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# Diethyl methyl chitosan as an intestinal paracellular enhancer: ex vivo and in vivo studies

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**Abstract**

Chitosan exhibits favorable biological properties such as no toxicity, biocompatibility and biodegradability; therefore, it has attracted great attention in both pharmaceutical and biomedical fields. Chitosan exhibits poor solubility at pH values above 6 that prevents enhancing effects at the sites of absorption of drugs. In the present work, *N*-diethyl methyl chitosan (DEMC) was prepared and the enhancing effect of this polymer was investigated. Ex vivo studies have shown a significant increase in absorption of brilliant blue in the presence of diethyl methyl chitosan in comparison with chitosan. DEMC with positive charges is able to interact with tight junctions of colon epithelial cells and hence increases permeability of brilliant blue across the tight junctions. In vivo investigations have exhibited the absorption enhancer effects of DEMC on the colon absorption of insulin in normal and diabetic rats. The insulin absorption from the rat's colon was evaluated by its hypoglycemic effect. A significant decrease in blood glucose was observed, when mixture of insulin and DEMC was introduced in ascending colon of rats.


*Keywords:* Colon; DEMC; Enhancer; Insulin; In vivo; Ex vivo

## 1. Introduction

Injection is the main route for protein or peptide administration to patients. This route has several disadvantages including patient dissatisfaction (Scott-Moncrieff et al., 1994). Therefore, many studies have been done to find other alternative pathways for peptide or protein delivery routes other than injection. These routes include: nasal, pulmonary, buccal, oral, colonic and rectal (Yamamoto et al., 1994). The intestinal absorption of peptides and proteins has a very low bioavailability due to extensive hydrolysis by the

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proteolytic enzymes and poor membrane permeability of GIT (Tozaki, 1997). For successful protein absorption from GIT route, both the proteins and peptides must overcome several barriers. First, the destructive acidic pH in stomach; second, the intensive proteolytic enzyme activity of the intestine; finally, the intestinal epithelial cells which prevent transport of macromolecules due to their structural properties (Hoffman and Ziv, 1997). Consequently, researchers have used different excipients for manufacturing tablets or developing drug carrier systems capable of controlling drug delivery after oral administration.

Colonic drug delivery (CDD) for either local or systemic effects has been the subject of much research over the last decade. This method of drug delivery has several advantages including protection of drug from harsh environment of stomach and small intestine, unwanted avoiding of drug absorption from upper GIT and increased bioavailability of some drugs.

Recent researches have determined that polymeric compounds are useful carriers for high molecular weight drugs (Domb and Bentolila, 1998). Biodegradable polymers such as chitosan have been used extensively in biomedical fields in the form of sutures—wound coverings and as artificial skin. Deacetylation of chitin, the second most abundant biopolymer isolated from insects, crustacea such as crab and shrimps as well as fungi, leads to poly(β-1,4-D-glucosamine) or so called chitosan (Illum, 1998). Chitosan with excellent biocompatible and biodegradable properties has been used extensively in the pharmaceutical industry as drug delivery systems (Roberts, 1992). Recently, complexation between oppositely charged macromolecules is widely used to prepare chitosan microspheres for controlled release drug formulations especially for peptide and protein drug delivery (Polk et al., 1994). Chitosan derivatives such as chitosan glutamate are also studied for their ability to enhance intestinal peptide drug delivery by opening epithelial tight junction; thus, allowing paracellular peptide drug transport (Felt et al., 1998). The influence of chitosan glutamate on the permeability of epithelial cell monolayer in vitro (caco-2 cell) has been demonstrated by measuring the transepithelial electrical resistance (Felt et al., 1998). Increased permeability is explained to be due to a direct interaction of the cationic polymer with the negatively charged cell membrane. It has also been shown that chitosan enhances the absorption of peptide and protein drugs across nasal and intestinal epithelia. Chitosan hydrochloride has been used as an intestinal absorption enhancer in vivo of the peptide drug buserelin when it was co-administered in rats (Lueßen et al., 1996). Chitosan with mucoadhesive properties and ability to interact with cell membrane is able to open the intercellular tight junctions of the epithelia and increase the paracellular permeation of hydrophilic macromolecules (Lueßen et al., 1996). It has also been shown that chitosan enhances the penetration of macromolecules across the intestinal barrier (Kotze et al., 1999a). However, chitosan is insoluble at neutral and alkaline pH values but forms salt with both inorganic and organic acids such as hydrochloric acid, lactic acid and acetic acid. The amine groups of the polymer are protonated in acidic medium and a soluble polysaccharide with positive charge is attained. Nevertheless, its poor solubility at pH values above 6 prevents its enhancing effect at the sites of absorption. At those pH values, chitosan loses its positive charge density, so it aggregates and precipitates (Kotze et al., 1999b).

