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Research paper

Synthesis, characterization and assessment of suitability of trehalose fatty acid esters as alternatives for polysorbates in protein formulation

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ABSTRACT

Nonionic polyethylene glycol-derived surfactants are today's choice as surfactants in protein formulations. Different groups discovered that although surface-induced stresses are reduced by these excipients, the long-term stability of different proteins decreased due to polyethylene glycol-related induction of oxidation processes under static storage conditions. In this paper, the potential of polyoxyethylene-free surfactants for protein formulation was evaluated. Three different sugar-based surfactants, 6-*O*-monocaprinoyl-α,α-trehalose, 6-*O*-monolauroyl-α,α-trehalose and 6-*O*-monopalmitoyl-α,α-trehalose, were synthesized in four reaction steps. These substances lack polyethylene glycol residues and can be produced from renewable resources.

The chemical and physical properties of these three surfactants were investigated and compared with polysorbate 20 and 80. 6-*O*-Monopalmitoyl-α,α-trehalose was insoluble in water at room temperature and was hence excluded from some of the further tests. The critical micellar concentration of all surfactants is in a comparable range of approximately 0.001–0.01% (m/V). The sugar-based surfactants showed slightly higher hemolytic activity than the polysorbate references. The surfactants with shorter chain length proved to be comparable to polysorbates in regard to physicochemical properties.

Finally for human growth hormone, the protein-stabilizing properties against shaking-induced stress were tested and compared to polysorbate-containing formulations. Whereas in the absence of surfactant, dramatic monomer loss and aggregate formation occurred, it was found that 100% monomer content was maintained when 0.1% (m/V) 6-*O*-monocaprinoyl-α,α-trehalose or 6-*O*-monolauroyl-α,α-trehalose was added to the formulation. Polysorbate 80 at a concentration of 0.1% (m/V) also significantly stabilized the protein. Lower amounts of surfactants result in only partial stabilization. Furthermore, adsorption of human growth hormone to the container surface is reduced in the presence of the surfactants. Thus, the new sugar-based surfactants offer a promising alternative and have potential for application in protein formulations.

1. Introduction

A large number of protein pharmaceuticals, both in liquid and in lyophilized forms, are formulated with the addition of nonionic surfactants such as block copolymers and polysorbates, for example the insulin Lantus® [1], pegylated erythropoetin Mircera® [2], a human growth hormone formulation Nutropin® [3] or the humanized monoclonal antibody Herceptin® [4]. Different groups have shown that these surfactants pose dual effects on the protein. On the one hand, they physically stabilize proteins against various kinds of surface-correlated stress, e.g. adsorption at container surfaces [5,6], denaturation at the air–water interface [7,8], or the ice–water interface [9–11]. On the other hand, these polyethylene glycol-based surfactants can put long-term chemical stability at risk [12–15]. Mostly, oxidizing reactions are described in the literature. These are explained by peroxide remnants in the technical products. An increase in hemolysis is referred to peroxide residues, too [16]. Adverse reactions in patients are known for some of these surfactants [17], which is another reason to search for other options for surface stabilization.

An alternative for these substances should show a good safety profile, the same surface-related stabilizing effects, but no impact on long-term chemical stability of pharmaceutical proteins. Sugar-based surfactants feature surface behavior similar to polysorbates. They are already in pharmaceutical and cosmetical use for topical and oral formulations [18–20]. In addition, they are known as reagents for the stabilization of membrane proteins [21,22]. Alkyl glycosides and sugar fatty acid esters are environmentally friendly [23,24] as only renewable components, sugars (mono- to

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oligosaccharides) for the hydrophilic and fatty alcohols or fatty acids for the hydrophobic part are required. Synthesis can be performed both chemically [25–27] and biochemically [25,26,28]. Technical mixtures are already on the market and are well characterized [25,29,30]. Of course, the lack of PEG-residues, and hence peroxides should pose the advantage regarding oxidizing behavior of this class of surfactants against the already approved substances.

In this paper, we describe the synthesis and scrutiny of trehalose fatty acid monoesters. Trehalose is a well-known stabilizer in protein formulations [31–33]. Having this in mind, the authors were looking for a good water-soluble surfactant. We designed a synthetic strategy in four steps resulting in highly pure products. These surfactants are unlike polysorbates, which are technical mixtures of all kinds of molecules with a broad heterogeneity. The authors were furthermore interested in a correlation of chain length and protein stability. Thus, we sought for substances that are comparable to Polysorbate 20 (PS 20) and Polysorbate 80 (PS 80) in regard to formulation feasibility. We characterized the physicochemical properties of trehalose esters, such as CMC, micelle size, viscosity and calorimetric behavior. Furthermore, they were tested for hemolytic activity as surfactants tend to rupture cell membranes [34–36]. Finally, the protein-stabilizing capacity of the trehalose esters was tested in agitation stress studies of human growth hormone.

2. Materials and methods

2.1. Materials

Palmitic acid, lauric acid, capric acid, anhydrous pyridine, trimethylsilyl chloride, hexamethyldisilazane, 4-(dimethylamino)pyridine (4-DMAP) and *N,N′*-dicyclohexylcarbodiimide (1,3-DCC) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Solvents for NMR were purchased from Euriso-Top SA (Saint-Aubin Cedex, France). α,α-Trehalose was purchased from Georg Breuer GmbH (Königstein, Germany). Flash column chromatography was performed using silica gel 60 (40–63 μm) (VWR International GmbH, Bruchsal, Germany). All other chemicals were of reagent grade and commercially available products. If indicated, solvents were dried using standard procedures. For the physicochemical analysis of the sugar-based surfactants, the water used in all experiments was purified in a PURELAB Plus system (ELGA labwater, Celle, Germany) and had a conductivity <0.055 μS/cm. Pyrene for synthesis and all buffer salts were obtained from Merck KGA (Darmstadt, Germany). Buffer salts were of analytical grade or higher.

Human growth hormone (hGH) was gratefully donated by Bernina Plus & HDS. Porcine blood was collected at the municipal slaughterhouse. Further purification and processing to an erythrocyte suspension is explained in Section 2.2.

2.1.1. Preparation of 2,3,4,6,2′,3′,4′,6′-octa-O-(trimethylsilyl)-α,α-trehalose (Fig. 1)

(**1**) α,α-Trehalose dihydrate (7.00 g, 18.5 mmol) was placed in a flask containing 100 ml of anhydrous pyridine. Trimethylsilyl chloride (20.0 ml, 158.0 mmol) was added over a period of 30 min, hexamethyldisilazane (40.0 ml, 188.0 mmol) was added the same way, and the mixture was mechanically stirred under nitrogen atmosphere for 18 h at room temperature. The solvent was removed by flash evaporation, and the resulting residue was treated with 100 ml ice water and extracted with hexane (3 × 50 ml). The combined organic extracts were dried over anhydrous $MgSO_4$, and then the solvent was removed by flash evaporation. The residue was crystallized in methanol to give 2,3,4,6,2′,3′,4′,6′-octa-*O*-(trimethylsilyl)-α,α-trehalose (**1**) (15.7 g, 17.0 mmol, 92%) as a white solid. MP: 82 °C (lit. [43]: 80–82 °C). IR (KBr): ν (cm^{-1}) = 2959, 1251, 1155, 1117, 1071, 1023, 901, 878, 840, 748, 682, 536, 457. ^{1}H NMR ($CDCl_3$): δ (ppm) = 4.91 (d, 2 H, J = 3.1 Hz, 1-H, 1′-H), 3.88 (t, 2 H, J = 9.0 Hz, 2-H, 2′-H), 3.78 (m, 2 H, 5-H, 5′-H), 3.66 (m, 4 H, 6-H, 6′-H), 3.42 (t, 2 H, J = 9,1 Hz, 4-H, 4′-H), 3.37 (dd, 2 H, J_1 = 3.2 Hz, J_2 = 9.3 Hz, 3-H, 3′-H), 0.14–0.09 (m, 72 H, 8 $SiMe_3$). ^{13}C NMR ($CDCl_3$): δ (ppm) = 96.8 (C-1, C-1′), 76.1 (C-2, C-2′), 75.7 (C-5, C-5′), 75.3 (C-3, C-3′), 74.3 (C-4, C-4′), 64.7 (C-6, C-6′), 3.6 (2 $SiMe_3$), 3.5 (2 $SiMe_3$), 2.7 (2 $SiMe_3$), 2.2 (2 $SiMe_3$). MS (ESI): *m/z* (rel. int. in%) = 941 $[M+Na]^+$ (87), 936 (100), 977 (47), 361 (40).

