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Research paper

Influence of PEGylation with linear and branched PEG chains on the adsorption of glucagon to hydrophobic surfaces

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ABSTRACT

PEGylation has proven useful for prolonging the plasma half lives of proteins, and since approval of the first PEGylated protein drug product by the FDA in 1990, several PEGylated protein drug products have been marketed. However, the influence of PEGylation on the behavior of proteins at interfaces is only poorly understood. The aim of this work was to study the effect of PEGylation on the adsorption of glucagon from aqueous solution to a hydrophobic surface and to compare the effects of PEGylation with a linear and a branched PEG chain, respectively. The 3483 Da peptide glucagon was PEGylated with a 2.2 kDa linear and a branched PEG chain, respectively, and the adsorption behaviors of the three proteins were compared using isothermal titration calorimetry, fixed-angle optical reflectometry and total internal reflection fluorescence. PEGylation decreased the number of glucagon molecules adsorbing per unit surface area and increased the initial adsorption rate of glucagon. Furthermore, the results indicated that the orientation and/or structural changes of glucagon upon adsorption were affected by the PEGylation. Finally, from the isothermal titration calorimetry and the reflectometry data, it was observed that the architecture of the PEG chains had an influence on the observed heat flow upon adsorption as well as on the initial rate of adsorption, respectively.

1. Introduction

PEGylation, which is the covalent attachment of polyethylene glycol (PEG) to biomolecules, has proven a useful approach for prolonging the plasma half lives of protein drugs [1,2]. The increased hydrodynamic radii of PEGylated proteins generally decrease the glomerular filtration [3], while the masking of the proteins by PEG chains decreases the proteins' immunogenicity [4] and subsequently reduces the proteolytic degradation of PEGylated proteins. Furthermore, PEGylation has been shown to increase the solubility and the physical and chemical stability of proteins [5,6].

The first PEGylated protein drug product was approved by FDA in 1990, and since then several PEGylated protein drug products have been marketed [7]. The early PEGylated protein drugs were PEGylated with linear PEG chains, while the next generation of PEGylated protein drugs was PEGylated with branched PEG chains with a central protein conjugation functional group surrounded by two or more identical methoxy-PEG chains [8,9].

Like other protein drugs, PEGylated protein drugs are exposed to a diversity of solid–liquid interfaces during purification, production, storage and use. PEGylation will affect the proteins' characteristics and is therefore likely to affect the adsorption behavior of proteins. The PEG chain is amphipilic with ethylene moieties conferring hydrophobicity to the PEG chain and oxygen that allows strong interactions with water. Studies have shown that PEG in aqueous solution tends to be excluded from the protein surface [10,11], but the conformation of PEG chains attached to proteins is poorly understood. Small angle X-ray scattering studies on the solution structures of PEGylated hemoglobin and antibody fragments indicate that the tertiary structure of proteins remain unchanged upon PEGylation and indicate that a part of the PEG chains protrudes away from the protein, whereas another part interacts with the protein [12,13]. The increase in the hydrodynamic radius of a protein upon PEGylation is likely to reduce the number of protein molecules adsorbing per unit surface area due to increased steric hindrance at the surface. Steric hindrance due to the presence of the PEG chain may also affect the orientation of the protein upon adsorption.

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Even though PEGylated protein drug products have been on the market since 1990, only few studies have been published on the effect of PEGylation on the adsorption behavior of proteins [14–16], and the influence of PEGylation on the adsorption behavior of proteins at solid–liquid interfaces is only poorly understood. Since the adsorption of proteins from solution to solid surfaces is likely to affect the stability of proteins in solution, a thorough knowledge about the effect of PEGylation on protein adsorption would be of great value in the future development of PEGylated protein drug products, with quality and safety equal to the un-modified protein drug products. To the best of our knowledge, the influence of PEGylation on the adsorption of a small rod-shaped protein from solution to a solid hydrophobic surface has not been studied. Furthermore, we are not familiar with studies comparing the influences on protein adsorption from PEGylation with linear and branched PEG chains of the same nominal weights, respectively.

The aim of this work was to study the influence of PEG chains with different architectures on the adsorption of glucagon to hydrophobic surfaces. The 3483 Da peptide glucagon that is used for treatment of hypoglycemia was chosen as a model protein and was PEGylated at the amino acid residue Lys12 with a linear or a branched PEG chain of 2.2 kDa. After purification and characterization of the PEGylated glucagon conjugates, adsorption isotherms were established for glucagon and glucagon PEGylated with linear and branched PEG chains, respectively. Finally, the adsorption behaviors of the three proteins were compared with isothermal titration calorimetry, fixed-angle optical reflectometry and total internal reflection fluorescence.

2. Materials and methods

2.1. Materials

Freeze dried glucagon (glucagon) was kindly donated by Novo Nordisk A/S, Denmark and used as received. Methoxy-poly(ethylene glycol)-succinimidyl propionate reagent with a nominal molecular weight of 2000 Da (PEG-L) was purchased from Nektar Therapeutics (Huntsville, AL, USA). (Methyl-PEG_{12})$_3$-PEG_4-NHS ester with a nominal molecular weight of 2400 Da (PEG-B) was obtained from Thermo Fisher Scientific (Rockford, IL, USA). The actual molecular weights for both PEG-L and PEG-B were estimated by MALDI-TOF to be 2.2 kDa. Sketches of the two PEG reagents are shown in Fig. 1. *N*-Hydroxysuccinimide is released upon PEGylation. All water used was Milli-Q grade, and all other chemicals were of analytical grade and were obtained from commercial sources.

2.2. PEGylation of glucagon

Glucagon was PEGylated with a 2.2 kDa linear PEG chain (PEG-L) and a branched PEG chain (PEG-B), respectively. These relatively small PEG chains were chosen because they are smaller than glucagon, which has a molar mass of 3483 Da. In comparison with larger PEG chains, smaller PEG chains are less likely to interfere with the activity of glucagon. Here, we only use glucagon as a model protein. However, seen from a pharmaceutical perspective, the rationale for PEGylation of glucagon would be to increase its stability, rather than to increase its plasma half-life.

