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Research paper

An oral oligonucleotide delivery system based on a thiolated polymer: Development and in vitro evaluation

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ABSTRACT

The purpose of this study was to develop and evaluate an oral oligonucleotide delivery system based on a thiolated polymer/reduced glutathione (GSH) system providing a protective effect toward nucleases and permeation enhancement. A polycarbophil–cysteine conjugate (PCP–Cys) was synthesized. Enzymatic degradation of a model oligonucleotide by DNase I and within freshly collected intestinal fluid was investigated in the absence and presence of PCP–Cys. Permeation studies with PCP–Cys/GSH versus control were performed in vitro on Caco-2 cell monolayers and ex vivo on rat intestinal mucosa. PCP–Cys displayed 223 ± 13.8 μmol thiol groups per gram polymer. After 4 h, 61% of the free oligonucleotides were degraded by DNase I and 80% within intestinal fluid. In contrast, less than 41% (DNase I) and 60% (intestinal fluid) were degraded in the presence of 0.02% (m/v) PCP–Cys. Permeation studies revealed an 8-fold (Caco-2) and 10-fold (intestinal mucosa) increase in apparent permeability compared to buffer control. Hence, this PCP–Cys/GSH system might be a promising tool for the oral administration of oligonucleotides as it allows a significant protection toward degrading enzymes and facilitates their transport across intestinal membranes.

1. Introduction

Recent advances in biotechnology have brought up antisense oligonucleotides as new therapeutic agents in order to improve the medical treatment of cancer, viral diseases, or genetic disorders [1]. Administration of oligonucleotides (ODNs) is currently limited to parenteral routes, mainly intravenous and subcutaneous, due to their poor oral bioavailability. Major reasons for this poor oral bioavailability are their rapid degradation in the GI-tract by nucleases, on the one hand, and limited intestinal permeability to hydrophilic macromolecules with a high negative charge density, on the other hand. Thus far, strategies to overcome the problem of degradation by nucleases are mainly based on chemical modifications, such as formation of phosphorothioates [2] or methoxyethyl phosphorothioates [3]. Coadministration of permeation enhancers such as medium chain fatty acids turned out to be a promising strategy to increase ODN uptake across gastrointestinal epithelia [4].

Thiolated polymers with excellent mucoadhesive features have been developed [5]. These so-called thiomers are hydrophilic polymers, such as polycarbophil (PCP), modified with thiol-bearing molecules, like L-cysteine, on their polymer backbone. Thiomers show enzyme inhibitory activity toward metalloenzymes [6]. Hence, it is assumed that they also exhibit inhibitory effects toward nucleases, which are abundantly present in the intestine. Their mechanism of enzyme inhibition is based on the deprivation of divalent metal cations from the enzyme structure. Thiomers also show excellent permeation-enhancing properties in combination with reduced glutathione (GSH) based on a reversible opening of tight junctions, which are mainly responsible for limited paracellular uptake of hydrophilic macromolecules [7]. In combination with their further advantages, including enhanced mucoadhesion and cohesive strengths, thiomers seem to be a promising candidate for oral ODN delivery.

Therefore, the aim of this study was to develop an oral delivery system for ODNs based on PCP–Cys/GSH and to determine its inhibitory activity toward nucleases and its permeation-enhancing effect. Enzyme inhibition studies were performed with DNAse I as well as fresh porcine intestinal fluid. Permeation studies were performed on differentiated Caco-2 cell monolayers and freshly excised rat intestinal mucosa. In these studies, a 30-mer phosphorothioate antisense oligonucleotide was chosen as model ODNs. The utilized ODN was used as a model since it was shown to be able to inhibit the growth of *Plasmodium falciparum* [8]. Therefore, it might be a valuable tool for the therapy of malaria diseases.

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2. Material and methods

Noveon® AA-1 USP Polycarbophil (PCP) was obtained as a kind donation from Lubrizol, Germany. The 30-mer model phosphorothioate oligonucleotide with the sequence 5′-ATG TAA TAT TCT TTT GAA CCA TAC GAT TCT-3′ (molecular mass 9608 Da) was purchased from VBC Biotech, Vienna, Austria. Caco-2 cells were obtained from German Collection of Microorganisms and Cell Cultures, Brunswick, Germany. Fetal calf serum was obtained from PAA Laboratories GmbH, Pasching, Austria. All other chemicals were of analytical grade and were purchased from Sigma Aldrich, Vienna, Austria.

