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# *N*-sulfonato-*N*,*O*-carboxymethylchitosan: A novel polymeric absorption enhancer for the oral delivery of macromolecules

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**Abstract**

Chitosan has been shown to act on the mucosal epithelial barriers mainly when protonated at acidic pH values in which it is soluble. Soluble chitosan is able to improve the permeation and absorption of neutral to cationic macromolecules only, as it forms polyelectrolyte complexes with anionic macromolecules. LMWH (Low Molecular Weight Heparin) is an anionic polysaccharide finding clinical application as an improved antithrombotic agent compared to Unfractionated Heparin (UFH). In this study we have employed *N*-sulfonato-*N*,*O*-carboxymethylchitosan (SNOCC) as a potential intestinal absorption enhancer of LMWH, Reviparin. SNOCC was prepared at 3 different viscosity grades 20, 40 and 60 cps and identified as SNOCC-20, SNOCC-40 and SNOCC-60, respectively. SNOCC materials were tested *in vitro* for their ability to decrease the Trans Epithelial Electrical Resistance (TEER) of Caco-2 cell monolayers. They were further tested as transport enhancers of hydrophilic compounds such as $^{14}$C-mannitol, FITC-Dextran (MW 4400 Da) and Reviparin (LMWH). Solutions of Reviparin, with or without SNOCC, were administered intraduodenally *in vivo* in rats and the absorption of the drug was assessed by measuring the Anti-Xa levels in rat plasma. *In vitro* studies showed that SNOCC materials were able to induce a concentration dependent decrease in the TEER of the Caco-2 monolayers. SNOCC-40 and -60 were shown to decrease resistance more readily compared to the low viscosity SNOCC-20. $^{14}$C-mannitol permeation data across intestinal epithelia were in agreement with the observed decrease in TEER; the higher viscosity SNOCC-60 was the most effective demonstrating a 51-fold enhancement of the permeation of the radiolabeled marker. Studies with both FITC-Dextran and Reviparin demonstrated significantly increased permeation across Caco-2 cell monolayers when they were co-incubated at the apical side of the monolayer. Intestinal absorption of Reviparin in rats was increased when it was co-administered with SNOCC-40 and -60, in agreement with *in vitro* data. Anti-Xa levels were elevated to and above the antithrombotic levels and were sustained for at least 6 h, giving an 18.5-fold increase in the AUC of LMWH in rats. In conclusion, SNOCC-40 and -60 have been shown to enhance both permeation and absorption of Reviparin across intestinal epithelia proving their potential as polymeric absorption enhancers.
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*Keywords:* SNOCC; Reviparin; LMWH; Chitosan; Oral delivery

## 1. Introduction

Oral administration of macromolecules is restricted by the intestinal epithelial barrier [1,2] which results in greatly reduced bioavailability and unpredictable pharmacokinetics and pharmacodynamics [3]. Hydrophilic macromolecules are not absorbed by the enterocytes unless their transport is carrier-mediated. The paracellular route appears to be the preferred pathway, however, this route is restricted by the tight junctions [4]. It is postulated that the transient and reversible opening of the tight junctions should increase the permeation and intestinal absorption of therapeutic macromolecules [1,5].

Oral administration of the anticoagulant heparin and LMWH would be beneficial for the prevention of deep vein thrombosis (DVT) [6]. A number of strategies have been suggested for the development of an “oral heparin” the most successful of which is the co-administration with small carriers [named SNACs; sodium *N*-(8-[7] amino) caprylate)] that are able to interact with macromolecules such as heparins to allow for a more absorbable conformation [5,8]. SNAC has delivered UFH (Unfractionated Heparin) efficiently and safely to patients undergoing elective

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total hip arthroplasty in clinical trials [9]. In these trials, Anti-Xa levels peaked at 40–60 min after oral absorption and returned to baseline by 4 h [9]. SNAC was tested with LMWH and was shown to decrease caval thrombosis in a porcine model of deep vein thrombosis and to increase plasma levels of antifactor Xa [10,11].

It has been suggested that molecules of the SNAC-type affect the transcellular rather than the paracellular transport of heparin across the intestinal epithelia. Using a series of fluorescent probes and Caco-2 cells, it was shown by microscopy that heparin transport in the presence of SNAC was selective and did not involve permeabilization of the plasma membrane or tight junction disruption [12].

LMW heparin conjugates with deoxycholate have been developed as a prodrug approach to permeation enhancement [13]. The conjugates were administered in mice in 10% DMSO and produced an increased oral bioavailability [14]. Assessment of the conjugate's mechanism of absorption showed increased particulate absorption by the ileum without damage or lesions [15]. In this latter study, the conjugate was co-administered with deoxycholate and it was suggested that free deoxycholic acid induced a decrease in particle size and a concomitant increase in absorption [15].

