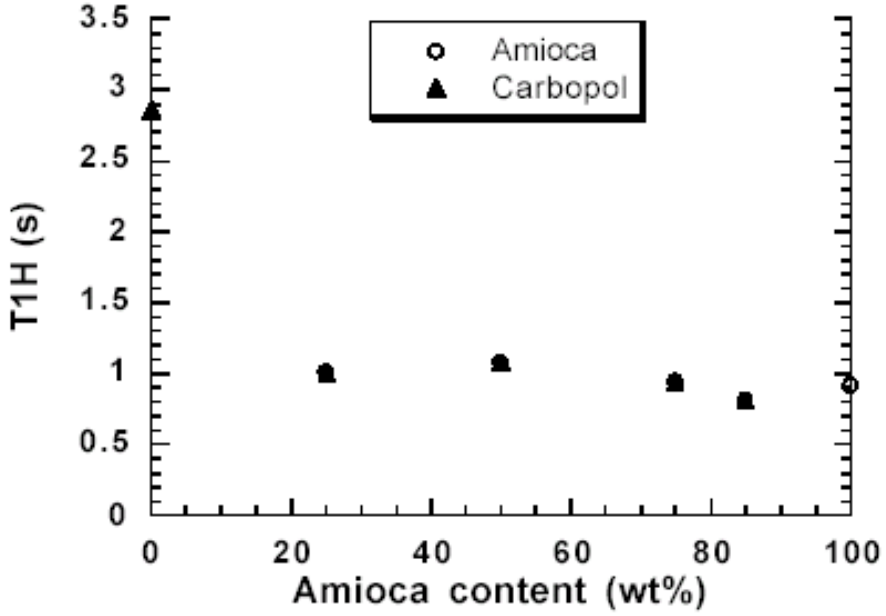

Figure 5. The T_{1H} relaxation decay time (s) as measured via the carbon signals of Amioca® starch (❍) and Carbopol® 974P (▲) vs. the composition (% w/w) of the spray-dried mixture.

Figure 5 shows a plot of the T_{1H} decay time as a function of the mixture composition for the spray-dried mixtures of Amioca® starch and Carbopol® 974P. As compared to the physical mixtures, the relaxation behaviour of the spray-dried mixtures is clearly different. Independently on the mixture composition, all signals of the carbon spectrum, these of Amioca® as well as those of Carbopol®, show the same T_{1H} decay time of which the value equals this of pure spray-dried Amioca® within experimental error.

In order to study the miscibility of Amioca® starch and Carbopol® 974P in the spray-dried mixtures on the nanometer length scale, also the $T_{1\rho H}$ decay times were determined. Figure 6 shows a plot of the $T_{1\rho H}$ decay times as a function of the mixture composition for the spray-dried mixtures. The same situation as for T_{1H} holds for the mixtures with high Amioca® content (≥ 50% w/w). A single $T_{1\rho H}$ decay time, for all signals in the carbon spectrum, is observed. A completely different situation occurs for a lower Amioca® content mixture (≤ 25% w/w). Although the observed $T_{1\rho H}$ decay times do not coincide with those of the pure components, the $T_{1\rho H}$ decay times found via the carbon resonances of Amioca® are clearly different from those obtained via the Carbopol® signals.

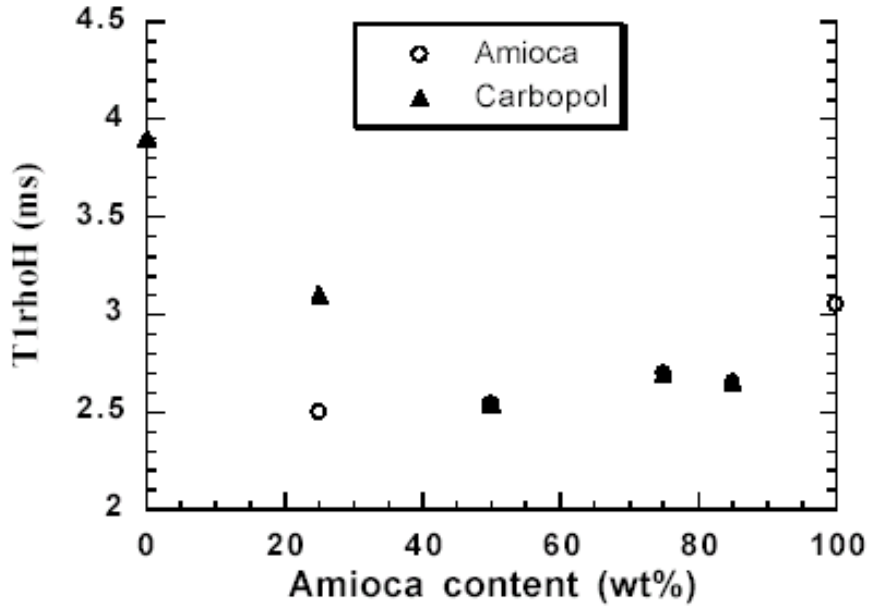

Figure 6. The $T_{1\rho H}$ relaxation decay time (ms) as measured via the Amioca® carbon signals (❍) and Carbopol® 974P signals (▲) vs. the composition (% w/w) of the spray-dried mixture.

In the physical mixtures, the T_{1H} decay time obtained via the Carbopol® carbon resonances is, independently on the mixture composition, clearly different from this observed via the Amioca® carbon resonances (Figure 4). This means that both components in the mixtures have to be phase separated into molecular domains of which the length scale exceeds several tens of nm. Based on the Carbopol® T_{1H} decay time and equation 1, it can be concluded that although the average size of the Carbopol® domains decreases upon lowering the Carbopol® content, it still exceeds 30 nm for the low Carbopol® content (15% w/w) mixture and 35 nm for the high Carbopol® content (75% w/w) mixture. This is completely in agreement with the observation of large scale phase separation in the SEM pictures (Figure 1).

With respect to the physical mixtures, the spray-dried mixtures behave completely different. Independently on the mixture composition, all signals of the carbon spectrum, these of Amioca® as well as those of Carbopol®, show the same T_{1H} decay time of which the value equals this of pure spray-dried Amioca® (Figure 5). This means that the process of spin-diffusion transfers the Carbopol® magnetisation efficiently toward Amioca® where it decays with the same time constant as the Amioca® magnetisation. Therefore, it can be concluded that if molecular domains of Carbopol® are present in the spray-dried mixtures, their dimension should be smaller than 25 nm (equation 1). Concerning the $T_{1\rho H}$ relaxation (Figure 6), a similar $T_{1\rho H}$ decay time is observed for all signals in the carbon spectrum for the SD 50/50 mixture. This means that both components of the mixture seem to be homogeneously mixed (L = 1.2 nm for a $T_{1\rho H}$ of 2.5 ms). Taking the high Carbopol® content into account, this can only be explained by the formation of a film of Carbopol® around the Amioca® granules. The same conclusion holds for the SD 75/25 and 85/15. A completely different situation starts to appear for higher Carbopol® contents. Starting from 75% w/w Carbopol® 974P the $T_{1\rho H}$ decay times found via the carbon resonances of Carbopol® are clearly different from those obtained via the Amioca® signals. It can be concluded that phase separation starts to take place for higher contents of Carbopol® ($\geq$ 75% w/w) resulting in molecular domains of Carbopol® that must exceed 1.5 nm. As a matter of fact, the Carbopol® domain size should be situated between 1.5 nm ($T_{1\rho H}$) and 25 nm (T_{1H}). If we assume that the Amioca® granules are still surrounded by a film of Carbopol®, this means that the film thickness has to exceed 1.5 nm. Another explanation can be found in phase separated Carbopol® nano-particles in addition to film formation, as shown in Figure 7.

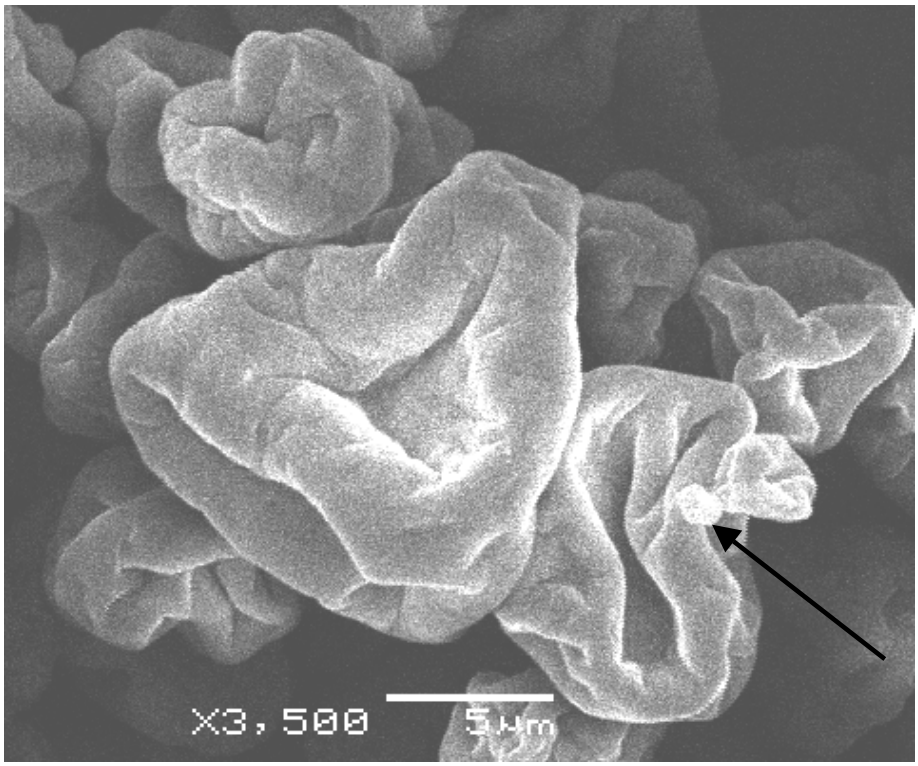

Figure 7. Scanning Electron Microscopy (SEM) picture of a spray-dried Amioca®/Carbopol® 974P 25/75 (w/w) mixture (SD 25/75). A phase separated Carbopol® 974P nano-particle is marked with the black arrow.

1.2.5 Conclusion

Scanning electron microscopy and solid state NMR spectroscopy and relaxometry analysis, revealed that by spray-drying Amioca® starch/Carbopol® 974P mixtures, Carbopol® films are formed around the starch granules. At higher Carbopol® concentrations (75% w/w), individual Carbopol® nano-particles can be found in addition to film formation.

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Chapter 2 Multifunctional Polymers for Oral Peptide Drug Delivery

CONTENTS:

Chapter 2.1 Introduction

2.1.1 Oral peptide and protein delivery

Recent advances in the structural elucidation of numerous natural peptides and proteins, in the understanding of their role in several physiological processes and in the use of biotechnological and recombinant DNA techniques for the production of a wide variety of biologically active peptides and proteins which are therapeutically applicable have stimulated considerable interest in establishing peptides and proteins as drugs in therapy. In most cases such compounds are indicated for chronic therapy where they will need to be administered by an appropriate delivery system. Different possible routes of peptide and protein administration, avoiding the parenteral route, are still investigated. They include nasal, transdermal, pulmonal, buccal and oral routes. (Lee, 1991; Lueβen et al., 1994)

Several challenges confront the delivery of peptide and protein drugs. A major challenge in using peptides and proteins as drugs is preservation of their structural integrity until they reach their sites of action, which are often remote from the site of administration. Another challenge in peptide and protein drug delivery, which is closely related to the first, is to understand the magnitude of the enzymatic barrier in degrading peptides and proteins that are administered orally, buccally, nasally, transdermally, etc. An understanding of the nature of this barrier is essential to the development of metabolically stable analogues, to the selection of protease inhibitors to control proteolytic activity, to the selection of a delivery route and to formulation of peptide and protein drug delivery systems. A third challenge is to overcome the resistance of the mucosal membranes to the penetration by peptide and protein drugs. This requires an understanding of the mechanisms of peptide and protein absorption from the mucosal routes and the development of effective and safe absorption promoters. (Lee, 1991)

The oral route is more accepted by patients than any of the other mentioned alternative routes. Successful oral peptide and protein delivery can only be achieved by taking care of the particular physiological conditions of the gastro-intestinal tract. The

stomach displays a very unfavourable environment for peptides and proteins due to the low pH and high proteolytic activity. The main absorption barriers are shown in Figure 1.

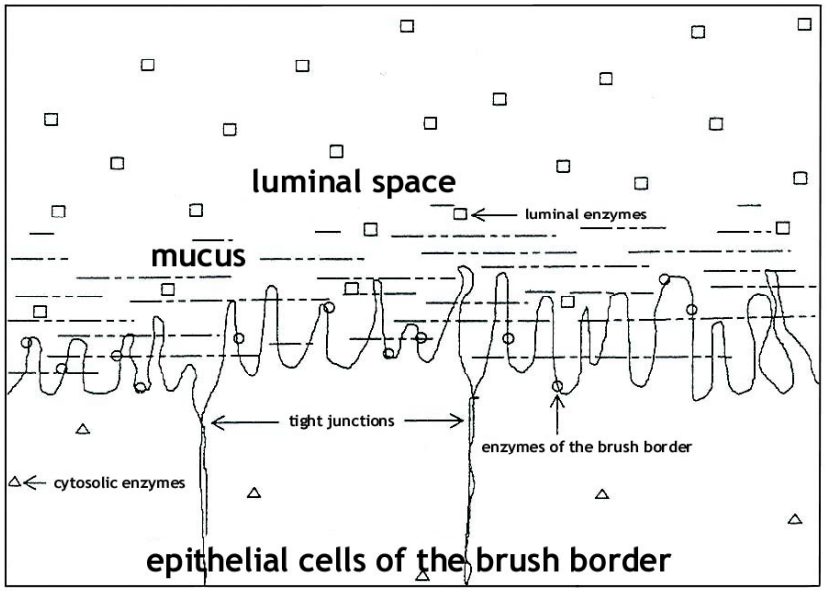

Figure 1: Schematic representation of the physiological barriers against peptide and protein absorption. (Lueβen, 1996)

Firstly, the metabolic barrier plays an important role by inactivating the peptide drug before it reaches its site of absorption. It consists of luminal proteases (such as trypsin, α-chymotrypsin, elastase, carboxypeptidase), brush border peptidases which are incorporated or attached to the membrane of epithelial cells and cytosolic enzymes. From these enzymes the luminal and the brush border enzymes play the most important role in the digestion of peptide drugs. Cytosolic enzymes are more relevant in the case of internalisation processes of the peptide drug into the epithelial cell such as endo- and transcytosis. The second barrier is presented by the paracellular epithelial integrity of the intestinal mucosa. Hydrophilic macromolecules, such as peptides, will most likely choose the paracellular route rather than the permeation through the lipophilic cell membranes. Passive diffusion of substances in between the cells is controlled by the integrity of intercellular junctions, such as tight junctions. Thirdly, the mucus, covering the epithelial cell surface, forms an efficient barrier against the diffusion of peptide drugs. The continuous secretion of glycoproteins into the intestinal lumen creates a highly viscous

gel which viscosity strongly increases in direction towards the cell surface. (Lueβen, 1996)

In general, poor absorption of peptides across mucosal surfaces is caused by the high polarity and high molecular weight of this class of compounds and their susceptibility to proteolytic degradation by luminal, brush border and cytosolic enzymes. Intestinal absorption is further reduced by the hostile environment in the gastro-intestinal tract caused by stong pH-extremes.

Next strategies for the oral absorption of peptides and proteins have been described:

- *Use of absorption enhancers, such as surfactants, bile salts, fatty acids, chelating agents, and salicylates (Aungst et al.).*
  Mesiha et al. (2002) investigated the hypoglycemic effect of orally given insulin using different bile salts as absorption promoters in rabbits. Significant hypoglycemic effects were achieved when insulin was orally given with palmitic acid combined with the bile salt in the form of aqueous fatty acid dispersions. The order of hypoglycemic enhancement was deoxycholate > cholate > glycholate > glycodeoxycholate > taurodeoxycholate > no bile salts. Hosny et al. (2002) studied the hypoglycemic effect of enteric-coated capsules containing sodium salicylate as absorption enhancer in dogs. 25-30% reduction in plasma glucose levels and a relative hypoglycemia of 12.5% relative to subcutaneous insulin injection could be achieved.
- *Use of enzyme inhibitors.*
  Lane et al. (1998) evaluated the effects of protease inhibitors aprotinin, bacitracin and soybean trypsin inhibitor (STI) on insulin stability and absorption in a perfused rat gut model. STI was less effective than bacitracin or aprotinin in promoting insulin stability. In line with these results, bacitracin and aprotinin produced higher plasma insulin levels than STI. Guggi et al. (2003) investigated in vitro the efficacy of chitosan-inhibitor conjugates towards calcitonin degradation by intestinal serine proteases. Protease inhibitors, Bowman-Birk inhibitor and elastatinal, were covalently attached to chitosan, resulting in polymer-inhibitor conjugates which showed excellent inhibitor efficacy towards trypsin/α-

chymotrypsin and elastase, respectively, and were able to reduce significantly the digestion of calcitonin caused by these proteases.

- *Encapsulation of the drug in particular carriers.*

  Sakuma et al. (1997) synthesised polysterene nanoparticles to increase the oral absorption of salmon calcitonin (sCT). The hypocalcemic effect after oral administration of a mixture of salmon calcitonin and polysterene nanoparticles in rats depended greatly on the administration schedule. Halved doses given 40 min. apart, enhanced markedly the sCT absorption. Minimun blood ionised clacium levels of 70% of the initial concentrations were obtained. Chung et al. (2002) encapsulated insulin in 'nano-cubicle' particles, produced by dispersion of a liquid formula in water, as carriers for peroral peptide delivery. Orally given 'nano-cubicles' encapsulating insulin to streptozotocin-induced diabetic rats, increased the serum insulin concentrations significantly compared to the basal concentrations during 4 to 6h. Cournarie et al. (2002) evaluated the biological activity and bioavailability of insulin given orally as insulin-loaded nanocapsules in streptozotocin-induced diabetic rats. Thirty minutes to 1 h after oral administration, significant plasma levels of insulin were detected, but no decrease of glycemia was observed.

- *Peptide analogues.*

  Based on the knowledge of the specificities and location of enzymes present in the gastro-intestinal tract, it is possible to identify the most vulnerable bonds in the structure of a therapeutic peptide and then to make chemical modifications to the peptide to prevent cleavage by intestinal enzymes (Woodley, 1994). Analogues of vasopressin (a nonapeptide) with a significant anti-diuretic effect have been synthesised. Potentiation and prolongation of biological responses have been achieved for enkephalins (pentapeptides with analgesic properties) by designing synthetic analogues (Lee et al., 1991). But, these strategy seemed to be limited to small peptides (10-12 amino acids) (Woodley, 1994).

Another approach consists in the use of mucoadhesive multifunctional polymers. Such polymers are multifunctional macromolecules which are both able to increase the permeability of epithelial tissues and simultaneously to inhibit proteolytic enzymes. With

their mucoadhesive properties, these polymers are expected to make close contact to the mucosa, thereby creating locally high drug concentrations in specified regions, improving and enhancing the bioavailability of the drug.

Chitosan, a linear polysaccharide derived by *N*-deacetylation of the natural polymer chitin, and its quaternised derivative *N*-trimethyl chitosan chloride have shown to be able to enhance the absorption of hydrophilic and macromolecular compounds across intestinal epithelia by interacting with components of the tight junctions, leading to opening of the paracellular transport route. (Thanou et al., 2001; Jonker et al., 2002)

Lueβen (1996) evaluated the effect of the mucoadhesive cross-linked poly(acrylic acid)s carbomer (Carbopol® 934P) and polycarbophil on the proteolytic activity of intestinal enzymes, such as trypsin, α-chymotrypsin and carboxypeptidases. Carbomer and polycarbophil were able to inhibit trypsin, α-chymotrypsin and carboxypeptidase A. Carbomer was found to be more efficient to reduce proteolytic activity than polycarbophil. The pronounced binding properties for bivalent ions such as calcium and zinc was found to be a major reason for the observed inhibitory effect (Lueβen et al., 1996). It was shown that carbomer was able to influence the permeability of epithelial cell monolayers in vitro. The depletion of extracellular Ca^{2+} was presumed to play the major role in opening the tight junctions and establishing paracellular transport. It was concluded that poly(acrylates) may be promising excipients to protect peptide drugs from intestinal degradation. In combination with their low toxicity they were expected to be suitable excipients to improve the peroral delivery of peptides (Borchard et al., 1996).

2.1.2 In vitro evaluation of multifunctional polymers: Trypsin inhibition and Calcium & Zinc ion binding

The enzymatic barrier is by far the most important of the multitude of barriers limiting the absorption of natural peptide and protein drugs from the gastro-intestinal (GI) tract. The enzymatic barrier is well designed to efficiently digest proteins and peptides to a mixture of amino acids and small quantities of peptides consisting of two to six amino acid residues prior to the appearance in portal circulation. In the GI tract, hydrolysis of peptides and proteins can occur at several sites: luminally, at the brush border and intracellularly. Protein and peptide digestion is initiated by pepsins, which are most active at pH 2-3 but which become inactive at a pH above 5. Although they are capable of doing so, pepsins rarely degrade proteins and peptides to amino acids. The partial digestion that results is then acted upon by pancreatic proteases in the duodenum and beyond. These proteases consist of trypsin, α-chymotrypsin, elastase and carboxypeptidase A. The first three are endopeptidases, whereas the last is an exopeptidase. The three endopeptidases have evolved to complement each other in cleaving almost all the internal peptide linkages likely encountered in a wide spectrum of peptides and proteins. Trypsin prefers to cleave peptide bonds near basic amino acids such as arginine and lysine. By contrast, α-chymotrypsin preferentially cleaves peptide bonds near hydrophobic linkages such as leucine, methionine, phenylalanine, tryptophan and tyrosine. Elastase complements the other two proteases by cleaving peptide bonds near alanine, glycine, isoleucine, leucine, serine and valine (peptide bonds of amino acids bearing smaller, unbranched, nonaromatic side chains). Carboxypeptidase A is a C-terminal exopeptidase. These pancreatic enzymes are responsible for only 20% of the enzymatic degradation of ingested peptides and proteins. The remainder of the degradation occurs upon contact with proteases of the brush border or following entry into the cell (cytosolic proteases). (Lee et al., 1991)

It is clear that trypsin plays a key role in initiating the degradation of orally administered peptide drugs and in activating the zymogen forms of a lot of pancreatic peptidases (Woodley, 1994). Many proteases have bivalent cations as calcium and zinc as essential co-factors within their structure. The endopeptidases trypsin and α-chymotrypsin are Ca^{2+} containing proteases, elastase is a zinc metalloprotease, while

carboxypeptidase A belongs to the group of Zn^{2+}-dependent exopeptidases (Lueßen et al., 1996). Several research groups have reported that the complexation of Ca^{2+} or Zn^{2+} from proteolytic enzymes reduces or totally inhibits the proteolytic activity in the lumen. The complexation of Ca^{2+} and Zn^{2+} by poly(acrylic acid) formulations (e.g. Carbopol® 934P) can be advantageous for peptide and protein drugs to prevent intestinal degradation and to enhance absorption. (Lueβen et al., 1995; Akiyama et al., 1996; Ooya et al., 2002)

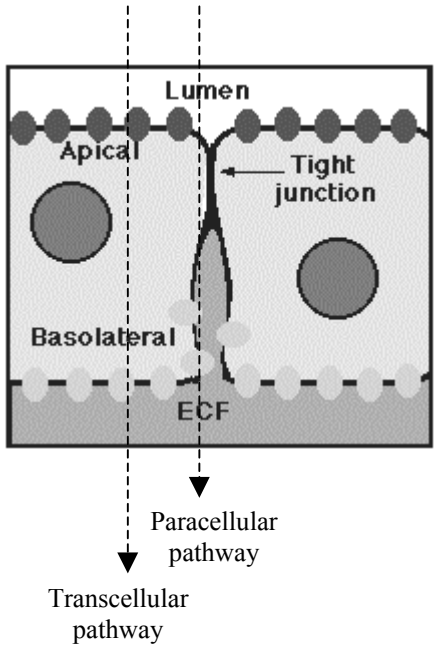

Figure 1. Schematic representation of the trans- and paracellular pathway in transporting epithelia with the tight junction.

The absorption barrier in the intestine is represented by epithelial cell membranes interconnected by tight junctions. The intactness of the tight junctions is linked to the presence of Ca^{2+} and Mg^{2+} ions. The reduction of extracellular Ca^{2+} concentration can result in an opening of the tight junctions, allowing paracellular peptide drug transport (Borchard et al., 1996). The so-called paracellular pathway is the aqueous pathway along the intercellular space of cells, which is restricted by tight junctions at the apical side of the cells. The aqueous nature of this pathway makes it the favourable route of transport across cell layers for hydrophilic compounds such as peptide and protein drugs, avoiding crossing the lipophilic cell membrane. The transport of the compounds via this route is by passive diffusion through the intercellular space. The main limitation in this transport is

the size of the tight junctional “channel” (Figure 1). The tight junction is a proteinaceous structure at the apical side of the intercellular space interconnecting the cell membranes of two adjacent cells. One of the main functions attributed to the tight junctions is to form a restriction in the permeability of solutes and water via the paracellular way. (Noach et al., 1994).

A critical ion necessary for the integrity of the tight junctions is Ca^{2+}. Reduction of the extracellular Ca^{2+}-concentration results in an opening of the tight junctions. The mucoadhesive poly(acrylic acid) Carbopol® 934P (carbomer) was able to influence the permeability of epithelial cell monolayers in vitro by depletion of extracellular Ca^{2+}. Taking the additional capabilities of Carbopol® 934P to inhibit proteolytic enzymes and its mucoadhesive properties into consideration, it might be concluded that multifunctional poly(acrylic acid) formulations can play an important role in the design of drug formulations for oral peptide drug delivery. (Borchard et al., 1996)

As the most proteolytic enzymes have Ca^{2+} or Zn^{2+} at their active sites and as a reduction of extracellular Ca^{2+} concentration results in an opening of the tight junctions, the in vitro inhibition potency of the newly synthesised starch-*g*-poly(acrylic acid) copolymers and freeze-dried / spray-dried starch/carboxylated polymer mixtures towards the proteolytic enzyme trypsin and the Ca^{2+} and Zn^{2+} ion binding capacity of these multifunctional polymers was investigated.

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Chapter 2.2 Optimisation of an In Vitro Procedure for the Determination of the Enzymatic Inhibition Potency of Multifunctional Polymers

2.2.1 Introduction

The two major barriers to peptide and protein absorption from the gastro-intestinal tract are enzymatic degradation and permeation across the intestinal epithelium. To overcome these barriers several approaches have been tried: coadministration of peptides with protease inhibitors and absorption enhancers, structural modifications of the peptide to prevent proteolytic attack, and carrier systems to protect the peptide from enzymatic attack and to release the drug at the site of the gut most favourable for absorption (Walker et al., 1999).

As peptides and proteins will most likely choose the paracellular route rather than the permeation through the lipophilic cell membranes, the luminal and membrane bound enzymes play the most important role in their digestion. Luminal enzymes such as the endopeptidases trypsin and α-chymotrypsin, often initiate the degradation of perorally administrated peptides. A variety of exopeptidases, such as carboxypeptidases and aminopeptidases, which are mainly embedded in the brush border membrane of the intestinal epithelium, but are also present in the lumen of the gut, will further digest the resulting fragments. (Lueßen et al., 1994; Lueßen et al., 1996; Woodley, 1994)

Several studies have already investigated the potency of multifunctional polymers to inhibit the proteolytic enzyme trypsin using an in vitro trypsin inhibition test. It has been shown that the mucoadhesive poly(acrylates) Carbopol® 934P and polycarbophil were able to inhibit some proteolytic enzymes like trypsin, due to Ca^{2+} depletion from the enzyme structure (Lueßen et al., 1996). The calcium binding capacity of poly(methacrylic acid) grafted with poly(ethylenegycol) (P(MAA-*g*-EG)) was related directly to a significant ability to inhibit the enzyme trypsin (Madsen and Peppas, 1999). In another study it was concluded that the trypsin inhibition by Carbopol® 934P was due to an

enzyme-polymer interaction (Walker et al., 1999). However, in the described inhibition assays the enzymatic reaction was never biochemically optimised.

In the present study an in vitro trypsin activity assay was biochemically optimised and validated for the evaluation of the inhibition capacity of newly synthesised starch-*g*-poly(acrylic acid) copolymers and freeze-dried / spray-dried starch/carboxylated polymer mixtures.

2.2.2 Materials and Methods

2.2.2.1 Materials

Trypsin (TPCK treated from bovine pancreas), N-α-benzoyl-L-arginine-ethylester (BAEE), N-α-benzoylarginine (BA) and 2-[N-morpholino]ethane-sulfonic acid (MES) were purchased from Sigma-Aldrich, Bornem, Belgium. Carbomer (Carbopol® 934P) was a gift from BF Goodrich (Cleveland, Ohio, USA). All other chemicals used were at least of analytical grade.

The buffer system used in the trypsin inhibition study was a 50 mmol/l 2-[N-morpholino]ethane-sulfonic acid (MES) / KOH buffer, pH 6.7, containing 250 mmol/l mannitol.

2.2.2.2 HPLC analysis

HPLC analysis was performed with a HPLC system consisting of an isocratic HPLC pump (type L-7110, Merck-Hitachi, Darmstadt, Germany), an injector with a loop of 20 µl (Valco 6 channel injector, Valco Instruments Corporation, Houston, USA), a UV detector (type L-7400, Merck-Hitachi, Darmstadt, Germany) and a software interface (type D-7000, Merck-Hitachi, Darmstadt, Germany). Data were calculated with the software package 'HPLC System Manager' (Merck-Hitachi, Darmstadt, Germany). The column was a Lichrosorb 7 RP 18 column (100 x 3.0 mm) equipped with a RP precolumn (10 x 2 mm) (Chrompack, Antwerpen, Belgium). The mobile phase, used as an isocratic eluent, consisted of 86 % (v/v) 10 mmol/l ammonium acetate buffer pH 4.2 with 10

mmol/l triethylamine and 14 % (v/v) acetonitrile. The analysis was performed at room temperature.

Amounts of 20 mmol/l N-α-benzoyl-L-arginine-ethylester (BAEE), the model substrate for trypsin, were dissolved in the polymer preparation containing 0.25 % (w/v) Carbopol® 934P dispersed in a 50 mmol/l MES/KOH buffer, pH 6.7, with 250 mmol/l mannitol. The pH of the test medium was 6.7 and was within the optimal activity range of trypsin, which is between pH 6 and 9 (Scharpé et al., 1997). At time zero 30 Enzymatic Units trypsin/ml (enzymatic activity determined according the Enzymatic Assay of Trypsin used by Sigma-Aldrich, Bornem, Belgium) were added to the polymer preparation, after which the solution was incubated at 37 °C for 1 h. 50 µl of sample was withdrawn at predetermined time intervals and diluted in 1.0 ml 0.1M HCl to stop the trypsin activity. The degradation of the substrate BAEE was studied by following the formation of the metabolite N-α-benzoylarginine (BA) by HPLC with UV detection at 253 nm. BA concentrations were calculated from a calibration curve of BA standards in distilled water. The retention time of the metabolite peak was 1.3 min after injection of 20 µl at a flow rate of 0.75 ml/min.

The degree of trypsin inhibition was expressed by the Inhibition Factor (IF):

$$IF = \text{reaction rate}_{control} \; / \; \text{reaction rate}_{polymer}$$

The IF is defined as the ratio of the reaction rate of the metabolite concentration time curve for the enzymatic reaction carried out without polymer (control) and with polymer, respectively. The reaction rate was calculated by linear regression analysis of the N-α-benzoylarginine (BA) concentration versus reaction time.

2.2.2.3 BAEE-Carbopol® complex

Formation of a complex between substrate and poly(acrylic acid) was quantitatively determined by filtration of polymer preparations containing different

BAEE concentrations over Ultrasart® D 20 filters with a cut off value of 20.000 (Sartorius AG, Göttingen, Germany).

Amounts of N-α-benzoyl-L-arginine-ethylester (BAEE) varying from 0 to 30 mmol/l were dissolved in the polymer preparation containing 0.25 % (w/v) Carbopol® 934P dispersed in a 50 mmol/l MES/KOH buffer, pH 6.7, with 250 mmol/l mannitol. After filtration, BAEE concentrations in the filtrate were spectrophotometrically analysed at 253 nm by means of a Perkin Elmer Lambda 12 UV-VIS double beam spectrophotometer (Zaventem, Belgium). BAEE concentrations were calculated from a calibration curve of BAEE standard solutions in the MES/KOH buffer (pH 6.7 + 250 mmol/l mannitol). Coefficients of variation on the filtration method were < 5%.

2.2.3 Results and Discussion

Trypsin inhibition assays have been described by Lueβen et al. (1996), Madsen and Peppas (1999) and Walker et al. (1999). However, in these assays the enzymatic reaction was not biochemically optimised. The used substrate concentrations were too low in proportion to the enzymatic activities or the enzymatic activity was too high for the used substrate concentration, so that the metabolite concentration versus time curves reached very fastly a plateau or post-steady state because of exhaustion of the substrate. Although Walker et al. (1999) stated that the formation of BA from BAEE in the presence and absence of Carbopol® 934P was constant over the period assayed, it is incorrect to describe these test conditions as the linear or steady state part of the metabolite versus time curve. Formation of the metabolite BA was followed by HPLC analysis (Lueβen et al., 1996; Madsen and Peppas, 1999) or by change in absorbance at 252 nm (Walker et al., 1999), where there is an interference of the substrate BAEE with BA. The degree of trypsin inhibition was expressed by the Inhibition Factor which was defined as the ratio of the AUC value of the metabolite concentration versus time curve for the reaction without polymer (control) and with polymer, respectively (Lueβen et al., 1996; Madsen and Peppas, 1999) or as a percentage of the control (Walker et al., 1999). Such an AUC value is obtained by integrating at varying reaction orders until the enzyme becomes inactive or the substrate is exhausted. When the shapes of the curves are not identical, great

uncertainties in data interpretation occur. These different trypsin inhibition assays make it very difficult to evaluate and to compare trypsin inhibition capacities.

In the present study a validated HPLC method was used to analyse BA concentrations and the enzymatic reaction was biochemically optimised.

2.2.3.1 Validation of the HPLC method

The HPLC analysis method was validated based on the International Conference Harmonisation (ICH) Harmonised Tripartite Guidelines for validation of analytical procedures (1994). The following validation characteristics were considered: specificity, linearity, accuracy, precision, detection and quantification limit.

2.2.3.1.1 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of other (interfering) components.

Specificity was assessed by comparing the chromatograms of N-α-benzoylarginine (BA) in distilled water (standard of the calibration curve) (a), in a control sample (b) and in a polymer sample (c) under inhibition test conditions (Figure 1). From Figure 1 it is clear that no interfering peaks could be observed. Thus, it was concluded that the method was specific for the determination of the metabolite N-α-benzoylarginine as there was no interference of the substrate BAEE, the enzyme, the buffer solution or dispersed polymer. The retention time of the N-α-benzoylarginine peak was 1.3 min after injection.

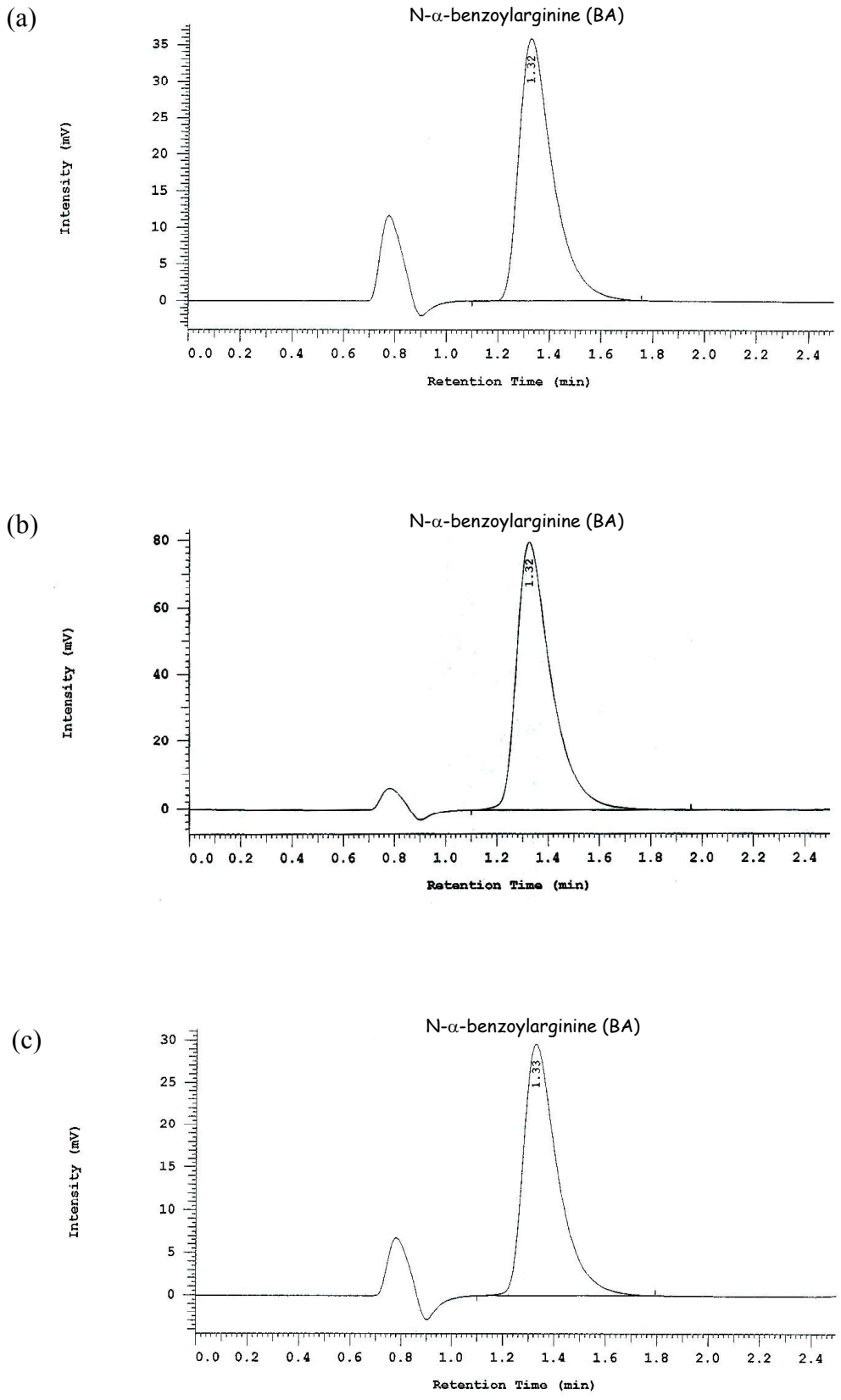

Figure 1. Chromatograms of N-α-benzoylarginine (BA) in distilled water (standard of the calibration curve) (a), in a control sample (b) and in a polymer sample (c) under inhibition test conditions.

2.2.3.1.2 Linearity

The linearity of an analytical procedure is its ability – within a given range – to obtain test results, which are directly proportional to the concentration of analyte in the sample.

Calibration curves of N-α-benzoylarginine (BA) were prepared in distilled water over a range from 0.01 mM to 0.4 mM (5 concentrations). A blank was included in the calibration curve. During validation and analysis different calibration curves were determined. Table 1 gives the slope, the Y-intercept and the determination coefficient (R^2) of the mean regression line (n = 6) determined within one day and over different days. The mean value (n = 6), the standard deviation (SD) and the coefficient of variation (CV) are given.

	Within 1 day variation			**Day-by-day variation**		
	mean	SD	CV (%)	mean	SD	CV (%)
Slope	6284682	16375	0.26	6295749	26008	0.41
Y-intercept	12672	1791	14.14	11928	2317	19.42
R^2	0.9999	0.0001	0.0074	0.9998	0.0001	0.0086

Table 1. Slope, Y-intercept and determination coefficient of the mean regression line (n = 6) determined within one day (within 1 day variation) and over different days (day-by-day variation).

From Table 1 it is clear that the relationship between response and concentration was linear and reproducible. The determination coefficients of all measured calibration curves were all higher than 0.9997 with coefficients of variation lower than 0.01%, both for within 1 day and day-by-day variation. The coefficients of variation on the slopes were lower than 0.5%.

2.2.3.1.3 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the value found and is expressed as the percent agreement between the mean determined value and the true concentration.

The accuracy was investigated at three concentration levels on standards containing a known BA amount covering the range of the calibration curve: 0.02 mM –

0.1 mM and 0.3 mM. The three concentration levels were submitted to the normal inhibition test procedure and were measured without polymer (control) and in presence of the reference polymer Carbopol® 934P (polymer). Each concentration was determined six times. The mean accuracies ± standard deviations (SD) are listed in Table 2.

	Accuracy (%)	
[BA]	Control	Polymer
0.02 mM	90.98 ± 0.82	88.37 ± 0.88
0.10 mM	100.12 ± 0.64	98.69 ± 0.98
0.30 mM	98.85 ± 1.01	97.06 ± 1.46

Table 2. Accuracy (%) measured without polymer (Control) and in presence of the reference polymer (Polymer). (n = 6, mean ± SD)

All mean values were within ± 15% of the actual concentration. (Acceptance criteria: within 15% of the actual value, Shah et al., 1992)

2.2.3.1.4 Precision

The precision expresses the closeness of agreement between repeated determinations of the same sample. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Repeatability or intra-assay precision expresses the precision under the same operating conditions over a short interval of time. Intermediate precision expresses within laboratory variations such as different days, different analysts, different equipment, etc. Reproducibility expresses the precision between different laboratories (inter-laboratory). Precision is expressed as the coefficient of variation (%) of a series of measurements of the calibration standards.