2.1. Synthesis of polycarbophil–cysteine conjugate

The polycarbophil–cysteine conjugate was synthesized according to a method described previously [9]. Polycarbophil (MW 3000 kDa) was neutralized with 5 M NaOH as described previously [10]. First, 2 g of neutralized polymer was hydrated in 500 ml of demineralized water. The carboxylic acid moieties of the polymer were activated by adding 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC) in a final concentration of 50 mM. This mixture was then stirred for 45 min. Afterward, 0.25 g of L-cysteine hydrochloride was added and pH was adjusted to 4.0 by adding 1 M HCl. The reaction mixture was stirred and incubated at room temperature for 3 h. Cysteine was covalently linked to polycarbophil via the formation of amide bonds between the primary amino group of cysteine and a carboxylic group of the polymer. Resulting conjugates were isolated by dialysis against 1 mM HCl containing 2 μM EDTA at 10 °C in the dark to avoid oxidation of the cysteine moieties, two times against the same medium but additionally containing 1% NaCl and one more time against 1 mM HCl. In order to prepare a non-thiolated control, polycarbophil was treated in exactly in the same manner but EDAC was omitted during the reaction. The purified polymers were lyophilized by freeze-drying aqueous polymer solutions at −30 °C and 0.01 mbar (Benchtop 2 K, VirTis, NY, USA). Freeze-dried polymers were stored at 4 °C until further use.

2.2. Determination of immobilized thiol groups

The amount of thiol groups on the polycarbophil–cysteine conjugate was determined via Ellman's reagent (5,5′-dithiobis(nitrobenzoic acid), DTNB) as described previously [5]. For this purpose, 1 mg polymer was hydrated in a 0.5 M phosphate buffer before a 0.03% solution of DTNB in the same buffer was added. Thiol concentrations were derived from a calibration curve that was obtained from solutions of different L-cysteine concentrations.

2.3. Intestinal stability of oligonucleotide

2.3.1. Protection against DNase I degradation

To evaluate the protection of ODNs from DNase degradation by PCP–Cys, mixtures of PCP–Cys and ODNs (1 mg/ml) were incubated with DNase I in artificial intestinal fluid (AIF). AIF is a physiological salt solution containing 20 mM bicarbonate, 139 mM chloride, 5 mM potassium, 140 mM sodium, 4 mM calcium and 3 mM magnesium ions (pH 7.0). Mixtures of 250 μl of PCP–Cys polymer solution (0.04% m/v) adjusted to pH 7.4, 250 μl of AIF containing 1 mg/ml of ODNs, and 3 μl of DNase I (3 Units) were incubated at 37 °C while shaking at 400 rpm (Thermomixer comfort 2 ml, Eppendorf, Germany). After predetermined time intervals of up to 4 h, 10 μl of 0.5 M EDTA solution, pH 8.0, was added to each sample in order to stop the reaction. ODNs were incubated with DNase I in the absence of PCP–Cys as control. After incubation, samples were centrifuged at 29,700*g* and 4 °C for 60 min (Sigma 3–18K centrifuge) and 30 μl of the supernatant was analyzed by a weak–base anion exchange HPLC assay, using a PRP-X600 Anion Exchange 4.6 × 100 HPLC column (Hamilton Co., Reno, Nevada, USA). A two-eluent gradient system was used with eluent A consisting of 80% 100 mM Tris (pH 8.0)/20% acetonitrile and eluent B consisting of 80% 100 mM Tris, 2.5 M LiCl (pH 8.0)/20% acetonitrile. A linear gradient from 100% eluent A to 100% eluent B in 15 min at a flow rate of 2 ml/min was performed. The amounts of ODNs were determined by measuring absorbance at 260 nm [11].