2.1.2. Preparation of 2,3,4,2′,3′,4′-hexa-O-(trimethylsilyl)-α,α-trehalose

(**2**) 2,3,4,6,2′,3′,4′,6′-Octa-*O*-(trimethylsilyl)-α,α-trehalose (**1**) (0.920 g, 1.00 mmol) was placed in a 250-ml flask containing 25 ml of methanol and cooled to 0 °C. A solution of potassium carbonate (0.140 mg, 1.0 mmol) in methanol (30 ml) was added dropwise over a period of 20 min. The reaction mixture was stirred for 5 h at 0 °C, complete conversion was checked by TLC. The reaction was stopped by adding glacial acetic acid (115 μL, 2.0 mmol). The solvent was removed by flash evaporation, and the crude product was treated with brine and extracted with diethyl ether (3 × 30 ml). The combined organic extracts were dried over magnesium sulfate, removing the solvent by flash evaporation yielded 2,3,4,2′,3′,4′-hexa-*O*-(trimethylsilyl)-α,α-trehalose (**2**) as a white solid (0.640 g, 0.82 mmol, 82%). MP: 114 °C (lit. [43] 115–118 °C). IR (KBr): ν (cm^{-1}) = 3503, 2959, 1405, 1251, 1171, 1126, 1110, 1076, 1011, 948, 897, 873, 843, 748, 683, 623, 589, 519, 456. ^{1}H NMR ($CDCl_3$): δ (ppm) = 4.90 (d, 2 H, J = 3.1 Hz, 1-H, 1′-H), 3.86 (t, 2 H, J = 9.0 Hz, 2-H, 2′-H), 3.84 (m, 2 H, 5-H, 5′-H), 3.71 (m, 4 H, 6-H, 6′-H), 3.48 (t, 2 H, J = 9.2 Hz, 4-H, 4′-H), 3.41 (dd, 2 H, J_1 = 3.2 Hz, J_2 = 9.3 Hz, 3-H, 3′-H), 0.16–0.12 (m, 54 H, 6 $SiMe_3$). ^{13}C NMR ($CDCl_3$): δ (ppm) = 94.8 (C-1, C-1′), 73.5 (C-2, C-2′), 73.0 (C-5, C-5′), 72.9 (C-3, C-3′), 71.5 (C-4, C-4′), 61.8 (C-6, C-6′), 1.1 (2 $SiMe_3$), 1.0 (2 $SiMe_3$), 0.2 (2 $SiMe_3$). MS (ESI): *m/z* (rel. int. in%) = 797 $[M+Na]^+$ (21), 792 (100), 379 (28), 361 (10), 289 (18).

2.1.3. Preparation of 6-O-monopalmitoyl-, 6-O-monolauroyl- and 6-O-monocaprinoyl-2,3,4,2′,3′,4′-hexa-O-(trimethylsilyl)-α,α-trehalose (**3a**, **3b**, **3c**)

2,3,4,2′,3′,4′-Hexa-*O*-(trimethylsilyl)-α,α-trehalose (**2**) (0.770 g, 1.00 mmol), together with 4-DMAP (0.160 g, 1.30 mmol) and the corresponding carboxylic acid (for **3a**: palmitic acid $C_{16}H_{32}O_2$; for **3b**: lauric acid $C_{12}H_{24}O_2$; for **3c**: capric acid $C_{10}H_{20}O_2$) (1.30 mmol) were placed in a flame-dried, 25-ml Schlenck flask, containing anhydrous dichloromethane (5 ml). A solution of 1,3-DCC (0.250 g, 1.20 mmol) in anhydrous dichloromethane (3 ml) was added dropwise. The mixture was mechanically stirred for 18 hours at room temperature under a nitrogen atmosphere. The resulting precipitate was removed by filtration under reduced pressure and washed with dichloromethane (5 ml). The crude product was obtained by removing the solvent from the combined, dried dichloromethane solutions by flash evaporation and purified by flash column chromatography (hexane, diethyl ether 7:3).

3a: 6-*O*-Monopalmitoyl-2,3,4,2′,3′,4′-hexa-*O*-(trimethylsilyl)-α,α-trehalose (0.330 g, 0.33 mmol, 33%, colorless oil). IR (NaCl): ν (cm^{-1}) = 3447, 2925, 2855, 2360, 2342, 1743, 1457, 1251, 1166, 1111, 1077, 1009, 965, 898, 874, 844, 748, 668, 518. ^{1}H NMR ($CDCl_3$): δ (ppm) = 4.93 (t, 2 H, J = 3.1 Hz, 1-H, 1′-H), 4.30 (dd, 1 H, J_1 = 11.8 Hz, J_2 = 2.3 Hz, 6-H), 4.07 (dd, 1 H, J_1 = 11.8 Hz, J_2 = 4.5 Hz, 6-H), 4.02 (m, 1 H, 5-H), 3.92 (m, 2 H, 2-H, 3-H), 3.85 (m, 1 H, 5′-H), 3.71 (m, 2 H, 6′-H), 3.47 (m, 4 H, 4-H, 2′-H, 3′-H, 4′-H), 2.35 (m, 2 H, 2″-H), 1.63 (m, 2 H, 3″H), 1.27 (m, 24 H, 4″-H–15″-H), 0.88 (m, 3 H, 16″-H), 0.15-0.12 (m, 54 H, 6 $SiMe_3$). ^{13}C NMR ($CDCl_3$): δ (ppm) = 173.7 (C-1″), 94.3 (C-1′), 94.2 (C-1), 73.3 (C-5), 73.1 (C-2), 72.8 (C-5′), 72.6 (C-4′), 72.5 (C-3′), 71.7 (C-2′),

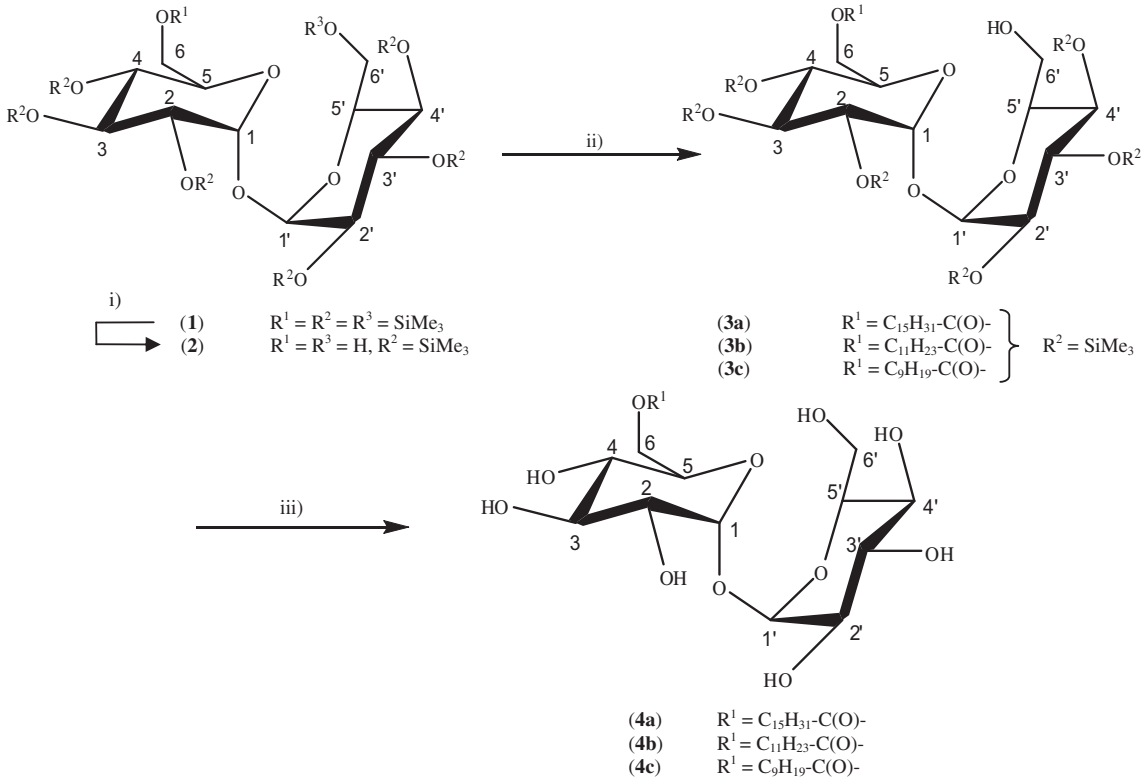

Fig. 1. Reaction scheme of the preparation of Mono-PT (**4a**), Mono-LT (**4b**) and Mono-CT (**4c**): (i) K_2CO_3, MeOH, 0 °C, 5 h; (ii) 1,3-DCC, 4-DMAP, carboxylic acid, dry dichloromethane, 18 h, rt; (iii) TFA, THF, H_2O, 1 h, rt.

71.2 (C-4), 70.6 (C-3), 63.1 (C-6), 61.5 (C-6′), 34.0 (C-2″), 31.8 (C-14″), 29.5 (C-4″–C-10″), 29.3 (C-11″), 29.2 (C-12″, C-13″), 24.7 (C-3″), 22.6 (C-15″), 14.0 (C-16″), 1.3–0.6 (6 $SiMe_3$).