Here, all analyses were performed in the same laboratory, so only the repeatability (within one day) and intermediate precision (day-to-day) were evaluated.

The repeatability and intermediate precision were calculated on six calibration curves (n = 6). The standard deviation (SD) and coefficient of variation (CV) were calculated. (Table 2, Table 3)

[BA]	Repeatability	
mM	Mean ± SD	CV (%)
0.01	0.0080 ± 0.0001	2.02
0.05	0.0499 ± 0.0003	0.29
0.10	0.1019 ± 0.0003	0.29
0.25	0.2518 ± 0.0013	0.48
0.40	0.3985 ± 0.0007	0.34

Table 2. Repeatability. (n = 6) (mean ± standard deviation (SD) and coefficient of variation (CV))

[BA]	Intermediate Precision	
mM	Mean ± SD	CV (%)
0.01	0.0079 ± 0.0002	2.97
0.05	0.0499 ± 0.0004	0.40
0.10	0.1019 ± 0.0004	0.30
0.25	0.2519 ± 0.0017	0.76
0.40	0.3984 ± 0.0009	0.45

Table 3. Intermediate precision. (n = 6) (mean ± standard deviation (SD) and coefficient of variation (CV))

As well for repeatability as for intermediate precision, all coefficients of variation did not exceed 15%. (Acceptance criteria: CV < 15%, Shah et al., 1992)

2.2.3.1.5 Detection and quantification limit

The detection limit of an analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value, while the quantification limit is the lowest amount which can be quantitatively determined with suitable precision and accuracy.

The detection limit was determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte could be reliably detected (CV < 20%) (Vermeire, 1998). The detection limit was 0.003 mM (CV = 12.25%, n = 10).

The quantification limit was determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte could be quantified with acceptable accuracy and precision. The quantification limit was 0.010 mM: accuracy < 15% of the actual concentration and precision around the mean value < 15% CV (n = 10). (Shah et al., 1992)

2.2.3.2 Biochemical optimisation of the enzymatic reaction

Several substrate (BAEE) concentrations and enzymatic activities of trypsin were evaluated aiming at extracting the linear or steady state part of the metabolite versus time curve of the enzymatic degradation reaction (Figure 2, Figure 3).

The reaction rate during steady state provides more consistent information about the initial rate of the enzymatic degradation than the AUC values, reported by Lueβen et al. (1996) and Madsen and Peppas (1999), which can only give an estimation of the average rate. As the initial rate is a better measure for the enzyme activity the Inhibition Factor (IF) is more accurately described by the ratio of reaction rates. By using the curves described by Walker et al. (1999), it is difficult to determine the correct inhibition strength by the reaction rates.

Working at steady state conditions has the advantage that both free enzyme and enzyme-substrate complex concentrations remain constant and that there is a constant decrease of free substrate and increase of product (Figure 2).

According to the Michaëlis-Menten theory $v = V \times S_0 / (K_m + S_0)$, in which v is the degradation rate as function of substrate concentration at t=0 (S_0), V is the maximum degradation rate and K_m is the Michaëlis-Menten constant of BAEE.

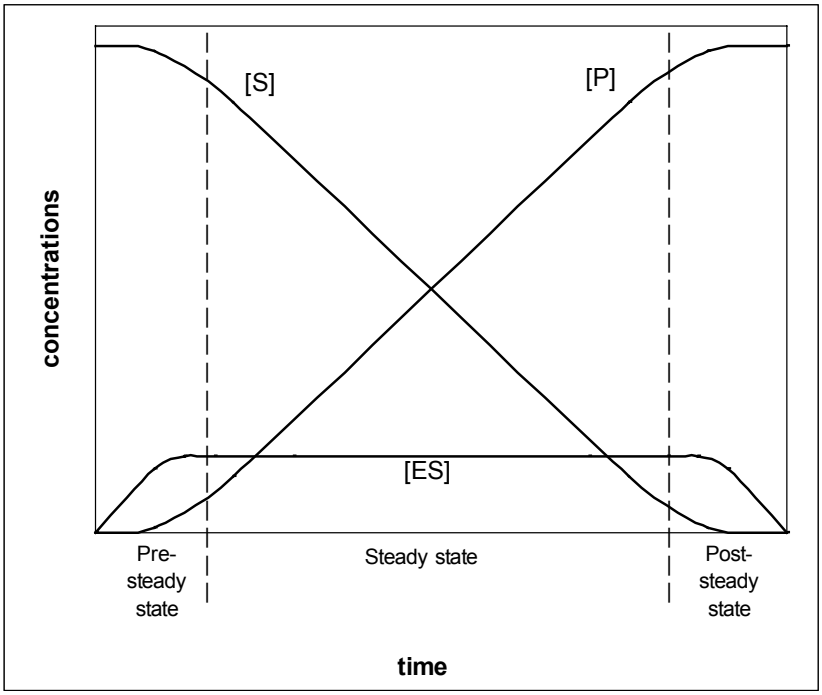

Figure 2: The steady state in enzyme kinetics. The concentrations of substrate [S], enzyme-substrate complex [ES] and product [P] vary with time. After a very brief initial period , [ES] reaches a steady state in which ES is consumed approximately as rapidly as it is formed. During steady state decrease of free substrate and increase of product is constant. (Mathews and Van Holde, 1996)

A BAEE concentration of 20 mmol/l and a trypsin activity of 30 Enzymatic Units/ml (enzymatic activity determined according the Enzymatic Assay of Trypsin used by Sigma-Aldrich, Bornem, Belgium) were used. As the Michaëlis-Menten constant equals 0.05 mmol/l (Barman, 1969), working at a substrate concentration of 20 mmol/l corresponds to 99.8% of the maximal reaction rate, and hence the degradation rate is independent of substrate concentration. With a substrate concentration of 20 mM BAEE and a trypsin activity of 30 Enzymatic Units/ml the determination coefficients for the control (without polymer) and the polymer were never below 0.999 and 0.995, respectively. Using enzyme activities between 0 and 40 Enzymatic Units/ml the reaction rate was directly proportional to the enzyme concentration (determination coefficient of 0.9999).

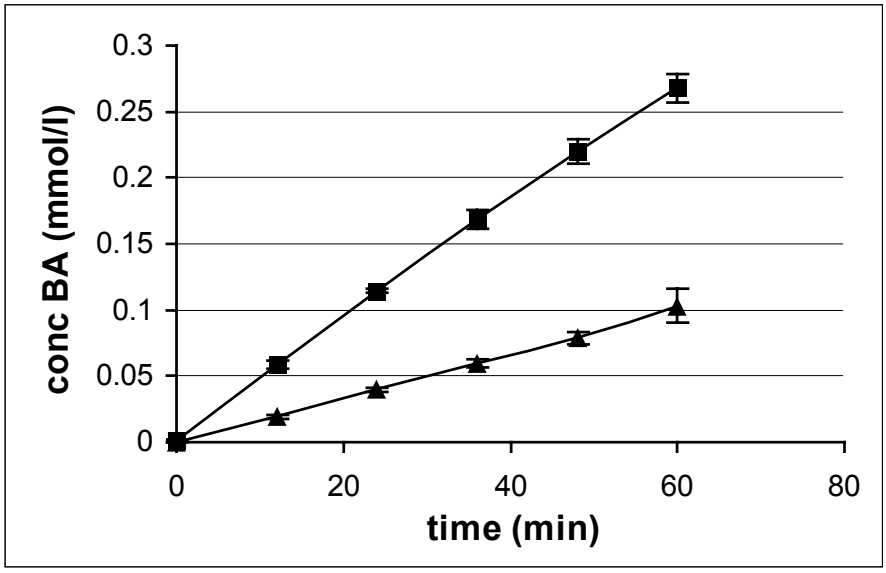

Figure 3: Formation of N-α-benzoylarginine (BA) due to the degradation of N-α-benzoyl-L-arginine-ethylester (BAEE) by trypsin in presence of Carbopol® 934P (carbomer) 0.25 % (w/v) ▲, and without any polymer (control) ■. (n = 3, mean ± SD)

Now the Inhibition Factor can be defined as the ratio of the reaction rate of the metabolite versus time curve without polymer (control) and with polymer (Figure 3). In the case of Carbopol® 934P the IF was 2.7.

$$IF = \text{reaction rate}_{\text{control}} / \text{reaction rate}_{\text{polymer}}$$

2.2.3.3 BAEE - Carbopol® complex

During the optimisation of the trypsin inhibition test formation of an ion complex in the MES/KOH buffer, pH 6.7, with 250 mmol/l mannitol, between the substrate BAEE and the carboxylic groups of the poly(acrylic acid) was observed. The formation of the complex was concentration dependent, reached a plateau at 20 mmol/l BAEE and was visually observed as with an increasing concentration of BAEE a higher degree of cloudiness of the solution was seen (Figure 4).

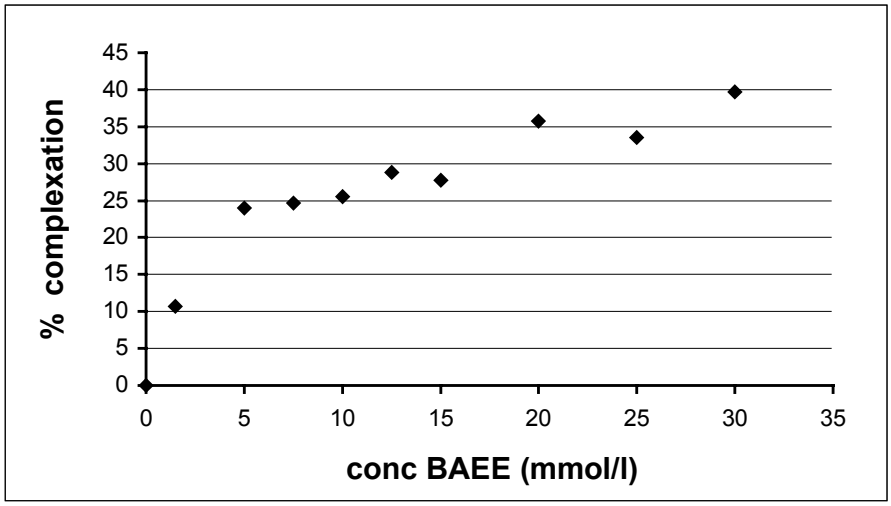

Figure 4: Formation of a concentration dependent complex between 20 mmol/l N-α-benzoyl-L-arginine-ethylester (BAEE) and 0.25 % (w/v) Carbopol® 934P.

After filtration a control sample (without polymer) containing 20 mM BAEE, 0 % complexation was found, indicating there was no binding of BAEE to the membrane during filtration.

When comparing the linear progress functions of metabolite formation (BA) during 1 h of a control sample containing 20 mM and 12.86 mM (20 mM – 35.71 % complexation) substrate BAEE, respectively, similar reaction rates were obtained. The reaction rates were 0.0052 (20 mM) and 0.0053 (12.86 mM). Also in presence of 0.25% (w/v) Carbopol® 934P, no differences in reaction rates were observed. 20 mM and 12.86 mM BAEE resulted in a reaction rate of 0.0019 and 0.0018, respectively.

Thus, as long as there is an excess of the substrate present in the given reaction circumstances and the maximal reaction rate is approached, the influence of this complex formation between substrate and polymer on the enzymatic reaction is nihil. Nevertheless when performing enzyme inhibition tests this complex formation has to be taken into consideration.

2.2.4 Conclusion

An in vitro trypsin inhibition test procedure was biochemically optimised and validated. This method allows to characterise, evaluate and compare the in vitro trypsin inhibition strength for most multifunctional polymers. The measurements are carried out in steady state conditions at 99.8% of the maximal reaction rate. Hence the Inhibition Factor (IF), defined as the ratio of reaction rate without polymer to the reaction rate with polymer, is a good measure of the in vitro inhibitory capacity of multifunctional polymers towards gastro-intestinal proteolytic activity.

2.2.5 References

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Chapter 2.3 Trypsin Inhibition, Calcium and Zinc Ion Binding Study

2.3.1 Introduction

As already mentioned, the two major barriers for successful oral peptide delivery are enzymatic degradation and permeation across the gastro-intestinal epithelium. The potential of the grafted starches and the freeze-dried / spray-dried starch/carboxylated polymer mixtures as excipients for oral peptide and protein delivery was in vitro evaluated by measuring the trypsin inhibition potency and the Ca^{2+} and Zn^{2+} binding capacity of these multifunctional polymers. Carbopol® 934P was used as a reference polymer.

2.3.2 Materials

Trypsin (TPCK treated from bovine pancreas), N-α-benzoyl-L-arginine-ethylester (BAEE), N-α-benzoylarginine (BA) and 2-[N-morpholino]ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (Bornem, Belgium). Carbopol® 934P (C 934P) and Carbopol® 974P (C 974P) were a gift from BF Goodrich (Cleveland, Ohio, USA). Calcium chloride dihydrate ($CaCl_2.2H_2O$) and zinc chloride ($ZnCl_2$) were purchased from Merck Eurolab (Leuven, Belgium). All other chemicals used were at least of analytical grade.

2.3.3 Methods

2.3.3.1 Trypsin inhibition study

The in vitro trypsin inhibition potency of the multifunctional polymers was determined using an optimised and validated trypsin inhibition assay (*Chapter 2.2*)

(Ameye et al., 2000) and was compared to Carbopol® 934P, the reference polymer (Lueßen et al., 1996).

Amounts of 20 mmol/l N-α-benzoyl-L-arginine-ethylester (BAEE), the model substrate for trypsin, were dissolved in the polymer preparation containing 0.25 % (w/v) polymer dispersed in a 50 mmol/l MES/KOH buffer, pH 6.7, with 250 mmol/l mannitol. At time zero 30 Enzymatic Units trypsin/ml (enzymatic activity determined according the Enzymatic Assay of Trypsin used by Sigma-Aldrich, Bornem, Belgium) were added to the polymer preparation, after which the solution was incubated at 37 °C for 1 h. 50 µl of sample was withdrawn at predetermined time intervals and diluted in 1.0 ml 0.1M HCl to stop the trypsin activity. The degradation of the substrate BAEE was studied by following the formation of the metabolite N-α-benzoylarginine (BA) by HPLC with UV detection at 253 nm.

HPLC analysis was performed with a HPLC system consisting of an isocratic HPLC pump (type L-7110, Merck-Hitachi, Darmstadt, Germany), an injector with a loop of 20 µl (Valco 6 channel injector, Valco Instruments Corporation, Houston, USA), a UV detector (type L-7400, Merck-Hitachi, Darmstadt, Germany) and a software interface (type D-7000, Merck-Hitachi, Darmstadt, Germany). Data were calculated with the software package 'HPLC System Manager' (Merck-Hitachi, Darmstadt, Germany). The column was a Lichrosorb 7 RP 18 column (100 x 3.0 mm) equipped with a RP precolumn (10 x 2 mm) (Chrompack, Antwerpen, Belgium). The mobile phase, used as an isocratic eluent, consisted of 86 % (v/v) 10 mmol/l ammonium acetate buffer pH 4.2 with 10 mmol/l triethylamine and 14 % (v/v) acetonitrile. The retention time of the metabolite peak was 1.3 min after injection of 20 µl at a flow rate of 0.75 ml/min. The analysis was performed at room temperature.

The degree of trypsin inhibition was expressed by the Inhibition Factor (IF). The IF is defined as the ratio of the reaction rate of the metabolite concentration time curve for the enzymatic reaction carried out without polymer (control) and with polymer, respectively. The reaction rate was calculated by linear regression analysis of the N-α-benzoylarginine (BA) concentration versus reaction time. The determination coefficients of the linear progress functions during 1h of incubation time were > 0.995.

$$IF = \text{reaction rate}_{control} / \text{reaction rate}_{polymer}$$

The IF values were statistically compared to the reference polymer using a non-parametric Kruskal-Wallis test followed by a post hoc Dunn procedure at a significance level of $p < 0.05$. (Rosner, 1995)

2.3.3.2 Calcium and Zinc ion binding study

The capability of the starch-*g*-poly(acrylic acid) copolymers and the freeze-dried starch/poly(acrylic acid) mixtures to bind the bivalent ions Ca^{2+} and Zn^{2+} was investigated at pH 6.7 and pH 6.0, respectively, and was compared to Carbopol® 934P (C 934P). The multifunctional polymers were dispersed at a concentration of 0.25% (w/v) in a 50 mM MES/KOH buffer, pH 6.7 or 6.0 (without mannitol). To a homogeneous polymer dispersion a buffered $CaCl_2.2H_2O$ solution or a $ZnCl_2$ solution in 0.01 N HCl was added to each preparation to give a final Ca^{2+} or Zn^{2+} concentration of 20 mM, and the samples were incubated for 2h at 37°C. After incubation the samples were centrifuged at 2578 g for 45 min. (Tehtnica® Centric 322 A, Novolab, Belgium). The Ca^{2+} and Zn^{2+} content in the supernatant was analysed by atomic absorbance spectroscopy. The measurements were performed on a Perkin-Elmer 280 flame atomic absorbance spectrometer (Perkin-Elmer, Zaventem, Belgium) in an air-acetylene flame. The absorption line used was 422.7 nm for Ca^{2+} and 213.9 nm for Zn^{2+}. The Ca^{2+} and Zn^{2+} content in the supernatant was quantified by means of a calibration curve [0 – 20 mg Ca^{2+}/l or 0 - 0.875 mg Zn^{2+}/l]. For calcium, standards and supernatant dilutions were prepared in distilled water, for zinc in 0.01 N HCl. The amount of polymer-bound calcium and zinc ions was calculated from the difference between the total amount of calcium or zinc ions added and the amount of free Ca^{2+} or Zn^{2+} measured in the supernatant.

The decrease in pH after binding of zinc ions to the polymer was followed by measuring the pH of the supernatant with a Consort SP28X electrode (Consort, Belgium).

2.3.4 Results and Discussion

2.3.4.1 Trypsin inhibition study

The enzymatic inhibition potency of the newly synthesised starch-*g*-poly(acrylic acid) copolymers and the freeze-dried / spray-dried starch/carboxylated polymer mixtures was measured and compared to Carbopol® 934P (C 934P).

The poly(acrylates) polycarbophil and Carbopol® 934P were shown to be able to inhibit the enzymatic activity of the Ca^{2+}-containing serine proteases trypsin and α-chymotrypsin and the Zn^{2+}-dependent exopeptidases carboxypeptidase A and cytosolic leucine aminopeptidase. The pronounced binding properties of polycarbophil and C 934P for the bivalent cations Ca^{2+} and Zn^{2+} was demonstrated to be a major reason for the observed inhibitory effect. These polymers were found to be able to deprive Ca^{2+} and Zn^{2+} from the enzyme structures, thereby inactivating the enzyme activity (Lueßen et al., 1996). Graft copolymer networks of poly(methacrylic acid-*g*-ethylene glycol) (P(MAA-*g*-EG)) could inhibit trypsin but to a lesser extent than C 934P and polycarbophil. The copolymers showed significant but again less binding of calcium ions compared to the above-mentioned poly(acrylates) (Madsen and Peppas, 1999).

The trypsin inhibition factors (IF) of the chemically modified (CM) and ^{60}Co irradiated (IR) grafted starches are presented in Table 1 and Figure 1.

Polymer	Inhibition Factor	Polymer	Inhibition Factor
C 934P	2.74 ± 0.19	CM 7	2.16 ± 0.12
CM 1	1.90 ± 0.04	CM 8	1.88 ± 0.03
CM 2	2.02 ± 0.08	CM 9	2.06 ± 0.05
CM 3	2.23 ± 0.06	CM 10	1.85 ± 0.06
CM 4	1.96 ± 0.12		
CM 5	/	IR 1	2.01 ± 0.06
CM 6	1.88 ± 0.03	IR 2	1.88 ± 0.05

Table 1. Inhibition Factors of the chemically modified (CM) and ^{60}Co irradiated (IR) grafted starches. (n=3, mean ± SD)

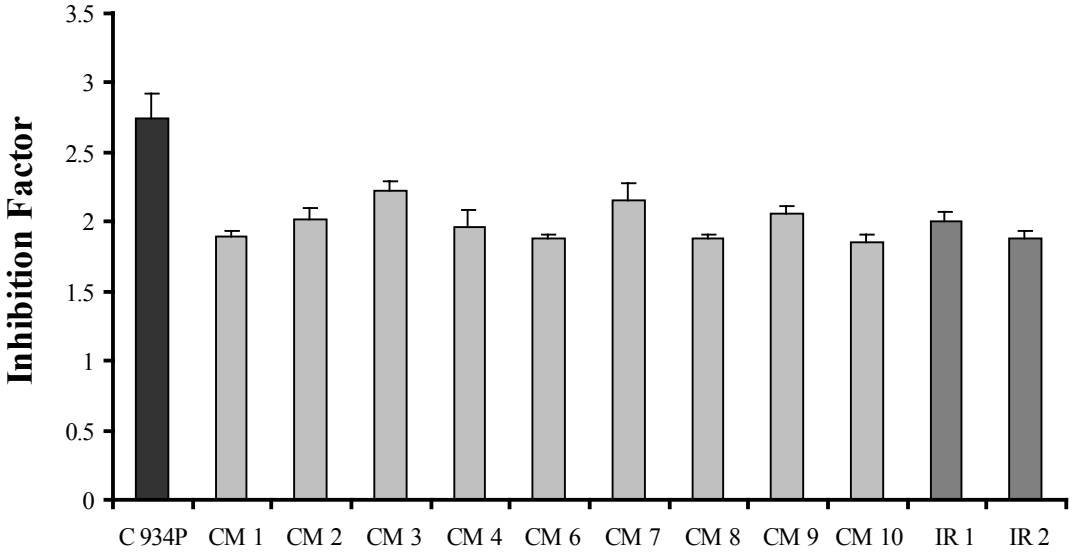

Figure 1. Inhibition Factors of the chemically modified (CM) and ^{60}Co irradiated (IR) grafted starches. (n=3, mean ± SD)

The trypsin inhibition factors of all chemically modified grafted starches (CM) were significantly lower than for C 934P. The differences in IF between the chemically modified grafted starches were not remarkable. Neither the origin of the starch (corn or rice starch), the AGE concentration (0.2 or 0.5%), the ratio starch/acrylic acid (1:3 or 1:5) or the degree of acrylic acid neutralisation or neutralisation technique after synthesis showed any influence on the IF. All tested chemically modified grafted starches showed IF values around 2. Polymer CM 5 was not evaluated due to precipitation problems during testing.

Also, the ^{60}Co irradiated grafted starches (IR) showed a trypsin inhibition which was significantly lower than for C 934P, but comparable to the chemically modified starches.

From all tested multifunctional polymers only the freeze-dried starch/poly(acrylic acid) (starch/PAA) mixtures showed inhibition factors comparable to the reference polymer C 934P. (Table 2, Figure 2)

Polymer	Inhibition Factor	Polymer	Inhibition Factor
C 934P	2.74 ± 0.19	SD PAA 50/50	2.05 ± 0.03
		SD PAA 50/50 HT	2.00 ± 0.03
FD 50/50	3.03 ± 0.64 *	SD PAA 25/75	2.02 ± 0.19
FD 50/50 HT	3.80 ± 0.40 *	SD PAA 25/75 HT	2.15 ± 0.16
FD 25/75	2.25 ± 0.08	SD 25/75	2.05 ± 0.05
FD 25/75 HT	2.65 ± 0.12 *	SD 25/75 HT	2.14 ± 0.11

Table 2. Inhibition Factors of the freeze-dried (FD) / spray-dried (SD) starch/carboxylated polymer mixtures. (n=3, mean ± SD)

[*no significant difference with C 934P (Kruskal-Wallis/Dunn test, $p < 0.05$)]

The IF values of the freeze-dried starch/poly(acrylic acid) 50/50 mixture, both heat treated or non-heat treated, and the heat treated 25/75 mixture were not significantly different from the reference polymer. The freeze-dried polymers with a starch/PAA ratio of 50/50 showed higher IF than those with a 25/75 ratio. Heat treatment (120°C, 15 min) after freeze-drying resulted in a higher IF.

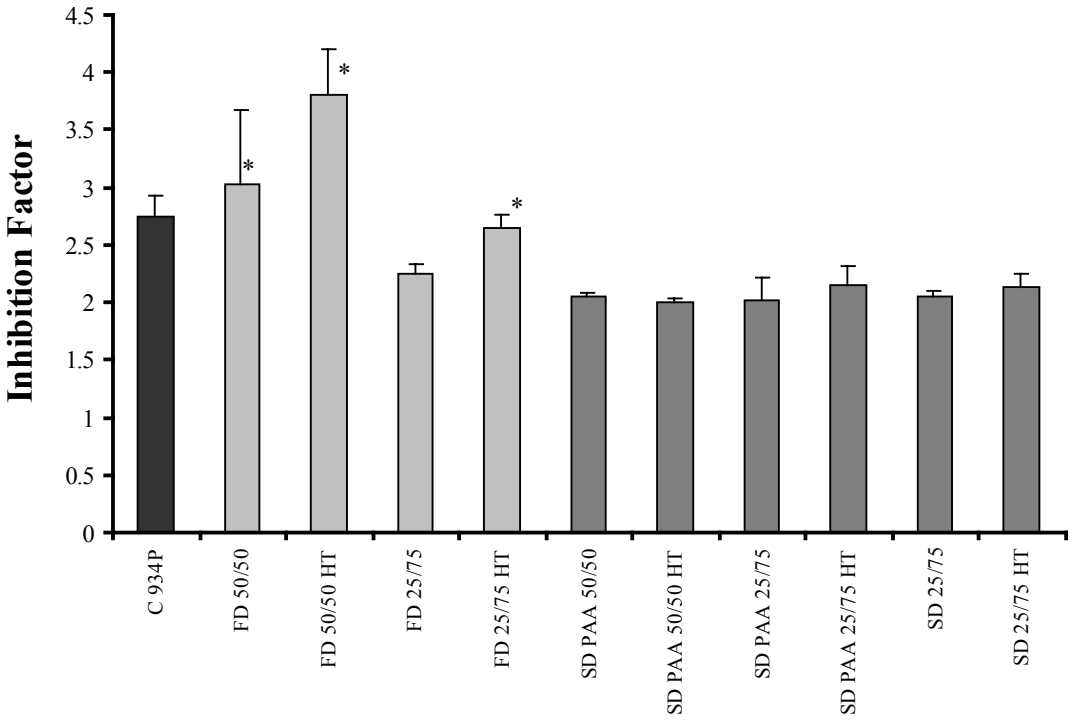

Figure 2. Inhibition Factors of the freeze-dried (FD) / spray-dried (SD) starch/carboxylated polymer mixtures. (n=3, mean ± SD)

[*no significant difference with C 934P (Kruskal-Wallis/Dunn test, $p < 0.05$)]

The spray-dried starch/poly(acrylic acid) mixtures (SD PAA) showed all a lower IF than the reference polymer C 934P, but comparable values to the grafted starches (CM and IR). The inhibition factors of mixtures containing a linear poly(acrylic acid) (PAA) in the same ratios were higher after freeze-drying than after spray-drying. Heat treatment after spray-drying had no influence on the trypsin inhibition potency. The use of the cross-linked Carbopol® 974P (C 974P) or the linear PAA in the spray-dried mixtures did not result in different inhibition factors.

2.3.4.2 Calcium and Zinc ion binding study

The amount of calcium and zinc ions bound to the polymers is shown in Table 3 and Figure 3.

Lueβen et al. (1996) and Madsen and Peppas (1999) reported a Ca^{2+} binding capacity at pH 6.7 for Carbopol® 934P (C 934P) of 240 mg and 167.5 mg Ca^{2+}/g polymer, respectively. In the present study the binding capacity for calcium ions for the reference polymers Carbopol® 934P and Carbopol® 974P (C 974P) was 204 and 207 mg Ca^{2+}/g polymer, respectively. The Zn^{2+} binding capacity for C 934P and C 974P was 374 and 390 mg Zn^{2+}/g polymer, respectively.

As a control, Carbopol® 974P was included in the calcium and zinc ion binding study to compare its ion binding capacity with Carbopol® 934P. Carbopol® 934P and 974P are both pharmaceutical grade cross-linked poly(acrylic acid) polymers. C 934P is cross-linked with allyl sucrose and is polymerised in benzene. C 974P is cross-linked with allyl pentaerythritol and is polymerised in ethyl acetate. C 974P was manufactured as an alternative to C 934P to meet the stricter requirements for toxicity and health. It was polymerised in ethyl acetate, a GRAS solvent, so avoiding toxic benzene residues. C 934P and C 974P have similar rheological properties. Both polymers had a similar Ca^{2+} and Zn^{2+} binding capacity: C 934P bound 204 mg Ca^{2+} and 374 mg Zn^{2+} per gram, while C 974P 207 mg Ca^{2+} and 390 mg Zn^{2+} per gram polymer (Table 3).

Polymer	mg Ca^{2+} bound/ g polymer	mg Zn^{2+} bound/ g polymer
C 934P	204.3 ± 1.7	374.4 ± 8.2
C 974P	207.3 ± 4.8	389.6 ± 3.5
CM 1	81.9 ± 4.3	232.9 ± 12.2
CM 2	100.5 ± 6.9	239.6 ± 16.1
CM 3	83.5 ± 3.7	217.0 ± 2.3
CM 4	10.0 ± 2.0	81.9 ± 2.3
CM 5	70.0 ± 1.8	175.9 ± 8.3
CM 6	93.9 ± 4.5	204.5 ± 24.3
CM 7	81.7 ± 2.8	262.7 ± 8.5
CM 8	75.8 ± 3.5	168.8 ± 45.5
CM 9	63.0 ± 5.3	232.8 ± 12.5
CM 10	94.2 ± 3.4	235.5 ± 7.1
IR 1	149.1 ± 1.5	306.3 ± 18.4
IR 2	181.8 ± 5.2	332.2 ± 6.2
FD 50/50	92.9 ± 4.1	203.1 ± 36.9
FD 50/50 HT	104.0 ± 4.6	208.5 ± 20.0
FD 25/75	163.3 ± 2.0	350.9 ± 8.3
FD 25/75 HT	162.2 ± 2.5	333.6 ± 32.0

Table 3. Ca^{2+} and Zn^{2+} binding capacity of the chemically modified (CM) and ^{60}Co irradiated (IR) grafted starches and the freeze-dried starch/poly(acrylic acid) mixtures (FD). (n=3, mean ± SD)

A same tendency in Ca^{2+} and Zn^{2+} binding capacity for all tested polymers was observed (Figure 3). The affinity for calcium and zinc ions was for all tested multifunctional polymers lower compared to Carbopol® 934P. The lower affinity of the chemically modified (CM) and ^{60}Co irradiated (IR) grafted starches for these bivalent ions was not surprising as the density of ionised carboxylic groups is much lower in these polymers. This explanation was also given by Madsen and Peppas (1999) for the lower binding capacity of their P(MAA-*g*-EG) gels.

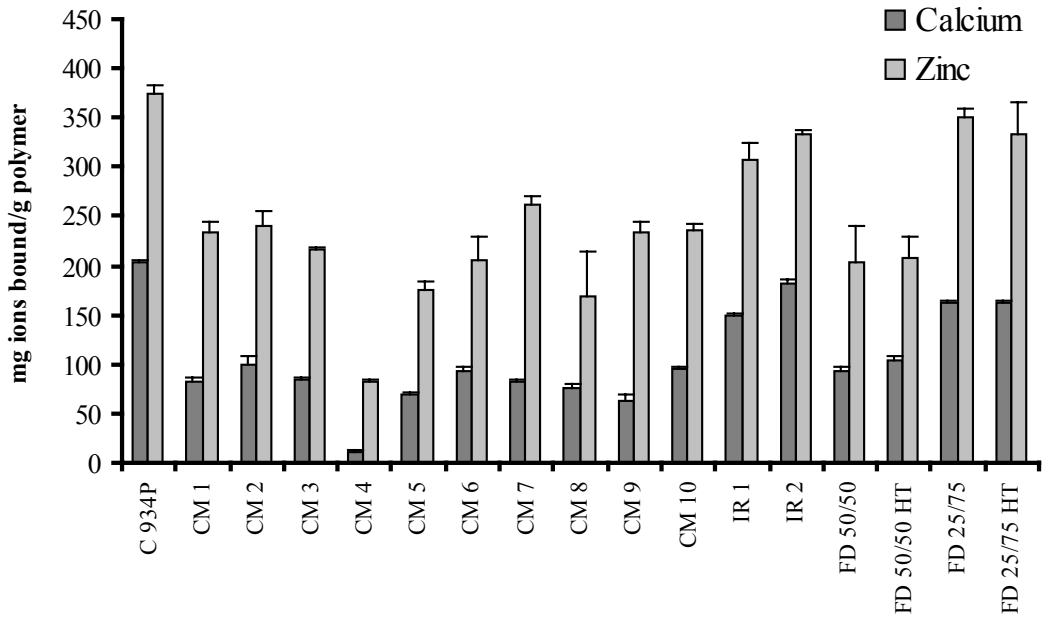

Figure 3. Ca^{2+} and Zn^{2+} binding capacity of the chemically modified (CM) and ^{60}Co irradiated (IR) grafted starches and the freeze-dried starch/poly(acrylic acid) mixtures (FD). (n=3, mean ± SD)

From Figure 3 it is clear that only the ^{60}Co irradiated copolymers and the freeze-dried mixtures with a starch/poly(acrylic acid) 25/75 ratio approached the Ca^{2+} and Zn^{2+} binding capacity of C 934P.

All chemically modified grafted starches showed a comparable Ca^{2+} and Zn^{2+} binding between 63 and 101 mg Ca^{2+}/g polymer and 169 and 263 mg Zn^{2+}/g polymer, except the non-neutralised chemically modified starch (CM 4), which only bound 10 mg Ca^{2+} and 82 mg Zn^{2+}/g polymer. The type of used starch (corn or rice starch), the AGE concentration (0.2 or 0.5%), the ratio starch/acrylic acid (1:3 or 1:5) and the degree of neutralisation or the neutralisation techniques after synthesis had no dramatic influence on the Ca^{2+} and Zn^{2+} binding capacity. Only between the non-neutralised sample (CM 4) and the partially neutralised starches a major difference in ion binding capacity was observed, although CM 4 could inhibit the proteolytic enzyme tryspin to the same extent compared to the other CM grafted starches (Figure 1). One should expect that a non-neutralised sample could bind more ions compared to a partially neutralised grafted starch. However, complexation of bivalent ions by these multifunctional polymers does not only depend on the degree of grafting, it also depends on the accessibility of the carboxylate functions (Kriwet and Kissel, 1996). Maybe, the lower ion binding capacity

of CM 4 can be explained by a lower grafting degree or less accessibility of the carboxylic acid functions, or due to both.

For the irradiated grafted starches no remarkable differences were observed in Ca^{2+} and Zn^{2+} binding capacity between the partially and non-neutralised polymers, although, as expected, the non-neutralised polymer showed both for Ca^{2+} and Zn^{2+} a higher affinity.

For the freeze-dried polymers the ratio starch/poly(acrylic acid) 25/75 resulted in a much higher ion binding capacity compared to the 50/50 ratio, due to the higher poly(acrylic acid) concentration. Heat treatment after freeze-drying did not influence the Ca^{2+} or Zn^{2+} binding capacity.

All tested polymers showed a certain in vitro trypsin inhibition activity. Only the freeze-dried powders approached or even exceeded the in vitro trypsin inhibition potency of the reference polymer Carbopol® 934P. Madsen and Peppas (1999) stated that their non-linear metabolite versus time profiles showed a decrease of degradation activity of trypsin with time and that this time dependency was an indication of a complex pattern of different kinetic parameters and that trypsin inhibition was more than just a rapid enzyme-inhibitor interaction. Lueβen et al. (1996) reported that trypsin inhibition by poly(acrylates) could be ascribed to deprivation of Ca^{2+} ions out of the trypsin structure, which leads to autodegradation of the enzyme. However, in this study the polymer with the highest inhibition factor (FD 50/50 HT) did not show the highest Ca^{2+} binding capacity. Besides, the irradiated starches showed lower IF values compared to the freeze-dried polymers, but had comparable Ca^{2+} and Zn^{2+} binding capacities. So, the trypsin inhibition potency can not be related to the Ca^{2+} binding capacity of the polymers alone. Many other mechanisms can play a role in the trypsin inhibition by multifunctional polymers: Ca^{2+} deprivation as described by Lueβen at al. (1996), enzyme-polymer interaction which reduces the free trypsin concentration and in part denaturates the enzyme (Walker et al.,1999) and a reduction of the pH below the optimum pH values of the pancreatic enzymes (Bai et al., 1996). A decrease of the pH in the supernatant after incubation with $ZnCl_2$ was observed (Figure 4). The decrease of the pH showed a similar

pattern of the Zn^{2+} binding capacity of the polymers. This pH reduction might contribute to the inhibition of pancreatic enzyme activity as described by Bai et al. (1996).

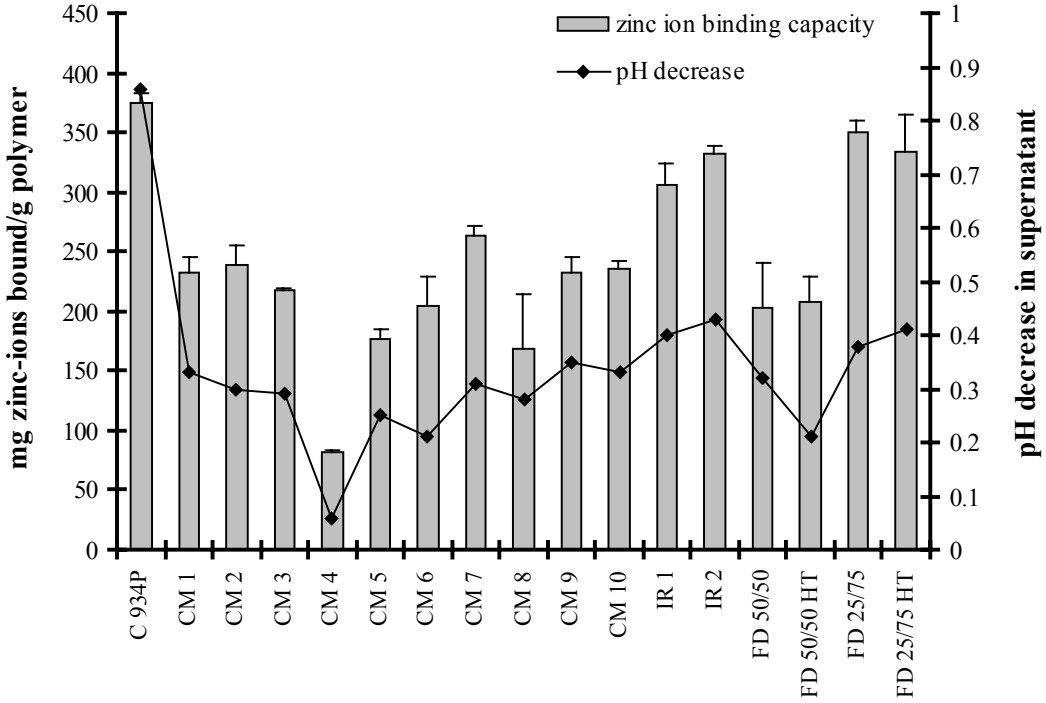

Figure 4. Zn^{2+} binding capacity compared to the decrease of pH in the supernatant. (n=3, mean ± SD)

Poly(acrylates) are thought to enhance paracellular transport of peptides such as insulin by a reduction of free extracellular Ca^{2+} concentration (Jung et al., 2000). The complexation of calcium ions by polycarbophil was found to be depending on the accessability of the carboxylate functions in the polymer (Kriwet and Kissel, 1996). In this study the calcium binding capacity increased with the degree of neutralisation (CM 3 – CM 4). For the chemically modified starches the Ca^{2+} and Zn^{2+} binding capacity was also depending on the degree of grafting with poly(acrylic acid) functions. From all tested polymers the irradiated starches and the freeze dried starches FD 25/75 and FD 25/75 HT showed the highest binding capacity for the bivalent ions Ca^{2+} and Zn^{2+}, which makes these polymers promising excipients to enhance the in vivo paracellular permeability for orally administered peptide drugs.

2.3.5 Conclusion

Newly synthesised starch-*g*-poly(acrylic acid) copolymers (chemical modified or ^{60}Co irradiated) and freeze-dried / spray-dried starch/carboxylated polymer mixtures were evaluated in vitro on their potential as excipients for oral peptide drug delivery. The heat treated freeze-dried powders showed the highest in vitro trypsin inhibition potency, which was comparable or even higher than for the reference polymer Carbopol® 934P. The calcium and zinc ion binding study showed that the freeze-drying and the ^{60}Co irradiation method could result in multifunctional polymers with the highest ion binding capacity. The combination of a high proteolytic inhibition and a pronounced binding ability for the bivalent ions calcium and zinc makes these polymers promising excipients for a successful oral peptide and protein drug delivery.

2.3.6 Addendum: In vivo insulin absorption after oral administration

In vivo insulin absorption after oral administration of spray-dried Amioca®/Carbopol® 974P based insulin formulations, was studied in rabbits and in dogs.

Insulin was delivered orally in rabbits using mini-tablets based on SD PAA 25/75 and SD 25/75, respectively. Insulin (50 IU per tablet) (Sigma-Aldrich, Bornem, Belgium) was mixed with the spray-dried powder, colloidal silica (0.2% w/w) (Alpha Pharma, Nazareth, Belgium) and sodium stearyl fumarate (1% w/w) (Edward Mendell Co. Inc., NY, USA). The mixture was compressed to mini-tablets on a Korsch compression machine (Type EK0, Berlin, Germany) equipped with 2 mm flat punches, at a pressure of 4.4 kN. The weight and the diameter of the tablets were 10 mg and 2 mm, respectively. An enteric coated (cellulose acetophtalate in acetone) gelatin capsule was filled with 10 mini-tablets (= 500 IU insulin per dose). One capsule was administered orally to New Zealand white rabbits (n = 4, 2.98 ± 0.10 kg), fasted during 16 h before administration, according to the protocol described by Callens et al. (2003). Blood samples were taken from the ear veins and analysed using an Enzyme-Linked Immunosorbent kit (Diagnostic Systems Laboratories, USA). Blood glucose levels were measured immediately after blood collection with Glucotouch test strips (Lifescan, Beerse, Belgium). Over a period of 8 hours no increased insulin serum levels nor a decrease in blood glucose was observed, although the high dose of 500 IU insulin used.