The PEGylation procedure described by Stigsnaes et al. [17] was used for PEGylation of glucagon. 20 mg of glucagon was dissolved in 1 mL 0.1 M NaOH, and subsequently, 4 mL N,N-dimethylformamide (DMF) was added. Then 28.6 mg of PEG-L was dissolved in 2 mL 10 mM H_3BO_3 pH 9.5 and immediately added to the glucagon solution under gentle rotation. To increase the reaction yield, another portion of freshly dissolved PEG-L was added after 10 min and allowed to react at room temperature for 30 min. The same method was used for PEGylation of glucagon with PEG-B, except for the dissolution of PEG-B. Because of its waxy texture, PEG-B was difficult to weigh out. Therefore, a stock solution of PEG-B was prepared by dissolving 100 mg PEG-B in 230 μL DMF directly in the vial in which it was received. 115 μL PEG-B stock was added to 2 mL 10 mM H_3BO_3 pH 9.5 and this solution was immediately transferred to the glucagon solution.

Glucagon PEGylated with PEG-L (Gluc-PEG-L) and PEG-B (Gluc-PEG-B), respectively, was purified by RP-HPLC using a Daiso C-18 column from FeF Chemicals A/S, Denmark, an eluent consisting of 61% 0.1% TFA in Milli-Q water and 39% 0.1% in acetonitril, and a flow rate of 2 mL/min. To the column, 1.8 mL reaction solution was applied per run, and UV at 280 nm was used to detect Gluc-PEG-L and Gluc-PEG-B. After collection, the fractions were stored at 4 °C for up to 60 h before they were freeze dried.

The freeze drying was carried out on a Christ Epsilon 2-4 LSC freeze-dryer (Martin Christ Gefrier Trocknungsanlagen GmbH, Germany). Initially, the shelf temperature was lowered to −55 °C over 1.5 h and kept there for 2.5 h to freeze the solutions. The primary drying was initiated by lowering the vacuum to 1.030 mbar and lasted 72 h. After this, the temperature was increased to −10 °C over 1 h, and this temperature was held for 4 h. A final drying was performed for 10 h at 0.0010 mbar and with a shelf temperature of 20 °C

2.3. Characterization of PEGylated glucagon

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was acquired on an Autoflex III

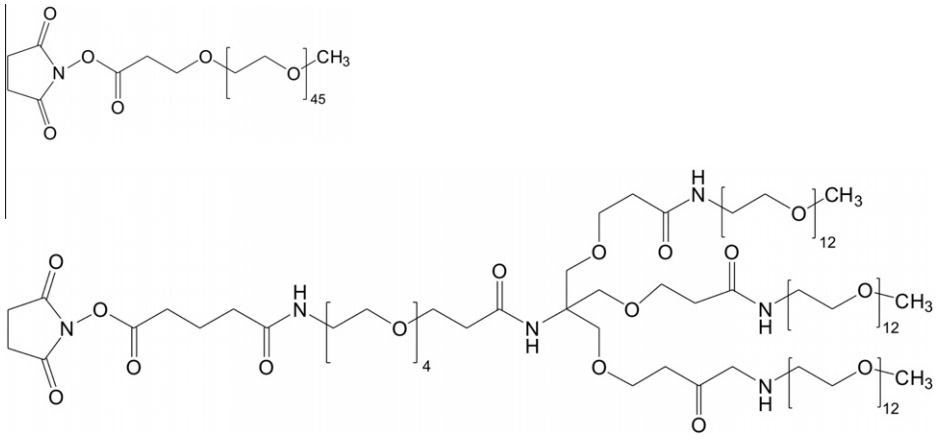

Fig. 1. Sketches of methoxy-poly(ethylene glycol)-succinimidyl propionate (top) and (Methyl-PEG_{12})$_3$-PEG_4-*N*-Hydroxysuccinimide ester (bottom). *N*-Hydroxysuccinimide is released upon PEGylation.

(Bruker) in linear mode to determine the molar masses of gluc-PEG-L and gluc-PEG-B. The proteins were dissolved in a mixture of 1% TFA and 50% acetonitrile in water and transferred to a stainless steel target plate, using α-cyano-4-hydroxycinnamic acid as matrix, and dried before measurement. The laser energy was adjusted for each sample.

The PEGylation sites were determined by Edman degradation from the N-terminus using a Procise 494 (Applied Biosystems, Foster city, CA, USA) protein sequencer. A PEGylated amino acid will elute outside the chromatographic window and produce a "blank" cycle.

2.4. Sample preparation

Glucagon, gluc-PEG-L and gluc-PEG-B were dissolved in 10 mM H_3BO_3 pH 9.5 and filtered through a 0.22 μm filter. The concentrations were determined using $\varepsilon_{280} = 8250\ M^{-1}cm^{-1}$. The 10 mM H_3BO_3 pH 9.5 buffer used for the PEGylation of glucagon was chosen for the CD and adsorption studies, because of increased solubility of glucagon, which is below 0.5 mg/ml in the pH-range 6–8 [18]. Glucagon solutions were used within 4 h, whereas gluc-PEG-L and gluc-PEG-B solutions were used within 10 h.

2.5. Surfaces

Three types of hydrophobic surfaces were used for the adsorption studies.

2.5.1. Polystyrene beads

Polystyrene beads with sulphate functional groups on the surface suspended in distilled de-ionized water were obtained from Interfacial Dynamics Corporation, USA. The beads had a diameter of 95 ± 5 nm. The surface of the polystyrene beads is overall hydrophobic with some distinctly distributed negatively charged spots due to the sulphate groups.

2.5.2. Silicon wafers coated with polystyrene

Silicon wafer with a 2–3 mm thick natural SiO_2 layer was cut into strips (4 cm × 1 cm) and subsequently rinsed with ethanol and water. The strips were dried and covered with a solution of 11 g/L vinyl-PS20/vinyl-PS200 86:14 in chloroform (M_W = 2100 Da, M_N = 1900 Da, M_W/M_N = 1.11 w/w% and M_W = 19,600 Da, M_N = 19,100 Da, M_W/M_N = 1.03 w/w%, Polymer Source Inc., Canada). After evaporation of the chloroform, the strips were heated for 72 h at 150 °C under vacuum, and then excess vinyl-PS20/vinyl-PS200 was washed off with chloroform. The vinyl-PS20 and vinyl-PS200 become covalently bound to the Si/SiO_2 surface [19], and a thicker polystyrene layer was obtained by spin-coating the surfaces with a solution of 13 g/L polystyrene (M_W = 876 kDa, Mw/M_N = 1.19 w/w%; Polymer Source Inc., Canada) in toluene at 2000 rpm for 30 s. The thickness of the polystyrene layer was determined to be approximately 100 nm with ellipsometry (SE 400, SENTECH Instruments GmbH, Germany). The surfaces were stored protected from dust and were used within a week after spin-coating.

