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Research paper

Nanosized insulin-complexes based on biodegradable amine-modified graft polyesters poly[vinyl-3-(diethylamino)-propylcarbamate-*co*-(vinyl acetate)-*co*-(vinyl alcohol)]-graft-poly-(L-lactic acid): Protection against enzymatic degradation, interaction with Caco-2 cell monolayers, peptide transport and cytotoxicity

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Abstract

Non-parenteral insulin delivery by the oral route is limited by epithelial barriers and enzymatic degradation. Nanosized insulin-complexes based on amine modified comb-like polyesters were designed to overcome these barriers. Protection of insulin in nanocomplexes (NC) from enzymatic degradation was investigated. The interaction with enterocyte-like Caco-2 cells in terms of cytotoxicity, transport through and uptake in the cell layers was evaluated by measuring transepithelial electrical resistance (TEER), release of lactate dehydrogenase (LDH) and insulin transport.

The protection capacity of NC and their interaction with Caco-2 cells varied strongly as a function of lactide-grafting (hydrophobicity). With increasing lactide-grafting (P(26) $\leqslant$ P(26)-1_{LL} $\leqslant$ P(26)-2_{LL}) NC protected up to 95% of the insulin against degradation by trypsin. Transport and uptake into cell monolayers increased with higher L-lactid grafting. About 25% of a 1.25 mg/ml TRITC-insulin NC dispersion with P(26)-2_{LL} was recovered in Caco-2 cells after 90 min. A concentration dependent cytotoxicity profile was observed showing elevated LDH release and decreased TEER values. The cytotoxicity correlates with the surfactant like character of the polymers, decreasing the surface tension to 46 mN/m for the amphiphilic P(26)-2_{LL}. The observed TEER decrease was reversible after 20 h, suggesting that the biodegradable comb-polyesters offer a promising approach to overcome mucosal barriers.

Keywords: Insulin; Nanocomplexes; Protein delivery; Enzymatic degradation; Caco-2 cells; Cytotoxicity

1. Introduction

Non-parenteral administration of peptides, such as insulin for the treatment of diabetes mellitus, remains a formidable challenge for drug delivery. To overcome inconvenient injection regimes in the intensified insulin therapy, several attempts have been made to exploit mucosal surfaces, especially the nasal, pulmonary and oral mucosa, for the delivery of macromolecules [1,2]. Of these routes, peroral administration would be the most desirable on a long-term basis. However, enzymatic degradation by proteolytic enzymes and low absorption rates limit the oral bioavailability of insulin in humans to <1% [3]. The potential for a successful implementation of novel meal-related insulin administration forms depends on improved time-action profiles, mimicking the physiological patterns of insulin secretion. This aspect may present a challenge for peroral insulin delivery because of the greater lag-time between administration and effects on blood glucose levels. Numerous strategies

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have been devised to enhance insulin absorption [4,5]. Most often these strategies have focused at addressing one of the barriers to intestinal absorption, either the epithelial cell layer or the digestive enzymes. These include the co-administration of insulin with penetration enhancers and/or protease inhibitors, enteric coatings, colloidal drug carriers, such as nanoparticles and liposomes and chemically modification of insulin by covalent attachment of amphiphilic oligomers to increase its lipophilicity [6]. Especially particles in the nanosize range, based on biodegradable and mucoadhesive polymers, are of particular interest, as they provide protection of sensitive proteins against enzymatic degradation and prolong intestinal residence time [7]. Their transport however, across the intestinal barrier is controversially discussed. Uptake can occur not only via micro-fold (M)-cells, highly specialized epithelial cells in the Peyer's patches, and isolated follicles of the gut associated lymphoid tissue (GALT), but also across the apical membrane of enterocytes [8]. Furthermore, non-covalent complexes formed between hydrophilic carrier molecules and insulin were thought to induce partial and reversible unfolding of the insulin molecule. These nanocomplexes of insulin and carrier molecules could possibly facilitate passive transcellular diffusion across cell membranes due to the increased flexibility and lipophilicity, before dissociation of the complex occurs beyond the epithelial barrier [9].