2.3.2. Incubation with intestinal fluid

The intestinal fluid from a freshly slaughtered pig was collected and frozen on dry ice. For this study, samples of 250 μl of ODN solution (1 mg/ml) were incubated with 250 μl of intestinal fluid containing 0.04% PCP–Cys at 37 °C under continuous shaking (400 rpm). At predetermined time points, aliquots of 50 μl were withdrawn and 10 μl of 0.5 M EDTA, pH 8.0, was added in order to stop any further enzymatic degradation. Withdrawn aliquots were substituted by 50 μl of intestinal fluid. ODNs without additional PCP–Cys were incubated with intestinal fluid in the same manner as a control. The degree of ODN degradation was determined via HPLC as described previously.

2.4. Caco-2 cell monolayer permeation studies

Caco-2 cells were employed to evaluate the effect of PCP–Cys on intestinal permeability. These cells originate from human adenocarcinoma and exhibit characteristics of intestinal epithelia, such as microvilli, intercellular tight junctions, enzymes, nutrients, and efflux transporters, and are appropriate models for evaluating the permeation of drug molecules across intestinal epithelia [12]. Cells were seeded in 75-cm^2 flasks and cultured until confluency. Caco-2 cells were maintained in MEM medium supplemented with 20% (v/v) of heat-inactivated fetal calf serum (FCS) and 0.01% (v/v) of penicillin–streptomycin solution. The pH was adjusted to 7.4 using 7.5% (m/v) sodium bicarbonate. Cells were maintained at 37 °C in an atmosphere of 5% CO_2. Cells in flasks were first washed with phosphate-buffered saline (PBS) and then treated with trypsin/EDTA solution (0.05%) at 37 °C for 20 min. The cell suspension was centrifuged at 900 rpm, and the supernatant removed in order to remove trypsin. Cells were resuspended in culture medium and thereafter seeded at 12 wells of a Transwell® plate (Corning Inc., Corning, NY, USA) before being cultivated for 3 weeks. The transepithelial electrical resistance (TEER) across cell monolayers was monitored by means of a volt ohmmeter with a chopstick electrode (World Precision Instruments, Berlin, Germany) during cell culturing and during the permeation experiment. Caco-2 cell monolayers were used at resistance readings between 600–700 Ω cm^2. After 2 h of pre-incubation with fresh medium, the medium in the donor chamber of Transwell® plate was substituted with MEM medium (1 ml) containing 1 mg/ml of ODNs and 0.5% (m/v) of PCP–Cys conjugate with or without 0.25% (m/v) reduced GSH. Permeation experiments with ODNs but without additives served as controls. In order to prevent enzymatic degradation of ODNs, 0.5 mM EDTA was added to each chamber prior to sampling. Samples of 100 μl were withdrawn from the acceptor chamber of Transwell® plates every 30 min over a time period of 2 h. The removed volume was immediately replaced by 100 μl fresh MEM. The EDTA concentration was kept constant in all experiments in order to allow investigation into the effect of PCP–Cys alongside the permeation-enhancing effect of EDTA. The amount of permeated ODNs was determined via HPLC as described previously. The apparent permeability coefficients (P_{app}) for ODNs were calculated according to the equation below. All sample solutions were removed after the permeation

experiment. Subsequently, the cells were washed with PBS and fresh MEM was finally added. TEER values were recorded for further 24 h in order to investigate the reversibility of the tight junction opening.

2.5. Rat intestinal mucosa permeation study

These permeation studies with ODNs were carried out in Ussing-type chambers displaying a permeation area of 0.64 cm^2. Small intestinal mucosa from the upper part of the ileum of a rat was excised immediately after sacrifice. Tissue samples of 3–4 cm^2 were prepared and mounted onto Ussing chambers without stripping off the underlying muscle layer. The donor and acceptor compartments of the Ussing-type chambers were both filled with 1 ml of *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethane-sulfonic acid) (HEPES) buffer (250 mM NaCl, 2.6 mM $MgSO_4$, 10.0 mM KCl, 40.0 mM glucose and 50 mM $NaHCO_3$, buffered with 40 mM HEPES, pH 7.0). To ensure oxygenation and agitation, a mixture of 95% O_2 and 5% CO_2 was bubbled through each compartment. The temperature within the chambers was maintained at 37 °C. The integrity of the membrane was checked by TEER measurements prior to the actual experiment. The viability of these tissues in the permeation studies was investigated before and after the experiment by staining with trypan blue. After a pre-incubation time of 20 min, the buffer medium in the donor chamber was substituted by a mixture of 0.5% (m/v) PCP–Cys and 0.1% ODN, either in the absence or presence of 0.25% GSH. In order to prevent enzymatic degradation of ODNs, 0.5 mM EDTA was added to each chamber prior to sampling. At predetermined time point, 100 μl of samples was withdrawn from the acceptor compartment and immediately replaced by 100 μl of buffer at 37 °C. The EDTA concentration was kept constant in all experiments in order to allow investigation into the effect of PCP–Cys alongside the permeation-enhancing effect of EDTA. Samples were frozen at −70 °C for further examination. The permeated amount of ODNs was determined using HPLC as described previously. The apparent permeability coefficients (P_{app}) for ODNs were calculated according to the equation, mentioned below.