2.5.3. Quartz wafers coated with Silane

Quartz slides from TIRF Technologies (US) were cleaned as previously described [20] and subsequently rinsed three times in water and ethanol. Clean quartz surfaces were silanized with (3,3,3-trifluoropropyl) chloromehtylsilane, using vapor deposition using vapor deposition as previously described [21], except that argon gas was used instead of nitrogen gas to fill the desiccator. The surfaces had a contact angle of 92 ± 1° [22].

2.6. Circular dichroism

Far-UV CD spectra were obtained on a Jasco-J-810 circular dichroism spectrophotometer (Jasco, Tokyo, Japan) calibrated with d-10-camphorsulfonic acid. Then 150 μM solutions of glucagon, gluc-PEG-L and gluc-PEG-B, respectively, were scanned in a 0.5 mm cell from 260 to 180 nm using a band width of 1 nm, a response time of 4 s, a data pitch of 0.2 nm and a scanning speed of 50 nm/min. Spectra of 10 mM H_3BO_3 pH 9.5 were recorded and subtracted from each protein spectrum, after which the data were converted to molar CD. Each spectrum was obtained as an accumulation of 10 scans, and all three spectra were obtained on the same day.

2.7. Adsorption isotherms

Adsorption isotherms for glucagon, gluc-PEG-L and gluc-PEG-B on sulphate-stabilized polystyrene beads in 10 mM H_3BO_3 pH 9.5 were established. Protein solutions of varying concentration were added to a suspension of beads, yielding a total surface area of 0.1 m^2. The samples were left to adsorb for 15 min, after which they were centrifuged at 200,000*g* at 15 °C for 30 min in a Centrikon T-1055 ultracentrifuge (Kontron Instruments, Milan, Italy) using a TFT 65.13 rotor (Kontron Instruments). The protein concentration in the supernatant was determined by UV spectroscopy, using $\varepsilon_{280} = 8250\ M^{-1}\ cm^{-1}$. Because of the low physical stability of glucagon in solution, a relative short adsorption time of 15 min was chosen, and for the same reason, desorption isotherms were not established. The adsorbed amounts were calculated by mass balance from the differences between the initial and the equilibrium concentrations in solution.

2.8. Isothermal titration calorimetry

Measurements were performed with a VP-ITC Micro Calorimeter from MicroCal LLC (Northhampton, MA). The reference cell was filled with 10 mM H_3BO_3 pH 9.5. In a typical experiment, the sample cell was filled with a suspension of polystyrene beads, yielding a surface area of 742 cm^2, and aliquots of 6 μL 200 μM glucagon, gluc-PEG-L or gluc-PEG-B were titrated into the cell. All experiments were initiated with a pre-titration of 2 μL titrant. The titration of glucagon into the bead suspension was replicated three times, whereas the experiments for gluc-PEG-L and gluc-PEG-B were performed once or twice due to the limited amounts of protein available and the high reproducibility observed for the glucagon experiment. The titrations of glucagon, gluc-PEG-L and gluc-PEG-B were performed at 5 °C, 15 °C and 25 °C, and the heat capacity change ΔC_p was calculated as $d\Delta H/dT$. Blank titrations of glucagon, gluc-PEG-L, gluc-PEG-B, PEG-L and PEG-B into 10 mM H_3BO_3 pH 9.5 were performed as were titrations of PEG-L and PEG-B into suspensions of polystyrene beads. Finally, glucagon was titrated into a suspension of polystyrene beads to which PEG-L and PEG-B, respectively, had been pre-adsorbed, and PEG-L and PEG-B were titrated into beads to which glucagon had been pre-adsorbed. These experiments were performed at 25 °C.

2.9. Reflectometry

The adsorption rates of glucagon, gluc-PEG-L and gluc-PEG-B were compared with fixed-angle optical reflectometry, which is described in detail elsewhere [23]. In short, a linearly polarized He–Ne light beam (λ = 632.8 nm) is reflected on the adsorbing surface, and the change in polarization as a result of adsorption is measured by simultaneous detection of the parallel (R_P) and the perpendicular (R_S) reflectance. R_P is divided by R_S to give the signal *S*. Changes in S can be related to the adsorbed mass per surface area Γ by Eq. (1):

$$\Gamma = Q \times (\Delta S/S_0), \quad (1)$$

where Q is the sensitivity factor, S_0 the baseline signal, and $\Delta S = S - S_0$. Before each measurement, 10 mM H_3BO_3 pH 9.5 was introduced into the cell until a stable baseline signal (S_0) was obtained. By switching of a two-way valve, adsorption was started by introducing 0.57 μM glucagon, gluc-PEG-L or gluc-PEG-B in 10 mM H_3BO_3 pH 9.5 into the cell, gradually replacing the 10 mM H_3BO_3 pH 9.5. Gravity was used to induce a flow rate of 82 μL/s, and the change in signal was measured with a sampling time of 2 s. The inner dimensions of the flow cell were 30 × 9.0 × 25 mm^3, while the radius of the inlet tube was 0.50 mm, and the distance between the outlet of the tube and the front of the polystyrene coated silicon wafer was 0.85 mm.

Q, which depends on the laser light's angle of incidence (θ), the refractive index (n), the thickness (d) of the layers on the silicon wafer and the refractive index increment (dn/dc) of the adsorbate, was calculated with "Prof. Huygens" 1.2b (Dullware Software) using the following values: $\theta = 70°$, $n_{silica} = 1.46$, $n_{polystyrene} = 1.59$, $n_{silicon} = 3.85$, $n_{H2O} = 1.33$, $d_{silica} = 2.0$ nm, $d_{PS} = 95$ nm and $d_{adsorbed}$ layer = 25 nm. The refractive index increment of glucagon, $dn/dc_{glucagon}$, was determined to 0.194 ml/g with a differential refractive index detector (Shodex RI-71, Separations) calibrated with NaCl, whereas for gluc-PEG-L and gluc-PEG-B, it was calculated from $dn/dc_{glucagon} = 0.194$ ml/g and $dn/dc_{PEG} = 0.134$ ml/g [24] using a mass average for glucagon, and PEG. $dn/dc_{gluc\text{-}PEG\text{-}L}$ and $dn/dc_{gluc\text{-}PEG\text{-}B}$ were calculated to 0.162 ml/g (due to limited amounts of gluc-PEG-L and gluc-PEG-B, the dn/dc was not determined experimentally). The corresponding Q factors were 46 mg/m^2 for glucagon and 52 mg/m^2 for gluc-PEG-L and gluc-PEG-B.