A new quaternized derivative of chitosan, triethyl chitosan (TEC) was synthesized and its absorption enhancing effect was evaluated by Avadi et al. (2003). Their studies showed that TEC is able to enhance the absorption of hydrophilic compounds similar to trimethyl chitosan. Hamman et al. (2002) have investigated the effect of the quaternization of *N*-trimethyl chitosan on absorption enhancement. These investigations have shown that administration of trimethyl chitosan polymers lead to enhancement of the absorption of hydrophilic model compounds in the nasal route of rats at both pHs 6.20 and 7.40. Present studies deal with synthesis of diethyl methyl chitosan. Moreover, the effect of DEMC on permeation of model drug (brilliant blue) across intestinal epithelia (ex vivo) was investigated. Furthermore, enhancing effect of DEMC enhancer on insulin absorption from ascending colon section of GIT in rats was investigated.

## 2. Experimental

### 2.1. Materials

Chitosan (viscosity 1%, w/v solution, 264 mPa s, MW = $10.5 \times 10^4$) was a gift from Primex (Iceland).

According to the producer, the chitosan sample was 98% deacetylated. Ethyl iodide, sodium borohydride, brilliant blue and formaldehyde were obtained from Sigma (Vienna, Austria). Sodium hydroxide, *N*-methyl pyrrolidone (NMP) and sodium iodide were purchased from Merck (Darmastadt, Germany). Insulin (actrapid beef and neutral insulin injection) and *ortho*-toluidine chromium tricarbonyl for measuring blood sugar were purchased from Fluka chemical company (Eschenstrasse, Germany) and the other materials were as pharmaceutical and analytical grade, and used as-received.

### 2.2. Preparation and characterization of diethyl methyl chitosan (DEMC)

Optimized DEMC was prepared by a two-step method reported previously for DEMC synthesis (Avadi et al., 2004). Briefly, chitosan was dissolved into 1% acetic acid. Then formaldehyde was added and after 1 h of stirring the pH of solution was adjusted to 4.5. Then, 2 mL of 10% $NaBH_4$ solution was added and stirred for 1.5 h. Finally methyl chitosan precipitate was obtained by adding 1 M NaOH solution and adjusting the pH of the solution to 10. The precipitate was washed with distilled water and soxhlet-extracted with ethyl alcohol and ether (1:1) for 4 days (yield 93% and H NMR-based degree of substitution 0.96). In the second step, 250 mg methyl chitosan was dispersed in 15 mL of NMP for 5 h. NaOH solution (1.5 mL of 15–30 wt.%), ethyl iodide (1.5–3 mL) and sodium iodide (500 mg) were then added to the dispersion. The reaction was carried out with stirring for 5 h at 60 °C. The precipitate of DEMC was obtained by adding acetone. For exchanging $I^-$ with $Cl^-$, the polymer was dissolved in 4 mL of 10% sodium chloride aqueous solution. The polymer was precipitated with acetone and dried to obtain a water-soluble white powder with quantitative yield (79%). The $^1H$ NMR spectrum of chitosan and DEMC were obtained in $D_2O$ and/or $CF_3COOD$ and degree of quaternization (DEMC) was calculated (Fig. 1). The FTIR spectrophotometer was used to record both chitosan and DEMC spectrums. The samples were prepared in 0.25 mm thickness (Fig. 2) KBr pellets (1 in 100 mg of KBr) and stabilized under controlled relative humidity before acquiring the spectrum.