In the cases of SNAC-Heparin complex and the Deoxycholate-LMWH conjugate, the absorption is achieved through increased transcytosis of either molecular assemblies or nanoparticles, respectively. With this type of absorption, the enhancer is absorbed by enterocytes as well as the drug, and though claimed to be metabolically inactive and safe, the enhancer follows its own pharmacokinetics.

Sodium caprate, a known paracellular permeation enhancer, has been tested as a permeation enhancer of heparin with Caco-2 cells and rats. A 2-fold increase in drug transport was observed with Caco-2 cells; whereas, when administered in rats, it provided a 27% oral bioavailability relative to subcutaneous administration [16]. Sodium caprate acts on the tight junctions and, therefore, alters the paracellular permeability of epithelia [17].

An alternative approach that precludes the permeation enhancer from being absorbed is the use of polymers as permeability enhancers. Polymers of high molecular weight can alter the permeability of intestinal epithelia [17–19]. Different structural polymers have been found to increase the absorption of LMWH: mucoadhesive Carbopol 934P or thiolated chitosans and polycarbophil, known as thiomers, have shown increased absorption of LMWH to anticoagulant levels [18,20–22].

Mono-*N*-Carboxymethylchitosan (MCC), a polyampholyte chitosan derivative, has been shown to increase the paracellular permeation and absorption of LMWH *in vitro* and *in vivo* in rats [19,23]. Although chitosan and its cationic derivatives are potent absorption enhancers, the polyampholytic derivative substantially enhanced the absorption of LMWH. MCC did not complex LMWH to form insoluble aggregates but rather formed a gel that provided sustained release and absorption of the macromolecule [19]. To investigate the effect of anionic chitosan derivatives on the paracellular permeation of intestinal epithelia, *N*-sulfonato-*N*,*O*-carboxymethylchitosan (SNOCC), a novel chitosan derivative, was designed and synthesized.

Reviparin (reviparin sodium) is a low molecular weight heparin (LMWH; 3900 Da) that catalyses the inactivation of factors Xa and IIa by binding to antithrombin which ultimately leads to the inhibition of the clotting cascade. It is administered subcutaneously for the treatment and/or prevention of DVT. Reviparin in dosages of 7000 to 12,600 Anti-Xa IU/day was found to be as effective as intravenous UFH in preventing the clinical recurrence of acute DVT and/or pulmonary embolism [7].

In general, LMWHs are considered to be improved anticoagulants and are preferred clinically for the prevention and treatment of DVT [6]. It has been recognized that LMWHs provide a more predictable anticoagulant response but their use is limited by the need for subcutaneous administration [24].

In this study, we investigated the use of polymeric absorption enhancers for the oral delivery of LMWH. Reviparin was used as the LMWH to be administered orally and *N*-sulfonato-*N*,*O*-carboxymethylchitosan (SNOCC), a novel chitosan derivative, was employed as the absorption enhancer. SNOCCs of different viscosity grades were investigated for their effect on the TEER and permeability of Caco-2 cells using a series of paracellular markers. Then, the most effective formulations of SNOCC were tested for their ability to affect the intestinal absorption of LMWH after intraduodenal administration in rats.

## 2. Materials and methods

### 2.1. Materials

LMWH, Reviparin (108 Anti-Xa U/mg, MW 3900 Da) was obtained from Knoll, Germany. Caco-2 cells were obtained from the ATCC. Trypsin, EDTA, FITC-Dextran, Dulbecco's Modified Eagle's Medium (DMEM), Hank's Balanced Salt Solution-w/o phenol red, (HBSS), *n*-(2-hydroxyethyl) piperazine-*N*-(2-ethanosulfonic acid) (HEPES), benzyl-penicillin G and streptomycin sulfate and $SO_3$-pyridine were all obtained from Sigma. Fetal calf serum was from Hyclone, The Netherlands. $^{14}C$-Mannitol (MW 182.2; specific radioactivity 57 mCi/mmol) was obtained from Amersham-Pharmacia, The Netherlands. Scintillation cocktail Ultima Gold, was obtained from Packard, The Netherlands. Chromostrate heparin Anti-Xa assay kits were purchased from Organon Teknika, The Netherlands.

