Electronic Supplementary Material (ESI) for MedChemComm.

Methods of Protein Surface PEGylation under Structure Preservation for the Emulsion-based Formation of Stable Nanoparticles

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Materials

Methoxypolyethylene glycol 2 kDa (mPEG), 2,4,6-trichloro-1,3,5-triazine (TsT), sodium hydride (60% dispersion in mineral oil), anhydrous benzene, hen egg white lysozyme [EC 3.2.1.17] (lyophilized powder, protein ≥90%, ≥40,000 units/mg protein), coomassie brilliant blue G, 2,4,6-trinitrobenzenesulfonic acid (TNBS), 4-methylumbelliferyl-*β*-D-N,N′,N″ triacetylchitotrioside ($(GlcNAc)_3MeU$), phosphate buffered saline (PBS), glycine and *n*-hexylamine (99%) were purchased from Sigma-Aldrich, St. Louis. Epichlorohydrin (pur. > 99%), 4-nitrophenyl chloroformate (pur. > 98%) and fluorescamine were purchased from TCI Europe N.V. *α*-Methoxy-*ω*-carboxy PEG 2000 was purchased from Rapp Polymere, Germany. 2,3,5,6-Tetrafluorophenol (97%) was purchased from Alfa Aesar, Germany. Zinc oxide and Rotiload® 1, Rotiphorese® Gel 30, tetramethylethylenediamine (TEMED) were purchased from Carl Roth, Germany. The PageRuler Prestained Protein Ladder for SDS-PAGE was purchased from Thermo Scientific, Germany. *N,N*′-Dicyclohexylcarbodiimide (DCC) was purchased from Acros Organics, Germany, and all used organic solvents were purchased from different suppliers and distilled once prior use.

Nuclear Magnetic Resonance Spectroscopy (NMR)

For standard analytical purpose ^{1}H-NMR and ^{13}C-NMR spectra were recorded at 300 MHz with the Bruker Topspin Fourier 300 MHz and at 600 MHz with the Bruker Avance III 600. The experiments were performed at room temperature using the indicated solvents mostly $CDCl_3$, D_2O or d_6-DMSO. The chemical shifts were measured against the solvent signal are reported in ppm from TMS. For the description of the signals the following abbreviations were used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Assignments were carried out according to the literature.

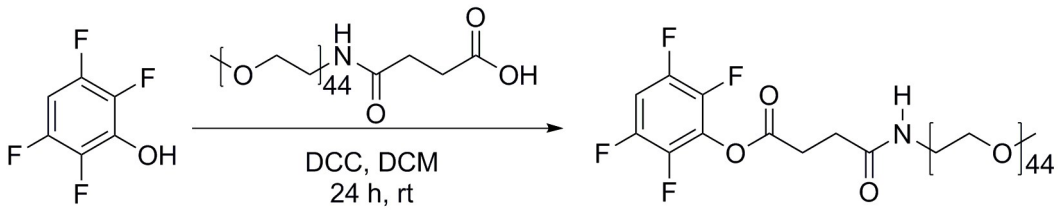
FT-Infrared Spectroscopy (FT-IR)

The compounds were measured pure on the diamond crystal of the device with the Nicolet Avatar 330-IR ATR-Unit. Thermo Electron Corporation. The types of signal are classified in four intensities: vs (very strong), s (strong), w (weak) and br (broad). The signals are reported in [cm^{-1}] and assignments were carried out according to the literature.

1. Activation of mPEG

1.1 Synthesis

1.1.1 Preparation of 2,3,5,6-Tetrafluorophenyl 4-((2-methoxypolyethylene glycol)amino)-4 oxobutanoate (TFP-mPEG)



mPEG was activated according to Zhang et al.[1] To a solution of *α*-methoxy-*ω*-carboxy PEG 2000 (200 mg, 0.1 mmol) and tetrafluorophenol (18.3 mg, 0.1 mmol) in dichloromethane (DCM) (15 mL) was added DCC (24.8 mg, 0.12 mmol) at room temperature. After being stirred for 24 h, the mixture was diluted with acetone (50 mL) and filtered. The solvent was removed under reduced pressure and the product was dried *in vacuo* (61.43%).

^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) *δ*(ppm) = 7.05–6.95 (m, 1H), 6.60–6.52 (m, 1H), 3.64 (s, 184H), 3.38 (s, 3H), 3.04 (t, *J* = 7.0 Hz, 2H), 2.64 (t, *J* = 7.2 Hz, 2H)

^{13}C NMR (600 MHz, $CDCl_3$, Me_4Si) *δ*(ppm) = 103.32, 70.69, 39.55, 30.54, 28.92

IR $\tilde{\nu}$ (max/cm^{-1}) = 2882vs (CH_3), 1651s (CO)

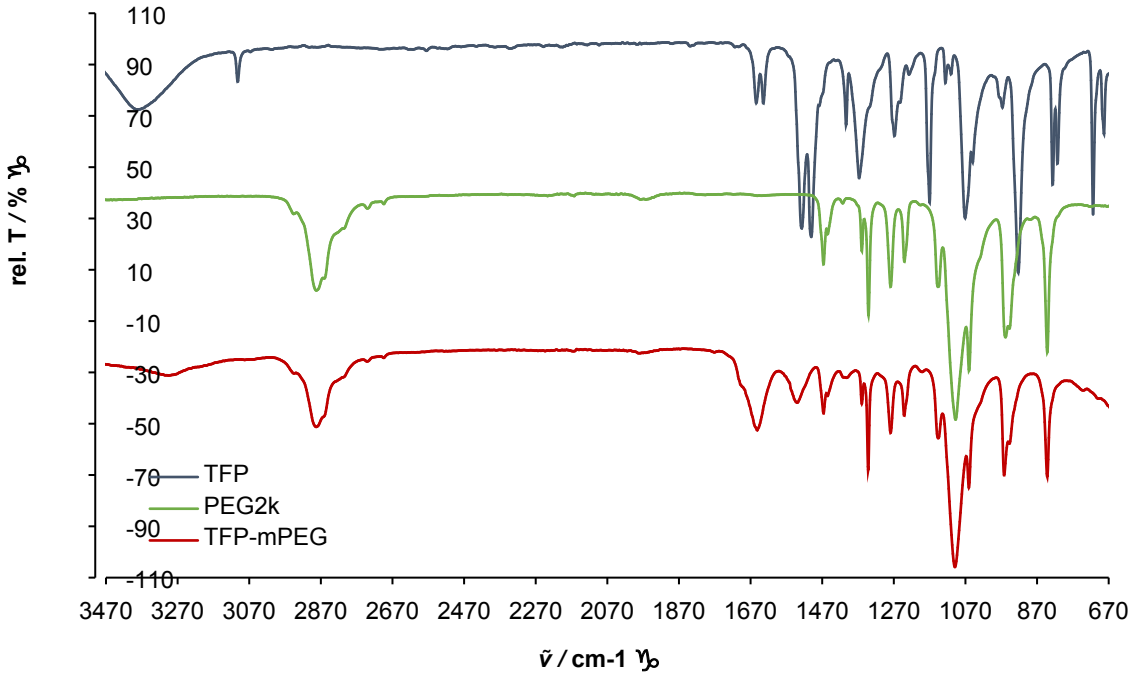
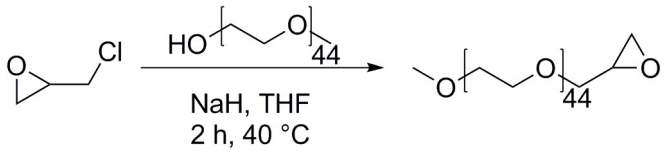


Figure 1: IR diagram of tetrafluorophenol (TFP, blue), mPEG 2000 (green) and TFP-mPEG (red). The activated mPEG shows one peak that can be assigned to TFP (1651 cm^{-1}).

1.1.2 Preparation of 2-Methoxypolyethylene glycol-oxirane (epoxy-mPEG)



The activation of mPEG 2000 was synthesized according to Park et al.[2] Sodium hydride (137 mg, 5.7 mmol) and epichlorohydrin (531 mg, 5.7 mmol) were added to 20 mL of dry tetrahydrofuran (THF) under argon atmosphere. Then mPEG (1 g, 0.5 mmol) was added at 40 °C and stirred for 2 h. After the reaction, the mixture was filtered and poured into 200 mL of cold diethyl ether. The light yellow solid was filtered, dissolved in THF and precipitated again with diethyl ether and dried *in vacuo* (92%).