3b: 6-*O*-Monolauroyl-2,3,4,2′,3′,4′-hexa-*O*-(trimethylsilyl)-α,α-trehalose (0.402 g, 0.42 mmol, 42%, colorless oil). IR (NaCl): *ν* (cm^{-1}) = 3522, 2957, 2926, 2856, 2361, 1744, 1455, 1385, 1327, 1251, 1165, 1110, 1076, 1009, 965, 898, 873, 842, 749, 681. ^{1}H NMR ($CDCl_3$): *δ* (ppm) = 4.92 (t, 2 H, *J* = 3.2 Hz, 1-H, 1′-H), 4.29 (dd, 1 H, J_1 = 11.9 Hz, J_2 = 2.5 Hz, 6-H), 4.06 (dd, 1 H, J_1 = 11.9 Hz, J_2 = 4.7 Hz, 6-H), 4.01 (m, 1 H, 5-H), 3.91 (m, 2 H, 2-H, 3-H), 3.85 (m, 1 H, 5′-H), 3.70 (m, 2 H, 6′-H), 3.45 (m, 4 H, 4-H, 2′-H, 3′-H, 4′-H), 2.35 (m, 2 H, 2″-H), 1.63 (m, 2 H, 3″H), 1.26 (m, 16 H, 4″-H–11″-H), 0.88 (m, 3 H, 12″-H), 0.14–0.10 (m, 54 H, 6 $SiMe_3$). ^{13}C NMR ($CDCl_3$): *δ* (ppm) = 173.7 (C-1″), 94.4 (C-1′), 94.3 (C-1), 73.4 (C-5), 73.2 (C-2), 72.8 (C-5′), 72.6 (C-4′), 72.5 (C-3′), 71.8 (C-2′), 71.3 (C-4), 70.7 (C-3), 63.2 (C-6), 61.6 (C-6′), 34.1 (C-2″), 31.8 (C-10″), 29.5–29.1 (C-4″–C9″), 24.7 (C-2″), 22.6 (C-11″), 14.0 (C-12″), 1.3–0.3 (6 $SiMe_3$). MS (ESI): *m/z* (rel. int. in%) = 979 $[M+Na]^+$ (34), 975 (100), 471 (43), 271 (90).

3c: 6-*O*-Monocaprinoyl-2,3,4,2′,3′,4′-hexa-*O*-(trimethylsilyl)-α,α-trehalose (0.420 g, 0.45 mmol, 45%, colorless oil). IR (NaCl): *ν* (cm^{-1}) = 3521, 2957, 2926, 2856, 1743, 1457, 1404, 1385, 1326, 1251, 1165, 1110, 1077, 1008, 964, 898, 874, 843, 749, 683. ^{1}H NMR ($CDCl_3$): *δ* (ppm) = 4.92 (t, 2 H, *J* = 2.55 Hz, 1-H, 1′-H), 4.29 (dd, 1 H, J_1 = 11.9 Hz, J_2 = 2.2 Hz, 6-H), 4.06 (dd, 1 H, J_1 = 11.7 Hz, J_2 = 4.6 Hz, 6-H), 4.01 (m, 1 H, 5-H), 3.89 (m, 2 H, 2-H, 3-H), 3.83 (m, 1 H, 5′-H), 3.70 (m, 2 H, 6′-H), 3.45 (m, 4 H, 4-H, 2′-H, 3′-H, 4′-H), 2.34 (m, 2 H, 2″-H), 1.63 (m, 2 H, 3″H), 1.26 (m, 12 H, 4″-H–9″-H), 0.88 (t, 3 H, *J* = 7.1 Hz, 10″-H), 0.13–0.10 (m, 54 H, 6 $SiMe_3$). ^{13}C NMR ($CDCl_3$): *δ* (ppm) = 173.9 (C-1″), 94.6 (C-1′), 94.5 (C-1), 73.6 (C-5), 73.4 (C-2), 73.0 (C-5′), 72.8 (C-4′), 72.7 (C-3′), 72.0 (C-2′), 71.5 (C-4), 70.9 (C-3), 63.4 (C-6), 61.7 (C-6′), 34.3 (C-2″), 32.0 (C-8″), 29.5–29.2 (C-3″–C-6″), 24.9 (C-3″), 22.8 (C-9″), 14.2 (C-10″), 1.3–0.3 (6 $SiMe_3$). MS (ESI): *m/z* (rel. int. in%) = 953 $[M+Na]^+$ (23), 946 (91), 361 (100), 279 (63).

*2.1.4. Preparation of 6-O-monopalmitoyl-, 6-O-monolauroyl- and 6-O-monocaprinoyl-α,α-trehalose (**4a**, **4b**, **4c**)*

The corresponding esters **3a**, **3b**, **3c** of 2,3,4,2′,3′,4′-hexa-*O*-(trimethylsilyl)-α,α-trehalose (0.74 mmol) were placed in a 25-ml flask, containing 8 ml of a mixture of trifluoroacetic acid, tetrahydrofuran and water in a ratio of 8:17:3. The resulting solution was mechanically stirred for 1 h at room temperature. **4a** was obtained as a white precipitate that was collected by filtration under reduced pressure and crystallized in acetone. **4b** and **4c** did not precipitate and thus were purified by removing the solvent using flash evaporation, followed by flash column chromatography (ethyl acetate, methanol 4:1).

4a: 6-*O*-Monopalmitoyl-α,α-trehalose (Mono-PT) (0.310 g, 0.53 mmol, 72%, white solid). MP: softening at 130–135 °C, melting at 203–207 °C; (lit. [42]: 198–200 °C). IR (KBr): *ν* (cm^{-1}) = 3421, 2926, 2854, 2361, 1740, 1636, 1467, 1378, 1150, 1109, 1078, 1046, 989, 939, 805, 721, 576, 527.^{1}H NMR ($CDCl_3$): *δ* (ppm) = 5.08 (dd, 2 H, J_1 = 11.2 Hz, J_2 = 3.6 Hz, 1-H, 1′-H), 4.36 (dd, 1 H, J_1 = 12.0 Hz, J_2 = 1.6 Hz, 6-H), 4.19 (dd, 1 H, J_1 = 12.0 Hz, J_2 = 5.1 Hz, 6-H), 4.02 (m, 1 H, 5-H), 3.79 (m, 4 H, 2-H, 2′-H, 5′-H, 6′-H), 3.66 (m, 1 H, 6′-H), 3.47 (m, 2 H, 3-H, 3′-H), 3.34 (m, 2 H, 4-H, 4′-H), 2.34 (t, 2 H, *J* = 7.4 Hz, 2″-H), 1.61 (m, 2 H, 3″-H), 1.29 (m, 24 H, 4″-H–15″-H), 0.90 (t, 3 H, *J* = 7.2 Hz, 16″-H). ^{13}C NMR ($CDCl_3$): *δ* (ppm) = 175.5 (C-1″), 95.2 (C-1′), 95.1 (C-1), 74.6 (C-2′), 74.4 (C-2), 73.9 (C-5′), 73.2 (C-3, C-3′), 71.9 (C-4, C-4′), 71.4 (C-5), 64.4 (C-6), 62.6 (C-6′), 35.0 (C-2″), 33.1 (C-14″), 30.9–30.3 (C-4″–C-13″), 26.1 (C-3″), 23.8 (C-15″), 14.5 (C-16″). MS (ESI): *m/z* (rel. int. in%) = 1183 $[2M+Na]^+$ (20), 603 $[M+Na]^+$ (100).

4b: 6-*O*-Monolauroyl-α,α-trehalose (Mono-LT) (0.294 g, 0.56 mmol, 76%, white solid). MP: softening at 130–134 °C, melting

at 159–163 °C; (lit. [40]: 156–158 °C). IR (KBr): ν (cm^{-1}) = 3421, 2926, 2854, 2361, 1740, 1636, 1467, 1378, 1150, 1109, 1078, 1046, 989, 939, 805, 721, 576, 527. ^{1}H NMR ($CDCl_3$): δ (ppm) = 5.08 (dd, 2 H, J_1 = 11.1 Hz, J_2 = 3.6 Hz, 1-H, 1′-H), 4.36 (d, 1 H, J = 11.7 Hz, 6-H), 4.19 (dd, 1 H, J_1 = 11.9 Hz, J_2 = 4.9 Hz, 6-H), 4.02 (m, 1 H, 5-H), 3.79 (m, 4 H, 2-H, 2′-H, 5′-H, 6′-H), 3.66 (m, 1 H, 6′-H), 3.47 (m, 2 H, 3-H, 3′-H), 3.33 (m, 2 H, 4-H, 4′-H), 2.34 (t, 2 H, J = 7.2 Hz, 2″-H), 1.61 (m, 2 H, 3″-H), 1.29 (m, 16 H, 4″-H–11″-H), 0.90 (t, 3 H, J = 6.6 Hz, 12″-H). ^{13}C NMR ($CDCl_3$): δ (ppm) = 175.5 (C-1″), 95.2 (C-1′), 95.1 (C-1), 74.6 (C-2′), 74.4 (C-2), 73.9 (C-5′), 73.2 (C-3, C-3′), 71.9 (C-4, C-4′), 71.4 (C-5), 64.4 (C-6), 62.7 (C-6′), 35.0 (C-2″), 33.1 (C-10″), 30.8-30.2 (C-4″–C-9″), 26.1 (C-3″), 23.8 (C-11″), 14.5 (C-12″). MS (ESI): m/z (rel. int. in%) = 547 $[M+Na]^+$ (41), 563 $[M+K]^+$ (100), 1071 $[2M+Na]^+$ (43), 1087 $[2{\cdot}M+K]^+$ (45).

