In Vitro and In Vivo Studies on HPMA-based Polymeric Micelles Loaded with Curcumin

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Table S1. Characteristics of the mPEG₅kDa-b-p(HPMA-Bz)ₓ block copolymers as determined by ¹H-NMR and GPC¹.

| Polymer                        | MI:M mol/mol | Mₙ ¹H-NMR (kDa) | Mₙ GPC (kDa) | Mₘ GPC (kDa) | Polydispersity (Mₘ/Mₙ) (GPC) | Weight fraction p(HPMA-Bz) (%) |
|-------------------------------|--------------|-----------------|--------------|--------------|-----------------------------|------------------------------|
| mPEG₅kDa-b-p(HPMA-Bz)₁₇.₁kDa  | 1:200        | 22.1            | 15.8         | 20.7         | 1.31                        | 77.4                         |
| mPEG₅kDa-b-p(HPMA-Bz)₁₀₀kDa   | 1:100        | 15.0            | 13.2         | 17.5         | 1.32                        | 66.7                         |
| mPEG₅kDa-b-p(HPMA-Bz)₅₂kDa    | 1:50         | 10.2            | 10.8         | 14           | 1.30                        | 51.0                         |

Abbreviations: MI:M, macro-initiator:monomer; Mn, the Number Average Molecular Weight; Mw, the Weight Average Molecular Weight.

Figure S1. ¹H-NMR spectrum of mPEG₅kDa-b-p(HPMA-Bz)₁₇.₁kDa block copolymer in DMSO-d₆.
Figure S2. $^1$H-NMR spectrum of curcumin in DMSO-$d_6$.

The GPC chromatograms of the fluorescently-labeled polymers are shown in Figure S3. The polymer eluted between 10 to 15 min (RI detector), which coincided with the elution time as measured by absorbance at 700 nm (Cy7) and 650 nm (Cy5). The results confirm that the dyes were conjugated to the polymer. On the other hand, free dye eluted at 18 min (UV detector). The fluorescently-labeled polymers did not show absorbance at this elution time, confirming that free dyes were completely removed by dialysis.

Figure S3. GPC chromatograms of fluorescently labeled mPEG-$b$-p(HPMA-Bz) polymers. (A) Cy7-labeled polymer: green and blue lines represent absorbance at 700 nm for free Cy7 dye and Cy7-labeled polymer, respectively. Red line is the refractive index (RI) signal of Cy7-labeled polymer under the
same chromatography conditions. (B) Cy5-labeled-polymer: green and blue lines are absorbance at 650 nm for free Cy5 dye and Cy5-labeled polymer, respectively. Red line is the RI signal of Cy5-labeled polymer under the same chromatography conditions. Abbreviations: AU, absorbance unit; MV, voltage offset in millivolts.

**Table S2.** Characterization of Cy7-labeled micelles loaded with curcumin.

| Polymer concentration (mg/mL) | Curcumin concentration (mg/mL) | Z-ave (nm) | PDI | EE (%) | LC (%) |
|------------------------------|--------------------------------|------------|-----|--------|--------|
| 5.7                          | 56                             | 56         | 0.11| 73.1   | 9.2    |

Abbreviations: Z-ave, Z-average hydrodynamic diameter; PDI, polydispersity index; EE, encapsulation efficacy and LC, loading capacity.

**The effect of dialysis on curcumin-loaded micelles**

Curcumin-loaded micelles (polymer 30.0 mg and curcumin 3.0 mg) were prepared using the method described in section 2.5. Dialysis was performed in HBS solution for one day at 4 °C using an 8-kDa dialysis membrane. Samples were taken before and after dialysis and analyzed for polymer and curcumin concentration.

**Table S3.** Characteristics of curcumin-loaded micelles in terms of curcumin content before and after the dialysis against HBS at 4 °C.

| Curcumin concentration (mg/mL) | Before dialysis | After dialysis |
|--------------------------------|-----------------|----------------|
|                                | Curcumin concentration (mg/mL) | Polymer concentration (mg/mL) | EE% | LC% | Curcumin concentration (mg/mL) | Polymer concentration (mg/mL) | EE% | LC% |
| 3.1 ± 0.1                      | 28.3 ± 0.4      | 103 ± 2.2      | 9.8 ± 0.2 | 2.3 ± 0.2 | 22.5 ± 1.5 | 77.4 ± 5.7 | 9.4 ± 0.3 |

Abbreviations: EE, encapsulation efficacy; LC, loading capacity.

Data are presented as mean ± SD (n = 5).

**HPLC method validation**

The method to extract curcumin from plasma was validated by spiking plasma (100 µL) with known amounts of curcumin-loaded micelles (10 µL). Samples were extracted by addition of 4 volumes of acetonitrile (ACN) to 1 volume of spiked plasma and vortexed for 2 min, followed by centrifugation at 15,000 × g for 10 min. The concentration of curcumin was determined in the supernatant using an isocratic HPLC method described in the Materials and Methods section 2.12 of the main text. The ratio of the measured curcumin concentration to the known curcumin concentration was used to calculate the recovery percentage (Figure S4 and Table S4).
**Figure S4.** Calibration curve of curcumin in ACN/water 80%/20%. Y-axis depicts area under the curve (AUC) of curcumin at 425 nm.

