IN VITRO EVALUATION OF THE TOXICITY PROFILE OF N-TRIMETHYL CHITOSAN CHLORIDE

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Abstract

The pharmaceutical scientist is confronted by numerous challenges in the area of developing new and effective delivery systems for new classes of drugs such as peptide and protein drugs. Due to the size and polarity of these drugs they are excluded from the normal transcellular transport pathway. The co-administration of absorption enhancers has proven to be an effective way of increasing the delivery of peptide and protein drugs. However, they have also been shown to cause significant cell damage and their use are considered rather impractical due to the fact that their toxicity was directly related to the mechanism of enhanced absorption.

Chitosan opens the paracellular pathway by acting on the tight junctions but is only effective in acidic environments. *N*-trimethyl chitosan chloride (TMC), a partially quaternised derivative of chitosan, has proven to be effective in neutral and basic environments for opening of tight junctions to increase paracellular transport. However, limited toxicity data is available for this absorption enhancing polymer. In this study TMC polymers with different degrees of quaternisation were synthesised (12 – 53 %) and evaluated for their possible toxic effects. *In vitro* cytotoxicity assays performed on human intestinal epithelial cell cultures (Caco-2) included the MTT and the LDH assay. Propidium iodide staining of Caco-2 cell monolayers was used to visualise possible cell membrane damage caused by the polymer.

Results of these assays indicated significant toxic effects for the synthesised polymers. A concentration dependent decrease in cell metabolic activity was seen with the MTT assay while the LDH assay and propidium iodide staining of the Caco-2 cell monolayers indicated that these polymers cause significant cell membrane damage. However, no direct correlation could be seen between the different toxicity assays. TMC-12 (degree of quaternisation = 12 %), which was not synthesised in our laboratory, showed no toxic effects. It was concluded that the polymers synthesised in our laboratory were not safe but should be investigated further to determine the cause of the toxicity.

Keywords: Absorption enhancers, N-trimethyl chitosan chloride, Toxicity, MTT assay, LDH assay, Propidium iodide staining

Uittreksel

Nuwe generasie geneesmiddels, soos proteïen- en peptiedgeneesmiddels, vereis die ontwikkeling van nuwe en effektiewe afleweringsisteme. Weens hierdie geneesmiddels se molekulêre grootte en polariteit is dit onmoontlik vir hulle om deur die transsellulêre transportroete opgeneem te word. Daar is egter bewys dat die gesamentlike toediening van absorpsiebevorderaars die absorpsie van hierdie middels bevorder. Ongelukkig beskik die meeste van hierdie absorbsiebevorderaars oor toksiese eienskappe wat direk met die megansime van absorbsiebevordering verband hou.

Chitosaan bevorder parasellulêre transport deurdat dit op die hegte aansluitingskomplekse tussen epiteelselle inwerk, maar egter net by `n lae pH. *N*-trimetiel chitosaan chloried (TMC), `n gedeeltelik gekwaternariseerde derivaat van chitosaan, beskik egter oor absorbsiebevorderende eienskappe in neutrale en alkaliese omgewings. Parasellulêre transport word op dielselfde wyse deur hierdie polimeer verhoog as deur chitosaan naamlik deur die opening van hegte aansluitingskomplekse. Om die beperkte toksisiteitdata van TMC aan te vul is hierdie polimeer met verskillende grade van kwaternarisering gesintetiseer. Die MTT en LDH analises, beide *in vitro* toksisiteitmetodes, is op selkulture van menslike afkoms uitgevoer. Die Caco-2 selkultuurlyn wat gebruik is boots epiteelmembrane teenwoordig in die liggaam na indien dit in `n monolaag gegroei word. Selskade aangerig deur die polimere is ook visueel ondersoek nadat die monolaag met propidiumjodied gekleur is.

Beduidende toksisiteit is vir alle gesintetiseerde polimere aangetoon. Propidiumjodied kleuring sowel as die LDH analise het getoon dat betekenisvolle skade aan die selmembrane van die Caco-2 selle aangerig word terwyl `n duidelike konsentrasie afhanklike afname in metaboliese aktiwiteit met die MTT analise waargeneem is. Daar was egter geen direkte verband tussen die verskillende toksisiteitstudies nie. Slegs een polimeer het geen toksiese effekte getoon nie, nl. TMC-12, met `n graad van kwaternarisering gelyk aan 12 %. Hierdie polimeer is egter nie in ons laboratorium

gesintetiseer nie. Ons gevolgtrekking uit hierdie resultate is dat alle polimere wat in ons laboratorium gesintestiseer is, onveilig is. Hierdie polimere moet egter verder ondersoek word om die oorsaak van hierdie toksiese effekte vas te stel.

Sleutelwoorde: Absorpsiebevorderaars, N-trimetiel chitosaan chloried, Toksisiteit, MTT analise, LDH analise, Propidium jodied kleuring.

Introduction and aim of study

Due to the enormous advances in drug design and biotechnology it is now possible to produce significant quantities of several new classes of drugs such as peptide and protein drugs. These substances could be seen as mere templates for more advanced peptidomimetic agents that will have a major impact on the treatment of medical disorders, now and in the future. Currently these hydrophilic macromolecules are mainly administered by invasive parenteral injection. Their size and physicochemical properties have excluded these hydrophilic compounds from solid dosage formulations for peroral delivery, which is the most patient compliant way of drug administration.

In recent years the pharmaceutical applications of chitosan and its derivatives has captured the attention of many researchers. The absorption enhancing effects of these polysaccharides have been intensively studied and it was shown that these compounds are potent absorption enhancers. Special emphasis has been placed on the absorption enhancing properties of *N*-trimethyl chitosan chloride (TMC), a partially quaternised derivative of chitosan, due to its solubility in neutral and basic environments. Chitosan is only soluble in acidic environments and is therefore incapable of enhancing absorption in the small intestine and colon, the main absorption areas in the gastrointestinal tract.

Before any foreign substance can be approved for human administration it must be subjected to extensive cytotoxicity testing to ensure the safety of these compounds. The use of cell cultures in the evaluation of the toxicity profiles of these substances have proved to be an effective and reliable way in determining toxicity profiles. Although there is extensive proof of the absorption enhancing properties of TMC, toxicity data on TMC is limited. Possible factors that might have an effect on its toxicity profile is the charge density, as determined by the degree of quaternisation, as well as the concentration in which the polymer is administered.

The general aim of this investigation was to synthesise TMC polymers with different degrees of quaternisation and to evaluate the effect that this increase in charge density might have on the viability of intestinal epithelial cells (Caco-2) *in vitro*.

Specific objectives of this study include:

1. A literature study on
   - Chitosan and TMC as absorption enhancers, and
   - the *in vitro* cytotoxicity evaluation of pharmaceutical compounds.
2. Synthesis and characterisation of TMC polymers with degree of quaternisation between 12 and 60 %.
3. Selection of suitable cytotoxicity assays.
4. Evaluation of the effect of the degree of quaternisation and concentration on the toxicity profile of TMC in neutral environments.

Chapter 1 will focus on the absorption enhancing properties of chitosan and TMC while chapter 2 will focus on the selection of suitable *in vitro* cytotoxicity assays. In chapter 3 the experimental procedures for synthesising TMC polymers with degrees of quaternisation between 12 and 60 % will be discussed. The procedures for the *in vitro* evaluation of the toxicity profiles of these TMC polymers will also be described in this chapter. In chapter 4 the results of these studies will be presented and discussed.

CHAPTER 1

CHITOSAN AND *N*-TRIMETHYL CHITOSAN CHLORIDE FOR INCREASED DELIVERY OF PEPTIDE AND PROTEIN DRUGS

1.1 INTRODUCTION

Peptides and proteins were initially of extractive origin, which gave way to synthesis and by the turn of the century development in biotechnology and recombinant DNA techniques facilitated the accessibility of a purer grade of proteins and peptides. Advances made in the understanding of the role of the multitude of these regulatory agents in physiology and pathology, coupled with their availability in sufficient quantities and a much purer form, at reasonable costs, with reduced immunogenecity and antigenecity, have expanded their field of application and now they represent an increasingly important group of drugs.

The critical issue when designing an effective delivery system is to ensure predictable and reproducible absorption without wasting up to 99 % of the drug. Normally poor bioavailability of peptides and proteins from oral and non-oral mucosal routes is a result of the interplay of poor permeability characteristics, instability towards proteolytic enzymes, cell metabolism and non-enzymatic clearance mechanisms such as the first pass effect and excretion in the bile (Vyas *et al.*, 1997:339). Protease inhibitors and absorption enhancers are means often considered to circumvent the enzymatic and absorption barriers to peptide and protein absorption from mucosal routes of administration.

In this chapter, the use of absorption enhancers will be discussed, with special emphasis on chitosan and *N*-trimethyl chitosan chloride as a means to increase the delivery of peptide and protein drugs.

1.2 THE USE OF ABSORPTION ENHANCERS

For most therapeutic agents, administration via a non-parenteral route is the preferred choice, with the oral route as the main preference (Lee & Yamamoto, 1990:172). However, poor absorption or highly variable absorption of the therapeutic agent may limit the development of a non-parenteral dosage form. If the limited absorption of a compound is due to it's inability to cross biological membranes, rather than any problems with instability or pre-absorptive metabolism, then the co-administration of an absorption enhancing agent offers a potential means for overcoming this barrier (Fix, 1987:151).

Since 1961, studies were performed on absorption enhancement in the gastrointestinal tract with the use of chelating agents and surfactants. EDTA and sodium lauryl sulphate have been shown to promote the intestinal absorption of impermeable drugs. However, they have also been shown to cause significant cell damage and their use are considered rather impractical due to the fact that their toxicity was directly related to the mechanism of enhanced absorption (Muranishi, 1990:2). Since then numerous absorption enhancers have been studied and classified but in most of the cases increased drug absorption is accompanied by damage to mucosal surfaces (Schipper *et al.*, 1996:1686). The ideal enhancer should be non-toxic and act in a reversible way and should furthermore be effective from the low pH in the stomach up to the very basic pH in the colon, being most effective in the small intestine (Kotzé *et al.*, 1999b:343).

Molecules can pass the intestinal epithelium by two parallel pathways: paracellularly (between the cells) or transcellularly (through the cells) as depicted in figure 1.1. The transcellular flux of compounds occurs by passive, facilitated or active processes, whereas the paracellular flux occurs strictly by passive diffusion. The properties of both solute (drug) and membrane will judge if the solute will be transported through the paracellular or transcellular route. Moderate and highly lipophilic compounds diffuse passively across the barrier set by epithelial membranes while polar, membrane-impermeable molecules diffuse through the paracellular route, which is controlled by tight junctions (Thanou, 2000:18 and Schipper *et al.*, 1996:1686).

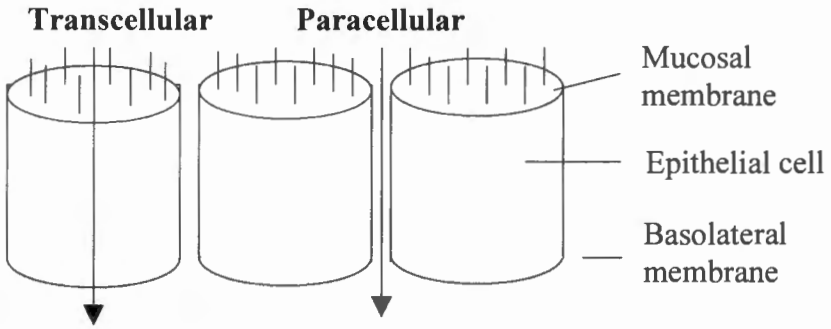


Figure 1.1 Schematic representation of the paracellular and transcellular pathways across intestinal epithelia (Thanou, 2000:18).

The paracellular pathway has gained interest in recent years because of the perception that it is deficient in proteolytic activity, maximizing the amount of peptides and proteins absorbed intact. The barrier to paracellular diffusion of molecules and ions across the epithelial cell layer is the tight junction or zonula occludens, whose integrity has long been known to depend on the extracellular Ca^{2+} concentration, which if lowered has been shown to enhance the permeability of the tight junction. Some other means of enhancing the permeability of the tight junctions include the enhancing of Na^{+} transport, promoting of glucose and amino transport or altering of the actin filaments with certain compounds (Lee, 1990:217).

In recent years chitosan has attracted a great deal of attention as a potential absorption enhancer across mucosal epithelia. This linear polysaccharide derived by *N*-deacetylation of the natural polymer chitin, which is the second most abundant naturally occuring polymer in nature, has already been approved as a food additive in Japan and is believed to be non-toxic. Except for the food industry, chitosan has also been applied in the agricultural and the cosmetic industry and advantages of this polymer include high availability, low cost, high biocompatibility, biodegradability and ease of chemical modification. Although chitosan has been widely used by these industries it was only in recent years that it's potential application in the pharmaceutical field has been identified by several scientists (Kotzé *et al.*, 1999b:344). The remainder of this chapter will focus

on chitosan and a chitosan derivative and their potential use in the delivery of peptide and protein drugs.

1.3 CHITOSAN

Chitosan, $(C_6H_{11}O_4N)_n$, depicted in figure 1.2, consists of linear 1-4 linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-β-D-glucopyranose (GlcN) units which is derived from chitin, the main component of shells of crab, shrimp and krill, by alkaline *N*-deacetylation (Kaş, 1997:689 and Borchard *et al.*, 1996:132). If the degree of *N*-acetylation of chitin is lowered to less than 50 % it becomes soluble in acidic solutions and is referred to collectively as chitosans (Le Dung *et al.*, 1994:209). The solubility of chitosans (pK_a 5.5 – 5.6) are obtained only under acidic conditions and is due to the protonation of the amino group of the D-glucosamine monomeric units (Domard, *et al.*, 1986:105). Chitosans are polysaccharides individually characterised by their ratio of acetylated to deacetylated units as well as their high molecular weight, both parameters being equally responsible for the properties of the polymer (Aspden *et al.*, 1995:69).

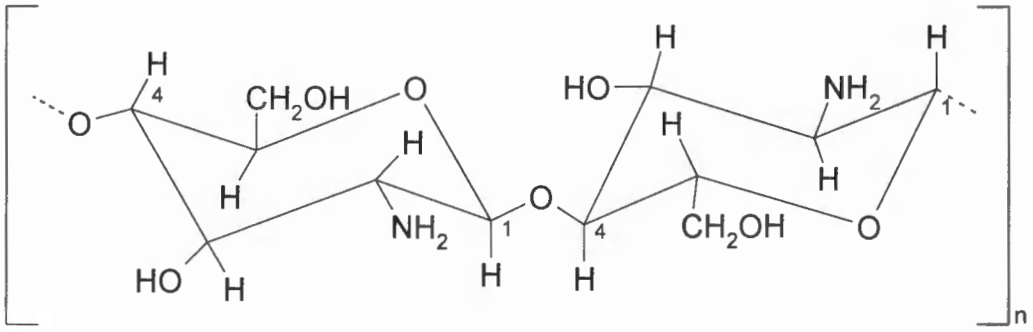


Figure 1.2 The chemical structure of chitosan.

Different grades of chitosans are commercially available; some of which are ultrapure and even well suited for implantation. These grades depend on the manufacturing process, which is briefly outlined in figure 1.3, and are assessed by determining the levels of heavy metals and proteins present in the chitosan, as well as the pyrogenicity and cytotoxicity of the chitosan (Kaş, 1997:690).

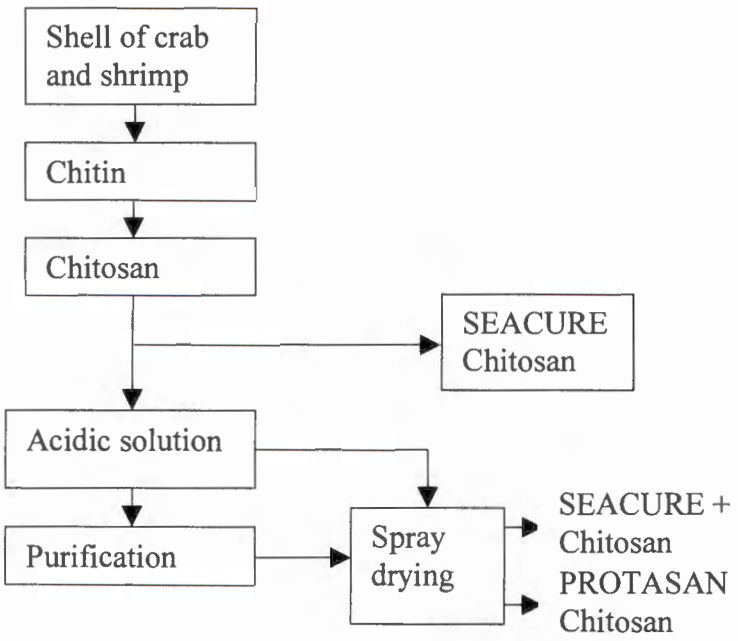


Figure 1.3 Production of chitosan (Skaugrud, 1995:103).

Chitosan is also known for it's biological properties namely, biocompatibility and degradability, wound healing acceleration, reduced blood cholesterol levels as well as it's immune stimulant effect (Kaş, 1997:690).

1.3.1 PHARMACEUTICAL APPLICATIONS OF CHITOSAN

As previously mentioned chitosan has been used for a range of applications ranging from a food additive, to a water purification agent as well as to numerous pharmaceutical applications (Aspden, 1995:70 and Hirano *et al.*, 1988:897). The popularity of this polymer is attributed to its high availability, low cost and ease of chemical modification due to the many functional groups available for modification. Chitosan has been used in the pharmaceutical industry as a tablet binder (Upadrashta *et al.*, 1992:1707) as well as a disintegrant (Ritthidej *et al.*, 1994:2130). In the low pH range chitosan has gel-forming properties and is used as a drug carrier in hydrocolloids and gel formulations (Knapczyk, 1993:233). If compared to other polysaccharides, which are usually neutral or negatively charged, the uniqueness of this acid soluble, cationic biopolymer is apparent when used in drug delivery systems. The main areas of application for chitosan in drug delivery

systems are its use as a constituent in matrix systems, as a bioadhesive material and its use in immobilisation and encapsulation. A preliminary trial with ocular studies in rabbits showed that the clearance rate for chitosan compared to saline solutions is significantly lower. It was evident that the chitosan in this trial was not easily washed off, but stuck to the negatively charged surface of the cornea and also influenced the drainage of the lacrimal fluid, resulting in a lowered clearance rate. These results were due to the bioadhesive properties of chitosan (Skaugrud, 1995:104). Under the general classification of bioadhesive properties, the more specific mucoadhesion properties is of great importance, especially in the light of chitosan's ability to enhance the absorption of large hydrophilic molecules.

1.3.1.1 Mucoadhesive properties of chitosan

Bioadhesion has been defined as the attachment of synthetic or biological macromolecules to a biological tissue. The term mucoadhesion is a special case of bioadhesion applicable in situations where the biological tissue is epithelium covered with mucus. Peppas and Buri (1985:258) came to the conclusion that a number of polymer characteristics are necessary for mucoadhesion which can be summarized as follows: (i) strong hydrogen bonding groups (-OH, -COOH), (ii) strong anionic charges, (iii) high molecular weight, (iv) sufficient chain flexibility and (v) surface energy properties favouring spreading onto mucus.

However chitosan, with significant mucoadhesive properties, does not have any anionic charges but a positively charged hydrogel is formed in acidic environments that could develop additional molecular attraction forces by electrostatic interactions with negatively charged mucosal surfaces (Lehr *et al.*, 1992:43). It was also shown that molecular weight played a significant role in the mucoadhesive properties of chitosan with the best results obtained at the higher molecular weights (Lehr *et al.*, 1992:46).

Chitosan showed weak, short-lasting mucoadhesive properties in artificial gastric fluid and this could be explained by the solubility of the pure polymer in acidic solutions.

However, cationic polymers are likely to be superior mucoadhesives in a neutral or slightly alkaline medium as would be desirable in the small or large intestines. It was observed that chitosan underwent minimal swelling in artificial intestinal fluid, which is explained by the poor water solubility of the free base. Lehr *et al.* (1992:48) suggested that the swelling could be improved by substitution of the free amino groups with short alkyl chains in order to increase the pK_a and hence the ionisation of these groups at a higher pH, thus possibly also increasing mucoadhesion.

1.3.1.2 Chitosan as an absorption enhancer for hydrophilic drugs

In recent years chitosan has attracted a lot of attention as a potential absorption enhancer across mucosal epithelia especially for peptide drugs (Kotzé *et al.*, 1999b:343). Chitosan shows some favourable properties, namely:

- it is not absorbed, due to its high molecular weight, and therefore are not expected to display systemic toxicity;
- it intensifies the contact between the dosage form and the site of absorption due to its mucoadhesive properties;
- it improves peptide transport across the epithelial barrier (Lueβen *et al.*, 1994:336).

Lueβen *et al.* (1997:19) evaluated the potential of chitosan glutamate to improve the intestinal transport of 9-desglycinamide, 8-L-arginine vasopressin (DGAVP) *in vitro* by using Caco-2 cell monolayers as well as a rat vertically perfused intestinal loop model. Chitosan glutamate at a pH of 5.60 proved to be able to decrease the transepithelial electrical resistance (TEER) of these human intestinal cells. The TEER was reduced to 45 ± 2 % of the control value by a 1 % (w/v) chitosan glutamate solution. At concentrations of 0.4 and 1 % (w/v) chitosan glutamate strongly increased the transport of DGAVP (MW = 1412) across the Caco-2 cell monolayers with transport of 1.2 % of the total dose applied after 4 hours. The similarity in the transport between the two different chitosan glutamate concentrations indicates that at 0.4 % (w/v) a maximum in transport rate is reached. Chitosan glutamate also showed a pronounced and comparable

improvement of DGAVP absorption across intestinal mucosae in the vertically perfused loop model.

Chitosan glutamate and chitosan hydrochloride, at a pH of 6.20, lead to a pronounced reduction in TEER across Caco-2 cell monolayers. The reduction in TEER, 1 hour after apical incubation with 1.5 % (w/v) solutions of the polymers was in the following order: chitosan hydrochloride (71 ± 4 % reduction) > chitosan glutamate (64 ± 6 % reduction). Prolonging of the incubation time only resulted in slight decrease in the initial TEER reduction measured after 1 hour of incubation. In agreement with the reduction in TEER with 1.5 % (w/v) of the chitosan salts, the increase in transport of the peptide drug buserelin up to 4 hours was in the order chitosan hydrochloride (4.3 ± 0.3 % of the total dose applied) > chitosan glutamate (3.0 ± 0.9 % of the total dose applied). Similar increases in the transport of peptide drugs across the Caco-2 cell monolayer was also observed with insulin at a pH of 4.40. The same trend was noticed in this study, namely the highest increase in the transport of insulin was obtained with chitosan hydrochloride (Kotzé *et al.*, 1997a:248, 249)

Lueβen *et al.* (1996:1668) also evaluated the *in vivo* absorption enhancing effects of chitosan hydrochloride on the peptide drug buserelin. In this study, the following polymers were tested: carbomer 934P (C934P), its freeze-dried neutralized sodium salt (FNaC934P) and chitosan hydrochloride. Of all the polymers tested chitosan hydrochloride (1.5 % w/v) in a gel formulation resulted in the highest absolute bioavailability for intraduodenally administered buserelin in rats (5.1 ± 1.5 %), even higher than the value reported for the commercial nasal formulation Suprecur® (3.3 %) in men.

It is known that the absorption enhancing effect of chitosan on epithelial permeability is dependent on the pH of the solutions and it was seen that the effect of chitosan on the transport of the marker molecule, [^{14}C]-mannitol is the best when the pH is well below the pK_a of 6.5. At a higher pH the chitosan molecules exists in a more coiled configuration but as the pH decreases and the molecule becomes more ionised the

molecule uncoiles and assumes a more elongated shape. Hence, at the lower pH values the chitosan has a higher charge density and will have a better possibility for intimate contact with the epithelial membrane. This suggests that charge density might be of importance for enhancement of mucosal absorption (Artursson *et al.*, 1994:1359).