^{1}H NMR (300 MHz, DMSO, Me_4Si) δ(ppm) = 3.52 (d, J = 8.1 Hz, 180H), 3.24 (s, 3H), 3.09 (m, J = 9.1, 3.3 Hz, 1H), 2.74–2.69 (m, 1H), 2.55–2.52 (m, 1H)

^{13}C NMR (300 MHz, DMSO, Me_4Si) δ(ppm) = 42.57, 49.96, 69.96

IR $\tilde{\nu}$ (max/cm^{-1}) = 2882vs (CH_3), 1466br (CO)

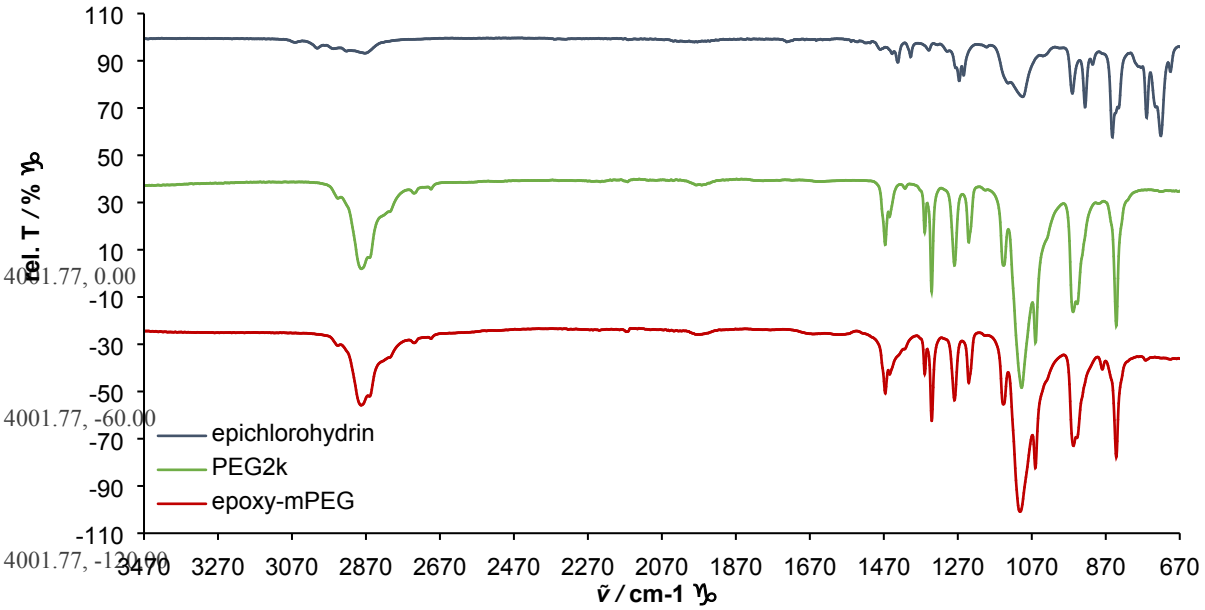
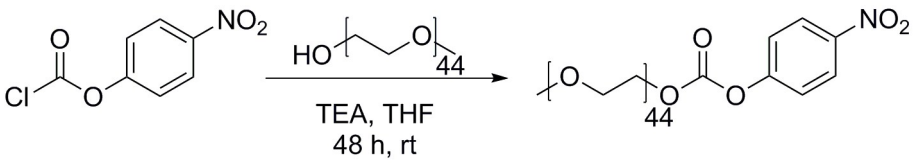


Figure 2: IR diagram of epichlorohydrin (blue), mPEG 2000 (green) and epoxy-mPEG (red). The activated mPEG shows one peak that can be assigned to epichlorohydrin (1466 cm^{-1}).

1.1.3 Preparation of 2-Methoxypolyethylene glycol-4-nitrophenyl carbonate (carbonate-mPEG)



Methoxypolyethylene glycol was activated according to Qi et al.[3] mPEG 2000 (1 g, 0.5 mmol) was dissolved in 100 mL of dry tetrahydrofuran (THF) at room temperature, and *p*-nitrophenyl chloroformate (300 mg, 1.5 mmol) and triethylamine (150 mg, 1.5 mmol) were added, followed by stirring for 48 h at room temperature. The obtained carbonate mPEG mixture was purified by recrystallization of the product from diethyl ether and dried *in vacuo* (70%).

^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ(ppm) = 8.28 (d, J = 9.2 Hz, 2H), 7.40 (d, J = 9.2 Hz, 2H), 4.51–4.37 (m, 2H), 3.64 (s, 178H), 3.38 (s, 3H)

^{13}C NMR (300 MHz, $CDCl_3$, Me_4Si) δ(ppm) = 70.90, 121.74, 125.25

IR $\tilde{\nu}$ (max/cm^{-1}) = 2882vs (CH_3), 1766s (CO), 1616vw and 1594vw (NO)

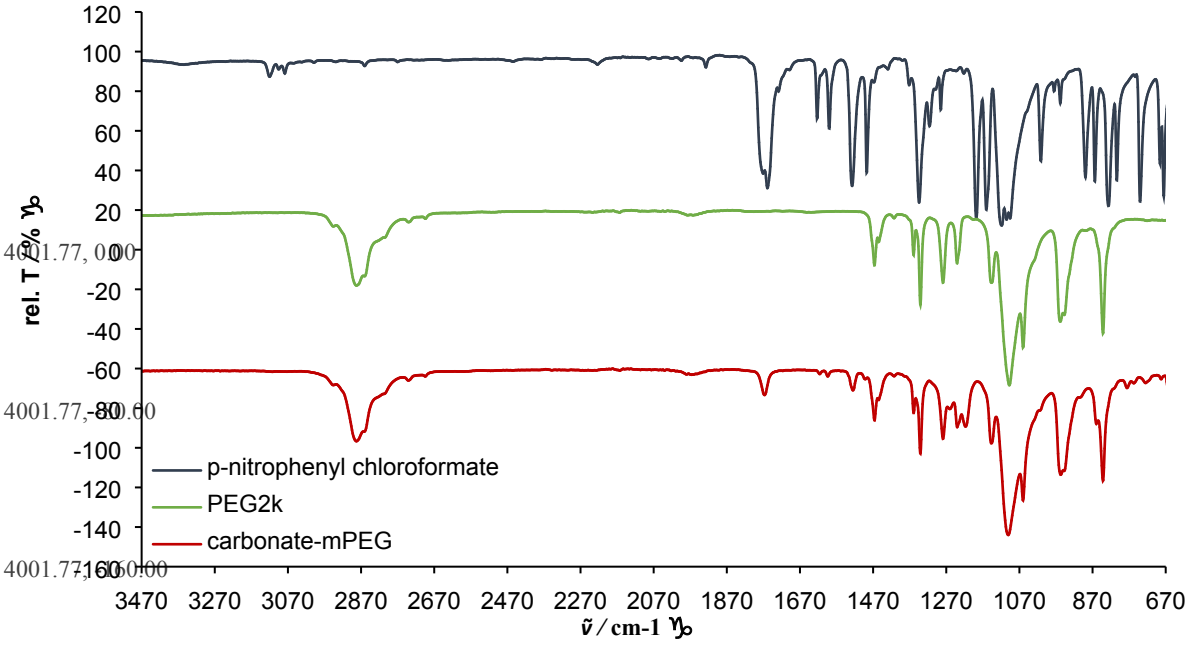
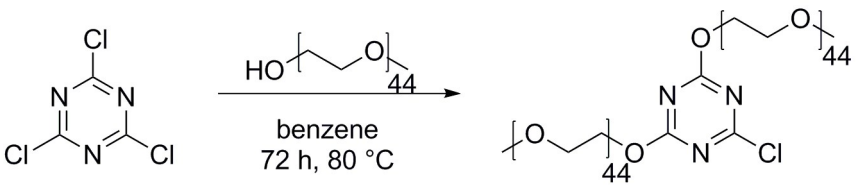


Figure 3: IR diagram of *p*-nitrophenyl chloroformate (blue), mPEG 2000 (green) and the activated mPEG with *p*-nitrophenyl chloroformate (carbonate-mPEG, red). Three peaks can assigned to *p*-nitrophenyl chloroformate (1766, 1616, 1594 cm^{-1}).

1.1.4 Preparation of 2,4-*ω*-Methoxypolyethylene glycol-6-chloro-triazine (TsT-mPEG)



Activated mPEG 2000 was synthesized according to Inada et al.[4] *α*-Methoxy-*ω*-hydroxy polyethylene glycol (mPEG) (1 g, 0.5 mmol), molecular sieve (4 Å, 1 g), zinc oxide (1.14 g, 14 mmol) and 2,4,6-trichloro-1,3,5-triazine (TsT) (46.1 mg, 0.25 mmol) were added to 3 mL of anhydrous benzene and stirred at 80 °C for 72 h. The mixture was centrifuged (15000 × g, 15 min.) and zinc oxide was separated. The supernatant was added drop wise with vigorous stirring to petroleum ether (30 mL). The product was isolated by centrifugation (15000 × g, 15 min). The resulting pellet was redissolved in benzene and precipitated in petroleum ether (30 mL) again. The washing step was repeated five times. The white solid was dried under reduced pressure and stored under argon atmosphere at -20 °C (74.28%).

Elemental analysis was performed to determine the nitrogen content (Found: C, 52.17; H, 10.88; N, 0.9. $C_{181}H_{358}N_3Cl$ requires C, 52.87; H, 8.71; N, 1.02%), indicates a modification of 88% of TsT with two mPEG chains per molecule.