2.10. Total internal reflection fluorescence

The adsorption of glucagon, gluc-PEG-L and gluc-PEG-B, as well as their fluorescence emission maxima, were compared with total internal reflection fluorescence. The principle of TIRF has been described elsewhere [25–27]. The experiments were performed in a TIRF flow cell from TIRF technologies (US) mounted in a Spex Fluorolog 3-22 (Jobin Yvon Horiba, France). The inner dimensions of the flow cell were 16 mm × 24 mm × 0.1 mm, yielding a flow cell volume of 38 μL. The experiments consisted of five steps. First, 10 mM H_3BO_3 pH 9.5 was pumped through the flow cell until a steady baseline signal was obtained. Then, the flow was stopped, and an emission scan was obtained of 10 mM H_3BO_3 pH 9.5 in the TIRF cell. Thereafter, a 29 μM sample solution was pumped into the flow cell with a flow rate of 4.17 μL/s and allowed to adsorb for 10 min. In order to remove protein from the bulk, as well as loosely adsorbed protein molecules, the cell was rinsed with 10 mM H_3BO_3 pH 9.5. Finally, an emission scan was obtained of the adsorbed protein. During baseline collection and rinsing, the flow rate was 16.67 μL/s. The fluorescence emission scans of 10 mM H_3BO_3 pH 9.5 were subtracted from the fluorescence emission scans of the three proteins, and the fluorescence emission maxima of the three adsorbed proteins were determined. The excitation and emission wavelengths were 295 nm and 350 nm, respectively. The excitation and emission slit widths were 5 nm, and the integration time was 0.1 s.

2.11. Fluorescence

Fluorescence emission scans of glucagon, gluc-PEG-L and gluc-PEG-B in 10 mM H_3BO_3 pH 9.5 and of 10 mM H_3BO_3 pH 9.5 were obtained on a Spex-Fluorolog 3.22 fluorescence spectrometer (Jobin Yvon Horiba, France). The settings were the same as for the TIRF experiments. The fluorescence emission scans of 10 mM H_3BO_3 pH 9.5 were subtracted from the fluorescence emission scans of the three proteins, and the fluorescence emission maxima of the three proteins in solution were determined.

3. Results

3.1. Characterization of PEGylated glucagon

Edman degradation showed that glucagon had been successfully mono-PEGylated at Lys12 with linear and branched PEG chains, respectively (data not shown). Mono-PEGylation was further supported by MALDI-TOF spectrometry analysis that revealed molar masses of 5500–5900 Da and 5700–5800 Da for gluc-PEG-L and gluc-PEG-B, respectively. With a molar mass of 3483 Da [28] for glucagon, the molar masses of the attached PEG-L and PEG-B chains constituted approximately 2000–2400 and 2200–2300 Da, respectively. The broader molar mass range for gluc-PEG-L compared to that for gluc-PEG-B was in accordance with the MALDI-TOF spectrometry analysis of PEG-L and PEG-B, which showed PEG-L to be more polydisperse than PEG-B (data not shown).

The secondary structures of glucagon, gluc-PEG-L and gluc-PEG-B were compared with far-UV CD (Fig. 2). All three proteins showed spectra with a minimum around 201 nm and a negative shoulder from approximately 215 to 245 nm, which may be due to a mixture of α-helix and random coil or due to 3_{10}-helix structure. The far-UV CD spectra for the three proteins are similar to spectra previously obtained for glucagon at alkaline pH, which have been reported to be mixtures of random coil and α-helix [29,30], but also similar to the far-UV CD spectrum found for a 3_{10}-helix structure [31]. With CD, it is not possible to distinguish a mixture of α-helix and random coil from a 3_{10}-helix, as α-helix has negative maxima at 208 nm and 222 nm, random coil structure has a positive maximum around 222 nm, and 3_{10} helix has a strong negative maximum at 208 nm and a weak transition at 222 nm.

No differences were observed in the far-UV CD spectra for gluc-PEG-L and gluc-PEG-B. However, the negative dichroism around 201 nm and from 201 to 235 nm was smaller than for glucagon. Furthermore, as a result of PEGylation, the crossover point at the x-axis and the positive band at 195 nm were blue-shifted, and the positive dichroism at 190 nm was weaker.

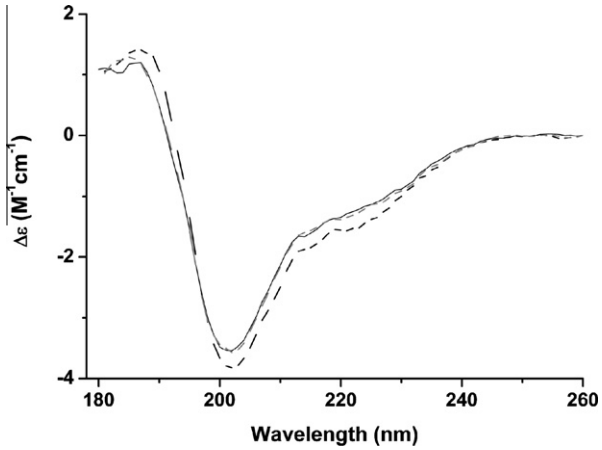

Fig. 2. Far-UV CD spectra of glucagon (black dashed line), glucagon PEGylated with a linear PEG chain (dark gray line) and glucagon PEGylated with a branched PEG chain (light gray dashed line). The Far-UV spectra were obtained on 150 μM solutions in 10 mM H_3BO_3 pH 9.5 in a 0.5 mm cell.