Insulin was administrated orally to dogs formulated in a lyophilised powder based on SD 80/20 and SD 25/75, respectively, and filled in an enteric coated capsule. The lyophilised formulation was prepared according to Callens et al. (2003). These authors reported an insulin bioavailability above 10% after nasal delivery of a lyophilised insulin-SD 25/75 formulation. One gram of the spray-dried powder was dispersed in distilled water and neutralised to pH 7.4 using a 2 M NaOH solution. After neutralisation, an insulin solution (Actrapid® HM 100, Novo-Nordisk, Bagsvaerd, Denmark) was added to obtain a dose of 1 IU insulin per mg powder. The dispersions were lyophilised in an Amsco-Finn Aqua GT4 freeze-dryer (Amsco, Brussels, Belgium). The lyophilised powder was filled in enteric coated (cellulose acetophtalate in acetone) gelatin capsule in

order to obtain a dose of 6 IU/kg. The dogs (n = 3, 38.0 ± 1.8 kg) were fasted during 12 h before administration. Insulin serum concentrations and blood glucose were measured as described above. Again no increased insulin serum levels nor a decrease in blood glucose was observed.

It is difficult to explain why no insulin was absorbed from the gastro-intestinal tract. Several problems are encountered during oral insulin delivery such as insulin stability in the gastro-intestinal tract, digestion by proteolytic enzymes and a poor absorption over the intestinal epithelium. From these trials, it is clear that in vitro inhibition and ion binding studies can be used to make a first selection of potential excipients, but they can not be used to predict in vivo absorption data. After a selection of potential excipients for oral peptide delivery, it is even more important to evaluate and optimise different formulation possibilities with respect to peptide stability in and absorption from the gastro-intestinal tract.

2.3.7 References

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Chapter 3 Multifunctional Polymers as Bioadhesive Drug Carriers

CONTENTS:

Chapter 3.1 Introduction

3.1.1 Bioadhesion

The term bioadhesion refers to any bond formed between two biological surfaces or a bond between a biological and a synthetic surface. In the case of bioadhesive drug delivery systems, the term bioadhesion is typically used to describe the adhesion between polymers, either synthetic or natural, and soft tissues (i.e. buccal mucosa). Although the target of many bioadhesive delivery systems may be a soft tissue cell layer (i.e. epithelial cells), the actual adhesive bond may form with either the cell layer, a mucous layer or a combination of the two. In instances in which bonds form between mucus and polymer, the term mucoadhesion is used synonymously with bioadhesion. In general, bioadhesion is an all-inclusive term used to describe adhesive interactions with any biological or biologically derived substance, and mucoadhesion is used only when describing a bond involving mucus or a mucosal surface. (Chickering and Mathiowitz, 1999)

The *mechanisms* responsible for the formation of bioadhesive bonds are not completely clear. Most research has been focused on analysing bioadhesive interactions between polymer hydrogels and soft tissues. The process involved in the formation of such bioadhesive bonds has been described in three steps: (1) wetting and swelling of the polymer to permit intimate contact with the biological tissue, (2) interpenetration of bioadhesive polymer chains and entanglement of polymer and mucin chains and (3) formation of weak chemical bonds between entangled chains (Duchêne et al., 1988; Ponchel et al., 1991). It has been stated that at least one of the following polymer characteristics are required to obtain bioadhesion: (1) sufficient numbers of hydrogen-bonding chemical groups (-OH and –COOH), (2) anionic surface charges, (3) high molecular weight, (4) high chain flexibility and (5) surface tensions that will induce spreading onto the mucous layer (Peppas and Buri, 1985). Each of these characteristics favours the formation of bonds that are either chemical or mechanical.

Types of *chemical bonds* include strong primary bonds (i.e. covalent bonds), as well as weaker secondary forces such as ionic bonds, van der Waals interactions and

hydrogen bonds. Both types of interactions have been exploited in developing bioadhesive drug delivery systems. Although systems designed to form covalent bonds with proteins on the surface of epithelial cells may offer strength advantages, three factors limit the usefulness of such permanent bonding. First, mucous barriers may inhibit direct contact of polymer and tissue. Second, permanent chemical bonds with the epithelium may not produce permanently retained delivery devices because most epithelial cells are exfoliated every 3 to 4 days. Third, biocompatibility of such binding has not been thoroughly investigated and could pose significant problems. For these reasons, many have focused on developing mucoadhesive systems that bond through either van der Waals interactions or hydrogen bonds. Although individually these forces are very weak, strong adhesions can be produced through numerous interaction sites. Therefore, polymers with high molecular weight and high concentrations of reactive, polar groups, such as –COOH and –OH functions, tend to develop intense mucoadhesive bonds. (Chickering and Mathiowitz, 1999)

Mechanical bonds can be thought of as physical connections between surfaces, similar to interlocking puzzle pieces. Macroscopically, they involve the inclusion of one substance in the cracks or crevices of another. On a microscopic scale, they can involve physical entanglement of mucin strands with flexible polymer chains and/or interpenetration of mucin strands into a porous polymer substrate. The rate of penetration of polymer strands into mucin layers is dependent on chain flexibility and diffusion coefficients of each. The strength of the adhesive bond is directly proportional to the depth of penetration of the polymer chains. Other factors that influence bond strength include the presence of water, the time of contact between the materials, and the length and flexibility of the polymer chains. (Chickering and Mathiowitz, 1999)

The same *theories* of adhesion that were developed to explain and predict the performance of glues, adhesives and paint can be and have been applied to bioadhesive drug delivery systems. In general, five theories have been adapted to the study of bioadhesion: the electronic, absorption, wetting, diffusion and fracture theories. Some are based on the formation of chemical bonds, whereas other focuses on mechanical bonds. (Chickering and Mathiowitz, 1999)

The hypothesis of the *electronic theory* relies on the assumption that the bioadhesive material and the target biological material have different electronic structures. On this assumption, when the two materials come in contact with each other, electron transfer occurs causing the formation of a double layer of electrical charge at the bioadhesive-biologic material interface. The bioadhesive force is believed to be due to attractive forces across this electrical double layer. The electrical theory has produced some controversy regarding whether the electrostatic forces are an important cause or the result of the contact between the bioadhesive and the biological surface.

The *adsorption theory* states that the bioadhesive bond formed between an adhesive substrate and tissue or mucosa is due to van der Waals interactions and hydrogen bonds. Although these forces are individually weak, the sheer number of interactions can as a whole produce intense adhesive strength. The adsorption theory is the most widely accepted theory of adhesion.

The *wetting theory* was developed predominantly in regard to liquid adhesives such as a bioadhesive gel. Using the wetting theory, it is possible to predict the ability of various bioadhesives to spread over biological tissues and the intensity of the bioadhesive bonds.

According the *diffusion theory* interpenetration and entanglement of bioadhesive polymer chains and mucous polymer chains produce semi-permanent adhesive bonds.

The *fracture theory* is perhaps the most applicable theory for studying bioadhesion through mechanical measurements. This theory analyses the forces required to separate two surfaces after adhesion.

In mucoadhesion, the adhering surface is a mucous membrane. Mucous membranes line the wall of various body cavities such as the oral, vaginal cavity or the gastro-intestinal and the respiratory tract. They are either single-layered epithelium (i.e. the stomach, small and large intestine, bronchi) or multi-layered stratified epithelium (i.e. oral cavity, vagina). The former contain goblet cells, which secrete mucus directly onto the epithelial surfaces, while the latter contain, or are adjacent to tissues containing, specialised glands such as salivary glands that secrete mucus onto the epithelial surface.

The major components of mucus, present as a gel layer adherent to the mucosal surface, are mucin glycoproteins, lipids, inorganic salts and water, the latter accounting for more than 95% of the gel weight, making it a highly hydrated system. The mucin glycoproteins are the most important components of the mucus gel, resulting in its characteristic gel-like, cohesive and adhesive properties. The thickness of this mucus layer varies on different mucosal surfaces from 50 to 450 μm in the stomach to 0.7 μm in the oral cavity. The major functions of mucus are protection and lubrication. (Smart, 1999)

The largest group of mucosal-adhesive materials are hydrophilic macromolecules containing numerous hydrogen bond-forming groups as carboxyl (i.e. poly(acrylic acid)s, Carbopols®) or amine (i.e. the chitosans) functions. These are called "wet" adhesives as they are activated by moistening. (Smart, 1999)

In many descriptions of the interactions between mucoadhesive materials and a mucous membrane, two basic steps have been identified: *contact stage* and *consolidation stage*. (Smart, 1999)

During the *contact stage*, an intimate contact is formed between the mucoadhesive and mucous membrane. The mucoadhesive and the mucous membrane initially have to form a close contact with each other. In some cases, these two surfaces can be readily brought together by placing and holding a delivery system (i.e. bioadhesive tablet) within the oral cavity or vagina.

During the *consolidation stage*, various physicochemical interactions occur to consolidate and strengthen the adhesive joint, leading to prolonged adhesion. The mucoadhesive point can be considered to contain three regions (Figure 1): the mucoadhesive, the mucosa and the interfacial region, consisting at least initially of mucus.

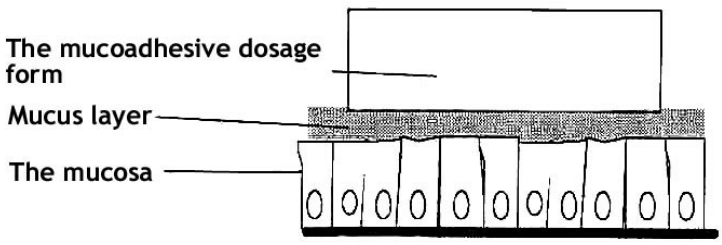

Figure 1. The three regions within a mucoadhesive joint. (Smart, 1999)

Adhesive joint failure will normally occur at the weakest component of this joint (Figure 2). For weak adhesives this will be the mucoadhesive-mucus interface; for stronger adhesives this will initially be the mucus layer but later may be the hydrating mucoadhesive material. (Smart, 1999)

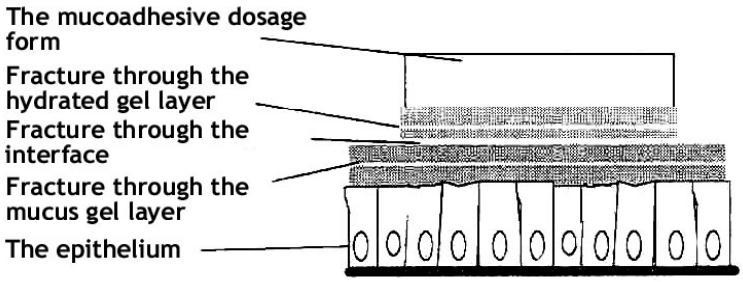

Figure 2. Possible regions of mucoadhesive joint failure. (Smart, 1999)

The strength of the adhesive joint will depend on the cohesive nature of the weakest region. For strong mucoadhesion, when a substantial mucus layer is present, an increase in the cohesive nature of this gel is necessary. There are two theories of how this gel strengthening occurs. One is based on a macromolecular interpenetration effect. In this theory, described by Peppas and Mikos (1990), the mucoadhesive molecules interpenetrate and bond by secondary interactions with mucus glycoproteins (Figure 3).

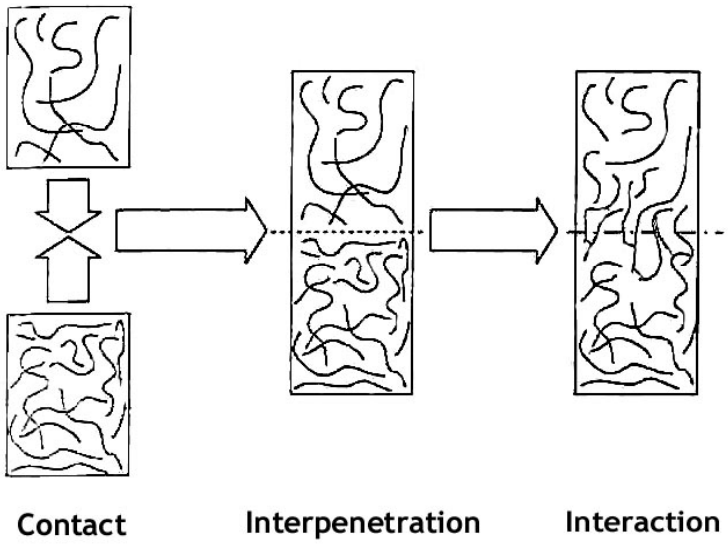

Figure 3. The interpenetration theory: interaction between a mucoadhesive polymer and mucin glycoprotein. (Smart, 1999)

The second theory says that consolidation arises from the ability of dry or partially hydrated mucoadhesive drug delivery systems (i.e. bioadhesive tablet formulations) to swell and dehydrate mucus gels and that this water movement drives the consolidation of the adhesive joint. (Smart, 1999)

3.1.2 Buccal drug delivery

The oral cavity has been investigated as a potential site for local and systemic drug delivery since more than 10 years. The oral cavity forms a convenient and easily accessible site for drug delivery.

The advantages of buccal drug delivery systems (i.e. patches, tablets) for *local* therapy of the oral mucosa can be easily described. Some inflammatory or infectious conditions of the mucosa, such as aphthae, herpes- or Candida-related stomatitis or physical injuries are most typically treated with local anesthetics, antimycotics, disinfectants, antiviral agents or corticosteroids. Buccal drug delivery systems have many advantages compared to the conventional therapies by means of oral gels and liquids. With these formulations the affected tissues are exposed to but a minor fraction of the drug dose for very short periods of time and most of the time no effective drug level is present at the site of action. In contrast, buccal drug formulations are applied directly to the affected mucosal region and have the potential to supply the site of action with effective drug levels and sustain these levels over a long period of time. (Guo and Cremer, 1999)

Amongst the various routes of *systemic* drug delivery, oral route is perhaps the most preferred to the patient. However, peroral administration of drugs has disadvantages such as hepatic first pass metabolism and enzymatic degradation within the gastro-intestinal tract, that prohibit oral administration of certain classes of drugs (i.e peptides, proteins, hormones, etc.). Consequently, other absorptive mucosae are considered as potential sites for drug administration. Transmucosal routes of drug delivery (i.e. the mucosal linings of the nasal, rectal, ocular, vaginal and oral cavity) offer distinct advantages over peroral administration for systemic drug delivery. These advantages include possible bypass of first pass effect, avoidance of presystemic elimination within

the gastro-intestinal tract, and, depending on the particular drug, a better enzymatic flora for drug absorption. (Shojaei, 1998)

The oral cavity has a number of features that make it a desirable site for drug delivery, including a rich blood supply that drains directly into the jugular vein, bypassing the liver and thereby protecting the drug from first pass metabolism. Furthermore, the oral transmucosal drug delivery avoids pre-systemic elimination in the gastro-intestinal tract and the oral cavity is highly acceptable by patients. These factors make the oral mucosal cavity a very attractive and feasible site for systemic drug delivery.

3.1.2.1 The oral cavity

The oral mucosa can be distinguished according to five major regions in the oral cavity: (de Vries, 1991[a])

- the floor of the mouth (sublingual region)
- the buccal mucosa (cheeks)
- the gum (ginigiva)
- the palatal mucosa
- the inner side of the lips.

Within the oral mucosal cavity, delivery of drugs can be classified into three categories: (de Vries, 1991[a])

- *sublingual delivery*, which is systemic delivery of drugs through the mucosal membranes lining the floor of the mouth
- *buccal delivery*, which is drug administration through the mucosal membranes lining the cheeks (buccal mucosa)
- *local delivery*, which is drug delivery into the oral cavity.

3.1.2.2 Structure of the oral cavity

The oral cavity is lined with mucous membranes with a total area of 100 cm². The oral mucosal tissues consist of a superficial layer of squamous epithelium covered with

mucus. At the proximal side the epithelium is bound to a connective tissue layer, called the lamina propria, by the basal lamina (1 μm), which separates the two layers (Figure 4).

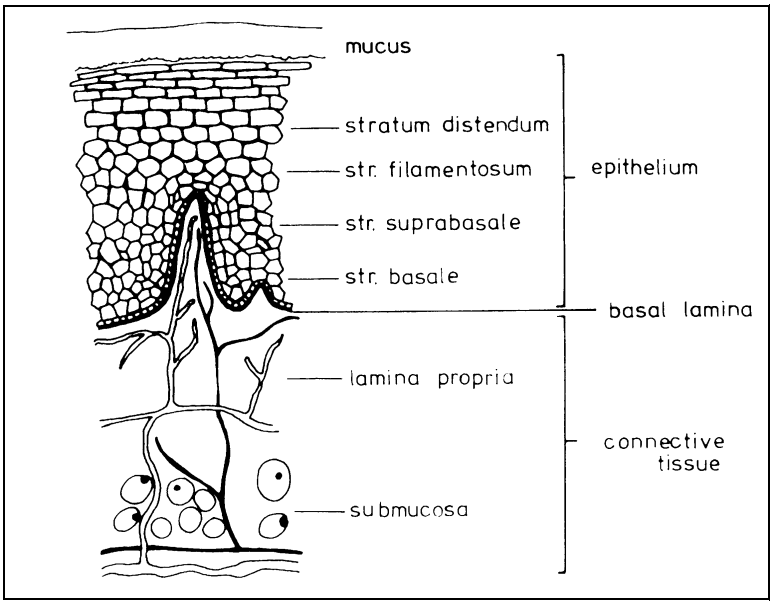

Figure 4. Schematic cross section through oral mucosa. (de Vries, 1991[a])

The mucous membranes are protective tissues and not primarily meant for drug absorption. The epithelium serves as a mechanical barrier, protecting the underlying tissues, whereas the lamina propria acts as a mechanical support and also carries the blood vessels and the nerves. Within the epithelium different layers can be distinguished. The inner, undifferentiated layer is called the stratum basale. Differentiation starts in the stratum suprabasale. In the stratum filamentosum cells excrete their contents to the intercellular space and differentiation is complete in the stratum distendum. Underneath the lamina propria a very loose submucosa can be present, depending on the membrane region. In case a submucosa is present, the oral mucosa is flexible. Where it is absent (gingiva) or fibrous (hard plate) the mucosa is fixed in position. (de Vries, 1991[a])

The mucous membranes show differences in structure, thickness and blood flow, depending on their location within the oral cavity. Figure 5 shows the different oral mucosae and their nature with respect to tissue keratinisation.

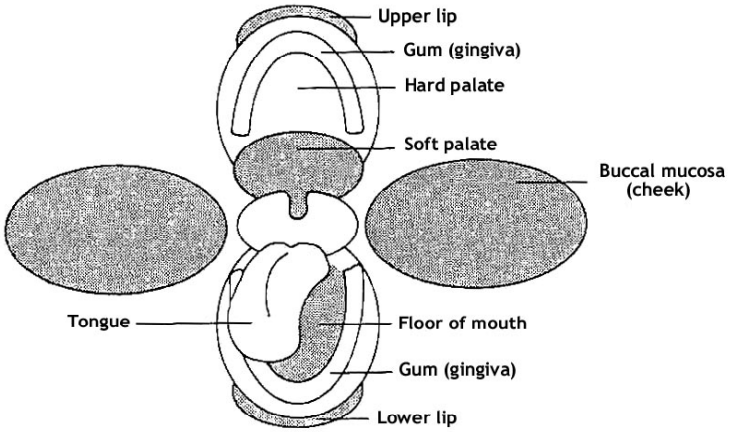

Figure 5. Schematic reproduction of an 'open' oral cavity showing the keratinised (white) and the non-keratinised (grey) regions. (de Vries, 1991[a])

Oral mucosae are divided into three types: masticatory, lining and specialised mucosa. The specialised mucosa is found at the top surface of the tongue, but is of minor importance for drug absorption. The major difference between masticatory and lining mucosa is the presence or absence, respectively, of a keratinised top layer as part of the epithelium. (de Vries, 1991[a])

In regions subjected to the mechanical forces of mastication – *masticatory mucosa*, such as the gingiva and hard palate – the epithelium is keratinised. In keratinised epithelium the surface layer is flattened, dehydrated, mechanically tough and chemically resistant. The outermost layer of keratinised epithelium is the stratum corneum and consists of an orderly array of flattened hexagonal cells, which are completely filled with aggregations of cytokeratins bounded by a cell envelope and surrounded by a complex mixture of lipids such as ceramides, cholesterol and fatty acids, extruded by the membrane-coating granules. These lipids are associated with the barrier function to the absorption of drugs. The turnover, necessary to stabilise the mucosal function and integrity, is 5 to 8 days. (Wertz and Squier, 1991; Wertz et al., 1993)

The *lining mucosa* is present in regions of the oral cavity that are stretched or compressed during speech and mastication. Lining mucosa is found in the buccal and sublingual area and at the inner side of the lips. The lining mucosa is non-keratinised and the surface is less able to resist mechanical abrasion, but is flexible. In non-keratinised epithelium, the accumulation of lipids and cytokeratins is less. The cells of the stratum

distendum flatten out towards the surface of this cell layer and the remaining layers, i.e. stratum granulosum en stratum corneum, are not present. In the superficial layers the cell membranes become more thickened, but nevertheless non-keratinised epithelium appears to be more permeable than keratinised tissue. Non-keratinised epithelium contains a large amount of polar lipids, such as glycosylceramides. (Wertz and Squier, 1991; Wertz et al., 1993)

In addition to the differences in epithelial structure, there are also differences in epithelial *thickness*. From Table 1 it can be seen that gingiva and floor of the mouth are comparable in thickness, whereas buccal epithelium is almost four times as thick.

The oral mucosa is highly *vascularised*. The blood supply to the oral tissues is delivered principally via the external carotid artery, which branches into the lingual, the facial and the maxillary arteries. The lingual artery supplies blood to the tongue and the sublingual and gingival areas, whereas the facial artery supplies blood to the soft palate and the lips. Hard palate and cheeks are supplied with blood by the maxillary artery. Blood from the capillary beds is collected by three main veins, which finally flow into the internal jugular vein. The blood flows through the different oral tissue are presented in Table 1. Buccal tissue is most perfused. Besides the permeability of the epithelium, the blood flow through a particular type of tissue is very important for drug absorption. (de Vries, 1991[a])

Tissue	Structure	Epithelial thickness (μm)[a]	Flow ($ml.min^{-1}.cm^{-2}$)[b]
Buccal	non-keratinised	500-600	2.40
Sublingual	non-keratinised	100-200	0.97
Gingival	keratinised	200	1.47
Palatal	keratinised	250	0.89

Table 1. Oral epithelium characteristics. [a] Thickness of human oral epithelium, [b] Blood flow in oral mucosa of the rhesus monkey (de Vries, 1991[a])

3.1.2.2.1 Saliva and mucus

The oral membranes are covered with mucus and continuously provided with fresh serous and mucous saliva. Saliva is essential in the prevention of oral mucosal cells from dehydrating and acts as a lubricant during the swallowing of food. The major salivary glands are the parotic, submandibular and sublingual glands. The submandibular and sublingual glands are situated in the lower region of the oral cavity whereas the parotic glands lie just below the ears, having its duct in the upper part of the cavity between cheek and upper jaw. Besides these major glands, there are small and minor glands on the tongue, the buccal, sublingual mucosa and on the palate.

Gland	Normal	Stimulated	Consistency
Parotic	25%	60%	serous
Submandibular	70%	30%	serous/mucous
Sublingual	5%	2%	serous/mucous

Table 2. Saliva production of the major salivary glands. (de Vries, 1991[a])

The amount of saliva produced throughout the day can be up to 1 litre. The saliva flow in rest is ± 0.5 ml/min, but can increase up to 7 ml/min upon stimulation (mechanically (pressure) or chemically (smell, taste)). The parotic glands produce only 25% of the total amount of saliva during rest, but is increased to 70% upon stimulation. These glands produce serous saliva, whereas the secretions of the other major glands is rather mucous (Table 2). The mucous secretions are viscous due to the presence of high molecular glycoproteins (mucins), while the serous secretions are aqueous and contain low molecular weight glycoproteins. Saliva contains 99.4% water and some important inorganic compounds like calcium, phosphate and bicarbonate ions. The pH varies between 5.8 and 7.1, and can be raised to 7.4 upon stimulation, due to an increase of bicarbonate content. The saliva secretion rate depends on gender, age, time of the day, illness and emotional condition. With increasing age, a decrease in saliva secretion can be observed. Saliva flow in women appears to be less than in men. It is the salivary fluid which is available to hydrate oral mucosal dosage forms. A main reason behind the selection of hydrophilic polymeric matrices as vehicles for oral mucosal drug delivery systems is this water rich environment of the oral cavity. (de Vries, 1991[a]; Voorspoels, 1997; Shojaei, 1998)

Mucins are the major components of mucus. Mucus is a highly viscous water-insoluble material that covers the mucosae as a protective film. Mucins from the saliva and other saliva compounds are absorbed to the oral mucosal surface and form a multi-layered structure. Mucus contains 95% water, 0.5 to 5% glycoproteins and small amounts of mineral salts (1%), proteins and lipids (0.5 to 1%). The major part of these glycoproteins are mucins which are responsible for the viscosity and adhesive properties of mucus. Mucin consists of a protein core with covalently attached oligosaccharide side chains. At the terminal ends of these oligosaccharides sialic acid or fucose are located. Some chains can contain sulphate groups. In contrast to these heavily glycosylated regions, mucin molecules also contain 'naked' regions. These regions are involved in intermolecular cross-linking via disulfide bonds due to the cysteins found in these parts of the molecules. In addition to disulfide cross-links, physical entanglement and secondary bonding also play a role in the formation of the mucus gel. It may be stated that mucus has excellent properties to act as a substrate in mucoadhesion due to its nature as a hydrated network of linear, flexible, random coil mucin molecules that is cross-linked (disulfide bonds and physical entanglement) and negatively charged (completely ionised - except at very low pH - sialic acid and sulphate groups). (Voorspoels, 1997; de Vries, 1991[b])

3.1.2.3 The oral cavity as a site for drug delivery

3.1.2.3.1 Buccal mucosa as a site for drug delivery

There are two categories of systemic drug delivery within the oral cavity (sublingual and buccal). Selecting one over another is mainly based on anatomical and permeability differences that exist among the various oral mucosal sites. The sublingual mucosa is relatively permeable, giving rapid absorption and acceptable bioavailabilities of many drugs, and is convenient, accessible, and generally well accepted (Harris and Robinson, 1992). Sublingual dosage forms are of two different designs, those composed of rapidly disintegrating tablets, and those consisting of soft gelatin capsules filled with liquid drug. Such systems create a very high drug concentration in the sublingual region before they are systemically absorbed across the mucosa. The buccal mucosa is considerably less permeable than the sublingual area, and is generally not able to provide

the rapid absorption seen with sublingual administration. However, besides the permeability other factors such as blood flow has also to be taken in account. Bloodflow through the buccal mucosa is higher than through the sublingual. Moreover, because of the smaller salivary flow in the buccal area, compared to the sublingual region, wash out of drug is less in the buccal area (de Vries, 1991[a]). The buccal mucosa is the more preferred route for systemic transmucosal drug delivery. The buccal mucosa is less permeable and is thus not able to give a rapid onset of absorption, but more suitable for a sustained release oral transmucosal delivery of less permeable molecules. (Shojaei, 1998)

3.1.2.3.2 Buccal drug absorption

The are two permeation pathways for passive drug transport across the oral mucosa: paracellular and transcellular routes. Permeants can use these two routes simultaneously, but one route is usually preferred over the other depending on the physicochemical properties of the diffusant. Since the intercellular spaces and cytoplasm are hydrophilic in character, lipophilic compounds would have low solubilities in this environment. The cell membrane, however, is rather lipophilic in nature and hydrophilic solutes will have difficulty permeating through the cell membrane due to a low partition coefficient. Therefore, the intercellular spaces pose as the major barrier to permeation of lipophilic compounds and the cell membrane acts as the major transport barrier for hydrophilic compounds. Since the oral epithelium is stratified, solute permeation may involve a combination of these two routes. The route that predominates is generally the one that provides the least amount of hindrance to passage. (Shojaei, 1998)

3.1.2.4 Buccal dosage forms

Many different dosage forms are suitable for buccal drug delivery such as tablets, patches, lozenges, sprays, hydrogels, chewing gums, powders and solutions. Most of these formulations will allow absorption through various parts of the oral cavity and will provide leakage into the gastro-intestinal tract by swallowing. In order to restrict the drug uptake to the buccal mucosa, mucoadhesive tablets or patches can be used. Hydroxypropylcellulose, hydroxyethylcellulose, polyacrylic resins, carboxy-

methylcellulose, polyvinylalcohol, polyvinylpyrrolidone, polyethyleneglycol, glycerin, agar and some oils are excipients that can be used in the manufacture of buccal formulations. Mixing with the active ingredient is succeeded by direct compression into tablets or patches, or formulation through a granulation step. In the case of multi-layered tablets or patches, the mucoadhesive layer can be separated from the drug-containing layer or a backing layer may be added to obtain an uni-directional release system. (Hoogstraate and Wertz, 1998)

Bioadhesive tablets can be used for the *local* delivery of antimycotics such as miconazole (Bouckaert et al., 1992, 1993), nystatin or clotrimazole (Knapczyk, 1992), sodium fluoride (Bottenberg et al., 1992) or corticosteroids (Mumtaz and Ch'ng, 1995). The bioadhesive tablet used by Bouckaert et al. (1992, 1993) and Bottenberg et. al (1992) were obtained by direct compression of a physical mixture of 5% Carbopol® 934P with a waxy corn starch. Knapczyk (1992) described a chitosan based bioadhesive carrier, obtained by direct compression too. Mumtaz and Ch'ng (1995) prepared a triamcinolone bioadhesive tablet based on different ratios poly(acrylic acid-2,5-dimethyl-1,5-hexadiene)/hydroxypropylmethylcellulose. Ahuja et al. (1998) formulated a bucco-adhesive tablet of metronidazole for the local treatment of oro-dental infections. The bucco-adhesive tablet was prepared by compression of a mixture of the drug, Carbopol® 934P, a cellulose ether derivative, mannitol and flavouring and sweetening agents. A buccal tablet formulation, based on spray-dried chitosan microparticles, containing the antimicrobial agent chlorhexidine was investigated by Giunchedi et al. (2002). All the above mentioned bioadhesive formulations, are single-layered tablets giving a multidirectional release in the oral cavity, which is likely in local oral treatment (Figure 6).

Minghetti et al. (1998) developed a two-layer bucco-adhesive acitretin tablet for local treatment of buccal keratinisation disorders. The inferior layer provided the bioadhesive properties (Carbopol® 934P and hydroxypropylmethylcellulose (HPMC)), while the upper layer was a slow-release matrix, based on HPMC and lactose, containing the drug.

Voorspoels et al. (1996) developed a buccal bioadhesive tablet for transmucosal *systemic* testosterone delivery in hypogonadal men. The bioadhesive carrier used was the

same multidirectional release matrix used by Bouckaert et al. (1992, 1993). A bucco-adhesive nifedipine tablet was formulated by Varshosaz and Dehghan (2002) for the treatment of angina pectoris and hypertension. The tablets were obtained by direct compression of a bioadhesive mixture based on carboxymethylcellulose and Carbopol® 934P. Singh and Ahuja (2002) developed a bucco-adhesive tablet of diltiazem hydrochloride (angina pectoris, hypertension), based on a physical mixture of Carbopol® 934P and HPMC. Choi et al. (2000) formulated an omeprazole (gastric acid secretion-inhibitor) buccal bioadhesive tablet, prepared by compressing the drug with sodium alginate and HPMC as bioadhesive agents.

Tsutsumi et al. (2002) investigated the buccal absorption of ergotamine tartrate (migraine treatment) from a bioadhesive tablet system, consisting of a adhesive backing and a drug core aiming at a unidirectional release (Figure 6). Alur et al. (1999) developed a unidirectional release buccal bioadhesive tablet for the transmucosal sustained delivery of chlorpheniramine (an anti-histaminic drug) by coating the tablet with Cutina® on all but one face. With a unidirectional release tablet the drug will only be absorbed over the mucosa on which the tablet is placed (i.e. the buccal mucosa). This results in a smaller absorbing surface, but on the other hand no drug is lost by swallowing.

Park and Munday (2002) developed and evaluated a biphasic buccal adhesive tablet for nicotine replacement. The biphasic tablet formulation contained a fast releasing layer and an adhesive controlled release layer.

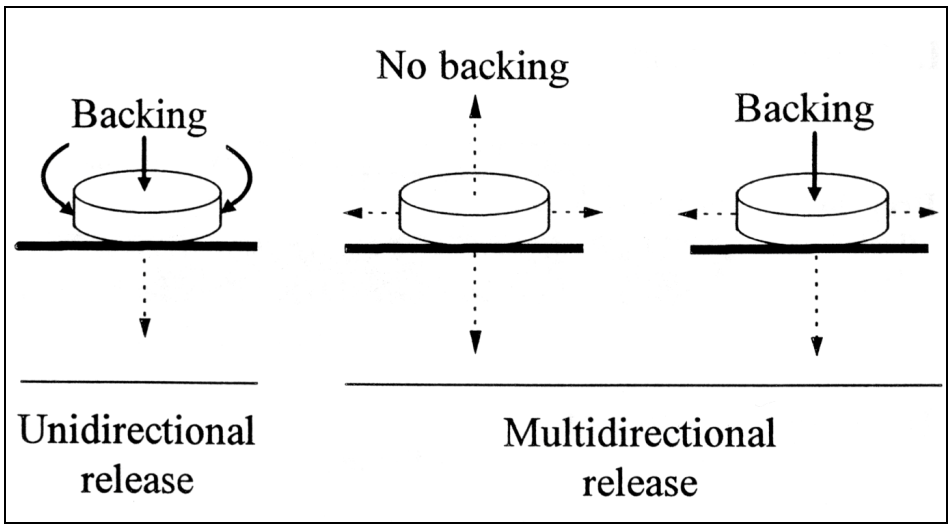

Figure 6. Unidirectional and multidirectional bioadhesive drug delivery systems. (DeGrande et al., 1996)

A mucoadhesive delivery system with a backing layer on one side can be used for local as well as systemic transmucosal drug delivery (Figure 6). Such a backing layer avoids sticking of the tablet to the finger during application in the oral cavity. Atrix Laboratories developed such a bioerodible mucoadhesive drug delivery system (BEMA™).

Aftac™ is a Japanese brand of mucoadhesive tablets for the treatment of inflammations in the mouth (Haas and Lehr, 2002). Tibozole™ (Tibotec-Virco) is a miconazole nitrate mucoadhesive tablet used for the treatment of oro-pharyngeal candidiasis, a common opportunistic infection in people with AIDS.

Nitrogard™ (Forest Pharmaceuticals, Inc.) is a nytroglycerin transmucosal buccal extended release tablet available on the U.S. market. It is used to relieve the pain of and to prevent angina attacks. Buccastem® (Reckitt & Colman) is a prochlorperazine maleate mucoadhesive buccal tablet and is used for the treatment of nausea, vomiting and migraine. Suscard Buccal (Pharmax, UK) is a prolonged release mucoadhesive buccal tablet of glyceryl trinitrate and is used in the treatment of angina pectoris.

Buccal patches are flexible and their shape is directly adapted to the structure of the oral cavity. A disadvantage is that patches have to be removed after drug delivery, while buccal tablets erode completely. The most patient friendly patches are ellipsoid in shape and have a surface of 1-3 cm², although they may be up to 10-15 cm². (Smart, 1993) Buccal patches can be used for systemic transmucosal drug delivery as well as for local treatment in the oral cavity. Depending on the backing, uni- or multidirectional release patches can be formulated (Figure 6).

Buccal patches consisted of a mucoadhesive drug reservoir formulation attached to an inert backing were described by Anders and Merkle (1989) and Nagai and Konishi (1987). 3M Pharmaceuticals developed a buccal patch (Cydot™) that can be used as a uni- or multidirectional drug delivery system, based on a mixture of polyisobutylene and Carbopol® 934P. (DeGrande et al., 1996)

3.1.2.5 Buccal peptide and protein delivery

In recent years, the buccal mucosa has been investigated as a potential site for controlled delivery of macromolecular therapeutic agents, such as peptides and proteins. The buccal mucosa offers an alternative route to the conventional, parenteral administration. The buccal route is good accessible, has a low enzymatic activity compared to the gastro-intestinal tract and avoids first-pass effect. However, peptides and proteins are generally not well absorbed through mucosae because of their molecular size, hydrophilic nature and the low permeability of the membrane. Peptide and protein transport across the buccal mucosa occurs via passive diffusion. Several approaches to improve buccal absorption of peptides and proteins have been described. They include the use of penetration enhancers, the addition of enzyme inhibitors, molecular modification or specific drug delivery systems such as bioadhesive delivery systems. (Veuillez et al., 2001)

Venugopalan et al. (2001) evaluated bioadhesive polymeric nanoparticles for the buccal delivery of insulin, while Yang et al. (2001) investigated the effects of various transmucosal absorption enhancers on buccal insulin delivery. Cui et al. (2002) reported buccal transmucosal delivery of calcitonin from mucoadhesive films.

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Chapter 3.2 Ex Vivo Bioadhesion Measurement

3.2.1 Introduction

As a first step in the pharmaceutical evaluation of the newly prepared multifunctional polymers, described in *Chapter 1 Multifunctional Polymers*, as potential bioadhesive drug carriers, the bioadhesive properties were measured using an ex vivo bioadhesion test method. The bioadhesion measurements were performed on placebo tablets according to a method previously described by Bouckaert (1994).

3.2.2 Materials and Methods

3.2.2.1 Materials

Drum Dried Waxy Maize (DDWM) was supplied by Cerestar (Vilvoorde, Belgium). Carbopol® 974P (C 974P) was supplied by BF Goodrich (Cleveland, Ohio, USA). Amioca®, Ultrasperse® A and National® 5730 were National Starch products (National Starch and Chemical Company, Bridgewater, New Jersey, USA). Sodium stearyl fumarate (NaSF) was given by Edward Mendell Co. Inc. (New York, USA). All other chemicals used were at least of analytical grade.

3.2.2.2 Production of tablets

For the bioadhesion measurements the powders were mixed with sodium stearyl fumarate (1%; w/w) as a lubricant and compressed on a Korsch compression machine (Type EK0, Berlin, Germany) equipped with 7 mm flat punches, at a pressure of 9.8 kN. The tablet weight was 100 mg.

The physical mixtures (PM) were prepared by physical blending, using pestle and mortar, granular Amioca® starch and Carbopol® 974P. Tablets were prepared as described above.

The reference formulation consisted of a physical mixture of 5% (w/w) Carbopol® 974P, 94% (w/w) DDWM and 1% NaSF (Bouckaert et al., 1993; Voorspoels et al., 1996). The reference tablets were prepared as described above.

3.2.2.3 Ex vivo determination of bioadhesion

The bioadhesive properties of the multifunctional polymers were determined on placebo tablets according to the validated ex vivo bioadhesion method developed and described by Bouckaert (1994).

The apparatus consisted of a tensile testing machine (type L1000R, Lloyd Instruments, Segenworth, Fareham, UK), equipped with a 20 N load cell. Porcine gingiva, the test substrate, was obtained at the slaughterhouse where they were excised directly after slaughtering. The mucosa was stored at –20°C in isotonic buffered saline pH 7.4 (2.38 g $Na_2HPO_4.H_2O$, 0.19 g KH_2PO_4, 8.0 g NaCl made up to 1000 ml with demineralised water).

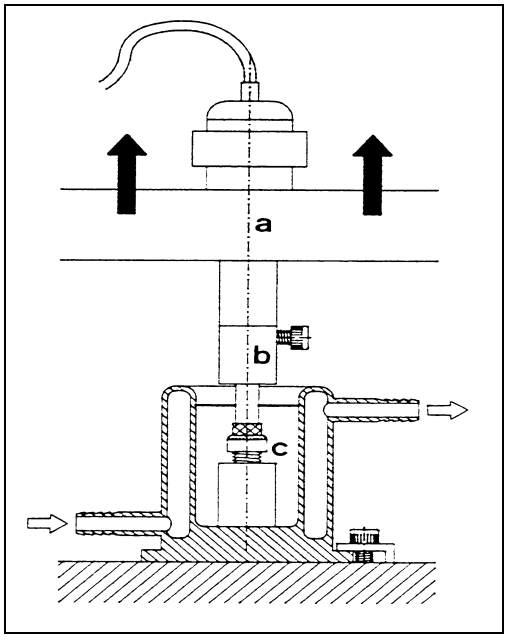

Figure 1. Schematic presentation of the ex vivo bioadhesion test set-up.

a is the superior cross-sectional bar which can be moved up and down.

b is the aluminium upper punch, connected to the superior cross-sectional bar, on which the tablet is attached with cyanoacrylate glue.

c is the Teflon support on which the porcine gingiva (± 1 cm²) is glued. The Teflon support is fixed in a 37°C thermostatic beaker, filled with an isotonic buffered saline pH 7.4 solution after contact between tablet and the porcine mucosa.

The porcine gingival tissue was attached with cyanoacrylate glue (Loctite®, Belgium) to a lower Teflon support, while the tablet was attached to an upper aluminium punch. After hydrating the mucosa with 15 µl of the isotonic phosphate buffered saline, the tablet was

fixed on the mucosa applying a force of 0.5 N for 5 min. After the initial contact, the thermostatic beaker (37°C) was filled with 125 ml isotonic buffered saline pH 7.4 at 37°C. Next, the tablet and mucosa were pulled apart at a speed of 5 mm.min^{-1} until a complete rupture of the tablet-mucosa bond was obtained. Figure 1 gives a schematic presentation of the used test set-up.