^{13}C NMR (300 MHz, DMSO, Me_4Si) *δ*(ppm) = 69.96

IR $\tilde{\nu}$ (max/cm^{-1}) = 2882vs (CH_3), 1729s (CO), 759w (CCl)

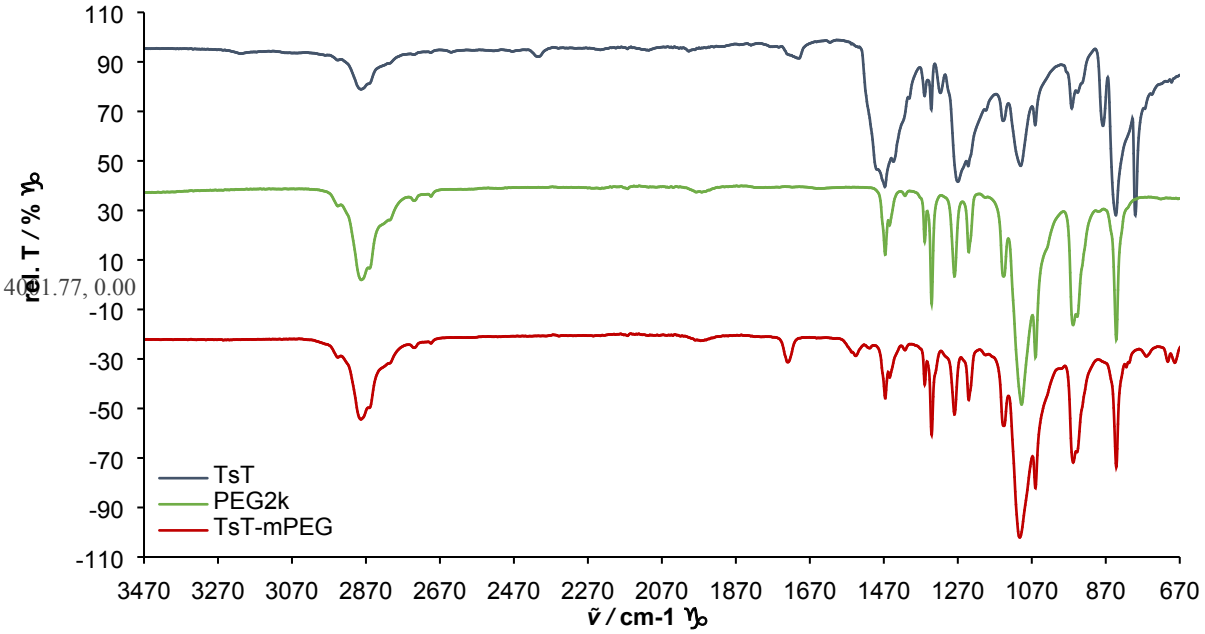


Figure 4: IR diagram of 2,4,6-trichloro-1,3,5-triazine (TsT, blue), mPEG 2000 (green) and TsT-mPEG (red). The activated mPEG shows two peaks that can be assigned to TsT (1729, 759 cm^{-1}).

1.2 Size-Exclusion Chromatography (SEC)

The activated mPEG was analyzed with size-exclusion chromatography (SEC). The measurements were performed in DMF containing 1 g/L lithium bromide (LiBr) with a sample concentration of 1-3 mg/mL on an Agilent 1100 Series, using PSS (Polymer Standards Service) as standard. A HEMA 300/100/40 column (*l*: 95.0 cm; *d*: 0.8 cm) was used with a flow rate of 1 mL/min at 50.0 °C (Agilent G1316A). A refractive index detector (Agilent G1362A) and an UV detector (Agilent G1314A) were used for detection and the results were obtained with the Fa. PSS WinGPC Unity software.

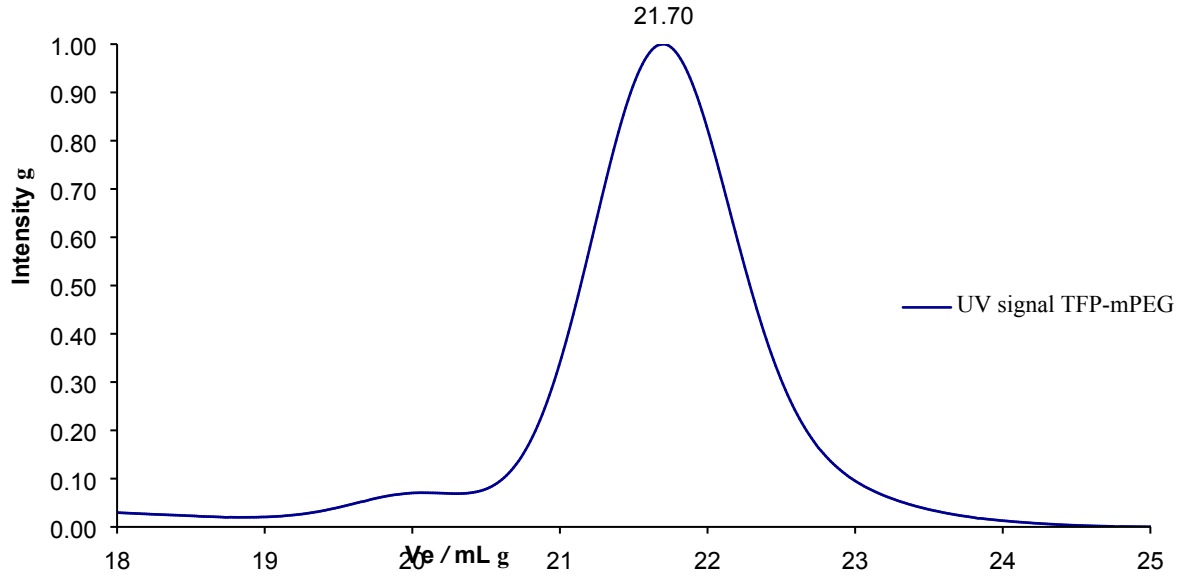


Figure 5: SEC diagram of TFP-mPEG. This diagram indicates that TFP is modified with one mPEG chain (V_e = 21.70 mL). mPEG itself produces a RI signal at 21.65 mL and no UV-signal and show a small “barrow” at V_e = 20.00 mL.

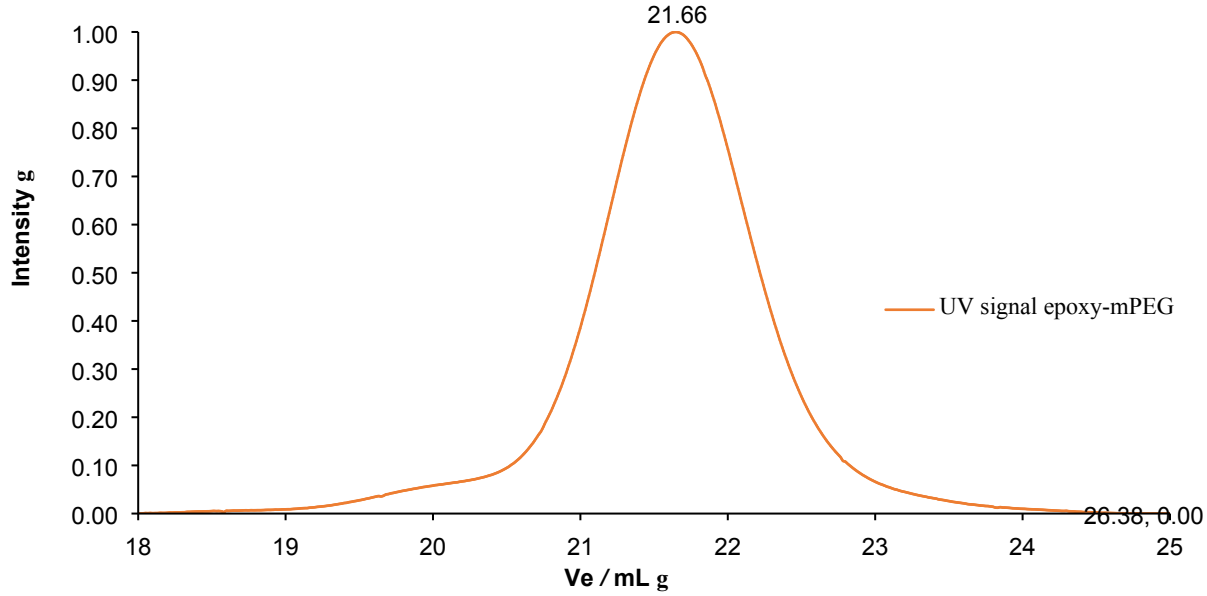


Figure 6: SEC diagram of epoxy-mPEG. The diagram indicates that epoxy-mPEG present one mPEG chain (V_e = 21.66 mL). mPEG itself produces a RI signal at 21.65 mL and no UV-signal.

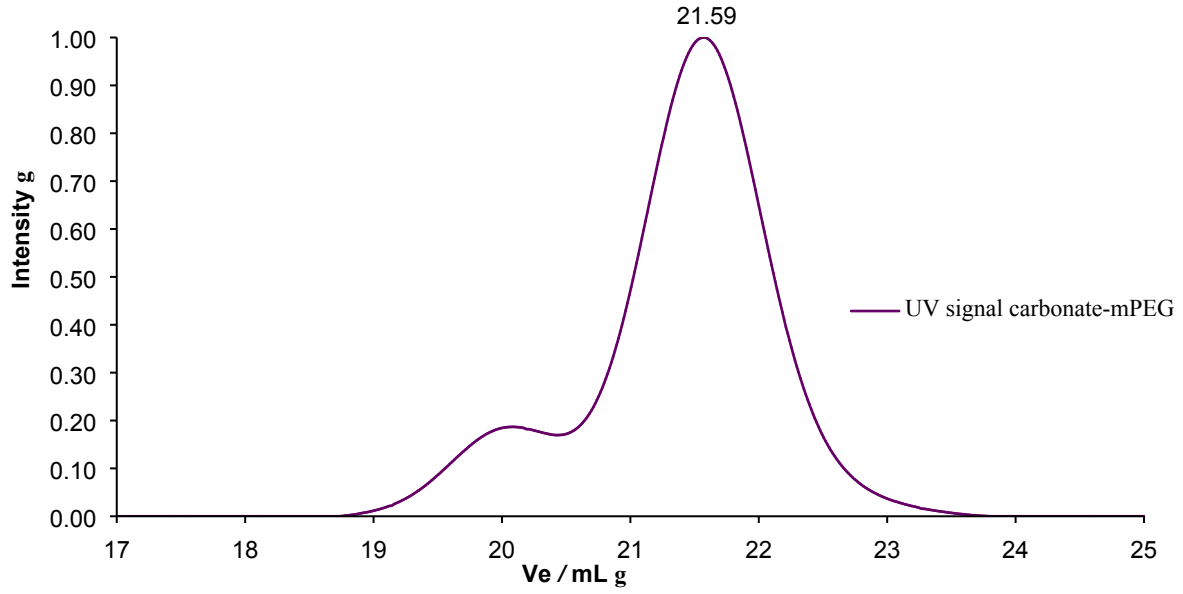


Figure 7: SEC diagram of carbonate-mPEG. An UV signal is measurable after the activation of mPEG with *p*-nitrophenyl chloroformate. The diagram indicates that carbonate-mPEG present one mPEG chain (V_e = 21.59 mL). At V_e = 20.00 mL a small "barrow" is present which can be ascribe to mPEG itself (see other SEC diagrams). The polymer produces a RI signal at 21.65 mL and no UV-signal.