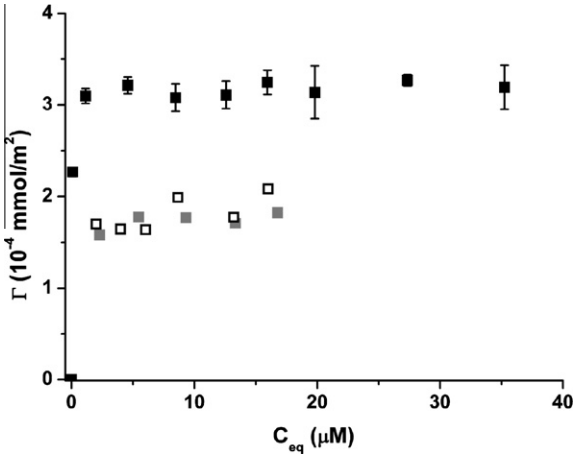

Fig. 3. Adsorption isotherms for adsorption of glucagon (■), glucagon PEGylated with a linear PEG chain (□) and glucagon PEGylated with a branched PEG chain (▩) on polystyrene beads in 10 mM H_3BO_3 pH 9.5.

3.2. Adsorption isotherms

Adsorption isotherms were established for glucagon, gluc-PEG-L and gluc-PEG-B (Fig. 3). All three proteins adsorbed with high affinity to the hydrophobic polystyrene beads, and they all reached adsorption plateaus at equilibrium concentrations between 5 and 10 μM. The adsorption plateau corresponded to $3.2 \pm 0.3 \times 10^{-4}$ mmol/m^2, $1.6–2.0 \times 10^{-4}$ mmol/m^2 and $1.7–1.8 \times 10^{-4}$ mmol/m^2 for glucagon, gluc-PEG-L and gluc-PEG-B, respectively. Although the adsorption plateau range was wider for gluc-PEG-L compared to gluc-PEG-B, their average adsorption plateau values were similar and approximately 1.8×10^{-4} mmol/m^2.

3.3. ITC adsorption studies

ITC was used to compare the adsorption of glucagon, gluc-PEG-L and gluc-PEG-B. Representative raw heat flow data for titration of glucagon into the polystyrene suspension in 10 mM H_3BO_3 pH 9.5 are shown in Fig. 4 (top). Titration of glucagon into the suspension of polystyrene beads was accompanied by exothermic heat flows, which, after a few injections, decreased gradually for each injection until a small endothermic heat flow was observed upon injection. Blank titrations of glucagon into 10 mM H_3BO_3 pH 9.5 revealed a small endothermic heat flow for each injection (data not shown). The area of the heat flow peaks was integrated to obtain the enthalpy change per mole of glucagon injected into the suspension of polystyrene beads (Fig. 4, bottom). The enthalpogram was s-shaped and could be fitted to a single process (fit is not shown), which implies that the enthalpy change is dominated by a single type of adsorption process.

The enthalpograms obtained for gluc-PEG-L and gluc-PEG-B differed from the enthalpogram obtained for glucagon, and differences were also observed between the enthalpograms of gluc-PEG-L and gluc-PEG-B (Fig. 5A). For glucagon, the initial heat flow was approximately 125 kJ per mole of glucagon injected. Upon titration of gluc-PEG-L into the suspension of polystyrene beads, a heat flow of 310 kJ per mole of gluc-PEG-L injected was observed, which gradually decreased before increasing slightly and leveling off to reach a plateau of approximately 90 kJ per mole injected gluc-PEG-L. After this, the heat flow leveled off until only the measured heat flow equaled that of the heat of dilution. An initial heat flow of approximately 200 kJ per mole of gluc-PEG-B injected was observed. The exothermic heat flow decreased and reached a plateau before it decreased again and leveled off. Eleven aliquots of 6 μL 200 μM gluc-PEG-L or gluc-PEG-B had to be titrated into the polystyrene bead suspension before the heat of dilution only was measured upon titration. In comparison, 21 aliquots of 6 μL 200 μM glucagon had to be added to the sample cell before saturation was reached. The error bars in the enthalpogram for glucagon into polystyrene beads are shown to give an indication of the overall reproducibility of the setup. Since gluc-PEG-L and gluc-PEG-B were freeze dried before use, whereas glucagon was used as it was received, the enthalpogram of glucagon used as received was compared with one of glucagon that had been freeze dried. No differences in the adsorption thermograms were observed (data not shown). The enthalpogram trends observed for gluc-PEG-L and gluc-PEG-B at 25 °C were replicated once, and the titration curve trends were also observed at 5 °C and 15 °C (data not shown). The heat capacity changes for the three proteins were negative, ranging from −21 to −39 kJ K^{-1} mol^{-1}. Such considerable reductions in heat capacity suggest that hydrophobic interactions are involved in the adsorption process [32].

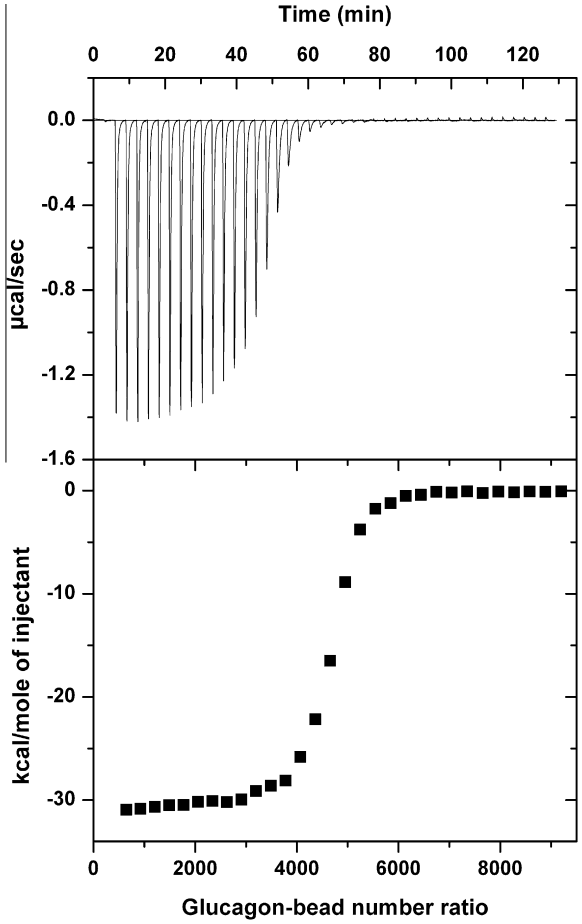

Fig. 4. A typical isothermal titration calorimetry experiment, with 6 μL aliquots of 200 μM glucagon titrated into a suspension of hydrophobic polystyrene beads in 10 mM H_3BO_3 pH 9.5 at 25 °C. The first injection was only 2 μL. Top: raw heat signal of the injections. Bottom: the enthalpogram obtained when each peak has been integrated to obtain the enthalpy change per mole of glucagon injected.