With the test method, the adhesion force and the work of adhesion can be measured when tablet and tissue are pulled apart. Adhesion force (N) and the work of adhesion (mJ) were determined as the height and the area under the curve of the force vs. extension diagram, respectively. Figure 2 shows such a force vs. extension diagram.

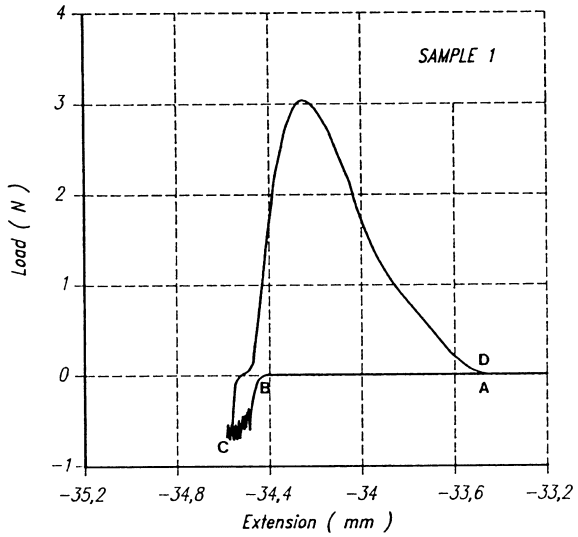

Figure 2. Force vs. extension diagram recorded during an ex vivo bioadhesion test.

Line A-B shows the descent of the upper punch with the bioadhesive tablet.
Line B-C shows the phase after initial contact where tablet and mucosa were pressed together with a force of 0.5 N for 5 min.
Line C-D depicts the phase where tablet and mucosa were pulled apart with a speed of 5 mm.min^{-1}.

The bioadhesion results were compared to a reference formulation, a physical mixture of 5% C 974P, 94% DDWM and 1% NaSF (Bouckaert et al., 1993; Voorspoels et al., 1996).

3.2.2.4 Statistical analysis

Statistical analysis was performed on the work of adhesion results. Statistically significant differences between the different multifunctional polymers were determined using a one-way ANOVA. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the Levene's test. The data were transformed to their logarithm, except for the carboxymethylcellulose

spray-dried samples. To compare the work of adhesion values of the different multifunctional polymers to a reference formulation, a Bonferroni test with $p < 0.05$ as significance level was used. To compare the different multifunctional polymers to each other, a multiple comparison was performed using a Scheffé test with $p < 0.05$ as significance level. The computer program SPSS version 10.0 was used for the statistical analyses.

3.2.3 Results and Discussion

As already mentioned in *Chapter 3.1 Introduction*, the bioadhesion phenomenon seems to be a composite of two mechanisms: a chemical interaction between the functional groups of the polymers and the mucus and a mechanical interpenetration of polymer chains into the mucus. Although adhesion force and work of adhesion values follow mainly the same tendencies and both parameters should be considered for a complete description of bioadhesion, previous studies by Bouckaert (1994) and Voorspoels (1997) using the same ex vivo bioadhesion method as used in this study, showed that the work of adhesion is a better parameter to compare the bioadhesion data. The work of adhesion is, generally considered, more accurate to quantify bioadhesion (Maggi et al., 1994). Ponchel et al. (1987) described that the energy of the rupture of the bioadhesive bond is more indicative of both chemical interactions and mechanical chain interpenetrations. Therefore, statistical analysis was only performed on the work of adhesion data.

3.2.3.1 ^{60}Co irradiated (IR) and chemically modified (CM) grafted starches

The ex vivo bioadhesion results for the ^{60}Co irradiated (IR) and the chemically modified (CM) starch-*g*-poly(acrylic acid) copolymers are shown in Figure 3. The bioadhesion values are compared to a reference formulation (REF). The reference formulation is a physical mixture of 5% (w/w) Carbopol® 974P, a cross-linked poly(acrylic acid), and 95% (w/w) DDWM, a drum dried waxy maize starch. This non-irritating bioadhesive carrier was shown to be effective in local buccal delivery of the

antimycotic drug miconazole (Bouckaert et al., 1993) as well as in transmucosal testosterone delivery (Voorspoels et al., 1996).

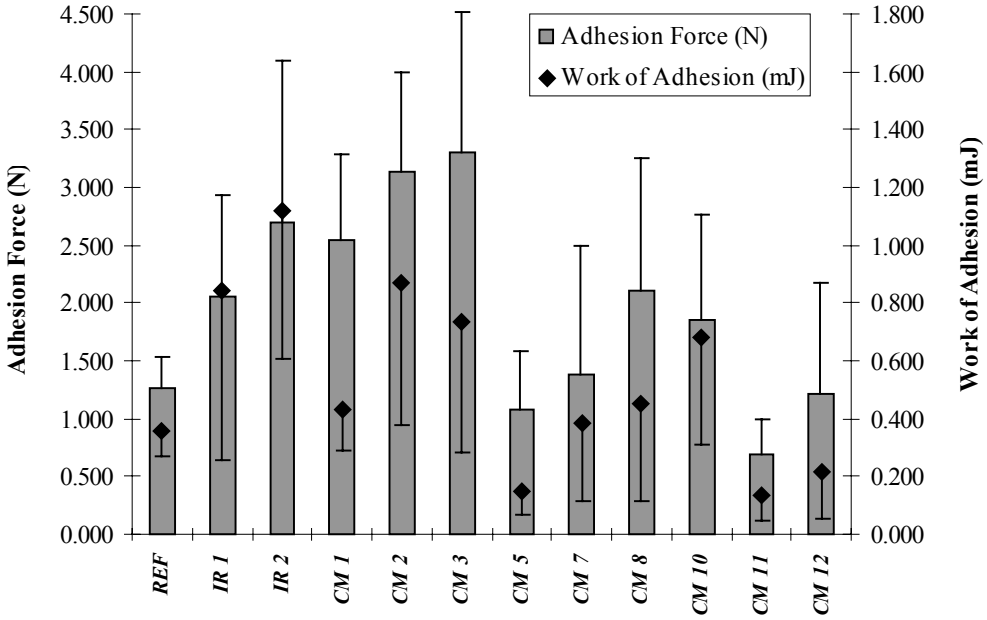

Figure 3. Ex vivo bioadhesion results – Adhesion Force (N) and Work of Adhesion (mJ) – for the ^{60}Co irradiated (IR) and chemically modified (CM) grafted starches compared to a reference formulation (REF). (mean ± SD, n = 10)

The two ^{60}Co irradiated grafted starches showed higher bioadhesive values both for adhesion force and work of adhesion than the reference tablet. The non-neutralised IR 2 performed better than the Na^+ partially neutralised IR 1, but the work of adhesion values were not significantly higher (Scheffé, $p < 0.05$). IR 2 showed compared to the reference for work of adhesion significantly higher values (Bonferroni, $p < 0.05$).
In summary one can say that grafted starch synthesised by ^{60}Co irradiation, using a starch/acrylic acid ratio 1 to 5, showed better ex vivo bioadhesive properties compared to a reference formulation. The non-neutralised sample performed better compared to a partially neutralised one.

Within the group of the chemically modified grafted starches, an Amioca® based copolymer (CM 3) performed better than when waxy rice starch (CM 8), waxy potato starch (CM 11) or tapioca (CM 12) was the starch basis. For the waxy rice starch the

difference was not significant, but the waxy potato and tapioca based grafted starches showed significant lower work of adhesion values compared to an Amioca® grafted starch (Scheffé, $p < 0.05$).

When we compare CM 1, CM 2 and CM 3, which are all three Amioca® based grafted starches, it is clear that a longer polymerisation process time (CM 2 compared to CM 1) and a higher AGE concentration (CM 3 to CM 1) resulted in higher bioadhesive capacities, but the differences were not significant (Scheffé, $p < 0.05$). The three CM grafted starches showed comparable work of adhesion results compared to the reference formulation (Bonferroni, $p < 0.05$).

Using a higher acrylic acid (AA) concentration in the grafting-polymerisation process (CM 5 – starch/AA ratio 1/5) resulted in significant lower bioadhesive properties (CM 3 – ratio 1/3) for the finally obtained grafted starch (Scheffé, $p < 0.05$).

The conversion of a partially (½) neutralised sample (CM 3) to a non-neutralised grafted starch by means of an ion exchange resin (CM 7) resulted in a powder with lower bioadhesive capacities, although not significantly (Scheffé, $p < 0.05$).

Partial neutralisation of the AA to ½ (CM 8) or ¼ (CM 10), did not result in remarkable differences in bioadhesion values.

Sample CM 4 (poor compressibility properties), CM 6 (large granules) and CM 9 (transparent films) could not be tested as they could not be compressed into tablets.

Summarising: The best bioadhesive properties were obtained with an Amioca® based grafted starch, a 0.5% AGE concentration, a starch/AA ratio of 1/3 and half-neutralised AA (CM 3).

3.2.3.2 Freeze-dried starch/poly(acrylic acid) mixtures (FD)

Figure 4 shows the ex vivo bioadhesion results of the freeze-dried starch/poly(acrylic acid) (starch/PAA) mixtures. Again the values are compared to the above mentioned reference formulation (REF).

Only the heat-treated 25/75 powder (FD 25/75 HT) performed better than the reference formulation and showed a significantly higher work of adhesion (Bonferroni, $p < 0.05$). The work of adhesion values for the FD 50/50 and FD 50/50 HT sample were even significantly lower compared to the reference (Bonferroni, $p < 0.05$).

The freeze-dried Amioca®/PAA mixtures with a higher linear PAA content (FD 25/75 and FD 25/75 HT, respectively) showed significantly higher adhesion values than the mixtures containing 50% PAA (FD 50/50 and FD 50/50 HT, respectively) (Scheffé, $p < 0.05$).

Heat treatment after freeze-drying did increase the bioadhesive properties, but only for the 25/75 mixture. The heat-treated FD 25/75 HT performed significantly better than the non-heat-treated FD 25/75 (Scheffé, $p < 0.05$).

It has to be noticed that the FD 50/50 samples did not have good compressibility properties. The obtained tablets were very soft and fragile. During the ex vivo bioadhesion test the tablets swelled as individual particles, which came loose from the tablet. These problems were not observed with the FD 25/75 powders.

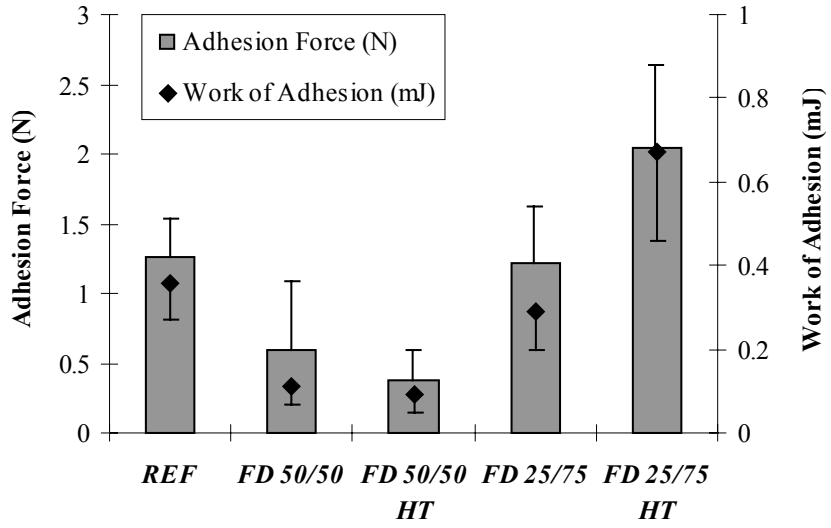

Figure 4. Ex vivo bioadhesion results – Adhesion Force (N) and Work of Adhesion (mJ) – for the freeze-dried starch/poly(acrylic acid) mixtures (FD) compared to a reference formulation (REF). (mean ± SD, n = 10)

Summarising: The freeze-dried mixture with the highest PAA content (75%) and additionally heat-treated after freeze-drying showed the best bioadhesive properties (FD 25/75 HT).

3.2.3.3 Spray-dried starch/carboxylated polymer mixtures (SD)

3.2.3.3.1 Spray-dried starch/poly(acrylic acid) mixtures

Figure 5 shows the ex vivo bioadhesion results of the spray-dried starch/poly(acrylic acid) (starch/PAA) mixtures. The values are compared to the above mentioned reference formulation (REF).

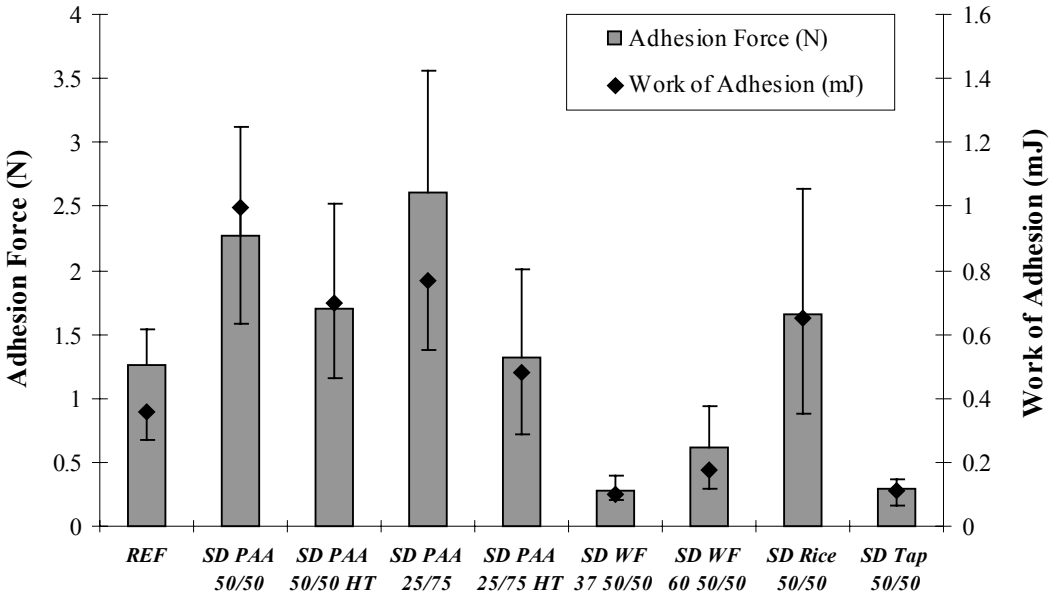

Figure 5. Ex vivo bioadhesion results – Adhesion Force (N) and Work of Adhesion (mJ) – for the spray-dried starch/poly(acrylic acid) mixtures (SD) compared to a reference formulation (REF). (mean ± SD, n = 10)

By spray-drying starch/poly(acrylic acid) mixtures, the best bioadhesive properties were obtained with Amioca® starch (SD PAA 50/50). Using rice starch (SD Rice 50/50) the bioadhesive capacities were still good and not significantly different from SD PAA 50/50, but with tapioca as starch basis (SD Tap 50/50) the work of adhesion values were significantly lower compared to SD PAA 50/50 (Scheffé, $p < 0.05$). SD PAA 50/50, SD PAA 25/75 and SD Rice 50/50 showed significantly higher work of adhesion compared to the reference formulation (Bonferroni, $p < 0.05$).

There was no significant difference between a 50% and a 75% poly(acrylic acid) sample. The additional heat treatment after spray-drying had a negative, but no significant influence on the bioadhesive properties. (Scheffé, $p < 0.05$)

In summary: the best bioadhesive properties were obtained with a non-heat treated Amioca®/PAA mixture containing 50 or 75% PAA.

3.2.3.3.2 Spray-dried starch/sodium carboxymethylcellulose mixtures

The ex vivo bioadhesion results of the spray-dried starch/sodium carboxymethylcellulose (starch/CMC) mixtures are shown and compared to the reference formulation (REF) in Figure 6. In Figure 6 sodium carboxymethylcellulose (CMC) is also compared to poly(acrylic acid) (PAA) as carboxylated polymer source in the spray-dried mixtures.

The best bioadhesive properties were obtained with a non-heat-treated 50/50 sample (SD CMC 50/50), which showed significantly higher work of adhesion compared to the reference (Bonferroni, $p < 0.05$). As with the PAA samples, a higher concentration of the carboxylated polymer did not increase the bioadhesive capacity and an additional heat treatment after spray-drying resulted for the SD 50/50 CMC sample in significantly lower work of adhesion values (Scheffé, $p < 0.05$).

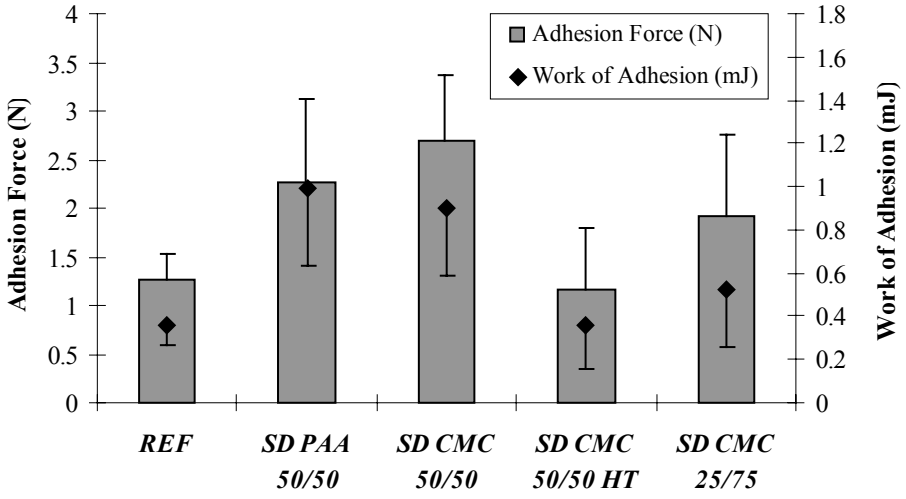

Figure 6. Ex vivo bioadhesion results – Adhesion Force (N) and Work of Adhesion (mJ) – for the spray-dried starch/sodium carboxymethylcellulose mixtures (SD CMC) compared to a reference formulation (REF) and to a spray-dried starch/poly(acrylic acid) 50/50 mixture (SD PAA 50/50). (mean ± SD, n = 10)

Sodium carboxymethylcellulose (SD CMC 50/50) or poly(acrylic acid) (SD PAA 50/50) as carboxylated polymer in a 50/50 spray-dried mixture gives no significant differences in bioadhesion values. Both samples showed good bioadhesive properties and performed significantly better than the reference formulation (Bonferroni, $p < 0.05$). Although, it has to be noticed that the tablets obtained with the CMC-mixtures were of poor quality. The strength of the tablets was too low compared to their bioadhesive capacities, which sometimes resulted in a horizontal crack of the tablet during the adhesion test. A higher CMC content and heat treatment after spray-drying worsened the tablet strength problem. The heat-treated 25/75 sample (SD CMC 25/75 HT) could even not be tested as the tablets were too soft and fragile. This problem was not encountered with the spray-dried starch/PAA mixtures.

3.2.3.3.3 Spray-dried starch/Carbopol® 974P mixtures

Figure 7 shows the ex vivo bioadhesion results of the spray-dried Amioca® starch/Carbopol® 974P mixtures. The values are compared to the above mentioned reference formulation (REF).

All spray-dried Amioca® starch/Carbopol® 974P mixtures showed a comparable or better bioadhesive capacity compared to the reference formulation. The Carbopol® 974P (C 974P) content ranged from 5 to 75% (w/w).

There was no significant difference in bioadhesive capacity between the heat treated SD 25/75 HT and its non-heat treated equivalent SD 25/75. As an additional heat treatment after spray-drying had no (positive or negative) effect on the bioadhesive properties, all other samples were not additionally heat treated.

Increasing the C 974P concentration up to 15% resulted in increasing bioadhesion values. The spray-dried Amioca®/C 974P mixtures containing 15% or more Carbopol® showed all significantly higher work of adhesion values compared to the reference formulation (Bonferroni, $p < 0.05$). Increasing the C 974P concentration above 15% up to 75% did not result in significantly higher bioadhesion values (Scheffé, $p < 0.05$). This observation is in accordance with Bouckaert and Remon (1993) and Park and Munday (2002). The bioadhesive properties of the 95/5 and 90/10 mixtures were not significantly (Bonferroni, $p < 0.05$) different compared to the reference tablet.

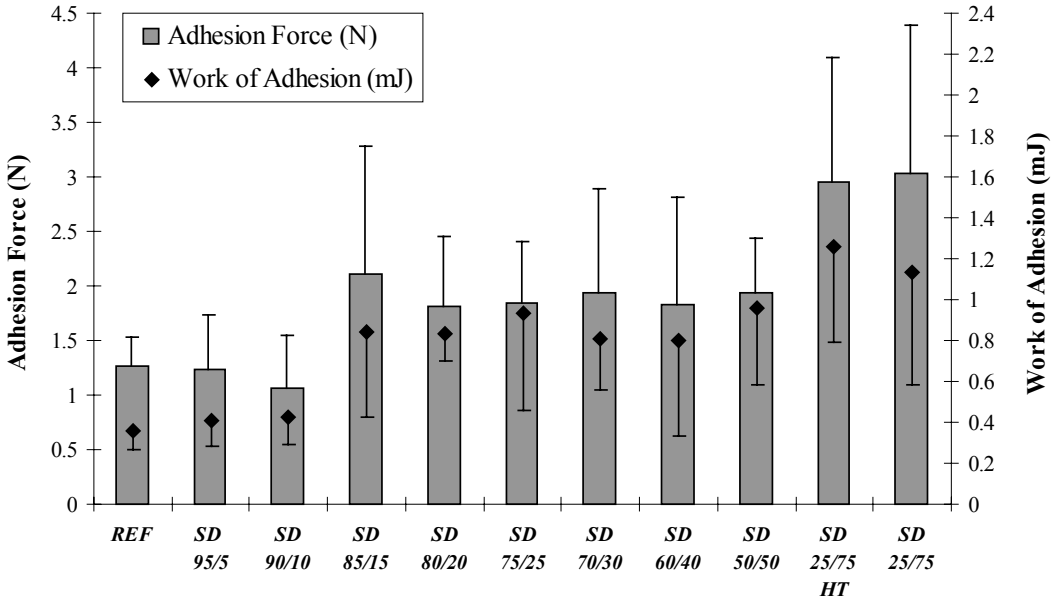

Figure 7. Ex vivo bioadhesion results – Adhesion Force (N) and Work of Adhesion (mJ) – for the spray-dried Amioca® starch/Carbopol® 974P mixtures (SD) compared to a reference formulation (REF). (mean ± SD, n = 10)

Mucoadhesion is, after the formation of an intimate contact between mucoadhesive and mucus, dependent on the hydration, swelling and interpenetration of the mucoadhesive polymers with the mucus macromolecules becoming so physically entangled. Secondly, polymer and mucus interact with each other via non-covalent bonds such as hydrogen bonds (Duchêne et al., 1988). Work of adhesion is suggested to be dependent on the interpenetration of the Carbopol® chains into the mucus, while the adhesion force is considered to be dependent on the formation of hydrogen bonds between the functional groups of the bioadhesive and the mucus (Park and Munday, 2002). Increasing the Carbopol® concentration and thus increasing the number of functional groups resulted up to 15% C 974P in better bioadhesive properties, but over 15% more functional groups did not significantly increase bioadhesion (Scheffé, $p < 0.05$).

Scanning electron microscopy and solid state NMR spectroscopy and relaxometry analysis, described in *Chapter 1.2*, showed that by spray-drying Amioca® starch/Carbopol® 974P mixtures, Carbopol® films are formed around the starch granules.

From a molecular point of view, film formation can explain the performance of the different spray-dried Amioca®/Carbopol® 974P mixtures in the ex vivo bioadhesion test. The significantly increased adhesion properties, starting from a Carbopol® concentration of 15 % (SD 85/15), can probably be explained by an optimal balance between Carbopol® coated and non-coated surface areas on the Amioca® granules. Amioca® granules are thought to be completely surrounded by Carbopol® in the spray-dried mixtures containing 25% or more C 974P (*Chapter 3.3 Mucosal Irritation Test using Slugs*). Increasing the C 974P concentration above 15% and thus complete C 974P coating of the Amioca® granules did not significantly increase the bioadhesive properties of the spray-dried mixtures. The slightly, but not significantly, increased bioadhesion values for the SD 25/75 mixture can be explained by individual Carbopol® 974P nano-particles in addition to an increased film thickness. Film thickness is probably also increased with increasing C 974P contents in the mixtures SD 85/15 – SD 50/50, but a complete coating or an increased film thickness had no additional positive effect on bioadhesion.

In Figure 8 Amioca® (SD 75/25) is compared to National® 5730 (SD Nat 75/25) and Ultrasperse® A (SD 75/25 Ultra) as starch in a 75/25 spray-dried starch/C 974P mixture.

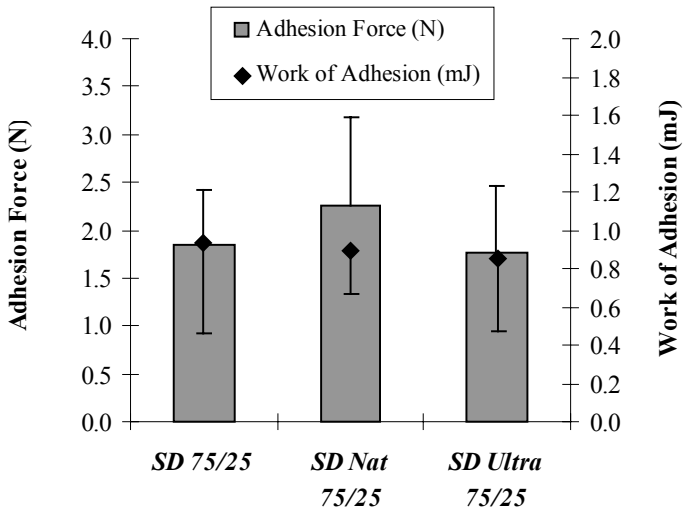

Figure 8. Ex vivo bioadhesion results – Adhesion Force (N) and Work of Adhesion (mJ) – for spray-dried starch/Carbopol® 974P mixtures with Amioca®, National® 5730 and Ultrasperse® A as starch, respectively. (mean ± SD, n = 10)

National® and Ultrasperse® are both pregelatinised waxy corn starches. Amioca® is a native waxy corn starch, but is pregelatinised by jet cooking before spray-drying (*Chapter 1 Multifunctional Polymers*). From the graph, it is clear that by using different types of waxy corn starch in a spray-dried 75/25 mixture similar bioadhesive properties were obtained. There was no significant difference between the three types of starches used (Scheffé, $p < 0.05$). The first selected Amioca® starch seemed, in comparison with other waxy corn starches, to be a good choice.

3.2.3.3.4 Poly(acrylic acid) (PAA), sodium carboxymethylcellulose (CMC) or Carbopol® 974P (C 974P) as carboxylated polymer in a spray-dried Amioca® starch/carboxylated polymer mixture

Figure 9 compares poy(acrylic acid) (PAA), sodium carboxymethylcellulose (CMC) and Carbopol® 974P (C 974P) as carboxylated polymer in a spray-dried Amioca® starch/carboxylated polymer mixture.

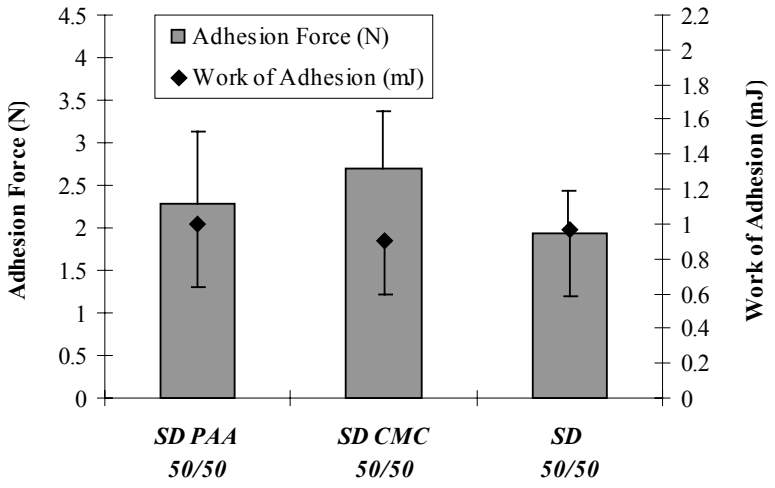

Figure 9. Ex vivo bioadhesion results – Adhesion Force (N) and Work of Adhesion (mJ) – for spray-dried (SD) Amioca® starch/poly(acrylic acid) (PAA), sodium carboxymethylcellulose (CMC) and Carbopol® 974P 50/50 mixtures. (mean ± SD, n = 10)

There are no significant differences in work of adhesion values between the spray-dried mixtures prepared with three different types of carboxylated polymer (Scheffé, $p <$

0.05). Although, as mentioned above CMC resulted in tablets of poor quality. Therefore CMC can not be used as carboxylated polymer in spray-dried mixtures with starch. With PAA and C 974P that problem was not encountered.

3.2.3.4 Freeze-drying versus spray-drying

The ex vivo bioadhesion results of freeze-dried (FD) and spray-dried (SD) Amioca®/Carbopol® 974P 50/50 and 25/75 mixtures are compared in Figure 10.

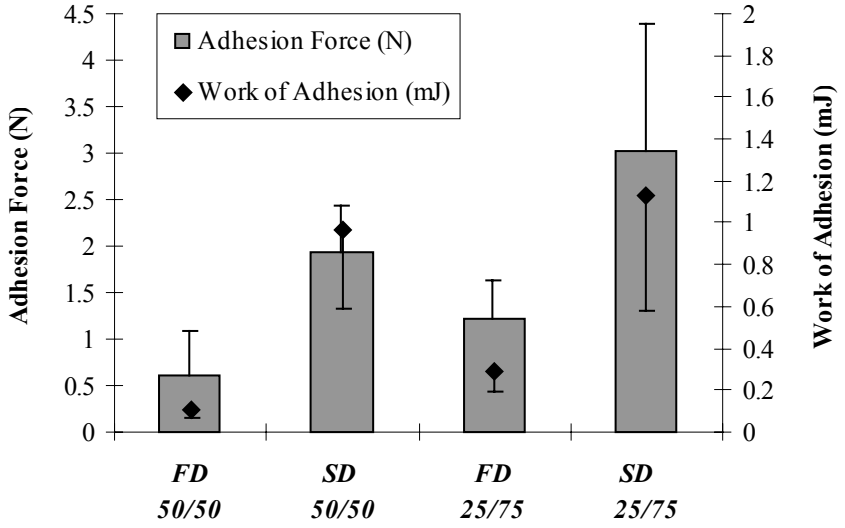

Figure 10. Ex vivo bioadhesion results – Adhesion Force (N) and Work of Adhesion (mJ) – for freeze-dried (FD) and spray-dried (SD) Amioca® starch/Carbopol® 974P 50/50 and 25/75 mixtures. (mean ± SD, n = 10)

From the graph, it is clear that by spray-drying powders are obtained with better bioadhesive properties than after freeze-drying the same mixtures. SD 50/50 and SD 25/75 showed significantly higher work of adhesion values compared to FD 50/50 and FD 25/75, respectively (Scheffé, $p < 0.05$).

As already mentioned, by freeze-drying a 50/50 mixture the obtained powder had poor compression properties and the tablets swelled into individual particles. The powders obtained by spray-drying had very good compression properties and after hydration a homogeneous gel layer was formed on the tablet surface, which progressed to the core of the tablet.

3.2.3.5 Influence of spray-drying on bioadhesive capacities

To evaluate the influence of the spray-drying process on the bioadhesive capacities of Amioca® starch/Carbopol® 974P mixtures, the ex vivo bioadhesive properties of spray-dried mixtures were compared to their equivalent physical mixtures. The bioadhesion results are shown in Figure 11.

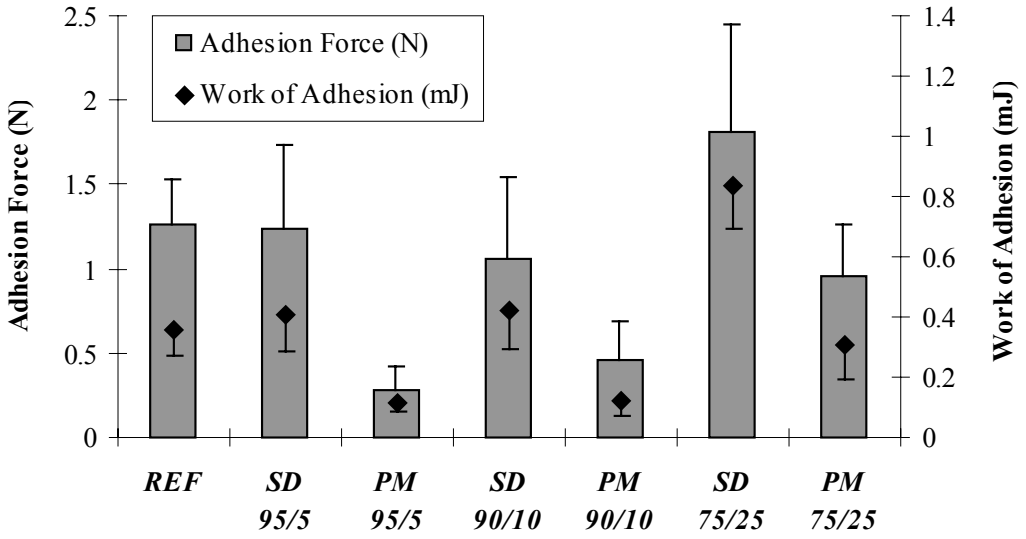

Figure 11. Ex vivo bioadhesion results – Adhesion Force (N) and Work of Adhesion (mJ) – for spray-dried Amioca® starch/Carbopol® 974P mixtures (SD) compared to their equivalent physical mixtures (PM), respectively, and to a reference formulation (REF). (mean ± SD, n = 10)

It is clear that the bioadhesive capacities of Amioca®/Carbopol® 974P mixtures were improved by spray-drying. All spray-dried mixtures showed significantly higher work of adhesion values compared to their equivalent physical mixtures (Scheffé, $p < 0.05$). The 5% C 974P physical mixture (PM 95/5) showed significantly lower adhesion values than the reference (Bonferroni, $p < 0.05$), also containing 5% Carbopol®. This can be explained by the difference in used starch. Drum dried waxy maize starch (DDWM), used in the reference formulation, and Amioca®, used in the physical mixture, are both waxy corn starches, but DDWM is a pregelatinised starch, while the Amioca® was a granular starch. In water, pregelatinised starches will hydrate and swell faster than granular starches. As hydration and swelling of the polymer is an important step in the formation of bioadhesive bonds between mucus/mucosa and polymer, it could be

expected that DDWM showed better bioadhesive properties than the granular Amioca® (Bouckaert, 1994). It should be noticed that the Amioca® in the spray-dried mixtures was pregelatinised by jet cooking. After pregelatinisation not only the bioadhesive properties are increased, but the starch can also be easier dispersed in water, which was required before spray-drying.

3.2.4 Conclusion

Grafted starches prepared by ^{60}Co irradiation as well as by chemical modification showed good ex vivo bioadhesive properties and some of them are potential bioadhesive drug carriers.

By freeze-drying starch/poly(acrylic acid) mixtures, only the powders containing 75% (w/w) poly(acrylic acid) showed good compression properties and performed good during the ex vivo bioadhesion test.

There was no difference observed in bioadhesive capacity between poly(acrylic acid), sodium carboxymethylcellulose (CMC) or Carbopol® 974P as carboxylated polymer in spray-dried starch/carboxylated polymer mixtures. However, the CMC based powders showed a very poor compression behaviour and can not be used in buccal bioadhesive tablet formulations.

It was observed that the powders obtained by spray-drying starch/poly(acrylic acid) mixtures showed better bioadhesive properties compared to the freeze-dried ones.

The most promising bioadhesive powders were obtained by spray-drying Amioca® starch/Carbopol® 974P mixtures. By spray-drying the bioadhesive capacities were significantly improved compared to equivalent physical mixtures of Amioca® and Carbopol® 974P.

3.2.5 References

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Chapter 3.3 Mucosal Irritation Test using Slugs

3.3.1 Introduction

The last decades the development of bioadhesive platforms for mucosal drug delivery has received considerable attention. Several bioadhesive formulations were developed for buccal, nasal, ocular, gastro-intestinal, vaginal and rectal drug delivery. Mucoadhesive polymers increase the residence time at the mucosal application site. Therefore, it is important to evaluate the possible adverse effects of these formulations on the mucosal surfaces. The efficacy of drug delivery systems containing polymers including poly(acrylic acid)s and cellulose derivatives have been extensively described (*Chapter 3.1 Introduction – Buccal dosage forms*). However, only limited toxicological data are available for these formulations and there exists no standard method to evaluate the possible adverse effects of bioadhesives on mucosal tissue. A variety of in vivo and in vitro models were used to investigate the irritation potential of these formulations. The in vitro biocompatibility of different bioadhesive polymers was assessed by the cytokine release from Callu-3 cells (Witschi and Mrsny, 1999). The ocular irritation potential of ophthalmic adhesive delivery systems was evaluated in rabbits (Srividya et al., 2001). The in vivo nasal toxicity of Carbopol®971P was assessed in rabbits and the ciliotoxicity was evaluated using primary human nasal epithelial cells (Ugwoke et al., 2000).The cellular effect of different chitosan polymers was investigated using Caco-2 monolayers and the influence on the ciliary beat frequency was determined with chicken embryonal trachea (Tengamnuay et al., 2000).

In general the use of vertebrates as test organism has been severely criticised based on ethical and financial considerations. The concept of the three Rs (refinement, replacement and reduction) stimulates the development of alternative methods such as in vitro methods and the use of 'lower' organisms (invertebrates, plants and micro-organisms) as test organism (Balls et al., 1995).

The mucosal irritation test using the slug *Arion lusitanicus* as model organism has been validated with reference molecules as an alternative test for screening the irritation potential of chemicals in solutions (Adriaens and Remon, 2002). The body wall of slugs

consists of a single-layered epithelium containing ciliated cells, cells with microvilli and mucus secreting cells. Slugs produce mucus to protect their skin against damage. A previous study showed that the irritation potential of bioadhesive formulations could be estimated with the mucosal irritation test using slugs (Callens et al., 2001). Adriaens et al. (2003) described that the mucosal irritation test using slugs can be used as a reliably and reproducible alternative method to study the biocompatibility of bioadhesive powder formulations. The amount of mucus produced by the slugs during a repeated contact period is a measure for irritation. Membrane damage can be estimated from the release of proteins, lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) from the body wall of the slugs.

In the present study the irritation potential of different multifunctional polymers described in *Chapter 1* was evaluated with the mucosal irritation test using slugs.

3.3.2 Materials and Methods

3.3.2.1 Chemicals

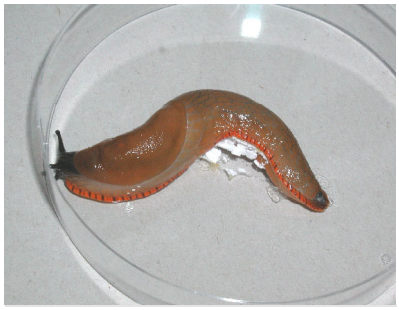
Drum dried waxy maize starch (DDWM) was obtained from Eridania-Béghin Say, Cerestar (Vilvoorde, Belgium) and Carbopol® 974P (C 974P) from BF Goodrich (Cleveland, OH, USA). Benzalkonium chloride was purchased from Sigma-Aldrich (Bornem, Belgium). All other reagents used were of analytical grade.

3.3.2.2 Test procedure mucosal irritation test

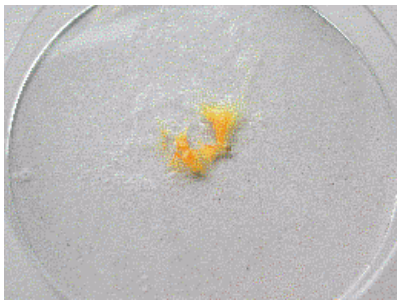
The mucosal irritation test according to the methodology described by Adriaens and Remon (1999) was modified for the bioadhesive powders (Callens et al., 2001).

Figure 1 gives a schematic description of the test procedure. Untreated slugs were used as negative controls (blanks) while slugs treated with DDWM/benzalkonium chloride (DDWM/BAC 95/5) were used as positive controls. The slugs were placed daily on 20 mg powder during 30 min. for 5 successive days.

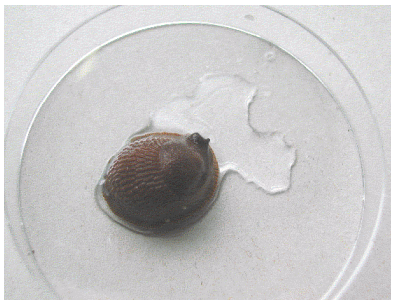
20 mg bioadhesive powder is weighed in a petri dish.



The slugs are placed daily on 20 mg powder during 30 min. for 5 successive days.



The amount of mucus produced during each contact period was measured by weighing the petri dishes with the test substance before and after the 30 min. contact period.



After each 30 min. contact period the slugs were transferred to a fresh petri dish containing 1 ml PBS. After 30 min. and 60 min. the PBS was collected and analysed for proteins and the enzymes LDH and ALP.

Figure 1. Schematic description of the alternative mucosal irritation test procedure using slugs.

