Supplementary Materials: Macrophage Targeting pH Responsive Polymersomes for Glucocorticoid Therapy

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Polymer Synthesis and Characterization

PMPC-b-PDPA was prepared by loading a round bottom flask (equipped with a magnetic stir bar) with 2-methacryloyloxyethyl phosphorylcholine (MPC, 25 eq.), 2-(4-morpholino)ethyl 2-bromoisobutyrate (ME-Br) initiator (1 eq.) and ethanol (final [MPC] = 2.8M), and this solution was deoxygenated by purging N₂ for at least 1 h under stirring at room temperature. Then, 2,2’-bipyridine (bpy) ligand (2 eq.) and Cu(I)Br (1 eq.) were added as solids whilst maintaining the flask under a mild positive N₂ pressure. The reaction was carried out under a N₂ atmosphere at 30 °C. After 90 min (MPC conversion > 99% from 1H-NMR), an ethanolic solution of 2-(diisopropylamino) ethyl methacrylate (DPA, 85 eq., [DPA] = 3.8 M), previously deoxygenated by purging N₂, was injected into the flask. After 48 h, the reaction solution was opened to air, diluted with ethanol and left stirring for 1 h. The solution was then passed through a silica column to remove the copper catalyst. After this step, the filtrate was concentrated by rotary evaporation and dialysed using a 3.5 kDa MWCO dialysis membrane (Spectrum Labs, Netherlands) against chloroform/methanol 2:1 (v/v) (2–3 × 500 mL), methanol (2–3 × 500 mL), and double-distilled water (4–6 × 2 L). After dialysis the copolymer was isolated by freeze-drying.

1H-NMR [CDCl₃/CD₃OD 3:1 (v/v), 600 MHz, H given in number per monomer unit, all broad signals]: PMPC₂₅-PDPA₆₈, δ = 4.24 (2H, PMPC); 4.14 (2H, PMPC) 3.98 (2H, PDPA), 3.84 (2H, PMPC), 3.69 (2H, PMPC), 3.24 (9H, PMPC) 3.00 (2H, PDPA), 2.64 (2H, PDA), 1.87–1.78 (2H, PMPC and 2H, PDPA), 1.01 (12H, PDPA), 0.89 (3H, PMPC and 3H, PDPA). GPC (H₂O + 0.25% TFA as eluent): PMPC₂₅-PDPA₆₈, Mₙ = 21.0 kDa, Mₘ/Mₙ = 1.39.

Cy5-labelled PMPC-b-PDPA was prepared as above but using bis[2-(2-bromoisobutryloxy)ethyl] disulfide as initiator [1]. After purification and isolation, an aliquot of the obtained polymer was reacted with Cyanine5 maleimide (1.1 eq.) and PPh₃ (2 eq.) in degassed chloroform/methanol [2:1 (v/v)]. The final polymer concentration was 1.6 mM, and the reaction was kept stirring under N₂ and in the dark at room temperature for 48 h. After this time, the reaction solution was opened to the air, filtered onto a silica column and dialysed using a 3.5 kDa MWCO dialysis membrane (Spectrum Labs, Netherlands) against chloroform/methanol 2:1 (v/v) (2–3 × 500 mL), methanol (4–6 × 500 mL), and double-distilled water (4–6 × 2 L). After dialysis the copolymer was isolated by freeze-drying.

GPC (H₂O + 0.25% TFA as eluent): Cy5-PMPC₂₅-PDPA₇₀, Mₙ = 23.0 kDa, Mₘ/Mₙ = 1.35.
Figure S1: Chemical structure of (a) PMPC\textsubscript{25}-PDPA\textsubscript{68} and (b) Cy5-PMPC\textsubscript{25}-PDPA\textsubscript{70}.
Polymersomes Characterization

Regarding the characterization study, HPLC analyses resulted in the drug encapsulation and loading efficiencies within PMPC-PDPA polymersomes. The drug encapsulation efficiency (EE) was calculated as the ratio between the final and initial mass of loaded prednisolone disodium 21-phosphate (PDP). The drug loading efficiency (LE) was determined according to a previously reported method [2] represented as the number of PDP molecules loaded within the total lumen volume of PMPC-PDPA polymersomes (which is related with the size of the vesicle and the actual amount of loaded drug).

|               | \(D_h\) (nm) | PDI       |
|---------------|-------------|-----------|
| Psome         | 117 ± 5     | 0.098 ± 0.01 |
| Psome:PDP     | 178 ± 4     | 0.166 ± 0.10 |

**Figure S2:** (a) DLS data on the hydrodynamic diameter (\(D_h\)) and polydispersity index (PDI) values of all formulations of unloaded and PDP loaded PMPC-PDPA polymersomes \((n = 3)\). Analysis on the PDI values below 0.2 indicates a formulation of polymersomes with monodisperse and homogeneous size distribution [3]. (b) TEM representative image of Cy5-PMPC-PDPA polymersomes produced via film rehydration method (200 nm scale bar). (c) DLS data on the number of PMPC-PDPA polymersomes as a function of the \(D_h\). Analysis on the drug loading capacity represented as the number of PDP molecules per polymersome as a function of their size. (d) Cryo-TEM representative image of PMPC-PDPA polymersomes produced via pH-switch method (200 nm scale bar). (e) Chemical structure and electrostatic surfaces of prednisolone disodium 21-phosphate (PDP) and respective representation of the electrostatic surfaces.
Drug Release study

To examine the kinetics and mechanism of PDP release from the PMPC-PDPA polymersomes, the data obtained from the in vitro drug release studies of each pH profile was analyzed using various models, including the zero and first order, Higuchi, Hixson-Crowell and Korsmeyer-Peppas models [4,5].

