Supplementary information

Synthesis of di-GOx Pluronic

A protein derivatization chemistry based on p-nitrophenol-activation was applied with small modifications.[18]

Synthesis p-nitrophenyl (pNP) Pluronic. 2 g of Pluronic F-127 (MW 12'600, Sigma) were dissolved in 6 ml dry benzene and added to 6.5 ml of dry benzene containing a 3 molar excess of *p*-nitrophenyl (pNP) chloroformate compared to Pluronic-OH-groups. After 24 h of stirring, the product was precipitated in 200 ml of cold diethylether, filtrated through a paper filter and redissolved in benzene three times. After the third filtration, the remaining solvent was evaporated under vacuum for 8 h. The degree of activation was determined by ^{1}H-NMR and hydrolysis experiments. For the latter, the absorbance at 400 nm of derivatized Pluronic in 0.1 M NaOH was measured after 1 h hydrolysis (Molar extinction coefficient of p-nitrophenol in 0.1 M NaOH = 18'100 $M^{-1}cm^{-1}$). The pNP-Pluronic was stored under nitrogen. ^{1}H-NMR ($CDCl_3$): = 1.1 (m, 3H, PPG CH_3), 3.4 (m, 1H, PPG CH), 3.5 (m, 2H, PPG CH_2), 3.65 ppm (m, 2H, PEG CH_2), 4.4 (t, 2H, C**H**$_2$-OCOO-), 7.5 (d, 2H nitrophenol-CH) and 8.3 (d, 2H nitrophenol-CH) ppm.

The yield of activation of Pluronic with p-nitrophenol was 82.5 % after the first precipitation step and finally 46.3 %. The degree of activation was determined from hydrolysis experiments and ^{1}H-NMR and was 89 and 90 %, respectively.

Conjugation of GOx with Pluronic. *Aspergillus Niger* GOx (Sigma) was dissolved in a 0.1 M potassium phosphate buffer and stirred gently. The pNP-Pluronic was dissolved in water (10 mg/ml) and added in drops to the GOx solutions to reach molar ratios of 1:5 to 5:1. The reaction was monitored by measuring the free *p*-nitrophenol absorbance at 400 nm, and stopped when this reached a plateau (about 50 h; extinction coefficient of *p*-nitrophenol at pH 8: 15,245 $M^{-1}cm^{-1}$). After the reaction, the emulsions were dialyzed against water through a membrane with a MWCO of 40 kDa (Spectra/ POR®) to remove free *p*-nitrophenol. The product was freeze-dried and stored in the freezer at -20°C. For analysis by size exclusion chromatography, a Superdex 200 HR 10/30 column (Amersham) was used on a Äkta FPLC system (Amersham) and the absorption of the fractions was measured at 280 nm. The concentration of the samples was about 1 mg/ml in the eluent PBS (phosphate buffered saline, 0.01 M, pH 7.4), and the flow rate was set at 0.25 ml/min. The FT-IR spectra of the freeze-dried samples were recorded in ATR mode with a Perkin Elmer Spectrum One spectrometer.

Enzyme Activity Measurement. GOx activity was assayed by a standard hydrogen peroxide determination in PBS (0.01 M, pH 6.0, bubbled with air for 30 min.). D-β-Glucose (0.1 M, mutarotated for 1 day at RT), ABTS (2,2-azinobis[3-ethyl-benzothiazoline-6-sulfonate] diammonium salt, Sigma, 1.35×10^{-7} M) and horseradish peroxidase type XII (HRP, Sigma, prepared as a 10 mg/ml solution in a 0.1 M PBS at pH 6 to prevent denaturation and diluted 1:1500 for the experiment) were shortly mixed with GOx (0.03 mg/ml) and the slope of the absorbance measured between 30 and 80 s at 420 nm with a Perkin Elmer MBA 2000 instrument. The increase in absorption is based on ABTS radical formation when hydrogen peroxide produced by GOx is reduced by HRP.

Nanoparticle Synthesis and Characterization. The nanoparticle synthesis was performed as described elsewhere[16] with small modifications. A total amount of 0.6 %w/v Pluronic was employed, containing 1 % (in weight) GOx-Pluronic of the batch with an original ratio of Pluronic to GOx of 2:1 or an equal amount (in weight) of unmodified GOx. The amount of base was reduced to 0.5 meq compared to thiol groups to limit enzyme-inactivation during the polymerization. The polymerization was stopped after 2 h by dialyzing the emulsion against water through a dialysis membrane with a MWCO of 6-8 kDa (for removal of remaining monomer) or 300 kDa (for removal of free GOx and Pluronic), respectively. The conversion of propylene sulfide was calculated based on the ratio of PPS to Pluronic as determined by ^{1}H-NMR of dried particles dialyzed through a 6-8 kDa MWCO membrane. The reaction mixture dialyzed through 300 kDa membrane was used for activity measurements, particle size analysis and further experiments. The particle dimensions were measured through hydrodynamic chromatography with a poly(styrene) latex calibration (PL-PSDA, Polymer Laboratories). ^{1}H-NMR ($CDCl_3$): δ = 1.35-1.45 (d, C**H$_3$** in PPS chain), 2.55-2.65 (m, 1 diastereotopic H of C**H$_2$** in PPS chain), 2.85-3.0 (m, C**H** and 1 diastereotopic H of C**H$_2$** in PPS chain) ppm, 1.1 (m, PPG CH_3), 3.4 (m, PPG CH), 3.5 (m, PPG CH_2), 3.65 (m, PEG CH_2) ppm.

Oxidative degradation, Drug Loading and Release. The activity of GOx as a producer of hydrogen peroxide for the oxidation of the poly(propylene sulfide) chains was tested by exposing nanoparticles (at a concentration of 1% wt.) to 0.2 M glucose in PBS. The oxidation of the particles was measured by optical density of the solutions at 600 nm with a Perkin Elmer MBA 2000. In parallel, the increase in mean particle size was analyzed as described above.

Loading of Doxorubicin-HCl (Dox, Fluka, Switzerland) was achieved by an adapted solvent evaporation method.[19] Briefly, a 13 mM Dox solution in chloroform containing 39 mM triethylamine was added in small aliquots (20 ml/ml/h) to a 1%wt. Nanoparticle dispersion to reach a final loading of 5 % w/w. The dispersions were continuously stirred in the dark at 30°C in order to evaporate the solvent and then further stirred at 4°C in the dark for 36 h. Free Dox was removed by gel filtration over a sephadex column (G-25, Fine) in 10 mM Tris pH 8.5. Fractions were collected until no more fluorescence was detected in the eluent. The collected fractions were analyzed for turbidity at 600 nm with a Perkin Elmer MBA 2000 spectrometer and fluorescence with a Perkin Elmer LS50B luminescence spectrometer with excitation at 487 nm and emission maximum between 545 and 555 nm. Turbidity measurements determined the nanoparticle concentrations in the fractions, while the concentration of free Dox was estimated from fluorescence measurements ($\varepsilon = 10^7 \times 10^6\ M^{-1}cm^{-1}$). A typical loading yield of >95% was achieved. Release of Dox from nanoparticles was also studied by measuring Dox fluorescence in the dialysates obtained through membranes with 25 kDa MWCO at RT in the dark against 10 mM sodium phosphate at pH 7.4 with or without 0.2 M glucose. Dialysates were replaced every 2 h to avoid Dox saturation (sink conditions).