### 2.2. Preparation of N-sulfonato-N,O-carboxymethylchitosan SNOCC polymers

Derivatives of NOCC, *N*,*O*-carboxymethylchitosan, containing sulfonate groups, $-SO_3^-Na^+$, were prepared by the reaction of sulfur trioxide–pyridine complex with NOCC in alkaline medium at room temperature. Typically, 10 g of NOCC (0.045 mol) dissolved in 0.60 l of water was treated with repeated additions of (12 g) $SO_3$-pyridine. The $SO_3$-pyridine was slurried in 50–100 ml of water and added dropwise, over 1 h (total mol $SO_3$-pyridine 0.075 mol). Both the NOCC solution and the sulfur trioxide reagent slurry were maintained at a pH above 9 by the addition of sodium hydroxide (5 M). Following the last addition of the sulfating reagent, the solution was stirred and NaOH was added until the pH stabilized (approx

40 min). The pH of the mixture was adjusted to 9 then the mixture was heated to 33 °C and held for 15 min. After filtration through a 110 μm nylon screen, the filtered SNOCC solution was poured into 6 l of 99% isopropanol. The resulting precipitate was collected and air dried overnight. The dried precipitate was dissolved in 0.45 l boiling water then the solution was poured into dialysis sacks (MWCO 12,000) and dialyzed for 3–4 days against deionized water. The contents of the sacks were lyophilized to yield the final product (6.2 g).

The degree of sulfonation was determined from the sulfur content of the derivative. SNOCCs were prepared from low molecular weight NOCCs with different viscosities. The viscosities of 1% w/v solutions in water at 20 °C were measured using a Brookfield RVT viscometer, spindle # 4 at speeds 50 and 100.

### 2.3. Intestinal epithelial Caco-2 cell lines

Caco-2 cell cultures were seeded initially from cryo-vials (stored in liquid nitrogen) to flasks and further passaged to passage numbers that are considered optimum for permeability studies (60–70). Costar Transwells® filters were purchased from Costar (Costar Europe, The Netherlands). After trypsin treatment, cells were seeded on the transwells at a seeding density of $10^4$ cells/cm$^2$. DMEM, (pH = 7.4), supplemented with 1% non-essential amino acids, 10% fetal calf serum, benzylpenicillin G (160 U/ml) and streptomycin sulfate (100 μg/ml) was used as culture medium, and added to both the donor and the acceptor compartment. The medium was changed every second day. The cell cultures were kept at a temperature of 37 °C, in a humidified atmosphere of 5% $CO_2$ and 95% air. For all the experiments, cells were used at 21 or 23 days after seeding.

#### 2.3.1. Transepithelial electrical resistance (TEER)

Four hours before application of the polymers at the apical compartment, the medium was replaced by DMEM buffered to pH 7.4 with 40 mM HEPES. TEER was monitored 1 h before application ($t$ = 00 min) and measurements were taken for 4 h post application at regular intervals. Alterations of the resistance are expressed as percentage of the resistance displayed at the zero time point. TEER was measured using a Millicell ERS meter (Millipore, MA, USA) connected to a pair of chopstick electrodes. SNOCC polymers of three different viscosities namely SNOCC-20, SNOCC-40 and SNOCC-60 were dissolved in DMEM medium buffered by 40 mM HEPES at increasing concentrations (1, 3, and 5% w/v). SNOCC solutions, at 37 °C, were applied on the apical compartment of the transwell. After the TEER measurements were completed, the polymers were removed from the monolayers (partly) and the cells were returned to media. Recovery of the TEER was measured 24 h later. Experiments were done in triplicate at 37 °C in an atmosphere of 95% humidity and 5% $CO_2$.

#### 2.3.2. Permeability studies

*2.3.2.1. $^{14}$C-mannitol radiolabeled paracellular marker.* The permeability of the monolayers under the influence of polymers was tested by measuring the transport of $^{14}$C-mannitol. The polymers were dissolved in DMEM-HEPES containing the radioactive marker at specific activity of 0.2 μCi/ml and the pH was adjusted to 7.4. SNOCC-20, -40, and -60 were dissolved in $^{14}$C-mannitol containing solutions at 3 and 5% w/v and samples of 200 μl were taken from the basolateral side for 4 h. Samples taken from the basolateral side were replaced with an equal volume of pre-warmed DMEM-HEPES. Control experiments were run in every setting with solutions containing the radioactive markers without any dissolved polymers. The radioactivity applied to the cells was predetermined in 200 μl samples of the solutions tested and background radioactivity was determined in 200 μl samples of DMEM-HEPES without the radioactive marker. The radioactivity present in samples was determined after adding 3 ml scintillation cocktail in a Tri-Carb 1500, Packard liquid scintillation counter. Results were corrected for dilution and expressed as cumulative transport with time. Apparent permeability coefficients were calculated according to the following equation:

$$P_{\text{app}} = (\mathrm{d}C/\mathrm{d}t) \times (1/A \times 60 \times C_0)$$

Where, $P_{\text{app}}$ is measured in cm/s, $\mathrm{d}C/\mathrm{d}t$ the permeability rate, $A$ the diffusion area of the monolayer (cm$^2$) and $C_0$ the initial concentration of the radiomarker.