Titration of both free PEG-L and PEG-B into the polystyrene bead suspension was accompanied by exothermic heat flows, which decreased gradually after a few injections until a small endothermic heat flow 55–65 kJ per mole of PEG injected were observed for the two PEG chains.

3.4. Reflectometry

Reflectometry was used to compare the initial adsorption rate of 0.57 μM glucagon, gluc-PEG-L and gluc-PEG-B (Fig. 6A). Glucagon

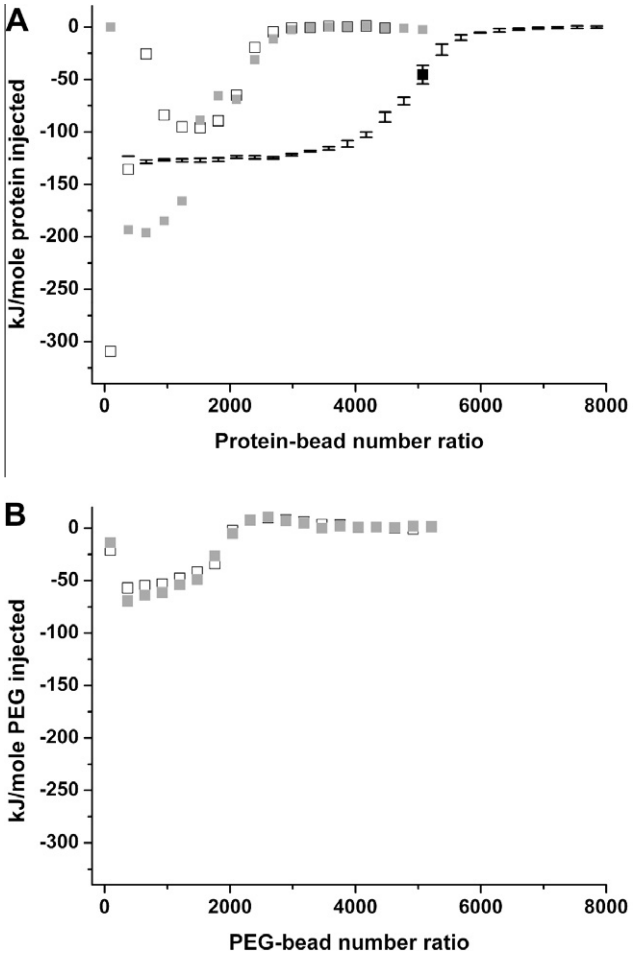

Fig. 5. Enthalpograms obtained at 25 °C for the titration of 6 μL aliquots of 200 μM protein or PEG into a suspension of hydrophobic polystyrene beads in 10 mM H_3BO_3 pH 9.5. (A) Glucagon (■), glucagon PEGylated with a linear PEG chain (□) and glucagon PEGylated with a branched PEG chain (▩). For glucagon, the ■ symbol is only shown for one point, whereas error bars are shown for all points. (B) A linear PEG chain (□) and a branched PEG chain (▩).

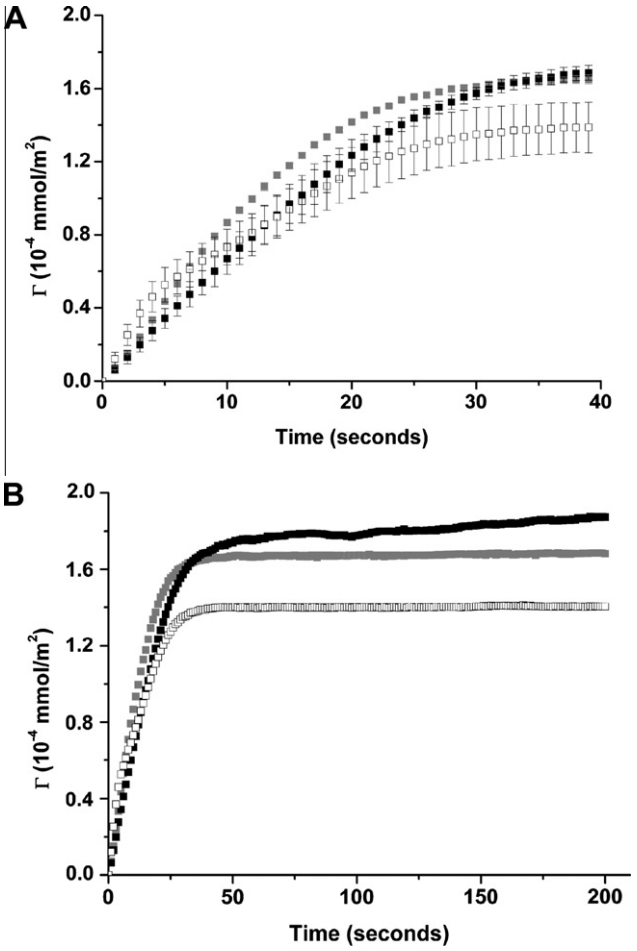

Fig. 6. Adsorption to a hydrophobic polystyrene coated silicon wafer studied with reflectometry. 0.57 μM glucagon (■), 0.57 μM glucagon PEGylated with a linear PEG chain (□) and 0.57 μM glucagon PEGylated with a branched PEG chain (▩). (A) The first 40 s of the experiment with error bars included. (B) All 200 s of the experiment.

had an initial adsorption rate of 6.8×10^{-6} mmol m^{-2} s^{-1}. This was slower than the initial adsorption rates for gluc-PEG-L and gluc-PEG-B, which were determined to 12×10^{-6} mmol m^{-2} s^{-1} and 8.4×10^{-6} mmol m^{-2} s^{-1}, respectively. After the initial adsorption phase, the adsorption rate of gluc-PEG-L decreased and became slower than the adsorption rate of both gluc-PEG-B and glucagon. Gluc-PEG-L and gluc-PEG-B reached adsorption plateaus after approximately 40 s, while the absorbed amount of glucagon continued to increase throughout the experiment (Fig. 6B). In general, the amount of protein molecules adsorbing per unit surface area increases with increasing concentration, until a plateau is reached. It has been proposed that at higher protein concentration in solution, the surface becomes covered more quickly allowing less time for the molecules to relax at the surface [33]. This may affect the orientation and the conformation of the adsorbed protein molecules and thereby the amount of molecules adsorbing per unit surface area [33]. Therefore, the number of molecules of each of the three proteins adsorbing per unit surface area in this setup was not expected to be similar to the saturation values obtained in the adsorption isotherm (Fig. 3). The larger standard deviations observed for gluc-PEG-L compared to gluc-PEG-B are likely caused by higher polydispersity of PEG-L compared to PEG-B.