As a new approach to non-parenteral insulin delivery, biodegradable nanocomplexes consisting of insulin and poly[vinyl-3-(diethylamino)-propylcarbamate-*co*-(vinyl acetate)-*co*-(vinyl alcohol)]-graft-poly(L-lactic acid) polymers, abbreviated as DEAPA-PVA-g-PLLA, were investigated. Preparation of NC by spontaneous self-assembly of DEAPA-PVA-g-PLLA and insulin was previously reported [10]. Subsequent studies of insulin-NC in healthy and diabetic rats demonstrated significant insulin uptake after nasal administration [11].

Here, we investigate the possible enhancing effect of NC on insulin absorption in a biological model environment to optimize the performance of DEAPA-PVA-g-PLLA nanocomplexes in vivo. The effect of NC on cellular interaction and insulin transport was thus evaluated using the well-established human intestinal cell line Caco-2 as a model for epithelial cell layers. Viability and integrity of the cell monolayers, which affect the permeability of cell monolayers for peptide molecules and transport behaviour through the cell monolayer, was examined. Furthermore, we investigated the protection capabilities of the NC against enzymatic degradation using trypsin, which contributes substantially to luminal insulin degradation in the GI tract.

2. Materials and methods

2.1. Materials

Polymers: poly[vinyl-3-(diethylamino)-propylcarbamate-*co*-(vinyl acetate)-*co*-(vinyl alcohol)]-graft-poly(L-lactic acid) polymers were synthesized and characterized as previously described [12]. As abbreviation for the polymers we use: P(X)-Y_{LL}. P(26) carried 26 amino-groups per PVA molecule (DP = 300). P(26)-1_{LL}/P(26)-2_{LL} had additional 32/48 short lactide chains (L-lactide) per backbone with an average chain length of about two/three lactic acid units. Human recombinant insulin (26.2 IU/mg) was a gift from Aventis Pharma AG (Germany). Trypsin (TPCK treated, from bovine pancreas, 10.000 U/mg), lactate dehydrogenase assay and FITC-WGA were obtained from Sigma (Taufkirchen, Germany). Tissue culture reagents and fetal calf serum (FCS) were obtained from Gibco (Eggenstein, Germany), and tissue culture articles from Nunc (Wiesbaden, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany) in analytical quality.

2.2. Nanocomplexes preparation

The colloidal polymer–insulin complexes were prepared as previously described [10]. Stock solutions of insulin (2.0–2.50 mg/ml) and polymers (1.0–12.75 mg/ml) in a Tris-buffer made of 87% (v/v) of 1.15×10^{-2} N–HCl and 13% (v/v) of 0.1 *N*-Tris(hydroxylmethyl)-aminomethane solution, supplemented with 18 mM glucose, 49 μM $MgCl_2$, and 170 μM $CaCl_2$ at a pH of 7.70 (0.01 M), were prepared. Only for the enzyme experiments a different Tris-buffer (pH 8.0) was used (see Section 2.3). The nanocomplexes (NC) were obtained by spontaneous self-assembly after mixing to defined ratios (m/m) of insulin and polymer in the final NC dispersions with ratios of 1:0.5 for Ins/P(26), 1:1.6 for Ins/P(26)-1_{LL}, 1:1.7 for Ins/P(26)-2_{LL} [10].

2.3. Enzymatic degradation

A stock solution of trypsin (4.83 mg/ml) was prepared in Tris-buffer, pH 8.0, made of 84% (v/v) of 1.19×10^{-2} N–HCl and 16% (v/v) of 0.1 *N*-Tris(hydroxymethyl)aminomethane solution, supplemented with 1.0 mM $CaCl_2$. Insulin-nanocomplexes solutions were prepared in the same buffer with corresponding concentrations of 0.5 mg/ml for P(26), 1.60 mg/ml for P(26)-1_{LL}, 1.70 mg for P(26)-2_{LL} and throughout 1.0 mg/ml for insulin. The solutions were pre-incubated for 15 min and tempered to 37 °C in a metal block thermostat (Rotatherm®, Fa. Liebisch, Germany). Afterwards 9.0 ml per NC-dispersion, respectively, insulin control solution (1.0 mg/ml) was mixed with about 50 μl enzyme stock solution resulting in a molar enzyme/insulin ratio of 1:173, according to the method of Schilling and Mitra [13]. The mixtures were filled in borosilicate tubes (Pyrex®) and again incubated in a thermostat at 37 °C. At time intervals of 5, 10, 15, 20, 25, 35, 45, 55 and 65 min, 400 μl samples were withdrawn and transferred to an ice-cold vial containing 15.0 μl Tris-buffer with 1% trifluoroacetic acid (TFA) to stop enzymatic degradation and to dissolve the remaining complexes. The experiments were performed in triplicate. Samples were analyzed by reversed phase HPLC using a

LiChrosphere®100 RP18, 5 μm, 250-4-column (Merck, Germany) as described earlier [10].