Enhancement ratios $R$ are calculated according:

$$R = P_{\text{app polymer}}/P_{\text{app control}}.$$

In these experiments, trimethylated chitosan (TMC; prepared according to [25]) and MCC (prepared according to [23]) were used for comparison since their enhancing properties have been reported.

*2.3.2.2. FITC-Dextran, FD-4, as a paracellular marker.* Similar to the radiolabeled marker, FITC-Dextran with MW 4400 Da was used as a hydrophilic macromolecule that is transported exclusively via the paracellular route across intestinal epithelia. FD-4 solution was prepared at 1 mg/ml concentration in HBSS-HEPES at pH 7.4. SNOCC-20, -40 and -60 were dissolved at 3% w/v and applied on the apical compartment of Caco-2 cell monolayers. Samples of 200 μl were taken from the basolateral side for 4 h.

Analysis of samples was performed by HPLC Size Exclusion Chromatography (SEC). For this, a Waters 515 HPLC pump, a Waters 474 Scanning Fluorescence detector and a Gilson 234 Autoinjector were used. The Column was a PSS Suprema 30 from Polymer Standard Service. The eluent was 15/85 Acetonitrile/$CH_3COONH_4$ (0.05 M pH = 9.0). Retention time of FD-4 was 7 min; however, chromatograms were run for 15 min to investigate the elution of possible degradation products (MW lower than 4000 Da). Samples for calibration curves were prepared with increasing concentration of FD-4. Permeability was expressed as cumulative transport with time.

*2.3.2.3. Transport of LMWH.* Reviparin solution was prepared at 1% (w/v) concentration in HBSS-HEPES at pH 7.4.

SNOCC-40 and -60 were dissolved at concentrations of 3% and 5% (w/v) in the LMWH containing medium and applied on Caco-2 cell monolayers. Samples of 200 µl were taken from the basolateral side for 4 h. Analysis of samples was performed using the Chromostrate heparin Anti-Xa assay (Organon Teknika, Netherlands). The assay measures the potentiating effect of Heparin on Anti-Xa activity in plasma and medium samples by an amidolytic method using a synthetic chromogenic substrate ($CH_3$OCO-D-Val-Gly-Arg-*p*NA.AcOH). Samples containing heparin are incubated in the presence of antithrombin III (ATIII; plasma cofactor) with an excess of factor Xa forming an ATIII–Heparin–Xa complex. The remaining Xa catalyses the release of *p*-nitroaniline (*p*NA) from the chromogenic substrate. The release of *p*NA was measured by an end-point method at 405 nm in an EL808 ultra microplate reader (Biotek Instruments Inc.). Analysis of LMWH was performed using a standard calibration curve in HBSS-HEPES at the range of 0.1–0.7 Anti Xa Units/ml ($R^2$ = 0.958). Where necessary, samples were diluted with HBSS-HEPES buffer to fit on the calibration curve. LMWH permeation was expressed as cumulative transport with time and $P_{app}$ was calculated according to the above mentioned formula.

### 2.4. In vivo intestinal administration of LMWH in rats

In order to investigate the *in vivo* effect of SNOCCs as absorption enhancers, a study in rats was performed. The protocol for the animal study was approved by the Ethics Committee of Leiden University. Male Wistar rats (SPF status) approximately 250 g were obtained from Charles River, the Netherlands. The animals were fasted for 16 h before administration but had free access to water. The animals were anesthetized with Hypnorm (fentanyl citrate) and Dormicum (midazolam). Body temperature was monitored during the operation and throughout the experiment and was kept at 36–37 °C.

Reviparin LMWH (Reviparin, 108 Anti-Xa U/mg) solution was prepared in physiological saline at 625 Anti Xa U/ml. SNOCC-40 and SNOCC-60 were dissolved at 3% (w/v) in the Reviparin solution to form very viscous solutions.

In order to administer the LMWH formulations, a thin teflon tube (~1 mm i.d.) was used for the control formulation (without polymer) while a polypropylene tube with i.d. ~3 mm was used to facilitate the application of the viscous SNOCC solutions. Both tubes were connected to a syringe.

The abdomen of the animals was opened by incision and the stomach was moved towards the incision. A small incision in the stomach wall was made to allow each tube to be guided via the pylorus to the beginning of the duodenum (first 2–3 cm.). Formulations were administered slowly over 5 min at +/−2 ml normalized for the body weight of each animal to assure administration of 5000 Anti-Xa U/kg.

Blood samples (~225 µl) were collected from the tail vein capillaries (after incision) in vials containing 25 µl sodium citrate (3.8%). Samples were centrifuged at 13,000 rpm for 20 min at 4 °C and plasma was collected and analyzed using the chromostrate heparin Anti-Xa assay with calibration curves prepared in clean plasma in the series between 0.1 to 0.7 Anti-Xa U/ml ($R^2$ = 0.975).