For each powder formulation 5 slugs were used. The amount of mucus produced during each contact period was measured by weighing the petri dishes with the test substance before and after the 30 min. contact period. The mucus production was expressed as % of the body weight. After each 30 min. contact period the slugs were transferred to a fresh petri dish containing 1 ml phosphate buffered saline solution (PBS). After 30 min. the PBS was collected with a micropipette and the slugs were placed in a fresh petri dish containing 1 ml PBS which was collected after 60 min. Then again the slugs were transferred in a fresh petri dish, 1 ml PBS was added and collected after 60 min. After sampling the slugs were placed in a petri dish on a membrane filter (cellulose acetate 0.45 µm, Sartorius AG, Germany) moistened with 2 ml PBS until the next contact period. The PBS samples were analysed for the presence of proteins, LDH and ALP released from the body wall.

The total protein concentration present in the PBS samples was determined with a NanoOrange^{TM} protein quantitation kit (Molecular Probes, Leiden, The Netherlands) and expressed in µg/ml per g body weight. The NanoOrange^{TM} reagent allowed accurate protein determination in a concentration range between 10 ng and 10 µg/ml. The fluorescent measurements were carried out on a fluorometer (Kontron Intruments SFM 25, Van Hopplynus, Brussels, Belgium) using excitation/emission wavelengths of 485/590 nm. Bovine serum albumine was used as a standard.

The lactate dehydrogenase activity (LDH, EC 1.1.1.27) was measured with an enzyme kit (DG 1340-K, Sigma Diagnostica, Belgium) and expressed as U/l per g body weight. The LDH reagents measure the enzyme activity based on the optimised standard method recommended by the German Society for Clinical Chemistry (1970, 1971, 1972). The LDH activity measurements were conducted on a Cobas Mira Plus analyzer (ABX, Brussels, Belgium).

The alkaline phosphatase activity (ALP, EC 3.1.3.1) was measured with an enzyme kit (DG 1245-K, Sigma Diagnostica, Belgium) and expressed in U/l per g body weight. The ALP reagents measure the enzyme activity based on the optimised standard method recommended by the German Society for Clinical Chemistry (1970, 1971, 1972). The ALP activity measurements were conducted on a Cobas Mira Plus analyzer (ABX, Brussels, Belgium).

3.3.2.3 Statistical analyses

For the statistical analysis the total mucus production, the mean protein, the mean LDH and the mean ALP release were calculated. Statistically significant differences between the different treatments were determined using a one-way ANOVA. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the Levene's test. If the variances were found not to be equal the data were transformed to their logarithm. To compare the effects of the different treatments a multiple comparison was performed using a Scheffé test with $p < 0.05$ as significance level. Acceptance criteria for the negative and positive controls were determined by calculating the prediction interval to contain future observations. For all the statistical analyses the computer program SPSS version 10.0 was used.

3.3.2.4 Test validity

Acceptance criteria for the negative (NC) and positive control (PC) were established to provide criteria for test validity. A lyophilised mixture of DDWM/BAC (95/5) was selected as a positive control. Previous research showed that this mixture induced severe membrane damage resulting in an increased mucus production, reduction in body weight and the release of protein and enzymes from the body wall of the slugs (Callens et al., 2001). Untreated slugs were used as negative control. The data of 8 repeated tests with the NC and PC were used to calculate the 95% prediction intervals to contain future observations. The results of the tested mucoadhesive polymers were only accepted if the following conditions were satisfied: the mean of the total mucus production (n=5) after a repeated 30 min. contact period was < 2% for the NC and > 10% for the PC slugs. Additionally none of the NC slugs may show LDH and ALP release. (Adriaens et al., 2003)

3.3.3 Results and Discussion

Some caution should be made about the interpretation of the mucus produced by the slugs. Upon contact with the body wall of the slugs dry powder formulations can absorb water by dehydration of the mucosal tissue which will be reported as an increased mucus production compared to untreated (blank) slugs. Therefore, the effect of the different multifunctional polymers on the mucus production was also compared to slugs treated with pure drum dried waxy maize starch (DDWM). The non-irritating potential of DDWM on buccal, nasal and ocular mucosal tissue has been reported by several authors (Callens et al., 2001; Bottenberg et al., 1991; Bouckaert et al., 1993; Bouckaert et al., 1996 and Ceulemans et al., 2001). DDWM was also non-irritating for the slugs as the mucus production was mainly the result of hydration of the dry bioadhesive powder formulations. The total mucus production and the mean protein release for 20 repeated experiments with DDWM treated slugs were not significantly different (Scheffé, $p < 0.05$) (Table 1).

3.3.3.1 ^{60}Co irradiated (IR) and chemically modified (CM) grafted starches

The results of the mucosal irritation test for the ^{60}Co irradiated (IR) and chemically modified (CM) grafted starches are summarised in Table 1.

All three grafted starches showed a significant increased mucus production compared to the blank slugs and the DDWM treated slugs. Moreover, the total mucus production was comparable to the positive control (PC) slugs. There was no significant difference in the amount of mucus produced between the ^{60}Co irradiated (IR) and the chemically modified (CM) grafted starches. The non-neutralised irradiated grafted starch (IR 2) showed a higher mucus secretion compared to the partially neutralised IR 1, but not significantly. The protein release profiles of the slugs treated with the different grafted starches were increased compared to the blank slugs and the DDWM slugs, although not significantly. Only the positive control slugs exhibited a significantly increased protein release and the protein release increased with a repeated contact period. IR 2 and CM 3 induced the release of cytosolic LDH, which is a sign of membrane damage, no ALP

release was detected. Only the PC slugs induced both cytosolic LDH and membrane bound ALP release.

	Total MP (%)[a]	Mean protein release (µg/ml.g)	Mean LDH release (U/l.g)	Mean ALP release (U/l.g)	N
Blank (NC)	-0.3 ± 1.4	12 ± 10	-	-	30
DDWM	3.6 ± 1.3 [a]	11 ± 7 [a]	-	-	20
DDWM/BAC 95/5 (PC)	18.7 ± 4.8 [b]	145 ± 83 [b]	6.37 ± 5.30	0.46 ± 0.58	30
IR 1	19.2 ± 2.4 [b]	22.05 ± 23.24 [a]	-	-	5
IR 2	23.4 ± 5.1 [b]	21.34 ± 16.73 [a]	0.26 ± 0.58	-	5
CM 3	23.9 ± 5.2 [b]	14.73 ± 7.64 [a]	0.17 ± 0.37	-	5

Table 1. Effect of the ^{60}Co irradiated (IR) and chemically modified (CM) grafted starches on the endpoints of the mucosal irritation test. (mean ± S.D)

[a] Treatment belongs to the same group as the DDWM slugs ($P > 0.05$, Scheffé test)

[b] Treatment belongs to the same group as the PC slugs ($P > 0.05$, Scheffé test)

MP: mucus production in % (w/w) of the body weight

The results indicated that the ^{60}Co irradiated (IR) and the chemically modified (CM) grafted starches were irritating to the mucosal tissue of the slugs since they induced an increased mucus production which is a sign of irritation. However no significantly increased protein release was detected, IR 2 and CM 3 induced the release of LDH from the slugs body wall, which is an indication of membrane damage and thus severe irritation. When using these grafted starches in bioadhesive formulations to be applicated on mucosal surfaces, i.e. the buccal mucosa, one has to take into account the irritating potential of these polymers. The mucosal irritation test has shown that the ^{60}Co irradiated and chemically modified grafted starches tested are excluded as mucoadhesive carriers.

3.3.3.2 Spray-dried Amioca®/poly(acrylic acid) and Amioca®/Carbopol® 974P mixtures

The results of the mucosal irritation test for the spray-dried starch/poly(acrylic acid) mixtures (SD PAA) are summarised in Table 2.

All the spray-dried mixtures induced a significant increased mucus production compared to the blank slugs and the slugs treated with DDWM. The amount of mucus produced increased with an increasing PAA concentration and the powders containing 75% (w/w) PAA showed comparable amount of mucus production compared to the positive control (PC) slugs. Heat treatment after spray-drying had no effect on the mucus production. The protein release profiles of the slugs treated with the different mixtures were comparable with the blank and the DDWM treated slugs. Only the positive control slugs exhibited a significantly increased protein release and the protein release increased with a repeated contact period. Enzyme release was only detected for the positive control slugs.

	Total MP (%)[a]	Mean protein release (µg/ml.g)	Mean LDH release (U/l.g)	Mean ALP release (U/l.g)	N
Blank (NC)	-0.3 ± 1.4	12 ± 10	-	-	30
DDWM	3.6 ± 1.3 [a]	11 ± 7 [a]	-	-	20
DDWM/BAC 95/5 (PC)	18.7 ± 4.8 [b]	145 ± 83 [b]	6.37 ± 5.30	0.46 ± 0.58	30
SD PAA 50/50	9.2 ± 3.7	9 ± 5 [a]	-	-	5
SD PAA 50/50 HT	9.7 ± 3.5	7 ± 5 [a]	-	-	5
SD PAA 25/75	14.1 ± 2.5 [b]	11 ± 2 [a]	-	-	5
SD PAA 25/75 HT	12.7 ± 2.0 [b]	13 ± 3 [a]	-	-	5

Table 2: Effect of the spray-dried starch/poly(acrylic acid) mixtures (SD PAA) on the endpoints of the mucosal irritation test. (mean ± S.D)

[a] Treatment belongs to the same group as the DDWM slugs ($P > 0.05$, Scheffé test)

[b] Treatment belongs to the same group as the PC slugs ($P > 0.05$, Scheffé test)

MP: mucus production in % (w/w) of the body weight

The results of the study indicated that the used PAA concentrations were irritating to the mucosal tissue of the slugs since all tested spray-dried Amioca®/PAA mixtures induced an increased mucus production, which is a sign of irritation, however no increased protein and enzyme release was detected. The irritation potential increased with an increasing PAA content in the spray-dried mixtures.

The effects of the spray-dried Amioca® starch/Carbopol® 974P (C 974P) mixtures on the endpoints of the mucosal irritation test are shown in Table 3.

	Total MP (%)[a]	Mean protein release (µg/ml.g)	Mean LDH release (U/l.g)	Mean ALP release (U/l.g)	N
Blank (NC)	-0.3 ± 1.4	12 ± 10	-	-	30
DDWM	3.6 ± 1.3 [a]	11 ± 7 [a]	-	-	20
DDWM/BAC 95/5 (PC)	18.7 ± 4.8 [b]	145 ± 83 [b]	6.37 ± 5.30 [b]	0.46 ± 0.58	30
SD 95/5	3.7 ± 0.8 [a]	5 ± 4 [a]	-	-	5
SD 90/10	4.8 ± 2.5 [a]	12 ± 15 [a]	-	-	10
SD 85/15	5.9 ± 1.7 [a]	13 ± 8 [a]	-	-	5
SD 80/20	5.3 ± 1.7 [a]	11 ± 6 [a]	-	-	15
SD 75/25	12.0 ± 3.2 [b]	17 ± 10 [a]	-	-	5
SD 70/30	12.4 ± 2.0 [b]	11.2 ± 5.4 [a]	-	-	5
SD 60/40	17.0 ± 2.2 [b]	28 ± 30 [a]	0.70 ± 0.85 [b]	-	5
SD 50/50	18.5 ± 8.3 [b]	27 ± 20 [a]	1.17 ± 1.13 [b]	-	5
Carbopol® 974P	21.4 ± 3.7 [b]	55 ± 30 [b]	0.43 ± 0.30	-	5

Table 3: Influence of the spray-dried Amioca® starch/Carbopol® 974P mixtures (SD) and pure Carbopol® 974P on the endpoints of the mucosal irritation test. (mean ± S.D)

[a] Treatment belongs to the same group as the DDWM slugs (P > 0.05, Scheffé test)

[b] Treatment belongs to the same group as the PC slugs (P > 0.05, Scheffé test)

MP: mucus production in % (w/w) of the body weight

All the spray-dried mixtures induced a significant higher total mucus production compared to the untreated slugs. However, the total mucus production induced after treatment with mixtures containing up to 20% C 974P was similar as in the DDWM treated slugs. The mucus secretion induced by the mixtures containing 25% (w/w) C 974P

or more was significantly higher than for the DDWM treated slugs and was comparable to positive control (PC) treated slugs. The spray-dried mixtures had no additional effect on the protein release in comparison with the DDWM treated slugs. However, the spray-dried mixtures containing 40% (w/w) or more C 974P induced the release of cytosolic LDH, which is a sign of membrane damage on the slugs' body wall. Pure Carbopol® 974P induced cytosolic LDH release and a comparable protein release to the PC treated slugs.

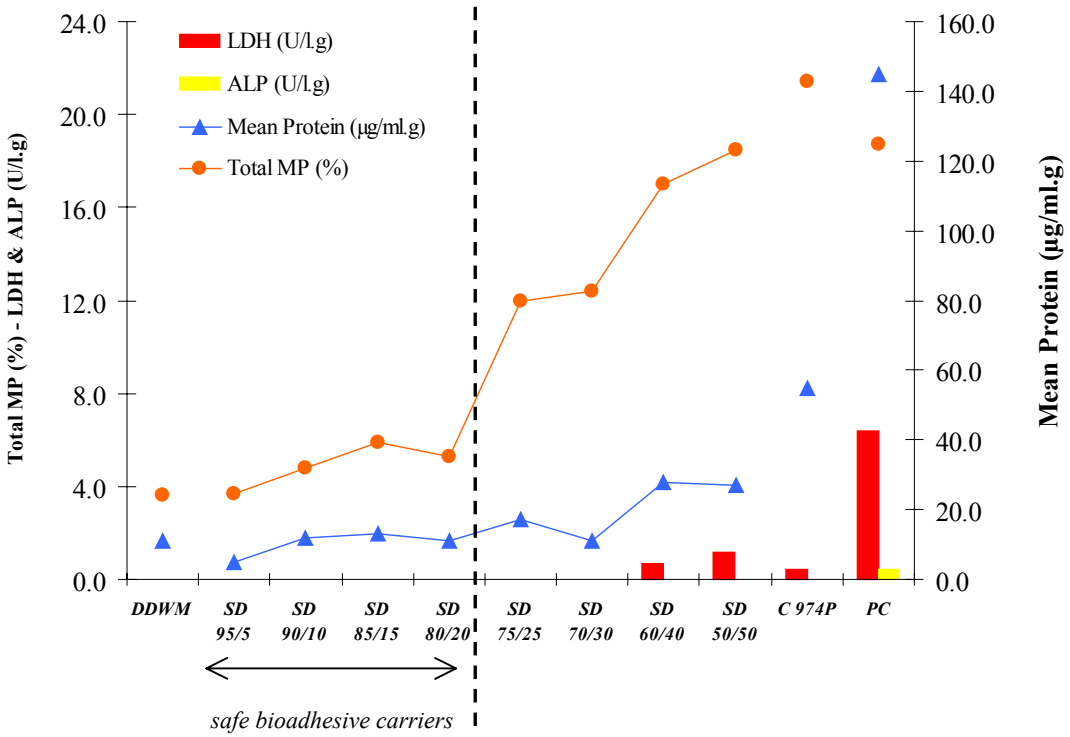


Figure 2. Overview of the influence of the spray-dried Amioca®/Carbopol® 974P mixtures (SD), pure DDWM, pure Carbopol® 974P (C 974P) and DDWM/BAC 95/5 (positive control, PC) on the endpoints of the mucosal irritation test.

The spray-dried Amioca®/C 974P mixtures containing 25% and 30% (w/w) Carbopol® 974P induced slight irritation of the mucosa as was demonstrated by the increased mucus secretion but no additional effect on the protein and enzyme release was detected. The spray-dried mixtures with 40% (w/w) or more C 974P induced LDH, which indicates membrane damage and severe irritation. On the other hand, the spray-dried Amioca®/Carbopol® 974P mixtures containing up to 20% Carbopol® 974P induced no irritation of the mucosal tissue of the slugs and are safe mucoadhesive carriers. (Figure 2)

Scanning electron microscopy and solid state NMR spectroscopy and relaxometry analysis, described in *Chapter 1.2*, showed that by spray-drying Amioca® starch/Carbopol® 974P mixtures, Carbopol® films are formed around the starch granules. Film formation can probably explain the mucosal irritation results from a molecular point of view. Total mucus productions after treatment with spray-dried mixtures containing 25% or more C 974P and pure C 974P belonged all to the same group and were significant higher compared to the DDWM treated slugs. Starting at a concentration of 25% C 974P (SD 75/25) the Amioca® granules are probably completely surrounded by Carbopol®. When the slugs are brought into contact with these powders the contact surface is 100% C 974P, resulting in irritation of the slugs' mucosa. Increasing the C 974P content above 25%, and thus an increased film thickness, and pure C 974P did not induce a significantly higher total mucus production, confirming this hypothesis. Spray-dried mixtures containing up to 20% C 974P are non-irritating, as the Amioca® granules are not completely surrounded with C 974P and as the ratio Carbopol® coated and non-coated surface areas on the Amioca® granules is to small to induce mucosal irritation.

When comparing linear poly(acrylic acid) (PAA) (SD PAA 50/50) and cross-linked poly(acrylic acid) Carbopol® 974P (C 974P) (SD 50/50) used in spray-dried Amioca® starch/poly(acrylic acid) mixtures, the irritation potential of the cross-linked poly(acrylic acid) C 974P on mucosal tissue was more pronounced. SD PAA 50/50 induced only an increased mucus production, while no enzyme release was detected. SD 50/50 resulted in a higher mucus production and cytosolic LDH release, which indicates membrane damage. The explanation for the higher irritation potential of the cross-linked Carbopol® 974P can probably be found in the higher molecular weight of the cross-linked polymer, which is in the billions, compared to the linear PAA (Mw 250.000). A higher molecular weight poly(acrylic acid) contains more carboxylic acid functions (-COOH), which are mainly responsible for the irritating effect.

3.3.3.3 Influence of spray-drying on the mucosal irritation potency of Amioca®/Carbopol® 974P mixtures

The effect of spray-drying Amioca®/Carbopol® 974P mixtures on the mucosal irritation potential was investigated by comparing spray-dried mixtures containing 5, 10 and 25% (w/w) Carbopol® 974P with their equivalent physical mixtures (Table 4). Since a Carbopol® 974P concentration of 25% (w/w) induced a significant increased mucus production compared to pure DDWM, only mixtures containing up to 25% (w/w) Carbopol® 974P were tested.

	Total MP (%)[a]	Mean protein release (µg/ml.g)	Mean LDH release (U/l.g)	Mean ALP release (U/l.g)	N
Blank (NC)	-0.3 ± 1.4	12 ± 10	-	-	30
DDWM	3.6 ± 1.3 [a]	11 ± 7 [a]	-	-	20
DDWM/BAC 95/5 (PC)	18.7 ± 4.8 [b]	145 ± 83	6.37 ± 5.30	0.46 ± 0.58	30
SD 95/5	3.7 ± 0.8 [a]	5 ± 4 [a]	-	-	5
PM 95/5	1.7 ± 1.8 [a]	7 ± 10 [a]	-	-	5
SD 90/10	4.8 ± 2.5 [a]	12 ± 15 [a]	-	-	10
PM 90/10	2.8 ± 1.0 [a]	6 ± 2 [a]	-	-	5
SD 75/25	12.0 ± 3.2 [b]	17 ± 10 [a]	-	-	5
PM 75/25	6.9 ± 2.5 [a]	7 ± 5 [a]	-	-	5

Table 4: Influence of spray-drying Amioca® starch/Carbopol® 974P mixtures (SD) compared to physical mixtures (PM) on the endpoints of the mucosal irritation test. (mean ± S.D)

[a] Treatment belongs to the same group as the DDWM slugs (P > 0.05, Scheffé test)

[b] Treatment belongs to the same group as the PC slugs (P > 0.05, Scheffé test)

MP: mucus production in % (w/w) of the body weight

Within the group of the spray-dried mixtures the 75/25 ratio showed a significantly increased mucus production compared to the 90/10 and 95/5 ratio, while the physical mixtures belonged all to the same group.

There was no significant difference in mucus production between the spray-dried and physical blended mixtures at the respective ratios, although an increased mucus production was observed for the spray-dried mixtures compared to their equivalent

physical mixtures, respectively. The protein release profiles of the slugs treated with the different spray-dried or physical blended mixtures were comparable with the blank and DDWM treated slugs. Only the positive control (PC) slugs exhibited a significantly increased protein release. Also enzyme release was only detected for the PC slugs.

The mixing process (spray-drying vs. physical blending) had no significant effect on the irritation potency of Carbopol® 974P, although spray-dried mixtures induced a slightly higher mucus production compared to equivalent physical mixtures.

From a molecular point of view this can be explained by the C 974P film formation around the Amioca® granules during spray-drying (*Chapter 1.2*). When the bioadhesive powders are brought into contact with the slugs' mucosa, it is mainly (partial coating) or 100% (total coating) Carbopol® 974P which will make direct contact with the mucosa, resulting in an increased irritative effect. In an Amioca®/Carbopol® 974P physical blend both components are homogeneously mixed and divided in the mixture.

Irritation studies in other models also indicated that Carbopol® was well tolerated when it was used in small amounts (< 10%). Buccal and ocular tablets containing 5% Carbopol together with non-irritating DDWM were well accepted by volunteers (Bottenberg et al., 1991; Bouckaert et al., 1996 and Ceulemans et al., 2001). A buccal erodible tablet containing 7.5% Carbopol® 974P induced no irritation over a period of 6 hours in human volunteers (Khanna et al., 1997). Daily administration of neutralised and lyophilised DDWM/Carbopol® 974P (90/10) during four weeks did not induce irritation of the nasal mucosa in rabbits (Callens et al., 2001). Rectal nicotine formulations with a Carbopol® concentration below 10% did not have any cytotoxic effect on Caco-2 cells (Dash et al., 1999) and suppositories containing 10% Carbopol® 934P administered to rabbits for 5 days did not irritate the tissue (Yahagi et al., 2000).

3.3.3.4 Correlation between the mucosal irritation test using slugs and in vivo irritation potential in dogs

To investigate the correlation between the results of the alternative mucosal irritation test using slugs and the irritation potential in vivo, SD PAA 50/50 and SD PAA 25/75 were evaluated as a buccal bioadhesive tablet in dogs (n = 2). One tablet was adhered daily on the gingiva above the right upper canine for one week.

SD PAA 25/75 resulted in irritation of the buccal mucosa after 4 days of application, while the SD PAA 50/50 showed the first signs of irritation after 6 days (Figure 3). As observed with the alternative mucosal irritation test, a higher PAA concentration resulted also in dogs in a higher degree of mucosal irritation, observed as an earlier onset of irritation. These data prove that the mucosal irritation test using slugs can be used as a reliably alternative method to study the biocompatibility of bioadhesive powder formulations.

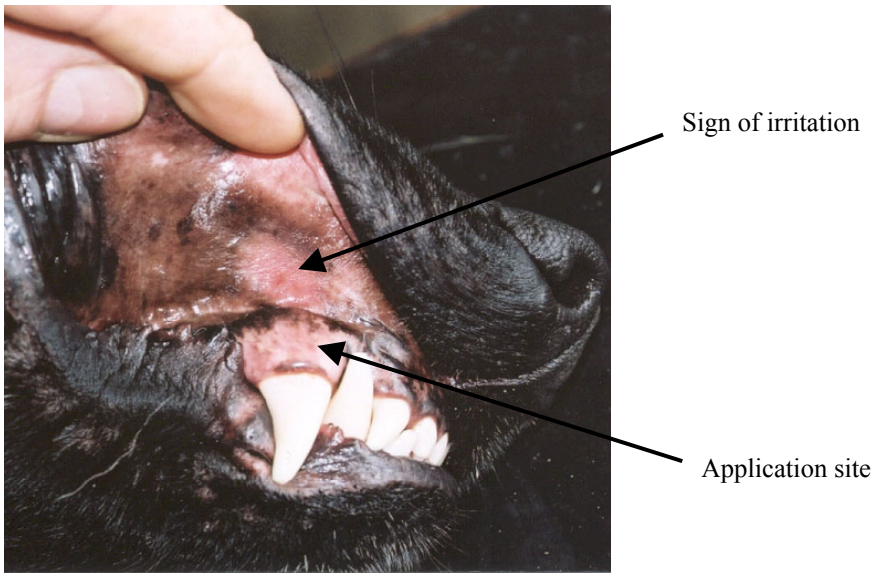


Figure 3. Irritation of the dogs buccal mucosa after application of a SD PAA 50/50 bioadhesive tablet.

3.3.4 Conclusion

It can be concluded that grafted starches prepared by ^{60}Co irradiation or chemical modification both induce irritation of mucosal tissues.

Spray-dried starch/poly(acrylic acid) mixtures containing 50 and 75% (w/w) poly(acrylic acid) were irritating on mucosal surfaces and the irritation potential increased with increasing poly(acrylic acid) concentrations. In vivo evaluation in dogs confirmed these findings.

Spray-dried Amioca® starch/Carbopol® 974P mixtures containing up to 20% Carbopol® 974P did not show a distinct sign of irritation. These powders can be incorporated in bioadhesive formulations without risk of irritation and are potential safe bioadhesive carriers.

3.3.5 References

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Chapter 3.4 Buccal Testosterone Absorption from a Bioadhesive Tablet

3.4.1 Introduction

The buccal absorption of testosterone from a bioadhesive tablet formulation based on a ^{60}Co irradiated or chemically modified grafted starch or on a freeze-dried starch/poly(acrylic acid) mixture was investigated in vivo in dogs.

In a previous study, a non-irritating buccal bioadhesive drug carrier was developed containing a physical mixture of 5% cross-linked poly(acrylic acid), Carbopol® 974P, with a thermally modified starch (Bouckaert and Remon, 1993). This buccal drug carrier has been shown to be effective for local (miconazole) delivery (Bouckaert et al., 1993) as well as for systemic drug delivery. Voorspoels et al. (1996) studied the buccal absorption of testosterone from this erodible bioadhesive tablet in dogs. They concluded that the application of a buccal bioadhesive tablet with 60 mg testosterone in dogs sustained plasma levels which were significantly higher than those obtained after the oral administration of the same dose of testosterone.

Testosterone (Figure 1), the main circulating androgen in men, is secreted predominantly by the testes. Normal serum concentrations are 10 – 35 nmol/l, and show a circadian variation with peak concentrations in the morning (Bremmer et al., 1983; Place and Nichols, 1991). In extragonadal tissues, circulating testosterone is enzymatically converted to dihydrotestosterone.

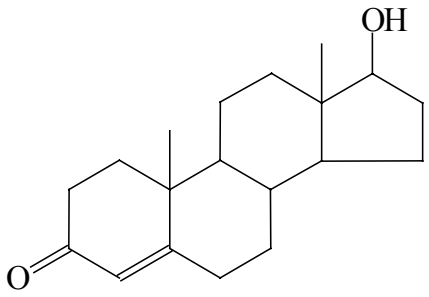

Figure 1. Chemical structure of testosterone.

When administered orally or parenterally, testosterone is rapidly absorbed and metabolised by the liver resulting in a very short circulating half-life. Nowadays, testosterone esters, such as testosterone proprionate, enanthate or decanoate), are injected

intramuscularly. They are more lipophilic than testosterone and are absorbed slowly when injected as an oil solution or suspension, yielding testosterone concentrations greatly above normal levels during the first days after administration, but they do not produce daily variation in testosterone concentrations. Testosterone undecanoate is an orally active ester formulated in a lipid-soluble preparation that is absorbed directly into the lymphatic system, thereby avoiding first-pass metabolism in the liver (Conway et al., 1988). However, because of the poor bioavailability of testosterone, the levels of circulating testosterone obtained with such treatment are unpredictable (Conway et al., 1988; Bagatelle and Bremner, 1996). Transdermal testosterone delivery, either via a patch or a gel, can yield physiological concentrations of testosterone and a circadian pattern close to that of healthy men (Meikle et al., 1996; Dobs et al., 1999). However, transdermal patches often cause local skin irritation (Arver et al., 1997; Parker and Armitage, 1999) and testosterone gels must be applied over a large surface area of the skin, which is not patient friendly (Wang et al., 2000).

In the first part of this study, the ^{60}Co irradiated or chemically modified grafted starches and a freeze-dried starch/poly(acrylic acid) mixture (*Chapter 1 Multifunctional Polymers*) were formulated as a buccal bioadhesive tablet, loaded with testosterone as a model drug, and the pharmacokinetics were evaluated in dogs.

In the second part of this Chapter spray-dried Amioca® starch/Carbopol® 974P mixtures were evaluated as potential buccal bioadhesive tablets. Different Amioca®/Carbopol® 974P ratios were tested in vivo in dogs as a placebo tablet and the influence of the Carbopol® content on the in vivo adhesion time was investigated. The in vitro drug release rate was evaluated in an USP III (BioDis) dissolution system by incorporating 10 mg miconazole nitrate as a marker. Miconazole nitrate is an antifungal drug and is frequently used in local oral drug delivery systems such as oral gels or bioadhesive buccal tablets (Bouckaert and Remon, 1993; Bouckaert et al., 1993; Nafee et al., 2003). The drug loading capacity and bioavailability of a selected spray-dried mixture was investigated in vivo in dogs using testosterone as model drug.

The dog was used as in vivo animal model. The dog, the monkey and the pig are suitable animals for drug delivery studies via the buccal mucosa as their buccal mucosa

has a non-keratinised epithelium, just like the human buccal mucosa (Hoogstraate et al., 1998). The pig is probably the most suitable model because there are essentially no differences between human and pig buccal mucosa, but the pig is very sensitive to stress and has to be sedated during the experiment. It is important that the animal is not sedated during the experiment as the drug is released by erosion of the buccal bioadhesive tablet, which is stimulated by the saliva flow and movement of the lips and cheeks. The dog must not be sedated during the experiments and is generally seen the most appropriate animal model for buccal drug delivery studies.

3.4.2 Grafted starches (IR & CM) and freeze-dried starch/poly(acrylic acid) mixtures (FD)

3.4.2.1 Materials

Testosterone was purchased from Diosynth (Oss, The Netherlands). Carbopol® 974P (C 974P) was supplied by BF Goodrich (Cleveland, Ohio, USA). Drum Dried Waxy Maize (DDWM) was supplied by Cerestar (Vilvoorde, Belgium). Sodium stearyl fumarate (NaSF) was given by Edward Mendell Co. Inc. (New York, USA). All other chemicals used were at least of analytical grade.

3.4.2.2 Methods

3.4.2.2.1 Production of tablets

To produce the tablets for the in vivo study the powder was first mixed with micronised testosterone (60 mg), next the sodium stearyl fumarate (1%) was added as a lubricant and mixed again. The tablets were compressed on a Korsch compression machine (Type EK0, Berlin, Germany). The weight and the diameter of the tablets was 200 mg and 9 mm, respectively. The compression force was 14.7 kN.

Because the surface of the powder particles of the irradiated starches was rough and because the bioadhesive tablets based on these powders swelled as large individual particles which came loose from the tablet, the IR 1 sample was first lyophilised. After hydration of the bioadhesive tablets formulated with the lyophilised powder, a homogeneous gel layer, that will be progressively eroded, is formed around the tablet. To lyophilise the powder, first a 3 % (w/w) gel in distilled water was formulated. Next the gel was filled into vials and lyophilised over 24 h in a Finn Aqua GT4 lyophilisator (Amsco, Brussels, Belgium). Then the lyophilised powder was milled in a powder mill (Fritsch, Germany). Tablets based on the lyophilised powder for the in vivo study were produced as described above.

3.4.2.2.2 In vivo study protocol

The bioavailability of the selected formulations was determined according to a previously described protocol by Voorspoels et al. (1996). The formulations were tested in 6 castrated male dogs (weight 34.2 ± 2.2 kg). The dogs were conscious and fasted from 12 h before until the end of the experiment. Drinking water was available at libitum. One tablet was placed on the gingiva above the right upper canine (Figure 2) and blood samples were collected before the administration and 0.5, 1, 2, 4, 8, 12, 16 and 24 h after the administration in heparinised tubes. The blood samples were centrifuged at 2000 g and the plasma was kept at –20°C until analysis. A time interval of at least 1 week was respected between each administration. To calculate the absolute bioavailability, 60 mg testosterone was administered intravenously to each dog. Blood samples were taken at 0, 2, 5, 10, 20, 30 min. and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24 h after administration. The approval of The Ethics Committee was obtained.

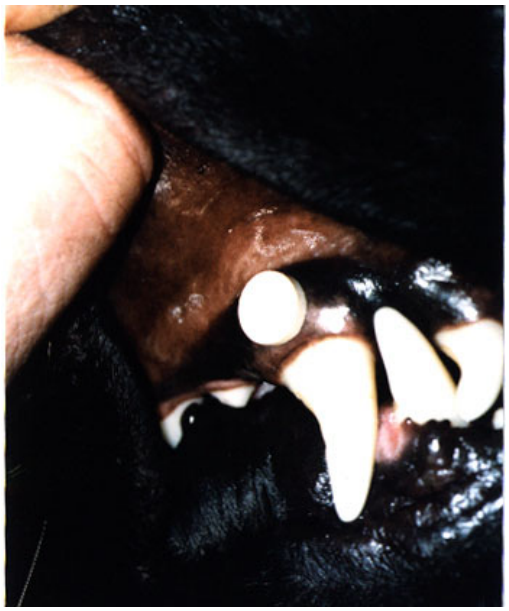

Figure 2. A bioadhesive tablet sticked on the gingiva above the right upper canine in the dogs' mouth.

3.4.2.2.3 Testosterone analysis

The testosterone plasma concentrations were determined by chemiluminescent immunoassay (Immulite® Total Testosterone, Diagnostic Products Corporation, Los Angeles, CA, USA). The analytical sensitivity was 10 ng/dl. The antibody was highly specific for testosterone, the cross reactivity was 0.79% for androstenedion and 9.1% for dihydrotestosterone. The interassay coefficient of variation varied between 6.5% and 16% depending on the concentration level.

3.4.2.2.4 Pharmacokinetics and in vivo adhesion time

The absolute bioavailability was calculated using the Kinbes® software (Proost and Meijer, 1992). The $T_{>3ng/ml}$ value (time during which the plasma testosterone concentration was above 3 ng/ml) was calculated from the individual graphs. The in vivo adhesion time of the bioadhesive tablet was determined visually. Adhesion was considered to be present until the complete erosion of the tablet.

3.4.2.3 Results and Discussion

Based on the results from the ex vivo bioadhesion measurements (*Chapter 3.2*) both ^{60}Co irradiated grafted starches IR 1, lyophilised IR 1 and IR 2, the chemically modified grafted starch CM 3 and the freeze-dried Amioca®/poly(acrylic acid) mixture FD 25/75 HT were selected to be investigated as potential buccal bioadhesive drug carriers in a bioavailability study of buccally administered testosterone in dogs.

The mean plasma concentration time profiles of the different formulations are shown in Figure 3. The absolute bioavailability, the time during which the plasma testosterone concentration was above the 3 ng/ml target concentration ($T_{>3ng/ml}$) (Mazer et al., 1992) and the mean adhesion time are shown in Table 1.

The reference formulation sustained the 3 ng/ml target concentration during 11.3 ± 3.9 h and adhered in vivo during 15.7 ± 8.6 h. The chemically modified grafted starch CM 3 showed a high testosterone plasma peak 2 hours after administration and the time during which the plasma concentration was above 3 ng/ml was 7.9 ± 2.1 h. The CM 3 tablet adhered in vivo during 9.7 ± 2.7 h. After application of the freeze-dried Amioca®/poly(acrylic acid) mixture FD 25/75 HT the mean plasma concentration time profile never exceeded the 3 ng/ml level, the $T_{>3ng/ml}$ was 5.0 ± 3.5 h. The tablet based on FD 25/75 HT adhered in vivo during 10.5 ± 3.1 h. With the non-neutralised IR 2 the mean plasma concentration time profile never reached the 3 ng/ml target concentration, although the tablet adhered during 13.7 ± 3.2 h. The partially neutralised IR 1 did not yield high plasma concentrations, but showed a $T_{>3ng/ml}$ of 8.5 ± 3.9 h and a adhesion time of 12.5 ± 2.5 h. The mean plasma concentration time profile of the lyophilised IR 1

fluctuated during 24 h around the 3 ng/ml target concentration. For the lyophilised IR 1 the $T_{>3ng/ml}$ was 13.5 ± 1.3 h and the tablet adhered during 22.0 ± 7.2 h. Only the lyophilised IR 1 approached the absolute bioavailability value of the reference formulation (C 974P/DDWM - 5/95). The absolute bioavailability of the other formulations was between 2.5 and 4.3%. None of the tested formulations showed any sign of irritation on the dogs gingiva or mucosa after single application.

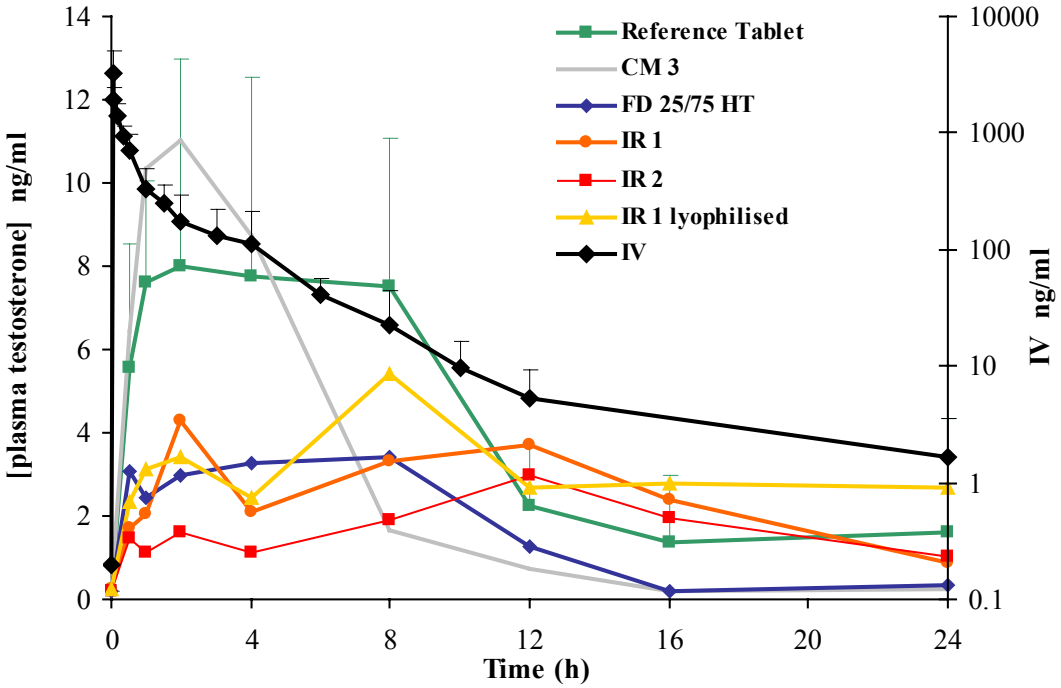

Figure 3. Plasma concentration time profiles for the ^{60}Co irradiated grafted starches IR 1, lyophilised IR 1 and IR 2, the chemically modified grafted starch CM 3 and the freeze-dried Amioca®/poly(acrylic acid) mixture FD 25/75 HT compared to a reference formulation. (n = 6, mean ± SD)

Using chemical modification grafted starches were synthesised that could sustain the testosterone target concentration during ± 8 h. The formulation based on the chemically modified starch CM 3 showed a shorter in vivo adhesion time compared to the reference formulation, which was due to a higher erosion rate. The faster erosion rate of the CM 3 tablet resulted in a high plasma peak concentration 2 h after administration and a shorter $T_{>3ng/ml}$. In case of the irradiated grafted starches, only the IR 1 formulation provided acceptable $T_{>3ng/ml}$ values. Lyophilisation of the partially neutralised ^{60}Co irradiated grafted starch IR 1, induced a longer in vivo adhesion time and a higher

$T_{>3ng/ml}$. The absolute bioavailability of the lyophilised formulation was similar to that of the reference formulation. The longer in vivo adhesion time of the lyophilised IR 1 formulation was the result of a slower erosion rate. A bioadhesive tablet formulation with a slow erosion rate does not provide high peak plasma concentrations, but can sustain (lower) plasma concentrations over a longer period of time.

Formulation	$T_{>3ng.ml}^{-1}$ (h)	F_{abs} (%)	Adhesion Time (h)
Reference	11.25 ± 3.86	6.97 ± 3.33	15.67 ± 8.62
IR 1	8.50 ± 3.90	3.90 ± 2.09	12.50 ± 2.51
IR 2	4.00 ± 6.48	2.46 ± 1.10	13.67 ± 3.20
IR 1 lyo	13.50 ± 1.32	6.46 ± 1.92	22.00 ± 7.21
CM 3	7.92 ± 2.11	4.25 ± 2.36	9.67 ± 2.73
FD 25/75 HT	5.00 ± 3.45	2.54 ± 1.11	10.50 ± 3.08

Table 1. Time during which the plasma testosterone concentration was above 3 ng/ml ($T_{>3ng.ml}^{-1}$), the absolute bioavailability (F_{abs}) and the in vivo adhesion time for the ^{60}Co irradiated grafted starches IR 1, lyophilised IR 1 and IR 2, the chemically modified grafted starch CM 3 and the freeze-dried Amioca®/poly(acrylic acid) mixture FD 25/75 HT compared to a reference formulation. (n = 6, mean ± SD)

3.4.2.4 Conclusion

The chemically modified grafted starch released the model drug testosterone rather fast and the 3 ng/ml target testosterone plasma concentration was sustained during 7 h. A partially neutralised grafted starch, synthesised by ^{60}Co irradiation, a lyophilized ^{60}Co irradiated grafted starch and a freeze-dried Amioca®/poly(acrylic acid) mixture did not reach the absolute bioavailability data of the reference formulation, but they did sustain the target testosterone plasma concentration during 8.5, 13.5, and 5.0 h, respectively.