4c: 6-*O*-Monocaprinoyl-α,α-trehalose (Mono-CT) (0.286 g (0.57 mmol, 78%, white solid). MP: 163–164 °C. IR (KBr): ν (cm^{-1}) = 3500, 3355, 2933, 2908, 2361, 2343, 1685, 1457, 1149, 1100, 1081, 1030, 998, 956, 803, 612. ^{1}H NMR ($CDCl_3$): δ (ppm) = 5.08 (dd, 2 H, J_1 = 11.1 Hz, J_2 = 3.7 Hz, 1-H, 1′-H), 4.36 (dd, 1 H, J_1 = 11.9 Hz, J_2 = 2.1 Hz, 6-H), 4.19 (dd, 1 H, J_1 = 12.1 Hz, J_2 = 5.2 Hz, 6-H), 4.01 (m, 1 H, 5-H), 3.79 (m, 4 H, 2-H, 2′-H, 5′-H, 6′-H), 3.66 (m, 1 H, 6′-H), 3.47 (m, 2 H, 3-H, 3′-H), 3.32 (m, 2 H, 4-H, 4′-H), 2.34 (t, 2 H, J = 7.6 Hz, 2″-H), 1.61 (m, 2 H, 3″H), 1.30 (m, 12 H, 4″-H - 9″-H), 0.90 (t, 3 H, J = 6.9 Hz, 10″-H). ^{13}C NMR ($CDCl_3$): δ (ppm) = 175.5 (C-1″), 95.2 (C-1′), 95.1 (C-1), 74.6 (C-2′), 74.4 (C-2), 73.9 (C-5′), 73.2 (C-3, C-3′), 71.9 (C-4, C-4′), 71.4 (C-5), 64.4 (C-6), 62.6 (C-6′), 35.0 (C-2″), 33.1 (C-8″), 30.6–30.3 (C-4″–C-7″), 26.1 (C-3″), 23.8 (C-9″), 14.5 (C-10″). MS (ESI): m/z (rel. int. in%) = 519 $[M+Na]^+$ (100), 516 (56), 509 (7).

2.1.5. Instruments used for identification of the synthesized products

IR spectra were recorded on a Perkin Elmer FT-Infrared Spectrometer Paragon 1000 (Perkin Elmer, Waltham, MA, USA). ^{1}H NMR and ^{13}C NMR spectra were taken on a Jeol JNMR-GX400 (400 MHz/100 MHz) and a Jeol JNMR-GX500 (500 MHz/125 MHz) (Jeol, Tokyo, Japan), and chemical shifts are reported in ppm, downfield from tetramethylsilane. ESI-mass spectroscopy was performed on an API 2000 (Applied Biosystems, Foster City, CA, USA). Melting points were taken on a Büchi Melting Point B-540 (BÜCHI Labortechnik GmbH, Essen, Germany). TLC was performed on POLYGRAM SIL G/UV_{254} plates (Macherey-Nagel, Düren, Germany).

2.2. Methods

2.2.1. Tensiometry

Surface tension was measured with a K100 MK2 tensiometer (Krüss, Hamburg, Germany) equipped with a F12 thermostat (Julabo, Ostfildern, Germany) and a 765 Dosimat (Metrohm, Leinfelden-Echterdingen, Germany) using LabDesk 3.1 software (Krüss, Hamburg, Germany). Before the surface tension of surfactants was determined, the system was tested with purified water. When surface tensions of 72.0 ± 0.1 mN/m were found for water in three consecutive measurements, surfactant solutions were tested. The critical micelle concentration (CMC) was determined from sharp breaks in surface tension vs. logarithm of surfactant concentration plots. A custom-made glass vessel and a platinum iridium plate were used for all experiments. A total of 11 concentrations were tested for each surfactant. One measurement lasted at least 300 s. Experiments (n = 3) were performed at 25 °C for Mono-LT and Mono-CT. Because of its low solubility at room temperature, Mono-PT was tested at 45 °C.

2.2.2. Pyrene interaction fluorescence

Pyrene fluorescence was applied to alternatively determine the CMC according to Kalyanasundaram and Thomas [37]. Fluorescence spectra were recorded, and the ratio I_1/I_3 was calculated, where I_1 was the emission at 373 nm, and I_3 was the emission at 393 nm. Excitation wavelength was 335 nm. Due to a change in polarity when micelles occur, the ratio becomes <1 at the CMC. Surfactant concentrations comprised the same range as applied to surface tension measurements. Experiments were performed in quartz cuvettes with 10-mm pathlength using a Cary Eclipse spectrofluorimeter (Varian, Palo Alto, USA). Ten microliters of a 200 mM pyrene solution in ethanol was added to 1990 µl surfactant solution (N = 3).

2.2.3. Hemolytic activity

Hemolytic activity was measured with porcine erythrocytes. Coagulation was prevented by adding 200 ml 3.8% (m/V) trisodium citrate buffer pH 7.4–800 ml fresh blood. Samples were washed six times with citrate buffer and centrifuged at 3.500g and 5 °C for 45 min. Different surfactant concentrations (in the same citrate buffer used for purification) were added to the porcine erythrocytes suspension at a 1:5 ratio. After incubating for 1 h at 37 °C under gentle shaking, samples were centrifuged (3.500g for 15 min). The supernatant was tested for free hemoglobin at A = 415 nm using an UV 8453 UV-spectrometer and UV-Visible ChemStation software (Agilent, Santa Clara, USA). Each experiment was performed with N = 5. The 100% hemolysis was determined by diluting 10 µl erythrocytes in 990 µl Millipore water. Then, 0% hemolysis was tested by adding pure buffer to the erythrocyte suspension. The degree of hemolysis produced by surfactants (%H) was calculated according to Eq. (1).

$$\%\mathrm{H} = \frac{\mathrm{Hb} - \mathrm{Hb}_0}{\mathrm{Hb}_{\mathrm{tot}}} \times 100 \quad (1)$$

Hb is the amount of hemoglobin found in the sample, Hb_0 is the amount of basal hemoglobin found in the blank, and Hb_{tot} is the amount of hemoglobin after 100% hemolysis, resp. their absorbance at 415 nm at 37 °C. Hemolytic activity ($HC_{5\%}$) was defined as the concentration at which at least 5% hemoglobin was found in the supernatant (adapted from [34,38]).

2.2.4. Photon correlation spectroscopy (PCS)

Size determination of micelles in 1% (m/V) surfactant solutions was performed on a Zetasizer nano ZS (Malvern Instruments, Herrenberg, Germany) with a 633-nm laser. The hydrodynamic diameter was calculated with DTS NANO v5.10 software (Malvern Instruments, Herrenberg, Germany). The sample, in single-use UV-plastic cuvettes (Brand GmbH and Co KG, Wertheim, Germany), was at first equilibrated for 1 min at 25 °C (and 5 min for 45 °C, respectively), and subsequently the time scale of the scattered light intensity fluctuations of the sample was measured. Each sample was recorded in triplicate, each with 20 runs. The size was obtained using the cumulants analysis by fitting a single exponential to the correlation function in order to obtain the volume-weighted mean size. The viscosity at 25 °C was used as input value for the fitting.

2.2.5. Viscometry

As viscosity is a crucial parameter for PCS measurements, the viscosity of the surfactant solutions was determined on an AMVn falling sphere viscometer (Anton Paar, Graz, Austria). For this, 800 µl of a 1% (m/V) surfactant solution was filled in a 1.6-mm-diameter tube, and viscosity was recorded from 20 °C to 45 °C ± 0.1 °C for every 5 °C. A 60° angle was applied, and the viscosity of every solution was determined 10 times.

2.2.6. Differential scanning calorimetry (DSC)

The 1% (m/V) aqueous surfactant solutions were analyzed using a DSC821 calorimeter (Mettler Toledo, Columbus, USA) and heated

from 10 °C to 75 °C at 10 °C/min. Transitions were analyzed using STAR software (Mettler Toledo, Columbus, USA) ($N = 1$).

2.2.7. Agitation stressing of human growth hormone formulations

Into 2R vials, 0.95 ml of a 0.2 mg/ml human growth hormone formulation was sterile filtrated. The protein was formulated in 10 mM phosphate buffer pH 7.2 containing different surfactant concentrations. Every concentration was tested in triplicate. A MM200 swinging mill (Retsch, Haan, Germany) was applied for a quick agitation stress for 10 min at 8 Hz. Consequently, protein recovery was studied using size exclusion chromatography (SEC), and protein aggregation was studied via turbidity (A_{350nm}), light obscuration and visual inspection for particulate matter.

2.2.8. Size exclusion chromatography (SEC)

The monomer content of the protein formulation as well as the dimer formation was investigated via SEC. A HP 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector and a 3000 SWXL gel filtration column (Tosoh Bioscience, Tokyo, Japan) was used for this experiment. Fifty microliters of a centrifuged protein sample was injected into the HPLC. Within the HPLC, a 0.2-μm online filter was used. The running buffer was composed of 50 mM NaH_2PO_4 and 150 mM NaCl at a pH of 7.2. The analytics were performed at a flow-rate of 0.5 ml/min with UV detection at 216 nm.