Besides pH, varying the degree of acetylation also controls the charge density of chitosan, since only the GlcN-units are positively charged. The influence of these parameters on the effects of chitosans on the epithelial permeability and toxicity were investigated in monolayers of the human intestinal epithelial cell line (Caco-2). Chitosan with a degree of acetylation between 1 and 49 % was used, each prepared in a low and high molecular weight form. In order to evaluate the toxicity profiles of the chitosans the mitochondrial dehydrogenase activity of the Caco-2 cells were measured (this method will be discussed in detail in chapter 2). The effect of the chitosans on cellular morphology was also studied with transmission electron microscopy.

The study revealed that the structural properties of chitosan, i.e. molecular weight and degree of acetylation, dictated absorption enhancing properties and toxicity largely. Chitosans with a low molecular weight (22 000) and a high degree of acetylation ($\geq$ 35 %) lacked absorption enhancement activity whereas chitosans with a low degree of acetylation and/or high molecular weight increased intestinal epithelial permeability. The correlation between molecular weight, degree of acetylation and effect on epithelial permeability is summarized in figure 1.4.

From these results it is clear that chitosans with a low degree of acetylation (1 and 15 %) are active as absorption enhancers at low and high molecular weights. However, these chitosans displayed a clear dose-dependent toxicity, which seemed to be influenced more by the degree of acetylation than by the molecular weight of the chitosans. Chitosans with degrees of acetylation of 35 and 49 % enhanced the transport of [^{14}C]-mannitol at high molecular weights only, with low toxicity. One chitosan, with a degree of acetylation equal to 35 % and a molecular weight of 170kD, was found to have especially

advantageous properties such as an early onset of action and very low toxicity (Schipper *et al.*, 1996:1686, 1689).

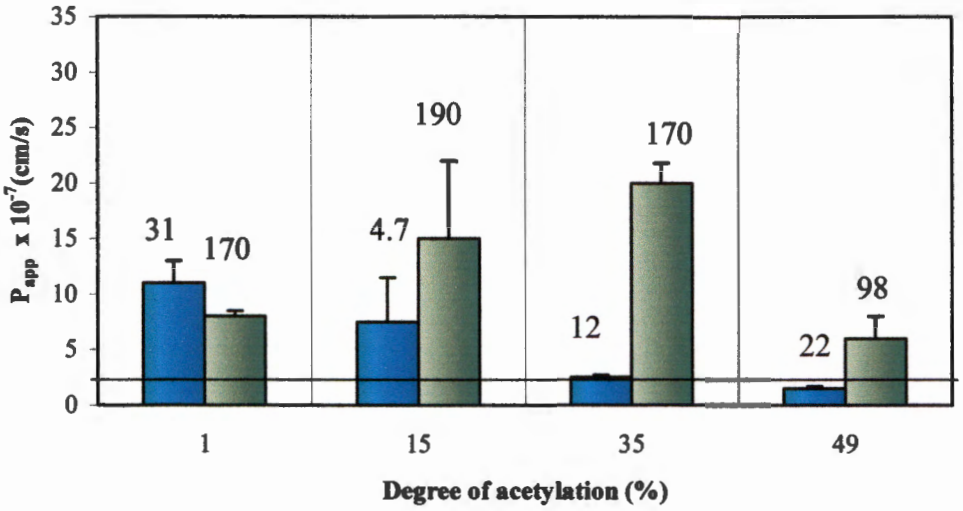


Figure.1.4 The mean apparent permeability coefficient (P_{app}) of [^{14}C]-mannitol across Caco-2 cell monolayers during 60 min exposure to 50 μg/ml chitosan. The numbers associated with the bars in the graph show the molecular weight of the studied chitosans in kD. The P_{app} of [^{14}C]-mannitol across untreated monolayers was 2.4 ± 0.2 (x 10^{-7}) cm/s, and is indicated in the figure by the horizontal line. Data represents the mean of 3-4 experiments and the error bars represent standard deviations (Schipper *et al.*, 1996:1691).

Schipper *et al.* (1996:1686) concluded that the structural features of chitosans determining absorption enhancement are not correlated with those determining toxicity, which makes it possible to select chitosans with maximal effect on absorption and minimal toxicity.

The absorption enhancing effects of chitosan are dependent on the concentration of the chitosan administered, however results showed that an unlimited increase in concentration did not lead to an unlimited increase in absorption enhancement. This saturable effect is in direct contrast to the non-saturable effect seen for surfactants and

bile salts, thus suggesting a different mechanism of absorption enhancement, namely paracellular absorption enhancement that depends on the opening of tight junctions. The increase in the transport of large hydrophilic compounds could be attributed to an interaction of a positively charged amino group on the C-2 position of chitosan with negatively charged sites on the cell membranes and tight junctions of the mucosal epithelial cells to allow opening of the tight junctions (Artursson *et al.*, 1994:1360). Confocal laser scanning microscopy has confirmed that chitosan is able to open the tight junctions to allow the paracellular transport of large hydrophilic compounds. It has also been reported that pharmacological agents, which interact with cytoskeletal F-actin simultaneously, increase the paracellular permeability. The redistribution of F-actin after the administration of chitosan was visualised by staining the F-actin with the fluorescent probe, rhodamine phalloidin (Schipper *et al.*, 1997:928).

1.3.2 THE NEED FOR CHITOSAN DERIVATIVES

In all the studies that have been mentioned, absorption enhancement was found only in acidic environments in which the pH was less or of the order of the pK_a value of chitosan (5.5 to 6.5). As previously mentioned, chitosan, a weak base, requires a certain amount of acid to transform the glucosamine units into the positively charged water-soluble form. Due to their charge loss in neutral and basic environments, chitosan precipitates from solution rendering it unsuitable as an absorption enhancer. At this pH, the molecule is most likely to exist in a coiled configuration (Kotzé *et al.*, 1999b:351).

In contrast to the reduction of TEER of Caco-2 cell monolayers found after the apical incubation with chitosan hydrochloride and chitosan glutamate at a pH of 6.20 no decrease in TEER, which is a good measurement of the tightness of the junctions between the cells, was observed at a pH of 7.40. At this pH, both chitosan salts did not form clear solutions. In agreement with the results of the TEER experiments, no increase in the transport of the hydrophilic model compound [^{14}C]-mannitol was found at a pH of 7.40 after incubation with these chitosan salts (Kotzé *et al.*, 1999a:149)

Most macromolecular pharmaceuticals such as peptide and protein drugs are indicated for chronic administration, and therefore possibilities for the potential use of chitosans in the more basic environments of the large intestine and colon, are limited. In this regard Kotzé *et al.* (1997b:1197) states that chitosan derivatives with different physicochemical properties, especially water solubility at neutral and basic pH values, will be of particular interest as they might prove to be useful as absorption enhancers in these environments. The pH of the nasal cavity is also in the order of 7.40, and therefore possibilities exist for the use of such chitosan derivatives as nasal absorption enhancers (Kotzé *et al.*, 1999b:351).

It was the hypothesis of Kotzé *et al.* (1999b:351) that polymers such as unmodified chitosan with a primary amino group may not be the optimal ones but that polymers or derivatives with different substituents, different basicities, or different charged densities will have the same or even increased efficacy in opening tight junctions.

As previously mentioned, chitosan is a versatile polymer with many functional groups available for chemical modification. In the past several chitosan derivatives have been synthesised, one of which is *N*-trimethyl chitosan chloride (TMC) (Domard *et al.*, 1986:105). However, these derivatives have only been evaluated for their pharmaceutical applications in the last few years. TMC, a partially quaternised derivative of chitosan, has intensely been studied and described by Kotzé *et al.* (1999b:351) for its absorption enhancing effects. It was concluded that the potential use of TMC, in neutral and basic environments where normal chitosan salts are ineffective as absorption enhancers, could contribute significantly to the effective delivery of hydrophilic compounds such as protein and peptide drugs. Recently another derivative of chitosan was also evaluated for its absorption enhancing ability, namely mono-N-carboxymethyl chitosan (MCC). The absorption enhancing effects of these two polymers will be discussed in more detail in the following sections.

1.4 N-TRIMETHYL CHITOSAN CHLORIDE (TMC)

1.4.1 PHYSICOCHEMICAL PROPERTIES OF TMC

Kotzé *et al.* (1998:37), based on the method of Domard *et al.* (1986:105), synthesized TMC. TMC, depicted in figure 1.5 is a partially quaternised derivative of chitosan which is prepared by reductive methylation with methyl iodide in a strong basic environment at an elevated temperature. The degree of quaternisation can be altered by increasing the number of reaction steps or by increasing the reaction time (refer to chapter 3).

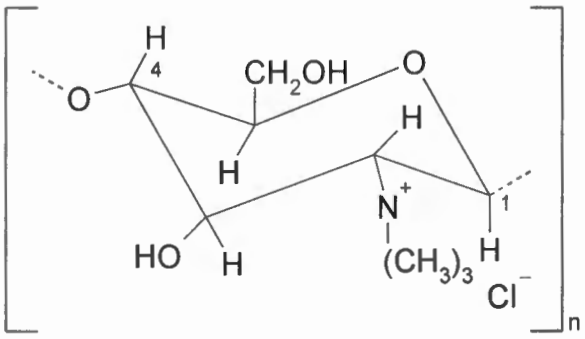


Figure 1.5 The chemical structure of TMC.

According to Kotzé *et al.* (1998:37) the initial chitosan used to synthesize TMC was only soluble in acidic solutions, but after quaternisation it became perfectly soluble in water. This increased solubility, either in basic or acidic medium, was even observed for a degree of quaternisation as low as 10 % as determined by ^{1}H-NMR spectra. As mentioned previously chitosan hydrochloride and chitosan glutamate are only soluble at acidic pH levels. Even at these low pH levels, it was difficult to prepare 1.5 % (w/v) solutions due to the high viscosity of the solutions. A pronounced decrease in the intrinsic viscosity of TMC, compared to the starting material, was observed and this correlated well with the strong reaction conditions in alkaline medium. TMC proved to be a derivative of chitosan with superior solubility and basicity, even at low degrees of quaternisation, compared to the chitosan salts. The increase in solubility was attributed to the replacement of the primary amino group on the C-2 position of chitosan with quaternary amino groups.

1.4.2 Effect of TMC on the transepithelial electrical resistance (TEER) of human intestinal epithelial cells (Caco-2)

As previously mentioned, tight junctions serve as barriers to paracellular diffusion and that the measurement of the transepithelial electrical resistance (TEER) is believed to be a good indication of the tightness of the junctions between epithelial cells (Kotzé *et al.*, 1998:38). It has also been suggested that measurement of TEER could be used to predict the paracellular transport of hydrophilic molecules (Boulenc *et al.*, 1995b:14).

Incubation of intestinal epithelial cells (Caco-2) with TMC with a degree of quaternisation of 12 % in concentrations of 1.5, 2.0 and 2.5 % (w/v) resulted in a pronounced reduction in TEER values. The reduction in TEER was respectively 9 ± 4 %, 52 ± 3 % and 79 ± 0.3 % respectively after 20 minutes. Prolonged incubation only resulted in a gradual decrease in resistance compared with the initial reduction in TEER after 20 minutes. The highest reduction in TEER was measured at a concentration of 2.5 % (w/v) thus indicating that the reduction in TEER is concentration dependent (Kotzé *et al.*, 1997b:1200)

With removal of the polymer solutions, repeated washing and substitution of the apical medium with fresh Dulbecco's Modified Eagles Medium, reversibility of the effects was noticed, especially at 1.5 and 2.0 % concentrations of TMC. The monolayers started to recover slowly and a slight increase in resistance, toward the initial values were found. According to Kotzé *et al.* (1997b:1200) complete removal of the polymer, without damaging the cells, proved to be difficult due to the high viscosity of the solutions and this might be the reason why the increase in resistance was only gradual. The viability of the monolayers was assessed, after incubation with TMC, by staining the monolayers with trypan blue. No visible uptake of the marker was observed and the authors concluded that the viability of the monolayers was not affected by incubation with TMC.

A comparison of the effect of the chitosan salts, chitosan hydrochloride and chitosan glutamate, and TMC (degree of quaternisation 12.28 %) on the TEER across Caco-2 cell monolayers revealed once again that all of these compounds were able to decrease the

TEER significantly. The decrease in TEER at 0.25 % (w/v) concentrations, 20 minutes after incubation, was in the order chitosan hydrochloride (71 ± 4 % reduction) > chitosan glutamate (56 ± 1 % reduction) > TMC (28 ± 1 % reduction), suggesting that the chitosan salts were more effective than TMC at similar weight concentrations.

According to the authors the difference in effect of these polymers could be explained in terms of the equivalent weights of each repeating unit in the polymer backbone of the respective polymers (theoretically 197.62 for chitosan hydrochloride, 308.30 for chitosan glutamate and 239.80 for TMC), thus determining the amount of free chitosan base and therefore the density of the amino groups available for protonation at similar weight concentrations. About 50 % of chitosan glutamate by weight is the glutamate salt whereas for chitosan hydrochloride the salt part only constitutes a small fraction (5 – 10 %). Additionally, the attached methyl groups on the C-2 position of TMC probably causes steric effects and also partially hide the positive charge on the quaternary amino groups, thereby altering the time needed for interaction with the negatively charged cell membranes and tight junctions (Kotzé *et al.*, 1998:41). However, at higher concentrations of TMC (2.0 – 2.5 % w/v) similar effects on the TEER, as seen with chitosan hydrochloride, was observed. The better solubility of TMC may therefore compensate for its lower effect at similar weight concentrations.

1.4.3 Effect of TMC on the absorption enhancement of hydrophilic model compounds

Mannitol and PEG-4000 are metabolically inert as well as highly hydrophilic in nature and mannitol has been used previously to follow changes in the intestinal epithelial integrity of mucosal cells. Both compounds do not diffuse to a large extent into the cell membranes, but are absorbed through the alternative aqueous paracellular pathway, and are therefore ideal substances to detect changes in permeability in studies of absorption enhancement.

Table 1.1 shows a comparison of the effect of TMC (degree of quaternisation 12.28%), chitosan hydrochloride and chitosan glutamate on the permeability of Caco-2 cells at a pH of 6.20 for the hydrophilic marker [^{14}C]-mannitol.

Table 1.1 Effect of TMC, chitosan glutamate and chitosan hydrochloride on the permeability of [^{14}C]-mannitol at a pH of 6.20 (Kotzé *et al.*, 1998:41).

Marker	**Concen-tration (% w/v)**	**TMC**		**Chitosan glutamate**		**Chitosan hydrochloride**	
		P_{app} x 10^{-7} (cm/s)[a]	**R**	**P_{app} x 10^{-7} (cm/s)[a]**	**R**	**P_{app} x 10^{-7} (cm/s)[a]**	**R**
[^{14}C]-Mannitol	Control	0.72 ± 0.08	1	0.72 ± 0.08	1	0.72 ± 0.08	1
	0.25	8.11 ± 0.21[b]	11	18.25 ± 1.10[b]	25	24.65 ± 2.13[b]	34
	0.50	9.26 ± 0.35[b]	13	14.17 ± 0.45[b,c]	20	23.28 ± 1.00[b]	32
	1.00	14.00 ±0.40[b]	19	20.82 ± 0.30[b]	29	25.56 ± 2.95[b]	36
	1.50	7.52 ± 0.86[b]	10	18.29 ± 1.53[b]	25	26.16 ± 1.86[b]	36
	2.00	12.35 ± 0.43[b]	17	n.d.	n.d.	n.d.	n.d.
	2.50	15.21 ± 1.37[b]	21	n.d.	n.d.	n.d.	n.d.

[a] Each value represents the mean ± S.D. of 3 experiments

[b] Significantly different from control ($P < 0.05$)

[c] Significantly different from all other treatments in group ($P < 0.05$)

n.d. = not determined due to insolubility of chitosan salts.

Exposure of the apical side of the monolayers to 0.25 % of the polymers resulted in a 34-fold (chitosan hydrochloride), 25-fold (chitosan glutamate) and 11-fold (TMC) increase in the absorption rate of [^{14}C]-mannitol, compared to the control group as indicated by the P_{app} values and absorption enhancement ratios (R). This changes to a 36-fold (chitosan hydrochloride), 25-fold (chitosan glutamate) and 10-fold (TMC) increase in the presence of 1.5 % concentrations of the respective polymers. Similar results were obtained for [^{14}C]-PEG-4000. At higher concentrations, TMC was able to increase the P_{app} and R-values further for both [^{14}C]-mannitol and [^{14}C]-PEG-4000. A 17-fold and 21-fold increase in R were found for [^{14}C]-mannitol at 2.0 and 2.5 % (w/v) concentrations of TMC, respectively. The same tendency was also seen with [^{14}C]-PEG-4000.

From these results, it is evident that TMC was not as effective at similar weight concentrations as chitosan hydrochloride and chitosan glutamate. These results are also similar to the results obtained in the TEER studies referred to in the previous section. The authors concluded that additional factors play a role in the absorption enhancement mechanism of TMC. This could most likely be explained in terms of the charge density, the equivalent weight of each repeating unit in the polymer backbone and possible steric effects of the attached methyl groups and partial hiding of the positive charge on the quaternary amino groups (Kotzé *et al.*, 1998:43).

Kotzé *et al.* (1997a:1200) also showed that TMC, with a degree of quaternisation of 12 %, was able to increase the transport of fluorescein isothiocyanate-labeled dextran (FD-4) across Caco-2 cell monolayers. The transport of this large hydrophilic model compound (MW = 4 400 Da) was increased 167-fold, 274-fold and 373-fold with 1.5, 2.0 and 2.5 % (w/v) concentrations of TMC respectively.

1.4.4 Effect of TMC on the absorption enhancement of peptide drugs.

As mentioned in a previous section, normally poor bioavailability of peptides and proteins from oral and non-oral mucosal routes is a result of the interplay of poor permeability characteristics, instability towards proteolytic enzymes, cell metabolism and non-enzymatic clearance mechanisms such as the first pass effect and excretion in the bile (Vyas *et al.*, 1997:339). Peptides and peptidomimetic agents are large, hydrophilic molecule pharmaceuticals that do not partition into the cell membranes, therefore they are mostly excluded from the transcellular pathway. The absorption of these compounds is for the most part limited to the alternative paracellular pathway which is primarily restricted by the tight junctions (Kotzé *et al.*, 1998:36).

TMC, with a degree of quaternisation of 12 %, was not only able to improve the transport of the hydrophilic model compounds [^{14}C]-mannitol, [^{14}C]-PEG 4000 and FD-4 across intestinal epithelial membranes (Kotzé *et al.*, 1997b:1197 and Kotzé *et al.*, 1998:35) but

was also able, in agreement with these results, to increase the transport of several peptide drugs across Caco-2 cell monolayers.

TMC, in concentrations of 1.5 and 2.5 % (w/v), was able to increase the transport of DGAVP (MW = 1 412), at a pH of 5.60, to 0.96 ± 0.28 % and 1.09 ± 0.08 % of the total dose applied respectively. The control group showed transport of 0.19 ± 0.29 % of the total dose applied. TMC was also able to increase the transport of insulin (MW = 5 778) at a pH of 4.40 compared to the control where no transport was observed. The transport of insulin was increased to 0.3 ± 0.1 % and 0.8 ± 0.1 % of the total dose applied at 1.5 and 2.5 % (w/v) concentrations of TMC respectively. An increase in the transport of buserelin (MW = 1 300) was also observed at a pH of 6.20. The transport was increased to 1.4 ± 0.2 % and 2.7 ± 0.3 % of the total dose applied with 1.5 and 2.5 % (w/v) solutions of TMC respectively (control group: 0.04 % of total dose applied) (Kotzé *et al.*, 1997a:243).

1.4.5 Effect of the degree of quaternisation of TMC on its absorption enhancing properties

As seen in the previous sections TMC was not as effective at the same weight per volume concentrations in increasing transport of hydrophilic compounds as the chitosan salts chitosan glutamate and chitosan hydrochloride. Its lesser efficacy was explained by its charge density, which was determined by the degree of quaternisation, and by a partial hiding of the positive charge on the amino group by the attached methyl groups. It was proposed that TMC with higher degrees of quaternisation might be more effective as an absorption enhancer for the increased paracellular transport of hydrophilic compounds in neutral environments (Kotzé *et al.*, 1999c:253).

In a study by Kotzé *et al.* (1999d:273) the cumulative transport of [^{14}C]-mannitol and [^{14}C]-PEG 4000 in the presence and absence of different concentrations of TMC, with degrees of quaternisation 12 % and 20 % respectively, was determined at a pH of 6.20 in Caco-2 cell monolayers. Both the TMC polymers were able to increase the transport of

[^{14}C]-mannitol as well as [^{14}C]-PEG 4000. However, it was noticed that the permeability decreased with an increase in molecular weight resulting in a much lower transport measured for [^{14}C]-PEG 4000 than for [^{14}C]-mannitol. It was suggested that the permeation of these compounds across intestinal epithelial cells depend on their molecular size and structural conformation. Overall, TMC with a degree of quaternisation of 20 % was able to increase permeability across the monolayers to a much higher extent than TMC with a degree of quaternisation of 12 %. The difference in effect between these two polymers was explained in terms of the charge density of each polymer, as determined by their respective degrees of quaternisation.

Kotzé *et al.* (1999c:253) also investigated the effect of TMC-H (degree of quaternisation 61.2 %), TMC-L (degree of quaternisation 12.28 %) and chitosan hydrochloride on the TEER and permeability of intestinal epithelial Caco-2 cell monolayers at pH values of 6.20 and 7.40. At a pH of 6.20 all the polymers caused a pronounced reduction (37-67 % at 0.5 % (w/v) concentrations) in the TEER of Caco-2 cells. On the contrary, at a pH of 7.40 only TMC-H was able to decrease the TEER values, even in a concentration as low as 0.05 % (w/v) (35 % reduction). Comparable results were obtained with the permeation of [^{14}C]-mannitol. Large increases in the transport rate (18 –23 fold at 0.5 % (w/v) concentrations) were found at pH 6.20, while only TMC-H was able to increase the permeation of [^{14}C]-mannitol at pH 7.40 (31 – 48 fold at 0.05 – 1.5 % (w/v) concentrations of TMC-H).

From the results in these studies it is clear that TMC, in contrast to the chitosan salts, is a promising absorption enhancer in neutral and basic environments. Although a low degree of quaternisation is not sufficient to produce noticeable absorption enhancement it is easily overcome by increasing the degree of quaternisation.

The results obtained in *in vivo* studies performed on rats were similar to the results obtained in the *in vitro* studies described above. After the nasal administration of semisynthetic human insulin (4 IU/kg body weight) at a pH of 4.40 with chitosan hydrochloride, TMC-L and TMC-H all the polymers were able to reduce blood glucose

levels. Major increases in plasma insulin levels were also found after co-administration with these polymers. No major differences in effect between TMC-L and TMC-H could be demonstrated. At a pH of 7.40 only TMC-H was able to decrease the blood glucose levels of the rats significantly. Neither chitosan hydrochloride nor TMC-L was able to produce any hypoglycemic response. A 0.5 % (w/v) solution of TMC led to a reduction in blood glucose levels, 30 minutes after co-administration with insulin, of about 34 % (Kotzé *et al.*, 1999d:381).

Ocreotide, a somatostatin analogue used for the control of endocrine tumours of the gastrointestinal tract, has limited oral absorption due to its limited permeation across the intestinal epithelium. This peptide was administered (pH 7.40) with or without the polymers chitosan hydrochloride and TMC (degree of quaternisation 60 %), intrajejunally in rats after which the serum peptide levels were measured by radioimmunoassay. A 1.0 % (w/v) TMC solution significantly increased the absorption of the peptide analogue, resulting in a 5-fold increase of ocreotide bioavailability compared to the controls (ocreotide alone). No increase in bioavailability was noticed with co-administration of a 1.0 % (w/v) solution of chitosan hydrochloride (Thanou *et al.*, 2000b:123).