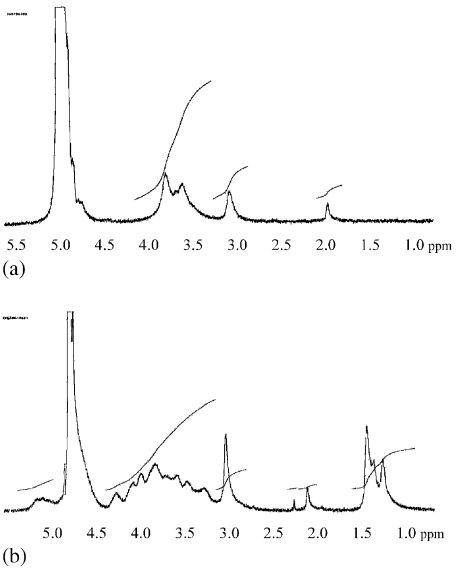


Fig. 1. $^1H$ NMR spectra of (a) chitosan in $CF_3COOD$ and (b) *N*-diethyl methyl chitosan (DEMC) in $D_2O$.

### 2.3. Blood glucose determination method

Glucose with *ortho*-toluidine in the presence of acetic acid, produce green glucosamine. Three grams of thiourea was mixed with 1.9 L of acetic acid and then 100 mL of *ortho*-toluidine was added. Prepared *ortho*-toluidine was stored in amber container (Moorehead and Sasse, 1970).

For preparation of glucose standard solutions, 200 mg of glucose was added to 100 mL of distilled water and the final solution was diluted to different rational concentrations. Blood glucose concentration was measured using UV–vis spectrophotometer at 630 nm and was compared with standard solutions.

### 2.4. Ex vivo study

The procedure used is a modification of Barr and Riegelman method (Barr and Riegelman, 1970) as shown in Fig. 3. At first, a section of large intestine (about 5 cm) was removed from a male rat under phenobarbital anesthesia and washed with Krebs–Ringer bicarbonate solution, pH 7.4. The lumen was inverted with a glass rod and a tube was inserted in one side of the intestine and tied securely with tape. The other side of the intestine was tied and 1 mL of Krebs–Ringer