The plasma heparin levels were expressed as AntiXaU/ml and plotted over the time of blood sampling. The AUC was measured and enhancement ratios were calculated from the ratio: $AUC_{polymer}/AUC_{control}$. *In vivo* experiments were performed with 6 animals per group.

## 3. Results

### 3.1. Preparation of SNOCC polymers

*N*,*O*-carboxymethylchitosan or NOCC retains one half of its nitrogen centers on the glucose rings as free amine groups, $-NH_2$ [26]. These amine centers were sulfonated using a sulfur trioxide–pyridine reagent to yield the derivative shown in Fig. 1. The percentage sulfur in these derivatives was determined by microanalysis to fall in the range 3.0–3.7% which corresponds to a degree of sulfonation of 0.25–0.32 (i.e. 2–3 sulphonate groups for every 10 sugar units).

NOCC materials that yielded Brookfield viscosities of 20, 40 or 60 cps, when prepared as 1% (w/v) solutions in water, were used to prepare SNOCC-20, -40 and -60. The NOCC-20 and -40 were prepared from chitosan that was depolymerized using $NaNO_2$ according to the method of Peniston et al. (US Patent 3,922,260) [27].

### 3.2. Effect of SNOCC polymers on cells

#### 3.2.1. Transepithelial electrical resistance (TEER)

Caco-2 cell monolayers were used as a model to evaluate SNOCC materials for their permeation enhancing properties. Reduction of the TEER across such monolayers was an indicator of increasing paracellular permeability. The effect of SNOCC polymers at various concentrations and viscosities is presented in Fig. 2. The results clearly showed a decrease of TEER with increasing concentration of the applied polymers. A similar correlation was observed with increasing viscosity of the biomaterials. SNOCC-20 slightly reduced TEER but not to levels where substantial transport could be expected. However, SNOCCs of higher viscosity showed a clear decrease of the initial resistance value that was sustained for 4 h. SNOCC-40 and -60 induced a decrease of >50% of the initial value indicating widening of the paracellular pathways. Both materials

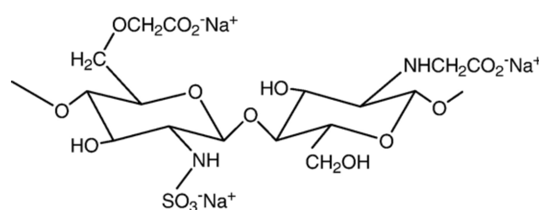


Fig. 1. Chemical structure of SNOCC materials.

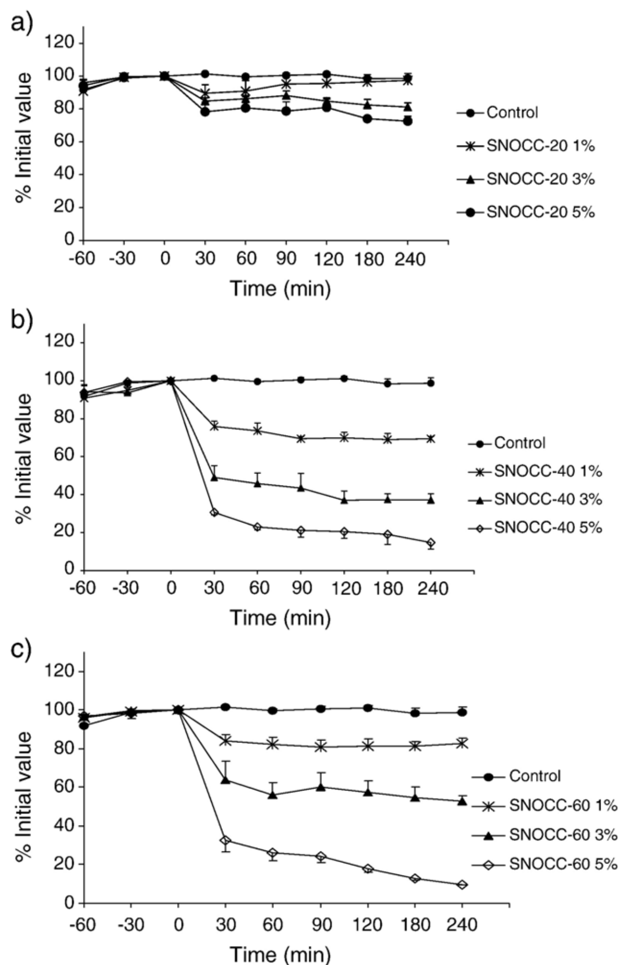


Fig. 2. Application of SNOCC materials of different viscosity at increasing concentrations (% w/v) on Caco-2 cell intestinal epithelia at pH 7.4 and their effect on the TEER, ($n$=3 +/− SD) a) SNOCC-20 ($\eta$=20 mPa s), b) SNOCC-40 ($\eta$=40 mPa s), c)SNOCC-60 ($\eta$=60 mPa s). Note: resistance was monitored 60 min prior application ($t$=0).