2.4. Labelling of insulin with tetra-methyl-rhodamine isothiocyanate (TRITC)

Insulin was dissolved at a concentration of 4.50 mg/ml in a sodium carbonate/hydrogen carbonate buffer, pH 9.30, made of 15% (v/v) of 0.1 M $Na_2CO_3 \times 10\,H_2O$ and 85% (v/v) of 0.1 M $NaHCO_3$. A solution of TRITC (1.0 mg/ml) in dimethyl sulfoxide (DMSO) was quickly added (molar ratio of insulin/TRITC 1:2.9) and the mixture stirred for 18 h at 4 °C under light exclusion. The reaction was quenched with an excess of ammonium chloride and stirred for another 4 h. Separation was performed on a PD-10 column Sephadex G-25 (Amersham Bioscience, Germany) with PBS, pH 7.40. For the uptake experiments the insulin stock-solution used for NC preparation was a mixture of labelled and non-labelled insulin solution according to a 1:10 (v/v) ratio.

2.5. Cell culture

Mycoplasma-free Caco-2 cells were used at passage numbers 42–50 (HD, DKFZ, German Cancer Research Institute, Heidelberg, Germany) under conditions described earlier [14]. Cells were seeded at a density of 6×10^4 cells/cm^2 either on cell culture dishes or on uncoated polycarbonate Transwell™ filter inserts (Costar, Bodenheim, Germany, 0.40 μm pore size, area: 4.71 cm^2) and cultivated over 21 days. Cells were cultured at conditions of 10% CO_2, 95% r.H. and 37 °C. The culture medium (Dulbecco's modified Eagle's medium, DMEM) supplemented with 10% fetal calf serum, 1% nonessential amino acids, 1% L-glutamine and 4.5 g/l glucose was changed every second day.

2.6. Release of lactate dehydrogenase (LDH)

Caco-2 monolayers, grown on 12-well dishes (polystyrene) for 21 days, were incubated with NC-dispersions (1.0 ml) in Tris-buffer supplemented with 18 mM glucose, 49 μM $MgCl_2$, and 170 μM $CaCl_2$ at a pH of 7.70 (0.01 M). Insulin-nanocomplexes dispersions were prepared in the same buffer with corresponding concentrations of 0.625 mg/ml for P(26), 2.0 mg/ml for P(26)-1_{LL}, 2.125 mg respective 6.375 mg for P(26)-2_{LL} and throughout 1.25 mg/ml for insulin. For the concentration dependence investigations of LDH release for Ins/P(26)-2_{LL} – NC, a concentration series with 6.25, 3.125 and 1.5625 mg/ml was made by dilution with supplemented Tris-buffer. As positive control 0.1% Triton X-100 in supplemented Tris-buffer was used and pure supplemented Tris-buffer as negative control. After 60 and 120 min of exposure, 100 μl samples were withdrawn and processed according to the manufacturer's instructions (Testkit: DG 1340-K, Sigma Diagnostics). All LDH concentrations were normalized relative to the values of the Triton X-100 controls and expressed as relative LDH release. Each experiment was performed in triplicate.

2.7. Transepithelial electrical resistance (TEER)

The integrity of the monolayers was checked at 21 days post seeding by measuring TEER before and after the transport studies. For measurement of initial monolayer resistance (timepoint 0 h; approximately 400 Ω cm^{-2}) the culture medium was removed and the filter inserts (Costar® Transwells, 6 well) were rinsed with PBS supplemented with $MgCl_2$ 0.10 g/l, $CaCl_2$ 0.13 g/l and 15 mM glucose, pH 7.40, and allowed to equilibrate for 15 min. Measurements were performed at room temperature with a EVOM™ voltohmmeter (World Precision instruments, Berlin) equipped with Endohm™ electrodes. Afterwards the transport experiments followed as described in Section 2.8 over a period of 2.5 h. The control was an insulin solution with 1.25 mg/ml in supplemented Tris-buffer. Directly after the transport period the remaining apical NC-dispersions were removed, followed by a threefold washing step with supplemented PBS and the TEER values were determined (timepoint 2.5 h). The monolayers were then allowed to regenerate in culture medium at 37 °C to study TEER reversibility at the timepoints 4.5, 7, 19.5 and 26 h. Therefore, culture medium was replaced against supplemented PBS without further pre-incubation. After measurement the PBS was replaced against fresh culture medium for further incubation. Percentage calculations are done considering the initial resistance values per monolayer as 100%. Each experiment was run in triplicate.