Some grafted starches, ^{60}Co irradiated or chemically modified, and a freeze-dried starch/poly(acrylic acid) mixture showed to be promising buccal bioadhesive drug carriers for systemic delivery. However, in *Chapter 3.3 Mucosal Irritation Test using Slugs* it was concluded that the ^{60}Co irradiated and the chemically modified grafted starches were irritating to the mucosal tissue of the slugs since they induced an increased mucus production. In the present study, the tested irradiated and chemically modified grafted starches did not show any sign of irritation on the dogs gingiva or mucosa after single application.

It can be concluded that the grafted starches and the freeze-dried starch/poly(acrylic acid) mixtures have a potential as (buccal) bioadhesive drug carriers, although they can only be used for single applications and not in chronic treatments.

3.4.3 Spray-dried Amioca® starch/Carbopol® 974P mixtures (SD)

3.4.3.1 Materials

Testosterone was purchased from Diosynth (Oss, The Netherlands). Miconazole nitrate was obtained from Janssen Pharmaceutica, Beerse, Belgium. Amioca® starch is a National Starch and Chemical Company product, Bridgewater, New Jersey, USA. Carbopol® 974P (C 974P) was supplied by BF Goodrich (Cleveland, Ohio, USA). Drum Dried Waxy Maize (DDWM) was supplied by Cerestar (Vilvoorde, Belgium). Sodium stearyl fumarate (NaSF) was given by Edward Mendell Co. Inc. (New York, USA). Econazole was purchased from Sigma-Aldrich (Bornem, Belgium), methanol HPLC-S grade was purchased from Biosolve BV (Valkenswaard, The Netherlands) and tetrahydrofuran (THF) was purchased from BDH, Laboratory Supplies, Poole, UK. All other chemicals used were at least of analytical grade.

3.4.3.2 Methods

3.4.3.2.1 Production of tablets

For the evaluation of the in vivo adhesion time placebo tablets (100 mg/7 mm) were used. The powders were mixed with sodium stearyl fumarate (1%; w/w), as a lubricant and compressed on a Korsch compression machine (Type EK0, Berlin, Germany) equipped with 7 mm flat punches, at a pressure of 9.8 kN.

The tablets used in the in vitro dissolution study contained miconazole nitrate. For the tablet production the spray-dried powder was firstly mixed with miconazole nitrate (10 mg), next the lubricant (1% sodium stearyl fumarate) was added and mixed again. The tablets were compressed as described above with a tablet weight of 100 mg and diameter of 7 mm.

To produce the tablets for the in vivo drug loading study the powder was firstly mixed with micronised testosterone (60 mg), next the sodium stearyl fumarate (1%) was added and mixed again. The weight and the diameter of the tablets was 100 mg/7 mm

(60% w/w drug concentration) and 200 mg/9 mm (30% w/w drug concentration) and the compression force was 9.8 and 14.7 kN, respectively.

3.4.3.2.2 In vivo adhesion time study

The in vivo adhesion time of each formulation was evaluated in 7 castrated male dogs (weight 29.07 ± 3.25 kg). The dogs were conscious during the whole test period. One tablet was placed on the gingiva above the right upper canine (Figure 2). The in vivo adhesion time was followed visually. The in vivo adhesion time was defined as the time until loss or complete erosion of the bioadhesive tablet.

3.4.3.2.3 In vitro drug release study (USP III)

To evaluate the in vitro drug release from the bioadhesive formulations based on the different spray-dried Amioca®/C 974P mixtures, miconazole nitrate was used as a model drug (Bouckaert and Remon, 1993; Bouckaert et al., 1993). The dissolution tests were performed in an automatic reciprocating cylinder dissolution apparatus USP III (United States Pharmacopeia XXIV, 2000) (VanKel BioDis III Release Rate tester, Cary, NC, USA). The dip speed was set at 21 dips per minute and the temperature at 37°C. The dissolution medium (250 ml) was a 0.1 N HCl solution containing 0.5% hydroxypropyl-β-cyclodextrine (Janssen Pharmaceutica, Beerse, Belgium) in demineralised water (De Spiegeleer et al., 2001).

Quantitative analysis of miconazole nitrate in the dissolution samples was performed with a validated HPLC method with UV-detection using econazole as the internal standard (De Spiegeleer et al., 2001). Analysis was performed with a HPLC system consisting of a gradient HPLC pump (type L-7100, Merck-Hitachi, Darmstadt, Germany), a solvent degasser (type L-7612, Merck-Hitachi, Darmstadt, Germany), an autosampler (type L-7200, Merck-Hitachi, Darmstadt, Germany) equipped with a Rheodyne injector and an injection loop of 100 µl (Rheodyne, California, USA), a column oven (type L-7360, Merck-Hitachi, Darmstadt, Germany), a UV detector (type L-7400, Merck-Hitachi, Darmstadt, Germany) and a software interface (type D-7000, Merck-Hitachi, Darmstadt, Germany). Data were calculated with the software package

'HPLC System Manager' (Merck-Hitachi, Darmstadt, Germany). The column was a Lichrospher® 100 RP-18 column (125 x 4 mm) equipped with a Lichrospher® 100 RP-18 guard column (4 x 4 mm) (Merck, Darmstadt, Germany). The mobile phase, used as an isocratic eluent, consisted of 75% (v/v) methanol, 20% (v/v) sodium acetate buffer (2.5 mM, pH 5.0) containing 5 mM triethylamine and 5% (v/v) tetrahydrofuran. The eluate was monitored at 220 nm. The retention time of the econazole and miconazole nitrate peak was 4.5 and 7.0 min, respectively, at a flow rate of 1.0 ml/min. The analysis was performed at 25°C.

Calibration samples were prepared in dissolution medium to obtain a standard curve ranging from 2.5 µg/ml to 50.0 µg/ml. To 1.0 ml of dissolution sample, 1.0 ml of a methanolic solution of econazole (0.01 mg/ml) was added as internal standard, mixed and centrifuged at 2578 g for 10 min (Tehtnica® Centric 322 A, Novolab, Belgium). 100 µl of the supernatant was injected onto the HPLC column.

The method was developed and validated by Tibotec-Virco (Mechelen, Belgium) for dissolution tests on Tibozole™ buccal tablets containing 10 mg miconazole nitrate. The standard curves (n=5) were linear with determination coefficients > 0.9995. The accuracy was < 15% (Shah et al., 1992). All within day (repeatability) and between day (intermediate precision) (n = 5) coefficients of variation were < 15% (Shah et al., 1992). The detection limit and quantification limit were 0.48 and 1.48 mg/L miconazole nitrate, respectively.

The exponential equation $M_t/M_\infty = kt^n$ which describes the Fickian and non-Fickian release behaviour of swellable systems that not swell more than 25% of their original volume, was used to evaluate the drug release mechanism (Ritger and Peppas, 1987). M_t is the amount released at time t, M_∞ is the overall released amount, k a release constant of the *n*th order. The exponent *n* gives information about the release mechanism. $n = 0.5$ indicates Fickian drug diffusion, while $n = 1.0$ for drug release controlled by polymer erosion.

3.4.3.2.4 In vivo study

The bioavailability of formulations containing 30 and 60% testosterone was determined according to the study protocol described in *3.4.2.2.2 In vivo study protocol*. The results were compared with reference formulations (DDWM/C 974P; 95/5) containing the same amounts of testosterone (Voorspoels et al., 1996). 60 mg testosterone was incorporated in a 100 mg (60% drug concentration) or a 200 mg tablet (30% drug concentration). The formulations were tested in 6 castrated male dogs (weight 30.0 ± 2.5 kg). Blood samples were collected before the administration and 0.5, 1, 2, 4, 8, 12, 16 and 24 h after the administration in heparinised tubes. To calculate the absolute bioavailability, 60 mg testosterone was administered intravenously to each dog. The approval of The Ethics Committee was obtained.

The testosterone plasma concentrations were determined by chemiluminescent immunoassay (Immulite® Total Testosterone, Diagnostic Products Corporation, Los Angeles, CA, USA) as described in *3.4.2.2.3 Testosterone analysis*.

The absolute bioavailability was calculated using the Kinbes® software (Proost and Meijer, 1992) as described in *3.4.2.2.4 Pharmacokinetics and in vivo adhesion time*. The $T_{>3ng/ml}$ value was calculated from the individual graphs. The in vivo adhesion time of the bioadhesive tablet was determined visually. Statistical analysis was performed on the absolute bioavailability and $T_{>3ng/ml}$ values. As Pearson correlation coefficients showed that the data for absolute bioavailability and $T_{>3ng/ml}$ were independent ($p > 0.05$) of the subject (dog), statistically significant differences were determined using a one-way ANOVA post hoc Scheffé test with $p < 0.05$ as significance level. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the Levene's test. The computer program SPSS version 10.0 was used for the statistical analyses.

3.4.3.3 Results and Discussion

3.4.3.3.1 In vivo adhesion time and in vitro dissolution

Figure 4 gives an overview of the in vivo adhesion time in dogs of placebo buccal bioadhesive tablets based on spray-dried Amioca®/Carbopol® 974P mixtures with a C 974P content ranging from 5 to 75 % (w/w).

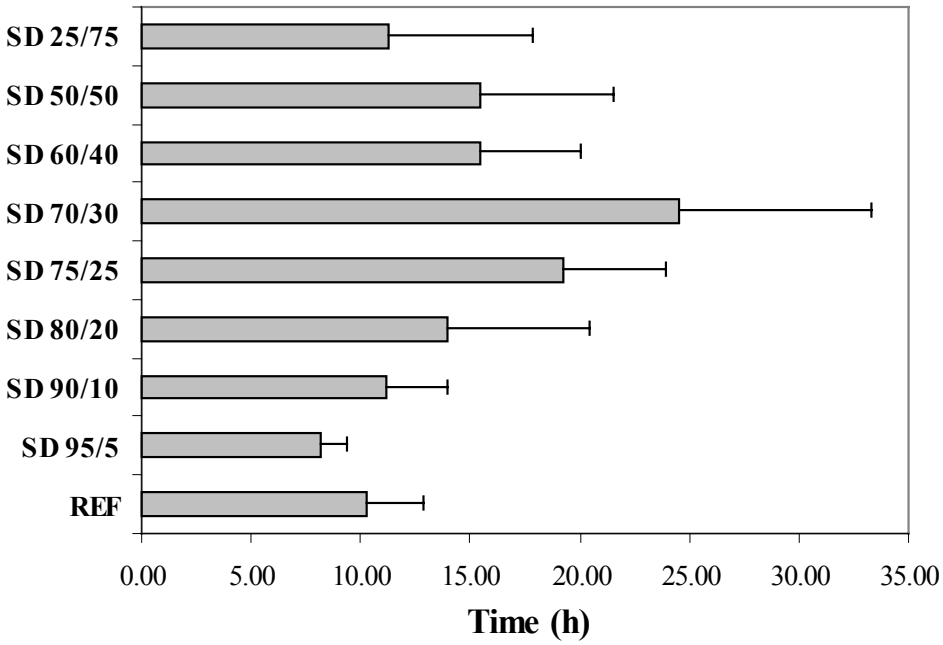

Figure 4. In vivo adhesion time for the spray-dried Amioca®/Carbopol® 974P mixtures (SD) and the reference formulation (Ref). (n = 7, mean ± SD)

Up to 30% C 974P the in vivo adhesion time increased with increasing C 974P amounts in the spray-dried mixtures. Over 30% C 974P the in vivo adhesion time decreased with increasing C 974P concentrations. The ratio Amioca®/C 974P 70/30 showed the longest in vivo adhesion time (24.5 ± 8.5 h) and is apparently the optimal ratio of Amioca® starch and Carbopol® 974P in terms of in vivo adhesion time. Although the SD 25/75 mixtures showed the highest ex vivo bioadhesive properties (*Chapter 3.2 Ex Vivo Bioadhesion Measurement*), it did not show the longest in vivo adhesion time. The ex vivo bioadhesion test can be used to evaluate the intrinsic bioadhesive properties and allows to compare different bioadhesive polymers or formulations, but the test seemed

unable to predict the in vivo adhesion time probably because the tablet is not subjected to frictional forces and erosion. The SD 25/75 formulation has high intrinsic bioadhesive capacities, but the polymer matrix eroded relatively fastly in vivo. This is in accordance with a previous study showing that a higher Carbopol® concentration did not result in a longer in vivo adhesion time (Bouckaert, 1994).

The in vitro drug release rate was evaluated by incorporating 10 mg miconazole nitrate as a marker. Miconazole nitrate is a poorly water soluble antifungal drug. It is frequently used in local oral drug delivery systems such as oral gels or bioadhesive buccal tablets (Bouckaert and Remon, 1993; Bouckaert et al., 1993; De Spiegeleer et al., 2001; Nafee et al., 2003).

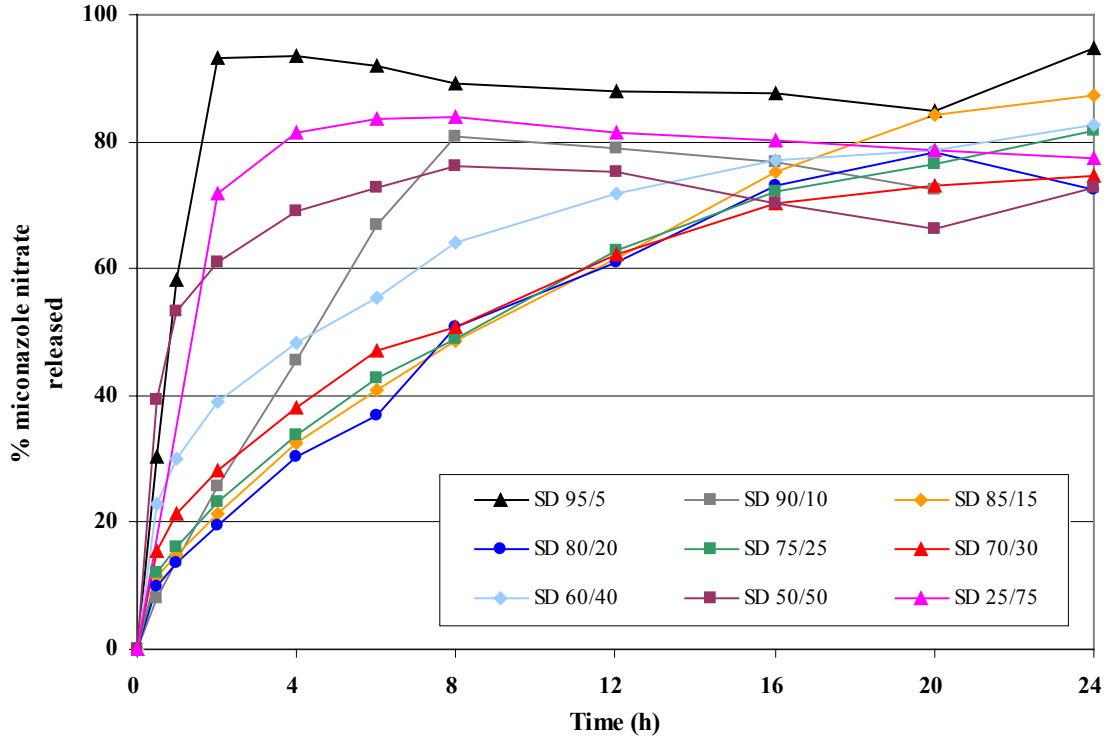

Figure 5. In vitro dissolution profiles for the different spray-dried Amioca®/Carbopol® 974P mixtures (SD). (n = 6, mean)

The in vitro dissolution profiles are shown in Figure 5. The spray-dried mixtures containing between 15 and 30% C 974P could all sustain the drug release over 20h. As well lower as higher C 974P concentrations in the spray-dried mixtures showed a faster in

vitro miconazole release. The drug release from the spray-dried mixtures with the lowest C 974P content, SD 95/5 and 90/10, is controlled by polymer erosion, as the diffusional exponents, n, were 1.01 ± 0.02 and 1.04 ± 0.05, respectively. The drug release was almost constant in relation to time. The release mechanisms from the spray-dried matrices containing 15% C 974P or more could not be described by the exponential equation $M_t/M_\infty = kt^n$ as the higher C 974P concentrations resulted in matrices which swelled more than 25% of their original volume. The miconazole release from these matrices was mainly controlled by diffusion as at the end of the dissolution an almost intact swollen translucent tablet gel matrix was found. The drug diffusion from the matrices with the highest C 974P concentrations (SD 60/40, SD 50/50 and SD 25/75) was faster than from the matrices containing between 15 and 30% C 974P, which sustained the drug release over the longest period. Drug diffusion through the swollen gel layer took the longest time for the polymer matrices of spray-dried combinations of 15 to 30% C 974P with Amioca® starch, resulting in the longest sustained release profiles. By increasing the C 974P concentration to 40% or more the diffusion rate of the drug through the polymer matrix was increased. It is well known that polymer matrices with high contents of Carbopol® exhibit short dissolution times (Khan and Zhu, 1999).

These results were in good correlation with the in vivo adhesion times of placebo tablets. The in vitro USP III dissolution test can be used to predict the in vivo adhesion time of buccal bioadhesive tablet formulations based on spray-dried Amioca®/Carbopol® 974P mixtures.

3.4.3.3.2 In vivo drug loading – bioavailability study

From the above mentioned results it is clear that by spray-drying Amioca® starch/Carbopol® 974P mixtures at different ratios a whole range of potential bioadhesive carriers can be prepared with improved bioadhesive properties. By modifying the C 974P concentration the in vivo adhesion time of the bioadhesive formulations can be influenced. As bioadhesive powder formulations are intended to stick to mucous membranes it is important to evaluate their mucosal irritation potency. In *Chapter 3.3. Mucosal Irritation Test using Slugs* the biocompatibility of the spray-dried Amioca®/Carbopol® 974P mixtures was evaluated using an alternative mucosal irritation

test using slugs. Spray-dried mixtures containing up to 20% C 974P induced no irritation of the mucosal tissue of the slugs and can be considered as safe bioadhesive carriers. On the other hand, mixtures containing higher amounts of C 974P induced mucosal irritation and membrane damage. This makes only the mixtures containing up to 20% C 974P useful as bioadhesive carriers. Nevertheless, by changing the C 974P content between 5 and 20% the in vivo adhesion time of 100 mg placebo tablets can be varied between 8 and 17 h (Figure 4) and the in vitro miconazole release between 2 and 20h (Figure 5).

The drug loading capacity of the non-irritating spray-dried mixture SD 80/20 was investigated in vivo in dogs. Testosterone, a lipophilic molecule which is known to be systemically absorbed over the buccal mucosa (Voorspoels et al., 1996), was used as model drug to investigate the in vivo bioavailability and drug loading capacity.

Figure 6 shows the mean testosterone plasma concentration time profiles for the 30% and 60% loaded SD 80/20 and the reference formulations (DDWM/C 974P; 95/5). The absolute bioavailability (F_{abs}), the $T_{>3ng/ml}$ and the in vivo adhesion time are shown in Table 2.

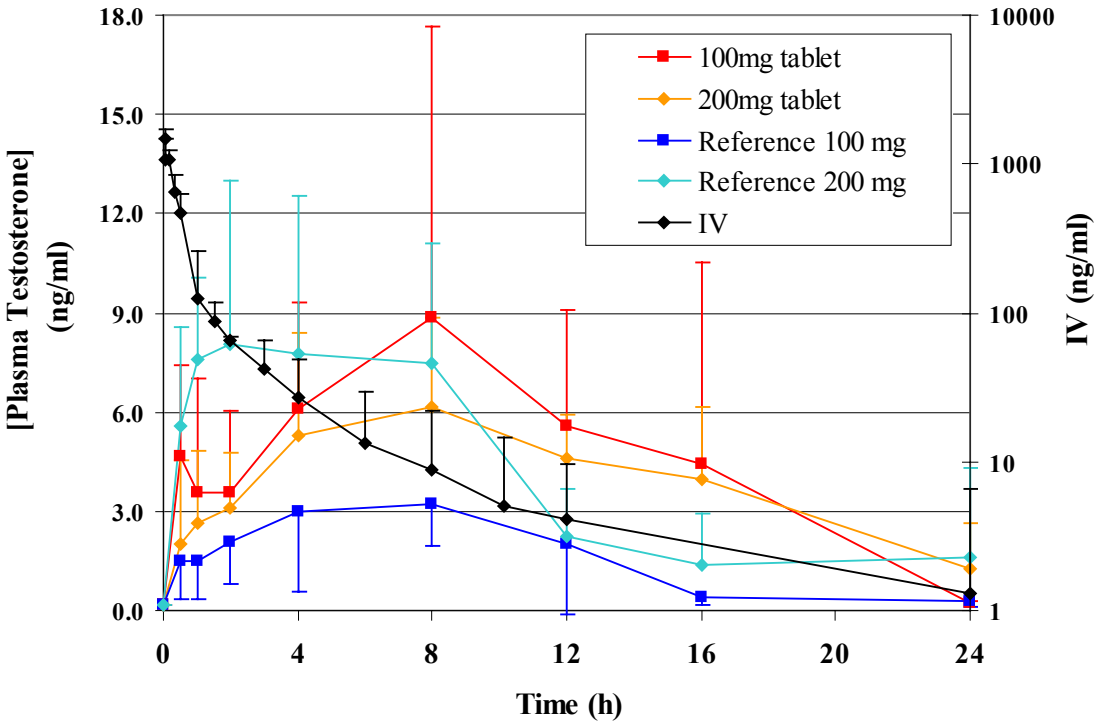

Figure 6. Plasma testosterone concentration time profiles for the 30 and 60% loaded spray-dried Amioca®/Carbopol® 974P 80/20 mixture (SD 80/20) compared to a reference formulation (Ref). (n = 6, mean ± SD)

	SD 80/20		REF	
Tablet Weight	**100 mg**	**200 mg**	**100 mg**	**200 mg**
Drug Load	**60 %**	**30 %**	**60 %**	**30 %**
F_{abs} (%)	14.28 ± 5.12*	11.31 ± 2.77	4.59 ± 2.16	6.97 ± 3.33
$T_{>3ng.ml}^{-1}$ (h)	14.00 ± 1.67*	15.83 ± 5.53	5.00 ± 5.06	11.25 ± 3.86
Adhesion Time (h)	15.25 ± 2.56	24.80 ± 5.31	13.00 ± 3.29	15.67 ± 8.62

Table 2. Absolute bioavailability (F_{abs}), time during which the plasma testosterone concentration was above 3 ng/ml ($T_{>3ng.ml}^{-1}$) and the in vivo adhesion time for a 30 and 60% loaded spray-dried Amioca®/Carbopol® 974P 80/20 mixture (SD 80/20) compared to a reference formulation (REF). (n = 6, mean ± SD)

* significantly higher compared to the similar loaded reference formulation (REF)

For both formulations the in vivo adhesion time decreased with a higher drug load, but the SD 80/20 formulation adhered for both drug concentrations longer compared to the reference formulation and resulted in a higher absolute bioavailability for the SD formulations. The F_{abs} was for the 60% loaded SD 80/20 formulation significantly higher than for the similar loaded reference formulation. Also the $T_{>3ng/ml}$ value, which gives a therapeutic indication (Mazer et al., 1992), was for the spray dried 60% loaded formulation significantly higher than for the 60% loaded reference formulation. From these results it is clear that using SD 80/20 as bioadhesive platform the buccal testosterone delivery was improved compared to a DDWM/C 974P 95/5 formulation (Voorspoels et al., 1996). Moreover the spray-dried formulation could be loaded with 60% drug without loss of its bioadhesive capacities and without major changes in plasma concentration time profiles and pharmacokinetic parameters F_{abs} and $T_{>3ng/ml}$.

3.4.3.4 Conclusion

By spray-drying Amioca®/Carbopol® 974P mixtures at different ratios a range of potential bioadhesive carriers was obtained with excellent bioadhesive properties. Up to 20% C 974P could be incorporated without any risk of mucosal irritation. By ranging the C 974P concentration between 5 and 20%, the in vivo adhesion time of placebo tablets could be varied between 8 and 17h. The data from the in vivo adhesion time study correlated well with the in vitro miconazole release profiles (USP III dissolution). A spray-dried Amioca®/C 974P 80/20 mixture could be loaded with 60% drug without loosing its in vivo bioadhesive and pharmacokinetic properties.

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Chapter 3.5 Local Vaginal Drug Delivery via a Bioadhesive Tablet

3.5.1 Introduction

In this clinical study the single application of a metronidazole vaginal bioadhesive tablet based on a spray-dried Amioca® starch/Carbopol® 974P 85/15 (w/w) mixture (SD 85/15) was evaluated in 12 female healthy volunteers. The vaginal bioadhesive tablet is applied on the uterine cervix and serves as a platform for local vaginal drug delivery. The in vivo tablet residence time was assessed as well as plasma and local vaginal metronidazole concentrations. SD 85/15 was selected as bioadhesive carrier based on the ex vivo bioadhesion (*Chapter 3.2 Ex Vivo Bioadhesion Measurement*) and mucosal irritation (*Chapter 3.3 Mucosal Irritation Test using Slugs)* test results. The spray-dried Amioca®/Carbopol® 974P mixture contains 15 % Carbopol® 974P and is expected to adhere better to the uterine cervix compared to a bioadhesive vaginal tablet based on a pregelatinised starch/Carbopol® 974P 95/5 (w/w) physical mixture (Voorspoels et al., 2002), as the SD 85/15 mixture showed significantly better adhesion properties in ex vivo bioadhesion measurements. The mucosal irritation study showed that SD 85/15 is a safe bioadhesive carrier. 20% Carbopol® 974P (SD 80/20) is the maximum concentration that can be used as a non-irritating bioadhesive carrier. SD 85/15 showed comparable ex vivo bioadhesion values as SD 80/20 and as the vaginal bioadhesive tablet is expected to adhere on the uterine cervix during several days, a safety margin was taken by selecting SD 85/15 as bioadhesive carrier.

Bacterial vaginosis (BV), the most prevalent infectious cause of vaginitis, is an imbalance of the bacterial vaginal flora. In women with BV, the normal vaginal flora is altered from a predominance of H_2O_2-producing *Lactobacillus* species to high concentrations of anaerobic bacteria, *Gardnerella vaginalis*, and *Mycoplasma hominis* (Joesoef et al., 1999). Bacterial vaginosis induces symptoms of odor, discharge and irritation in 50% of affected patients. Metronidazole is the drug of choice in the treatment of bacterial vaginosis, but dosage and duration of therapy are still controversial (Voorspoels et al., 2002). Nowadays there are two regimen of oral treatment, including

500 mg metronidazole twice daily for 7 days (Centres for Disease Control, 1989) and a 2 g single dose (Lugo-Miro et al., 1992), however the 7-day regimen is recommended for the treatment of BV (Joesoef et al., 1999). Bioavailability after oral administration is almost complete (Hoffmann et al., 1995). However, oral metronidazole has some side effects, such as gastric intolerance and a sharp metallic unpleasant taste. Hence, there is growing interest in alternative treatments such as intravaginal clindamycin cream and metronidazole gel for 5-7 days, which have been proved to be as efficacious as the 7-day oral regimen (Joesoef et al., 1999). However, these long vaginal applications result in poor patient compliance. Other alternative treatments are vaginal ovules (single or multiple administration) (Borin et al., 1999) and vaginal tablets (multiple administration) (Hoffmann et al., 1995).

Interest has grown in the development of vaginal bioadhesive tablets because of the advantages of maintaining local drug levels, enabling lower dosing frequency and a lower amount of drug administered, as well as less systemic side effects (Voorspoels et al., 2002; Bouckaert et al., 1995; Robinson and Bologna, 1994). In a preliminary efficacy study where a single 100 mg metronidazole bioadhesive vaginal tablet was administered, similar cure rates were obtained as for oral metronidazole (500 mg doses daily for 7 days), although only one-seventieth of the drug was administered locally (Bouckaert et al., 1995). In a second dose finding study different doses of metronidazole in a single bioadhesive vaginal tablet were compared to a placebo tablet. A cure rate of 64%, 61.5% and 68% was obtained with 100 mg, 250 mg and 500 mg metronidazole, respectively (Voorspoels et al., 2002).

3.5.2 Materials

Metronidazole was purchased from Certa (Braine-l'Alleud, Belgium). Amioca® starch was from National Starch and Chemical Company, Bridgewater, New Jersey, USA. Carbopol® 974P (C 974P) was supplied by BF Goodrich (Cleveland, Ohio, USA). Sodium stearyl fumarate (NaSF) was given by Edward Mendell Co. Inc. (New York, USA). Tinidazole was purchased from Sigma-Aldrich (Bornem, Belgium). Methanol HPLC-S grade and acetonitrile HPLC-S grade were purchased from Biosolve BV (Valkenswaard, The Netherlands), potassium dihydrogen orthophosphate (KH_2PO_4) from VWR International (Leuven, Belgium). All other chemicals used were at least of analytical grade.

3.5.3 Methods

3.5.3.1 Composition and preparation of the tablets

The tablets were produced by firstly mixing the Amioca®/C 974P 85/15 powder with metronidazole (500 mg per tablet), next sodium stearyl fumarate (1%; w/w) was added as a lubricant. The mixture was compressed on a Korsch compression machine (Type EK0, Berlin, Germany) equipped with 20 mm special designed punches, at a pressure of 19.6 kN. The weight and the diameter of the tablets were 1500 mg and 20 mm, respectively. To increase the adherence to the genital tract, the design of the tablet was specially shaped to fit better to the uterine cervix. The tablet had a flat bottom and a concave upper face aiming at an increase of residence time of the tablet.

3.5.3.2 Study protocol

3.5.3.2.1 Volunteers

The study was performed at the *Drug Research Unit Ghent (D.R.U.G.), Ghent University Hospital, De Pintelaan 185, Gent, Belgium*. The approval of The Ethics Committee of the Ghent University Hospital was obtained. Twelve (12) female healthy

volunteers (age 21 - 39 years, body weight 52 - 74 kg, body height 157 - 179 cm) were included into the clinical trial, after giving an informed consent.

3.5.3.2.2 Pharmacokinetic protocol

The study was an open uncontrolled 5-day study. At study start, the gynaecologist applied one bioadhesive vaginal tablet to each volunteer. On day 5, 108h after drug administration, a vaginal examination to the presence of the tablet or tablet remainings was performed by the gynaecologist. Remaining parts of the bioadhesive vaginal tablet were removed. Venous blood samples were taken before and 12h, 24h, 36h, 48h, 60h, 72h, 84h, 96h and 108h after drug administration. At the same time points vaginal swabs were obtained by the volunteer herself using a standardised vaginal swab (Sterilin vaginal swab, Copan, Italy). The swabbing procedure was standardised by swabbing depth and technique and the women were told to insert the swab 5 cm in the vagina as marked on the swab. The plasma samples and vaginal swabs were stored at –20°C until drug analysis. At each blood sampling point the volunteers were asked if they lost the tablet. The in vivo adhesion time of the vaginal tablet was reported as the tablet residence time.

3.5.3.2.3 Quantitative drug analysis

3.5.3.2.3.1 Plasma samples

Quantitative analysis of metronidazole in the plasma samples was performed with a validated HPLC method with UV-detection using tinidazole as internal standard. HPLC analysis was performed with a HPLC system consisting of a gradient HPLC pump (type L-7100, Merck-Hitachi, Darmstadt, Germany), a solvent degasser (type L-7612, Merck-Hitachi, Darmstadt, Germany), an autosampler (type L-7200, Merck-Hitachi, Darmstadt, Germany) equipped with a Rheodyne injector and an injection loop of 50 µl (Rheodyne, California, USA), a column oven (type L-7360, Merck-Hitachi, Darmstadt, Germany), a UV detector (type L-7400, Merck-Hitachi, Darmstadt, Germany) and a software interface (type D-7000, Merck-Hitachi, Darmstadt, Germany). Data were calculated with the software package 'HPLC System Manager' (Merck-Hitachi, Darmstadt, Germany). The column was a Lichrospher® 100 RP-18 column (250 x 3 mm) equipped with a

Lichrospher® 100 RP-18 guard column (10 x 2 mm) (Merck, Darmstadt, Germany). The mobile phase, used as an isocratic eluent, consisted of 85 % (v/v) potassium dihydrogen orthophosphate (0.002 M, pH 4.8), 7.5% (v/v) methanol and 7.5% (v/v) acetonitrile. The eluate was monitored at 320 nm. The retention time of the metronidazole and tinidazole peak was 4.8 and 8.9 min, respectively, at a flow rate of 0.85 ml/min. The analysis was performed at room temperature.

The HPLC method described by Rajnarayana et al. (2002) was modified and validated. Calibration samples were prepared by spiking blank plasma with methanolic metronidazole solutions to become standard plasma concentrations from 0.25 µg/ml to 10.0 µg/ml. To 300 µl of plasma sample, 50 µl of a methanolic solution of tinidazole (1.2 µg/50 µl) was added as internal standard, and shaken well. Then 350 µl of acetonitrile was added for protein precipitation, mixed and centrifuged at 2578 g for 10 min (Tehtnica® Centric 322 A, Novolab, Belgium). 100 µl of supernatant was mixed with 200 µl mobile phase. 50 µl of this mixture was injected onto the HPLC column.

3.5.3.2.3.2 Vaginal swabs

Quantitative analysis of metronidazole in vaginal swabs was performed using the USP 24 Official Monograph for metronidazole analysis in tablets (USP XXIV, 2000). The HPLC method was slightly modified and validated. The HPLC system consisted of a isocratic HPLC pump (type L-7100, Merck-Hitachi, Darmstadt, Germany), an autosampler (type L-7200, Merck-Hitachi, Darmstadt, Germany) equipped with a Rheodyne injector and an injection loop of 10 µl (Rheodyne, California, USA), a UV detector (type L-7400, Merck-Hitachi, Darmstadt, Germany) and a software interface (type D-7000, Merck-Hitachi, Darmstadt, Germany). Data were calculated with the software package 'HPLC System Manager' (Merck-Hitachi, Darmstadt, Germany). The column was a Lichrospher® 100 RP-8 column (125 x 4 mm) (Merck, Darmstadt, Germany). The mobile phase, used as an isocratic eluent, consisted of 80 % (v/v) distilled water and 20 % (v/v) methanol. The eluate was monitored at 254 nm. The retention time of the metronidazole peak was 3.0 min at a flow rate of 1.0 ml/min. The analysis was performed at room temperature.

Calibration samples from 10.0 to 500.0 µg/ml were prepared in mobile phase. Each vaginal swab was brought into a test tube containing 2.0 ml mobile phase, shaked on a IKA MTS 4 shaker (VWR International, Leuven, Belgium) at 1000 rpm for 1 h and then centrifuged at 2578 g for 10 min (Tehtnica® Centric 322 A, Novolab, Belgium). 10 µl of the supernatant was injected onto the HPLC column.

3.5.4 Validation of the HPLC method – Plasma samples

The HPLC analysis method was validated based on the International Conference Harmonisation (ICH) Harmonised Tripartite Guidelines for validation of analytical procedures (1994). The following validation characteristics were considered: specificity, linearity, accuracy, precision, detection and quantification limit.

3.5.4.1 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of other (interfering) components.

Specificity was assessed by comparing the chromatograms of blank plasma (a) and plasma spiked with metronidazole (standard of the calibration curve) (b), both with tinidazole added as internal standard (Figure 1). From Figure 1 it is clear that no interfering peaks could be observed. Thus, it was concluded that the method was specific for the determination of metronidazole with tinidazole as internal standard as there was no interference of other components. The retention time of metronidazole and tinidazole was 4.8 min and 8.8 min after injection, respectively.

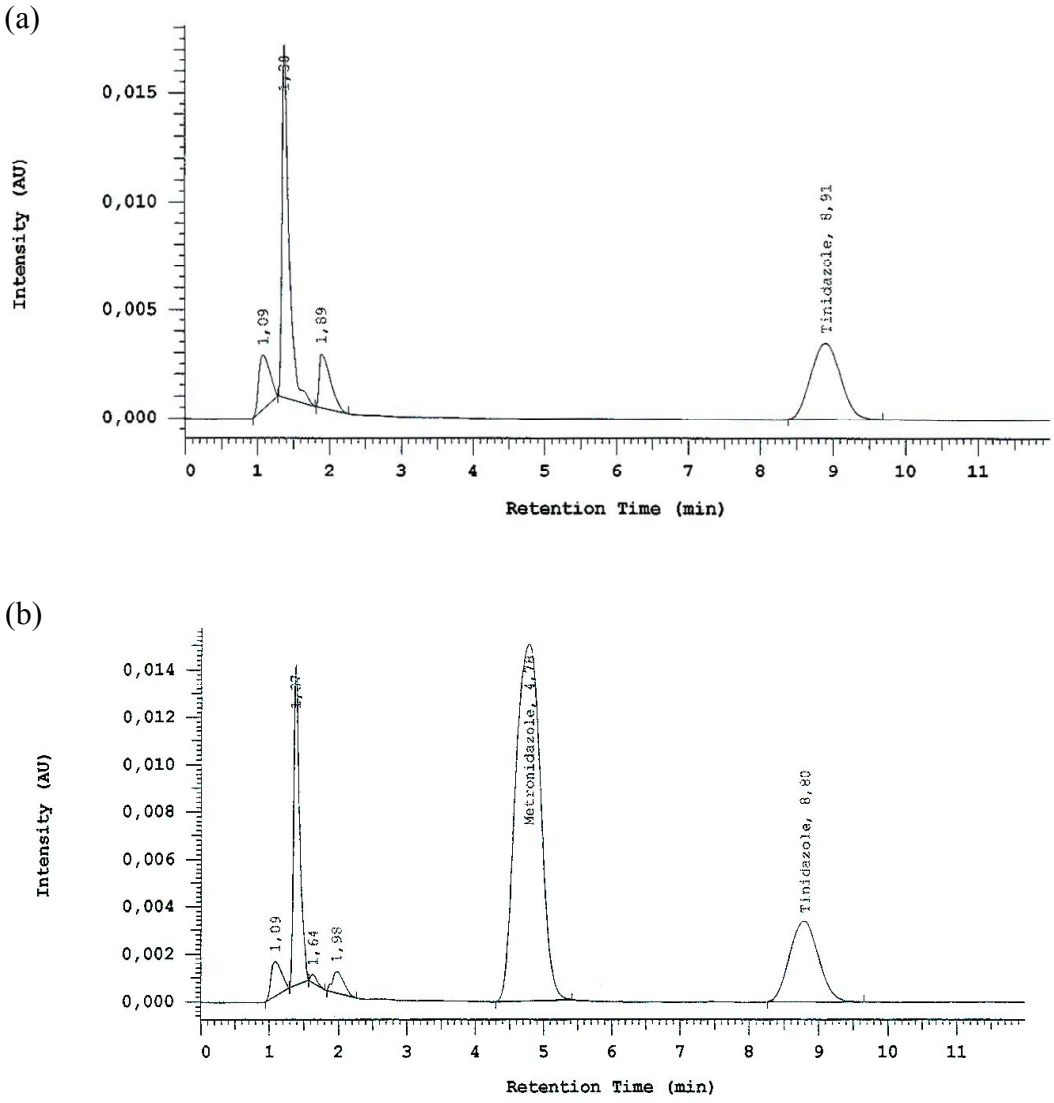

Figure 1. Chromatograms of blank plasma (a) and plasma spiked with metronidazole (standard of the calibration curve) (b), both with tinidazole added as internal standard.

3.5.4.2 Linearity

The linearity of an analytical procedure is its ability – within a given range – to obtain test results, which are directly proportional to the concentration of analyte in the sample.

Calibration curves were prepared by spiking blank plasma with a methanolic metronidazole solution over a range from 0.25 µg/ml – 10.0 µg/ml (6 concentrations). A blank was included in the calibration curve. During validation and analysis different calibration curves were determined. The linearity was evaluated by the determination coefficient R^2 of the mean regression line (n = 7). The mean R^2 was 0.9966 ± 0.0052 and

the coefficient of variation (CV) was 0.52%. It is clear that the relationship between response and concentration was linear and reproducible.

3.5.4.3 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the value found and is expressed as the percent agreement between the mean determined value and the true concentration.

The accuracy was investigated at three concentration levels on standards containing a known metronidazole concentration covering the range of the calibration curve: 0.25 µg/ml – 1.0 µg/ml and 10.0 µg/ml. The three concentration levels were submitted to the normal analysis procedure. Each concentration was determined six times. The mean accuracies ± standard deviations (SD) are listed in Table 1.

[metronidazole]	**Accuracy (%)**
0.25 µg/ml	91.95 ± 3.27
1.00 µg/ml	103.83 ± 8.57
10.00 µg/ml	93.08 ± 2.48

Table 1. Accuracy (%). (n = 7, mean ± SD)

All mean values were within ± 15% of the actual concentration. (Acceptance criteria: within 15% of the actual value, Shah et al., 1992)

3.5.4.4 Precision

The precision expresses the closeness of agreement between repeated determinations of the same sample. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Repeatability or intra-assay precision expresses the precision under the same operating conditions over a short interval of time. Intermediate precision expresses within laboratory variations such as different days, different analysts, different equipment, etc. Reproducibility expresses the precision between different laboratories (inter-laboratory). Precision is expressed as the coefficient of variation (%) of a series of measurements of the calibration standards.

Here, all analyses were performed in the same laboratory, so only the repeatability (within one day) and intermediate precision (day-to-day) were evaluated.

The repeatability and intermediate precision were calculated on three calibration curves (n = 6). The standard deviation (SD) and coefficient of variation (CV) were calculated. (Table 2 and Table 3)

[metronidazole]	**Repeatability**	
µg/ml	Mean ± SD	CV (%)
0.25	0.27 ± 0.01	14.77
1.00	0.97 ± 0.09	9.86
10.00	10.75 ± 0.28	3.58

Table 2. Repeatability. (n = 6) (mean, standard deviation (SD) and coefficient of variation (CV))

[metronidazole]	**Intermediate Precision**	
µg/ml	Mean ± SD	CV (%)
0.25	0.25 ± 0.01	13.31
1.00	1.04 ± 0.06	7.35
10.00	9.96 ± 0.15	1.53

Table 3. Intermediate precision. (n = 6) (mean, standard deviation (SD) and coefficient of variation (CV))

As well for repeatability as for intermediate precision, all coefficients of variation did not exceed 15%. (Acceptance criteria: CV < 15%, Shah et al., 1992)

3.5.4.5 Detection and quantification limit

The detection limit of an analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value, while the quantification limit is the lowest amount which can be quantitatively determined with suitable precision and accuracy.