2.2.9. Turbidity measurement

Turbidity of the shaken samples was quantified by means of UV absorption at 350 nm. Two hundred microliters of samples was placed in a 96-well plate and tested in a Fluostar Omega reader with analysis software (BMG Labtech, Offenburg, Germany). Pure buffer was used as a blank sample.

2.2.10. Light obscuration

Subvisible particle counting was carried out via a light obscuration method. The particles are deduced by the size from the amount of light blocked as the particles pass in a single-file fashion. According to Ph. Eur. method 2.9.19 and USP method ⟨788⟩, the experiment was adapted using a reduced sample volume. The analysis was performed using a SVSS-c particle counting system (Pamas, Stuttgart, Germany). At fixed fill and ejection rates of 10 ml/min, each sample was measured at a volume of 0.3 ml per injection. A first injection of 0.5 ml was discarded to assure that the sample will end up directly in the Hamilton syringe, and the mean value was obtained from three injections. Between each sample analysis, the system was rinsed with water to the point at which the 1-μm particle count of the apparatus was less than 20. Subvisible particles bigger than or equal to 10 and 25 μm are presented as cumulative counts per ml.

2.2.11. Visual inspection

A visual inspection for particulate matter was performed in front of a black-and-white screen manually adapted to Ph. Eur. method 2.9.20.

3. Results and discussion

3.1. Synthesis of sugar-based surfactants Mono-PT, Mono-LT and Mono-CT

Few papers have previously been published on the preparation of fatty acid monoesters of α,α-trehalose, in any cases the primary hydroxyl group at C-6 was esterified [28,39,40]. Due to the symmetrical character of the disaccharide α,α-trehalose, both primary hydroxyl groups at C-6 and at C-6′ show equal reactivity. Consequently, the two monoesters at C-6-OH or C-6′-OH can be considered equivalent, but double acylation to give a 6,6′-diester has to be avoided. Chen et al. [28] obtained monolinoleoyl α,α-trehalose by a lipase-catalyzed condensation of trehalose with linoleic acid under sophisticated reaction conditions, Raku et al. [40] performed transesterifications of α,α-trehalose with a couple of fatty acid vinyl esters catalyzed by *Bacillus subtilis* protease to obtain various α,α-trehalose monoesters. In contrast, a lipase-catalyzed esterification of α,α-trehalose with a fatty acid, accelerated by microwave irradiation, gave mainly the 6,6′-diester [41]. In an alternative approach, Datta and Takayama [42] prepared fatty acid monoesters of α,α-trehalose in a non-enzymatic manner using a convenient application of silyl protective groups.

For the preparation of the target surfactants, an improved protocol based on Datta's work was developed (Scheme 1). Fully silylated 2,3,4,6,2′,3′,4′,6′-octa-*O*-(trimethylsilyl)-α,α-trehalose (**1**) was prepared from α,α-trehalose dihydrate using the silylating agents trimethylsilyl chloride and hexamethyldisilazane in anhydrous pyridine following the method described by Toubiana et al. [43], but with improved stoichiometry. Product **1** was easily purified by crystallization from methanol. Selective deprotection of both primary hydroxyl groups to give 2,3,4,2′,3′,4′-hexa-*O*-(trimethylsilyl)-α,α-trehalose (**2**) was performed using a methanolic potassium carbonate solution. After complete conversion, the reaction was stopped by the addition of a defined amount of glacial acetic acid to neutralize the reaction mixture. Using an excess of glacial acetic acid results in complete deprotection of the silylated α,α-trehalose. The crude product **2** could be used for the subsequent acylation step without further purification. Predominant monoacylation of the symmetrical diol **2** to give the monoesters **3a**, **3b**, **3c** of 2,3,4,2′,3′,4′-hexa-*O*-(trimethylsilyl)-α,α-trehalose was achieved using Steglich conditions [44]. In this reaction, the carboxylic acids add 1,3-DCC formatting *O*-acylisoureas. Esterification of the hydroxyl group is facilitated by an acyl group carrier like 4-DMAP. As a side reaction, the *O*-acylisourea can undergo a 1,3-rearrangement to give a non-reactive *N*-acylurea [45]. Typically, this side reaction is very slow, but if sterically ambitious molecules like the sixfold silylated α,α-trehalose and long chained fatty acids are used, this side reaction can cause a significant loss in yield. We found that using stoichiometric instead of catalytic amounts of 4-DMAP leads to a significant increase in yields of the monoacyl products. But still, this step gives only moderate yields (33–45%). The products were separated from unreacted starting material **2** and from 6,6′-diesters by flash column chromatography and were obtained as colorless oils. The aspired monoesters of α,α-trehalose **4a**, **4b**, **4c** were prepared by complete removal of the remaining six silyl protecting groups with trifluoroacetic acid in aqueous tetrahydrofuran. Mono-PT precipitated from the reaction mixture and could be separated by filtration. This compound could not be purified by silica gel flash column chromatography, since it is able to dissolve significant amounts of silica gel. Datta and Takayama [42] report on comparable observations with this monoester. Finally, crystallization from acetone gave pure Mono-PT (**4a**) as a white solid. Mono-LT (**4b**) and Mono-CT (**4c**) did not precipitate from the solution after deprotection. Removing the solvent using flash evaporation resulted in oily crude products which could be purified by flash column chromatography, giving Mono-LT and Mono-CT as white solids. The structures of all compounds were confirmed by the data from IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy.

Potential impurities within the trehalose-based excipient could be there agents used during synthesis of the excipient. Furthermore, the excipient could contain 6,6′-diester which is obtained due to double acylation of the 2,3,4,2′,3′,4′-hexa-*O*-(trimethylsilyl)-α,α-trehalose. After purification of the product, none of these substances could be detected by NMR spectroscopy or mass spectrometry.

3.2. Physical characterization

3.2.1. Surface tension and critical micelle concentration

Both surface tension measurements and pyrene interaction spectroscopy yielded comparable CMC results in water (Table 1). An increase in hydrophobic chain length resulted in a decrease in the CMC. This is in accordance with other publications on sugar-based surfactants [27,46]. The general rule that a two-carbon-atoms longer fatty acid results in an approximately 10-fold lower CMC could be applied to the trehalose monoesters [46]. The CMC at 25 °C was not significantly affected by the buffer salts used for hemolytic activity and protein stability tests (data not shown). This was expected as the surfactants are nonionic and should show only minor interaction with ions in the solution [47], when salts are used in concentrations to control pH and regulate osmolarity of the final drug product. Mono-CT showed a CMC of 1.92 mg/ml and a decrease in surface tension to 29 mN/m. Mono-LT reached a decrease in surface tension of ~39 mN/m at a CMC of 0.33 mg/ml. For polysorbates, similar values were observed, as PS 20 has its CMC at 0.15 mg/ml with a decrease in surface tension ~38 mN/m and PS 80 has a CMC of 0.014 mg/ml with surface tension values of ~45 mN/m [48].

3.2.2. Micelle size characterization and rheometry

PCS experiments were performed at 25 °C and 45 °C. Due to the relatively poor solubility at 25 °C, Mono-PT could not be analyzed at the lower temperature. As reported by Soderman for other sugar-based surfactants, all hydrodynamic diameters were in the range of 5–15 nm [49]. Mono-CT had a size of 5.0 nm at 25 °C and 5.5 nm at 45 °C, Mono-LT had a size of 6.3 nm at 25 °C and 7.3 nm at 45 °C, Mono-PT had a size of 10.1 nm at 45 °C (Table 2). The size increased with fatty acid chain length. The size range was comparable to other surfactants such as PS 20 (6.9 nm at 25 °C and 7.9 at 45 °C) or PS 80 (7.4 nm/9.1 nm) and is in good agreement with the data reported for other nonionic Macrogol-based surfactants [50]. Results were strongly dependent on viscosity and consequently on temperature. Temperature-dependent viscosities are displayed in Fig. 2a and b. For both Mono-CT and Mono-LT, viscosities of 1% (m/V) solutions were almost identical, with marginally lower values for Mono-CT. Mono-PT was only measured at 45 °C and in this area, the increase in viscosity was comparable with all three trehalose monoesters and in the range of the viscosity of water. The ratio of viscosity of a 1% (m/V) surfactant solution to pure water at any temperature between 20 and 45 °C was between 1.025 and 1.038. The maximum increase could be detected at 30 °C. In contrast, Sodermann et al., who worked with the same concentrations of tetradecylmaltoside, found a far more pronounced maximum of >1.2 at 60 °C (with 1.1 at 30 °C), indicating a shape transition of the micelles. Thus, the surfactants described here are expected to form spherical micelles and to preserve the structure in the temperature range evaluated.