2.8. Insulin transport studies in Caco-2 cell monolayers

Transport studies. Transport studies were performed 21 days post seeding. Filter inserts (Costar® Transwells, 6 well) were rinsed with PBS supplemented with $MgCl_2$ 0.10 g/l, $CaCl_2$ 0.13 g/l and 15 mM glucose, pH 7.40, and allowed to equilibrate for 15 min at 37 °C. Experiments were started by replacing the apical buffer with 1.50 ml of either the nanocomplex dispersions or the control solution (1.25 mg/ml insulin in supplemented Tris-buffer) and the basolateral buffer with 2.50 ml PBS. Before replacing the buffer with NC dispersions, monolayers were washed once with supplemented Tris-buffer. After 2.5 h a sample (1.50 ml) was drawn from the basolateral chamber and analyzed by reversed-phase HPLC as previously described [10]. Permeability in % was calculated from the concentration profiles using the following equation: (cumulative amount transported/initial amount) × 100.

Uptake studies. After incubation of polystyrene 12 wells with supplemented PBS buffer for 15 min at 37 °C and intermediate washing with supplemented Tris-buffer, the test solutions were applied (1.0 ml) and incubated for 90 min. After removal of the NC solutions and a threefold washing with PBS, the cells were dried up at ambient

conditions for 24 h under light exclusion. Each layer was afterwards dissolved in 300 μl of 2.0% Triton X-100 with 50 mM EDTA, pH 8.0, solution and the insulin-TRITC concentration measured with a Luminescence Spectrometer LS50B (Perkin-Elmer). Results were expressed as mean values ± SD of three experiments. Significance between mean values was calculated using ANOVA one way analysis (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, USA). Probability values $p < 0.05$ were considered significant.

2.9. Surface tension

The surface tension was measured according to the Noüy Ring method [15]. A ring made of platinum/iridium alloy with a diameter of 2.0 cm was immersed in the test solution with an upward force applied to it. Maximum force exerted on the ring by the surface is measured in mN/m using a spring dynamometer against purified water as reference. The concentrations of the polymer solutions were 1.0 mg/ml. All measurements were performed in triplicate at 20 °C.

3. Results and discussion

3.1. Preparation of nanocomplexes

Branched polyesters consisting of a poly (vinyl alcohol) (PVA) backbone grafted with side chains of poly (L-lactic acid) (PLLA) represent a novel class of biodegradable polymers showing significant potential for the development of a variety of drug delivery vehicles [16]. The modification of the PVA backbone by introduction of diethylamino-propylamine (DEAPA)-groups creates a polymer with positively charged functions and increases its hydrophilicity. Additional grafting leads to the amphiphilic DEAPA-PVA-g-PLLA polymer, characterized by a three-dimensional comb-like architecture with hydrophobic and hydrophilic moieties. By reduction of the average side chain length to PLLA units (⩽3) for a PVA backbone with 8.5% amine substitution, corresponding to 26 DEAPA-groups, water-soluble polymers were obtained [12]. We recently demonstrated the spontaneous formation of nanocomplexes by self-assembly from these polymers with insulin [10]. The resulting NC were in the size range of 200–400 nm, exhibiting a positive zetapotential between +15–35 mV and a high insulin association efficiency.

3.2. Protection from enzymatic degradation of insulin in NC

In addition to promoting its epithelial absorption, insulin must also be protected from digestive enzymes to provide the greatest amount of intact, biologically active insulin to be available for absorption. The utilized Caco-2 cells exhibit a high degree of differentiation resulting in a distinctive brush border enzyme pattern, like peptidases and hydrolases [17]. Nevertheless, the enzymatic degradation during epithelial transit is still moderate in comparison to the enzymatic environment during gastrointestinal passage. Especially α-chymotrypsin and trypsin, the major proteolytic enzymes secreted by the pancreas into the intestinal lumen, are responsible for the degradation of orally administered insulin [13]. Therefore, we utilized trypsin to investigate the protection of insulin by NC from enzymatic degradation. As demonstrated in Fig. 1, in the presence of trypsin, free insulin was found to be markedly degraded. Under the experimental conditions about 23.0 ± 2.8% of the free insulin control solution with 1.0 mg/ml is degraded within 60 min.