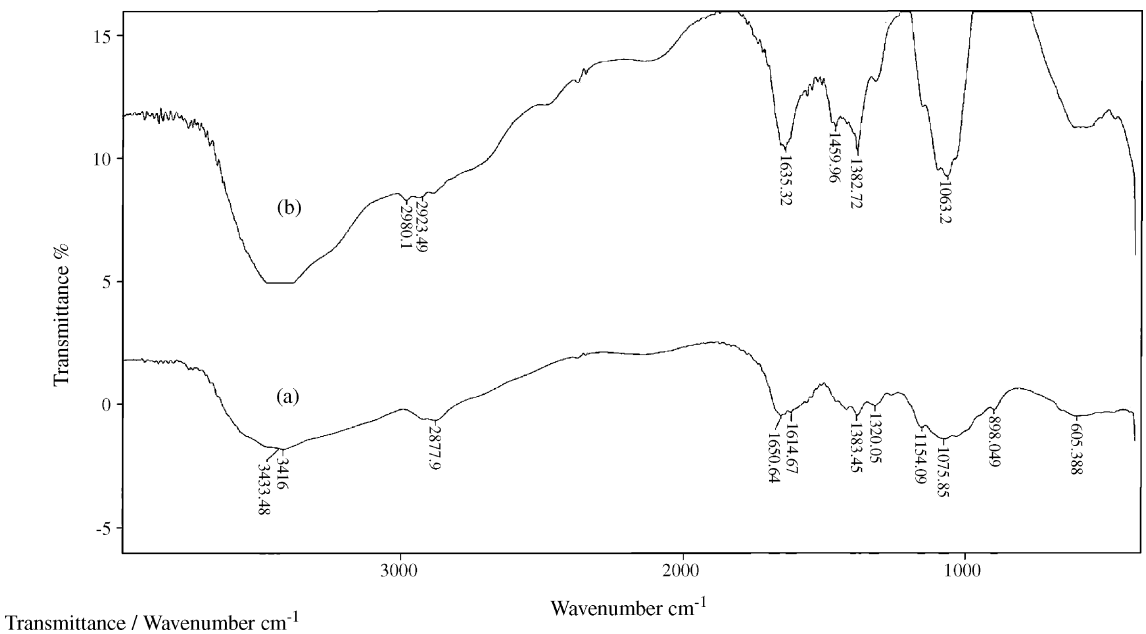


Fig. 2. FTIR spectra of chitosan (a) and diethyl methyl chitosan chloride (b).

bicarbonate solution was poured through the hypodermic needle in the tube. The intestine was placed in a medium saturated with 95% $O_2$, 5% $CO_2$ and containing $10^{-3}$ M of brilliant blue and 1% PDMC or DEMC in Krebs–Ringer bicarbonate solution at 37 °C. In absorption studies, $O_2$ and $CO_2$ mixture was bubbled into the intestinal mucosal to obtain intestinal peristaltic movement. In certain periods of time, samples with known volume were drawn from inside of intestine and were assayed spectrophotometrically at $\lambda_{max} = 30$ nm and amount of brilliant blue absorption was calculated.

### 2.5. In vivo study

Male rats (200–250 g) were maintained in groups containing 5–6 rats, under 12 h light–dark cycle duration and constant temperature (21–22 °C). For induction of diabetes in rat, 90 mg $kg^{-1}$ Alloxan was administered intravenously and blood glucose levels more than 300 mg $dL^{-1}$ indicated as hyperglycemia. After 72 h, rats were anesthetized with intraperitoneal pentobarbital (50 mg $kg^{-1}$) for surgical procedure with small incision in abdomen cavity. Prior to the start of each experiment, rats were fasted for 12 h but were allowed to have unlimited access to water. After drawing blood sample at time 0 min, the mixture of insulin (25 units) and DEMC (1%) in phosphate buffer solution (PBS), pH 7.2 was injected into the ascending colon portion. In certain periods (30 and 60 min) blood samples with known volume were drawn from portal vein and blood glucose concentration was assayed according to *ortho*-toluidine.

### 2.6. Statistical analysis

The data are expressed as means ± S.E.M. For statistical analysis, linear regression (in vivo studies) and non-linear regression (ex vivo studies) were used. $P < 0.05$ was considered significant.

## 3. Results and discussion

### 3.1. Characterization of DEMC chloride

The FTIR spectrum of chitosan (Fig. 2a) shows peaks assigned to the saccharide structure at 898 and 1154 $cm^{-1}$ and a strong amino characteristic peak at around 1614 $cm^{-1}$. The absorption bands at 1650 and 1320 $cm^{-1}$ are characteristic of *N*-acetylated chitin and