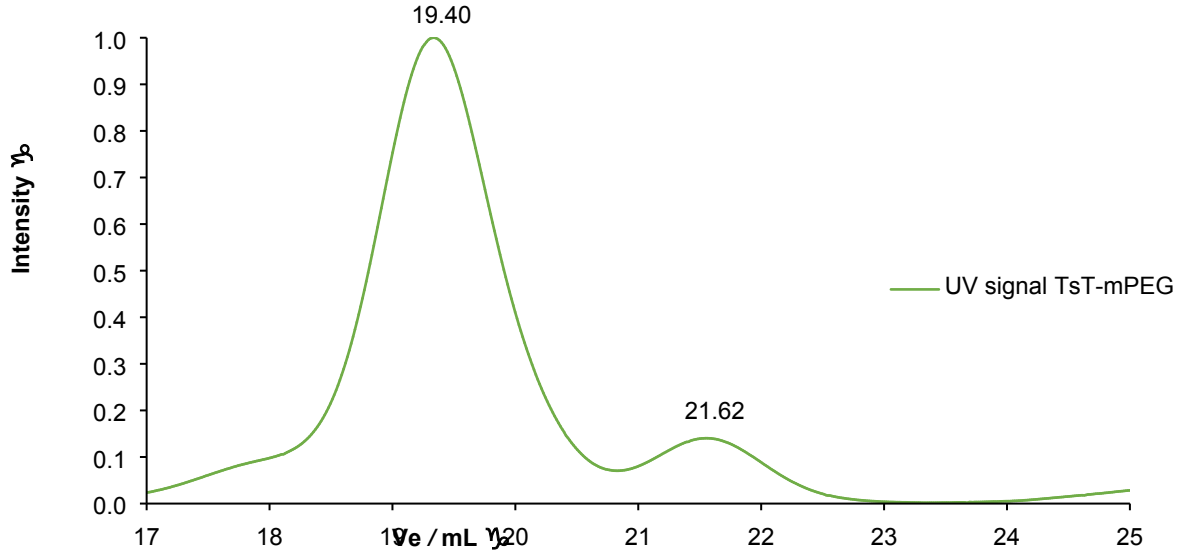
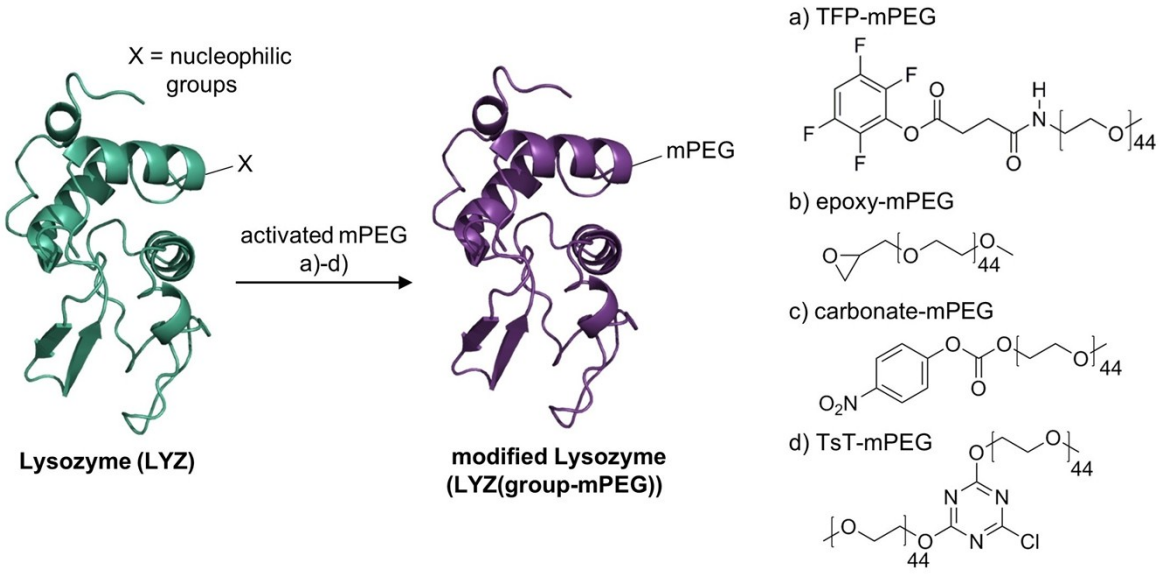


Figure 8: SEC diagram of TsT-mPEG. An UV signal is measurable after the activation of mPEG. This diagram indicates, that most TsT molecules are modified with two mPEG chains (V_e = 19.40 mL; compare elementary analysis). As byproduct, TsT with one mPEG chain exists (V_e = 21.62 mL). mPEG itself produces a RI signal at 21.65 mL and no UV-signal.

2. Protein Modification and Analysis

2.1 Protein Modification



2.1.1 Modification of LYZ with TFP-mPEG

Lysozyme [EC 3.2.1.17] (15.0 mg, 1.0 *µ*mol) was dissolved in 3 mL of 0.1 M carbonate buffer (pH 8) and combined with TFP-mPEG (48.4 mg, 22.0 *µ*mol). The mixture was stirred at room temperature for 48 h. The product was purified by dialysis (Slide-A-Lyzer® Dialysis Cassettes, 10000 MWCO) against water for 3 days and was then lyophilized (ALPHA 1-2 LD plus). The molecular weight of the protein after modification was approx. 20-24 kDa (MALDI-ToF MS), which indicates that one protein was modified with around 4 mPEG chains.

2.1.2 Modification of LYZ with epoxy-mPEG

Lysozyme [EC 3.2.1.17] (15.0 mg, 1.0 *µ*mol) was dissolved in 3 mL of 0.1 M carbonate buffer (pH 8) and combined with epoxy-mPEG (44.0 mg, 22.0 *µ*mol). The mixture was stirred at room temperature for 48 h. The product was purified by dialysis (Slide-A-Lyzer® Dialysis Cassettes, 10000 MWCO) against water for 3 days and was then lyophilized (ALPHA 1-2 LD plus). The molecular weight of the protein after modification was approx. 30-34 kDa (MALDI-ToF MS), which indicates that one protein was modified with around 9 mPEG chains.

2.1.3 Modification of LYZ with carbonate-mPEG

Lysozyme [EC 3.2.1.17] (15.0 mg, 1.0 *µ*mol) was dissolved in 3 mL of 0.1 M carbonate buffer (pH 8) and combined with carbonate-mPEG (44.0 mg, 22.0 *µ*mol). The mixture was stirred at room temperature for 48 h. The product was purified by dialysis (Slide-A-Lyzer® Dialysis Cassettes, 10000 MWCO) against water for 7 days and was then lyophilized (ALPHA 1-2 LD

plus). The molecular weight of the protein after modification was approx. 26-30 kDa (MALDI-ToF MS), which indicates that one protein was modified with around 7 mPEG chains.

2.1.4 Modification of LYZ with TsT-mPEG

Lysozyme [EC 3.2.1.17] (5.0 mg, 0.3 *µ*mol) was dissolved in 1 mL of 0.1 M borate buffer (pH 10) and combined with activated TsT-mPEG (195.7 mg, 48.9 *µ*mol). After 2 h at 40 °C the reaction was stopped with 2 mL phosphate buffer (pH 6). The excess of mPEG was removed with Microsep™ centrifugal devices (MWCO 30 kDa, PALL Corporation) and the resulting solution was lyophilized (ALPHA 1-2 LD plus). The molecular weight of 33-40 kDa was determined *via* MALDI-ToF MS, leading to the assumption that 10-12 mPEG chains per protein are attached.

2.2 SDS-PAGE

SDS-PAGE was performed as described elsewhere[5] using a 15% polyacrylamide gel (Rothiphorese® 30 gel mix) with a thickness of 0.75 mm (C.B.S. Scientific) and stained with Coomassie Brilliant Blue G. 7 µL of PageRuler Pre-Stained Protein Ladder (10-170 kDa) were used as marker. Samples of lysozyme (1 mg/mL), LYZ(TFP-mPEG), LYZ(epoxy-mPEG), LYZ(carbonate-mPEG) and LYZ(TsT-mPEG) in a concentration of 2 mg/mL, were dissolved in water. The proteins were denaturized by adding 5 µL of Roti®-Load 1 (Carl Roth) to 15 µL protein solution and heating in a boiling water bath for 10 min. 20 µg was loaded on each bag. Images of SDS-PAGE gels were taken with the GelDoc™ XR+ (Bio-Rad Laboratories Inc.) and processed with the Image Lab™ Software (camera filter 1).