demonstrated an effect after 30 min which became reasonably stable throughout the experiment. Due to the viscosity and adhesion properties of the materials they could not be completely removed from the cells to assess cell viability by dye exclusion assays. However, at the end of the experiments the cells were returned to medium and TEER was measured after 24 h. For all cases TEER had returned to 90–100% of initial value after one day (data not shown).

Table 1
Permeability parameters of $^{14}$C-mannitol across Caco-2 cells as an effect of SNOCC

| Application | Conc (w/v) | $P_{app} \times 10^{-81}$ cm/sec | $R$ |
|---|---|---|---|
| Control | | 2.3±0.2 | 1 |
| SNOCC-20 | 3 | 4.5±0.2 | 1.9 |
| | 5 | 11.7±2.7 | 5 |
| SNOCC-40 | 3 | 25.9±0.4 | 11.2 |
| | 5 | 71.5±11.6 | 31.0 |
| SNOCC-60 | 3 | 20.5±1.85 | 8.9 |
| | 5 | 119±15.35 | 50.7 |

Table 2
$^{14}$C-mannitol permeation enhancement ratio across Caco-2 induced by MCC and TMC chitosan derivatives

| Chitosan derivative | $R$ |
|---|---|
| MCC 3% (w/v) | 15.1 |
| TMC 0.5% (w/v) | 13.0 |
| TMC 1% (w/v) | 26.38 |

### 3.2.2. $^{14}$C-mannitol paracellular marker transport

Caco-2 cell monolayers and radiolabeled mannitol were used to assess the quantitative alteration of paracellular permeability [28]. $P_{app}$ was calculated from cumulative mannitol transport for both treated and untreated monolayers. Results are presented in Table 1. In this experiment, SNOCCs were used at concentrations of 3 and 5% (w/v). All SNOCCs induced an increase in the permeation of $^{14}$C-mannitol as a function of concentration and viscosity, in agreement with the TEER data. SNOCC-20 induced a minor effect on the permeability ($R$=1.9 and 5 for 3 and 5% respectively). However, SNOCCs of higher viscosity showed higher ratios ($R$). SNOCC-60 gave an increase of 8.9 and 50.7 for 3 and 5% concentrations respectively (Table 1).

Table 2 lists the enhancement ratios induced by the structurally different chitosan derivatives including MCC and TMC. From a comparison of the data in Tables 1 and 2, it can be seen that SNOCC-40 and -60 are potent permeability enhancers of radiolabeled paracellular marker mannitol and their effect is comparable to 1% w/v TMC.

### 3.2.3. FITC-Dextran, FD-4 as a paracellular marker

FD-4 is a dextran conjugate with FITC and has been tested in this study as a model for the LMWH polysaccharide. FD-4 is anionically charged and is often used as a fluorescent paracellular marker. The results of measurements of FD-4 transport across Caco-2 monolayers, when co-applied with SNOCC derivatives, are shown in Fig. 3. The maximum permeation is induced by SNOCC-40 which is inconsistent with previous data. In this experiment, all SNOCCs increased FD-4 permeation; the enhancement ratios calculated for SNOCC-20, -40, and -60 are 4.1, 15.2, and 8.5. The lower enhancement ratio of SNOCC-60 compared to SNOCC-40 could be attributed to an interaction between the FD-4 macromolecule and the higher molecular weight SNOCC polymer. It is likely that larger molecules transport slower through viscous solutions due to entanglement with polymer chains.

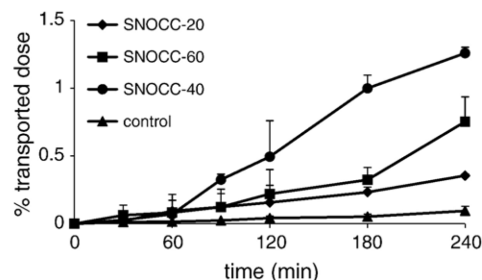


Fig. 3. Effect of different viscosity SNOCCs on the cumulative transport of fluorescent dextran, FD-4 across Caco-2 intestinal monolayers over 4 h. SNOCCs were applied at 3% (w/v) ($n$=3;+/− SD).