3.2.3. DSC characterization

Calorimetric measurements of 1% (w/w) solutions showed no transitions for Mono-CT and Mono-LT in the range between 10 °C and 75 °C. In contrast, Mono-PT exhibited an endothermic transition at 43 °C. In this temperature range, the solubility also increases substantially. At this point, the flexibility of the fatty acid increases, and melting occurs. Therefore, the applicability of Mono-CT and Mono-LT in a temperature range which is relevant for biopharmaceuticals is given. In an additional test, no cloud point could be detected for 1% (m/m) aqueous solutions between 20 and 90 °C for Mono-CT and Mono-LT (data not shown).

Table 1
CMC of Mono-CT (**4c**), Mono-LT (**4b**) and Mono-PT (**4a**) in water as derived from surface tension measurements (STM) and pyrene fluorescence (PF).

	CMC_{STM} (mg/ml)	CMC_{PF} (mg/ml)	CMC_{STM} (mM)	CMC_{PF} (mM)
Mono-CT	2.14	1.92	4.32	3.87
Mono-LT	0.21	0.33	0.40	0.62
Mono-PT	0.006	n.d.[a]	0.01	n.d.

[a] Not determined.

Table 2
Volume mean diameter of different sugar surfactants and PS 20 and PS 80 at 25 and 45 °C.

	Volume mean diameter (nm) at 25 °C	Volume mean diameter (nm) at 45 °C
Mono-CT	5.0	5.5
Mono-LT	6.3	7.3
Mono-PT	n.d.[a]	10.12
PS 20	6.9	7.9
PS 80	7.4	9.1

[a] Not determined.

Table 3
Hemolytic activity of Mono-CT, Mono-LT and Mono-PT.

	$HC_{5\%}$ (mg/ml)	$HC_{5\%}$ (mM)
Mono-CT	0.1984	0.400
Mono-LT	0.0524	0.140
Mono-PT	0.025	0.05

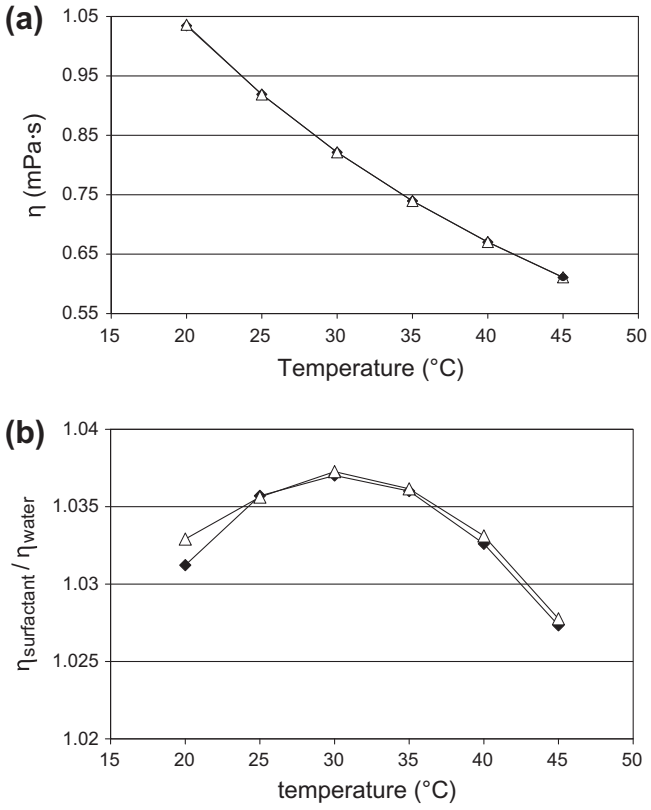

Fig. 2. Temperature dependence of the kinematic viscosity (a) and relative viscosity $\eta_{Surfactant}/\eta_{water}$ (b) of 1% solutions of Mono-CT (♦) and Mono-LT (△) (✸ Mono-PT at 45 °C).

3.2.4. Hemolytic activity

An important safety concern of surface-active substances is possible rupture of cell membranes and formation of mixed micelles with membrane lipids. All trehalose-based surfactants tended to destabilize blood cell membranes below their CMC. The $HC_{5\%}$ values were approximately 10 and 5 times below the CMC for Mono-CT and Mono-LT, respectively. Mono-PT shows a $HC_{5\%}$ at a concentration four times above CMC (Table 3). For comparison, PS 20 shows the same hemolytic activity at a concentration of 1 mg/ml, which is factor 10 higher than the CMC. Concentrations usually applied in protein stabilization are approximately at or slightly below CMC. Hence, Mono-CT and Mono-LT appear to be suitable candidates for further testing concerning protein stabilization, but hemolytic side effects should be carefully evaluated. For Mono-PT, the calculated HLB was 12.7, for Mono-LT 14.0 and for Mono-CT 14.8. In comparison, PS 20 has a HLB-value of 16.7 and PS 80 has a value of 15.0. Hence, both polysorbates are slightly more hydrophilic than the trehalose monoesters. In the literature, HLB-values are described as a less significant factor for hemolytic activity; however, the membrane interaction appears to be more dependent on the structural alignment of the surfactants [51,52]. Somfai et al. studied other sugar-based surfactants and found similar results for both highly pure substances and technical products, but the cell compatibility could be improved by redesigning the hydrophobic chain, e.g. branching at the hydroxyl group of 12-hydroxystearic acid [35,36,53]. In general, determining hemolytic activity of substances using washed red cells might result in lower $HC_{5\%}$ values than in whole blood, as other substances present in blood could compete with cell membranes for binding to surfactants.

3.3. Agitation stress of human growth hormone

Due to its poor solubility at 2–8 °C and 25 °C, Mono-PT was not included in the stability testing of hGH. The expected required concentration of surfactant for protein stabilization is in the range of 0.1–1 mg/ml (0.01–0.1% (m/V)) as PS 20 and 80 tend to show their best stabilizing behavior at these concentrations, just below and approximately at CMC. Consequently, a range of 0.001–5 mg/ml was included in the studies.

3.3.1. Particle formation

Visible particles were found in all formulations with surfactant concentrations ⩽0.1 mg/ml. Analysis of subvisible particles via light obscuration also indicated strong formation of particles >10 μm and >25 μm upon shaking (Fig. 3). During agitation for a short period, concentrations of 0.1 mg/ml PS 80 and Mono-CT suppress the formation of visible and subvisible aggregates. Samples with lower Mono-CT concentrations showed higher particle numbers than all other formulations but also a high standard deviation. For Mono-LT, 1 mg/ml was necessary for the stabilization. The turbidity measurements, detecting particles in the sub-μm range with high sensitivity [13], indicated the same trend. As for visible and subvisible particles, extensive protein aggregation occurs at concentrations below 1 mg/ml for Mono-CT and Mono-LT and below 0.1 mg/ml for PS 80 (Fig. 4). Thus, particle analysis demonstrates the stabilizing effect of Mono-CT and Mono-LT. Similar or slightly higher concentrations compared to PS 80 appear to be necessary for sufficient stabilization of hGH. hGH is known for its high aggregation tendency during agitation stress, and the stabilizing effects of surfactants have been described, for example, by Katakam et al. [54].

3.3.2. Size exclusion chromatography

Results on SEC show a clear dependency of monomer recovery from surfactant concentration for all surfactants (Fig. 5). Very

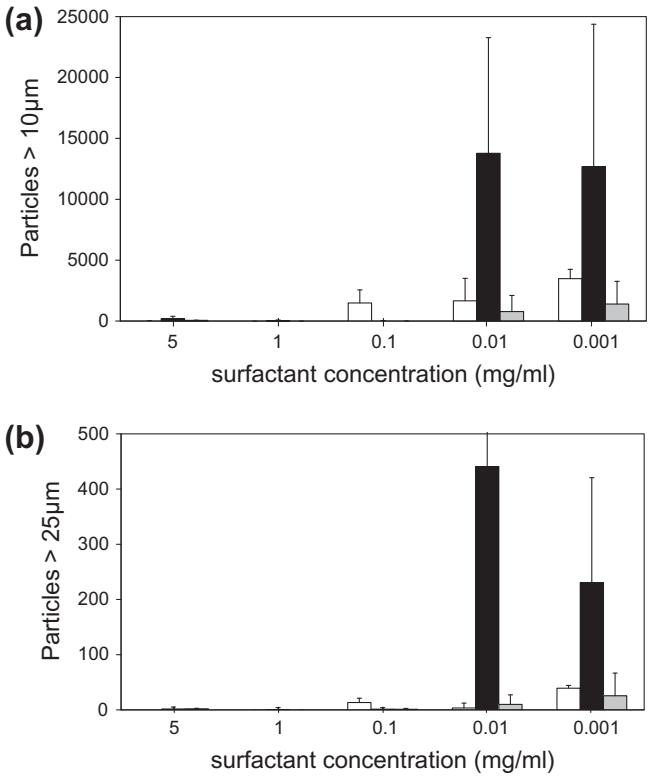

Fig. 3. Subvisible particles >10 μm (a) and >25 μm (b) of hGH solutions after agitation in the presence of Mono-LT (□), Mono-CT (■) and PS 80 (▩).