1.4.6 MECHANISM OF ACTION OF TMC AND VISUALISATION OF THE TRANSPORT PATHWAY

Schipper *et al.* (1997:923) found that the effect of chitosan on the paracellular permeability is initiated by its direct and specific binding at the cell membrane. This binding could be inhibited by heparin, indicating that the positive charge is important for the binding properties of chitosan. TMC, at all degrees of quaternisation, bears positive charges, independently of the environmental pH. It can therefore be speculated that TMC will also bind to the cell surfaces in a similar way as chitosan.

In a study by Thanou *et al.* (2000a:91) the tight junction's membrane protein occludin was visualised by immunocytochemistry staining in the presence and absence of TMC-60

(degree of quaternisation 60 %) using confocal laser scanning microscopy (CLSM). Additionally, the effects of TMC-60 on cytoskeletal F-actin were determined by visualisation using CLSM.

The transmembrane protein occludin displayed a disrupted pattern after incubation with 1.0 % (w/v) TMC-60, suggesting that the interaction of TMC-60 with the tight junctions' proteins is the major mechanism for opening the tight junctions and subsequently increased paracellular permeability. These observations were quite similar to images of Caco-2 cells with 0.1 % (w/v) chitosan, but the effect appeared to be stronger than that reported for 0.1 % (w/v) chitosan. Chitosan treated cells showed a thickened pattern of occludin at the cell periphery and not a disrupted one, which might be due to the 10-fold difference in concentration or to an effect exclusively related to the quaternised derivative of chitosan, TMC-60. Schipper *et al.* (1997:923) stained the protein ZO-1 (a protein related to occludin) in order to study the effects of chitosans on tight junctions. A distinctive disruption of the ZO-1 patterns similar to the ones observed after occludin staining was observed. In the study by Thanou *et al.* (2000a:104) it was observed that TMC-60 provoked a redistribution of the cytoskeletal F-actin, a phenomenon that appeared to correlate well with the opening of epithelial tight junctions.

In order to visualise the transport pathway of FD-4 (MW = 4 400), a fluorescent hydrophilic compound, across Caco-2 cell monolayers Kotzé *et al.* (1998:44) used CLSM. After 60 min incubation with 0.5 % (w/v) concentrations of chitosan hydrochloride, chitosan glutamate and TMC (degree of quaternisation 12 %) at a pH of 6.20, fluorescence was detected in the intercellular spaces. After incubation with the control solution containing only FD-4, no intracellular or intercellular fluorescence was observed. The fluorescence observed after incubation with TMC clearly demonstrated the ability of these polymers to open these intercellular (paracellular) spaces. Similar results were obtained after incubation with fluorescein isothiocyanate-labeled dextran with a molecular weight of 19600 Da (FD-20). A 0.5 % (w/v) solution of TMC was able to open the tight junctions resulting in the transport of FD-20 through these paracellular spaces. Similar results were obtained by Thanou *et al.* (2000c:85) with 1.0 % (w/v)

TMC-60 and a texas red-labeled dextran with a molecular weight of 10 000 Da. Transport of these large hydrophilic molecule suggests that other high molecular weight compounds such as peptides and proteins could pass through the paracellular transport pathway.

1.4.7 CYTOTOXIC EVALUATION OF TMC

For the evaluation of novel absorption enhancers, safety studies are required in order to guarantee the absence of tissue damaging effects of the compound under investigation. As previously mentioned Schipper *et al.* (1996:1686) observed some toxic effects for certain chitosans, although chitosan in general is considered safe, biodegradable and a non-toxic polymer. Thanou *et al.* (2000c:77) selected Caco-2 cell monolayers to study the possible membrane damaging effects of TMC. The fluorescent probe YO-PRO-1 was used in this cytotoxicity study which only emits fluorescence upon binding with the nuclei of cells. Cells, which do not take up this fluorescent probe, were considered viable. CLSM horizontal cross sections of Caco-2 cell monolayers treated with 1.0 % (w/v) TMC-60 for 4 hours showed no nuclei staining. The effect of 1.0 % (w/v) TMC-60 on the cilliary beat frequency of chicken embryo trachea resulted in a slight decrease in this frequency. This decrease in frequency was however less pronounced than the decrease observed after incubation with physiological saline (0.9 % NaCl) (Thanou *et al.*, 2000c:87). It was therefore concluded by Thanou *et al.* (2000c:89) that TMC is a safe absorption enhancer for hydrophilic macromolecules such as peptide and protein drugs across nasal and other mucosal tissues.

1.5 CONCLUSION

In the previous sections an overview of the diverse pharmaceutical applications of chitosan was given and special emphasis was placed on the use of chitosan as an absorption enhancer. Chitosan was able to allow paracellular transport by opening the tight junctions of epithelial cells. This absorption enhancing effects was however only possible in acidic environments due to the insolubility of this polymer in neutral and

basic environments. There is, however, a need to use chitosan in more neutral and basic environments such as those found in the large intestine and colon.

The partially quaternised derivative of chitosan, *N*-trimethyl chitosan chloride (TMC), showed excellent solubility over a wide pH range suggesting that it could be used as an absorption enhancer in the neutral and basic environments. These absorption enhancing effects were demonstrated by a decrease in TEER values across epithelial cell monolayers (Caco-2) as well as the increase in transport of large hydrophilic compounds across these monolayers at neutral pH values. Similar results were also obtained in *in vivo* experiments where an increase in peptide drug delivery was observed. It was also shown that the degree of quaternisation of TMC plays an important role in its absorption enhancing properties, especially in neutral environments.

Although it was concluded that TMC is non-toxic, it should be mentioned that there are many cytotoxicity assays described in literature in order to evaluate the toxicity profiles of compounds meant for human administration. In chapter 2, toxicity assays, which are performed on cell cultures, will be discussed in detail, and it will be clear that different mechanisms of toxicity do exist. It will be evident that there is an obvious need to perform other cytotoxicity assays in order to ensure that TMC is a non-toxic absorption enhancer fit for human administration, due to the fact that the limited toxicity results available for TMC is insufficient to conclude that this polymer is non-toxic.

CHAPTER 2

IN VITRO CYTOTOXICITY EVALUATION OF PHARMACEUTICAL COMPOUNDS - THE USE OF CELL CULTURES

2.1 *INTRODUCTION*

Under current legislation, new drugs, cosmetics, food additives, etc., must go through extensive cytotoxicity testing before they are released, this being a prerequisite for human use. It usually involves a large number of animal experiments, which are very costly and raise considerable public concern. In this regard there is much pressure, both emotional and economically, to perform at least part of cytotoxicity testing *in vitro*. The introduction of assays performed on cell cultures have proved to be a reliable alternative to other *in vitro* assays, and are characterized by their low cost, simplicity and reproducibility. These assays also provide results corresponding well to results obtained in *in vivo* assays. Due to these advantages, which these *in vitro* assays hold, they are widely applied in cytotoxicity assays performed in the development stage of drugs, cosmetics and food additives.

2.2 *CLASSIFICATION OF CYTOTOXICITY ASSAYS*

Since their introduction by Goodman in 1961, ^{51}Cr-release assays have been extensively used to measure cell-mediated cytolysis. In this technique, cells are preloaded with a radioactive isotope of chromium, $^{51}CrO_7^{2-}$, which after passing through the cell membrane, is reduced to $^{51}Cr_3^{+}$ that forms complexes with cytoplasmic proteins. The assay determines the leakage of $^{51}Cr^{3+}$ from the cells when membrane damage is present (Krüger-Krasagakes *et al.*, 1992:1). However, ^{51}Cr-release assays have some unfavorable characteristics, including (i) appreciable spontaneous release of ^{51}Cr from target cells during long term assays, (ii) the time required for labeling target cells and the loss of

target cells during washes following labeling, (iii) the expense of the radioisotope and instrumentation required for its measurement, and (iv) problems of radiation safety and disposal of the isotope (Korzeniewski & Callewaert, 1983:313). These characteristics prompted researchers to develop other methods to determine cytotoxicity and this method is therefore no longer frequently used.

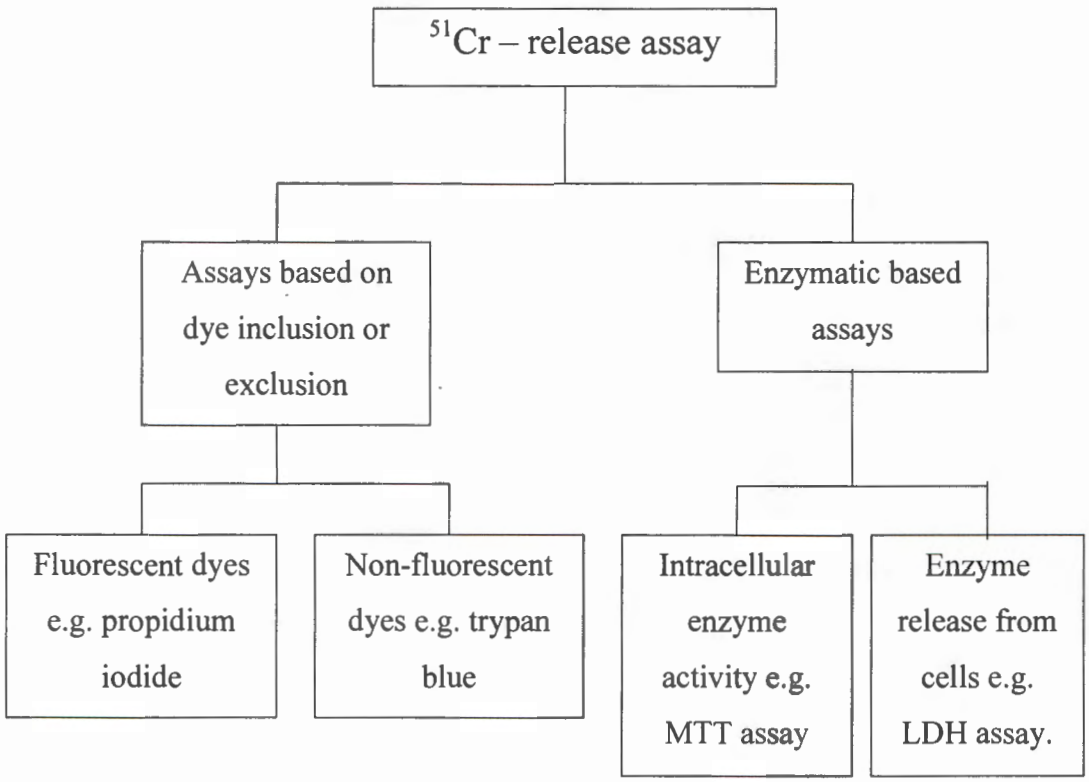


Figure 2.1 Schematic representation of the development in cytotoxicity assays performed on cell cultures.

The development of cytotoxicity assays devided mainly into two streams as illustrated by figure 2.1. One of which is based either on the measurement of dye, either excluded from or included into cells, and the other based on an enzymatic mechanism present in the cells. The first type of assay based on the inclusion or exclusion of dyes could also be devided into two categories, namely the use of fluorescent probes, for example propidium iodide, or the use of non-fluorescent probes like trypan blue. The use of fluorescent probes allows the researcher to obtain the results not only through fluorescence

microscopy but also through fluorometry or flow cytometry, making it possible to quantify the data. Non-fluorescent probes are assessed normally either by optical microscopy or by colorimetry, the latter method simplifying quantification.

Assays based on an enzymatic mechanism could also be categorized into two classes. The first is the assessment of intracellular enzymatic activity through the conversion of usually a colourless probe, absorbed by the cell, into a coloured complex. The tetrazolium salts are mainly used in these assays and monitors the mitochondrial dehydrogenase activity of cells. The formazan product formed by reduction of these salts has a linear relation to the number of viable cells in the total cell population, and is easily determined by using colorimetry.

When cell membranes are damaged the amount of enzymes released from the cells can be used to determine the number of viable cells in a cell population. This forms the base of the second type of enzymatic assay, of which the release of lactate dehydrogenase (LDH) from cells has been used in many cytotoxicity assays. The LDH activity is determined by the decrease in NADH measured at 340 nm or in a coupled assay with *p*-INT where the formation of NADH is determined by the production of a formazan product that is measured spectrophotometrically.

All the assays discussed in the following paragraphs are performed on cell cultures. These cell cultures are either analyzed as a suspension of cells or as monolayers grown on filters in culture plates. An example of the latter culture method is Caco-2 cells. These Caco-2 cells have extensively been used to assess the effect that new drugs and other pharmaceutical compounds such as absorption enhancers might have on intestinal epithelia, including their potential cytotoxicity. All the assays mentioned above can either be performed on cell monolayers or cell suspensions. There is however, an assay applicable only on cell monolayers namely the measurement of transepithelial electrical resistance (TEER).

A cytotoxicity assay that has not been used as extensively as the above-mentioned assays is the bioluminescence assay used to determine the release of ATP from membrane damaged cells. Luciferin in combination with the enzyme luciferase generates 560 nm light in the presence of ATP and is detected by using a luminometer. Of all the assays described in the following paragraphs this is the only assay that has not yet been used in combination with Caco-2 cell monolayers.

2.3 CYTOTOXICITY ASSAYS BASED ON THE INCLUSION OR EXCLUSION OF DYES IN CELL CULTURES

2.3.1 THE PROPIDIUM IODIDE ASSAY

Propidium iodide (PI) (figure 2.2) is a phenantrene derivative and, like ethidium bromide, binds to DNA. It has an excitation spectrum of between 400 and 540 nm and gives an emission spectrum between 560 and 620 nm, but when intercalated between nucleic acid bases its fluorescence increases up to 20-fold. Used alone it complexes with ribonucleoprotein and thus binds to ribosomes and nucleoli as well as to nuclear and mitochondrial DNA, and in combination with ribonuclease digestion it can be used as a DNA specific cytochemical probe (Ockleford *et al.*, 1981:261). When dissolved in phosphate-buffered saline, the dye is excluded by the plasma membrane and hence, is only found in dead cells. The dead cells exhibit red nuclear fluorescence and are distinguished from the viable cells on this basis (Jacobs & Pipho *et al.*, 1983:101).

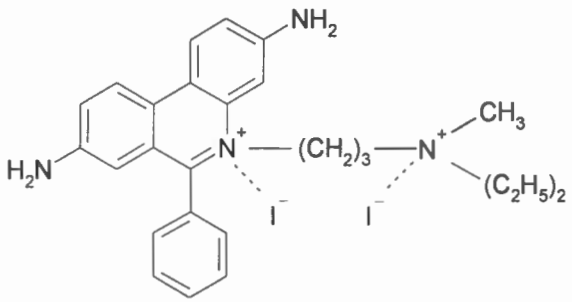


Figure 2.2 Chemical structure of propidium iodide (Ockleford *et al.*, 1981:262).

When comparing the PI assay with the standard ^{51}Cr release assay it was found that if the incubation time is shorter, the ^{51}Cr release assay appears to give an underestimation of cell death. Apparently PI is taken up more quickly in dead cells than ^{51}Cr is released from these cells (Kroesen *et al.*, 1992:53).

Fluorescence microscopy is mainly used to detect the fluorescence of PI upon binding with the cell nucleus. Although this is qualitative data it was quantified by Lindmark *et al.* (1995:959). The number of PI-stained cells was determined from the monolayers by analyzing images taken with a TV camera connected to the fluorescence microscope. The images were stored and analyzed by image analysis software (Lindmark *et al.*, 1995:959).

The fluorescence intensity of PI can also be determined by using a microplate fluorometer, which automatically measures the fluorescence intensity in each well of the microplate. Construction of a linear standard curve of relative fluorescence units versus the number of cells (x 10^3/well) allows the analyst to determine the number of cells stained by PI in a particular well (Wan *et al.*, 1994:269).

The percentage of viable cells can also be determined by using the following equation:

$$\% \text{ viable cells} = \left[1 - \frac{F_t - F_0}{F_f - F_0}\right] \times 100$$

where F_0 is the fluorescence measured immediately in the wells at the beginning of the assay, F_t is the fluorescence measured at the optimum time of incubation with the substance of which the toxicity profile is being determined and F_f the fluorescence measured after all the cells are lysed (Wrobel *et al.*, 1996:245).

PI is a known mutagen, and should be handled with care. However, disposal of this substance is easy. PI may be removed from various solutions by filtration with activated charcoal, which can then be incinerated. It can also be completely degraded in buffer by reaction with sodium nitrite and hypophosphorous acid (Wan *et al.*, 1994:271).

In various studies PI was not used alone but in combination with other fluorescent or non-fluorescent dyes. A typical fluorescent dye used in combination with PI is 3,3-dioctadecyloxacarbocyanide perchlorate (DiO). The lipophilic DiO, due to its membrane specificity, is used to provide a general view of the detailed structure of the plasma membrane. By making use of confocal laser scanning microscopy, PI and DiO can simultaneously be detected due to their similar absorption spectrums ($\lambda_{max} \approx 490$ nm) and widely separated emission peaks ($\lambda_{max} \approx 639$ and 507 nm, respectively). Argon laser (the light source used in confocal systems) excites the molecules at the same time while the different emission wavelengths make it possible to distinguish between the two fluorescence signals, also at the same time. The use of DiO in combination with PI allows a more sensitive detection of membrane damage (Rojanasakul *et al.*, 1990:132). PI emits a red fluorescence upon binding with nucleus, as previously mentioned, while the DiO emits a green fluorescence when bound to the cell membrane (Kroesen *et al.*, 1992:48).

It should also be mentioned that the confocal method offers several advantages over conventional microscopy including (a) improved resolution, contrast, and rejection of out-of-focus interference, (b) by optical sectioning through the interior structures of the tissue, serial images of deeper layers of a thick specimen, that are completely obscured in conventional imaging, can be obtained and most importantly, (c) it gives the opportunity to study the living tissue without interfering artifacts caused by tissue processing, i.e., chemical fixation, dehydration, embedding, and sectioning, that is normally required in conventional microscopy (Rojanasakul & Robinson, 1990:132).

The combination of PI and DiO can also be analyzed by using flow cytometry. The method is based on sorting the cells by the difference of their fluorescence that they emit, by using a fluorescence activated cell sorter. The fluorescence data of each cell is localized on a two-dimensional plot according to the amount of green (abscissa) and red (ordinate) fluorescence that it exhibits. The plot permits distinguishing between viable (green) and non-viable (green and red) cells (Jacobs & Pipho *et al.*, 1983:102). However, for this method the cells have to be in suspension (Mattis *et al.*, 1997:137), but does have

the advantage of being a fast, versatile, and inexpensive procedure (Johann *et al.*, 1995:210).

For fluorometric assays, serum free culture media is usually recommended since serum induced high values of background fluorescence for the fluorochromes used by previous researchers. Some substances in the culture media as well as the cellular content can emit autofluorescence with excitation and emission wavelengths common to many fluorochromes (Wan *et al.*, 1994:271). Wrobel *et al.* (1996:245) used culture medium free of phenol red indicating that phenol red has an unwanted effect on the fluorescence units measured by the fluorometer. When performing this assay it should also be noted that PI itself becomes cytotoxic at concentrations higher than 50 μg/ml and incubation times longer than 6 hours (Wrobel *et al.*, 1996:247).

In an experiment performed by Wrobel *et al.* (1996:245) a lower LD_{50} value was determined for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay than for the PI assay. This is however, counterbalanced by the fact that both methods show a similar detection limit, and more importantly, the analytical working range of the PI method (0.3-300 pg/ml) is much wider than that of the MTT method (0.3-50 pg/ml). Moreover, the total analysis time for the proposed method (about 7 h) is much shorter than that of the MTT assay (about 48 h). An additional advantage of the proposed PI assay is its possible application to kinetic studies of cytotoxicity since sequential measurements of cell viability may be carried out in the same sample over a longer period of time (Wrobel *et al.*, 1996:247).

2.3.2 THE NEUTRAL RED ASSAY

Neutral red (NR) (figure 2.3) is a weakly cationic dye, which accumulates after uptake in a cell (Twiss *et al.*, 1994:699). The mechanism by which the dye penetrates cell membranes and accumulates into lysosomes is not fully understood. The entry could be accomplished by micropinocytosis with subsequent fusion of vesicles with secondary lysosomes or this dye could enter cells by non-ionic diffusion and bind to anionic

carboxylic or phosphoric groups of the lysosomal matrix (Zhang *et al.*, 1990:220). Viable cells might also take up the dye by active transport incorporating it into the lysosomes. The assay is based on the fact that viable cells do take up the dye in contrast to non-viable cells that don't. These differences in the amount of NR incorporated by the cells could indicate either a variation in the number of cells, or simply a change in their physiological state (Manna *et al.*, 1996:288). The uptake of NR is therefore proportional to the number of viable cells.

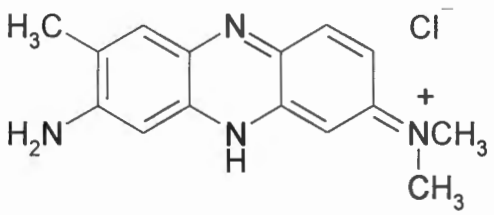


Figure 2.3 Chemical structure of Neutral Red (Sigma, 1999:741).

Filman *et al.* (1974:190) presented data which shows that cells which have the ability to adhere to surfaces, in cell culture, and to divide are characterized by the ability to accumulate NR locally in discrete intracytoplasmic granules and vacuoles and to exclude trypan blue. Early stages of damage are associated with delocalization of NR so that it appears as a diffuse stain of both the cytoplasm and nucleus. Cells damaged to this extent are incapable of either adherence or replications but still do not stain with trypan blue. For this reason Zhang *et al.* (1990:220) stated that the vital stain trypan blue is incapable of detecting sublethal injury to cells. Cells damaged to a somewhat greater extent lose the ability to retain NR at a higher concentration than that of the surrounding medium and consequently appear 'unstained'. At this extent of damage, cells are only seen to take up trypan blue. These three characteristic staining patterns formed the base of the rapid and sensitive cytotoxic assay capable of reflecting the levels of cell damage simultaneously in the same cell population (Filman *et al.*, 1974:191).

In recent years the approach to the NR assay has been changed somewhat. Filman *et al.* (1974:191) obtained their results through optical microscopy, which can be quantified by counting the cells, using an analysis program linked to the microscope. However, this is

a tedious process and human error can not be excluded, thus increasing the amount of mistakes. For this reason some researchers have turned to using colorimetry. To measure the dye taken up, the cells are lysed whereafter the cell lysis products are centrifuged and the supernatants are colorimetrically measured at 540 nm (Müllbacher *et al.*, 1984:205).

It should be emphasised that the NR method measures cell survival by staining cells at the end of the assay. In contrast, the radioactive assay estimates cell destruction by labeling the cells at the start of the assay and measuring release of ^{51}Cr from dead cells. This difference in methodology probably explains the increased sensitivity of the colorimetric method as ^{51}Cr is slowly released from the dead cells, whereas lysed cells are immediately unable to take up NR. A time course experiment supported this interpretation where the colorimetric method detected cell lysis more rapidly than the ^{51}Cr release procedure (Parish & Müllbacher, 1983:236). The increase in sensitivity ranged from 6- to greater than 25-fold (Parish & Müllbacher, 1983:235).

Theoretically, NR could be used like ^{51}Cr in a release assay where target cells being labeled with the dye before addition of the possible cytotoxic substance. The sensitivity was however, proved to be lower through preliminary experiments performed by Parish & Müllbacher (1983). It was noticed that the released dye tended to be re-utilized by the surviving cells. Such problems are not encountered when targets are stained at the end of the assay (Parish & Müllbacher, 1983:236).

2.3.3 OTHER ASSAYS BASED ON THE INCLUSION OR EXCLUSION OF DYES

Ethidium bromide (EB) (figure 2.4), a phenantridine dye, has been used in many cytotoxicity assays (Brawn *et al.*, 1975:7). Like PI it is excluded by living cells but penetrates dead cells, giving bright red fluorescence of the nucleus (Tanke *et al.*, 1982:91 and Dobersen & Scharff, 1982:79). It also undergoes a 20- to 25-fold fluorescence enhancement on binding with nucleic acids (Brawn *et al.*, 1975:7).