The detection was determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte

could be reliably detected (CV < 20%) (Vermeire, 1998). The detection limit was 0.25 µg/ml (CV = 14.8%, n = 6).

The quantification limit was determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte could be quantified with acceptable accuracy and precision. The quantification limit was 0.25 µg/ml: accuracy < 15% of the actual concentration and precision around the mean value < 15% CV (n = 10). (Shah et al., 1992)

3.5.4.6 Stability of metronidazole in plasma

The stability of metronidazole in plasma at the storage temperature of –20°C was established by investigating the influence of two freeze/thaw cycles on the analyte stability at three concentration levels (0.25 – 1.0 – 10.0 µg/ml) according to Shah et al. (1992). After two freeze/thaw cycles 97.5%, 97.4% and 99.7% of the actual concentration was found in a 0.25, 1.0 and 10.0 µg/ml metronidazole standard, respectively. (< 15%, Shah et al., 1992)

3.5.5 Validation of the HPLC method – Vaginal swabs

3.5.5.1 Specificity

Specificity was assessed by comparing the chromatograms of metronidazole in mobile phase (standard of the calibration curve) (a) and an unknown vaginal swab (b) (Figure 2). From Figure 2 it is clear that no interfering peaks were observed. Thus, it was concluded that the method was specific for the determination of metronidazole as there was no interference of other components. The retention time of metronidazole was 2.9 min after injection.

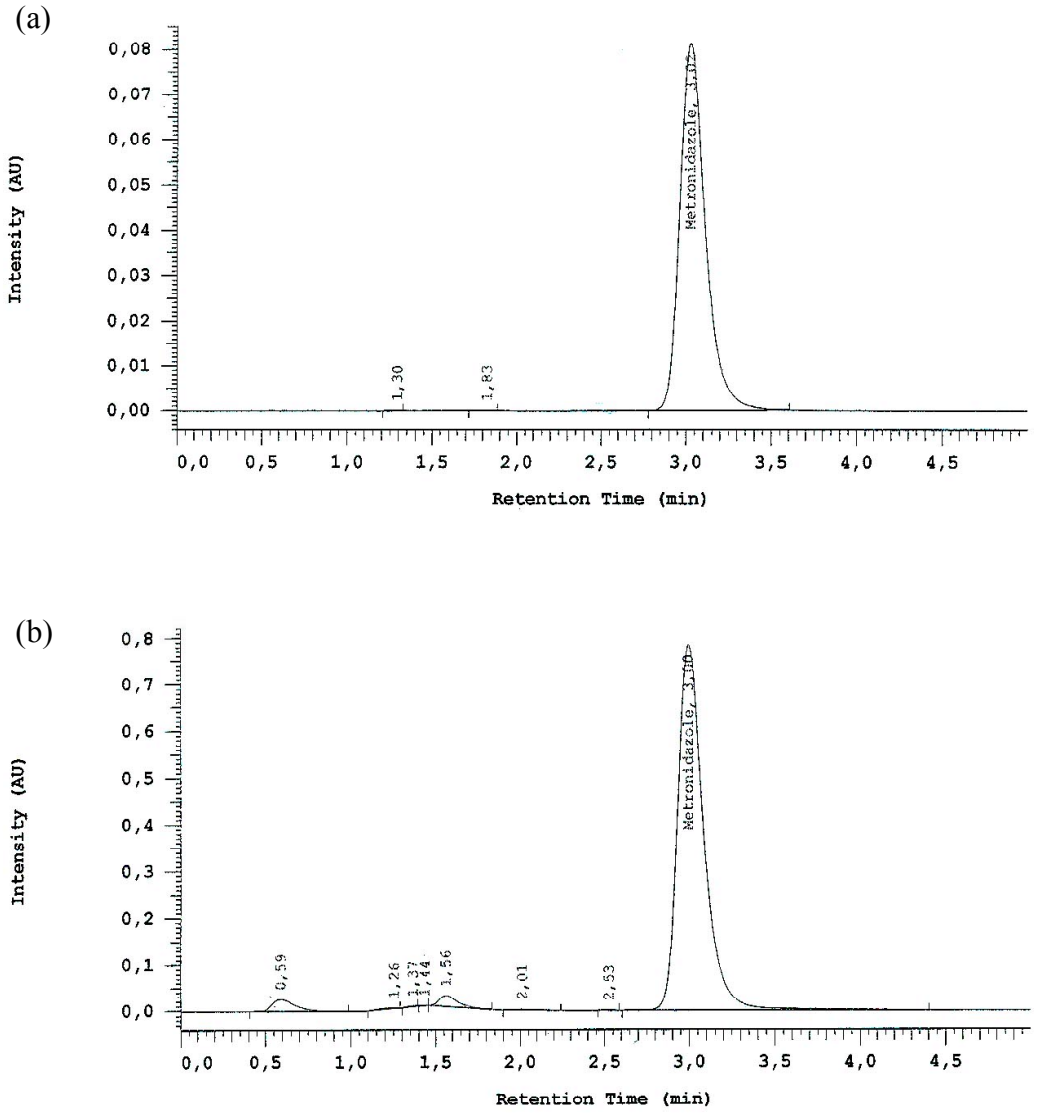

Figure 2. Chromatograms of metronidazole in mobile phase (standard of the calibration curve) (a) and an unknown vaginal swab (b).

3.5.5.2 Linearity

Calibration curves were prepared in mobile phase over a range from 10.0 µg/ml – 500.0 µg/ml (6 concentrations). A blank was included in the calibration curve. During validation and analysis different calibration curves were determined. The linearity was evaluated by the determination coefficient R^2 of the mean regression line (n = 6). The mean R^2 was 0.9999 ± 0.0001 and the coefficient of variation (CV) was 0.01%. It is clear that the relationship between response and concentration was linear and reproducible.

3.5.5.3 Accuracy

The accuracy was investigated at three concentration levels on standards containing a known metronidazole concentration covering the range of the calibration curve: 10.0 µg/ml – 100.0 µg/ml and 500.0 µg/ml. The three concentration levels were submitted to the normal analysis procedure. Each concentration was determined six times. The mean accuracies ± standard deviations (SD) are listed in Table 4.

[metronidazole]	**Accuracy (%)**
10.0 µg/ml	96.84 ± 1.27
100.0 µg/ml	96.68 ± 2.35
500.0 µg/ml	100.26 ± 1.44

Table 4. Accuracy (%). (n = 6, mean ± SD)

All mean values were within ± 15% of the actual concentration. (Acceptance criteria: within 15% of the actual value, Shah et al., 1992)

3.5.5.4 Precision

Here, all analyses were performed in the same laboratory, so only the repeatability (within one day) and intermediate precision (day-to-day) were evaluated.

The repeatability and intermediate precision was calculated on three calibration curves (n = 6). The standard deviation (SD) and coefficient of variation (CV) were calculated. (Table 5 and Table 6)

[metronidazole]	**Repeatability**	
µg/ml	Mean ± SD	CV (%)
10.0	10.33 ± 0.14	1.26
100.0	103.49 ± 2.52	2.23
500.0	498.79 ± 7.11	1.30

Table 5. Repeatability. (n = 6) (mean, standard deviation (SD) and coefficient of variation (CV))

[metronidazole]	Intermediate Precision	
µg/ml	Mean ± SD	CV (%)
10.0	9.88 ± 0.37	3.93
100.0	97.99 ± 2.27	2.33
500.0	480.55 ± 17.48	3.64

Table 6. Intermediate precision. (n = 6) (mean, standard deviation (SD) and coefficient of variation (CV))

As well for repeatability as for intermediate precision, all coefficients of variation did not exceed 15%. (Acceptance criteria: CV < 15%, Shah et al., 1992)

3.5.5.5 Detection and quantification limit

The detection was determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte could be reliably detected (CV < 20%) (Vermeire, 1998). The detection limit was 10.0 µg/ml (CV = 4%, n = 6).

The quantification limit was determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte could be quantified with acceptable accuracy and precision. The quantification limit was 10.0 µg/ml: accuracy < 15% of the actual concentration and precision around the mean value < 15% CV (n = 10). (Shah et al., 1992)

3.5.5.6 Recovery

The recovery of metronidazole from spiked swabs was measured at three concentration levels: 25.0, 50.0 and 200.0 µg/ml. All mean values were within 15% of the spiked concentration (n = 3). (Shah et al., 1992)

3.5.6 Results and Discussion

An overview of the in vivo tablet residence time is given in Figure 3. The individual metronidazole plasma concentration-time profiles after administration of a single bioadhesive vaginal tablet are shown in Figure 4. The vaginal swab concentrations are shown in Table 7.

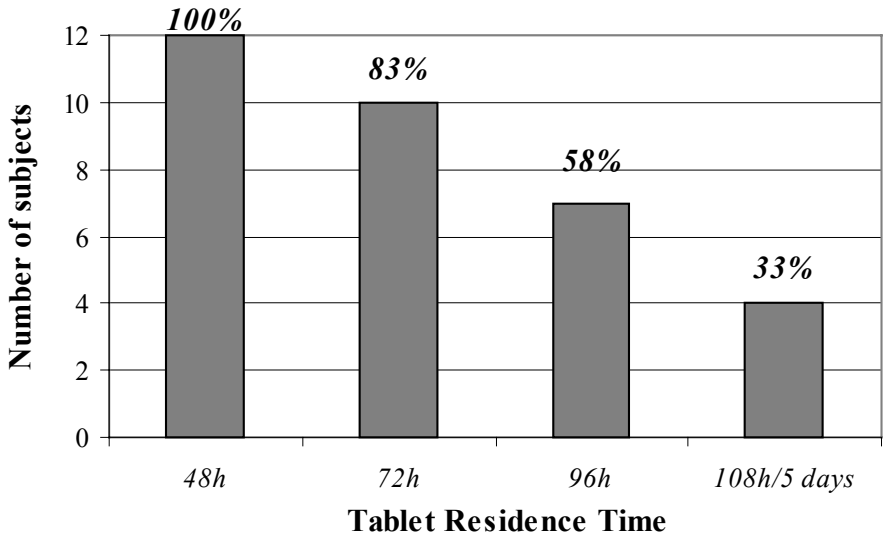

Figure 3. Individual in vivo residence time of a 500 mg metronidazole vaginal bioadhesive tablet.

None of the volunteers lost the tablet within the first 48h after application. 17% of the subjects (n=2) reported having lost the tablet on the third day (48h–72h), while 25 % (n=3) lost the tablet during the fourth day (72h-96h). 58% of the volunteers (n=7) lost the tablet on day 5 (96h-108h) (25%; n=3) or did not lose the tablet during the 5-day study (33%; n=4).

The individual metronidazole plasma concentrations varied between 0.2 and 1.0 µg/ml. For 17% (n=2) of the subjects the metronidazole plasma concentration decreased to 0.0 µg/ml at 72h, while the plasma concentrations for 25% (n=3) returned back to 0.0 µg/ml after 84h. In 58% (n=7) of the subjects plasma metronidazole concentrations could still be measured at 108h after application. From these data it is clear that by measuring metronidazole plasma concentrations the vaginal residence time of the bioadhesive tablet could be well followed. Only for 2 of the volunteers (subject 2 and 10) the tablet residence time could be clearly followed by vaginal swabbing (Table 7). The swab concentrations showed very large person-to-person differences probably as a result of

Quantity of Metronidazole per swab (µg/swab)												
Time	**Subjects**											
h	1	2	3	4	5	6	7	8	9	10	11	12
0	-	-	-	-	-	-	-	-	-	-	-	-
12	-	328.48	-	27.09	-	-	-	-	-	64.17	39.35	-
24	-	476.12	-	-	-	-	-	-	27.35	118.76	-	-
36	-	150.98	-	20.55	-	-	-	32.16	-	17.49	375.30	-
48	-	378.15	-	24.62	-	-	-	43.75	36.69	1086.13	570.32	-
60	196.90	19.31	-	33.36	28.71	-	-	71.06	18.65	32.37*	-*	-
72	-*	-*	-	41.22	21.53	-	-	-*	33.46	-	-	-
84	-	-	-	55.51	138.15	-	-	-	30.90	-	-	-
96	-	-	-	32.30*	968.06	-	39.47	-	95.58	-	-	30.75
108	-	-	62.59	-	22.09*	-	46.57	-	155.63*	-	-	69.67

Table 7. Quantity of metronidazole determined in the vaginal swabs (µg).

* Tablet lost

'-' = no metronidazole measured

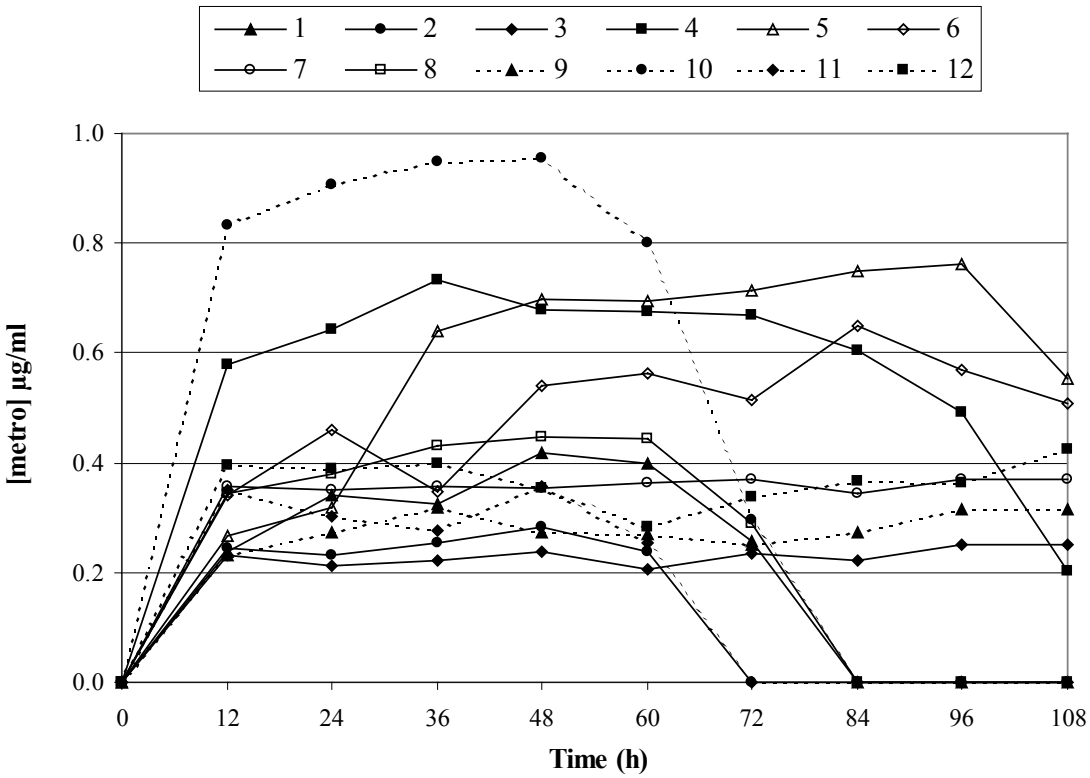

Figure 4. Individual metronidazole plasma concentration time profiles after administration of a 500 mg metronidazole vaginal bioadhesive tablet.

differences in individual swabbing effectiveness, although the swabbing depth and technique were standardised. For subject number 2 and number 10 the local vaginal

metronidazole concentrations could be used to follow the in vivo tablet residence time. For all other subjects there were one or more swabs wherein no drug was detected although the tablet still adhered to the uterine cervix, concluding that vaginal swabbing is not effective in following in vivo adherence of a bioadhesive vaginal tablet. Nevertheless, the measured swab concentrations confirmed the presence of distinguishable local metronidazole concentrations.

In Figure 5 mean systemic metronidazole concentrations after oral dosing of a 500 mg immediate release tablet (Hoffmann et al., 1995) are compared to the mean plasma concentrations measured after single application of a 500 mg metronidazole vaginal bioadhesive tablet described in this study. After oral administration metronidazole is rapidly absorbed from the gastro-intestinal tract and reached a mean maximum metronidazole concentration of 11 μg/ml after 2 hours. The mean metronidazole plasma concentrations measured with the vaginal bioadhesive tablet never reached 0.5 μg/ml. The plasma concentration time profiles did not show peak concentrations, but a constant systemic absorption as long as the tablet adhered to the uterine cervix (Figure 4). The measured plasma concentrations are comparable to those obtained after vaginal administration of a film coated 500 mg metronidazole tablet (Hoffmann et al., 1995) and are of marginal importance for systemic side effects and drug safety. Hoffman et al. (1995) reported that the absorption into systemic circulation after vaginal administration of a 500 mg metronidazole film coated tablet yielded mean values of about 30% of an adequate oral dose, that maximal concentrations are bactericidal only for the most susceptible anaerobic micro-organisms and are only of minor importance for the development of microbial resistance.

From the metronidazole plasma concentration time profiles in Figure 4, it is clear that the used spray dried Amioca®/C 974P 85/15 mixture is a suitable bioadhesive carrier for the local vaginal delivery of metronidazole as for 58% (n=7) of the subjects the drug release was sustained over 5 days, resulting in prolonged local metronidazole concentrations after application of a single tablet. In a previous study in bacterial vaginosis patients, application of a 500 mg metronidazole bioadhesive vaginal tablet based on a physical mixture of pregelatinised starch and 5% (w/w) Carbopol® 974P resulted in a cure rate of 68% with comparable in vivo tablet residence times to our

formulation (Voorspoels et al., 2002). The in vivo erosion rate of our spray dried Amioca®/C 974P formulation seemed slower, as for 33% (n=4) of the subjects still parts of the tablet were present on day 5.

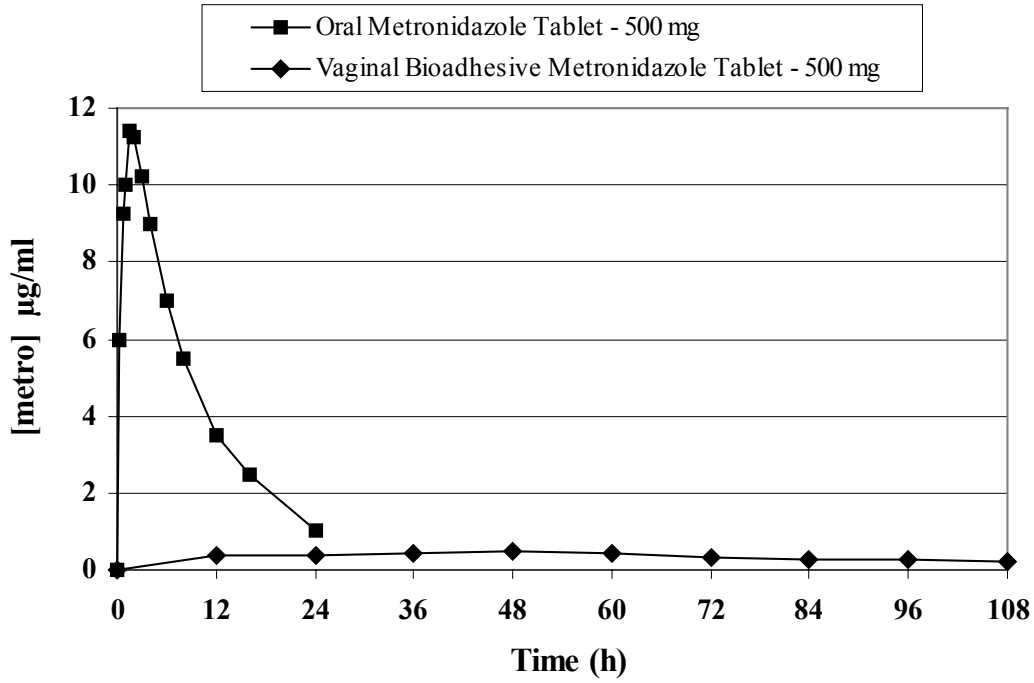

Figure 5. Mean serum concentration time profile after oral administration of a 500 mg metronidazole immediate release tablet (■) (n = 16) and mean plasma concentration time profile after local vaginal administration of 500 mg metronidazole via a bioadhesive tablet (♦) (n = 12).

Systemic absorption of metronidazole from a single bioadhesive vaginal tablet was about 5 times prolonged compared to a 500 mg oral tablet given twice daily for 7 days recognised as the treatment of choice for BV (Cunningham et al., 1994). A 500 mg oral dose results in a fast and high peak plasma concentration, but cannot sustain local drug concentrations. Although cure rates with the 7-day regimen are over 80%, the frequent occurrence of systemic adverse effects may complicate oral metronidazole therapy and reduce patient compliance. A safer alternative can be the twice a day intravaginal administration of a 0.75% metronidazole gel (Hillier et al., 1993). Systemic exposure is reduced with local vaginal administration, as serum concentrations after intravaginal administration of 5 g of a 0.75% metronidazole gel (37.5 mg metronidazole) were only 2% of the concentrations seen after administration of the 500 mg standard dose (Cunningham et al., 1994). Notwithstanding a cure rate of 81% (Joesoef et al., 1999), a twice daily vaginal application of a gel or cream is a difficult and time-consuming task,

resulting in poor patient compliance. A major advantage of a single application of a 500 mg metronidazole loaded bioadhesive tablet is that the tablet can be applied directly after the clinical diagnosis of bacterial vaginosis, resulting in optimal patient compliance. As expected with a local metronidazole treatment, none of the volunteers reported related (systemic) side effects, indicating that the measured plasma concentrations did not induce metronidazole related side effects. The gynaecologist reported no vaginal lesions. This proves the good tolerability and the potential of the vaginal bioadhesive tablet as vaginal drug delivery system. It is also proved that a spray dried Amioca® starch/Carbopol® 974P 85/15 mixture can be used as such a bioadhesive vaginal carrier. In the future more research has to be done on tablet shape, tablet weight and drug load aiming to reduce tablet loss before complete erosion. Also cure rates in patients have to be investigated.

3.5.7 Conclusion

The present study has proved that a spray-dried Amioca® starch/Carbopol® 974P 85/15 mixture can be used as a bioadhesive vaginal carrier and can prolong the drug release over 5 days, resulting in prolonged local vaginal drug concentrations. The in vivo residence time of a vaginal bioadhesive metronidazole tablet was assessed by measuring plasma concentrations, while local metronidazole concentrations collected by vaginal swabbing did not reflect tablet adherence due to large person-to-person variabilities in swabbing effectiveness. The bioadhesive tablet formulation has a large potential as (vaginal) bioadhesive drug delivery system, although further research is needed for optimization of tablet shape, tablet weight and drug load.

3.5.8 References

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Chapter 3.6 Effect of a Buccal Bioadhesive Nystatin Tablet on the Device Life of a Provox™ Silicone Tracheoesophageal Voice Prosthesis

3.6.1 Introduction

In the present study the efficacy of a buccal bioadhesive nystatin tablet on the device life of a Provox™ tracheoesophageal voice prosthesis was investigated in postlaryngectomy patients. A spray-dried Amioca®/Carbopol® 974P mixture containing 10% (w/w) Carbopol® 974P (SD 90/10) was selected as bioadhesive carrier. The selection was based on the in vivo adhesion time results obtained in dogs with placebo tablets (*Chapter 3.4 Buccal Testosterone Absorption from a Bioadhesive Tablet*), aiming an in vivo adhesion time of ± 8 h with a drug loaded tablet.

Restoration of speech after total laryngectomy is generally considered to be a primary task in the rehabilitation of the laryngectomee. Throughout the years, a variety of alaryngeal voice techniques have been advocated to restore speech and voice. Alaryngeal speech production requires the establishment of an alternative vibratory source in the reconstructed pharyngoesophageal region. Esophageal speech and artificial larynges are known as primary methods of alaryngeal communication. Since the introduction of the Blom-Singer duckbill prosthesis in 1979, several silicone tracheoesophageal valve prostheses have been developed with promising results in voice rehabilitation after total laryngectomy. These one-way valve prostheses allow sufficient shunting of expiratory air to the esophagus without leakage of esophageal contents into the trachea and compared to the other methods of voice rehabilitation consistent high success rates of shunt esophageal speech were obtained. Further research was directed toward the development of a device that could be easily applied after primary (during the total laryngectomy) or secondary (after a total laryngectomy) puncture, that enables short-term acquisition of functional laryngeal speech, and that requires only simple maintenance for optimal performance.

This resulted in the development of indwelling, biocompatible silicone prostheses with a low-resistance one-way valve mechanism. (Weissenbruch et al., 1997 [a,b])

Device life of the voice prosthesis is known to be limited due to dysfunction of the valve mechanism and one of the drawbacks of voice prostheses is their limited device life due to deterioration of the silicone valve. Colonisation and invasion of silicone material with *Candida* species has been reported to correlate with valve dysfunction (Mahieu et al., 1986; Izdebski et al., 1987). The latter may be caused by leakage of esophageal contents through the prosthesis into the trachea as a result of insufficient valve closure, or by an increased expiratory airflow resistance during phonation. This will gradually lead to increased efforts to produce fluent shunt esophageal speech. Topical agents (i.e. lozenges and semi-solid formulations) might adequately resolve superficial fungal infections, but must be applied frequently to maintain effective saliva concentrations. Successful decontamination of the oropharynx with 10 mg amphothericin B lozenges four times daily was associated with a prolonged device life and lower intratracheal phonatory pressures, but the frequent daily applications lead to poor patient compliance. Application of a buccal bioadhesive miconazole slow-release tablet has proven to maintain effective salivary drug concentrations for 10-12 hours (Bouckaert et al., 1992). A bioadhesive slow-release tablet diminishes the need for frequent drug applications with lower daily doses. (Weissenbruch et al., 1997 [a,b])

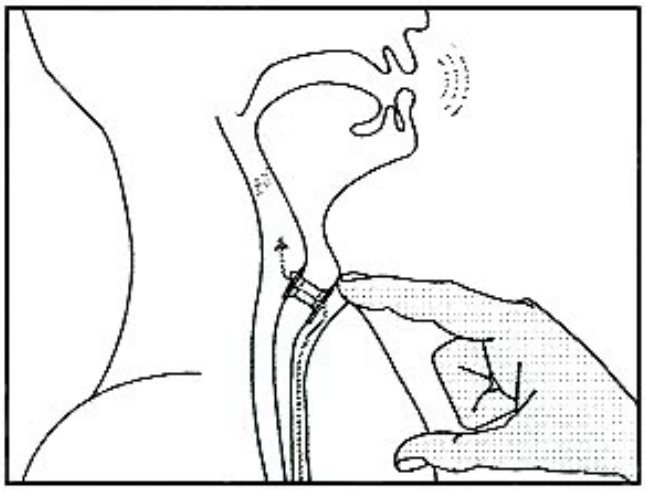

Figure 1. The Provox™ 2, retained in the tracheoesophageal fistula.

Although miconazole has been to date the first-choice topical agent for the prophylaxis of the colonisation of tracheoesophageal voice prostheses by yeasts, Bauters et al. (2002) questioned its position as preferred prophylactic antimycotic. A broad range of minimal inhibitory concentrations (MIC) of albicans and non-albicans *Candida* species was observed for miconazole, while nystatin showed narrowly distributed MIC values for all isolates suggesting uniform sensitivity. Based on these findings nystatin was incorporated in a slow-release buccal bioadhesive tablet in stead of miconazole (Bouckaert et al., 1992; Weissenbruch et al., 1997[b]) and the effect of a nystatin bioadhesive tablet on the Provox™ device lifetime was investigated. The bioadhesive carrier used by Bouckaert et al. (1992) and Weissenbruch et al. (1997) is a physical mixture of 5% (w/w) Carbopol® 974P with a pregelatinised starch, in *Chapter 3.2 Ex Vivo Bioadhesion Measurement* used as reference formulation. The selected bioadhesive carrier, SD 90/10, showed comparable ex vivo bioadhesion values and in vivo adhesion times as for the reference formulation (*Chapter 3.4 Buccal Testosterone Absorption from a Bioadhesive Tablet*).

Nystatin is an antifungal agent used to treat a variety of fungus infections, but it is exceptionally popular to treat skin and mucosal membrane infections (i.e. in the mouth) caused by varieties of *Candida albicans*, a yeast-like fungus that is commonly found in the gastro-intestinal tract. Nystatin is a polyene antibiotic (Figure 2) and works by binding to sterols in the fungal cellular membrane altering the permeability to allow leakage of the cellular contents and destroying the fungus. Nystatin is administered by mouth, by means of vaginal suppositories or tablets, and topically (on the skin), through the use of a cream, powder, or ointment. Nystatin for oral use is available as a liquid suspension, as tablets, and as lozenges.

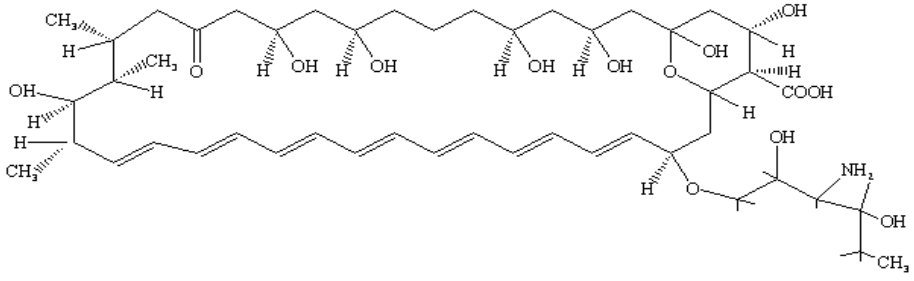

Figure 2. Chemical structure of nystatin, an antifungal polyene antibiotic.

3.6.2 Materials

Nystatin (5658 IU/mg) and colloidal silica were purchased from Alpha Pharma (Nazareth, Belgium). Sodium stearyl fumarate (NaSF) was given by Edward Mendell Co. Inc. (New York, USA). All other chemicals used were at least of analytical grade.

3.6.3 Methods

3.6.3.1 Production of tablets

The tablets were produced by firstly mixing the spray-dried Amioca®/Carbopol® 974P 90/10 mixture (SD 90/10) with nystatin (100.000 IU per tablet), next 1% (w/w) colloidal silica was added as a glidant and the mixture was mixed for 5 min. in a Turbula blender (Type T2A, W.A. Bachofen, Bazel, Switzerland). Finally, sodium stearyl fumarate (2%; w/w) was added as a lubricant and mixed for 2 min. in a Turbula blender. The mixture was compressed on a Korsch compression machine (Type EK0, Berlin, Germany) equipped with 6.5 mm punches, at a pressure of 7.4 kN. The weight and the diameter of the tablets were 750 mg and 6.5 mm, respectively.

3.6.3.2 Study protocol

The study was performed at the *Head and Neck Surgery Department, Ghent University Hospital, De Pintelaan 185, Gent, Belgium* in co-operation with the *Laboratories for Pharmaceutical Technology and Pharmaceutical Microbiology, Ghent University, Harelbekestraat 72, Gent, Belgium*. The approval of The Ethics Committee of the Ghent University Hospital was obtained.

Nineteen (19) patients (age 42-79 years/ 1 female) who underwent total laryngectomy and prosthesis insertion were included, after appropriate explanation of the trial procedures and signature of an informed consent document. All patients received a new Provox™ 2 indwelling low-resistance voice prosthesis (Atos Medical AB, Hörby, Sweden) as a standard outpatient procedure. The patients were selected to start participation in the trial at the time when replacement of their voice prosthesis was

required due to internal leakage of esophageal contents and/or increased phonatory efforts to produce fluent shunt esophageal speech. The device lifetime of the prosthesis was followed and expressed in days. The voice prosthesis was replaced by a new one when required due to leakage and/or increased efforts to phonate. The patients were divided at random into three groups.

Group one (n = 7) cleaned the voice prosthesis locally by means of ten (10) drops of an oral nystatin suspension (Nystatine Labaz® suspension, 100.000 IU/ml, Sanofi-Synthelabo, Brussels, Belgium) on a brush once a day in the evening. After replacement, the patients cleaned their new prosthesis in the same way with nystatin oral suspension on a brush. No other antimicrobial agents were used simultaneously.

Group two (n = 7) cleaned the prosthesis locally by means of ten (10) drops of an oral nystatin suspension (Nystatine Labaz® suspension, 100.000 IU/ml, Sanofi-Synthelabo, Brussels, Belgium) on a brush once a day in the evening. After replacement of the voice prosthesis, the patients applied one nystatin bioadhesive tablet per day, after breakfast, on the gingiva above the upper canine, altering left and right side. Patients with teeth prostheses sticked the tablet on the inside of the cheek opposed to the gingiva above the upper canine, altering left and right side. No other antimicrobial agents were used simultaneously.

The third group (n = 5) was a control group. These patients did not use any anti-microbial agent to clean their voice prosthesis.

Any occurring adverse effect or complication was recorded. A questionnaire with possible side effects was included for the patients at each replacement.

The device lifetime of the different groups was statistically compared by one-way analysis of variance at a significance level of $p < 0.05$ using a post hoc Scheffé test. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the Levene's test.
To statistically compare the device lifetime within one group a paired T-test was used at a significance level of $p < 0.05$. The computer program SPSS version 10.0 was used for the statistical analyses.

3.6.4 Results and Discussion

The device lifetime, expressed in days, of the patients' voice prostheses in the three groups are shown in Table 1.

	Voice Prosthesis Lifetime (days)	
	Period 1	**Period 2**
	Treatment	**Treatment**
Group 1 (n = 7)	*nystatin on brush*	*nystatin on brush*
	112 ± 46	**149 ± 119**
Group 2 (n = 7)	*nystatin on brush*	*nystatin bioadhesive tablet*
	143 ± 42	**262 ± 86**
Group 3 (n = 5)	*Control*	/
	83 ± 23	/

Table 1. Voice prosthesis lifetimes, expressed in days, with the different treatments in the three patient groups. (mean ± sd)

There was no significant difference in the prosthesis lifetime of the first period between group 1 and group 2, both treated with nystatin oral suspension on a brush (Post Hoc Scheffé, $p < 0.05$). The mean prosthesis lifetime of the control group was lower, however not significantly (Post Hoc Scheffé, $p < 0.05$), compared to group 1 and 2. Within group 1 there was no significant difference (Paired T-test, $p < 0.05$) in lifetime between the first and the second period. Within group 2, the nystatin bioadhesive tablet significantly (Paired T-test, $p < 0.05$) increased the lifetime of the Provox™ compared to the local treatment with nystatin on a brush. The bioadhesive tablets were well tolerated by the patients, no irritation or side effects were reported.

Bauters et al. (2002) reported that the lifetime of prostheses ranged from 2 weeks to more than 1 year, depending on the personal hygiene and maintenance (i.e. cleaning with an antimicrobial agent on a brush), with an average lifetime of 4 months (120 days). These data are in accordance with our findings, namely an average lifetime of 112 and 143 days in the first period of group 1 and 2, respectively, during which the prosthesis was daily cleaned with nystatin oral suspension on a brush. The relationship between the colonisation of tracheoesophageal voice prostheses by yeasts, particularly *Candida*

species, and their physical deterioration and dysfunction is well described (Bauters et al., 2002; Weissenbruch et al., 1997 [a,b]; De Carpentier et al., 1996). This is confirmed in the present study, as the average lifetime of control group was lower compared to the nystatin-brush treated group 1 and 2 (1st period). Application of a nystatin bioadhesive tablet, one per day, significantly increased the lifetime of the Provox™ indicating that a sustained release of nystatin in the oral cavity, by erosion of the tablet during ± 8 hours, is more effective in preventing microbial colonisation of the prosthesis compared to a local cleaning with nystatin suspension on a brush. By daily application of a nystatin bioadhesive tablet, the average prosthesis lifetime was increased to almost 9 months (262 days) compared to 4 months (120 days) reported by Bauters et al. (2002). However, daily application of a bioadhesive tablet is, especially for these post-laryngectomy patients who are irradiated, an intensive task. Moreover, irradiated patients suffer from mouth dryness which might prevent the adhesion and erosion of the bioadhesive tablet due to insufficient hydration. Although this might result in a poor patient compliance, a significant increase of the Provox™ lifetime was observed with a daily application of a nystatin bioadhesive tablet.

3.6.5 Conclusion

Daily application of a nystatin bioadhesive slow-release tablet (100.000 IU per tablet) significantly increased the lifetime of a Provox™ voice prosthesis in laryngectomised patients compared to the conventional local cleaning of the prosthesis with an antimicrobial agent on a brush. However, patient compliance was rather poor as a daily application of a bioadhesive tablet is an extra charge for post-laryngectomy patients and as irradiated patients suffer from mouth dryness.

3.6.6 References

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Chapter 3.7 Stability Study of a Spray-Dried Amioca®/Carbopol® 974P mixture

3.7.1 Introduction

The spray-dried Amioca®/Carbopol® 974P 80/20 mixture (SD 80/20) was selected for stability testing according to the USP guidelines (United States Pharmacopeia XXIV, 2000) for accelerated stability testing. SD 80/20 was stored during 12 months as a powder and as placebo tablets in open containers at 40 ± 2 °C and 75 ± 5 % relative humidity (RH). The ex vivo bioadhesion, tablet hardness, in vitro drug release and moisture content were determined as a function of storage time.

3.7.2 Materials

Sodium stearyl fumarate (NaSF) was given by Edward Mendell Co. Inc. (New York, USA). Miconazole nitrate was obtained from Janssen Pharmaceutica, Beerse, Belgium. Econazole was purchased from Sigma-Aldrich (Bornem, Belgium), methanol HPLC-S grade was purchased from Biosolve BV (Valkenswaard, The Netherlands) and tetrahydrofuran (THF) was purchased from BDH, Laboratory Supplies, Poole, UK. All other chemicals used were at least of analytical grade.

3.7.3 Methods

3.7.3.1 Storage conditions

SD 80/20 was stored under two different physical forms: as bulk powder and as tablets. The bulk powder was then compressed to placebo or miconazole tablets, as described in the next paragraph (3.7.3.2), directly after storage to evaluate ex vivo bioadhesion/tablet hardness and drug release, respectively.

The bulk powder and the tablets were stored in an oven (Memmert U 60, Schwabach, Germany) at 40 ± 2°C in open containers in a sealed chamber above a saturated NaCl solution to obtain a relative humidity of 75 ± 5 %.

3.7.3.2 Production of tablets

To prepare tablets the SD 80/20 powder was mixed with sodium stearyl fumarate (1%; w/w) as a lubricant, and compressed on a Korsch compression machine (Type EK0, Berlin, Germany) equipped with 7 mm flat punches, at a pressure of 9.8 kN. The tablet weight was 100 mg.

The tablets used in the in vitro drug release study contained miconazole nitrate. The tablets were prepared by firstly mixing the SD 80/20 powder with miconazole nitrate (10 mg), next the lubricant (1% sodium stearyl fumarate) was added and mixed again. The tablets were compressed as described above with a tablet weight of 100 mg and diameter of 7 mm.

3.7.3.3 Measurements

Ex vivo bioadhesion, tablet hardness, in vitro drug release and moisture content were measured before storage (point 0 – p 0) and after 2 weeks, 1 month, 2 months, 3 months, 9 months and 12 months of storage at 40°C/75%RH.

3.7.3.3.1 Ex vivo bioadhesion measurements

Ex vivo bioadhesion was measured on placebo tablets, prepared as described above, according to the ex vivo bioadhesion method described in *Chapter 3.2 - 3.2.2.3 Ex vivo bioadhesion determination*.

Statistical analysis was performed on the work of adhesion values, as work of adhesion is, generally considered, more accurate to quantify bioadhesion than adhesion force (*Chapter 3.2 – 3.2.3 Results and Discussion*). Statistically significant differences were determined using a one-way ANOVA. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the

Levene's test. The data of stored tablets were transformed to their logarithm, the data of stored bulk powder were transformed to their square root. The data were compared to point 0 (before storage) using a Bonferroni test with $p < 0.05$ as significance level. The computer program SPSS version 10.0 was used for the statistical analyses.

3.7.3.3.2 Tablet hardness test

Tablet hardness was measured with a Pharma Test hardness tester (Type PTB 311, Heinburg, Germany). The test modus was set at 20 $N.s^{-1}$. Tablet diameter (mm), thickness (mm) and hardness (N) were measured. Tablets were prepared as described above.

3.7.3.3.3 In vitro drug release

The in vitro drug release was evaluated in an automatic reciprocating cylinder dissolution apparatus USP III (BioDis) (United States Pharmacopeia XXIV, 2000) (VanKel BioDis III Release Rate tester, Cary, NC, USA) on tablets containing 10 mg miconazole nitrate as a marker. Tablets were prepared as described above. Quantitative analysis of miconazole nitrate in the dissolution samples was performed with a validated HPLC method with UV-detection using econazole as internal standard. Dissolution and HPLC method are described in *Chapter 3.4 – 3.4.1.1.1 In vitro drug release study (USP III)*.

The time for 50% drug release, $t_{50\%}$, was calculated from the mean (n = 6) miconazole nitrate release profiles.

3.7.3.3.4 Moisture content

The moisture content was measured on SD 80/20 powder using a Mettler DL 35 Karl Fisher titrator (Mettler-Toledo, Beersel, Belgium) equipped with a double platinum electrode for endpoint determination. Hydranal® composite 5 (Riedel-de Haën, Seelze, Germany), a one-component reagens, was used as titrant solution. The reaction medium was anhydrous methanol (Riedel-de Haën, Seelze, Germany). The reagens titer

(theoretical 2 mg H_2O/ml) was calibrated and verified with 100% distilled water. Directly after storage, stored tablets were crushed with pestle and mortar to obtain a powder.