**Table S4.** Determination of curcumin concentration in plasma samples spiked with curcumin-loaded micelles at different concentrations.

| Area            | Curcumin concentration measured (µg/mL) | Curcumin concentration added (µg/mL) | Recovery (%) |
|-----------------|----------------------------------------|--------------------------------------|--------------|
| 5004168 ± 47875 | 186 ± 2                                 | 203.9                                | 91 ± 1       |
| 1648332 ± 8911  | 61.4 ± 0.3                              | 68.0                                 | 90 ± 1       |
| 472586 ± 3349   | 17.7 ± 0.1                              | 19.4                                 | 91 ± 1       |
| 104090 ± 975    | 4.02 ± 0.04                             | 4.0                                  | 100 ± 1      |
| 47849 ± 653     | 1.93 ± 0.02                             | 2.0                                  | 96 ± 1       |

Data are presented as mean ± SD (n = 3).

**Table S5.** Physicochemical characteristics of mPEG5kDa-b-(HPMA-Bz)17.1kDa micelles loaded with 2.0 and 4.8 % w/w curcumin.

| Curcumin-loaded micelles | Polymer                           | Z-ave (nm) | PDI   | EE (%) | LC (%) |
|--------------------------|-----------------------------------|------------|-------|--------|--------|
|                          | 2.0% curcumin-loaded micelles    | 53 ± 1     | 0.05 ± 0.01 | 98 ± 2 | 1.9 ± 0.0 |
|                          | 4.8% curcumin-loaded micelles    | 55 ± 1     | 0.08 ± 0.02 | 96 ± 2 | 4.5 ± 0.0 |

Abbreviations: Z-ave, Z-average hydrodynamic diameter; PDI, polydispersity index; EE, encapsulation efficacy and LC, loading capacity. Data are presented as mean ± SD (n = 3).
Figure S5. (A) Curcumin retention in mPEG$_{5kDa}$-b-p(HPMA-Bz)$_{17.1kDa}$ micelles composed of polymers with varying molecular weight of the hydrophobic block (17.1, 10.0, and 5.2 kDa). Micelles contained 9% (w/w) curcumin and were dispersed in PBS, pH = 7.4, at 37 °C during 168 h. (B) Curcumin retention of mPEG$_{5kDa}$-b-p(HPMA-Bz)$_{17.1kDa}$ micelles as a function of curcumin loading (9%, 4.8%, and 2.0% w/w) during 168 h incubation in PBS, pH = 7.4, at 37 °C. Data are presented as mean ± SD (n = 3).
Figure S6. Representative HPLC chromatograms of curcumin samples at different time points collected during the stability study of curcumin-loaded mPEG_{5kDa-b-p(HPMA-Bz)} micelles in PBS at 37 °C.

**Ratio of HPMA-Bz/curcumin**

The exemplary calculation to obtain the ratio of HPMA-Bz/curcumin (mol/mol) for mPEG_{5kDa-b-p(HPMA-Bz)}_{17.1kDa} is provided as an illustration.

Feed ratio: 3.0 mg/mL curcumin and 30 mg/mL polymer

Weight fraction% of p(HPMA-Bz): 77.4%

p(HPMA-Bz): 30 mg/mL × 77.4% = 23.2 mg/mL

p(HPMA-BZ): 23.2 (mg/mL) / 247.4 (HPMA-Bz monomer Mw g/mol) × 10³ = 94 mM

Curcumin: 3.0 (mg/mL) × 91% (EE%) / 368.38 (curcumin Mw g/mol) × 10³ = 7.4 mM

Ratio HPMA-Bz/curcumin: 94 (mM) / 7.4 (mM) = 12.7
Figure S7. Cytotoxicity of free curcumin, curcumin-loaded mPEG$_{5kDa}$-b-(HPMA-Bz)$_{17.1kDa}$ micelles, and empty mPEG$_{5kDa}$-b-(HPMA-Bz)$_{17.1kDa}$ micelles in EGI-1, Mz-ChA-1, Sk-ChA-1, and TFK-1 cells incubated for 24 h, 48 h, and 72 h. Cell viability was measured with the SRB total protein assay. Data were normalized to the average value of the control (untreated) cells at the respective incubation time. The highest curcumin concentration in mPEG$_{5kDa}$-b-(HPMA-Bz)$_{17.1kDa}$ micelles (200 µM) corresponds to a polymer concentration of 800 µg/mL. Data were fitted using a nonlinear regression model (curve fit) based on inhibitor vs. normalized response curve (variable slope) and are presented as mean ± SD (n = 4 per time interval).