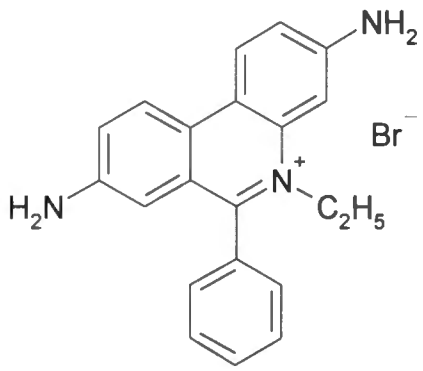


Figure 2.4 Structure of ethidium bromide (2,7-diamino-9-phenylphenantridine-10-ethyl bromide) (Brawn *et al.*, 1975:14).

When this assay is performed certain factors should be considered when the concentration of EB which will be used is determined. Firstly, if many dye molecules are adjacent to one another, it is possible that a kind of dimer or polymer formation could occur with a subsequent alteration in fluorescence properties. This kind of interaction is felt to take place when acridine dye molecules intercalate in the helix of nucleic acids and is probably a manifestation of the well-known tendency of dye molecules to aggregate in solution. Excimer formation could also be responsible for anomalies at high concentrations of this dye. Excimers are dimers, which are stable only in the excited state and are known to account for some anomalies in the fluorescence properties of aromatic hydrocarbons at high concentrations (Brawn *et al.*, 1975:15).

The use of EB was compared to other specific fluorescent dyes such as Hoechst 33258 and (4,6-diamidino-2-phenylindole) DAPI. This was determined by using an automatic plate reader and it was found that EB was superior to these dyes. Not only do these dyes penetrate living cells slowly, but their fluorescence spectra are also significantly overlapped by the fluorescence spectrums of the plates (Beletsky & Umansky, 1990:204).

Another cytotoxic assay is the trypan blue exclusion assay. The cytotoxic effect of the trypan blue assay is measured by the failure of damaged cells to exclude this vital dye. The readout of this assay is by visual enumeration of cells, which have or have not

excluded the dye to a degree, which is visually detectable, but this method is laborious and the readout is subjective (Brawn *et al.*, 1975:22).

When the trypan blue exclusion assay was compared with the EB exclusion assay, it was found that the ethidium EB is much more sensitive than the trypan blue exclusion assay when used in a lymphocytotoxic test system. It seemed that this is due to the earlier penetration of EB in the lymphocytes relative to trypan blue penetration (Darke & Gay, 1974:170). Both of these assays are however, clearly much less sensitive than the NR stain delocalization cytotoxicity assay described by Filman (Brawn *et al.*, 1975:24).

PicoGreen®, a membrane-impermeable fluorescent nucleic acid stain, was recently used in a cell proliferation assay. It was compared to other fluorochromes such as PI and Hoechst 33342 and was found to have a sensitivity far above the limit of sensitivity of these two fluorochromes. It was also noticed that PicoGreen® has an autofluorescence much lower than both of these flurochromes (Blaheta *et al.*, 1998:159) do. Although this fluorescent stain was used in a proliferation assay it could in future be a good substitute in cytotoxicity assays where PI is used, due to its membrane-impermeable character.

Molecular Probes, Inc. (Leiden, The Netherlands) developed a new series of DNA-intercalant dyes of which YO-PRO is one. This dye is also excluded by viable cells and does not exhibit any toxic effects on its own. The latter effect makes it possible to sort a cell suspension into living and dying cells if a flow cytometer is used, thus enabling examining of the physiological properties of the viable cells. The YO-PRO assay is a simpler assay and has the advantage that it does not enter all cells, making it an excellent stain for cytotoxicity assays (Idziorek *et al.*, 1995:256).

2.4 *ENZYMATIC BASED ASSAYS USED TO DETERMINE CYTOTOXICITY*

2.4.1 THE TETRAZOLIUM SALTS

2.4.1.1 The MTT assay

As mentioned earlier the number of viable cells can be determined by using any of several staining methods. In many of these methods washing of the samples increases processing time and increases sample variation. Multiwell scanning spectrophotometers can measure large numbers of samples with a high degree of precision which prompted Mosmann in 1983 to investigate the possibility of using a colour reaction as a measure of viable cell number (Mosmann, 1983:55). Ideally, a colorimetric assay for living cells should utilize a colourless substrate that is modified to a coloured product by any living cell, but not by dead cells or tissue culture medium. Tetrazolium salts are attractive candidates for this purpose, since they measure the activity of various dehydrogenase enzymes. The tetrazolium ring is cleaved in active mitochondria and the reaction thus only occurs in living cells (Mosmann, 1983:56).

Mosmann made use of the tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), as seen in figure 2.5, to develop this assay.

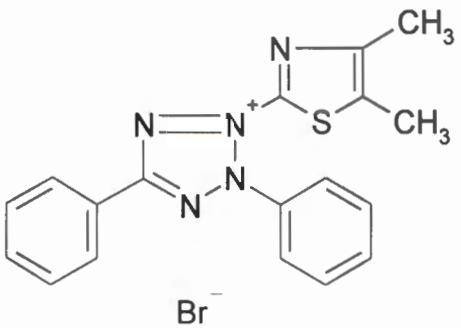


Figure 2.5 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, 1999:723).

MTT, a pale yellow substrate, produces a dark blue crystalline formazan product when incubated with live cells (Mosmann, 1983: 57). To accurately determine the optical density of the formazan product produced by the living cells one should ensure that the entire product is dissolved before analysis. Mosmann investigated several organic solvents for dissolving the formazan crystals and isopropanol was found to be the most suitable solvent at that time. Several years later Van de Loosdrecht produced data showing that dimethyl sulfoxide (DMSO) with glycine buffer (pH = 10.5) is a much better solvent than isopropanol and produced a signal twice as high as the standard curve obtained with isopropanol (Van de Loosdrecht *et al.*, 1991:18).

During the development of the original assay, Mosmann (1983:57) also determined that normal tissue culture medium has a variable colour due to pH changes, and the red colour of phenol red (found in many tissue culture mediums) interfered at the wavelength most suitable for blue MTT formazan measurement. Therefore, when adapting the MTT assay for use in experiments this is a very important point to consider. Mossman (1983:57) did minimize the interference produced by phenol red by converting the phenol red to the fully acidic, yellow form at the end of the assay by the addition of HCl (Mosmann, 1983:57).

The addition of HCl, as suggested by Mosmann (1983:57), partly solved the problem, but not entirely. It was determined that the decrease in sensitivity of the MTT assay after addition of HCl was also accompanied by the appearance of a new absorption peak at 400 nm indicating that the acid treatment changed the spectral properties of the formazan. The solution to this problem is to use an incubation medium lacking phenol red. It is however possible to include phenol red in the original cell culture medium, since any slight carryover of this indicator during medium change is insignificant, if care is taken to remove most of the supernatant from the cells in this procedure (Denizot & Lang, 1986:274).

Another factor found to decrease the sensitivity of the original assay was the choice of the reference wavelength in the original procedure. In the original assay developed by Mosmann (1983:57) a reference wavelength of 630 nm was used. By choosing a reference wavelength of 690 nm the sensitivity of the adopted procedure was 3.7-fold more sensitive than the original assay.

The data obtained by Mosmann (1983:57) showed that there is a linear correlation between the absorbency values after spectrophotometric analysis and the number of viable cells. The range over which this linearity extended (50 000 to 200 cells/well) indicated that the assay is able to detect very small numbers of living cells. It is reasonable to assume that this is however, not always the case and is dependent on the type of cells used in the assay. Cells with low metabolic activity (e.g. lymphocytes) must be used in high numbers to achieve a measurable MTT reduction (Garn *et al.*, 1994:253).

It was noticed that a direct application of the original procedure (Mosmann, 1983) produced a sensitivity below that of the [^{3}H]thymidine-uptake assay, an assay used extensively to assess cytotoxicity, and similar to the ^{51}Cr-release assay. Lappalainen *et al.* (1994:1130) compared toxicity assays using two cationic liposomes on a human cervical cancer cell line containing human papilloma virus. They state that although the thymidine assays measure DNA synthesis and MTT assays measure mitochondrial activity, the results obtained from these two assays were similar. In another study performed by Gieni *et al.* (1995:91) they reported a 2 to 16-fold increase in sensitivity compared to thymidine incorporation-based approaches when performed at low cytokine levels. Although there is not a definite trend in the comparison between the sensitivity of the assays, the MTT assay has several advantages over the thymidine assay. These advantages include savings in the cost of the reagents and equipment, reduced labour by elimination of sample processing steps required for liquid scintillation counting, as well as avoiding problems of safety and waste disposal associated with the use of radioisotopes. These problems are also encountered, as previously mentioned, when performing the ^{51}Cr-release assay (Roehm *et al.*, 1991:257).

Reduction of MTT remains the most common assay for tetrazolium salt-based viability testing and has been used extensively since its development in 1983 by Mosmann (Cruz *et al.*, 1997:73; Carreño-Gómez & Duncan, 1997:232; Wrobel *et al.*, 1996:243; Boulenc *et al.*, 1995:74a; Hovgaard *et al.*, 1994:143; Lappalainen *et al.*, 1994:1127; Nouri *et al.*, 1994:63; Jørgensen *et al.*, 1993:211; Anderberg *et al.*, 1992:880; Twentyman *et al.*, 1989:19 and Green, *et al.*, 1984:257). This assay can provide reproducible and accurate measurements of cell death and proliferation, it is safe, economical, simple and sensitive enough to handle a large number of samples in a short space of time, and it can be used for studying as few as 3000 cells (Hussain *et al.*, 1993:95).

2.4.1.2 The XTT and MTS assays

Unlike MTT's purple formazan product, the extremely water-soluble, orange formazan product of XTT does not require solubilization prior to quantification, thereby reducing assay time in many viability assay protocols. Moreover, sensitivity of the XTT reduction assay is reported to be similar to or better than that of the MTT reduction assay (Haugland, 1996:382 and Roehm *et al.*, 1991:258). When XTT was used in combination with the electron coupling agent phenazine methosulfate (PMS), it was observed that the absorption spectra of the XTT formazan product had an absorbance maximum at wavelengths between 440-490 nm. In contrast the culture medium (Dulbecco's modified eagle's medium) and XTT/PMS substrate mixture had a low absorbance in this wavelength range. This choice eliminated the need to acidify the cultures or to use phenol red free culture medium because of phenol red interference as occurs with MTT at 570 nm. (Roehm *et al.*, 1991:260). Based on the kinetics of formazan production, cells appear to remain metabolically active for longer periods when pulsed with XTT/PMS than with MTT (Roehm *et al.*, 1991:265). As for MTT, poor bioreduction of XTT by cells with low metabolic activity (e.g. lymphoid cells) limits the use of this cytotoxicity assay (Jost *et al.*, 1992:163).

Although the use of PMS solved the problem with phenol red, present in most culture mediums, it was demonstrated that XTT forms an unstable reagent mixture with PMS.

This instability resulted in a time dependent depletion of PMS from the reagent mixture, which led to a subsequent decrease in formazan production. It seems when using XTT that the depletion in PMS is due to the formation of charge-transfer complexes between PMS and this tetrazolium salt. These complexes could arise from an interaction between the positively charged quaternary nitrogen on PMS and the SO_3^- groups, which are on two of the aromatic rings in the XTT structure. These two SO_3^- groups have been specifically introduced to increase the water solubility of the XTT-formazan. The formation of such charge-transfer complexes would be favoured by the electron donor/acceptor characteristics of PMS and XTT (Goodwin *et al.*, 1995:101).

The decrease in PMS was also accompanied by the formation of fine yellow crystals in the reagent mixture only seconds after its preparation. Previous studies reported crystal formation only after addition of the XTT/PMS mixture to the cells. Crystal formation was attributed to a reaction between the positively charged quaternary nitrogen on PMS and the sulfhydryl group of glutathione, which was present in the cell system. It is now known that nucleation and crystal deposition can occur in the XTT/PMS reagent mixture before addition to the cells, and it seems unlikely that this is the reason for the crystal deposition (Goodwin *et al.*, 1995:101).

Another tetrazolium salt with similar advantages over MTT as XTT, is MTS. An additional advantage that this salt has over MTT and XTT/PMS is the storage stability of the MTS/PMS solution, but this advantage is overshadowed by the fact that assays carried out with MTS are more than five times expensive than those using MTT, even if both reagents are purchased in the most economical powdered form. This consideration may be relevant for laboratories that make extensive use of such assays (Gieni *et al.*, 1995:93).

2.4.2 THE LDH ASSAY

Measurement of alkaline phosphatase activity is also a method to determine cytotoxicity, according to Korzeniewski & Callewaert (1983:314). While the target cells, most frequently used in studies of natural cytotoxicity do not contain substantial amounts of alkaline phosphatases, these authors found that lactic acid dehydrogenase (LDH) is released in sufficient amounts in short-term cytotoxicity assays to provide a sensitive and quantitative measure of natural cytotoxicity (Korzeniewski & Callewaert, 1983:314). The reason for the release of this enzyme is due to the fact that one of the primary sites of initial interaction of macromolecules is the plasma membrane (Choksakulnimitr *et al.*, 1995:234).

Lactate dehydrogenase (LDH) is a common cytosolic enzyme present in all eukaryotic cells which catalyses the oxidation of L-lactate to pyruvate in the presence of NAD^+:

$$\text{L-lactate} + NAD^+ \longrightarrow \text{Pyruvate} + NADH + H^+$$ (Sepp *et al.*, 1996:176).

Any agent that damages the cell membrane will cause the release of this enzyme into the extracellular fluid (Choksakulnimitr *et al.*, 1995:234).

It is possible to measure LDH activity spectrophotometrically in the near UV range by recording the decrease in the optical density of NADH at 340 nm. This assay is based on the oxidation of NADH when pyruvate is converted to lactate. When measuring the decrease in absorbance of NADH, the measurements should not be delayed for more than 1 hour since NADH is sensitive to photolysis and subject to chemical decay at neutral and acid pH values (Decker & Lohmann-Matthes, 1988:63).

LDH activity is also determined by measuring the formation of NADH in a coupled assay where the 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-tetrazolium (*p*-INT) salt is reduced to a red formazan product in the presence of either diaphorase or phenazine methosulphate (PMS), acting as a catalyst. The assay is fast and simple, as the optical density of the formazan can be recorded spectrophotometrically at 490 nm. Although the procedure

used to measure oxidation of NADH is quicker and cheaper it was determined that the INT reduction assay has a superior linear range (Decker & Lohmann-Matthes, 1998:68). For a larger number of samples, microplate reader based protocols have been established and are available from several commercial sources (Sepp *et al.*, 1996:176).

Korzeniewski and Callewaert (1983:319) found that the assay developed by them was more rapid than the ^{51}Cr-release assay in that: (i) preincubation of target cells with ^{51}Cr and subsequent washes are eliminated, (ii) following addition of substrate, the enzymatic assay is completed within 20 min whereas gamma counting may require several hours, and (iii) a computer interface to the microtitre plate reader allows for rapid and accurate data processing.

Comparison of kinetic parameters and percent cytotoxicity values obtained in parallel ^{51}Cr and enzymatic assays demonstrated equivalent results. However, the spontaneous release of LDH is significantly less than spontaneous release of ^{51}Cr. Thus, the enzymatic LDH assay provides a sensitive, rapid, inexpensive, and quantitative measure of natural cytotoxicity (Korzeniewski & Callewaert, 1983:319).

In concurrence with the sensitivity mentioned by Korzeniewski & Callewaert (1983:319), Lappalainen *et al.* (1994:1127) found that the LDH assay appears to be more sensitive in determining earlier damage of cell membranes than the trypan blue assay. However, the LDH assay is plagued by the same problem as the MTT assay, namely the high background resulting from phenol red when a culture medium containing phenol red is used (Lappalainen *et al.*, 1994:1129). As previously mentioned the formazan produced in the LDH assay absorbs light at 490 nm, as does phenol red causing significant loss in sensitivity (Sepp *et al.*, 1996:176). This problem was overcome by Quan *et al.* (1998:615) who washed the monolayers three times with Hanks balanced salt solution (HBSS), which does not contain phenol red, and performing all the following toxicity experiments in the HBSS buffer. Fetal calf serum, a common addition to culture mediums used in the culturing process, contains detectable LDH activity, yet another point indicating that the assay should not be performed in culture medium containing

phenol red or fetal calf serum (Franke & Porstmann, 1994:260, Duval *et al.*, 1990:180 and Decker & Lohmann-Matthes, 1988:65).

It is noteworthy that the LDH assay reagents are stable for several months at -20°C. This is an additional advantage if only a limited number of assays are to be performed over long time periods (Sepp *et al.*, 1996:180).

Korzeniewski & Callewaert (1983:319) stated that this toxicity assay that they developed should be applicable to many different cell culture lines, by determining the range of concentrations of target cells for which the assay is linear. In later years this assay was adapted with success for determining the cytotoxicity of different substances on Caco-2 cell lines (Quan *et al.*, 1998:615, De Angelis *et al.*, 1994:484 and Schasteen *et al.*, 1992:49).

2.4.3 OTHER TOXICITY ASSAYS DEPENDENT ON ENZYMATIC ACTIVITY

Six 4-methylumbelliferyl esters were tested for their ability to determine cell viability. These fluorogen substrates readily diffuse through the cell membrane and are hydrolysed by intracellular esterases or sulphatases resulting in the production of highly fluorescent 4-methylumbelliferone. 4-methylumbelliferyl heptanoate (MUH) has proven to be superior among the six candidates with respect to providing sufficient activity and low background fluorescence. The production of a fluorescent compound from a fluorogen substrate in this assay is rather similar to the widely used colorimetric MTT assay, where a tetrazolium salt is cleaved and converted to a blue formazan by the mitochondrial dehydrogenases of living cells. Due to this similarity the MTT and the MUH assay was compared. It was noticed that the MUH assay is a more rapid method and can be completed in 5 hours. Its simplicity is another advantage and is due to the avoidance of centrifugation and washing steps, but it should be noted that medium without phenol red, once again, should be used in order to avoid high background fluorescence. Avoidance of the washing may also contribute to the high accuracy (Virag *et al.*, 1995:207). The MUH assay was also compared to the ^{51}Cr-release assay and it was found that the MUH

assay shows reliable reproducibility and higher sensitivity than the ^{51}Cr-release assay (Krüger-Krasagakes *et al.*, 1992:1).

Another cytotoxicity assay performed by Wrobel *et al.* (1996:245) additional to propidium iodide staining, is the BCECF assay. BCECF is a fluorescent dye commonly used as an indicator of cell membrane integrity. With a pK_a of 6.98, BCECF is also used as an intracellular fluorescent pH indicator. The hydrophobic derivative BCECF-AM freely crosses the cell membrane thus, facilitating loading of the cells. Once inside the cell, BCECF-AM is hydrolysed by esterases to release BCECF, which is retained by the cell. When a decrease in fluorescence is observed this is an indication that the cell membrane has been damaged (Wrobel *et al.*, 1996:246).

2.5 *THE ATP BIOLUMINESCENCE ASSAY*

Molecular Probes (Leiden, The Netherlands) offers a convenient ATP Determination Kit for the sensitive bioluminescence-based detection of adenosine 5'-triphosphate (ATP) with recombinant firefly luciferase and its substrate luciferin. This assay is based on luciferase's requirement for ATP to produce light. In the presence of Mg^{2+}, luciferase catalyzes the reaction of luciferin, ATP and O_2 to form oxyluciferin, AMP, CO_2, pyrophosphate and ~560 nm light (figure 2.6).

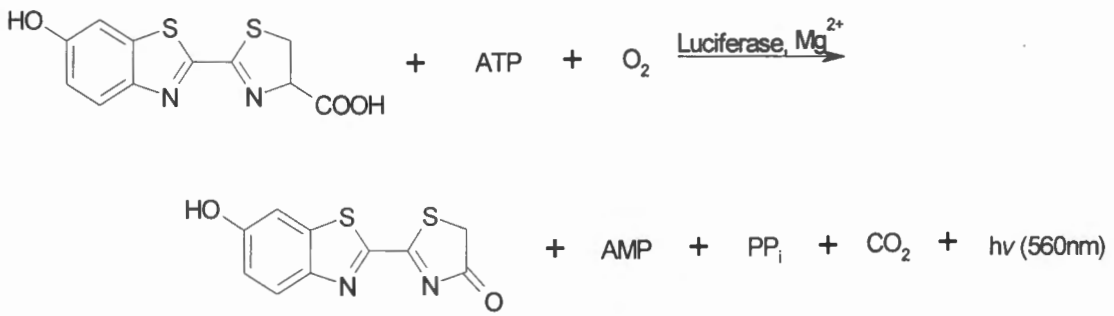


Figure 2.6 Reaction scheme for bioluminescence generation via luciferase-catalyzed conversion of luciferin to oxyluciferin.

The luciferin-luciferase assay is extremely sensitive as most luminometers can detect as little as 1 picomole of pre-existing ATP, or ATP as it is generated in kinetic systems. This sensitivity has led to its widespread use for detecting ATP in various enzymatic reactions, as well as for measuring viable cell numbers (Haugland, 1996:376). Since cellular lysis is accompanied by ATP release and ATP is required as a cofactor of the luciferase reaction, the detection of released ATP has been suggested as the readout system in cytotoxicity assays. This assay also avoids the use of radioisotopes, is quick, simple and reproducible. Results can be obtained within 15 minutes of the termination of cell incubation. (Crouch *et al.*, 1993:87).

Petty *et al.* (1995:29) compared the MTT assay with the ATP bioluminescence assay and reported a sensitivity and reproducibility for this assay superior to that of the MTT assay. The inferior sensitivity of the MTT assay can be attributed to the robustness of mitochondria increasing the possibility of injured cells to reduce MTT. The necessity to use more control wells for the MTT assay, than the ATP assay, and to optimize the concentration of MTT used, often means that fewer test samples per microplate can be accommodated in the MTT assay.

In contrast to the advantages of the assay it also has some major disadvantages: ATP is very unstable in tissue cultures and ATP is rapidly consumed by the assay, and therefor detection is not as sensitive as in a system where the enzyme (luciferase) is measured and ATP is provided externally or in combination with coenzyme A (Schäfer *et al.*, 1997:96). Compared to the MTT assay the ATP assay is also very expensive. The MTT assay uses reagents costing approximately £5/plate, while the ATP assay uses reagents costing about £30/plate. Furthermore most laboratories have spectrophotometers or microtitre plate readers, while the number with luminometers is much smaller. Although the ATP assay is more sensitive and reproducible than the MTT assay, the cost and the availability of equipment and reagents rather than the assays sensitivity and reproducibility, will influence many laboratories decision (Petty *et al.*, 1995:32).

2.6 *MEASUREMENT OF THE TRANSEPITHELIAL ELECTRICAL RESISTANCE (TEER)*

The measurement of transepithelial electrical resistance (TEER) is a method that has widely been used to assess the effect that absorption enhancers have on Caco-2 cell monolayers. In epithelia such as those found in the small intestine, the majority of passive ion flow is presumed to be through the paracellular space. Therefore, as TEER is a measure of the ion permeability of the epithelial layer, it is also a measure of the integrity of tight junctions found between the cell membranes of the cells (Tötterman *et al.*, 1997:47).

A reduction in TEER is believed to be an indication of the opening of the tight junctions in the epithelium resulting in increased paracellular absorption. This is however, not the only application for TEER measurements but it is also used to determine the reversibility of the effect that these substances have on the monolayer. An irreversible effect on the lowering of the TEER of a monolayer could be an indication of non-specific epithelial damage and thus cytotoxicity (Quan *et al.*, 1998:619; Werner *et al.*, 1996:1225; Anderberg & Artursson, 1993:397, Borchard *et al.*, 1996:136). If a complete vanishing of TEER is noticed it could be due to necrotized areas of the Caco-2 monolayer. (Twiss *et al.*, 1994:703).

2.7 *CONCLUSION*

Cytotoxicity assays performed on cell cultures proved to be a quick and easy way to determine the cytotoxicity of a substance. Since the introduction of the ^{51}Cr-release assay in 1961, many other cytotoxicity assays have been developed. In the development of these assays it was attempted to reduce the disadvantages connected with the ^{51}Cr-release assay. Mainly two types of assays were developed, namely assays based on the inclusion or exclusion of a dye from cells and assays based on the measurement of enzymatic activity.