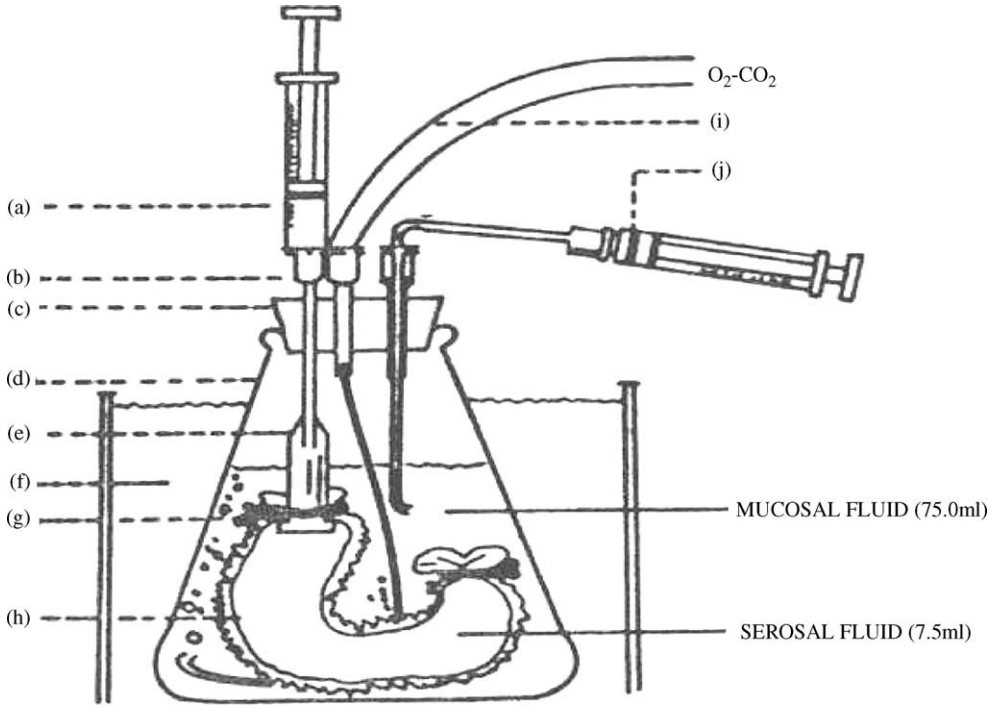


Fig. 3. Apparatus used in the ex vivo everted intestine: (a) disposable syringe for collection of serosal fluid; (b) hypodermic needle; (c) rubber stopper; (d) conical flask; (e) polyethylene centrifuge tube; (f) water bath (37°C); (g) tape used to fasten intestine to tube; (h) everted intestine; (i) mixture of gas inlet ($O_2$ 95% and $CO_2$ 5%); (j) disposable plastic syringe used to collect mucosal fluid.

have been reported to be the amide I and III bands, respectively (Zhishen et al., 2001). Disappearance of the peak at 1614 $cm^{-1}$ in Fig. 2b is due to conversion of $NH_2$ to *N*-diethyl methyl.

The $^1H$ NMR spectrum of the initial chitosan and the prepared DEMC chloride is shown in Fig. 1a and b, respectively. The signal at 1.3 ppm is attributed to $CH_3$ groups of the ethyl substituted, while H2–H6 protons of the polysaccharide backbone superimpose the $CH_2$ groups. The intense band at 4.8 ppm is related to HDO (solvent). In this region, as observed more clearly from an extended spectrum, some different anomeric protons (H 1s) are appeared at 4.05, 4.23 and 5.1 ppm. They can be attributed to mono-*N*-acetyl glucoseamine unit, mono-*N*-substituted and di-substituted glucoseamine units, respectively. The integral of $CH_3$ of ethyl groups versus the other protons was used to calculate the degree of quaternization (Desbrieres et al., 1996).

As a complementary experiment, it was shown that the peaks at 1.3 and 3 ppm, assigned to alkyl of the quaternized amino group, did not shift when a droplet of $CF_3COOD$ was added to the solution. This is a good proof that the amount of other possible derivatives of *N*-alkyl under our preparative conditions were negligible.

### 3.2. Ex vivo study

Intercellular tight junction is the main barrier to the passage of macromolecules and hydrophilic agents. DEMC, by involving actin filaments of intestinal epithelium, is able to increase permeability of agents through tight junction. Absorption profiles of brilliant blue from standard samples and formulations containing chitosan and diethyl methyl chitosan are depicted in Fig. 4. It is clearly demonstrated that diethyl methyl chitosan, as an enhancer, is able to increase permeation of brilliant blue into the serosal region much more in comparison with chitosan ($P < 0.01$). These experiments have suggested that diethyl methyl chitosan with high degree of quaternization (more than 80%) is able to take more negative charged groups from tight junctions of colonic epithelium.