2.6. Data analysis

Apparent permeability coefficients (P_{app}) for ODNs were calculated according to the following equation:

$$P_{app} = Q/Act \quad (1)$$

where P_{app} (cm/s) is the apparent permeability coefficient, Q (mg) is the total amount permeated ODNs throughout the incubation time, A (cm^2) is the diffusion area of the Ussing-type chambers or the Transwell®, c (mg/cm^3) is the initial concentration of the test compound in the donor compartment, and $t(s)$ is the total time of the experiment.

Transport enhancement ratios (R) were calculated from P_{app} values by:

$$R = P_{app}(\text{sample})/P_{app}(\text{control}) \quad (2)$$

Statistical data analyses were performed using the Student's *t*-test, with $p < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Characterization of polycarbophil–cysteine conjugate

The covalent attachment of cysteine to polycarbophil (PCP) was achieved by the formation of amide bonds between the primary amino group of the amino acid and the carboxylic acid group of the polymer as shown in Fig. 1. To minimize the formation of disulfide bonds without exclusion of oxygen at this step, a pH of 4.0 was chosen. At that pH, the activity of the reactive form of thiol groups (thiolated-anions) that tend to oxidize is low and the formation of disulfide bonds can be minimized [13]. The lyophilized polymer was white, odorless and of fibrous structure. It was quickly swellable in water and buffer solution forming transparent gels above a pH 5.0 [14]. The PCP–Cys in this study displayed 223 ± 13.8 μmol thiol groups per gram polymer. Negligible amounts of sulfhydryl groups were detected for the control polymer, where the coupling reagent EDAC was omitted. This indicates that the utilized purification method is appropriate to remove unbound L-cysteine. The amount of covalently attached cysteine on the thiomer is crucial for its permeation-enhancing properties [15].

3.2. Inhibitory effect toward nucleases

A major limitation to oral gene delivery is the vulnerability of DNA to nucleases that are present in small intestinal fluid [16]. ODNs were incubated with nucleases in the presence of PCP–Cys in order to investigate its protective effect toward nucleolytic degradation. Within this system, ODN and the thiomer will undergo ionic repulsion due to the same charge. Therefore, it is assumed that ODN and thiomer are independently distributed and do not form any complexes. However, polycarbophil–cysteine forms quite strong gels that will reduce the motility and rapid dissolution of the ODN. For preliminary experiments, the inhibitory effect of PCP–Cys was analyzed toward DNase I. Free ODNs were used as a control. Results are displayed in Fig. 2A. The remaining amount of ODNs (59%) was significantly increased in the presence of PCP–Cys compared with free ODNs (39%). The polymer conjugates demonstrated an obvious inhibitory effect toward DNase I.

In order to examine the protective properties of the PCP–Cys conjugate in the harsh environment of the intestinal tract, the ODNs and PCP–Cys were incubated with freshly collected intestinal fluid. The amount of ODNs remaining after predetermined time points was determined via HPLC. The results are presented in Fig. 2B. Within this study, phosphorothioate ODNs were used. This modification improves the stability of ODNs in biological systems [17]. In these molecules, a sulfur atom replaces an oxygen of each phosphate group in the chain. This causes increased resistance toward nucleases [18]. This substitution retains the charge and hence the aqueous solubility characteristics of the parent sequence [19]. However, unprotected ODNs in the control experiment were degraded to a significantly higher extent than in the presence of PCP–Cys. Only 20% of intact ODNs were found after 4 h of incubation without PCP–Cys whereas more than 40% were still intact when PCP–Cys was present in the experiment. It is evident that the PCP–Cys conjugate was effective in protecting ODNs from enzymatic degradation in the intestine. In Fig. 2A and B, it is shown that the free ODNs are not completely degraded after incubation with DNase I or intestinal fluid within 4 h. It is most likely that denaturation of the enzyme occurred during the experiment. This would explain the significantly decelerated degradation after