Fluorescent probes are extensively used to assess the process of dye uptake in the cells after membrane damage. The different instruments that could be used in measurements also add to the popularity of these fluorescent probes. Many fluorescent probes are applicable in these studies, increasing the popularity of this type of assay. Of these fluorescent probes PI has proven to be very popular, and is extensively used to assess cytotoxicity.

NR is actively absorbed by the cell membrane into a cell. The assay is based on the fact that viable cells do take up the dye in contrast to non-viable cells that don't. With the use of NR, sublethal injury to cells can be detected in contrast to trypan blue, a dye used to detect cell membrane damage. Viable cell numbers are easily determined through colorimetric measurement.

The second type of assay used to determine cytotoxicity is based on determining the activity of certain intracellular enzymes. Mitochondrial dehydrogenase converts colourless tetrazolium salts to coloured formazan crystals, which are spectrophotometrically analyzed after dissolving the crystals. This process however, only occurs in living cells with active mitochondria. From all the terazolium salts, MTT is the most commonly used. Compared to the propidium iodide assay the sensitivity of the MTT assay is superior, although propidium iodide has a much wider analytical working range and a shorter analysis time.

Lactate dehydrogenase is released from cells after the cell membranes are damaged. The release of this enzyme can be determined spectrophotometrically, either directly by measuring the decrease in the optical density of NADH or in a coupled assay where *p*-INT is reduced to a red formazan product. The release of LDH should be seen as an indication of severe toxicity.

A new development in cytotoxicity assays is the measurement of the amount of ATP released from lysed cells. ATP in combination with luciferin and luciferase produces light that is detectable with a luminometer. This is a very sensitive assay and results can be obtained in a short period of time.

All of the above assays are applicable to cell monolayers as well as cell suspensions. The measurement of transepithelial electrical resistance (TEER) can be used to determine toxicity, but only on cell monolayers. An irreversible decrease in TEER or a complete vanishing of TEER is seen as an indication of cytotoxicity.

None of the disadvantages experienced in performing the ^{51}Cr-release assay is experienced with any of the above mentioned assays. These assays also have a higher sensitivity than the ^{51}Cr-release assay, are simpler and more reliable, but should preferably be performed in medium free of phenol red because several problems may be encountered when culture medium containing phenol red is used.

CHAPTER 3

IN VITRO EVALUATION OF THE TOXICITY PROFILE OF *N*-TRIMETHYL CHITOSAN CHLORIDE : EXPERIMENTAL DESIGN

3.1 INTRODUCTION

In chapter 1 the absorption enhancing properties of *N*-trimethyl chitosan chloride (TMC), the partially quaternised derivative of chitosan, were discussed. It was shown that TMC was able to decrease transepithelial electrical resistance (TEER) across Caco-2 cell monolayers as well as to increase the transport of hydrophilic macromolecules both *in vitro* and *in vivo* (Kotzé *et al.*, 1998:45). These absorption enhancing effects of TMC were dependent on the degree of quaternisation of the polymer. It was also shown that in the neutral environments, where chitosan hydrochloride is ineffective as an absorption enhancer, TMC was able to reduce the TEER of Caco-2 cell monolayers and to increase the transport of several hydrophilic compounds across the monolayers.

In chapter 2 different cytotoxicity assays performed on cell cultures were discussed. It was clearly illustrated that chemical compounds could have different effects on the viability of cells, either by damaging cell membranes or by inhibiting intracellular enzymatic processes. Thanou *et al.* (2000:77) came to the conclusion that TMC is non-toxic towards Caco-2 cells due to the absence of fluorescence, after staining the monolayers with the fluorescent probe YO-PRO®. Although this was an indication that cell membrane damage was minimal it cannot be concluded that there is no effect on the intracellular enzymatic processes.

As mentioned earlier the aim of this study is to evaluate the toxicity profile of TMC by studying the effect of the degree of quaternisation and concentrations of TMC on the viability of intestinal epithelial cell cultures (Caco-2).

In order to achieve the aim set TMC polymers with different degrees of quaternisation were synthesised and characterised. These polymers were applied to Caco-2 cells in different concentrations and the viability of the cells were determined by the MTT assay, LDH assay as well as staining of the cultures with propidium iodide. In the following sections of this chapter, the synthesis and characterisation of the TMC polymers as well as the methodology in performing these cytotoxicity assays will be described in more detail. Results of these assays will be discussed in chapter 4.

3.2 SYNTHESIS AND CHARACTERISATION OF N-TRIMETHYL CHITOSAN CHLORIDE (TMC) WITH DIFFERENT DEGREES OF QUATERNISATION

In chapter 1 the absorption enhancing effects of chitosan and TMC on hydrophilic macromolecules, such as peptide and protein drugs, were discussed. It was shown that the charge density of TMC played an important role in its absorption enhancing effects and that the charge density of TMC is determined by the degree of quaternisation. According to Sieval *et al.* (1998:157) the degree of quaternisation can be increased by increasing the number of reaction steps as well as the duration of each step combined with the amount of methyl iodide used as reagent. The degree of quaternisation can be calculated from nuclear magnetic resonance spectra (^{1}H-NMR).

3.2.1 EXPERIMENTAL PROCEDURES

3.2.1.1 Synthesis of TMC polymers

Five TMC polymers with different degrees of quaternisation were synthesised in our laboratory according to the method of Sieval *et al.* (1998:157-158). TMC was synthesised from Seacure 244, which is 93 % deacetylated chitosan (Pronova Biopolymer A.S., Drammen, Norway). In order to promote the solubility of the starting polymer it was milled in a Retsch-mill (Retsch KG, West Germany) to obtain a powder with relative

small particles. The process of reductive methylation in which the chitosan is converted to TMC is depicted in figure 3.1.

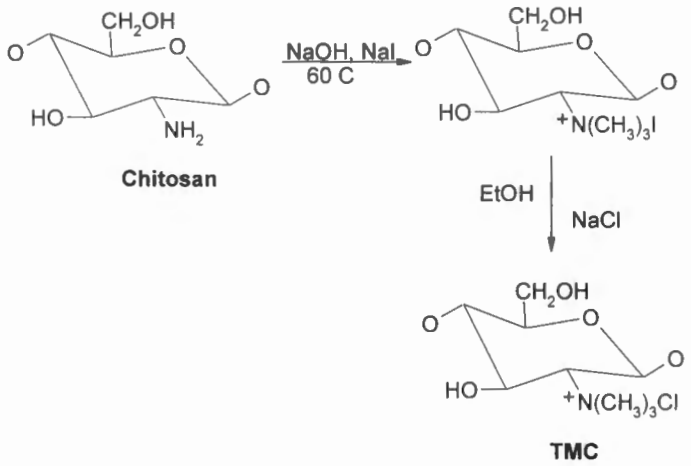


Figure 3.1 Synthesis of TMC polymers by reductive methylation of chitosan.

From the reaction depicted in figure 3.1 it can be seen that the primary amino group at position C-2 is changed to a quaternary amino group. The difference in degree of quaternisation was obtained by varying the number as well as the length of reaction steps. The different reaction steps in the synthesis of TMC are described in the following paragraphs.

Reaction step 1: A mixture of 2 g chitosan, 4.8 g sodium iodide (Saarchem, Midrand, R.S.A.), 11 ml of 15 % (w/v) aqueous sodium hydroxide solution (Merck, Midrand, R.S.A.) and 11.5 ml of methyl iodide (Merck, Midrand, R.S.A.) in 80 ml of *N*-methylpyrrolidone (Saarchem, Midrand, R.S.A.) was magnetically stirred on a water bath at a temperature of 60 °C. The methyl iodide was kept in reaction by a Liebig's condenser. The product was carefully precipitated with 99 % ethanol (Merck, Midrand, R.S.A.) by slowly adding the ethanol to the mixture.

Reaction step 2: The product obtained in the previous reaction step was dissolved in 80 ml of *N*-methylpyrrolidone. 4.8 g sodium iodide, 11 ml of 15 % (w/v) aqueous sodium hydroxide solution and 7 ml of methyl iodide was added to the mixture after which it was stirred on a water bath at a temperature of 60 °C for 30 min.

Later adding step: Preceding precipitation at the end of a reaction step an additional 2 ml of methyl iodide and 0.6 g sodium hydroxide pellets were added to the mixture while stirring. This process was continued for another 30 minutes at a temperature of 60 °C. The product was precipitated and isolated by centrifugation.

Reaction step 3: The product was dissolved in 80 ml *N*-methylpyrrolidone. 4.8 g sodium iodide, 11 ml of 15 % (w/v) aqueous sodium hydroxide solution and 11.5 ml of methyl iodide was added to the mixture after which it was stirred on a water bath at a temperature of 60 °C for 30 min. The product was precipitated and isolated by centrifugation.

Iodide-exchange step: After washing with ethanol and diethylether (Merck, Midrand, R.S.A.) the product was dissolve in 40 ml of a 5 % NaCl solution to exchange the iodide-ion with a chloride-ion. The polymer was precipitated using ethanol and isolated by centrifugation.

The product was dissolved in 40 ml water and precipitated with ethanol to remove the remaining NaCl from the material. The final product was dried under vacuum at 40 °C.

The previous paragraphs describe the different steps in the synthesis of the TMC polymers, however it was mentioned that different degrees of quaternisation is obtained by varying the number of reaction steps as well as the reaction time. Table 3.1. is a summary of the number of reaction steps and duration of these steps of all the polymers synthesised. Please note the order in which the reaction steps were carried out for each polymer. It should also be noted here that TMC-12 was not synthesised in our laboratory but was kindly donated by Dr. A.F. Kotzé of the Potchefstroomse Universiteit vir

Christelike Hoër Onderwys (South Africa). However, the procedure described above was also followed for the synthesis of TMC-12 except for the chitosan used as starting material which was 80 % deacetylated.

Table 3.1 Number of reaction steps used to synthetize TMC polymers with different degrees of quaternisation.

Polymer	Number of reaction steps	Duration of reaction steps
TMC-12	One reaction step	Step one: 60 min
TMC-22	One reaction step	Step one: 45 min
TMC-38	Two reaction steps	Step one: 45 min Step two: 30 min
TMC-43	Two reaction steps with later adding step	Step one: 45 min Step two: 30 min Later adding step: 30 min
TMC-48	Three reaction steps with later adding step	Step one: 45 min Step two: 30 min Later adding step: 30 min Step three: 30 min
TMC-53	Three reaction steps with later adding step	Step one: 45 min Step two: 30 min Later adding step: 45 min Step three: 30 min

3.2.1.2 Characterisation of TMC

^{1}H-NMR was used to characterise the synthesised TMC polymers. The ^{1}H-NMR spectra were recorded in D_2O with a Bruker 600 MH spectrometer (Bruker, Switzerland) at 80 °C. The following equation was used in order to calculate the degree of quaternisation for each TMC polymer from the ^{1}H-NMR spectra:

$$DQ\ (\%) = [(TM/H) \times 1/9] \times 100 \qquad [1]$$

Where DQ (%) = degree of quaternisation expressed as a percentage,

TM = integral of the trimethyl amino group peak, and

H = integral of the ^{1}H peaks.

3.2.2 RESULTS AND DISCUSSION

The ^{1}H-NMR obtained for each polymer is depicted in figures 3.2 – 3.8. Sieval *et al.* (1998:158) assigned the peak at 3.3 ppm to the trimethyl amino group and the peaks between 4.7 and 5.7 ppm to ^{1}H. The integrals of these peaks were substituted into equation 1 to calculate the degree of quaternisation for each synthesised polymer.

The degree of quaternisation for each TMC polymer is shown in table 3.2.

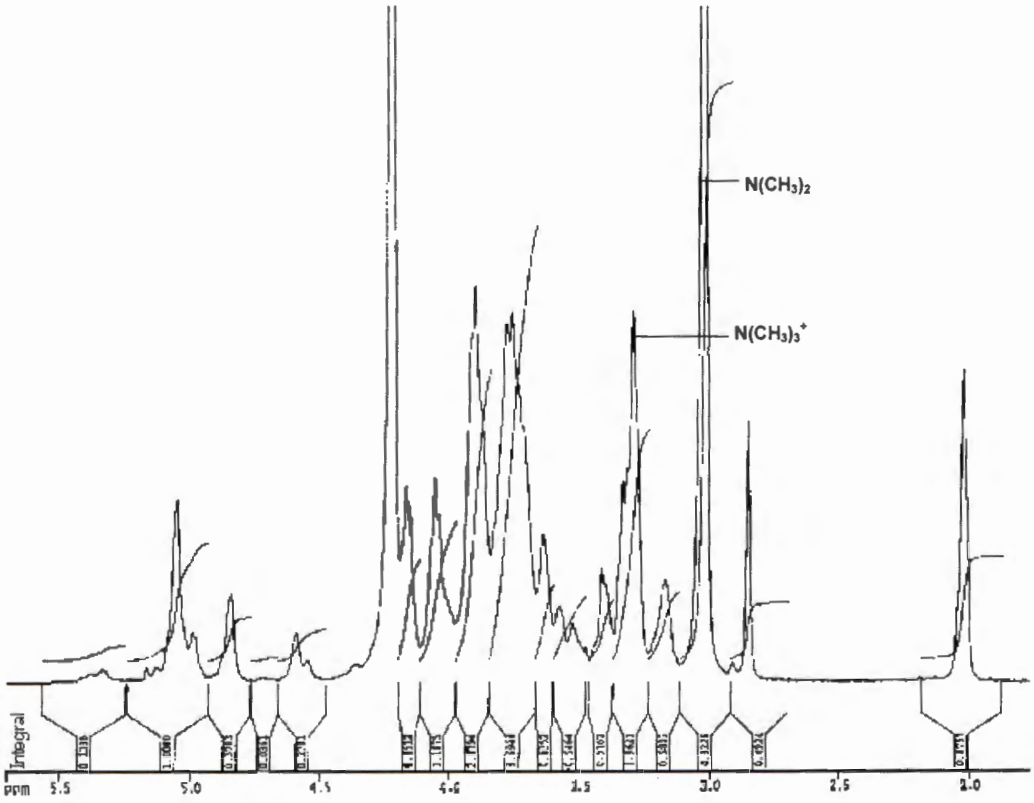


Figure 3.2 ^{1}H-NMR spectra of TMC with a degree of quaternisation of 12.28 %.

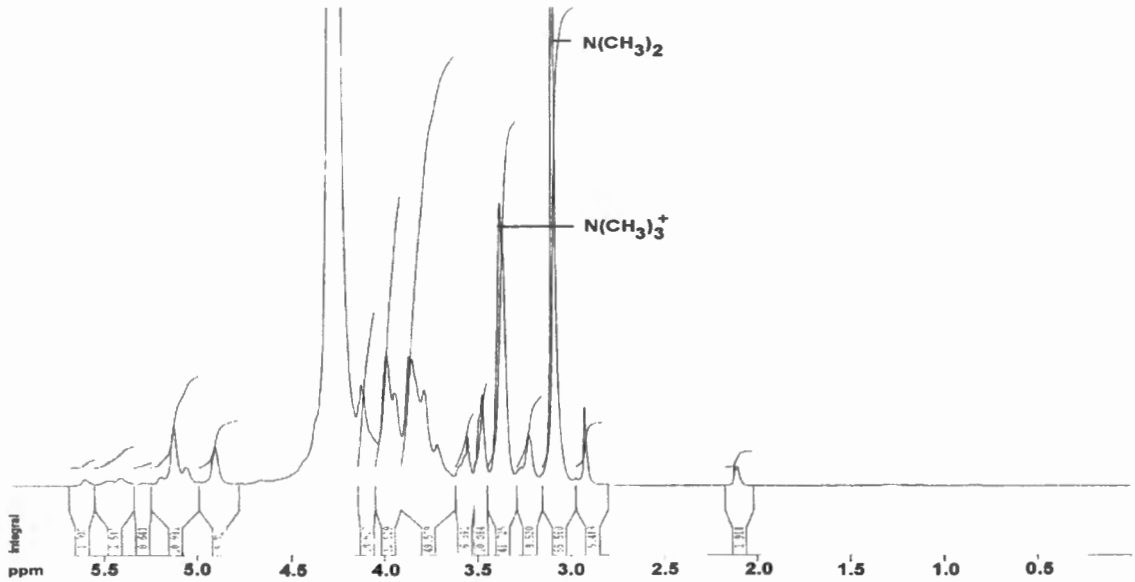


Figure 3.3 ^{1}H-NMR spectra of TMC with a degree of quaternisation of 22.15 %.

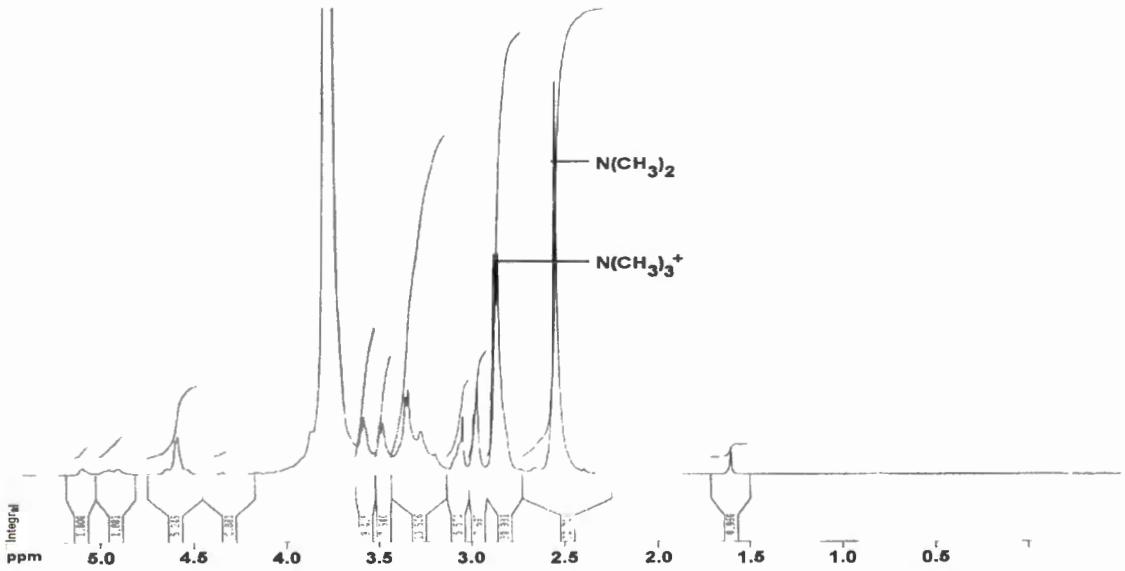


Figure 3.4 ^{1}H-NMR spectra of TMC with a degree of quaternisation of 38.14 %.

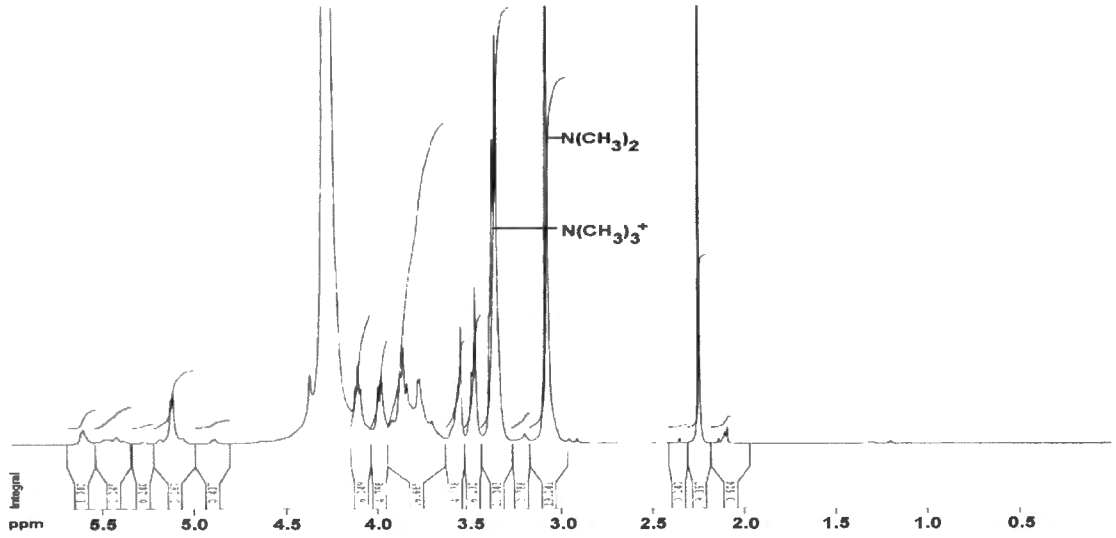


Figure 3.5 ^{1}H-NMR spectra of TMC with a quaternisation of 42.75 %.

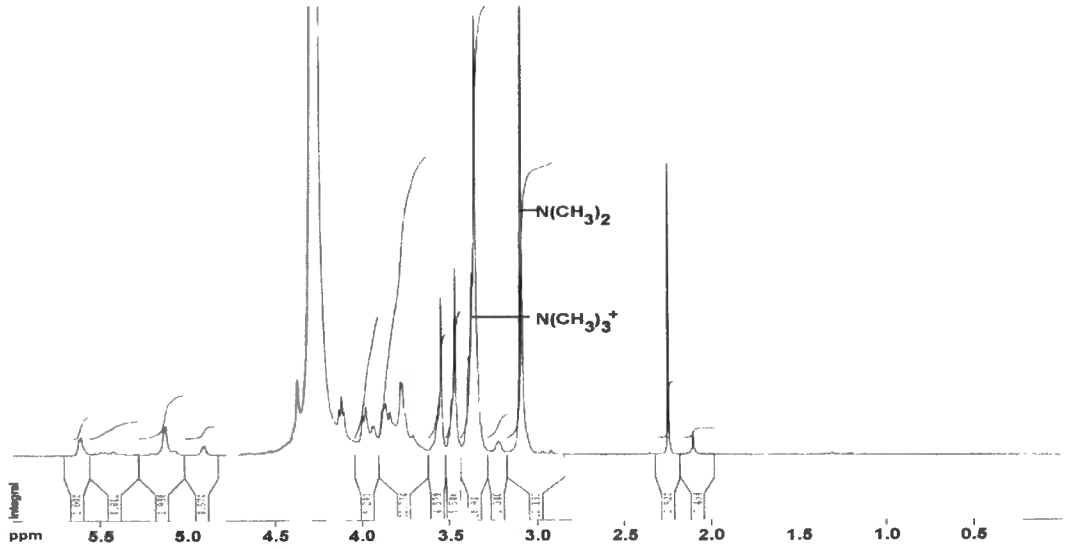


Figure 3.6 ^{1}H-NMR spectra of TMC with a degree of quaternisation of 48.75 %.

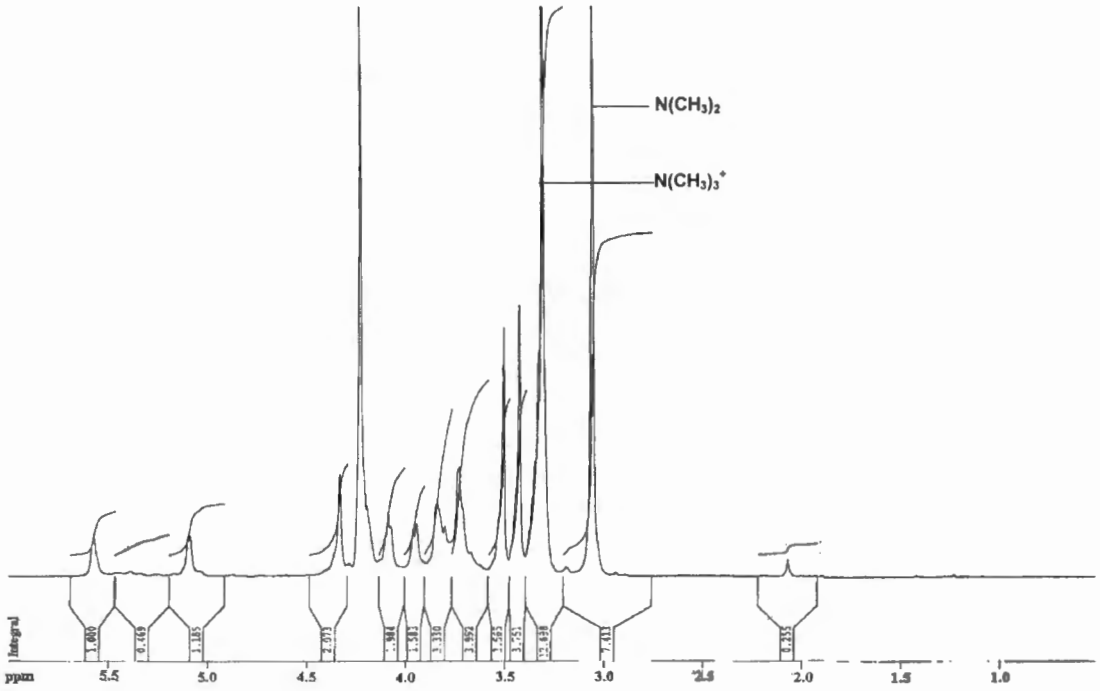


Figure 3.7 ^{1}H-NMR spectra of TMC with a degree of quaternisation of 53.16 %

Table 3.2: Degrees of quaternisation for the different TMC polymers calculated from ^{1}H-NMR spectra.