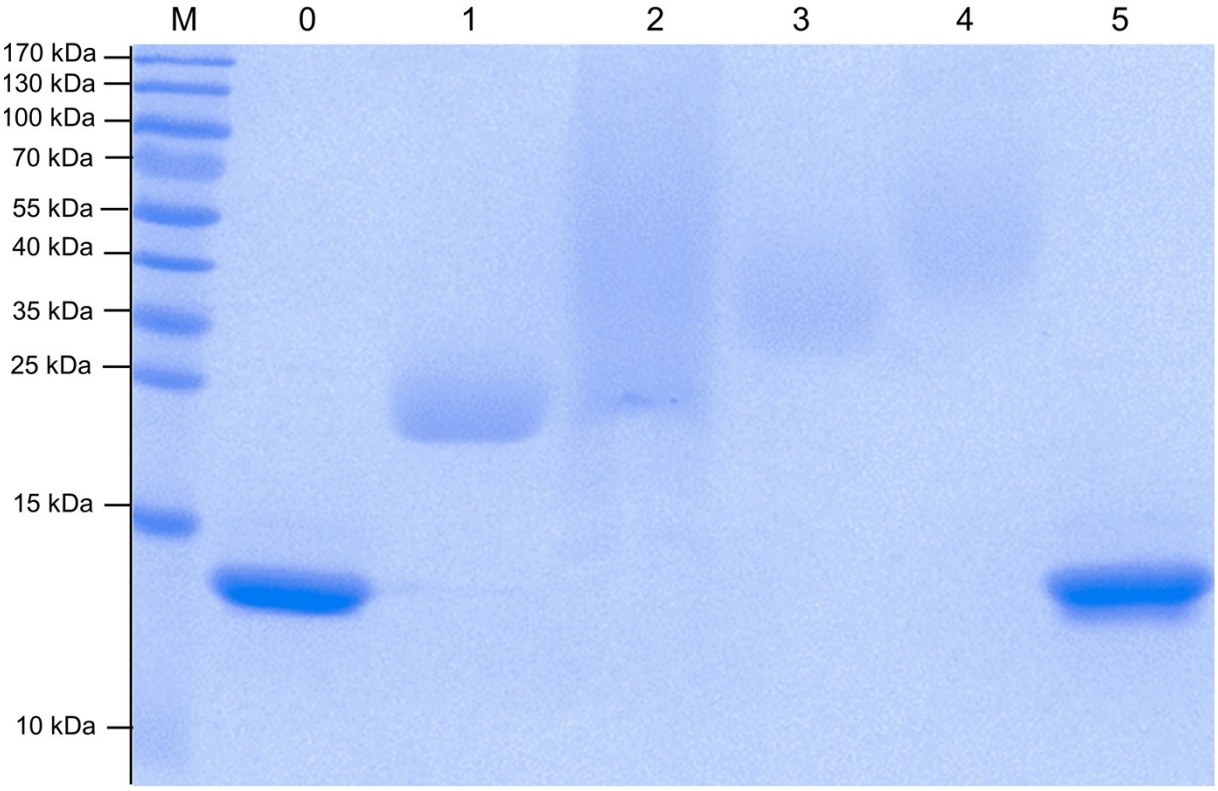


Figure 9: SDS-PAGE (15%) of non-PEGylated (Lane 0 and 5) and PEGylated hen egg white lysozyme (lanes 1-4). PEGylation was done either with TFP-mPEG (lane 1), epoxy-mPEG (lane 2), carbonate-mPEG (lane 3) or TsT-mPEG (lane 4). The marker (M) is a PageRuler Pre-Stained Protein Ladder. On each lane 20 µg protein sample was loaded. The gel was stained with Coomassie brilliant blue G.

2.3 MALDI-ToF MS

MALDI-ToF MS measurements were obtained with a Shimadzu Axima CFR MALDI-ToF mass spectrometer, equipped with a nitrogen laser delivering 3 ns laser pulses at 337 nm. Samples were prepared by dissolving the sample in CH3CN/TFA 0.1% at a concentration of ~ 1 mg/mL. 1μL of this mixture was applied to a multistage target to evaporate and create a thin analyte film. Sinapic acid was used as matrix and applied in the target before adding the sample. The samples were measured in positive ion and in linear mode of the spectrometer.

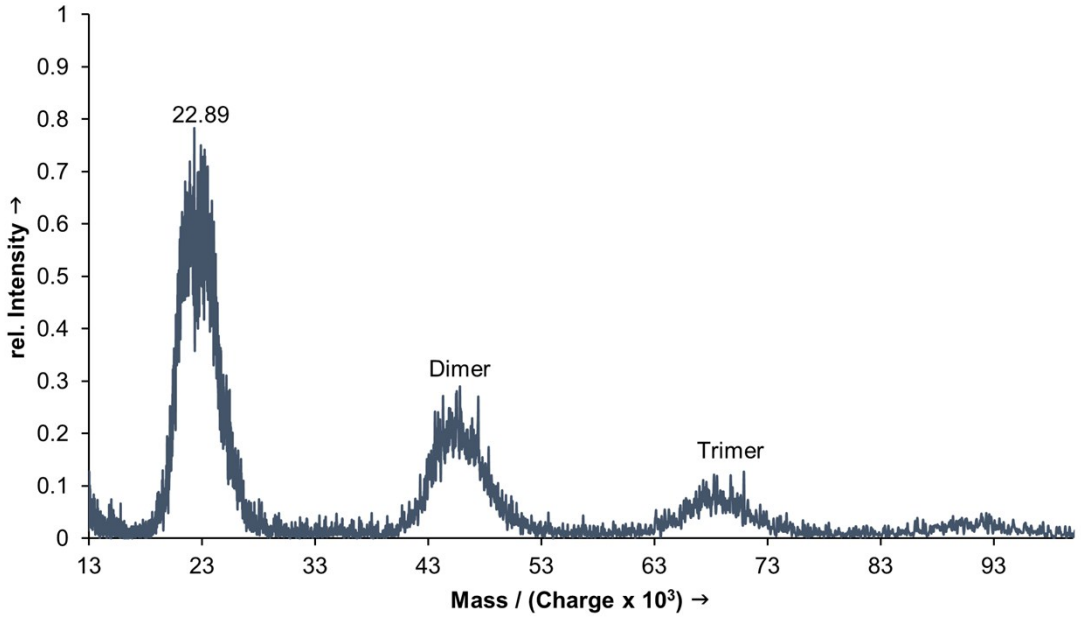


Figure 10: MALDI-ToF MS of LYZ(TFP-mPEG). After the PEGylation the protein has a molecular weight of 20-24 kDa, leading to the assumption that 4 mPEG chains are attached. The peaks at 46 kDa and at 67 kDa can be assigned to the MALDI-dimer and -trimer of the PEGylated protein.

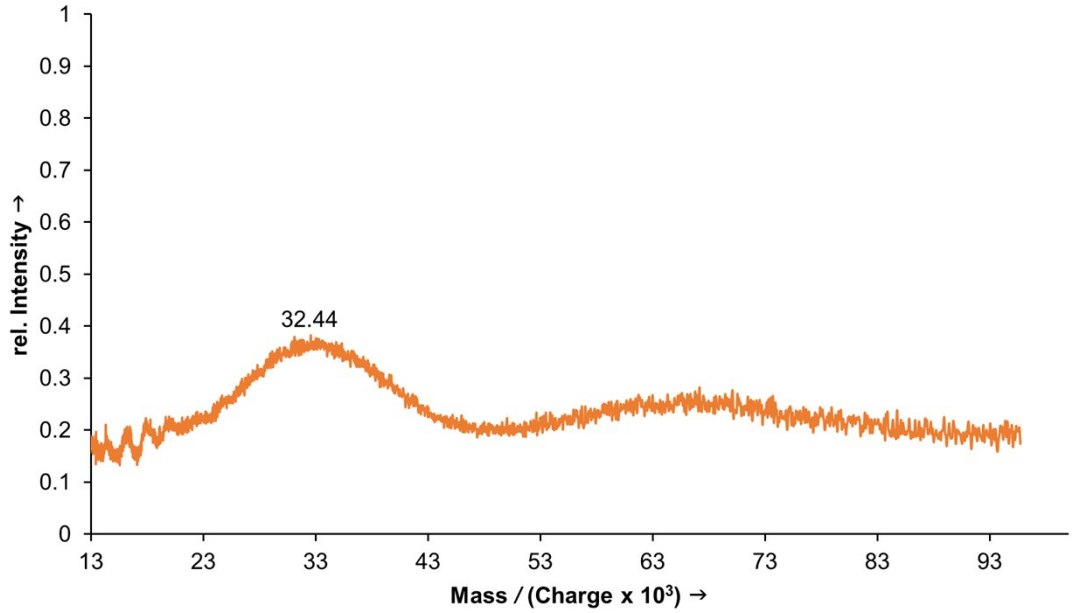


Figure 11: MALDI-ToF MS of modified lysozyme with epoxy-mPEG. The main peak is broad and localized at 30-34 kDa with a maximum at 32 kDa. This indicates that in average approx. 9 mPEG chains are attached on the protein surface.