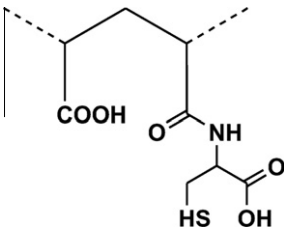

Fig. 1. Schematic diagram of a PCP–Cys substructure.

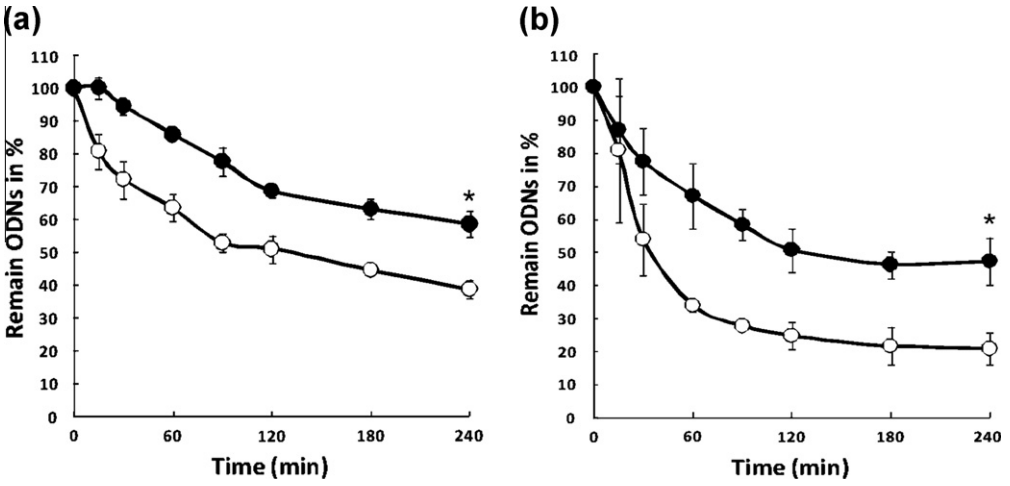

Fig. 2. (A) Degradation of ODN by DNAse I in AIF in the presence of 0.02% PCP–Cys (closed circles) and control without PCP–Cys (open circles). (B) Degradation of ODN within freshly collected porcine intestinal fluid in the presence of 0.02% PCP–Cys (closed circles) and control without PCP–Cys (open circles). Indicated values are means of at least three experiments ± standard deviation. *Differs from control ($p < 0.001$).

120 min. As shown in Fig. 2A and B, the coadministration of PCP–Cys to ODNs significantly improves the protective effect toward nucleases in comparison with free ODNs during the initial 2 h of degradation. PCP–Cys has an inhibitory effect toward aminopeptidase N by chelation of Zn^{2+} from the protein structure [6]. Like aminopeptidase N, DNase I is a divalent ion (Ca^{2+}/Mg^{2+})-dependent nuclease [20]. Possible mechanisms for the observed inhibition may include binding of the polymer to the enzyme via Ca^{2+}/Mg^{2+} within the active site or simple deprivation of these ions from the enzyme by chelation and subsequent denaturation. This is supported by other studies showing that thiolated polymers display enzyme inhibitory properties due to the complexation of divalent metal ions from metalloenzymes [6].