TMC polymer	Degree of quaternisation (%)
TMC-12	12.28
TMC-22	22.15
TMC-38	38.14
TMC-43	42.75
TMC-48	48.75
TMC-53	53.16

The results presented above are clear evidence that a range of TMC polymers can be synthesised by varying the reaction conditions during the reductive methylation of chitosan. The ^{1}H-NMR spectra were in good agreement with previously published results (Sieval *et al.*, 1998:158). The polymers obtained in this synthesis procedures were

used for cytotoxicity evaluation and it is concluded that low, intermediate and high degrees of quaternisation of TMC are equally represented to compile toxicity profiles for TMC with different degrees of quaternisation.

3.3 CULTURING AND MAINTENANCE OF INTESTINAL CACO-2 CELLS

3.3.1 INTRODUCTION

In chapter 2 different cytotoxicity assays were discussed as a means to determine the potential damaging effect that chemicals might have on the human body. Most of these assays can be performed on a variety of cell culture lines and therefore different factors influence the choice of the most appropriate cell line in order to perform these assays. The Caco-2 cell line has been widely used as an *in vitro* model for the evaluation of intestinal epithelial permeability. Due to it's favourable characteristics for these permeability assays, and the fact that the aim of this study is to evaluate the toxicity profile of an absorption enhancer, which increases the permeability of substances known to have a low permeability across epithelial membranes, it was decided that the Caco-2 cell line was most suited for these cytotoxicity assays. In the following paragraphs the culturing and maintenance of this cell line will be discussed.

3.3.2 CACO-2 CELL CULTURING PROCEDURES.

All cell culture procedures were performed under aseptic condition in a model 834 laminar flow chamber (Labotec, Halfway House, R.S.A.). All cultures were incubated in a water jacketed CO_2 incubator (Forma Scientific Inc., Marietta, Ohio, U.S.A.) at 37 °C in a humidified atmosphere of 5 % CO_2. All chemicals that came into contact with the cultures were preheated to 37 °C in a series SWBD water bath (Stuart Scientific, Surrey, U.K.).

All culture medium used, unless otherwise specified, consisted of Dulbecco's Modified Eagle's Medium (DMEM) (Bio Whittaker, Walkersville, Maryland) supplemented with 10 % (v/v) foetal bovine serum (Delta Bioproducts, Kemptonpark, R.S.A.), 1 % (v/v) non-essential amino acids (Bio Whittaker, Walkersville, Maryland) and 1% (v/v) Penstrep Fungizone® solution (Bio Whittaker, Walkersville, Maryland).

3.3.2.1 Growth medium replacement and monitoring of growth in culture flasks

The Caco-2 cells were grown in 25 cm^2 culture flasks (Corning Costar Corporation, Cambridge, U.S.A.). In order to obtain optimal growth of the Caco-2 cells the culture medium was removed and replaced every second day with 10 ml of fresh preheated media. Caco- 2 cells were also cultivated as monolayers on filters of which the procedure will be described later (section 3.4.4). The cells multiplied logarithmically in the culture flask, which was constantly monitored for confluency in order to determine when the cells should be subcultured or seeded out for experimental use. This was usually done 5 to 7 days after the cells were seeded in the culture flasks.

3.3.2.2 Trypsination of cell layers

As mentioned in the previous paragraph the cell counts in the culture flasks were high enough to proceed with after 5 to 7 days by either subculturingting the cells in a 1:3 ratio or by seeding the cells onto filters or microtitre plates. After sterilisation of the aspirator the used growth medium was removed from the healthy confluent monolayer grown in the 25 cm^2. The monolayer was washed twice with 5 ml of preheated Hank's Balanced Salt Solution (HBSS) (without calcium and magnesium) (Bio Whittaker, Walkersville, Maryland) taking care not to disturb the cell layer. In order to loosen the cells from the culture flask and from each other the cell monolayers were incubated for 10 minutes at 37 °C with 0.5 ml of preheated Trypsin-Versene® solution (Bio Whittaker, Walkersville, Maryland). After incubation with Trypsin-Versene® a small amount of culture medium was added to the flask. The cells were loosened from the bottom by rapid agitation with a pasteur pipette. The cells were ready for seeding onto microtitre plates or filters for

subsequent cytotoxicity evaluation. These procedures will not be described at present but will be discussed in the appropriate sections of cytotoxicity evaluation.

3.4 CYTOTOXICITY EVALUATION OF THE DIFFERENT SYNTHESISED TMC POLYMERS

3.4.1 SELECTION OF APPROPRIATE ASSAYS

In chapter 2 the different assays which can be performed on cell cultures was discussed in detail, which assisted with the selection of appropriate assays to evaluate the toxicity profile of TMC. It was decided to perform an enzymatic based assay, an assay where cell damage is measured by the uptake of a fluorescent probe and an assay where cell damage is measured by the leaking of a intracellular compound from the cells. The following assays were chosen:

- MTT assay
- LDH assay and
- Propidium iodide staining of monolayers.

As mentioned in chapter 2 all of the above mentioned assays have been performed on Caco-2 cell monolayers in the past. It was therefore assumed that these assays would be well suited for the evaluation of the toxicity profile of TMC. In the following sections the experimental procedures for these assays will be described in detail.

3.4.2 THE MTT ASSAY

As discussed in chapter 2 mitochondrial dehydrogenase converts colourless tetrazolium salts to coloured formazan crystals, which are spectrophotometrically analyzed after dissolving the crystals. This process however, only occurs in living cells with active mitochondria. One of these salts that are easily converted is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). In this assay the viability of the cell culture, after performing the assay, is expressed as a percentage of the control value (cells which were not subjected to the substance). Due to this expression of results as a percentage of a control value it is evident that the relation between the amount of formazan crystals formed and the number of viable cells should be linear.

3.4.2.1 Proof of linearity

A culture flask with Caco-2 cells at a passage number of 21 was incubated with Trypsin–Versene® as described in section 3.3.2.2. Special care was taken in loosening the cells from the flask and each other in order to prevent damage to the cells. The cells were seeded onto 96 well tissue culture treated flat bottom microtitre plates (Corning Costar Corporation, Cambridge, U.S.A.) in the following concentrations: 80 000, 60 000, 48 000, 40 000, 20 000 and 0 cells/well. The final seeding volume for each well was 200 µl and for each of these concentrations 4 wells were seeded. The plate was incubated at 37 °C for 24 hours for the cells to adhere to the bottom of the plate. MTT (Sigma-Aldrich, Atlasville, R.S.A.) solution, at a concentration of 5 mg/ml in phenol red free HBSS (Bio Whittaker, Walkersville, Maryland) was prepared and stored at -20 °C until it was used. After the 24 hour incubation period the plates were inverted onto tissue paper to remove the culture medium. The cells were washed twice with preheated phenol red free HBSS in order to remove the phenol red and foetal bovine serum present in the culture medium. After each wash the plate was once again inverted onto tissue paper. 20 µl of the prepared MTT solution (preheated) was added to each well and the plate was incubated at 37 °C for another 5 hours in order for the formazan crystals to form. 100 µl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Atlasville, R.S.A.) was added to each well to dissolve the crystals. The optical density of each well was measured with a Biotek FL600 microtitre plate reader (Bio-Tek Instrument Inc., Winooski, Vermont,

U.S.A.) at a wavelength of 560 nm. The backround measured at 630 nm was automatically subtracted by the plate reader. A graph was constructed with the average of the optical densities and the cells/well. The correlation coefficient was determined for the graph in order to prove linearity.

3.4.2.2 Cytotoxicity evaluation of the TMC polymers

Solutions of the synthesised polymers were prepared in the following concentrations: 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.015625 % (w/v) in serum free DMEM supplemented with 1 % Penstrep Fungizone® mixture and 1 % non essential amino acids. Caco-2 cells (passage 21) were seeded onto 96 well tissue culture treated microtitre plates (refer to previous section) at a concentration of 50 000 cells/well. After the 24 hour incubation period the plate was inverted and 200 µl of the prepared TMC solutions concentrations was added to a quadruplicate set of wells. The culture medium without any TMC was added to a quadruplicate set of wells, which functioned as the control. The plates were incubated for 4 hours with the TMC solutions at 37 °C. After this incubation period the polymer solutions were removed from the cells by inverting the plates onto tissue paper. From this point onward all wells were treated in the same manner as described in the previous section.

3.4.2.3 Data analysis and statistical evaluation

The viability of the cells were expressed as a percentage of the control values. For each well the percentage of viable cells was determined and the data was expressed as the mean with its standard deviation. Statistical differences between the means of the values between the different treatments and concentrations were evaluated with 2 way variance analysis [The SAS system for Windows Release 6.12 (1996), SAS Institute, Cary, NC, U.S.A.)]. To determine where the statistical differences between the means of the different concentrations, treatments and interactions were, Tukey intervals were calculated (Statistica for Windows release 5.5 (1999), StatSoft, Inc., Tulsa, U.S.A.). Results were considered statistically significant if $p < 0.01$.

3.4.3 THE LDH ASSAY

The LDH assay measures the amount of lactate dehydrogenase leaking from damaged cells. To perform this assay a CytoTox 96® non- radioactive assay kit was obtained from Promega (Madison, Wisconsin, U.S.A.). The specifications of the manufacturer were followed in the performance of this assay and will be described in the following sections. It should however be noted that the protocol supplied by the manufacturer only states the procedure in which cell mediated cytotoxicity is evaluated. The protocol was therefore adapted in order to evaluate cytotoxicity mediated by chemicals, in this case TMC polymers. The TMC solutions were prepared in the same concentrations as for the MTT assay, however, phenol red free DMEM (Bio Whittaker, Walkersville, Maryland) was used instead of the normal DMEM.

3.4.3.1 CytoTox 96 assay controls

In this assay it is important to note that 5 control values are of importance, namely:

- Cell spontaneous LDH release control
- Cell maximum LDH release control
- Volume correction control
- Culture medium background control
- Test compound background control

3.4.3.2 Optimization of target cell number for use in the CytoTox 96® assay.

3.4.3.2.1 Assay plate setup

Serial dilutions of Caco-2 cells (passage 23) were prepared in quadruplicate set of wells in V-bottom 96 well tissue culture plates (Corning Costar Corporation, Cambridge, U.S.A.) using serum free and phenol red free DMEM. This was done by the seeding of 0, 5 000, 10 000 and 20 000 cells/100 µl into these wells. A quadruplicate set of wells was prepared containing no cells for the culture medium control.

3.4.3.2.2 Cell lysis and supernatant harvest.

10 µl of lysis solution (10 x) (supplied in kit) was added per 100 µl of medium to all wells to lyse cells after which the plates were incubated at 37 °C, in a humidified atmosphere of 5 % CO_2 for 45 minutes. The plate was centrifuged at 250 x g for 4 minutes in a model 3-15 laboratory centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) specially equipped for centrifuging of the microtitre plates. 50 µl aliquots from all wells were transferred to a clean 96 well flat bottom (enzymatic assay) plate.

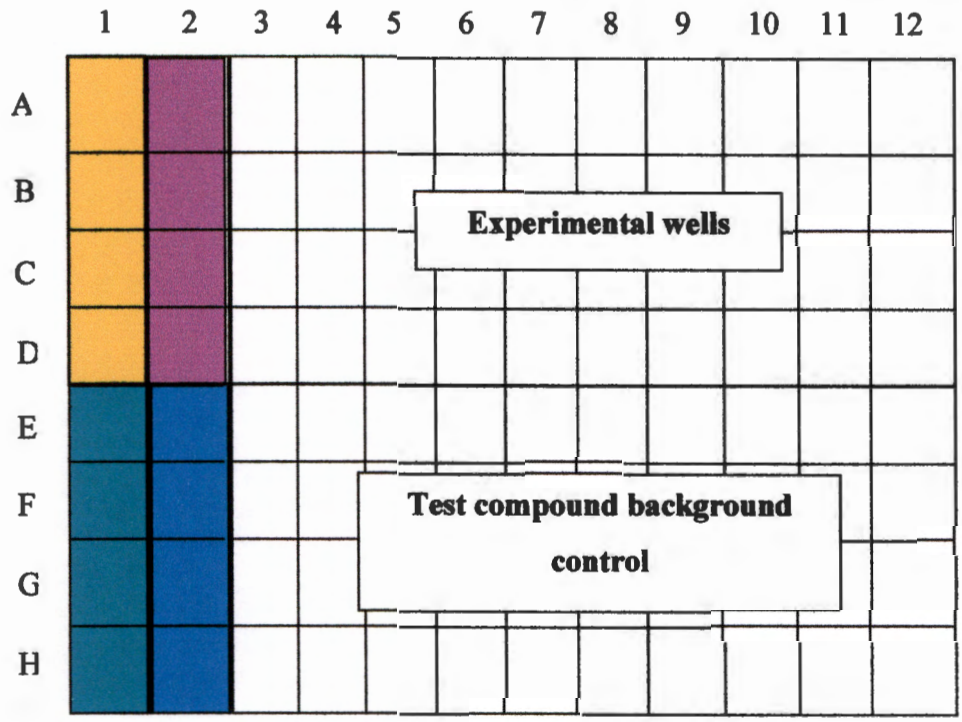
3.4.3.2.3 LDH measurement

The assay buffer supplied in the kit was thawed and 12 ml were removed. The unused portion was promptly stored at –20 °C. The 12 ml assay buffer was warmed to room temperature, while being **protected from light**, after which it was added to a bottle of substrate mix (supplied in kit). The bottle was gently shaken in order to dissolve the substrate mix, which was used immediately. Special care was taken in protecting the substrate mix from light. Unused portions were promptly stored at –20 °C for future use.

50 µl of the reconstituted substrate mix was added to each well of the plate. The plate was covered with foil and incubated at room temperature for 30 minutes. After this incubation period 50 µl of stop solution (supplied in kit) was added to each well. The absorbance was recorded immediately at 495 nm with a Biotek FL600 microtitre plate reader, as stability is only guaranteed within one hour of adding the stop solution. The concentration of cells, which yielded an absorbance of at least two times the background absorbance of the culture medium, was determined. All concentrations were able to yield absorbance values twice the absorbance of the culture medium control. It was decided to perform the assay with 10 000 cells/100 µl.

3.4.3.3 Cytotoxicity assay

3.4.3.3.1 Assay plate setup




Cell spontaneous release control


Culture medium background control


Volume correction control


Cell maximum LDH release control

Each experimental and control reaction was performed in quadruplicate.

3.4.3.3.2 Experimental wells

10 000 Caco-2 cells (passage 25) per 50 µl of culture medium were seeded in the experimental wells of V-bottom 96 well plates, thus all wells in row A-D from position 3 to 8 (section 3.4.3.3.1). For each TMC polymer a different plate was seeded containing all the concentrations of the particular test compound. In all of these cases one culture flask of Caco-2 cells were used per plate and all controls were determined for each plate. The same number of cells were seeded in the wells that were used for determining the cell spontaneous LDH release control as well as the cell maximum LDH release control. At this point it should be noted that the agitating process used to loosen the cells from each other does damage the cell membranes of the cells to a certain extent. In order to keep the cell spontaneous LDH release control as low as possible the original suspension medium was replaced with fresh medium before the suspension was diluted to 200 000 cells per ml. This resulted in very low values for this specific control.

To all of the wells, except the wells used to determine these two control values, 50 µl of the appropriate TMC polymer in different concentrations was added. Four wells per concentration were allocated vertically on the plate. For the wells of the two mentioned control values the volume was adjusted to 100 µl with culture medium containing no TMC.

In the bottom half of the plate (rows E to F) 100 µl of the TMC solution corresponding with the concentration of the TMC solution located in the 4 wells directly above these wells, were added. For the volume correction control and culture medium background control, each of the appropriate wells were filled with 100 µl of culture medium containing no cells and no TMC polymers. All plates were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 for 4 hours.

3.4.3.3.3 Cell maximum LDH release and volume correction control

45 minutes prior to harvesting the supernatants 10 µl of lysis solution was added to the wells used to determine cell maximum LDH release. The same amount of lysis solution was also added to the volume correction control wells.

3.4.3.4 Cell culture and supernatant harvest

After the appropriate incubation period, 4 hours for each plate, the plates were centrifuged at 250 x g for 4 minutes. All wells were subsequently treated in the same way as described from section 3.4.3.2.3. For the transfer of the 50 µl aliquots as well as the addition of the 50 µl substrate mix an 8-channel Eppendorf Research Pro® pipette (Eppendorf, Hamburg, Germany) was used. The addition of the stop solution was also done with this specific pipette. The absorbance was recorded immediately at 495 nm after adding the stop solution with a Biotek FL600 microtitre plate reader.

3.4.3.5 Data analysis

The average absorbance value (A) for each quadruplicate set of wells was calculated and subsequently substituted into equation 2 and cytotoxicity was expressed as a percentage value.

$$\% \text{ Cytotoxicity} = \frac{(A_{experimental} - A_{spontaneous}) - (A_{test\ compound\ control} - A_{medium})}{(A_{maximum\ release} - A_{volume\ correction})} \times \frac{100}{1} \qquad [2]$$

3.4.4 PROPIDIUM IODIDE STAINING OF CACO-2 CELL MONOLAYERS

3.4.4.1 Preparation of Caco-2 cell monolayers

Caco-2 cells (passage 23) were seeded onto tissue culture treated clear polyester filters (area 4.7 cm^2) in Costar Transwell 6 well plates (Corning Costar Corporation, Cambridge, U.S.A.) at a seeding density of 10^4 cells/cm^2. DMEM supplemented with 10 % (v/v) foetal bovine serum, 1 % (v/v) non-essential amino acids and 1 % (v/v) Penstrep Fungizone® solution was used as culture medium. Cell culture were kept at a temperature of 37 °C in an atmosphere of 95 % air and 5 % CO_2. The culture medium was replaced every second day with fresh preheated medium in order to sustain maximum growth of the culture.

3.4.4.2 Staining of filters with propidium iodide

The monolayers were confluent and ready for use 21 days after seeding of the cells. TMC polymer solutions were prepared at 0.5 and 0.125 % (w/v) concentrations in serum free DMEM. The apical and basolateral medium was removed from the filters and replaced with medium containing no TMC basolaterally and medium containing TMC apically. The filters were incubated for 1 hour at 37 °C in an atmosphere of 95 % air and 5 % CO_2. All medium was removed from the filters, both apically and basolaterally. The filters were washed twice with preheated phosphate buffered saline (PBS) (Bio Whittaker, Walkersville, Maryland) to remove the polymer solutions and the remaining phenol red which was present in the DMEM. A preheated solution of propidium iodide (Sigma-Aldrich, Atlasville, R.S.A.) at a concentration of 50 μg/ml in PBS was applied on the apical side of the monolayer for 5 minutes. The propidium iodide solution was removed from the monolayer and the monolayer was prepared for fluorescence micrography.

3.4.4.3 Preparation of control filters

As a positive control for this assay, a filter was incubated with 0.1 % sodium lauryl sulphate for one minute on the apical side of the filter. A filter incubated for 1 hour with DMEM containing no TMC was used as a negative control. The filters were stained in exactly the same way as the experimental filters.

3.4.4.4 Fluorescence micrography

The support filter with the monolayer was carefully cut loose from the plastic insert and sandwiched between two round coverslips. The sandwiched filter was placed in a chamber specially designed for this purpose and was mounted on a heated stage (37 °C) of an Olympus IX70 inverted fluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Before any fluorescence micrography was performed each filter was inspected with normal brightfield microscopy to ensure that the monolayer was still confluent. The propidium iodide was exited with the appropriate fluorescence filter and the images were captured with a Colourview-12 digital camera (Soft Imaging Systems, Münster, Germany) with the assistance of an analySIS® software package (Soft Imaging Systems, Münster, Germany).

3.5 CONCLUSION

In this chapter the synthesis and characterisation of TMC polymers with different degrees of quaternisation were described. TMC polymers, with degrees of quaternisation between 12 and 53 %, were synsthesised by the process of reductive methylation of chitosan by varying the reaction time and number of reaction steps. The rest of the chapter gives a detailed description of the toxicity assays performed to evaluate the toxicity profile of the TMC polymers. The MTT assay and LDH assay were performed. Caco-2 cell monolayers were also stained with the fluorescent probe, propidium iodide, to visualise cell membrane damage. Results of the toxicity assays will be presented in chapter 4.

CHAPTER 4

TOXICITY PROFILES OF *N*-TRIMETHYL CHITOSAN CHLORIDE: EFFECT OF THE DEGREE OF QUATERNISATION

4.1 INTRODUCTION

In chapter 1 the problems associated with peptide and protein drug delivery were discussed. Numerous absorption enhancers have been studied in the past as a way to increase the transport of these large hydrophilic compounds across epithelial membranes. However, most of these absorption enhancers cause significant cell membrane damage, which is directly related to their mechanism of action. *N*-trimethyl chitosan chloride was shown to be one of the most promising absorption enhancers to increase peptide drug absorption across intestinal epithelial cells. It was also shown that the degree of quaternisation of TMC played an important role in its absorption enhancing properties, especially in neutral environments. However, limited data is available on the toxicity profile of this partially quaternised derivative of chitosan.

Toxicity assays performed on cell cultures have been shown to be reliable alternatives to *in vivo* toxicity assays (chapter 2). TMC polymers were synthesised with different degrees of quaternisation and their effects on Caco-2 cells were evaluated with the MTT and LDH assays. Caco-2 cell monolayers were also stained with propidium iodide to study the possible cell damaging effect of these polymers.

4.2 EXPERIMENTAL DESIGN

The synthesis of TMC polymers with different degrees of quaternisation and the *in vitro* procedures for studying the toxicity profiles of these polymers have been described in chapter 3. All TMC polymers were prepared in a series of concentrations which ranged from 0.015625 up to 0.5 % (w/v). Table 4.1 gives a summary of the concentrations used

in each toxicity assay. Statistical evaluation was done only for the MTT assay as the other two assays are unsuitable for statistical evaluation.

Table 4.1 Summary of the different concentrations tested for each TMC polymer.

TMC CONCENTRATION (% w/v)	MTT ASSAY	LDH ASSAY	PROPIDIUM IODIDE STAINING
0.5	✓	✓	✓
0.25	✓	✓	×
0.125	✓	✓	✓
0.0625	✓	✓	×
0.03125	✓	✓	×
0.015625	✓	✓	×

✓ Tested

× Not tested

4.3 RESULTS AND DISCUSSION

4.3.1 THE MTT ASSAY

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is converted to a purple formazan crystal by active mitochondria. This formazan production is an indication of the viability of the cells. To quantify the crystal formation the optical densities (absorbance) were measured with a microtitre plate reader after the crystals had been dissolved. In this particular assay the optical densities were recorded at a wavelength of 560 nm with background subtraction at 630 nm. As mentioned in chapter 3 proof of linearity between the optical densities and the amount of cells should be established. This is to ensure that the results can be expressed as a percentage of the control.