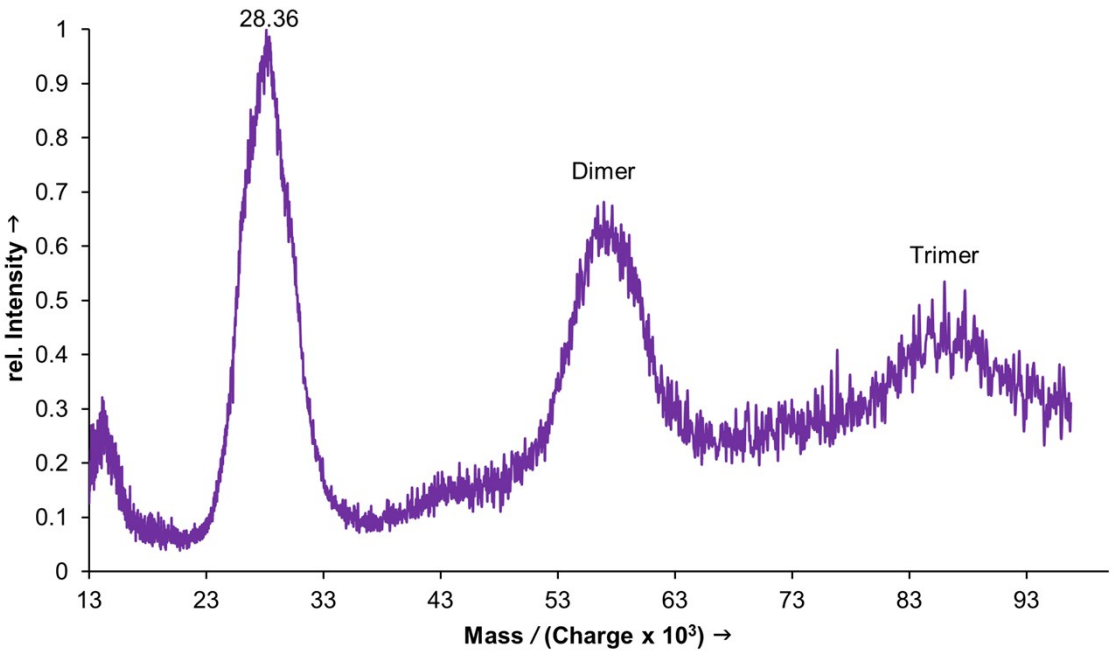


Figure 12: MALDI-ToF MS of LYZ(carbonate-mPEG). The peak at 28.36 kDa, indicates that the protein are modified with around 7 mPEG chains. The peaks at 58 kDa and 85 kDa can be assigned to the MALDI-dimer and trimer of the sample.

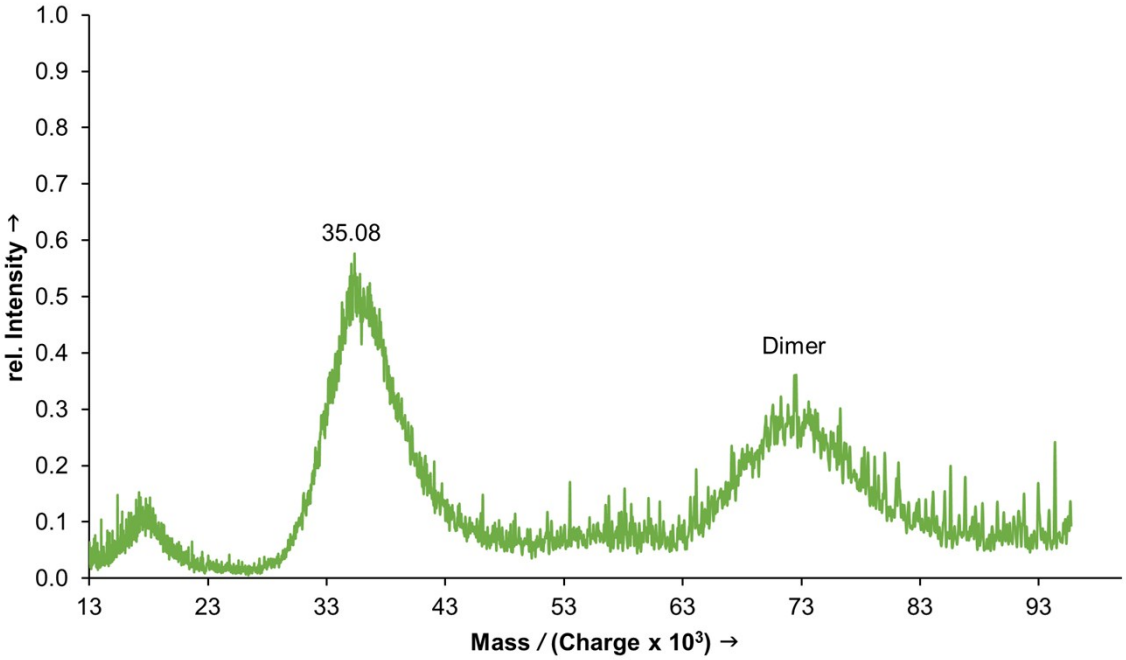


Figure 13: MALDI-ToF MS of modified lysozyme with TsT-mPEG. One peak is found at 33-40 kDa, leads on to the assumption that 10-12 mPEG chains were attached to the protein surface. The peak at 73 kDa is the MALDI-dimer of the PEGylated lysozyme.

2.4 Fluorescamine Assay

A protocol for quantification of primary amines on the protein surface, on the basis of fluorescence, was carried out. All samples were dissolved in PBS pH 7.4 at a concentration of 2 mg/mL and native lysozyme at a concentration of 0.2 mg/mL. Hexylamine was used as external standard in a concentration range of 19–40 μg/mL and was prepared similar. Then 125 μL PBS pH 7.4 was pipetted into each well of a 96-black-well-microplate (flat bottom). 25 μL sample dispersion or 25 μL PBS (blank) or 25 μL standard were added in triplets to each well. Finally, 50 μL of 0.3 mg/mL fluorescamine solution (in acetone) was added, mixed and measured immediately. The emission was measured with a Tecan Plate Reader (Infinite R pro M200, Tecan Group Ltd., Switzerland). For all measurements the excitation was set to 380 nm, the emission was set to 460 nm.

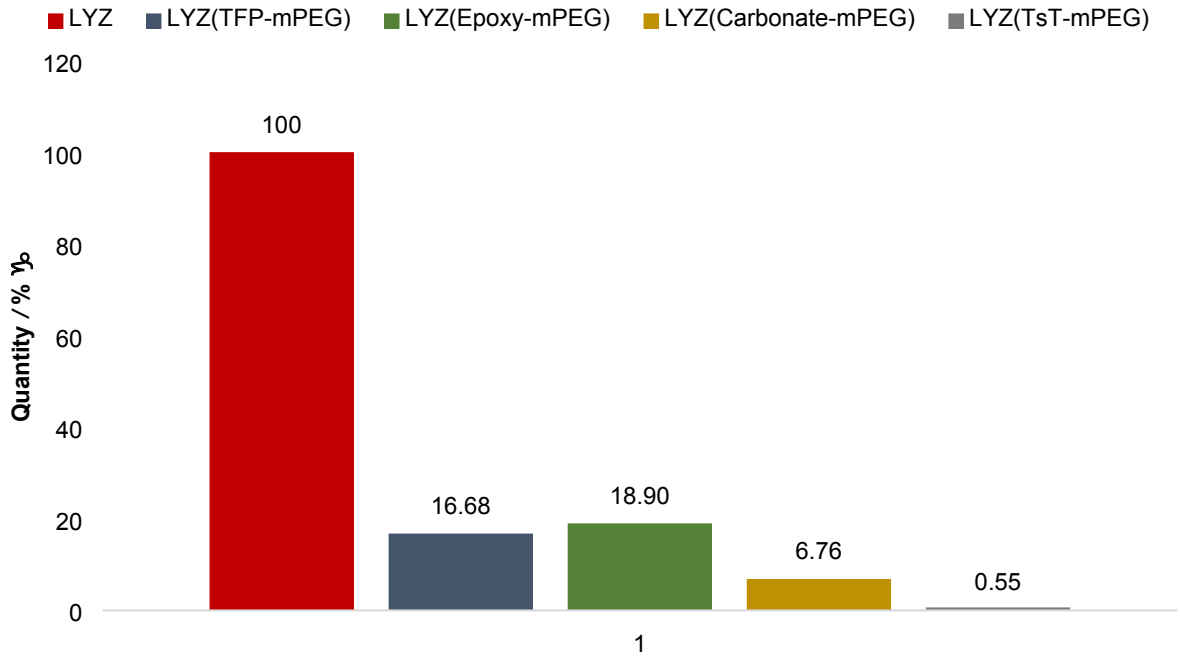


Figure 14: A fluorescamine assay with the modified proteins indicates an almost complete saturation of all surface primary amines (either by steric shielding or actual modification). For the LYZ sample modified with TFP-mPEG and epoxy-mPEG only one free amine was measured. For LYZ(carbonate-mPEG) only 0.5 free amines were calculated in average. No amines can be detected in the LYZ(TsT-mPEG) sample. Unmodified lysozyme contains 7 free amines on its surface (red bar on the left).

2.5 Protein Activity Assay

PEGylated LYZ was dissolved in 0.1 M phosphate buffer (pH 5.2) and diluted to a final protein concentration of 2 μM. A solution of 4-methylumbelliferyl-β-D-N,N′,N″ triacetyl-chitotrioside ((GlcNAc)$_3$MeU) (20 μM in the same buffer) was preheated to 42 °C for 5 min. 200 μL of each solution were combined and further incubated at 42 °C. Every 30 min 50 μL of each sample was transferred to 300 μL 0.5 M glycine buffer pH 12 to stop the catalytic activity of the protein and enhance the fluorescence intensity of methylumbelliferone. The fluorescence of the samples was determined with a Tecan Plate Reader (Infinite R pro M200, Tecan Group Ltd., Switzerland, ex. 380 nm; em. 460 nm). A solution of native lysozyme (2 μM), treated under the same conditions, was used as a reference. The time dependent change in fluorescence for native lysozyme and the modified LYZ is shown in Figure 15. The remaining activity of the PEGylated protein was calculated in comparison to native lysozyme (see Table 1).