3.7.4 Results and Discussion

3.7.4.1 Moisture content

The moisture content of SD 80/20, stored as bulk powder, is shown in Figure 1. After 2 weeks of storage the water content increased from 8 to 12 % (w/w). A longer storage time did only slightly increase the moisture content to a maximum value of 13 % (w/w). The water content in placebo tablets after 12 months of storage was 9.9 ± 0.4 % (w/w). The lower water uptake by stored tablets is due to a smaller relative surface of tablets compared to bulk powder.

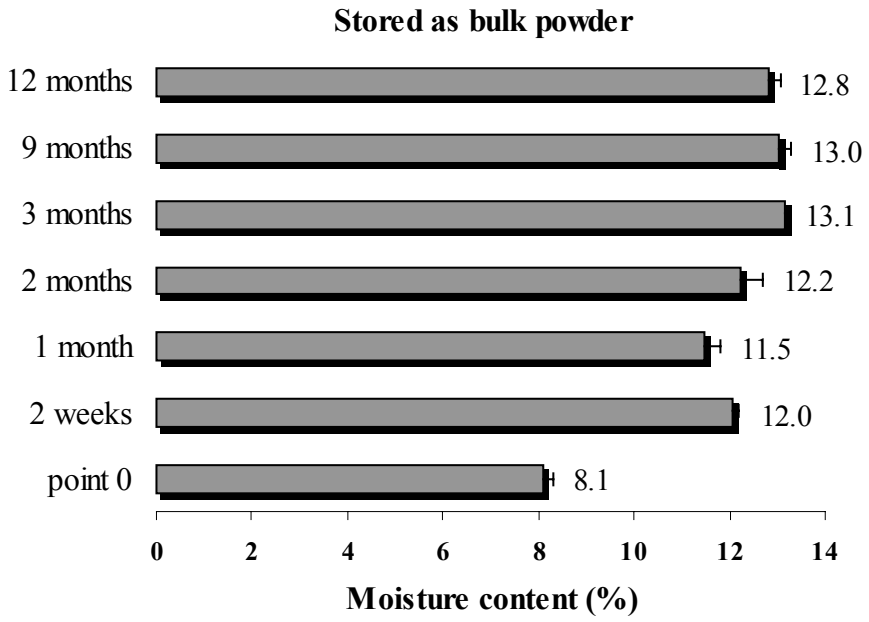

Figure 1. Moisture content (% w/w) of SD 80/20, stored as bulk powder, as function of storage time at 40°C/75%RH. (n = 3, mean ± sd)

3.7.4.2 Tablet hardness

Tablet hardness, thickness and diameter for SD 80/20, stored as bulk powder and next tabletted or stored as tablets over a period of 12 months, are given in Table 1 and Table 2, respectively.

Stored as bulk powder			
Time	**Hardness (N)**	**Thickness (mm)**	**Diameter (mm)**
Point 0	136.6 ± 9.6	2.30 ± 0.04	7.08 ± 0.02
1 month	118.4 ± 2.0	2.18 ± 0.03	7.00 ± 0.02
2 months	127.6 ± 4.8	2.15 ± 0.03	6.99 ± 0.02
3 months	130.5 ± 4.9	2.06 ± 0.02	6.91 ± 0.01
9 months	79.8 ± 9.4	2.15 ± 0.03	6.96 ± 0.02
12 months	92.9 ± 5.7	2.11 ± 0.06	6.95 ± 0.02

Table 1. Tablet hardness (N), thickness (mm) and diameter (mm) for SD 80/20, stored as bulk powder. (n = 6, mean ± sd)

Stored as tablets			
Time	**Hardness (N)**	**Thickness (mm)**	**Diameter (mm)**
Point 0	136.6 ± 9.6	2.30 ± 0.04	7.08 ± 0.02
2 weeks	103.8 ± 2.5	2.56 ± 0.03	7.24 ± 0.01
1 month	117.8 ± 7.7	2.53 ± 0.03	7.22 ± 0.01
2 months	110.7 ± 9.7	2.54 ± 0.05	7.24 ± 0.03
3 months	139.2 ± 7.6	2.46 ± 0.02	7.37 ± 0.55
9 months	123.8 ± 14.4	2.47 ± 0.04	7.16 ± 0.03
12 months	117.0 ± 4.6	2.47 ± 0.02	7.20 ± 0.03

Table 2. Tablet hardness (N), thickness (mm) and diameter (mm) for SD 80/20, stored as tablets. (n = 6, mean ± sd)

Under both conditions, stored as bulk powder or as tablet, the tablet hardness decreased as a function of storage time. Storage as a powder (93 N after 12 months) had a much more pronounced negative influence on tablet hardness compared to storage as a tablet (117 N after 12 months). When the bulk powder was stored at 75% RH, water was absorbed on the surface of the individual powder particles (*3.7.4.1 Moisture content*). The so formed water films affect the compression and prevent inter-particular binding,

resulting in softer tablets. Water uptake by stored tablets induced small tablet deformations, which were observed by an increased tablet thickness and diameter (Table 2). These deformations were already measured after 2 weeks and were next stabilised. Tablets, prepared from stored bulk powder, were tested immediately after compression and no deformations were observed.

Although storage of bulk powder and tablets at 40°C/75%RH had a negative influence on the tablet hardness, the tablets still showed after 12 months acceptable hardness values of 93 N and 117 N, respectively.

3.7.4.3 Ex vivo bioadhesion

The ex vivo bioadhesion results for SD 80/20 tablets as function of time, stored as bulk powder and next compressed or compressed prior to storage (stored as tablets), are shown in Figure 2 and Figure 3, respectively.

The ex vivo bioadhesion capacity of SD 80/20 tablets decreased as function of storage time as well when stored as bulk powder as when stored as tablets. The work of adhesion was, compared to point zero (before storage), significantly decreased after 1 month for stored bulk powder and after 2 months for stored tablets.

As already described in *Chapter 3.1 Introduction*, bioadhesion strength depends on the ability of dry mucoadhesive drug delivery systems (i.e. bioadhesive tablets) to swell and dehydrate mucus and consolidation of the formed adhesive joint is driven by this water movement (Smart, 1999). As well the powder as the tablets will take up water during storage at 75% RH (*3.7.4.1 Moisture content*). A possible explanation for the decreasing bioadhesive properties, is a lower swelling and dehydration potential after storage at a high relative humidity. On the other hand, Voorspoels (1997) reported that it was difficult to find a correlation between an increased water content and a decrease of the bioadhesive properties due to tablet deformations (*3.7.4.2 Tablet hardness*), which negatively influenced the ex vivo bioadhesion results. However, tablets prepared from stored bulk powder were compressed and tested immediately after storage and did not show deformations (Table 1). Nevertheless, it is clear that the bioadhesive properties of the

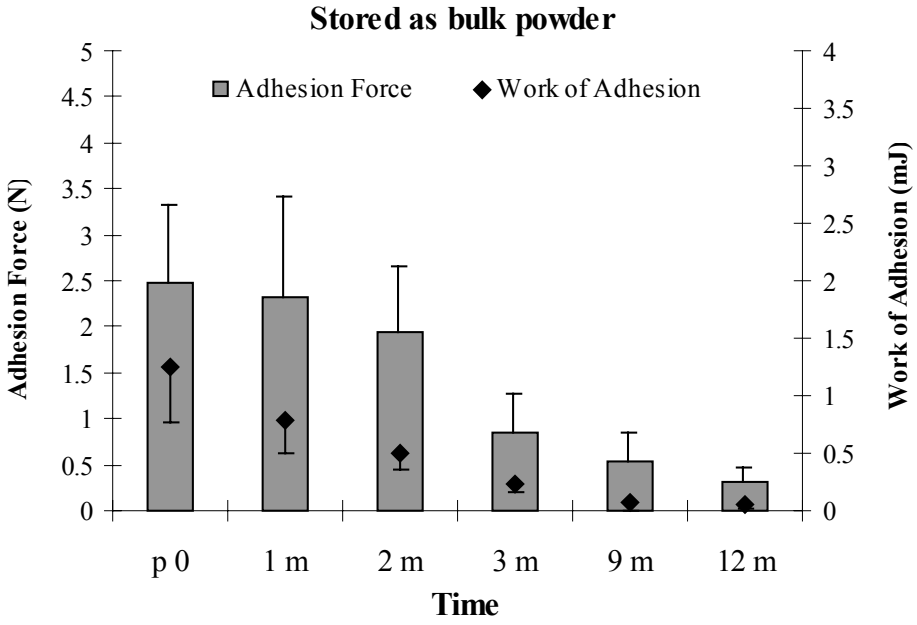

Figure 2. Ex vivo bioadhesion results – Adhesion Force (N) and Work of Adhesion (mJ) – before storage (point 0 – p 0) and after 1 month (1 m), 2 months (2 m), 3 months (3 m), 9 months (9 m) and 12 months (12 m) storage of SD 80/20 as bulk powder at 40°C/75%RH. (n = 10, mean ± sd)

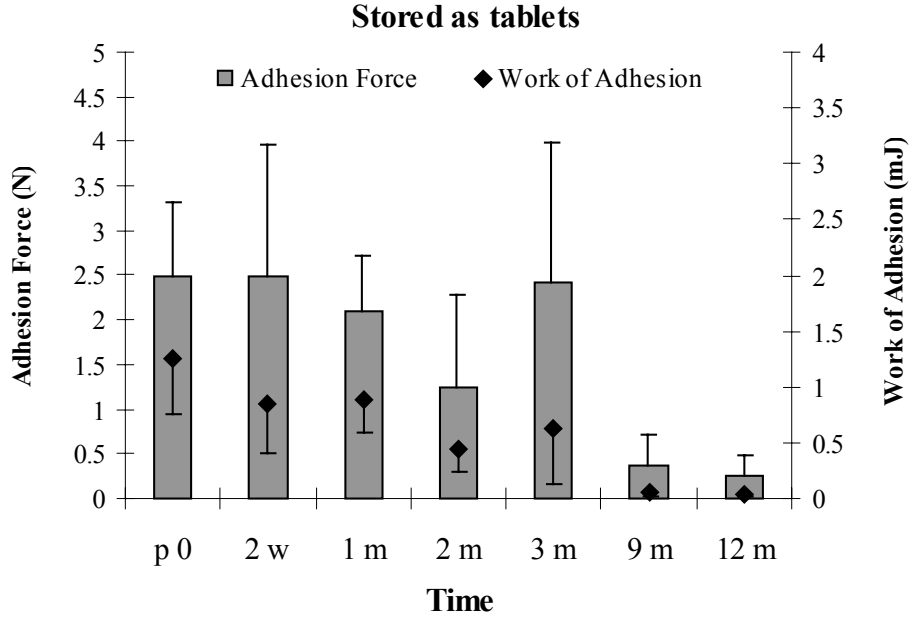

Figure 3. Ex vivo bioadhesion results – Adhesion Force (N) and Work of Adhesion (mJ) – before storage (point 0 – p 0) and after 2 weeks (2 w), 1 month (1 m), 2 months (2 m), 3 months (3 m), 9 months (9 m) and 12 months (12 m) storage of SD 80/20 as tablets at 40°C/75%RH. (n = 10, mean ± sd)

spray-dried Amioca®/Carbopol® 974P mixture significantly decreased after 1 and 2 month, respectively, when stored at 40°C/75%RH as bulk powder and as tablets.

3.7.4.4 In vitro drug release

The in vitro drug release profiles from a SD 80/20 miconazole nitrate bioadhesive tablet formulation as a function of storage time are shown in Figure 4 (stored as bulk powder prior to compression) and Figure 5 (stored as a tablets).

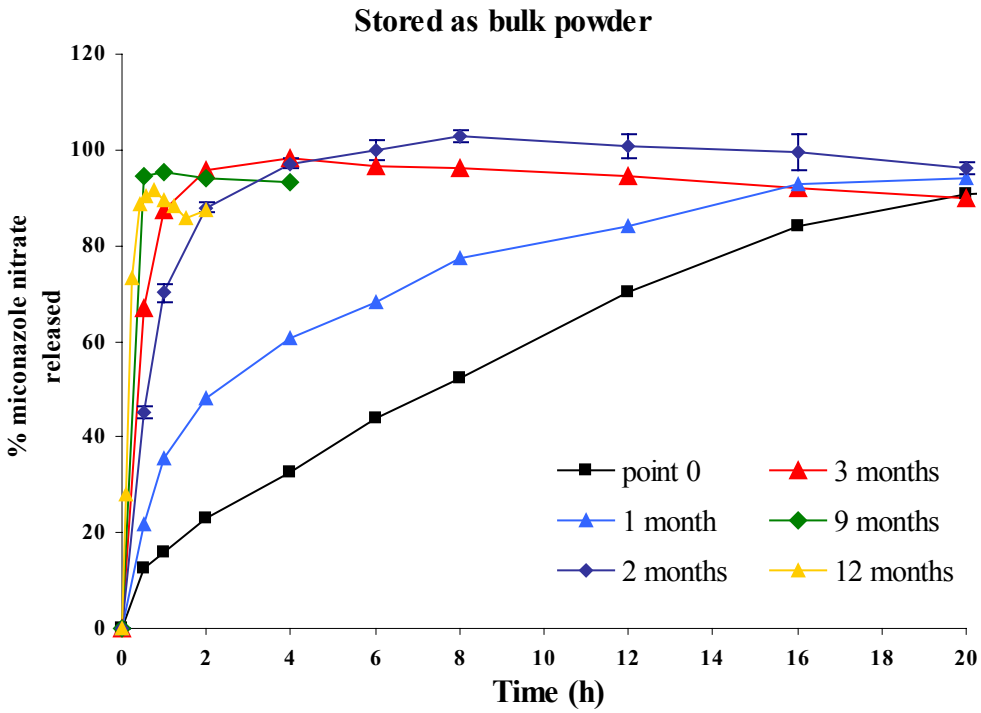

Figure 4. In vitro miconazole nitrate release profiles from a SD 80/20 bioadhesive tablet formulation, stored as bulk powder, as function of storage time. (n = 6, mean)

When stored as bulk powder, the in vitro miconazole release already increased after 1 month of storage. Before storage at 40°C/75%RH the $t_{50\%}$ value was 7.5 hours, while after 1 month these value already decreased to 2.5 hours and after 2 months 50% miconazole nitrate was released after 0.5h. These in vitro release data clearly indicate that a spray-dried Amioca®/Carbopol® 974P mixture has to be stored with caution for temperature and relative humidity.

When stored as tablets, the in vitro drug release was stable for the test period of 12 months. The $t_{50\%}$ values varied between 7.5 and 9.5 hours. Only a slight tendency of retardation of drug release as function of storage time could be observed.

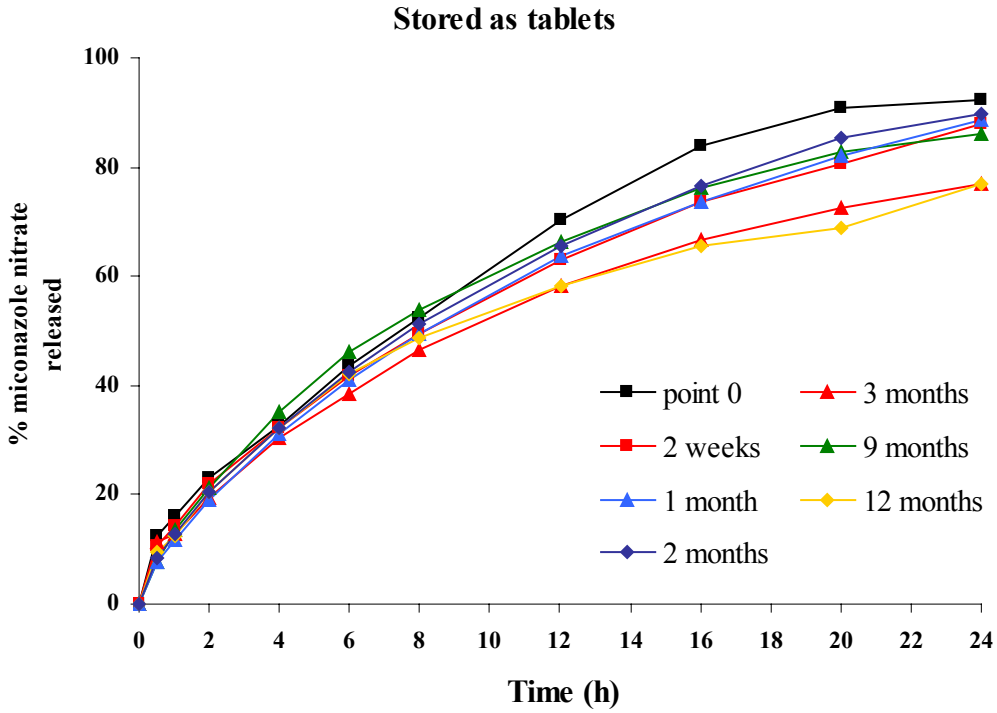

Figure 5. In vitro miconazole nitrate release profiles from a SD 80/20 bioadhesive tablet formulation, stored as tablets, as function of storage time. (n = 6, mean)

As already mentioned above, due to a larger relative surface bulk powder is more sensitive for moisture uptake compared to tablets. During storage at 75% RH, the individual powder particles will absorb water on their surface. The so formed water layers prevent inter-particular binding, resulting in a lower tablet hardness and faster erosion times and corresponding dissolution profiles.

3.7.5 Conclusion

Storage of a spray-dried Amioca®/Carbopol® 974P mixture at 40°C/75%RH, both stored as bulk powder and next compressed and stored as tablets from the start, resulted in a significant decrease of bioadhesive properties due to water uptake and tablet deformations. The in vitro drug release profiles of stored tablets were stable for the whole test period (12 months). When stored as bulk powder prior to compression, after 1 month 50% of the drug was released after 2.5 h compared to 7.5 h before storage. Spray-dried Amioca®/Carbopol® 974P mixtures, as bulk powder as well as formulated to tablets, have to be stored under controlled conditions.

3.7.6 References

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United States Pharmacopeia 24/The National Formulary 19, United States Pharmacopeial Convention, INC., 2000.

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Summary

Multifunctional polymers have been used in developing controlled release systems, peroral peptide delivery systems and bioadhesive platforms for buccal, nasal, vaginal and oral drug delivery. Multifunctional polymers are hydrophilic macromolecules. They have good bio- or mucoadhesive properties, they can prolong the residence time of the drug delivery system at the site of drug absorption and can increase the contact between delivery system and absorbing mucosa, resulting in a concentration gradient which can favour drug absorption. Drug delivery systems based on multifunctional polymers can be applied on specified mucosal tissues, such as the nasal, buccal and vaginal mucosa, to improve and enhance the bioavailability of the drug. Multifunctional polymers are supposed to improve the absorption of peptides across mucosal surfaces by increasing the permeability of epithelial tissues and inhibiting proteolytic enzymes.

In this doctoral thesis multifunctional polymers were prepared by grafting starches with poly(acrylic acid) (*Chapter 1.1 Synthesis and Process Details*). Two grafting methods were evaluated: ^{60}Co irradiation and chemical modification. By irradiation and chemical modification poly(acrylic acid) chains were grafted onto the starch molecules. Different types of starch, starch/acrylic acid ratios and degrees of neutralisation were used.

A second series of multifunctional polymers was prepared by freeze-drying or spray-drying starch/carboxylated polymer mixtures. Amioca® (waxy corn) was used as starch. A linear poly(acrylic acid), a cross-linked poly(acrylic acid) (Carbopol® 974P) and sodium carboxymethylcellulose were used as carboxylated polymers.

In *Chapter 1.2* the spray-dried Amioca®/Carbopol® 974P mixtures were analysed using Scanning Electron Microscopy (SEM) and solid state NMR (Nuclear Magnetic Resonance) spectroscopy. SEM and NMR analysis showed that by spray-drying Amioca® starch/Carbopol® 974P mixtures, Carbopol® films are formed around the starch granules. At higher Carbopol® concentrations (75% w/w), individual Carbopol® nano-particles can be found in addition to film formation.

The starch-*g*-poly(acrylic acid) copolymers, the freeze-dried and spray-dried starch/carboxylated polymer mixtures were evaluated as potential excipients for oral peptide delivery (*Chapter 2 Multifunctional Polymers for Oral Peptide Drug Delivery*). The two major barriers for successful oral peptide delivery are enzymatic degradation and permeation across the gastro-intestinal epithelium. The in vitro inhibition potency of the polymers towards the proteolytic enzyme trypsin was investigated. As most proteolytic enzymes have Ca^{2+} or Zn^{2+} at their active sites and as a reduction of extracellular Ca^{2+} concentration results in an opening of the tight junctions the calcium and zinc ion binding capacity of the multifunctional polymers was determined.

In *Chapter 2.2* an in vitro trypsin activity assay was biochemically optimised and validated for the evaluation of the inhibition capacity of the multifunctional polymers. In previously described assays the enzymatic reaction was not biochemically optimised. The used substrate concentrations were too low in proportion to the enzymatic activities or the enzymatic activity was too high for the used substrate concentration, so that the metabolite concentration versus time curves reached very fastly a plateau or post-steady state because of exhaustion of the substrate. Several substrate concentrations and enzymatic activities of trypsin were evaluated aiming at extracting the linear or steady state part of the metabolite versus time curve of the enzymatic degradation reaction. The inhibition measurements were carried out in steady state conditions and hence the degradation rate is independent of substrate concentration. The enzyme inhibition potency was expressed by the Inhibition Factor (IF), which was defined as the ratio of reaction rate without polymer to the reaction rate with polymer. The IF value is a good measure of the in vitro inhibitory capacity of multifunctional polymers towards gastro-intestinal proteolytic activity.

In *Chapter 2.3* the potential of the grafted starches and the freeze-dried / spray-dried starch/carboxylated polymer mixtures as excipients for oral peptide and protein delivery was in vitro evaluated by measuring the trypsin inhibition potency and the Ca^{2+} and Zn^{2+} binding capacity of these multifunctional polymers. Carbopol® 934P was used as a reference polymer. Heat treated freeze-dried starch/poly(acrylic acid) mixtures showed the highest in vitro trypsin inhibition potency, which was comparable or even higher than for the reference polymer. The ^{60}Co irradiated or chemically modified grafted starches showed lower IF values than the reference. Also the spray-dried starch/poly(acrylic acid) mixtures showed all a lower IF than the reference polymer. The

use of the cross-linked Carbopol® 974P or the linear poly(acrylic acid) in the spray-dried mixtures did not result in different inhibition factors. The calcium and zinc ion binding study showed that the freeze-drying and the ^{60}Co irradiation method could result in multifunctional polymers with the highest ion binding capacity. The combination of a high in vitro proteolytic inhibition and a pronounced binding ability for the bivalent ions calcium and zinc makes these polymers promising excipients for a successful oral peptide and protein drug delivery. However, in vivo studies with insulin as model peptide revealed that in vitro inhibition and ion binding studies can be used to make a first selection of potential excipients, but they can not be used to predict in vivo absorption. After a selection of potential excipients for oral peptide delivery, it is even more important to evaluate and optimise different formulation possibilities with respect to peptide stability in and absorption from the gastro-intestinal tract.

The starch-*g*-poly(acrylic acid) copolymers, the freeze-dried and spray-dried starch/carboxylated polymer mixtures were evaluated as potential buccal and vaginal bioadhesive drug carriers (*Chapter 3 Multifunctional Polymers as Bioadhesive Drug Carriers*).

In *Chapter 3.2* the bioadhesive properties of the multifunctional polymers were measured using an ex vivo bioadhesion test method and compared to a reference formulation (physical mixture of 5% Carbopol® 974P with a pregelatinised waxy corn starch). Grafted starches prepared by ^{60}Co irradiation as well as by chemical modification showed good ex vivo bioadhesive properties. By freeze-drying starch/poly(acrylic acid) mixtures, only the powders containing 75% (w/w) poly(acrylic acid) showed good compression properties and performed well during the ex vivo bioadhesion test. There was no difference observed in bioadhesive capacity between linear poly(acrylic acid), sodium carboxymethylcellulose (CMC) or Carbopol® 974P as carboxylated polymer in spray-dried starch/carboxylated polymer mixtures. However, the CMC based powders showed a very poor compression behaviour and can therefore not be used in buccal bioadhesive tablet formulations. It was observed that the powders obtained by spray-drying starch/poly(acrylic acid) mixtures showed better bioadhesive properties compared to the freeze-dried ones. The most promising bioadhesive powders were obtained by spray-drying Amioca® starch/Carbopol® 974P mixtures. By spray-drying the bioadhesive

capacities were significantly improved compared to equivalent physical mixtures of Amioca® and Carbopol® 974P.

In *Chapter 3.3* the mucosal irritation potential of the different multifunctional polymers was evaluated with a mucosal irritation test using slugs. It was concluded that grafted starches prepared by ^{60}Co irradiation or chemical modification both induced irritation of mucosal tissues. Spray-dried starch/poly(acrylic acid) mixtures containing 50 and 75% (w/w) poly(acrylic acid) were irritating on mucosal surfaces and the irritation potential increased with increasing poly(acrylic acid) concentrations. In vivo evaluation in dogs confirmed these findings. Spray-dried Amioca® starch/Carbopol® 974P mixtures containing up to 20% Carbopol® 974P did not show a distinct sign of irritation. These powders can be incorporated in bioadhesive formulations without risk of irritation and are potential safe mucoadhesive carriers. With 25% or more Carbopol® 974P the Amioca® granules are probably completely surrounded by Carbopol®. When the slugs are brought into contact with these powders the contact surface is 100% Carbopol® 974P, resulting in irritation of the slugs' mucosa.

In *Chapter 3.4*, the buccal absorption of testosterone from a bioadhesive tablet formulation based on the different multifunctional polymers was investigated in vivo in dogs and compared to the above mentioned reference formulation. The chemically modified grafted starch released the model drug testosterone rather fastly. A ^{60}Co irradiated grafted starch and a freeze-dried Amioca®/poly(acrylic acid) mixture did not reach the absolute bioavailability data of the reference formulation. The grafted starches and the freeze-dried starch/poly(acrylic acid) mixtures showed to be promising (buccal) bioadhesive carriers, although, due to their mucosal irritation potential, they can only be used for single applications and not in chronic treatments. By spray-drying Amioca®/Carbopol® 974P mixtures at different ratios a range of potential bioadhesive carriers was obtained. By ranging the Carbopol® 974P concentration between 5 and 20%, the in vivo adhesion time of placebo tablets could be varied between 8 and 17h. The data from the in vivo adhesion time study correlated well with the in vitro miconazole release profiles (USP III dissolution). A spray-dried Amioca®/Carbopol® 974P 80/20 mixture could be loaded with 60% drug without loosing its in vivo bioadhesive and pharmacokinetic properties.

In *Chapter 3.5* the single application of a metronidazole vaginal bioadhesive tablet based on a spray-dried Amioca® starch/Carbopol® 974P 85/15 mixture was evaluated in

12 female healthy volunteers. The vaginal bioadhesive tablet is applied on the uterine cervix and serves as a platform for local vaginal drug delivery. The clinical study proved that a spray dried Amioca® starch/Carbopol® 974P 85/15 mixture can be used as a bioadhesive vaginal carrier and can prolong the drug release over 5 days, resulting in prolonged local vaginal drug concentrations. The in vivo residence time of a vaginal bioadhesive metronidazole tablet could be assessed by measuring plasma concentrations.

In *Chapter 3.6* the efficacy of a buccal bioadhesive nystatin tablet on the device life of a Provox™ tracheoesophageal voice prosthesis was investigated in postlaryngectomy patients. Aiming at an in vivo adhesion time of ± 8 h, a spray-dried Amioca®/Carbopol® 974P 90/10 mixture was selected as bioadhesive carrier. The study revealed that a daily application of a nystatin bioadhesive slow-release tablet could significantly increase the lifetime of a Provox™ voice prosthesis in laryngectomised patients compared to the conventional local cleaning of the prosthesis with an anti-microbial agent on a brush.

In *Chapter 3.7* the spray-dried Amioca®/Carbopol® 974P 80/20 mixture (SD 80/20) was selected for accelerated stability testing during 12 months at 40°C and 75% relative humidity (RH). Storage of SD 80/20 at 40°C/75%RH, both stored as bulk powder and next compressed and stored as tablets from the start, resulted in a significant decrease of bioadhesive properties. The in vitro drug release profiles of stored tablets were stable for the whole test period (12 months). When stored as bulk powder prior to compression, after 1 month 50% of the drug was released after 2.5 h compared to 7.5 h before storage. Spray-dried Amioca®/Carbopol® 974P mixtures, as bulk powder as well as formulated as tablets, have to be stored under controlled conditions.

Samenvatting

Multifunctionele polymeren worden gebruikt als excipiëntia voor de ontwikkeling van gecontroleerde vrijgavesystemen, bij orale toediening van peptiden, en als bioadhesieve dragers voor buccale, nasale, vaginale en orale toediening van geneesmiddelen. Multifunctionele polymeren zijn hydrofiele macromoleculen. Sommigen bezitten goede bio- of mucoadhesieve eigenschappen, verlengen de verblijftijd van het toedieningssysteem ter hoogte van de absorptieplaats en versterken het contact tussen toedieningssysteem en mucosa. Toedieningssystemen op basis van multifunctionele polymeren kunnen aangebracht worden op specifieke mucosale weefsels, zoals de buccale, nasale en vaginale mucosa, om zo de biologische beschikbaarheid van het geneesmiddel te verhogen. Multifunctionele polymeren zouden ook de absorptie van peptiden over mucosale weefsels kunnen verbeteren door het verhogen van hun permeabiliteit en de inhibitie van proteolytische enzymes.

In dit doctoraatsproefschrift werden multifunctionele polymeren aangemaakt door het enten (*grafting*) van poly(acrylzuur) ketens op zetmelen (*Hoofdstuk 1.1 Synthesis and Process Details*). Twee verchillende methodes werden geëvalueerd om dergelijke *grafted starches* te bekomen: bestraling met een ^{60}Co bron en chemische modificatie. Door bestraling of door chemische modificatie kunnen poly(acrylzuur) ketens geënt worden op de zetmeelmolecules. Verschillende zetmeelsoorten, verschillende verhoudingen zetmeel/acrylzuur en een verschillende graad van acrylzuurneutralisatie werden geëvalueerd.

Een tweede reeks multifunctionele polymeren werd aangemaakt door het vriesdrogen of sproeidrogen van mengsels van zetmeel en polymeren met carboxylfuncties. Amioca® (amylopectine maïs) werd als zetmeel gebruikt. Een lineair poly(acrylzuur), een vernet poly(acrylzuur) (Carbopol® 974P) en natriumcarboxymethylcellulose werden gebruikt als polymeren.

In *Hoofdstuk 1.2* werden de gesproeidroogde Amioca®/Carbopol® 974P mengsels geanalyseerd met *Scanning Electron Microscopy* (SEM) en *solid state NMR (Nuclear Magnetic Resonance) spectroscopy*. SEM en NMR analyse toonden aan dat tijdens het sproeidrogen van Amioca® zetmeel/Carbopol® 974P mengsels, er Carbopol® films

worden gevormd rond de zetmeelgranules. Bij hogere Carbopol®-concentraties (75% w/w), worden naast filmvorming ook individuele Carbopol®-nanopartikels teruggevonden.

De *grafted starches*, de gevriesdroogde en de gesproeidroogde zetmeel/polymeer mengsels werden geëvalueerd als potentiële excipiëntia voor orale toediening van peptiden (*Hoofdstuk 2 Multifunctional Polymers for Oral Peptide Drug Delivery*). De twee grootste barrières voor een succesvolle orale peptidetoediening zijn enzymatische afbraak en de absorptie over het gastro-intestinaal epithelium. De *in vitro* inhibitie van het proteolytisch enzyme trypsine door de diverse multifunctionele polymeren werd onderzocht. Daar de meeste proteolytische enzymes Ca^{2+} of Zn^{2+} ionen gebonden hebben ter hoogte van hun actieve plaatsen en aangezien een reductie van de extracellulaire Ca^{2+} concentratie de *tight junctions* kan openen, werd de Ca^{2+} en Zn^{2+} bindingscapaciteit van de multifunctionele polymeren bepaald.

In *Hoofdstuk 2.2* werd een *in vitro*-trypsine-inhibitietest biochemisch geoptimaliseerd en gevalideerd. In eerder beschreven trypsine-activiteitstesten werd de enzymatische reactie biochemisch nooit geoptimaliseerd. De gebruikte substraatconcentraties waren te laag in verhouding tot de enzymatische activiteit of de enzymatische activiteit was te hoog voor de aanwezige hoeveelheid substraat, met als gevolg dat de metabolietconcentratie-tijdsprofielen zeer snel een plateau of *post-steady state* bereikten door uitputting van het substraat. Verscheidene substraatconcentraties en trypsine-activiteiten werden geëvalueerd met als doel het lineair of *steady state* stuk van de metabolietconcentratie-tijdscurve van de enzymatische reactie uit te vergroten. De inhibitietesten werden dan uitgevoerd onder *steady state* condities waarbij de afbraaksnelheid onafhankelijk is van de substraatconcentratie. De mate van enzyme-inhibitie werd weergegeven door de inhibitiefactor (IF), die werd gedefinieerd als de verhouding van de reactiesnelheid zonder polymeer tot de reactiosnelheid in aanwezigheid van polymeer. De IF-waarde is een goede maat voor de *in vitro* inhibitiecapaciteit van multifunctionele polymeren tegenover gastro-intestinale proteolytische enzymen.

In *Hoofdstuk 2.3* werden de *grafted starches*, de gevriesdroogde en de gesproeidroogde zetmeel/polymeer mengsels geëvalueerd als potentiële excipiëntia voor orale toediening van peptiden door het meten van de *in vitro* trypsine inhibitie- en Ca^{2+} en

Zn^{2+} bindingscapaciteit van deze multifunctionele polymeren. Carbopol® 934P werd als referentiepolymeer gebruikt. Thermisch behandelde gevriesdroogde zetmeel/poly(acrylzuur) mengsels hadden het grootste *in vitro* trypsine inhibitiepotentieel, gelijk aan of zelfs groter dan voor het referentiepolymeer. De ^{60}Co bestraalde en de chemisch gemodificeerde *grafted starches* hadden lagere IF-waarden dan de referentie. Ook de gesproeidroogde zetmeel/poly(acrylzuur) mengsels hadden allen een lagere IF-waarde dan de referentie. Vernet Carbopol® 974P of lineair poly(acrylzuur) in de gesproeidroogde mengsels gaf geen verschil in inhibitiefactor. De calcium- en zinkionenbindingsstudie toonde aan dat vriesdrogen en ^{60}Co bestraling resulteerde in multifunctionele polymeren met de grootste ionenbindingscapaciteit. De combinatie van een grote *in vitro* proteolytische enzyme-inhibitie en een uitgesproken ionenbindingscapaciteit maakt van deze polymeren potentiële excipiëntia voor een succesvolle orale toediening van peptiden. *In vivo* studies met insuline als modelpeptide toonden echter aan dat *in vitro* inhibitie- en ionenbindingsstudies kunnen gebruikt worden om een eerste selectie van potentiële excipiëntia te maken, echter zonder predictieve waarde naar *in vivo* absorptie toe.

De multifunctionele polymeren werden in *Hoofdstuk 3 Multifunctional Polymers as Bioadhesive Drug Carriers* geëvalueerd als buccale en vaginale bioadhesieve dragers.

In *Hoofdstuk 3.2* werden de bioadhesieve eigenschappen gemeten met een *ex vivo* bioadhesietest en vergeleken met een referentieformulatie (een fysisch mengsel van 5% Carbopol® 974P met een gepregelatiniseerd maïszetmeel). *Grafted starches*, zowel aangemaakt via ^{60}Co bestraling als met behulp van chemische modificatie, vertoonden goede bioadhesieve eigenschappen. Van de gevriesdroogde zetmeel/poly(acrylzuur) mengsels, had alleen het mengsel met 75% (w/w) poly(acrylzuur) goede compressie-eigenschappen en vertoonde een goede *ex vivo* bioadhesie. Er was geen verschil in bioadhesieve capaciteit tussen lineair poly(acrylzuur), natriumcarboxymethylcellulose (CMC) of Carbopol® 974P in de gesproeidroogde mengsels. De CMC-poeders lieten zich moeilijk comprimeren waardoor deze niet geschikt zijn voor het formuleren van (buccale) bioadhesieve tabletformulaties. Gesproeidroogde zetmeel/poly(acrylzuur) mengsels waren beter adhesief dan de gevriesdroogde. De beste resultaten werden bekomen met de gesproeidroogde Amioca®/Carbopol® 974P mengsels. De bioadhesieve capaciteiten van

Amioca®/Carbopol® 974P mengsels waren significant verhoogd na sproeidrogen vergeleken met gelijkaardige fysiche mengsels.

In *Hoofdstuk 3.3* werd de mucosale irritatie, geïnduceerd door de verschillende multifunctionele polymeren geëvalueerd met een mucosale irritatietest op naaktslakken. De *grafted starches*, zowel deze gesynthetiseerd via ^{60}Co bestraling als via chemische modificatie, induceerden beiden irritatie van de mucosale weefsels. Gesproeidroogde zetmeel/poly(acrylzuur) mengsels met 50 en 75% (w/w) poly(acrylzuur) waren irriterend voor de mucosa en het irriterend effect werd groter met een stijgende poly(acrylzuur) concentratie. *In vivo* evaluatie bij de hond bevestigde dit. Gesproeidroogde Amioca®/Carbopol® 974P mengsels die tot 20% Carbopol® 974P bevatten, toonden geen enkel teken van mucosale irritatie. Deze poeders kunnen zonder enig gevaar voor irritatie in bioadhesieve formulaties worden gebruikt. Bij 25% of meer Carbopol® 974P zijn de Amioca® granules meer dan waarschijnlijk volledig omgeven met Carbopol®. Wanneer de slakken met deze poeders in contact worden gebracht, bestaat het contactoppervlak uit 100% Carbopol®, met mucosale irritatie tot gevolg.

In *Hoofdstuk 3.4* werd de buccale absorptie van testosteron uit een bioadhesieve tablet op basis van de verschillende multifunctionele polymeren bestudeerd bij de hond en vergeleken met de hierboven beschreven referentieformulatie. Met een chemisch gemodificeerd *grafted starch* werd het modelgeneesmiddel, testosteron, vrij snel vrijgegeven. Een ^{60}Co bestraald *grafted starch* en een gevriesdroogd Amioca®/poly(acrylzuur) mengsel konden de absolute biologische beschikbaarheid verkregen met de referentieformulatie niet evenaren. De *grafted starches* en de gevriesdroogde zetmeel/poly(acrylzuur) mengsels bezitten een potentieel als (buccale) bioadhesieve dragers, alhoewel, daar ze mucosale irritatie induceerden, ze enkel kunnen gebruikt worden voor éénmalige applicaties en dus zeker niet bij chronische behandelingen. Door Amioca®/Carbopol® 974P mengsels te sproeidrogen in verschillende verhoudingen, bekomt men een brede reeks potentiële bioadhesieve dragers. Met een Carbopol® 974P-concentratie tussen 5 and 20%, kan de *in vivo* adhesietijd gevarieerd worden tussen 8 en 17 uur. De *in vivo* adhesietijden van placebo-tabletten bij de hond correleerden goed met de *in vitro* miconazole-vrijstellingsprofielen bekomen in een USP III dissolutiesysteem. Een gesproeidroogd Amioca®/Carbopol® 974P 80/20 mengsel kon worden beladen met 60% testosteron zonder verlies van zijn *in vivo* bioadhesieve en pharmacokinetische eigenschappen.

In *Hoofdstuk 3.5* werd de éénmalige toediening van een metronidazole vaginale bioadhesieve tablet, geformuleerd op basis van een gesproeidroogd Amioca®/Carbopol® 974P 85/15 mengsel, geëvalueerd bij 12 vrouwelijke gezonde vrijwilligsters. De vaginale bioadhesieve tablet werd op de *portio uteri* gekleefd en zorgt voor lokale vaginale metronidazolevrijgave. De klinische studie toonde aan dat een gesproeidroogd Amioca®/Carbopol® 974P 85/15 mengsel kan gebruikt worden als bioadhesieve vaginale drager. De metronidazole vrijgave kon gedurende 5 dagen worden aangehouden. De verblijftijd van de vaginale bioadhesieve tablet kon worden gevolgd via plasma metronidazoleconcentraties.

In *Hoofdstuk 3.6* werd het effect van een buccale bioadhesieve nystatine tablet op de levensduur van een Provox™ spraakprothese onderzocht in gelaryngectomiseerde patienten. Een gesproeidroogd Amioca®/Carbopol® 974P 90/10 mengsel werd geselecteerd als bioadhesieve drager, met als doel een *in vivo* adhesietijd van ongeveer 8 uur. De studie toonde aan dat een dagelijkse applicatie van een nystatine bioadhesieve tablet de levensduur van de spraakprothese significant kon verlengen vergeleken met de conventionele lokale reiniging van de prothese met een nystatinesuspensie.

In *Hoofdstuk 3.7* werd het gesproeidroogd Amioca®/Carbopol® 974P 80/20 mengsel (SD 80/20) geselecteerd voor een versnelde stabiliteitsstudie over 12 maanden bij 40°C and 75% relatieve vochtigheid (RV). Bewaring van SD 80/20 bij 40°C/75%RV, zowel als bulk poeder als onder de vorm van tabletten, resulteerde in een significante daling van de bioadhesieve eigenschappen. De *in vitro* miconazole-vrijstellingsprofielen uit bewaarde tabletten waren stabiel gedurende 12 maanden, terwijl de vrijstelling uit bewaard bulk poeder reeds na 2 weken versneld was. Gesproeidroogde Amioca®/Carbopol® 974P mengsels, zowel als bulk poeder als onder de vorm van tabletten, moeten bewaard worden onder gecontroleerde omstandigheden.