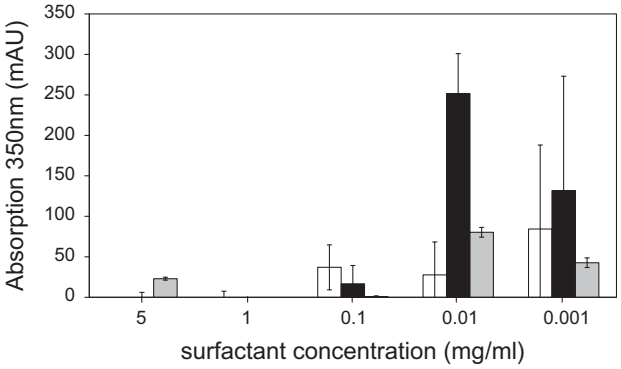

Fig. 4. Turbidity of hGH solutions after agitation in the presence of Mono-LT (□), Mono-CT (■) and PS 80 (▩).

low recovery of less than 40% resulted in the formulations containing 0.01 or 0.001 mg/ml surfactant as well as in the 0.1 mg/ml mono-LT and PS80 containing formations. This compared to a recovery of only 24.6 ± 8.6% in surfactant-free formulations. The amount of dimers and trimers recovered in SEC after agitation was negligible. This loss in recovery corresponded to substantial formation of large aggregates that were excluded from SEC, but detected by particle analysis and turbidity measurements. This could also be prevented by the new trehalose-based surfactants. For hGH, adsorption plays a major role and has been shown to occur at filter materials, silica or methylated silica surfaces [55–58]. In order to study this effect, hGH was also stored statically without shaking in surfactant-free formulation. The recovery of hGH decreased over time and leveled off after 6 h. After 24 h, 80% hGH were lost due to adsorption. Addition of 0.1% (m/V) PS 80, Mono-CT and Mono-LT could suppress this effect providing up to 95% recovery. Thus, the

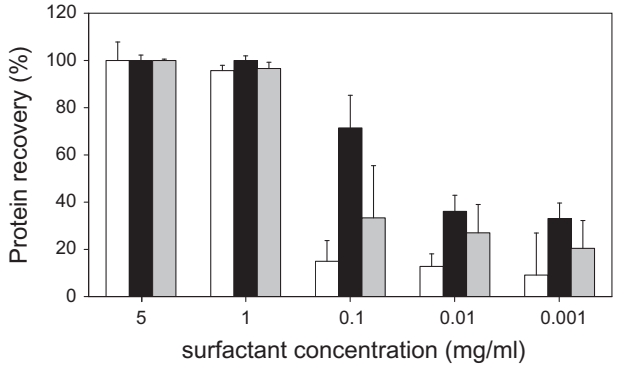

Fig. 5. SEC monomer recovery of hGH solutions after agitation in the presence of Mono-LT (□), Mono-CT (■) and PS 80 (■).

loss in monomer recovery in the shaken samples was due to both aggregation and adsorption. Recovery higher than 95% could only be assured at concentrations of 1 mg/ml and 5 mg/ml for all three surfactants. These concentrations were slightly above the minimum concentration necessary to suppress particle formation. Overall, Mono-CT trends to provide the highest recovery values.

Mono-CT as well as Mono-LT are comparable to PS 80 with regard to their protein-stabilizing ability in this case study with hGH. A concentration of 1 mg/ml seems to be sufficient to stabilize this hydrophobic protein against stress from harsh agitating with a high headspace volume. Polysorbate is usually applied in concentrations between 0.017 mg/ml and 1.6 mg/ml [59–62] for this purpose. This corresponds to the results of this study with a stabilizing concentration for both trehalose esters and PS 80 of 1 mg/ml. SEC revealed that hGH did not form dimers nor trimers upon agitation. Instead, marked formation of larger particles as demonstrated by turbidity measurement, subvisible and visible particles occurs. This corresponds to results seen by other groups [54,63]. Depending on the type of stress, different types of aggregates can be produced [13,64]. In this case, shaking induced the formation of very large vesicles.

4. Conclusions

Three trehalose-based surfactants were synthesized in high purity. Mono-PT with the lowest HLB value in this group shows insufficient water solubility at 2–8 °C and 25 °C and therefore appears to be less suitable for protein formulation. The two substances with shorter fatty acid chains, Mono-CT and Mono-LT, seem to have the potential to be applied in biopharmaceutical formulations. The formation of micelles and the reduction in surface tension are comparable to PS 80 and PS 20. Both these sugar-based surfactants show slightly higher hemolytic activity compared to polysorbates. A change from a linear to a branched hydrophobic part of the surfactant might lead to an improvement in regard to the ratio of CMC to hemolytic activity as described by Somfai et al. for other sugar-based surfactants [35]. Mono-CT and Mono-LT form transparent and colorless aqueous solutions and do not show phase transitions or separation at pharmaceutically applied temperatures. Due to their high purity and their molecular structure, induction of chemical instability of biopharmaceuticals is less likely compared to polysorbates. Similar to polysorbates, Mono-CT and Mono-LT were able to stabilize hGH against aggregation upon shaking as well as adsorption. As trehalose is known to be a good bulking agent and lyoprotector [65], these substances might show an even better performance in freeze-dried formulations. Consequently, trehalose-based surfactants offer great potential for application in biopharmaceutical formulations.

Potential use requires large toxicological studies, and enourmous regulatory hurdles would have to be met, but some manufacturers of sugar-based surfactants got approval for clinical use after only a few animal studies [66], and increased IP space by application of new excipients could trigger this.

References

[1] Sanofi-Aventis, Lantus, full prescribing information (03/2007).
[2] Roche, Mircera, full prescribing information.
[3] Genentech, Nutropin AQ, full prescribing information (01/2008).
[4] Genentech, Herceptin, full prescribing information (03/2009).
[5] D. Wu, Y.-Y. Hao, et al., Inhibition of degradation and aggregation of recombinant human consensus interferon-alpha mutant expressed in Pichia pastoris with complex medium in bioreactor, Appl. Microbiol. Biotechnol. 80 (6) (2008) 1063–1071.
[6] S.A. Charman, K.L. Mason, W.N. Charman, Techniques for assessing the effects of pharmaceutical excipients on the aggregation of porcine growth hormone, Pharm. Res. 10 (7) (1993) 954–962.
[7] N.B. Bam, T.W. Randolph, et al., Stability of protein formulations: investigation of surfactant effects by a novel EPR spectroscopic technique, Pharm. Res. 12 (1) (1995) 2–11.
[8] N.B. Bam, J.L. Cleland, J. Yang, M.C. Manning, J.F. Carpenter, R.F. Kelley, T.W. Randolph, Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions, J. Pharm. Sci. 87 (12) (1998) 1554–1559.
[9] L. Kreilgaard, L.S. Jones, et al., Effect of Tween 20 on freeze-thawing- and agitation-induced aggregation of recombinant human factor XIII, J. Pharm. Sci. 87 (12) (1998) 1597–1603.
[10] S.D. Webb, J.L. Cleland, et al., A new mechanism for decreasing aggregation of recombinant human interferon-gamma by a surfactant: slowed dissolution of lyophilized formulations in a solution containing 0.03% polysorbate 20, J. Pharm. Sci. 91 (2) (2002) 543–558.
[11] B.S. Chang, B.S. Kendrick, et al., Surface-induced denaturation of proteins during freezing and its inhibition by surfactants, J. Pharm. Sci. 85 (12) (1996) 1325–1330.
[12] W. Wang, Y.J. Wang, et al., Dual effects of Tween 80 on protein stability, Int. J. Pharm. 347 (1-2) (2008) 31–38.
[13] S. Kiese, A. Papppenberger, et al., Shaken, not stirred: mechanical stress testing of an IgG1 antibody, J. Pharm. Sci. 97 (10) (2008) 4347–4366.
[14] V.M. Knepp, J.L. Whatley, A. Muchnik, T.S. Calderwood, Identification of antioxidants for prevention of peroxide-mediated oxidation of recombination human ciliary neurotrophic factor and recombinant human nerve growth factor, J. Pharm. Sci. Technol. 50 (1996) 163–171.
[15] E. Ha, W. Wang, et al., Peroxide formation in polysorbate 80 and protein stability, J. Pharm. Sci. 91 (10) (2002) 2252–2264.
[16] E. Azaz, R. Segal, et al., Hemolysis caused by polyoxyethylene-derived surfactants. Evidence for peroxide participation, Biochim. Biophys. Acta 646 (3) (1981) 444–449.
[17] E. Azaz, R. Segal, I. Milo-Goldzweig, Hemolysis caused by polyoxyethylene-derived surfactants. Evidence for peroxide participation, Biochim. Biophys. Acta 646 (3) (1981) 444–449.
[18] S. Savic, G. Vuleta, et al., Colloidal microstructure of binary systems and model creams stabilized with an alkylpolyglucoside non-ionic emulsifier, Colloid Polym. Sci. 283 (4) (2005) 439–451.
[19] A.-K. Kirchherr, A. Briel, et al., Stabilization of indocyanine green by encapsulation within micellar systems, Mol. Pharm. 6 (2) (2009) 480–491.
[20] M. El-Laithy Hanan, Novel transdermal delivery of Timolol maleate using sugar esters: preclinical and clinical studies, Eur. J. Pharm. Biopharm. 72 (1) (2009) 239–245.
[21] O. Lopez, M. Cocera, et al., Applications of alkyl glucosides in the solubilization of liposomes and cell membranes, Surfact. Sci. Ser. 143 (2009) 585–626. Sugar-Based Surfactants.
[22] G.G. Prive, Detergents for the stabilization and crystallization of membrane proteins, Methods 41 (4) (2007) 388–397.
[23] M.T. Garcia, I. Ribosa, et al., Ecological properties of alkylglucosides, Chemosphere 35 (3) (1997) 545–556.
[24] I.J.A. Baker, B. Matthews, et al., Sugar fatty acid ester surfactants: structure and ultimate aerobic biodegradability, J. Surfact. Deterg. 3 (1) (2000) 1–11.
[25] W. von Rybinski, K. Hill, Alkyl polyglycosides – properties and applications of a new class of surfactants, Angew. Chem. Int. Ed. 37 (10) (1998) 1328–1345.
[26] K. Hill, Industrial development and application of biobased oleochemicals, Pure Appl. Chem. 79 (11) (2007) 1999–2011.
[27] J. Chen, Y. Kimura, et al., Surface activities of monoacyl trehaloses in aqueous solution, LWT-Food Sci. Technol. 40 (3) (2006) 412–417.
[28] J. Chen, Y. Kimura, et al., Synthesis of linoleoyl disaccharides through lipase-catalyzed condensation and their surface activities, J. Biosci. Bioeng. 100 (3) (2005) 274–279.
[29] T. Rades, C.C. Mueller-Goymann, Electron and light microscopical investigation of defect structures in mesophases of pharmaceutical substances, Colloid Polym. Sci. 275 (12) (1997) 1169–1178.
[30] A.V. Kuehn, R.H.H. Neubert, Characterization of mixtures of alkyl polyglycosides (plantacare) by liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry, Pharm. Res. 21 (12) (2004) 2347–2353.