3.3. In vitro permeation studies

The permeation of ODNs across mucosal membranes is rather low [21]. The use of thiolated polymers (PCP–Cys) and the addition of GSH appear to be an alternative strategy for permeation enhancement [22]. Permeation studies were executed on Caco-2 monolayer cell cultures and freshly excised rat intestinal mucosa in order to evaluate the influence of PCP–Cys/GSH on the permeation of ODNs across these tissues. ODNs in buffer solution without thiomer were used as control. As shown in Fig. 3A and B, PCP–Cys in combination with GSH significantly ($p < 0.001$) increased the permeation of ODNs across Caco-2 monolayers and rat intestine compared to free ODNs. The apparent permeability coefficient on Caco-2 monolayer and rat intestinal mucosa was 8-fold and 10-fold higher than that of the control (Table 1). By using these polymers in combination with GSH, the permeation of ODNs across Caco-2 cell monolayers and small intestinal mucosa was enhanced significantly. Corresponding permeation studies with GSH but without a thiolated polymer showed only a slight enhancement in the permeation of hydrophilic compounds [22].

EDTA that was present within these experiments certainly influences the permeation as well. Nevertheless, the addition of PCP–Cys and GSH significantly increased the amount of permeated ODN compared to EDTA-containing controls Thus, it can be concluded that PCP–Cys/GSH adds a further permeation-enhancing effect to this system. The resultant strongly increased permeation

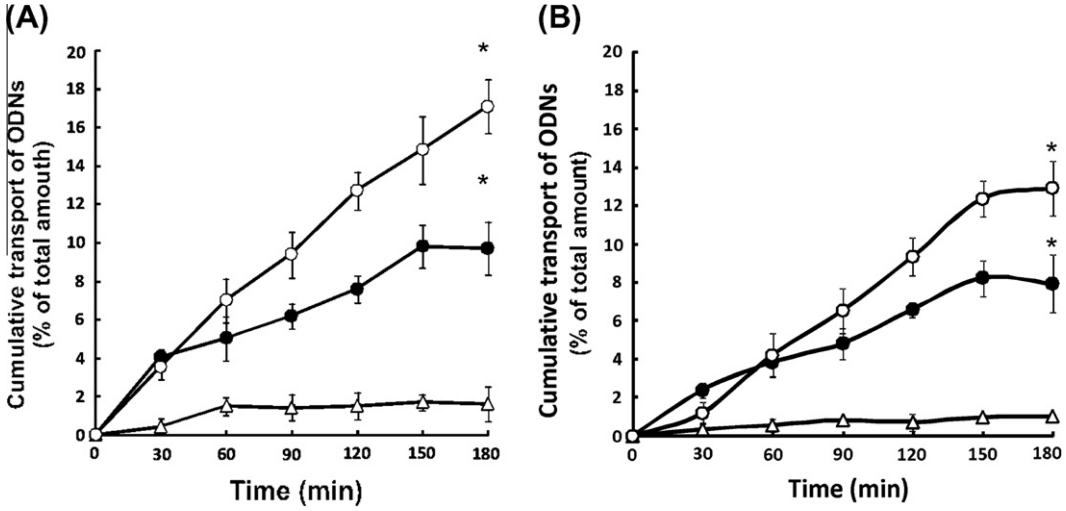

Fig. 3. (A) Cumulative transport of ODN across Caco-2 cell monolayers in the presence of 0.5% PCP–Cys/0.25% GSH (open circles), 0.5% PCP–Cys (closed circles), and without additives as control (triangles). (B) Cumulative transport of ODN across rat intestinal mucosa in the presence of 0.5% PCP–Cys/0.25% GSH (open circles), 0.5% PCP–Cys (closed circles), and without additives as control (triangles). Indicated values are means of at least three experiments ± standard deviation. *Differs from control ($p < 0.001$).

Table 1
Apparent permeability coefficients and transport enhancement ratio of ODNs without and with polymer on rat intestinal mucosa and Caco-2 cell monolayer (means ± SD, $n = 3–4$).