3.5. Fluorescence emission maxima and TIRF

Fluorescence and total internal reflection fluorescence were used to compare the fluorescence emission maximum of glucagon,

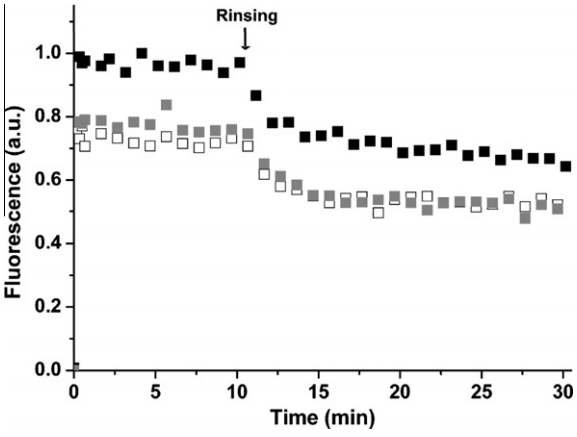

Fig. 7. TIRF experiment showing the adsorption of 29 μM glucagon (■), glucagon PEGylated with a linear PEG chain (□) and glucagon PEGylated with a branched PEG chain (▩). Rinsing with 10 mM H_3BO_3 pH 9.5 was initiated at t = 10 min.

gluc-PEG-L and gluc-PEG-B in solution and after adsorption to a hydrophobic silanized quartz surface. In solution, all three proteins had fluorescence emission maxima of 360 nm. Upon adsorption, the maximum fluorescence emission for glucagon was blue-shifted to 357 nm, whereas for gluc-PEG-L and gluc-PEG-B, it was blue-shifted to 353 nm.

The adsorption of glucagon, gluc-PEG-L and gluc-PEG-B to a hydrophobic silanized quartz surface was compared with TIRF (Fig. 7). Within one minute of adsorption, all three proteins reached an adsorption plateau, which was higher for glucagon than for gluc-PEG-L and gluc-PEG-B. Since the three proteins have equal fluorescence quantum yields in solution (data not shown), more glucagon molecules adsorbed per unit surface area compared to PEGylated glucagon molecules. Upon rinsing with 10 mM H_3BO_3 pH 9.5, the fluorescence signal for all three proteins decreased approximately 20% before new plateaus were reached.

4. Discussion

4.1. Primary driving forces for adsorption

The interactions considered to be the major driving forces for adsorption of proteins from aqueous solution to solid surfaces are electrostatic interactions, hydrophobic interactions and structural changes in the protein upon adsorption [34]. The *pI* value of glucagon was calculated to 6.75 using the ProtParam program on the ExPASy proteomics server: http://www.expasy.org/ [35]. With a *pI* value of 6.75, glucagon will have a net negative charge at pH 9.5. Gluc-PEG-L and gluc-PEG-B will also be net negatively charged at pH 9.5, and because their PEG chains are attached to the side chain of Lys12, which is positively charged in glucagon, both PEGylated glucagon conjugates will have a negative charge that is one higher than the charge of glucagon. Overall, the polystyrene beads are hydrophobic but are stabilized by sulphate ions and will therefore have negative charges on the surface. The finding that all three proteins adsorbed with high affinity to the hydrophobic polystyrene beads under conditions of electrostatic repulsion suggests that hydrophobic interaction is the dominant driving force for adsorption of glucagon and PEGylated glucagon to the hydrophobic polystyrene surface. This is further supported by the negative slopes observed for ΔH versus temperature (ΔC_p) for all three proteins that points to the hydrophobic effect as a driving force [32]. A previous TIRF study showed that neither glucagon nor glucagon PEGylated with a linear 5 kDA PEG chain adsorbed to a hydrophilic quartz surface at pH 7.4 [18], indicating that electrostatic interactions are negligible.

4.2. Number of molecules adsorbing per unit surface area

The decreased number of gluc-PEG-L and gluc-PEG-B molecules adsorbing to the hydrophobic polystyrene beads per unit area when compared to glucagon molecules (Figs. 3 and 7) is likely a result of increased steric hindrance at the surface due to the PEGylation. PEGylation has previously been shown to reduce the number of RNase A and the number of lysozyme molecules adsorbing per unit surface area to a PLG surface [14] and silica [15], respectively. No difference in the amount of gluc-PEG-L and gluc-PEG-B molecules adsorbing per unit surface area was detected, which indicates that a gluc-PEG-L and a gluc-PEG-B molecule take up approximately the same space on the hydrophobic polystyrene and hydrophobic silanized quartz surfaces. This is supported by two studies reporting that PEGylation with linear and branched PEG chains of the same nominal weight increases the protein size to the same extent [36,37]. In the first study, no difference was observed for the hydrodynamic volumes and viscosity radii for α-Lactalbumin and bovine serum albumin PEGylated with linear and branched forms of PEG having the same molecular weight, regardless of the degree of PEGylation [36]. In the second study, PEGylation of interferon alpha 2b with linear and branched PEG chains of the same nominal weight was found to have the same size when determined with size exclusion chromatography, while a smaller hydrodynamic radius for branched conjugates was found with dynamic light scattering [37].

4.3. Heat flow

Different heat flows were observed for adsorption of gluc-PEG-L and Gluc-PEG-B to hydrophobic polystyrene beads (Fig. 5A), whereas similar heat flows were observed for adsorption of PEG-L and PEG-B to the polystyrene beads (Fig. 5B). Different conformational changes in gluc-PEG-L and gluc-PEG-B upon adsorption can contribute to the observed differences in heat flow. Variation in the flexibility of the linear and branched PEG chains in gluc-PEG-L and gluc-PEG-B, respectively, could also add to the observed difference in heat flow. However, the data do not indicate whether only one or both of these parameters have an influence on the observed heat flow.