[31] J.L. Cleland, X. Lam, et al., A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody, J. Pharm. Sci. 90 (3) (2001) 310–321.
[32] M. Uritani, M. Takai, et al., Protective effect of disaccharides on restriction endonucleases during drying under vacuum, J. Biochem. 117 (4) (1995) 774–779.
[33] W.Q. Sun, P. Davidson, Protein inactivation in amorphous sucrose and trehalose matrixes: effects of phase separation and crystallization, Biochim. Biophys. Acta, Gen. Subj. 1425 (1) (1998) 235–244.
[34] T. Reinhart, K.H. Bauer, Investigations on the hemolysis and solubilization behavior of nonionic polymeric surfactants, Pharmazie 50 (6) (1995) 403–407.
[35] E. Soederlind, L. Karlsson, Haemolytic activity of maltopyranoside surfactants, Eur. J. Pharm. Biopharm. 62 (3) (2006) 254–259.
[36] E. Soderlind, M. Wollbratt, et al., The usefulness of sugar surfactants as solubilizing agents in parenteral formulations, Int. J. Pharm. 252 (1-2) (2003) 61–71.
[37] K. Kalyanasundaram, J.K. Thomas, Environmental effects on vibronic band intensities in pyrene monomer fluorescence and their application in studies of micellar systems, J. Am. Chem. Soc. 99 (7) (1977) 2039–2044.
[38] U.T. Seyfert, V. Biehl, et al., In vitro hemocompatibility testing of biomaterials according to the ISO 10993-4, Biomol. Eng. 19 (2-6) (2002) 91–96.
[39] K. Suzuki et al., Structural requirements for alkyl glycoside-type renal targeting vector, Pharm. Res. 16 (7) (1999) 1026–1034.
[40] T. Raku, M. Kitagawa, H. Shimakawa, Y. Tokiwa, Enzymatic synthesis of trehalose esters having lipophilicity, J. Biotechnol. 100 (1) (2003) 203–208.
[41] M. Gelo-Pujic, E. Guibé-Jampel, A. Loupy, S.A. Galema, D. Mathé, Lipase-catalysed esterification of some α-D-glucopyranosides in dry media under focused microwave irradiation, J. Chem. Soc. Perkin Trans. 1 (1996) 2777–2780.
[42] A.K. Datta, K. Takayama, An improved synthesis of trehalose 6-mono- and 6,6′-di-corynomycolates and related esters, Carbohydr. Res. 218 (1) (1991) 95–109.
[43] R. Toubiana, B.C. Das, J. Defaye, B. Mompon, M.J. Toubiana, Étude du cord-factor et de ses analogues, Carbohydr. Res. 44 (2) (1975) 308–312.
[44] G. Höfle, W. Steglich, H. Vorbrüggen, 4-Dialkylaminopyridines as highly active acylation catalysts, Angew. Chem. Int. Ed. 17 (8) (1978) 569–583.
[45] E.F.V. Scriven, 4-Dialkylaminopyridines: super acylation and alkylation catalysts, Chem. Soc. Rev. 12 (1) (1983) 129–161.
[46] R. Aveyard, B.P. Binks, et al., Surface and colloid chemistry of systems containing pure sugar surfactant, Langmuir 14 (17) (1998) 4699–4709.
[47] M.V. Thorsteinsson, J. Richter, et al., 5-Dodecanoylaminofluorescein as a probe for the determination of critical micelle concentration of detergents using fluorescence anisotropy, Anal. Biochem. 340 (2) (2005) 220–225.
[48] l.S. Wan, P.F. Lee, CMC of polysorbates, J. Pharm. Sci. 63 (1) (1974) 136–137.
[49] A. Ericsson Caroline, O. Soderman, et al., Effects of temperature, salt, and deuterium oxide on the self-aggregation of alkylglycosides in dilute solution. 2, n-Tetradecyl-beta-D-maltoside 21 (4) (2005) 1507–1515.
[50] P. Becher, H. Arai, Nonionic surface-active compounds. XI. Micellar size, shape, and hydration from light-scattering and hydrodynamic measurements, J. Colloid Interface Sci. 27 (4) (1968) 634–641.
[51] M. Ohnishi, H. Sagitani, The effect of nonionic surfactant structure on hemolysis, J. Am. Oil Chem. Soc. 70 (7) (1993) 679–684.
[52] B.Y. Zaslavsky, N.N. Ossipov, et al., Action of surface-active substances on biological membranes. II. Hemolytic activity of nonionic surfactants, Biochim. Biophys. Acta 507 (1) (1978) 1–7.
[53] K. Neimert-Andersson, S. Sauer, et al., Synthesis of new sugar-based surfactants and evaluation of their hemolytic activities, J. Org. Chem. 71 (9) (2006) 3623–3626.
[54] M. Katakam, L.N. Bell, et al., Effect of surfactants on the physical stability of recombinant human growth hormone, J. Pharm. Sci. 84 (6) (1995) 713–716.
[55] Y.-F. Maa, C.C. Hsu, Membrane fouling in sterile filtration of recombinant human growth hormone, Biotechnol. Bioeng. 50 (3) (1996) 319–328.
[56] J. Buijs, D.W. Britt, et al., Human growth hormone adsorption kinetics and conformation on self-assembled monolayers, Langmuir 14 (2) (1998) 335–341.
[57] Y.-F. Maa, C.C. Hsu, Investigation on fouling mechanisms for recombinant human growth hormone sterile filtration, J. Pharm. Sci. 87 (7) (1998) 808–812.
[58] J. Buijs, V. Hlady, Adsorption kinetics, conformation, and mobility of the growth hormone and lysozyme on solid surfaces, studied with TIRF, J. Colloid Interface Sci. 190 (1) (1997) 171–181.
[59] A. Hawe, W. Friess, Development of HSA-free formulations for a hydrophobic cytokine with improved stability, Eur. J. Pharm. Biopharm. 68 (2) (2008) 169–182.
[60] M.J. Treuheit, A.A. Kosky, et al., Inverse relationship of protein concentration and aggregation, Pharm. Res. 19 (4) (2002) 511–516.
[61] A. Hawe, V. Filipe, et al., Fluorescent molecular rotors as dyes to characterize polysorbate-containing IgG formulations, Pharm. Res. 27 (2) (2010) 314–326.
[62] W. Wang, Instability, stabilization, and formulation of liquid protein pharmaceuticals, Int. J. Pharm. 185 (2) (1999) 129–188.
[63] R.J. St. John, J.F. Carpenter, et al., High pressure refolding of recombinant human growth hormone from insoluble aggregates: structural transformations, kinetic barriers, and energetics, J. Biol. Chem. 276 (50) (2001) 46856–46863.
[64] H.-C. Mahler, F. Senner, et al., Surface activity of a monoclonal antibody, J. Pharm. Sci. 98 (12) (2009) 4525–4533.
[65] W. Wang, Lyophilization and development of solid protein pharmaceuticals, Int. J. Pharm. 203 (1-2) (2000) 1–60.
[66] E.T. Maggio, Novel excipients prevent aggregation in manufacturing and formulation of protein and peptide therapeutics, BioProcess Int. (11/2008), vol. 6, 2008.