In contrast, the NC attenuated the degradation of insulin dependent on lactide-grafting of the polymers. The complexes prepared from P(26) polymer reduced the degraded amount to 16.9 ± 1.5%, P(26)-1_{LL} – NC to 12.0 ± 0.8% and the best protection was realized with P(26)-2_{LL} – NC, with a reduction to only 5.1 ± 0.8% degraded insulin of the initial 1.0 mg/ml.

In general, the insulin molecule consists of two chains, the A-chain with 21 amino acids and the B-chain with 30 amino acids, linked by two disulfide bonds. Trypsin itself cleaves insulin initially at only two sites, at the carboxyl side of residues B29-Lys and B22-Arg [18]. Since the bonds susceptible to tryptic cleavage are located at the carboxyl terminus of the B-chain, which is known as hydrophobic domain of insulin, it is obvious that the longer hydrophobic PLLA chains of P(26)-2_{LL} could better interact and shield this segment.

3.3. In vitro cytotoxicity studies

A colloidal drug carrier designated for routine application should display as little cytotoxicity as possible and polycationic molecules have often been linked to cytotoxic reactions [19,20]. Therefore, we investigated the in vitro cytotoxicity of NC using various techniques. Membrane damaging effects were evaluated by the release of the cytosolic enzyme lactate dehydrogenase (LDH). As shown in Fig. 2, left, only a negligible release of LDH was observed after 60 and 120 min for P(26)- and P(26)-1_{LL} – NC in comparison to the buffer control. However, P(26)-2_{LL} – NC showed a significant increase of LDH-release, reaching 16.6 ± 7.2% after 60 min and 31.1 ± 0.1% after 120 min. This effect was concentration dependent and decreased linearly with decreasing concentration (Fig. 2, right).

Since all DEAPA-polymers have the same degree of amine-substitution with about 26 amine groups per PVA, the cytotoxic effects of P(26)-2_{LL} – NC must be due to other properties. A large number of amphiphilic, surfactant-like molecules used for absorption enhancing have been shown to have cytotoxic effects [21]. We investigated the polymers in terms of surface activity with the Noüy Ring method (Fig. 3). Each of the polymer solutions, with a concentration of 1.0 mg/ml, exhibited a decrease of the surface tension from 55 mN/m for P(26) polymer to

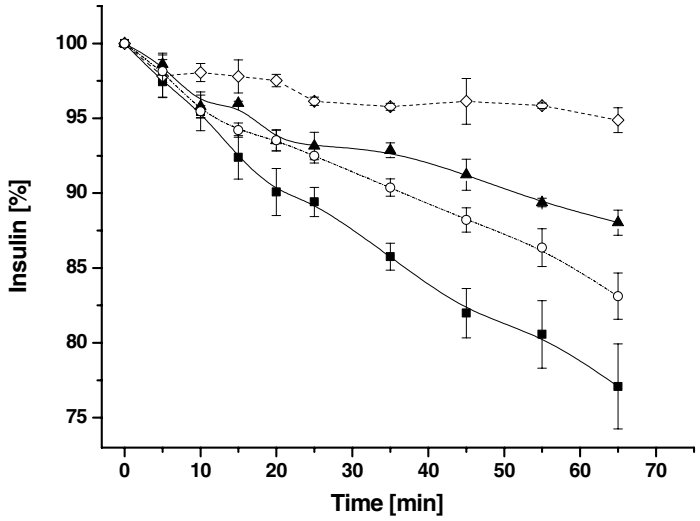

Fig. 1. Enzymatic degradation of insulin (1.0 mg/ml) by trypsin. The nanocomplexes of insulin and P(26)- (·-·○·-·), P(26)-1_{LL}- (—▲—), respective P(26)-2_{LL} – polymer (--◇--) exhibited a significant protection against enzymatic attack in comparison to the unprotected insulin control solution (—■—).