4.4. Adsorption rate

The higher adsorption rate for gluc-PEG-L and gluc-PEG-B when compared to glucagon (Fig. 6) was unanticipated. The adsorption rate of a protein will depend on the sticking probability of the protein and the rate at which the protein arrives at the surface. The amphipilic character of the linear and branched PEG chains makes it difficult to predict their effect on the sticking probability of glucagon to the hydrophobic polystyrene coated silicon surface. It has previously been shown that the PEG part of a block copolymer does not adsorb to hydrophobic surfaces [38], and ITC measurements showed no heat flow when PEG-L or PEG-B was titrated into an ITC cell containing polystyrene beads to which glucagon had been pre-adsorbed (data not shown). In contrast, when glucagon was titrated into an ITC cell containing polystyrene beads that had been mixed with either PEG-L or PEG-B heat signals that resulted in s-shaped enthalpograms were obtained (data not shown). This indicates that glucagon has a higher affinity for the hydrophobic polystyrene beads than PEG-L and PEG-B). In contrast, PEG has also shown to be surface active at a hydrophobic surface [39], and another study has shown that PEGylation of RNase A increases the initial rate of adsorption of RNase A [14].

In the reflectometry setup used to compare the adsorption rates, transport of the proteins towards the surface is controlled by invoking a stagnation point flow that results in a constant thickness of the diffusion boundary layer. Therefore, the protein flux toward the surface will be proportional to the protein concentration in solution and can be described as follows:

$$J_0 = 0.53(D^2 \alpha \Phi R^{-4})^{1/3} c_p \quad (2)$$

where D is the diffusion coefficient, α is a flow intensity parameter that is constant for a certain cell geometry and flow rate, Φ is the flow rate, R is the radius of the circular hole through which the solution enters the cell, and c_p is the protein concentration in solution [40]. The same molar concentration of the three proteins was used, and therefore, c_p was the same for the three proteins. PEGylation increases the hydrodynamic radii of proteins, and therefore, gluc-PEG-L and gluc-PEG-B will have smaller diffusion coefficients and thus slower fluxes than glucagon. In aqueous solutions at concentrations below 287 μM, glucagon is flexible and has little helical structure [29,30], but at higher concentrations, the helical content increases [30,41] and glucagon forms trimers [42,43]. It is possible that the higher adsorption rate observed for PEGylated glucagon when compared to glucagon is a result of impeded trimer formation

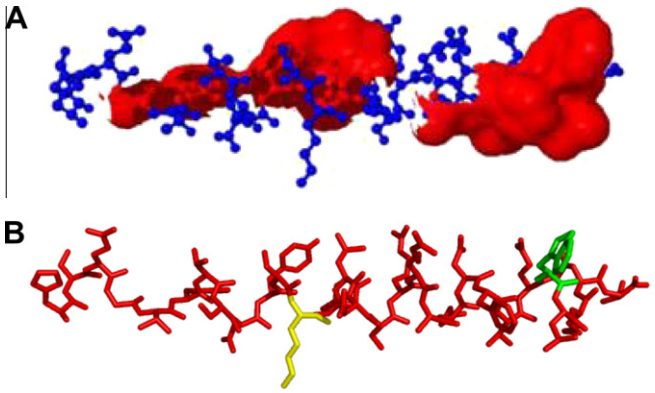

Fig. 8. Crystal structure of glucagon (Pdf entry 1GCN[34]). (A) Hydrophobic and hydrophilic amino acid residues are shown in red and blue, respectively (modified in JmolScript). (B) The primary structure of glucagon is shown in red, and the Lys12 and Trp25 amino acid residues are shown in yellow and green, respectively (modified in Pymol). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of gluc-PEG-L and gluc-PEG-B, due to steric hindrance from the PEG chains. However, at the concentration of 0.57 μM, at which the study was performed, the solution is likely dominated by the monomer form of glucagon.

4.5. Orientation upon adsorption

Fluorescence emission of tryptophan residues in proteins is sensitive to the polarity of the surroundings, and a decrease in polarity in the local environment will result in a blue-shift in the fluorescence emission spectra [44]. The blue-shift observed upon adsorption of the three proteins showed that the hydrophobicity in the local environment around Trp25 increased upon adsorption, indicating that Trp25 was exposed to the hydrophobic silanized quartz surface and/or was shielded from the aqueous solution due to structural changes in the proteins. The crystal structure of glucagon has two hydrophobic patches [42] (Fig. 8A), one in the C-terminal, which includes Trp25, and one in the middle of the helix on the opposite side of Lys12 (Fig. 8B). Therefore, it is likely that Trp25 is exposed to the hydrophobic silanized surface for all three proteins upon adsorption. The lower blue-shift for glucagon compared to PEGylated glucagon may be due to glucagon adsorbing with either of the two hydrophobic patches towards the surface while steric hindrance at the surfaces of the PEGylated glucagon molecules impedes them from adsorbing with the hydrophobic patch in the middle of the helix toward the surface. However, it cannot be ruled out that different structural changes can cause the observed difference in blue-shift between glucagon and PEGylated glucagon.

5. Conclusions

Glucagon with a molar mass of 3483 Da was PEGylated with linear and branched PEG chains yielding conjugates with molar masses of 5500–5900 Da and 5700–5800 Da, respectively. PEGylation was shown to reduce the number of glucagon molecules adsorbing per unit surface area to hydrophobic polystyrene beads and to a hydrophobic silanized quartz surface. Furthermore, PEGylation resulted in a larger blue-shift in the fluorescence emission maximum of glucagon at a hydrophobic silanized quartz surface, indicating that PEGylation affected the orientation and/or the structural changes induced in glucagon upon adsorption. The initial adsorption rate of glucagon to a hydrophobic polystyrene coated silicon surface was increased by PEGylation, and the enthalpy of adsorption to hydrophobic polystyrene beads was also affected by PEGylation. The linear and the branched PEG chains had different effects on the initial adsorption rate and on the heat of adsorption.

Our findings will support the interpretation of the effect of PEGylation with linear and branched PEG chains on other protein stability parameters as well as the design of chromatographic separation of PEGylated protein drugs.

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