Table 3
Permeability parameters of LMWH Reviparin across Caco-2 cells with or without the SNOCC materials

| Application | Conc. (%w/v) | $P_{app} \times 10^{-11}$ cm/sec |
|---|---|---|
| Control | | – |
| MCC | 3 | 435.02±31.76 |
| | 5 | 250.40±18.78 |
| SNOCC-40 | 3 | 629.89±158.6 |
| | 5 | 1042.6±212.8 |
| SNOCC-60 | 3 | 620.85±26.0 |
| | 5 | 978.8±183.3 |

MCC is used for comparison.
Note: no enhancement ratios are presented since LMWH was not transported at non-treated cells.

### *3.2.4. Transport of LMWH across Caco-2*

The permeation characteristics of LMWH, Reviparin (4500 Da), were investigated by co-applying it with SNOCC polymers on Caco-2 cell monolayers. Table 3 displays the resulting $P_{app}$ values for LMWH as assessed by the colorimetric assay. In this experiment, the presence of Reviparin at the basolateral compartment of the untreated or control cells was undetectable; hence, no enhancement ratio is presented. MCC was included in the study for comparison and the results in Table 3 show that MCC increased Reviparin's $P_{app;}$ however, the enhancement ratio decreased with increasing MCC concentration. By comparison, both SNOCC-40 and -60 demonstrated substantially higher $P_{app}$ than MCC 5% (w/v). SNOCC-40 at 5% (w/v) yielded the highest $P_{app}$ ($1042.6 \times 10^{-11}$) although not significantly different from that for SNOCC-60 (*t*-test $p<0.05$). These findings were in agreement with the FD-4 permeation data and confirmed that SNOCC materials with intermediate viscosity were most effective for LMWH transport. The $P_{app}$ values in Table 3 were substantially smaller than the comparable values for $^{14}$C-mannitol; however, this was expected due to the anionic character and large size of LMWH.

### *3.3. In vivo intestinal administration of LMWH in rats*

LMWH solutions with and without SNOCC-40 and -60 were administered intraduodenally in rats under anesthesia and blood samples were taken to monitor LMWH absorption. Fig. 4 presents the Anti-Xa plasma levels achieved after the administration of LMWH. Animals that received only Reviparin solution showed minimal LMWH absorption and below 0.05 Anti-Xa U/ml. However, animals that received 3% SNOCC along with the Reviparin showed substantial absorption. The Anti-Xa levels increased to 0.4 Anti-Xa U/ml and remained above 0.2 Anti-Xa U/ml throughout the experiment. Such Anti-Xa levels are considered antithrombotic since blood clotting is inhibited [29]. These levels were sustained throughout the experiment without exceeding levels that could induce bleeding. The AUC was calculated for the three treatment groups and enhancement ratios were also estimated (see Table 4). Both SNOCC formulations had similar AUC's in agreement with the *in vitro* data and both increased LMWH absorption by 16 to 18 times.

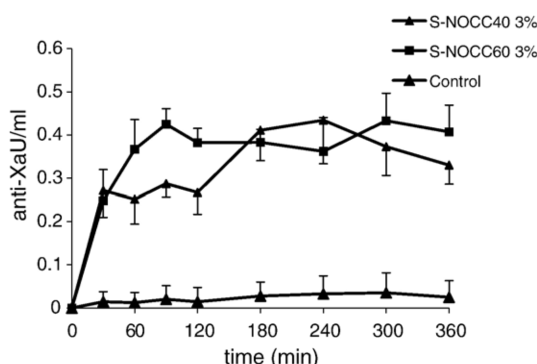


Fig. 4. Plasma levels of LMWH Reviparin after intraduodenal administration at 5000 AntiXaU/kg with or without polymers (3%w/v). ($n=6\pm$SEM).

Table 4
Intestinal absorption of LMWH Reviparin in rats ($n=6\pm$SEM)

| Polymer | AUC AntiXa U/ml min | Enhancement ratio (*R*) |
|---|---|---|
| Control | 7.34±2.27 | 1 |
| SNOCC-40 3% | 119.32±16.87 | 16.25±2.30 |
| SNOCC-60 3% | 136.20±19.12 | 18.54±2.60 |

## 4. Discussion

In this study, we investigated SNOCC, a sulfonated derivative of *N*,*O*-carboxymethyl chitosan, as a novel polymeric absorption enhancer and assessed its potential for the oral delivery of LMWH Reviparin. Reviparin sodium (Clivarine®) is a low molecular weight heparin (LMWH) with an average MW of 4400 Da and mean peak molecular weight of 3900 Da: it is characterized by a narrow molecular weight distribution profile and an anti-factor Xa (Anti-Xa):anti-factor IIa (anti-IIa) ratio of ≥3.6. When Reviparin was administered s.c. to healthy human volunteers, plasma Anti-Xa activity increased to five times higher and lasted three times longer than treatment with UFH [30]. Results from various clinical trials have been summarized and it has been found that $C_{max}$ was between 0.2 and 5.8 AntiXaU/ml and MRT (mean residence time) between 6.4 and 5.8 h, indicating a slow elimination [7]. The use of Reviparin was also associated with similar or lower incidence of bleeding complications compared to UFH [7]. Further, Reviparin was found to be absorbed following oral administration in rats in a dose dependent way. Even when low traces were detected in the blood, an antithrombotic effect was observed [31]. The development of an oral Reviparin regime that would reproducibly achieve antithrombotic levels, should be beneficial for the treatment and prevention of DVT after surgical procedures [10].