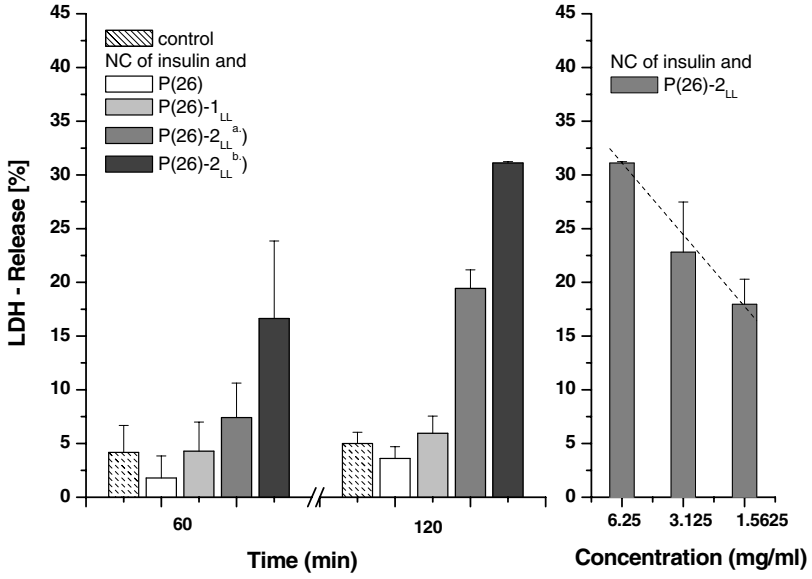

Fig. 2. Left plot: LDH release after 60 and 120 min of buffer control and NC composed of insulin and the respective polymers. NC's of P(26)-2_{LL} with (a) 2.125 mg/ml and (b) 6.25 mg/ml exhibited a concentration dependent increase of LDH release. Right plot: In the investigated dilution series with 100%, 50% and 25% of 6.25 mg/ml the dependence of LDH release is nearly linear to polymer concentration (---- linear fit with $R = 0.9982$).

46 mN/m for P(26)-2_{LL} polymer in comparison to the pure Tris-buffer control with 69 mN/m.

The basic structure of the polymers is a polyvinyl alcohol, whose properties are mainly governed by the degree of polymerisation and the degree of hydrolysis. For the polymer synthesis we utilized a PVA with a hydrolysis degree of 86–89%. Aqueous solutions of such partially hydrolysed PVA decrease the surface tension of water and are therefore utilized as protective colloids [22]. Even the very hydrophilic amine-modified P(26) backbone maintained these properties. The hydrophobic modification by grafting with lactide side chains resulted in a more amphiphilic, surfactant-like character of the polymer. A similar increase in surface activity, depending on the chain length of the introduced hydrophobic groups to partially hydrolysed PVA, was reported for PVA modifications with alkyl groups [23] or fatty acids [24].

The integrity of cell monolayers upon contact with the NC was assessed by measuring the transepithelial electrical resistance (TEER) of Caco-2 cells. NC from the ungrafted backbone P(26) caused a decrease to 66% of the initial resistance values and exhibited a moderate influence on TEER in comparison to the buffer control with still 90% of initial after 2.5 h. The transepithelial resistance was significantly decreased with an increasing degree of lactide-grafting, being highest for P(26)-2_{LL} – NC (Fig. 4). Similar to the release of LDH this effect was concentration dependent. The P(26)-2_{LL} – NC formulation with 2.125 mg/ml