**Table S1. Mathematical models for drug-release kinetics.**

| Release Model       | Equation 1 | Information                                                                 |
|---------------------|------------|------------------------------------------------------------------------------|
| Zero–Order          | Q = Q0 + K₀t | refers to the process of constant drug release from a drug delivery device |
| First–Order         | Log C = Log C₀ – (kt / 2.303) | represents a system where the release rate of the drug depends on the concentration of the drug in the system |
| Hixson–Crowell      | Q₀t/3 – Q₁/3 = KₘC t | describes the release from systems where there is a change in surface area and diameter of particles |
| Higuchi             | Qt = k₀t (t)₀.₅ | assumes that the drug’s release is caused primarily by a diffusion mechanism |
| Korsmeyer–Peppas    | F=M₀/M N = Kᵗ | provides insight into the type of drug release mechanism taking place from swellable devices |

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1 Q is the amount of drug released or dissolved; Q₀ is initial amount of drug in solution; C₀ is the initial concentration of drug; t is the time in hours; F is the fraction of drug release at time t; M₀/M is the fraction of drug released at time t; K are the rate constants for each models.

**Table S2. Correlation coefficient (r²) from various drug release mathematical models for each pH profile.**

|          | Zero–Order | First–Order | Hixson–Crowell | Higuchi | Korsmeyer–Peppas |
|----------|------------|-------------|----------------|---------|------------------|
| pH 5.0   | 0.935      | 0.635       | 0.643          | 0.995   | 0.172            |
| pH 6.5   | 0.984      | 0.657       | 0.757          | 0.959   | 0.503            |
| pH 7.4   | 0.636      | 0.419       | 0.410          | 0.758   | 0.348            |

**Cell Viability Study**

**Figure S3:** Cell viability assay after 48 h incubation with increasing concentrations of (a) unloaded PMPC-PDPA polymersomes, (b) either free PDP or PDP-loaded polymersomes (Psome:PDP).
Gene Expression Study

For the RT-qPCR experiments, the ribosomal protein L13A (RPL13A) was used as reference gene, because it was stably expressed in THP-1, both in stimulated and unstimulated cells (data not shown).

Table S3. Forward (Fw) and reverse (Rv) gene sequences of designed primers (PRIMER-BLAS; Sigma-Aldrich) used for gene expression studies.

| Gene   | Primers          | Classification       |
|--------|------------------|----------------------|
| RPL13A | Fw CTTCCTTTCCAGTTTGCTGC | ribosomal protein    |
|        | Rv TCTCGCAGTCCACTTCTTT |                      |
| TNFα   | Fw GGAAGGGTGACCGACTCA  | tumor necrosis       |
|        | Rv CTGCCAGACTCGGCAA   | factor               |
| IL8    | Fw TCCAAACCTTTCACCCCAAA | chemokine           |
|        | Rv ACCCTCTGCACCCAGTTTT   |                      |
| IL6    | Fw TGCAATAACCACCCCTGACC | interleukin         |
|        | Rv AGCTGCCGAGAATGAAGTA   |                      |
| IL1β   | Fw CCAAAGAAAGATGGAAGACC | interleukin         |
|        | Rv GGGACGCGCCAGACTCAAA  |                      |

RT-qPCR data was analysed using the comparative cycle threshold (Ct) method, also known as the \( \Delta \Delta \text{Ct} \) method. The Ct value of each target gene (TNFα, IL1β, IL6 and IL8) was normalized to the reference gene (RPL13A), obtaining the \( \Delta \text{Ct} \) value (Equation 1) of treatment and control (i.e., non-treated). Then, the change in Ct is compared against the control to obtain the \( \Delta \Delta \text{Ct} \) value (Equation 2) using the following equations:

\[
\Delta \text{Ct} = \text{Ct (target gene)} - \text{Ct (RPL13A)} \quad (1)
\]

\[
\Delta \Delta \text{Ct} = \Delta \text{Ct (treated)} - \Delta \text{Ct (non-treated)} \quad (2)
\]

Then, the \( -\Delta \Delta \text{Ct} \) values corresponds to the folds in gene expression change of the treated compared to the non-treated group.

References

1. Gaitzsch, J.; Delahaye, M.; Poma, A.; Du Prez, F.; Battaglia, G. Comparison of metal free polymer-dye conjugation strategies in protic solvents. Polym. Chem. 2016, 7, 3046–3055, doi:10.1039/c6py00518g.
2. Wang, L.; Chierico, L.; Little, D.; Patikarnmonthon, N.; Yang, Z.; Azzouz, M.; Madsen, J.; Armes, S.P.; Battaglia, G. Encapsulation of biomacromolecules within polymersomes by electroporation. Angew Chem. Int. Ed. Engl. 2012, 51, 11122–11125, doi:10.1002/anie.201204169.
3. Blanco, E.; Shen, H.; Ferrari, M. Principles of nanoparticle design for overcoming biological barriers to drug delivery. Nat. Biotechnol. 2015, 33, 941–951, doi:10.1038/nbt.3330.
4. Mircioiu, C.; Voicu, V.; Anuta, V.; Tudose, A.; Celia, C.; Paolino, D.; Fresta, M.; Sandulovici, R.; Mircioiu, I. Mathematical Modeling of Release Kinetics from Supramolecular Drug Delivery Systems. Pharmaceutics 2019, 11, doi:10.3390/pharmaceutics11030140.
5. Mhlanga, N.; Ray, S.S. Kinetic models for the release of the anticancer drug doxorubicin from biodegradable polylactide/metal oxide-based hybrids. Int. J. Biol. Macromol. 2015, 72, 1301–1307, doi:10.1016/j.ijbiomac.2014.10.038.

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