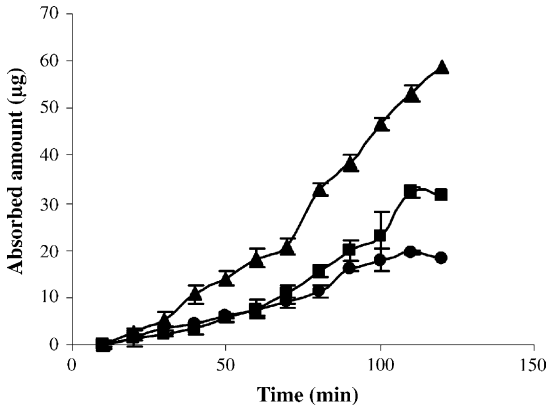


Fig. 4. Profile of the amount of brilliant blue absorption as function of time: (●) brilliant blue, (■) brilliant blue with chitosan and (▲) brilliant blue with DEMC. Experiments were carried out as triplicate.

### 3.3. In vivo study

The mean blood glucose concentrations after ascending colon administration of insulin with and without DEMC is shown in Table 1. Normal blood glucose concentration in these diabetic rats ranged from $599.88 \pm 134.74\,\mathrm{mg\,dL^{-1}}$ at time 0 min to $567.20 \pm 130.74\,\mathrm{mg\,dL^{-1}}$ after 60 min. As it has been shown in Table 1, there was no decrease in blood glucose level after colon injection of insulin. This demonstrates that insulin was not able to permeate from cell membrane on intestine. In contrast, there was a significant change in blood glucose, when mixture of insulin and DEMC was injected into ascending colon from $532.62 \pm 111.89\,\mathrm{mg\,dL^{-1}}$ (at time 0 min) to $241.19 \pm 184.78\,\mathrm{mg\,dL^{-1}}$ (after 60 min). Adjacent to the tight junction in the cytoplasm is an actin–myosin ring that circumscribes the cell and associates with the plasma membrane. This ring can contract, thereby pulling tight junction components and inducing separations from the junctional complexes of neighboring cells. Agents that disrupt actin filaments perturb the structure and integrity of tight junctions. The mechanism by which DEMC is able to open epithelial tight injection is not completely clear. DEMC may induce a redistribution of cytoskeleton of F-actin. Furthermore, it may be possible that DEMC is able to interact with tight junction proteins (e.g. occludin and claudin) leading to disruption of tight junction integrity. This study has shown that DEMC with cationic character is compatible with the insulin properties and no precipitation or aggregation phenomena was observed during the preparation of insulin-DEMC mixture in phosphate buffer solution at pH 7.2.

Table 1
Blood glucose concentration ($\mathrm{mg\,dL^{-1}}$; $n = 6$) at different times

| Group | Time | | |
|---|---|---|---|
| | 0 min | After 30 min | After 60 min |
| 1[a] | 301.49 ± 25.33 | 296.25 ± 14.91 | 285.30 ± 17.62 |
| 2[b] | 599.88 ± 134.74 | 605.36 ± 94.95 | 567.20 ± 130.74 |
| 3[c] | 532.62 ± 111.89 | 314.82 ± 131.37 | 241.19 ± 184.78 |

[a] In normal rats.
[b] In diabetic rats after 10 unit/kg of insulin in PBS, pH 7.2.
[c] In diabetic rats after 10 unit/kg of insulin and 1% DEMC in PBS, pH 7.2.

## 4. Conclusion

Results showed that DEMC is able to enhance absorption of hydrophilic models through tight junction. Our preliminary studies have shown that like DEMC, TMC is able to enhance absorption of hydrophilic models through tight junction. Furthermore, this study has clearly demonstrated the DEMC enhancing effect in opening tight junction of colonic epithelia in ex vivo (brilliant blue) and in vivo (insulin) model studies. Understanding the structure and function of proteins that involved in the formation of intercellular junctions may create a new field for developing different mechanisms of tight junction permeation. Further investigations are required and are in progress in our laboratory to fully characterize and optimize these systems.

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