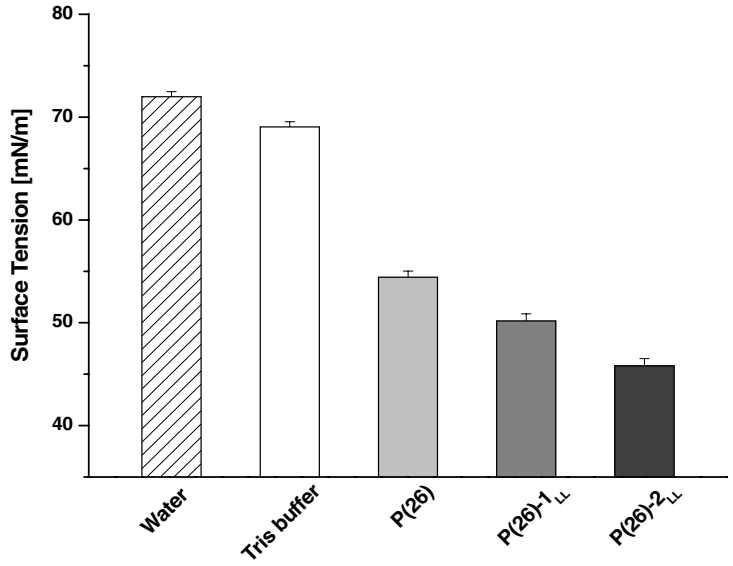

Fig. 3. Surface tension of polymer solutions with 1.0 mg/ml and buffer control.

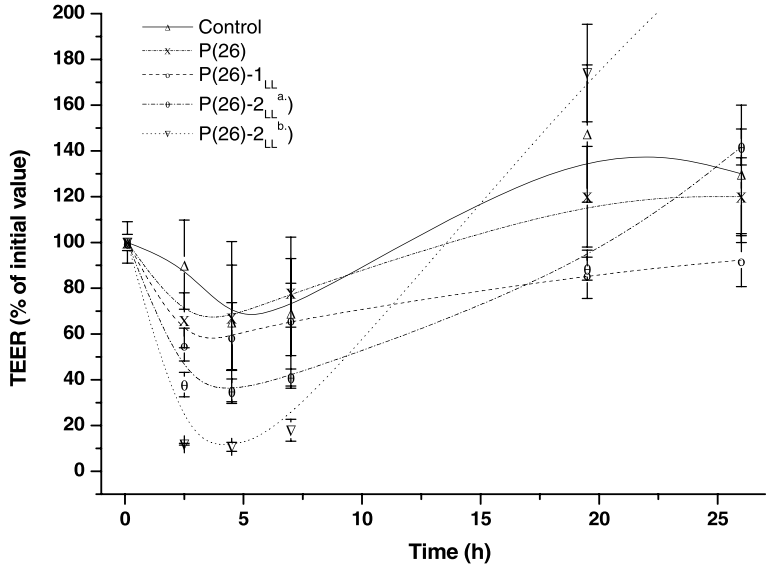

Fig. 4. Reversibility of TEER decrease over 26 h. Layer were incubated with nanocomplexes for 150 min followed by removal of NC, washing three times and further incubation with culture medium. Applied were a control solution (—△—) and NC composed of insulin and/P(26) polymer (·-·X·-·), /P(26)-1_{LL} polymer (—○—), /P(26)-2_{LL} polymer (a) with 2.125 mg/ml (—θ—·), (b) with 6.25 mg/ml (···▽···).

polymer concentration decreased the resistance down to 38%, while a formulation with a threefold higher concentration decreased it to about 12% of the initial resistance value. Again, this ranking is more likely related to the higher amphiphilic character of the grafted DEAPA-polymers with increasing number of lactic side chains, than to the cationic character caused by amine modification.

Both, the influence of positive charge and surface activity, were reported to decrease epithelial resistance: for instance, a comparable reduction in transepithelial resistance was found for NP made from chitosan [25], a biocompatible polymer with positive charges, and for solutions, respectively, in a concentration dependent manner [26]. In the case of chitosan its positive charges are thought to interact with epithelial cells, resulting in structural reorganisations of tight junction associated proteins [27]. The observed decrease in TEER upon contact with chitosan NP was reversible after removal of the carrier [25]. Furthermore, the effects of several absorption enhancing agents exhibiting surface activity on epithelial integrity were studied in Caco-2 cells [21,28]. All these surfactants induced a concentration dependent decrease of TEER, resulting from micro-disturbances and local damage to cells, whereby at intermediate concentrations the effects were found to be reversible. Likewise we observed for NC made of grafted polymers no irreversible disrupture