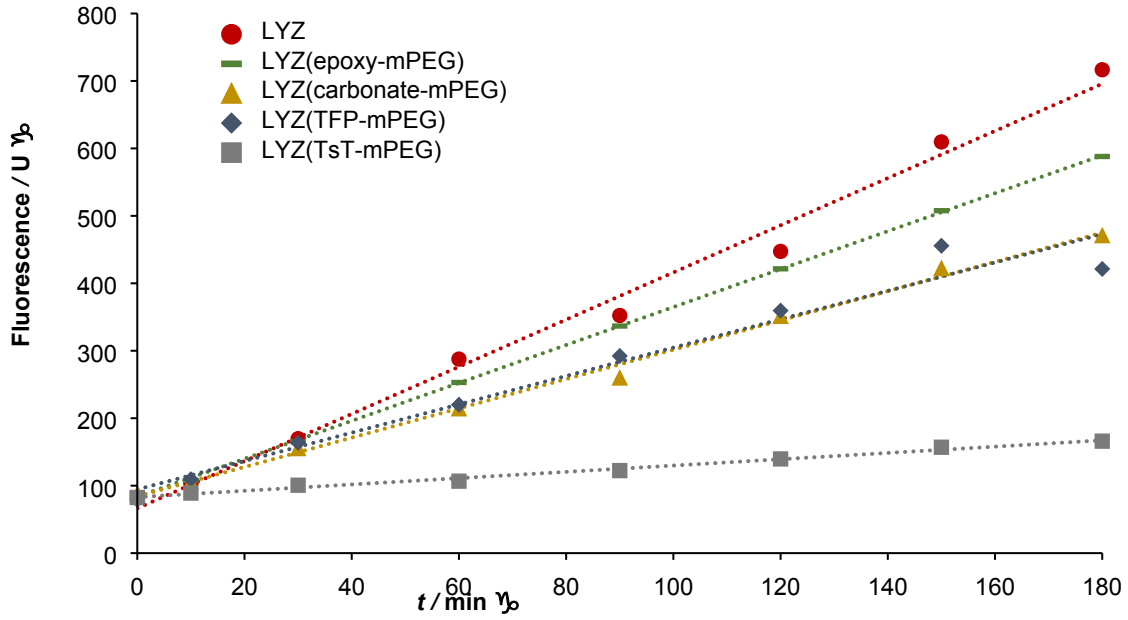


Figure 15: Activity assay of the modified LYZ samples. Depending on the modification method, the remaining activity of the PEGylated proteins yield. The calculated activity in comparison to native lysozyme can be found in Table 1.

Table 1: Protein activity (%) of LYZ and modified samples. The activity of the modified proteins was calculated referred to native lysozyme.

sample	protein activity / %
LYZ	100
LYZ(epoxy-mPEG)	80
LYZ(carbonate-mPEG)	62
LYZ(TFP-mPEG)	60
LYZ(TsT-mPEG)	13

2.6 Circular Dichroism (CD)

CD spectra were recorded on a J-815 (JASCO) using the software Spectra Manager 2.08.04. All spectra were recorded at 20 °C with a total concentration of 0.1 mg/mL in 10 mM potassium phosphate/50 mM Na_2SO_4 buffer using quartz cells with a path length of 1 mm. All spectra were corrected by subtraction from the background (buffer). Data points were collected at a resolution of 1 nm. Secondary structure contents were predicted with DICHROWEB using the CONTIN-LL method (reference set 7).[6]

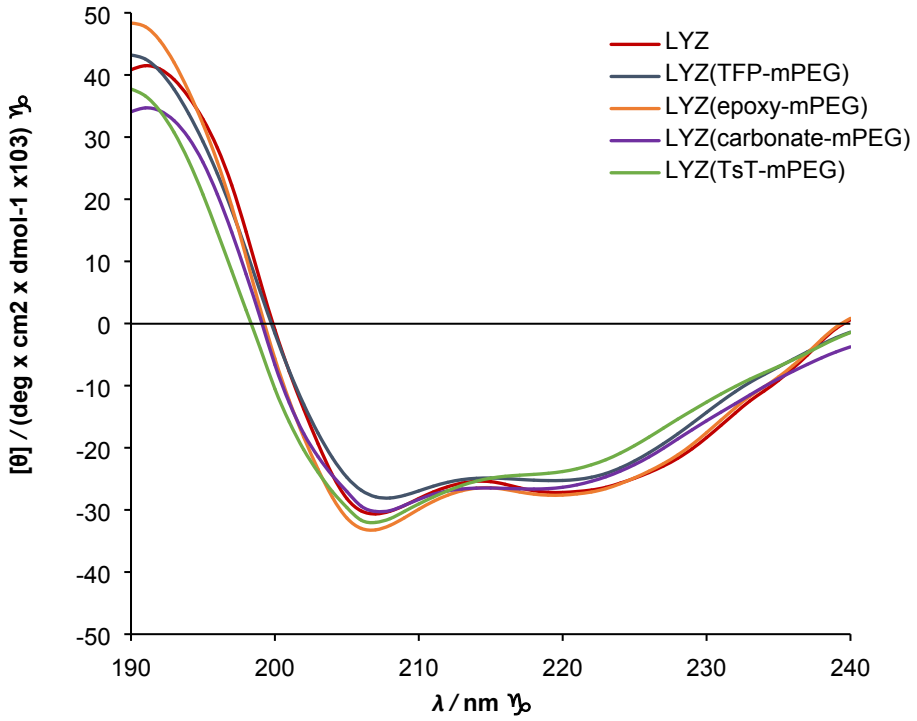


Figure 16: CD spectra of the different modified LYZ and the unmodified LYZ (red). The spectra indicate no significant change in the secondary structure of the modified proteins. A detailed analysis can be found in Table 2.

Table 2: Calculated amounts of secondary structure elements for LYZ and the modified proteins (in %)

	LYZ	LYZ(epoxy-mPEG)	LYZ(TsT-mPEG)	LYZ(carbonate-mPEG)	LYZ(TFP-mPEG)
α-helix	32.54	33.5	22.3	26.6	30.6
β-sheet	15.2	12.9	22.1	15.1	12.7
turns	13.4	13	15.4	13.6	13.1
unordered	38.86	40.6	40.2	44.7	43.6

3. Nanoparticle Preparation

3.1 Preparation

Nanoparticles of PEGylated LYZ were prepared using a single emulsion method.[7] LYZ(TFP-mPEG), LYZ(epoxy-mPEG), LYZ(carbonate-mPEG) and LYZ(TsT-mPEG) were dissolved in a concentration of 2.5 mg/mL in ice cold dichloromethane (DCM, 0.5 mL) and added to 2.5 mL of ice cold PBS buffer (pH 7.4). The mixture was sonicated for 45 s on ice, using an ultrasonicator (Bandelin Ultrasonic Homogenisator Sonoplus UW 70, microtip MS 73 SH70G Stufenhorn 20 kHz, Germany). The emulsion was stirred in a well-ventilated hood overnight for the evaporation of DCM.

3.2 Nanoparticle Tracking Analysis (NTA)

Nanoparticle Tracking Analysis (NTA) was performed on a NanoSight LM 14 equipped with a green laser (532 nm) and a marlin charged coupled device (CCD) camera. Samples (stock solution 1 mg/mL in PBS) were diluted in a ratio of 1:100 in PBS and loaded into the measurement cell. Movements of particles in the samples were recorded as videos for 30 seconds at 25 °C. The videos were analyzed with the NanoSight NTA 3.1 software. Figure 17 shows the mean values of five individual measurements. The nano-emulsions were stored at 4 °C and NTA size measurements of the 2 months old samples are shown in Figure 18.

Table 3: Detailed size values obtained by NTA measurements of the modified LYZ nanoparticles. Particle size (diameter) was determined with five individual measurements per sample.

Sample	Mean / nm	Mode / nm	SD / nm
LYZ(TFP-mPEG)-NP	149.4 ± 5.8	107.6 ± 1.6	61.5 ± 4.4
LYZ(epoxy-mPEG)-NP	147.0 ± 6.0	112.7 ± 2.7	57.2 ± 5.6
LYZ(carbonate-mPEG)-NP	164.0 ± 5.9	131.8 ± 3.3	66.7 ± 6.3
LYZ(TsT-mPEG)-NP	199.6 ± 7.0	158.1 ± 15.9	76.0 ±7.12

Mean size and SD (standard deviation) correspond to the arithmetic values calculated based on the sizes of all particles detected in the NTA measurement. Mode values describe the average size of the main particle population.