Fluorescence spectrum of curcumin-loaded micelles

Curcumin-loaded micelles were prepared at 30 mg/mL polymer and varying curcumin concentrations (0.06-3.0 mg/mL) using the method described in section 2.5. The samples were diluted to a curcumin concentration of 40 µg/mL. The fluorescence spectra were recorded using a Jasco FP8300 spectrofluorometer (Tokyo, Japan). The excitation wavelength was 429 nm.
Figure S8 shows that the fluorescence spectra of curcumin-loaded micelles changed with increasing the curcumin loading. The fluorescence intensity decreased with curcumin loadings > 0.4%. Micelles with a curcumin loading of 9% (w/w) were used for the in vitro cell studies and Figure 8 demonstrates that the curcumin fluorescence was substantially quenched.

Cytotoxicity of Empty Micelles in Human Pancreatic Stellate Cells (hPSC)

The cytotoxicity of mPEG$_{5kDa}$-$b$-p(HPMA-Bz)$_{17.1kDa}$ micelles was investigated in hPSC as the main precursor of pancreatic cancer-associated fibroblasts. hPSC cells were seeded in 96-well plates at a density of 2500 cells/well. Empty micelles were added to the cells in the range of 0-800 µg/mL. Also, HEPES buffer equivalent to the highest concentration of micelles diluted in the medium was used as control. After 24, 48, and 72 h of incubation, the cells were washed with PBS and AlamarBlue (×1 in cell culture medium) was added to the cells. After 4 h of incubation, the medium was removed and collected in a 96-well plate. The absorbance of the medium was measured with a microplate reader (Tecan Group, Männedorf, Switzerland).
Figure S9. Cytotoxicity of empty micelles in human pancreatic stellate cells (hPSC) using AlmarBlue test. Data were normalized to the average value of the control (untreated) cells at the respective incubation time. Data are presented as mean ± SD (n = 4 per time point).

Figure S10. Cytotoxicity of mPEG<sub>5kDa</sub>-b-p(HPMA-Bz)<sub>17.1kDa</sub> micelles in primary human umbilical vein endothelial cells (HUVECs) incubated for 24 h, 48 h, and 72 h. Cell viability was measured with the SRB total protein assay. Data were normalized to the average value of the control (untreated) cells at the respective incubation time. Data are presented as mean ± SD (n = 4 per time point).
**Figure S11.** Mouse body weight before and 24 h after treatment with 100, 300, and 500 mg/kg empty mPEG$_{5\text{KDa}}$-$b$-p(HPMA-Bz)$_{17.1\text{KDa}}$ micelles vs control group receiving HBS. Data are presented as mean ± SD (n = 3 per concentration).

**Figure S12.** Red blood cell (RBC) and white blood cell (WBC) counts in mice after intravenous administration of 100, 300, and 500 mg/kg empty mPEG$_{5\text{KDa}}$-$b$-p(HPMA-Bz)$_{17.1\text{KDa}}$ micelles vs control group receiving HBS. Blood samples were taken after 24 h. Data are presented as mean ± SD (n = 3 per concentration).
Figure S13. Accumulation of curcumin-loaded Cy7-labeled mPEG$_{5kDa}$-$b$-p(HPMA-Bz)$_{17.1kDa}$ micelles in tissues of BALB/c mice at 4 and 24 h after intravenous injection. Data are presented as mean ± SD (n = 3 per time interval).

Figure S14. Therapeutic efficacy of curcumin-loaded micelles in Neuro2A tumor-bearing mice. Tumors were grown from cells subcutaneously injected (3 x 10$^6$ cells/100 µL PBS, pH = 7.4) into the right flank of female A/J mice. Treatment consisted of 10 consecutive intravenous injections (starting 9 days after inoculation) of HEPES-buffered saline (HBS), empty mPEG$_{5kDa}$-$b$-p(HPMA-Bz)$_{17.1kDa}$ micelles (500 mg/kg, polymer), and curcumin-loaded mPEG$_{5kDa}$-$b$-p(HPMA-Bz)$_{17.1kDa}$ micelles (500 mg/kg micelles, equivalent to 50 mg/kg curcumin) dispersed in HBS. The injection volume was between 100-200 µL and infusions were performed slowly. Arrows point to the days on which intravenous injections were administered. (A) Mean ± SD tumor size (n = 5). (B) Mean ± SD relative body weight of Neuro2A tumor-bearing mice (n = 5). The body weights were normalized to the animal’s body weight on day 9 (considered as 100%).
Figure S14. Therapeutic efficacy of curcumin-loaded micelles in Neuro2A tumor-bearing mice. Tumors were grown from cells subcutaneously injected (1 × 10⁶ cells/100 μL PBS, pH = 7.4) into the right flank of female A/J mice. Treatment consisted of 10 consecutive intravenous injections (starting 7 days after inoculation) of HEPES-buffered saline (HBS), empty mPEG₅kDa-b-p(HPMA-Bz)₁₇.₁kDa micelles (500 mg/kg, polymer), and curcumin-loaded mPEG₅kDa-b-p(HPMA-Bz)₁₇.₁kDa micelles (500 mg/kg micelles, equivalent to 50 mg/kg curcumin) dispersed in HBS. The injection volume was between 100-200 μL. Arrows point to the days on which intravenous injections were administered. Data are presented per individual mouse.

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