Chitosan has been reported to increase paracellular permeation [32]. However, the physicochemical properties of chitosan prevented this material from being employed for the co-administration of anionic heparins. To overcome this problem, a series of chitosan modifications have been suggested [22,23]. Anionic chitosan derivatives have been found to increase permeation of anionic macromolecules though to a lesser extent than cationic derivatives. The mechanism of absorption

enhancement is unknown but it is possible that mucoadhesion together with paracellular permeation enhancement are promoting absorption of anionic macromolecules.

In this study, highly anionic (approx. 1.3 negative charges/sugar), water soluble SNOCC derivatives have demonstrated a dose-dependent and reversible ability to decrease the TEER of Caco-2 cell monolayers. The most effective SNOCC materials were those of nominal viscosities 40 and 60 cps and not the lowest viscosity material. This finding suggests that the SNOCC polymer must be of a certain size ($M_w$) to attach to and disrupt the junctions between the epithelial cells.

From TEER recovery data obtained 24 h post-removal of the formulation, it can be hypothesized that SNOCCs are not likely to affect cell viability and that their effect on paracellular permeability is mainly due to the opening of tight junctions. Unfortunately, the adhesive nature of SNOCC did not allow complete removal of the polymer and the assessment of cell viability will require the use of another method such as the nucleic acid stain exclusion assay. However, these materials due to their polymeric and anionic character are not expected to induce any membrane perturbation that could lead to cytotoxicity.

SNOCC polymers increased the permeation of $^{14}$C-mannitol compared to previously studied MCC and TMC (1% w/v concentration). It is likely that the mechanism of permeation enhancement by SNOCC polymers is different from that for chitosan and further investigation is required to delineate the exact pathway.

The Caco-2 system was chosen as a predictive model to screen and develop SNOCC materials as it has been well established and widely used [33]. Caco-2 cells were used as a predictive tool for the absorption enhancement effects of sodium caprate for ardeparin [16] and Tetradecylmaltoside for enoxaparin [34].

Sodium caprate, a known paracellular permeation enhancer, increased ardeparin absorption substantially when administered orally in rats. Administration of 4800 Anti-Xa U resulted in blood levels of 0.5 Anti-Xa U/ml that were sustained just above the 0.4 AntiXa U/ml level for 8 h [16]. These values are similar to the Anti-Xa levels obtained in the current study.

In an attempt to make oral heparin particle formulations, LMWH was formulated in polymeric nanoparticles (NPs) of biodegradable poly-ε-caprolactone (PCL) and poly(lactic-co-glycolic acid) (PLGA) and non-biodegradable positively charged polymers (Eudragit RS and RL). These nanoparticles were evaluated *in vitro* and *in vivo* after a single oral administration in rabbits. The AntiXa levels peaked at 0.16 AntiXa U/ml at 6 h post-administration and a 23% absolute bioavailability was calculated [35]. These levels appear lower to the ones achieved in the current study suggesting that in the case of SNOCC elevated oral bioavailabilities can be considered.

In the current study, SNOCC, following intestinal co-administration with LMWH in rats, increased the Anti-Xa content of the blood samples to levels above that necessary to prevent thrombosis [7]. These results indicate that SNOCC is a potential absorption enhancer of Reviparin and that dosage-form design may yield an efficient oral formulation of LMWH. In addition, SNOCC offers the advantage of not being absorbed, compared to other permeation enhancers, because of its high molecular weight which is in excess of 100 KDa.

In this study LMWH was administered intraduodenally to mimic a future oral dosage form designed to deliver the therapeutic to the site of absorption. Currently an oral dosage form based on SNOCC is being evaluated *in vivo* for its efficiency to control–release LMWH exclusively in the intestine.

## 5. Conclusions

SNOCC is a novel polymeric permeation enhancer that decreased the TEER of Caco-2 cell monolayers and increased the paracellular permeability of hydrophilic markers and LMWH Reviparin. SNOCC demonstrated a potent Reviparin absorption enhancing effect in rats, with AntiXa levels reaching those required to prevent thrombus formation. Because SNOCC is a hydrophilic, anionic, non-absorbed polymer, it represents a potentially efficient and safe approach to oral LMWH delivery.

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