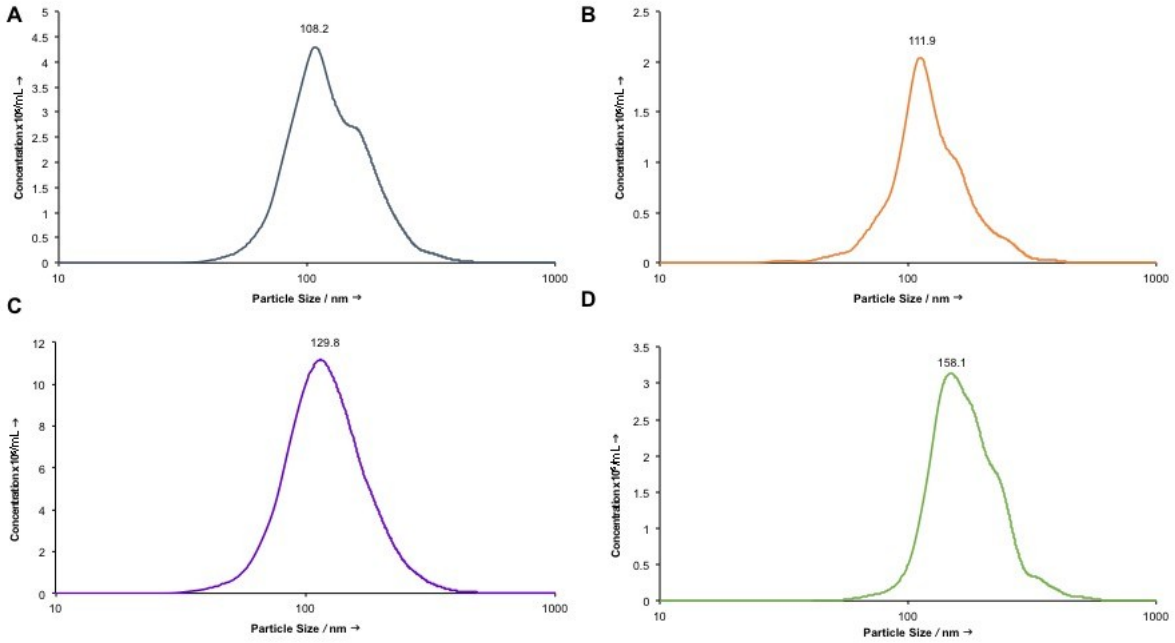


Figure 17: NTA diagrams of LYZ(TFP-mPEG) (**A**), LYZ(epoxy-mPEG) (**B**), LYZ(carbonate-mPEG) (**C**) and LYZ(TsT-mPEG) (**D**). The nanoparticles were prepared *via* single emulsion and result in average diameters of around 100-130 nm. There are no significant differences among the samples.

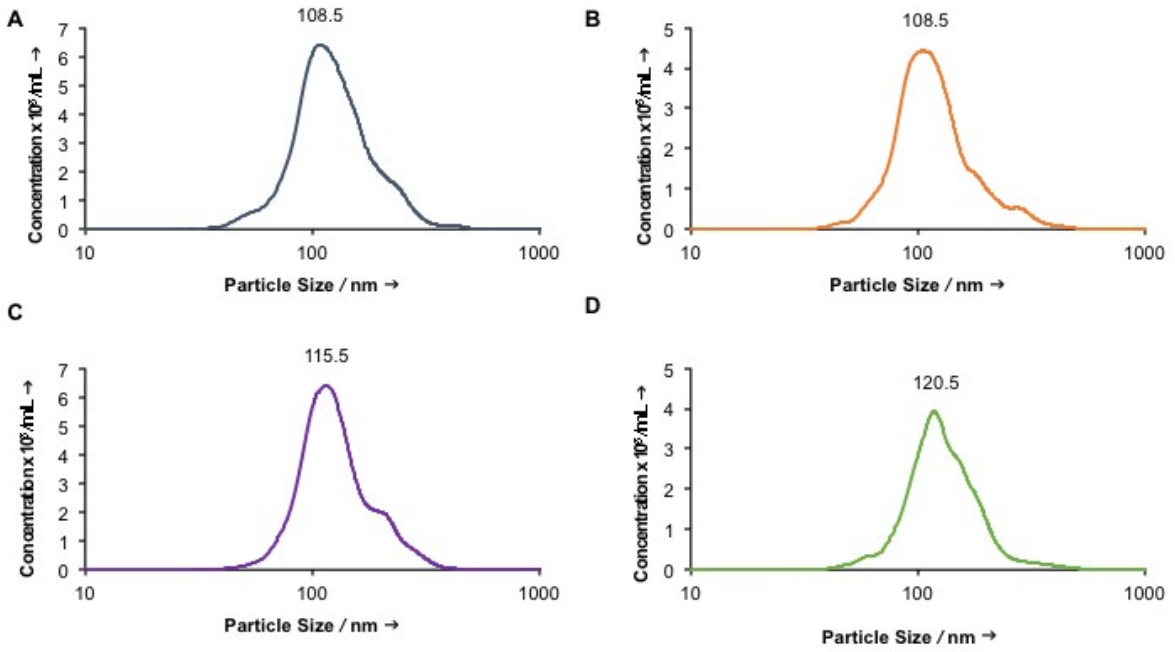


Figure 18: NTA diagrams of LYZ(TFP-mPEG) (**A**), LYZ(epoxy-mPEG) (**B**), LYZ(carbonate-mPEG) (**C**) and LYZ(TsT-mPEG) (**D**) of two months old samples. There are no significant differences regarding to the size or concentration between the fresh and old samples. The nano-emulsion are stable at 4 °C for the observed time and retain their initial size of around 100-130 nm in diameter.

3.4 Zeta-Potential

ζ-potential measurements were performed on a Zetasizer Nano ZS instrument (Malvern) using a clear disposable capillary cell. Three measurements with fifteen individual runs were performed. The refractive index (RI) of the dispersant (preset: water) was set to 1.330 and the viscosity to 0.8872 cP, respectively. The RI of the particle was set to 1.45 with a dielectric constant of 78.5. All samples had a concentration of 1 mg/mL. The results of the measurements for native lysozyme, LYZ(TFP-mPEG), LYZ(epoxy-mPEG), LYZ(carbonate-mPEG) and LYZ(TsT-mPEG), and the prepared particles (abbreviate with NP) are shown in Table 3. The protein PEGylation leads to a negative zeta potential due to the functionalization of positive surface amino acids of the protein (unmodified lysozyme has a positive zeta potential). The following nanoparticle preparation further increases the zeta potential to overall negative values between -10 and -20 mV.

Table 4: *ζ*-potential of native lysozyme, the PEG-modified proteins and the prepared nanoparticles (abbreviated with NP).

protein/particle	*ζ*-potential (mV)
lysozyme	+5.46
LYZ(TFP-mPEG)	-3.21
LYZ(epoxy-mPEG)	-1.71
LYZ(carbonate-mPEG)	-3.27
LYZ(TsT-mPEG)	-9.09
LYZ(TFP-mPEG)-NP	-15.47
LYZ(epoxy-mPEG)-NP	-13.33
LYZ(carbonate-mPEG)-NP	-19.67
LYZ(TsT-mPEG)-NP	-11.80

3.5 Transmission Electron Microscopy (TEM)

Nanoparticles have been diluted in PBS buffer (1mg/mL) and drop-casted on a 400 mesh copper carbon grid from Plano GmbH for TEM measurements. The image acquisition was done with a transmission electron microscope Technai T12 (FEI, acceleration voltage: 120 kV, electron source: LaB6 BIO-TWIN cathode) equipped with a 4K CCD camera (Tietz). PEGylated proteins have an average diameter of around 17 nm. The native protein has a diameter of 3.5 nm.

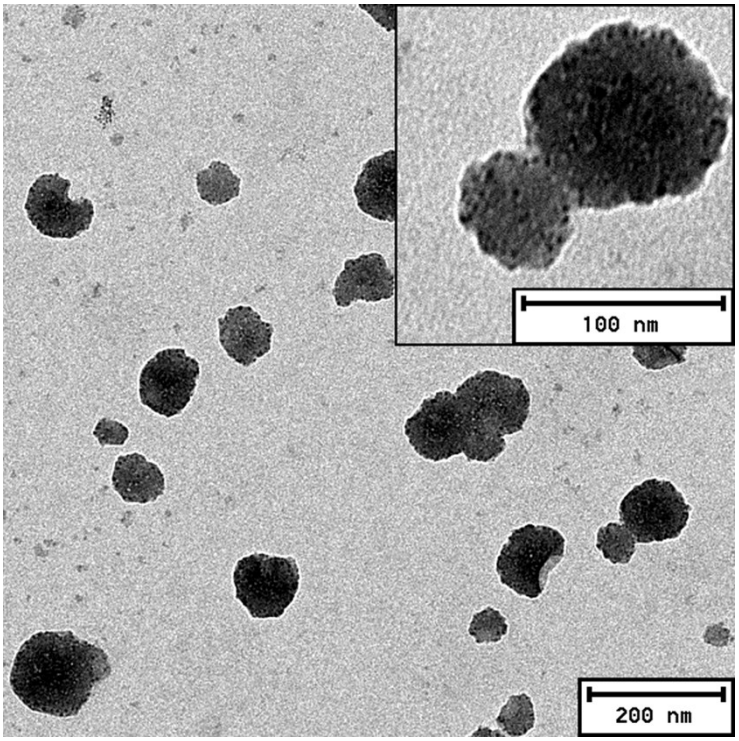


Figure 19: TEM images of nanoparticles prepared *via* single emulsion. The particles are assemblies of around 250-450 modified individual proteins (small black dots within the particle). Exemplary shown here: LYZ(epoxy-mPEG) nanoparticles.

4. Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) as part of the collaborative research center SFB 1066 (Project A2).

We thank *Monika Schmelzer* of the working group of *Prof. Dr. H. Frey* (JGU Mainz, Germany) for the SEC measurements.

Additionally we thank *Prof. Dr. P. Besenius, Prof. Dr. T. Efferth, Prof. Dr. B. Epe, Prof. Dr. T. Schirmeister* (JGU Mainz, Germany) for the kind permission to use some of their analytical equipment.

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