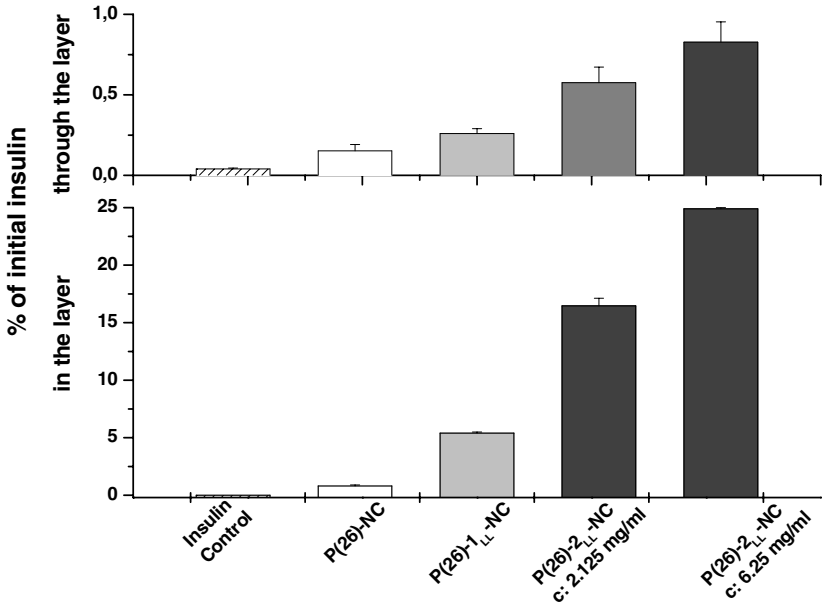

Fig. 5. Transport studies of TRITC labelled insulin. Upper plot: percentage of initial concentration of insulin found in the Costar Transwell® acceptor part. Lower plot: percentage of initial concentration of insulin internalized in Caco-2 cell monolayers after 2.5 h.

of cell structures, which is in accordance with the microscopic investigations of rat nasal epithelia after NC application, where no cell perturbation was observed [11]. In order to test the reversibility of NC modulation on TEER, the recovery of the initial monolayer electrical resistance was monitored over 26 h following washing with buffer and the incubation of cells with culture medium (Fig. 4). The time course of TEER observed for the controls and P(26) – NC were not significantly different and resulted from the influence of TRIS-buffer used. The increase of TEER for the control after 20 and 27 h is probably connected to the conditions during measurement. While initial TEER measurements were performed after pre-incubation with supplemented PBS buffer for 15 min, the following measurements were performed directly after the replacement of medium with buffer. In general, all NC prepared from grafted polymers exhibited a progressive reversibility of the epithelial resistance. The P(26)-2_{LL} – NC, having shown the most distinctive decrease after 150 min, displayed a progressive and complete recovery after 17 h. TEER-levels even increased to $234 \pm 32\%$ after 26 h. This final increase might be due to a reorganisation of junctional-complexes after they have been damaged by the exposure to NC.

3.4. Transport studies

After having demonstrated that insulin-NC interacted with cell membranes, we wanted to quantify the internalization in and the transport through the monolayers using transport studies (Fig. 5). Caco-2 monolayers were incubated with NC or pure insulin as a control. After 2.5 h, the amount of insulin permeating in the acceptor compartment was quantified using HPLC (Fig. 5, upper plot). The uptake into Caco-2 cell monolayers was determined after removal of the NC solutions and rigorous washing of the apical side followed by layer dissolution and TRITC fluorescence measurements of the cell lysis (Fig. 5, lower plot).

Although transport remained below 1%, it was demonstrated that all NC-formulations were able to significantly enhance the transport through as well as the uptake in Caco-2 cells compared to pure insulin. For internalization, we observed again a ranking dependent on amount of lactic grafting for NC, being highest for P(26)-2_{LL} – NC with about 25% of the applied insulin dose internalized after 2.5 h.

4. Conclusion

Colloidal carriers prepared from positively charged DEAPA polyesters revealed some interesting properties as drug delivery system for insulin. The physico-chemical properties of NC determined the interaction with Caco-2 monolayers and were mainly depending on the polymer composition. The most effective NCs were obtained by combination of a hydrophilic backbone and hydrophobic side chains. This combination led to a higher amphiphilic, surfactant-like character of the NC, which probably caused transient membrane perturbations and reversible opening of tight junctions, as indicated by the release of LDH and a decrease in TEER values. These effects seemed to be reversible and NC with a higher lactide-grafting showed the best protection against enzymatic degradation, the highest internalization and transport through Caco-2 monolayers. NCs are attractive carriers for insulin therapy since they seem to facilitate uptake through mucosal surfaces.

Further optimization of the polymer structure DEAPA-PVA-g-PLLA is under way to increase colloidal stability of NC and to reduce surfactant properties.

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