Sample	Apparent permeability coefficient [$P_{app} \times 10^{-6}$ (cm/s)]		Transport enhancements ratio [P_{app}(sample)/P_{app}(control)]	
	Intestinal rat mucosa	Caco-2 monolayer	Intestinal rat mucosa	Caco-2 monolayer
ODNs (control)	0.9 ± 0.4	1.6 ± 0.6	–	–
ODNs + PCP–Cys	6.9 ± 3.3[a]	8.7 ± 3.4[a]	7.7	5.4
ODNs + PCP–Cys/GSH	9.6 ± 6.7[a]	13.3 ± 7.3[a]	10.7	8.3

[a] Differs from control ($p < 0.001$).

of the drug in the presence of the PCP–Cys/GSH system can be explained by the interaction of the mediator GSH with thiol groups of the enzyme protein tyrosine phosphatase (PTP). PTP is able to dephosphorylate tyrosine residues of occludin, which is believed to play an essential role in the opening of the tight junctions. This dephosphorylation results in the closing of the tight junctions, leading consequently to a decreased permeation of hydrophilic macromolecules [23]. According to this theory, the inhibition of PTP must lead to an opening of tight junctions and further to an increase in permeability. The presence of the thiolated polymer is essential as it prevents the oxidation of GSH on the surface of the mucosa. This effect can be explained by the higher reactivity of cysteine residues (p*K*a 7.0) that have a lower p*K*a than glutathione (p*K*a 9.25). Hence, thiol groups of the polymer should be present as reactive thiolate anions to a much higher degree than those of glutathione [22].

In case of the PCP–Cys/GSH system, the permeation across the mucosa was accompanied by a relatively strong decrease in the transepithelial electrical resistance (TEER), indicating a loss of tightness of the intercellular junctions [22]. This in turn indicates the opening of the paracellular route across the epithelium for ODNs. After removing the PCP–Cys/GSH system, the TEER increased again and reached almost the same level as in the beginning of the study, indicating a reversible mechanism of tight junction opening (Fig. 4). These findings are in good agreement with previous studies focusing on the permeation-enhancing effect of PCP–Cys on ODN transport across biological membranes [24]. Certainly, this decrease in TEER cannot be attributed to PCP–Cys exclusively. However, it is quite obvious that PCP–Cys/GSH does not introduce a further irreversible effect. Moreover, it is quite unlikely that PCP–Cys is taken up from the intestine. Riley et al. demonstrated that polyacrylates of 140 kDa and higher molecular mass cannot be detected in the blood after oral administration [25]. Polycarbophil, which has been used within in this study, has a molecular mass of 3000 kDa. This enormous molecular weight and the fact that polycarbophil is approved as a bulk laxative for patients with irritable bowel syndrome suggest a minimum risk of systemic side effects as it appears highly unlikely that polycarbophil is absorbed from the gut. Native GSH is present on the apical side of the mucosa and is involved in detoxification processes [26]. Concerning local tissue damage, staining of rat intestinal mucosa with trypan blue after permeation studies with PCP–Cys/GSH did not show any dead cells (data not shown).

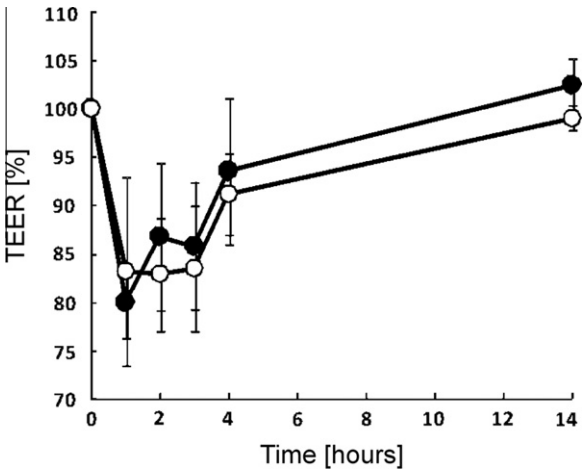

Fig. 4. TEER decrease and recovery after treatment of Caco-2 cell monolayers with 0.5% PCP–Cys/0.25% GSH (open circles) and 0.5% PCP–Cys (closed circles). Indicated values are means of at least three experiments ± standard deviation.

4. Conclusion

A PCP–Cys system has improved the stability of the ODNs toward degradation by nucleases that are abundant in the gastrointestinal track. As shown in permeation studies on Caco-2 monolayer and freshly collected rat intestinal mucosa, this PCP–Cys/GSH system has a strong permeation-enhancing effect for ODNs. PCP–Cys/GSH enhanced the intestinal permeability to ODNs by effectively decreasing the resistance along the paracellular pathway. Together with their low toxicity, the thiomer/GSH delivery system therefore represents a promising tool for oral administration of ODNs.

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