4.3.1.1 Proof of linearity

Figure 4.1 is a graphical representation of the difference in optical densities (delta optical density) measured at 560 and 630 nm respectively vs. concentration of cells/well.

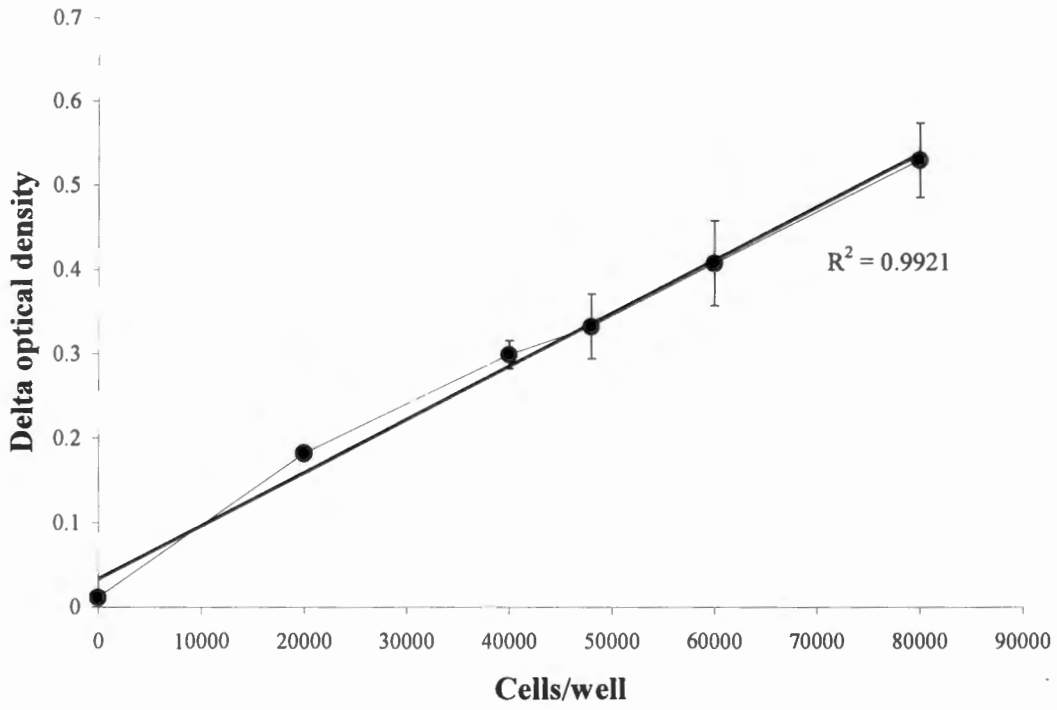


Figure 4.1 Proof of linear relationship between the amount of formazan crystals produced expressed as optical density (mean ± S.D. of 4 wells) and the number of cells/well seeded.

The calculated R^2-value for this linear relation is 0.9921. From this value it can be concluded that there is a definite linear relationship between formazan production and the number of viable cells. The optical densities depicted in this graph are the mean of the values measured for 4 wells with the same number of seeded cells. It should however be noted at this point that it is impossible to draw a direct correlation between the formazan production in a single well and the number of cells seeded. This can be explained by the fact that it is impossible to determine the exact number of cells which is seeded per well as the cells are suspended in culture medium and are therefore expressed as number of cells per volume of culture medium when seeded onto the plates. The cells adhered to

the plates within 24 hours after which the culture medium was removed before the MTT solution was applied.

4.3.1.2 The effect of TMC on the viability of Caco-2 cells

Table 4.2 shows the viability of the Caco-2 cells as a percentage of the control after incubation with the TMC polymers at the concentrations listed in table 4.1. The results are expressed as a % of the control value (100 %). Figure 4.2 is a graphical representation of these results.

Table 4.2 Viability of Caco-2 cells (% of control) after an incubation time of 4 hours.

Concentration (% w/v)	**Viable cells (% of control)**					
	TMC-12	***TMC-22***	***TMC-38***	***TMC-43***	***TMC-48***	***TMC-53***
0.015625	131.72 ± 0.92	145.34 ± 3.09	90.61 ± 2.62	76.49 ± 5.86	96.19 ± 1.07	68.07 ± 3.24
0.03125	135.01 ± 5.72	120.73 ± 2.29	69.06 ± 2.41	67.01 ± 3.12	56.59 ± 1.37	55.29 ± 1.63
0.0625	149.81 ± 3.80	111.29 ± 0.06	53.26 ± 1.28	45.93 ± 6.98	35.75 ± 0.56	45.95 ± 2.84
0.125	141.75 ± 4.04	99.64 ± 6.28	34.10 ± 2.76	41.03 ± 1.53	18.65 ± 1.68	25.39 ± 1.90
0.25	133.43 ± 6.41	97.39 ± 1.32	28.99 ± 2.63	32.55 ± 1.85	14.39 ± 1.75	18.07 ± 0.77
0.5	120.21 ± 4.79	80.33 ± 5.20	19.78 ± 2.27	27.07 ± 1.33	8.34 ± 0.99	13.85 ± 2.27

Indicated is the mean ± S.D. of 4 experiments

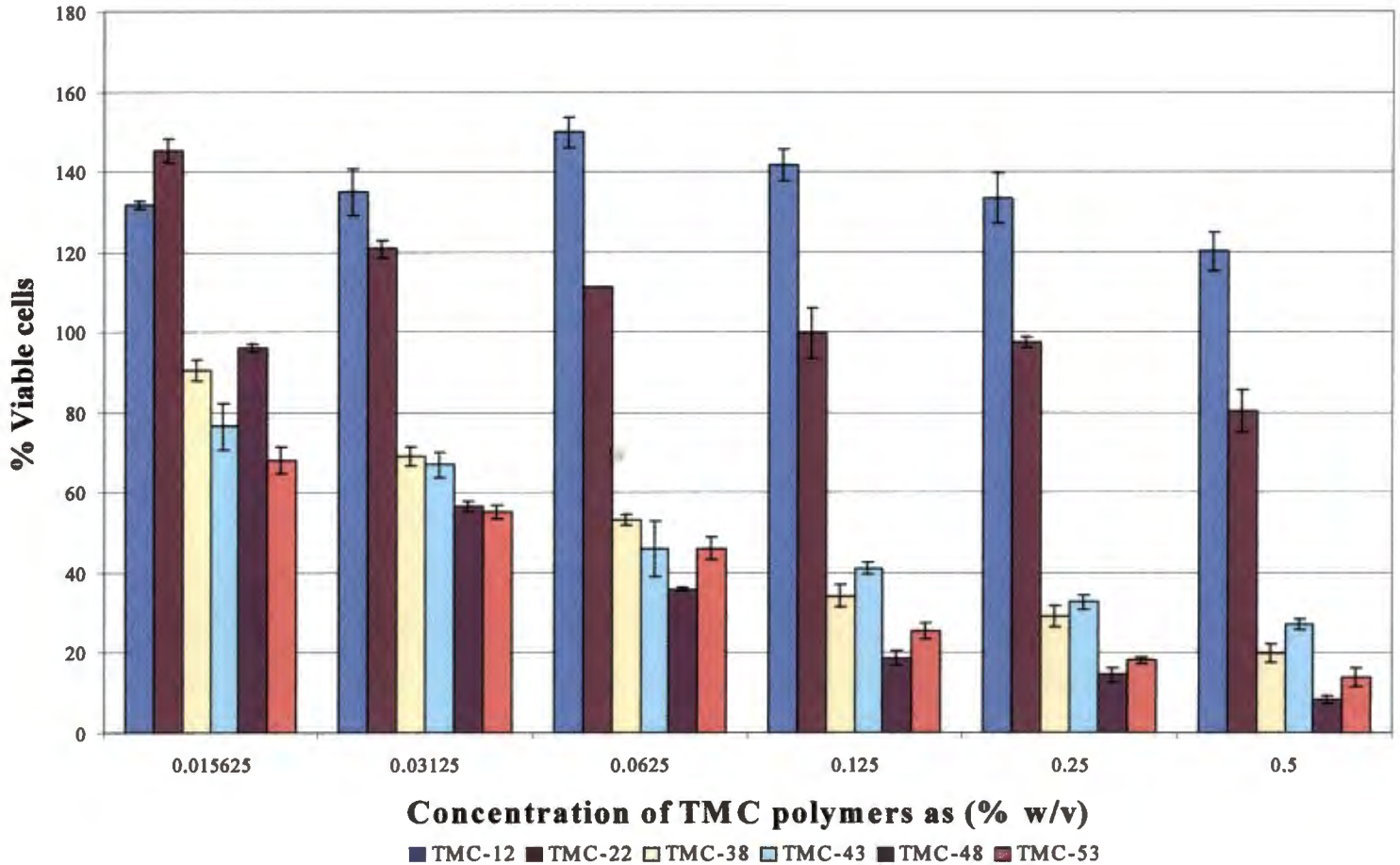


Figure 4.2 The effect of TMC on the viability of Caco-2 cells (MTT assay).

From table 4.2 and figure 4.2 it is clear that most of the TMC polymers decrease mitochondrial activity in the Caco-2 cells resulting in decreased viability compared to the control values. This is however not observed for all the polymers. According to these results TMC-12 has a stimulating effect on the mitochondrial activity of the Caco-2 cells as all values are > 100 %. The cell viability after incubation with this polymer ranges from 120.21 ± 4.79 % up to 149.81 ± 3.80. There is however no direct correlation between the concentration of TMC-12 applied and the viability (%) calculated for these cells. The results obtained for this polymer correlates well with results published by Florea *et al.* (1999:572). According to these published results TMC polymers with degrees of quaternisation of 20 and 60 % respectively, had a stimulating effect on the metabolic activity of COS-1 monkey kidney cell cultures. The authors are of the opinion that the higher metabolic activity may be a result of the action of TMC on the cell membranes.

The results for TMC-22 shows a slight decrease in metabolic activity for the Caco-2 cells compared to the results obtained with TMC-12. At a concentration of 0.5 % (w/v), 80.33 ± 5.20 % of the cells seeded are viable. Viability values ≥ 99 % were observed for concentrations ranging from 0.015625 up to 0.125 % (w/v). The viability determined at a concentration of 0.015625 % (w/v) for TMC-22 is 145.34 ± 3.09 %. From figure 4.2 it seems that the viability of the cells depend on the concentration of the polymer (TMC-22) applied. The results obtained suggest that TMC-22 is non-toxic in concentrations of 0.015625 – 0.25 % (w/v) and that the concentration of 0.5 % (w/v) only shows a minor effect on mitochondrial activity.

From the results depicted in figure 4.2 and table 4.2 it is clear that all TMC polymers with a degree of quaternisation above 22 % are extremely toxic at certain concentrations. At a concentration of 0.5 % (w/v) the viability (%) determined for TMC-38, TMC-43, TMC-48 and TMC-53 is 19.78 ± 2.27, 27.07 ± 1.33, 8.34 ± 0.99 and 13.85 ± 2.27 % respectively. These results suggest that all these polymers are extremely toxic at this weight concentration. However, at very low concentrations (0.015625 and 0.03125 % w/v) more than 50 % of the cells seeded were still viable. It might be possible to utilise

these polymers as absorption enhancers at these concentrations if they are effective in increasing permeability of hydrophilic compounds across epithelial membranes in such low concentrations, but this should be intensely investigated beforehand. Utilisation of these polymers at higher concentrations will however not be possible due to the extreme toxic effects that they exert as indicated by the MTT assay performed in this study.

4.3.1.3 Statistical evaluation of MTT results

Variance analysis (2–way) showed that there is statistically significant differences between the means of the viability percentages obtained of the different treatments and concentrations with $p = 0.0001$ in both cases. Tukey intervals calculated to determine the significant difference between the means of the different treatments, thus the TMC polymers, revealed that the effect of most of the polymers are significantly different from each other. The exceptions to the rule are TMC-38 and TMC-43 with $p = 0.912961$ and TMC-48 and TMC-53 with $p=0.992410$. Tukey intervals showed there is statistically significant differences between all means of the concentrations with $p < 0.01$. For all the TMC polymers applied, in the concentration range defined, Tukey intervals revealed that most of the means for these interactions are significantly different from the other interactions. Exceptions to the rule are summarised in table 4.3.

From the statistical evaluation it can be concluded that the sythesised polymers decrease the metabolic activity of the Caco-2 cells in significantly different amounts except for TMC-12 (not synthesised in our laboratory) which stimulates metabolic activity in these cells and TMC-22 which also stimulates metabolic activity at some (lower) concentrations (0.0625 – 0.015625 % w/v)

Table 4.3 Summary of treatments with no significant difference ($p > 0.01$).

A		*B*		*p – value*
Polymer degree of quaternisation	**Concentration % (w/v)**	**Polymer degree of quaternisation**	**Concentration % (w/v)**	
12	0.500000	22	0.031250	1.000000
12	0.250000	12	0.125000	0.139457
12	0.250000	12	0.015625	1.000000
12	0.250000	12	0.031250	1.000000
12	0.125000	12	0.062500	0.183129
12	0.125000	12	0.031250	0.554045
12	0.062500	22	0.015625	0.993091
22	0.500000	43	0.015625	0.999504
22	0.250000	38	0.015625	0.536225
22	0.250000	49	0.015625	1.000000
22	0.125000	22	0.250000	1.000000
22	0.125000	38	0.015625	0.583160
22	0.125000	49	0.015625	0.999940
22	0.062500	12	0.500000	0.674690
38	0.500000	43	0.500000	0.373850
38	0.500000	49	0.250000	0.920448
38	0.500000	49	0.125000	1.000000
38	0.500000	53	0.500000	0.808361
38	0.500000	53	0.250000	1.000000
38	0.500000	53	0.125000	0.880935
38	0.250000	38	0.125000	0.956194
38	0.250000	43	0.50000	1.000000
38	0.250000	49	0.062500	0.545350
38	0.250000	56	0.125000	0.999861
38	0.125000	43	0.500000	0.454594
38	0.125000	43	0.250000	1.000000
38	0.125000	43	0.125000	0.489590
38	0.125000	49	0.062500	1.000000
38	0.125000	53	0.125000	0.087668
38	0.062500	43	0.062500	0.362480
38	0.062500	48	0.031250	0.999974
38	0.062500	53	0.062500	0.368428
38	0.031250	49	0.015625	0.887943
38	0.015625	43	0.031250	1.000000
38	0.015625	43	0.015625	0.340788

Treatments in column A not statistically different from treatments in Column B

Indicated are only the different treatments as the means of these treatments are summarised in table 4.2

4.3.2 THE LDH ASSAY

The release of lactate dehydrogenase (LDH) from cells in culture is an indication of the amount of damage caused to the cell membranes of these cells. With the aid of a commercially available kit (CytoTox 96®) the % cytotoxicity was determined for Caco-2 cells after treatment of these cells with TMC polymers. At this point it should be noted that results from the MTT assay (section 4.3.1) is expressed differently from this assay. The MTT assay determines the percentage of viable cells after treatment with the polymers compared to the LDH assay which measures the percentage of cytotoxicity which is an indication of the decrease in the viability of the cells.

4.3.2.1 The effect of TMC on the viability of Caco-2 cells

Table 4.4 summarises the % cytotoxicity calculated after incubation of Caco-2 cells with the different TMC polymers. These results are graphically represented in figure 4.3. All polymers were incubated for 4 hours (similar to the MTT assay). A summary of the concentrations tested was given in table 4.2 (section 4.3).

Table 4.4 Cytotoxicity values determined with the CytoTox 96® non-radioactive assay (LDH assay).

Concentration (% w/v)	**Cytotoxicity (%)**					
	TMC-12	***TMC-22***	***TMC-38***	***TMC-43***	***TMC-48***	***TMC-53***
0.015625	-16.775	15.147	15.910	19.902	19.191	22.455
0.031250	-18.537	16.567	29.593	41.226	39.723	37.364
0.062500	-12.667	28.874	36.029	56.582	37.017	28.979
0.012500	-29.866	31.666	47.495	73.975	34.155	35.653
0.250000	-10.985	80.201	33.699	58.477	35.083	35.083
0.500000	-8.538	85.747	31.802	72.186	23.741	26.530

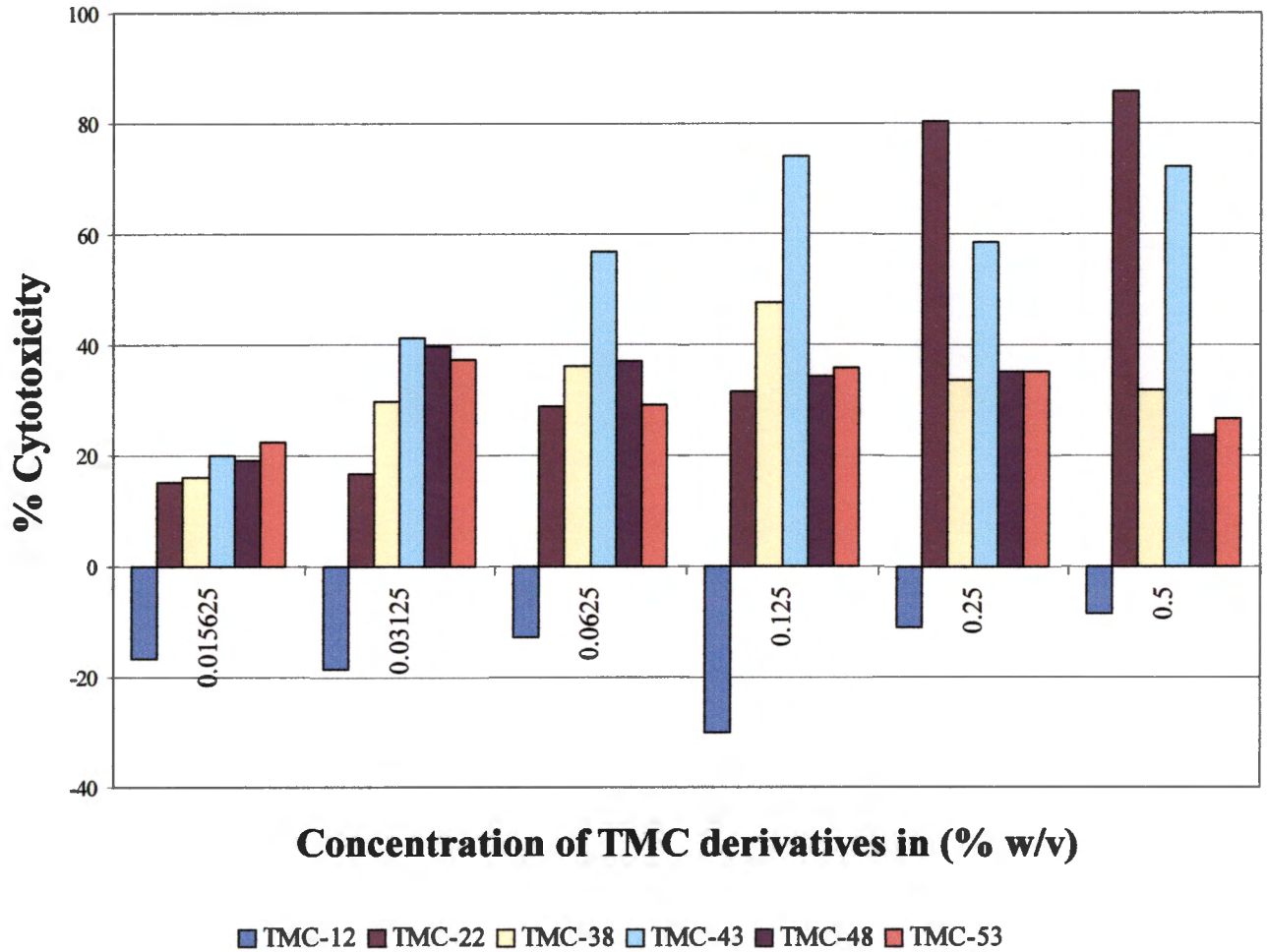


Figure 4.3 The effect of TMC on the viability of Caco-2 cells (LDH assay)

The results for TMC-12 after 4 hours incubation resulted in negative cytotoxicity values as seen in table 4.4 and figure 4.3. These values range from -8.538 (0.5 % w/v) to –29.867 (0.125 % w/v). No clear correlation between the concentration of polymer applied and the toxicity values could be established. The MTT assay showed an increase in cell viability for TMC-12 with values > 100 % which correlated well with the values determined from this assay. The LDH assay does not indicate an increase in cell viability but it does seem that the polymer has an effect on the cell membrane resulting in a decrease in the spontaneous release of LDH from the cell. As uptake of LDH from the surrounding environment by the cell seems unlikely due to the size of the molecule it seems that the suggested explanation is the most acceptable for the negative toxicity values observed.

All the other polymers showed positive cytotoxicity results. In contrast to the MTT assay there is no definite concentration dependency. TMC-22 shows an increases in toxicity with an increase in concentration. Toxicity values for this polymer range from 15.147 (0.5 % w/v) to 85.747 % (0.015625 % w/v). Compared to the MTT assay where a minimal decrease in cell viability was found only at 0.5 % (w/v) concentrations of TMC-22 this data shows excessive damage to the cell membranes at high concentrations (0.25 and 0.5 % w/v) All other values obtained with lower concentrations of this polymer are lower than 30.0 %.

Toxicity value for TMC-38, TMC-48 and TMC-53 were in the same order ranging between approximately 15 and 40 %. TMC-43 shows pronounced toxicity values between 0.0625 % (w/v) and 0.5 % (w/v) with values of 56.582 % and 72.186 % respectively. A slight decrease in toxicity was observed with a 0.25 % (w/v) solution (58.477 %) of TMC-43.

The results of this assay for the polymers synthesised in our laboratory indicate significant cell membrane damage with concentration dependency demonstrated only for TMC-22 and TMC-43. The concentration independence found for the other polymers is in direct contrast to the results of the MTT assay. Although it seems that the effect on the

cell membrane is less (decrease in damage) than the effect on the metabolic activity of the cells, the toxicity observed is still pronounced and should be considered in possible administration of the polymers.

4.3.2 PROPIDIUM IODIDE STAINING

Propidium iodide is used to visualise cell membrane damage. This probe emits fluorescence upon binding with the nuclei of cells with damaged cell membranes, after excitation at an appropriate wavelength. The results obtained after Caco-2 monolayers were stained with propidium iodide are depicted in figures 4.4 – 4.10. Table 4.5 gives an explanation of the TMC polymers and the concentrations tested. All micrographs represent enlargements of 200 times.

Table 4.5 Micrograph outlay of figures 4.4 – 4.10.

Figure number	TMC polymer	Concentration (% w/v)
4.4 A	Positive control	-
4.4 B	Negative control	-
4.5 A	TMC-12	0.500
4.5 B	TMC-12	0.125
4.6 A	TMC-22	0.500
4.6 B	TMC-22	0.125
4.7 A	TMC-38	0.500
4.7 B	TMC-38	0.125
4.8 A	TMC-43	0.500
4.8 B	TMC-43	0.125
4.9 A	TMC-48	0.500
4.9 B	TMC-48	0.125
4.10 A	TMC-53	0.500
4.10 B	TMC-53	0.125

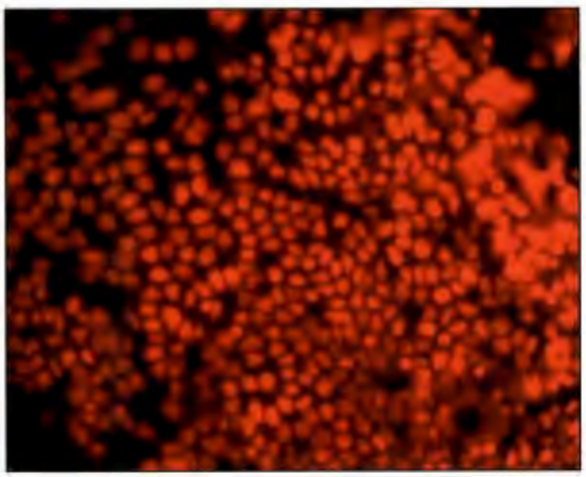


Figure 4.4 A) Positive control

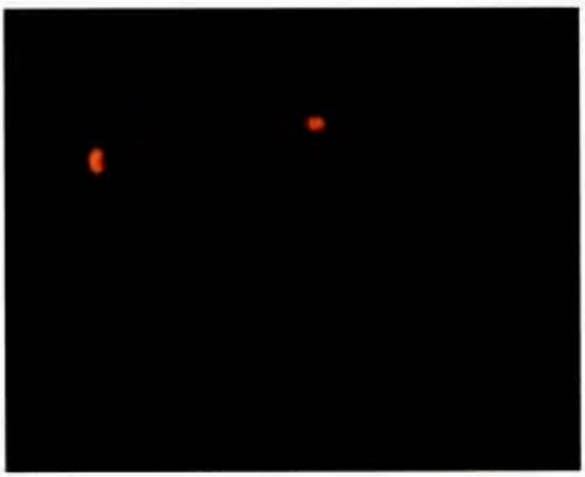


Figure 4.4 B) Negative control

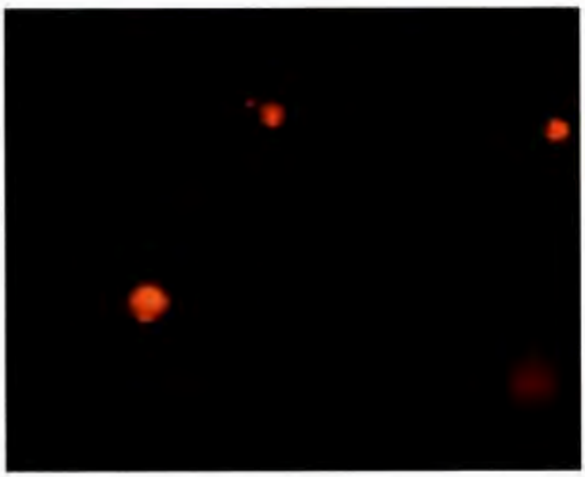


Figure 4.5 A) TMC-12 (0.5 %)

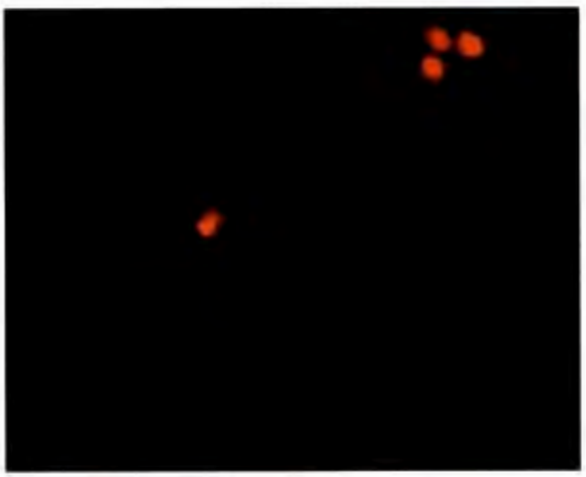


Figure 4.5 B) TMC-12 (0.125 %)

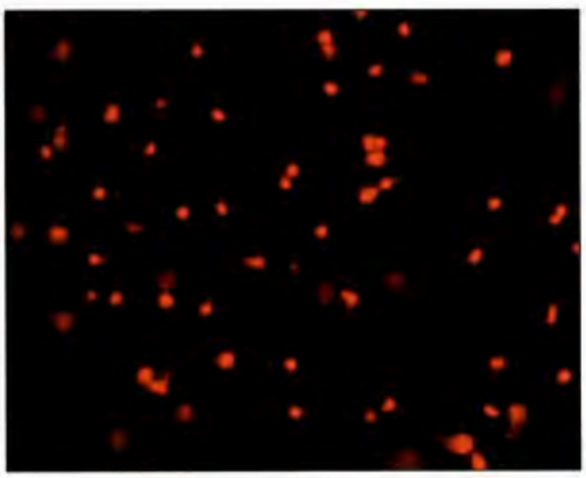


Figure 4.6 A) TMC-22 (0.5 %)

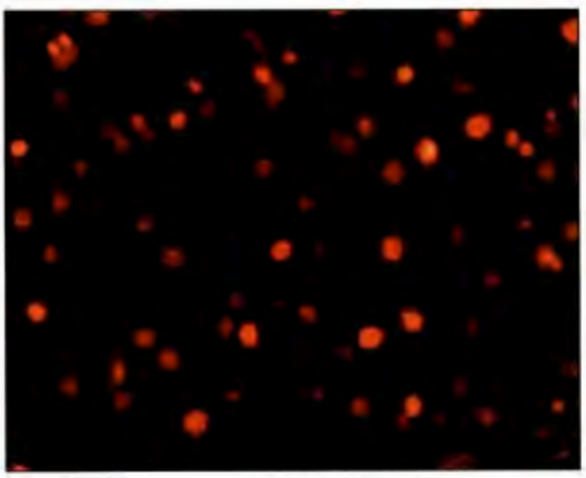


Figure 4.6 B) TMC-22 (0.125 %)

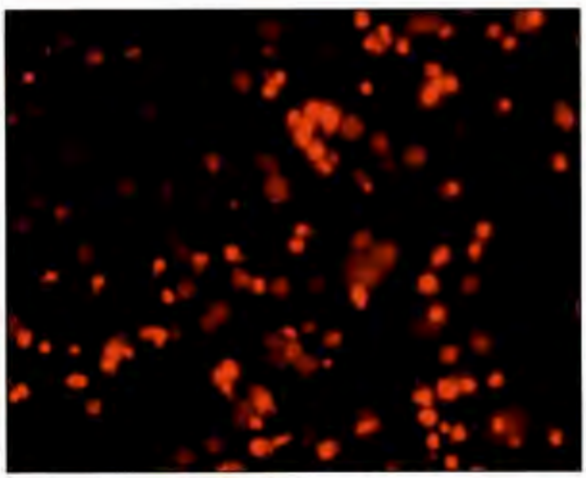


Figure 4.7 A) TMC-38 (0.5 %)

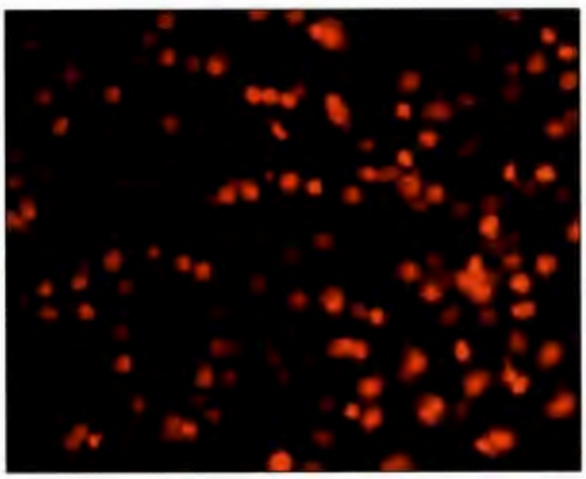


Figure 4.7 B) TMC-38 (0.125 %)

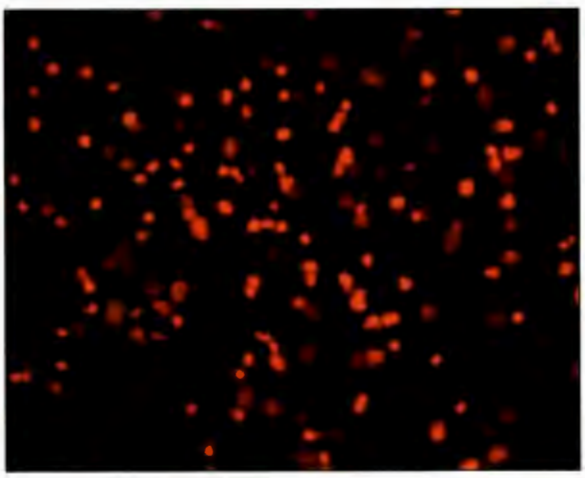


Figure 4.8 A) TMC-43 (0.5 %)

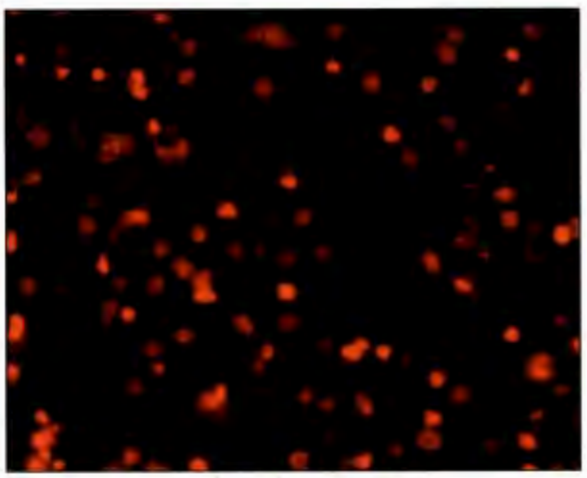


Figure 4.8 B) TMC-43 (0.125 %)

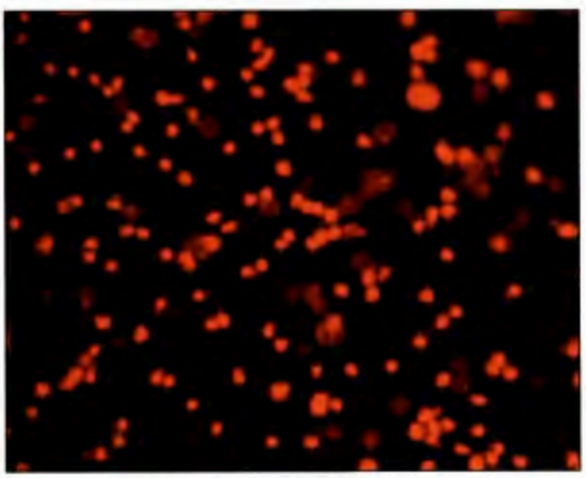


Figure 4.9 A) TMC-48 (0.5 %)

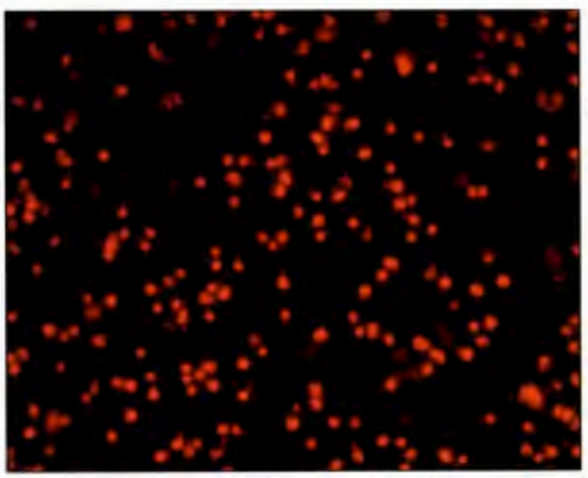


Figure 4.9 B) TMC-48 (0.125 %)

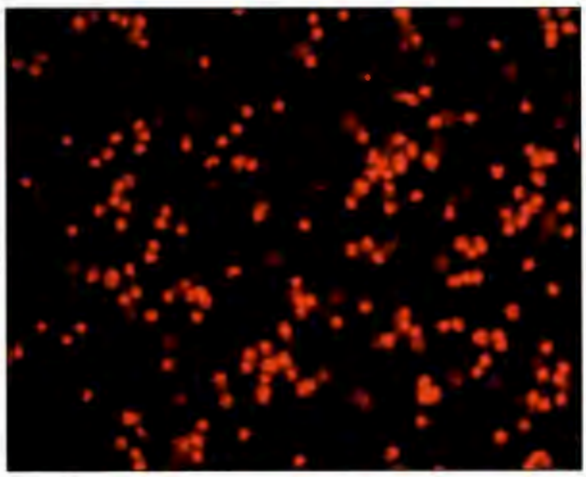


Figure 4.10 A) TMC-53 (0.5 %)

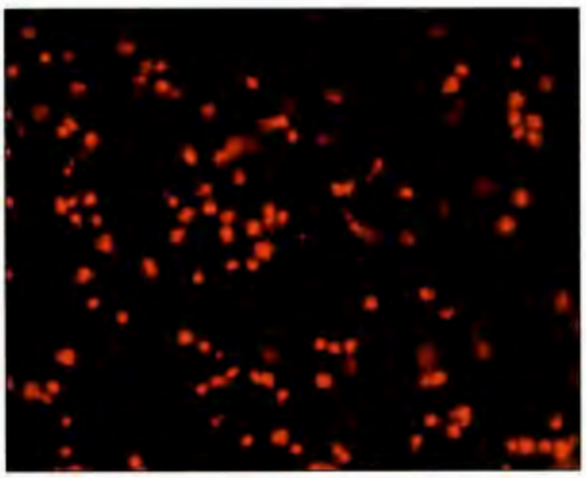


Figure 4.10 B)TMC-53 (0.125 %)

Figure 4.4 A and B: The positive control resulted in the lysis of all the cells culture on the filter. The areas where no fluorescence is visible is not an indication of cells with intact cell membranes but is an indication of the severe toxicity of the sodium lauryl sulphate. It was observed with brightfield microscopy that the cells completely disintegrated at these spaces. The negative control was of good standard.

Figure 4.5 A and B: From these micrographs it is clear that TMC-12 does not cause any significant cell membrane damage. The micrographs for this polymer correlates well with the negative control as depicted in figure 4.3 B (monolayer only treated with culture medium).

Figure 4.6 A and B: Higher levels of cellular fluorescence, caused by TMC-22, are visible in these micrographs which indicate increased cell membrane damage. There is only a slight decrease in fluorescence observed at a 0.125 % (w/v) concentration compared to a 0.5 % (w/v) concentration.

Figure 4.7 A and B: At a 0.5 % (w/v) concentration TMC-38 caused significant cell damage as seen by the many flourescing cells. No decrease in the number of fluorescing cell is observed at a 0.125% (w/v) concentration.

Figure 4.8 A and B: An increase in fluorescence is observed for TMC-43 compared to TMC-38 at the same weight concentrations. A decrease in the number of stained cells is visible at a concentration of 0.125 % (w/v) compared to a 0.5 % (w/v) concentration.

Figure 4.9 A and B: TMC-48 caused significant cell membrane damage as seen from the number of fluorescing cells in the micrographs. At 0.125 % (w/v) concentration no significant decrease in cell membrane damage occurred.

Figure 4.10 A and B: TMC-53 caused significant cell membrane damage at both concentrations. However a decrease in the number of fluorescent cells was observed at a concentration of 0.5 % (w/v) compared to a concentration of 0.125 % (w/v).

Although fluorescent staining of these monolayers were not quantifiable the extreme toxicity observed for all polymers, except TMC-12, is a point of concern. TMC-12 micrographs indicates that this is the only polymer that does not cause cell membrane damage which directly correlates to the results of the MTT and the LDH assay.

4.2 CONCLUSION

In this chapter results on the toxicity profile of TMC was supplied. The MTT assay showed a clear dose dependent decrease in cell viability for all polymers except TMC-12 and TMC-22. These results were indicative of severe inhibition of the metabolic activity of the cells. TMC-12 increased cell viability at all concentrations with values > 100 %. Although the results showed that some concentrations administered did not cause a major decrease in cell viability the overall profile still indicates that these polymers are toxic.

Similar results were obtained with the LDH assay. Results of this assay indicated that the polymers have highly damaging effects on the cell membranes. Values in this assay may seem to indicate that some of these polymers are not as toxic as suggested by the MTT assay but it should be noted, as mentioned in chapter 2, that the leakage of LDH from cells is an indication of severe toxicity. From these assays it does seem that TMC-12 has a stabilising effect on the cell membranes of the Caco-2 cells which leads to a significant decrease in the spontaneous release of this enzyme from the cells.

Propidium iodide staining of the monolayers indicated that, with the exception of TMC-12, TMC at higher degrees of quaternisation is responsible for, in some cases, severe cell membrane damage.

No direct correlation could be drawn between the assays except that TMC-12 and TMC-22 in concentrations below 0.25 % (w/v) is totally non-toxic compared to TMC-38, TMC-43, TMC-48 and TMC-53, with extreme toxicity at certain concentrations. It is also clear from the results that before it can be concluded that a substance is non-toxic, multiple toxicity assays should be performed as the results from some assays may

indicate less toxic effects towards biological tissue than is actually true for the particular compound.

SUMMARY AND FUTURE PROSPECTS

Recent advances in the field of drug design and biotechnology have made it possible to produce significant quantities of several new classes of drugs such as peptide and protein drugs. Due to their inherent characteristics, namely size and polarity, most of these drugs are poorly absorbed and are currently mainly administered by invasive parenteral injection. However, the most patient compliant route of administration is still the peroral route. Absorption of these substances is mostly limited to the paracellular transport route, which is transport through intercellular spaces between cells. Tight junctions which restrict the passage of these large hydrophilic compounds, seals the intercellular spaces at their apical surfaces thus restricting the transport through these intercellular spaces. The co-administration of absorption enhancers offers a potential means of overcoming this barrier.

Many absorption enhancers have been identified and studied since 1961. However, most of these drugs have been shown to cause significant cell damage. The use of these absorption enhancers have been considered to be impractical due to the fact that their toxicity is directly related to the mechanism in which absorption is enhanced. In recent years the absorption enhancing properties of chitosan and its partially quaternised derivative, *N*-trimethyl chitosan chloride (TMC), has captured the attention of many researchers.

The chitosan salts, chitosan glutamate and chitosan hydrochloride, proved to be able to improve the transport of 9-desglycinamide 8-L-arginine vasopressin (DGAVP), buserelin and insulin both *in vitro* and *in vivo* as well as the transport of several hydrophilic model compounds *in vitro* across human intestinal epithelial cells (Caco-2). The results of these studies showed that these chitosan salts were able to decrease transepithelial electrical resistance (TEER) significantly, which is a good indication of opening of the tight junctions between cells. The opening of intercellular spaces were visualised with the administration of the fluorescent probes fluorescein isothiocyanate-labeled dextran

(FD-4). Due to the size of this probe transport was only achieved through the paracellular route. This was also demonstrated with confocal laser scanning micrography (CLSM).

In all of these studies the increase in transport was only obtainable under acidic conditions. Chitosan, a weak base, requires a certain amount of acid to transform the glucosamine units into the positively charged water-soluble form. It was proposed that the positively charged amino group on the C-2 position of the polymer subunit interacts with the negatively charged cell membranes and tight junctions. This interaction compromises the integrity of the tight junctions thus allowing paracellular transport.

The partially quaternised derivative of chitosan, TMC, was also able to improve the transport of various peptide drugs such as DGAVP, buserelin, insulin and ocreotide. The transport of hydrophilic model compounds such as mannitol and polyethylene glycol 4000 were also significantly increased *in vitro* across Caco-2 cell monolayers. Studies have shown that the charge density of this polymer plays an important role in its absorption enhancing effects, especially in neutral environments, and is dependent on the degree of quaternisation of the polymer.

Although the efficacy of TMC as absorption enhancer was shown through various studies limited toxicity data was available for this polymer. Before any foreign substance can be approved for human administration it must be subjected to extensive cytotoxicity testing to ensure the safety of the compound. In this regard cell cultures have proved to be effective and reliable in determining toxicity profiles. Possible factors that could have an effect on the toxicity of this polymer are the charge density and the concentration in which the polymer is administered.

It was therefore the aim of this study to synthesise TMC polymers with degrees of quaternisation between 12 and 60 % and to evaluate the effect that this increase in charge density, and concentration, might have on the viability of intestinal epithelial cells.

TMC with different degrees of quaternisation were synthesised by varying the number of reaction steps, duration of each step and the volume of methyl iodide used as reagent. Five TMC polymers with degrees of quaternisation of 22.15, 38.14, 42.75, 48.75 and 53.16 % were synthesised. TMC with a degree of quaternisation of 12.28 % was kindly donated by Dr. A.F. Kotzé (Potchefstroomse Universiteit vir Christelike Hoër Onderwys, R.S.A.).

Possible toxic effects of these polymers were extensively evaluated with the MTT assay, which evaluates cell metabolic activity, and the LDH assay, which evaluates the release of lactate dehydrogenase from cells with damaged cell membranes. The staining of Caco-2 cell monolayers with the fluorescent probe, propidium iodide, was also used to visualise the cell membrane damage.

Results from these studies showed that all of the TMC polymers synthetized in our laboratory were extremely toxic on the Caco-2 cell cultures, used as model for these assays, except for TMC-22 which was only slightly toxic at a 0.5 % (w/v) concentration. The MTT assay showed that with an increase in the degree of quaternisation there was a decrease in cell viability. Statistical evaluation confirmed that all the TMC polymers were significantly different from each other. It was also noticed that an increase in concentration also resulted in a decrease in cell viability. In direct contrast to these results it was shown that TMC-12 and TMC-22 stimulated cell viability and that this phenomenon correlated with results previously published. It was suggested that the interaction of the polymer with the cell membranes of COS-1 cells resulted in an increase in the metabolic activity of these cells.

There was no direct correlation between the results of the LDH assay, performed with a CytoTox 96® non-radioactive assay kit, and the MTT assay. Results from this assay showed that toxicity of these polymers were not directly related to the weight concentrations applied to the cell suspension. Only TMC-22 and TMC-43 proved to be highly toxic at high concentrations. Toxicity values for TMC-38, TMC-48 and TMC-53 ranged from approximately 15 to 40 % and was, compared to TMC-22 and TMC-43,

much less toxic. However, it should be noted that the release of lactate dehydrogenase from cells is an indication of severe toxicity. The results obtained for TMC-12 indicated that this polymer might have a stabilising effect on the cell membrane resulting in a decrease in the spontaneous release of LDH from the cells. Propidium iodide staining of Caco-2 cell monolayers indicated that severe cell membrane damage occurs with all synthesised polymers. The fluorescence observed with TMC-12 correlated well with the fluorescence observed for the negative control.

Severe toxicity is indicated for the synthetized polymers by either two or more assays performed. From this it can be speculated that the synthesis procedure might have an effect on the toxicity caused by these polymers. In the synthesis process NaCl is used to exchange the iodide-ion for a chloride-ion but it is a known fact that NaCl precipitates in the presence of ethanol, which was used to precipitate the polymer. All culture medium used in cell culturing procedures and these assays are isotonic. If any NaCl is present in the final product it could cause cell lysis due to culture mediums that becomes hypertonic. Better control over the synthesis procedure is advised and analysis of the products for Na^+, Cl^- and I^- content is proposed.

Schipper *et al.* (1996:1686) showed that chitosan with a high molecular weight was non-toxic and it is known that reaction conditions during the TMC synthesis, such as the strong alkaline environment and elevated reaction temperature, decreases the molecular of the starting polymer (Domard *et al.*, 1986:105). Therefor further studies should be performed to determine the possible effect of a decrease in molecular weight on the toxicity profile of this polymer. Schipper *et al.* (1996:1686) however attributed the toxic effects observed with the different chitosans tested more to a decrease in the degree of acetylation and it is therefor also suggested that effect of the degree of acetylation of the starting polymer on the toxicity profile of TMC should also be investigated.

Furthermore, although the toxicity results on TMC is limited, published results available indicate that TMC is completely non-toxic. All these assays performed, which indicated the non-toxic effects of this polymer were performed on Caco-2 cell cultures with

passage numbers between 73 and 82 (Thanou *et al.*, 2000:64). These cell cultures are also used at high passage numbers in transport studies, especially at pH values below 7.4 (Kotzé *et al.*, 1997b:248, 249), as these cells do not respond well to acidic environments at low passage numbers. It is therefore suggested that these assays should be repeated at higher passage numbers to exclude the possibility that the cultures are to sensitive at low passage numbers. Caco-2 cells at higher passage numbers might be able to resist the toxic effects of the TMC polymers with high degrees of quaternisation.

If better control over the synthesis procedure and toxicity evaluation at higher passage numbers indicate that these polymers are non-toxic the next step should be the development of dosage forms for the co-administration of this potent absorption enhancer with numerous peptide drugs. The many functional groups of chitosan available for chemical modification makes this polymer an excellent candidate for the synthesis of other derivatives. These derivatives might also prove to be effective absorption enhancers in neutral environments.

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