In this study, a rationally designed nanocomposite (BUDPDA@MAP) composed of polydopamine (PDA) nanoparticle and anti-inflammatory drug budesonide (BUD) encapsulated in a pH-responsive endosomolytic polymeric (poly(butyl methacrylate-co-methacrylic acid) grafted acetalated dextran, denoted by MAP), is proposed. The uniform nanocomposite is prepared using a microfluidic device. At low endosomal pH (5.5), MAP destabilizes the endosomal membranes for the cytoplasmic delivery of PDA, and releases BUD simultaneously, resulting in a greater reactive oxygen species scavenging capability than both the free drug and PDA alone. The combined therapeutic efficacy from PDA and BUD also leads to a successful macrophage phenotype switch from pro-inflammatory M1 to anti-inflammatory M2.

1. Introduction

The persistent activation of pro-inflammatory macrophages is the hallmark of nonresolving inflammation, which drives the development of many prevalent chronic diseases, including rheumatoid arthritis, atherosclerosis, diabetes, and some types of cancer. The pro-inflammatory macrophages (M1 phenotype) secrete high levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-12, and IL-23, which contribute to inflammation progression. M1 macrophages also produce increased amount of reactive oxygen species (ROS), which may cause protein denaturation, lipid oxidation, and DNA damage. It has been found that the oxidative stress induced by ROS can further elevate inflammation by stimulating the intracellular signaling pathways, such as NF-κB, to produce more pro-inflammatory cytokines. Considering the critical role of M1 macrophages, switching the pro-inflammatory M1 phenotype to anti-inflammatory M2 phenotype, which secretes anti-inflammatory cytokines (e.g., IL-10 and transforming growth factor-β) and promotes tissue repair, is a promising strategy for anti-inflammatory therapy.

Polydopamine (PDA), a natural biomacromolecule, has been intensively used as biosensing, bioimaging, photothermal agent, and surface coating material in the biomedical field, due to its high biocompatibility, biodegradability, and molecular adsorption ability. In addition, the abundant reductive pedant groups, such as phenol, catechol, and imine, enable...
PDA to scavenge multiple ROS effectively for the treatment of inflammation-induced injuries both in vitro and in vivo.\textsuperscript{17–20} Despite the impressive ROS scavenging capability, PDA-based nanoparticles (NPs) were found to accumulate in endosomes and lysosomes, followed by the endocytic uptake, and exocytosed eventually after prolonged treatment.\textsuperscript{21} The endo/lyso-somal entrapment and the exocytosis may limit the availability of PDA NPs for cytoplasmic ROS scavenging, thus impairing its efficacy.

Endosomolytic polymeric carriers can facilitate the cytoplasmic delivery by disrupting endo/lyso-somal membranes.\textsuperscript{22} Commonly used endosomolytic polymers include polyethylenimine and polyamidoamine, which are cationic at endosomal pH. The prevalent positive charges cause osmotic swelling of endosomes, resulting in “proton sponge” effects.\textsuperscript{23} Despite the high efficiency, these cationic polymers also have high cytotoxicity, due to the nonspecific binding to cell membranes.\textsuperscript{24} In comparison, anionic pH-responsive polymers which only disrupt lipid membranes at endo/lyso-somal pH via a different endosomolytic mechanism have been developed.\textsuperscript{25} With pendent carboxylic acid groups, these polymers are negatively charged at physiological pH, and not disrupt lipid membranes due to electronic repulsions.\textsuperscript{26} At endosomal pH when the carboxylic acid groups become protonated, these polymers gradually lose their negative charges and become more hydrophobic, which enhance the interaction with endosomal membranes.\textsuperscript{27} Subsequently, the endosomal membrane permeability increases, resulting in successful endosomal escape of payloads.\textsuperscript{28} The pH-controlled membrane interaction strategy dominated by anionic pH-responsive polymers has been used to deliver various payloads, including peptides, genes, and proteins.\textsuperscript{29–32}

Herein, we develop a nanocomposite composed of PDA NPs and a common anti-inflammatory corticosteroid drug, budesonide (BUD), co-encapsulated in a pH-responsive endosomolytic polymer, for macrophage phenotype switch from M1 to M2. The endosomolytic polymer, poly(butyl methacrylate-co-methacrylic acid) grafted acetalated dextran (AcDx) (MAP, i.e., membrane-active polymer), facilitates the endosomal escape of PDA for cytoplasmic ROS scavenging. The BUD, co-loaded in MAP, contributes to modulating the macrophages polarity from pro-inflammatory phenotype to anti-inflammatory phenotype, by regulating the expression of critical inflammatory-related genes.\textsuperscript{33,34} By rationally designed the PDA and BUD co-loaded nanocomposite (BUDPDA@MAP), we aim to combine ROS scavenging capability from PDA and the anti-inflammatory efficacy from BUD, simultaneously delivered to cytoplasm by MAP as a carrier.

The MAP polymer was synthesized by grafting pH-responsive endosomolytic moieties (poly(butyl methacrylate-co-methacrylic acid), pBMAMAA) on AcDx, which can accommodate hydrophobic drugs with pH-controlled release.\textsuperscript{35,36} pBMAMAA has been reported to have pH-dependent membrane-lytic activity.\textsuperscript{26} After polymer synthesis, BUDPDA@MAP NPs were fabricated by nanoprecipitation on a glass capillary-based microfluidic device with uniform size and morphology characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The cytotoxicity, cellular uptake, and endosomal escape of NPs were evaluated on murine macrophages (RAW 264.7). Finally, the antioxidant and the macrophage phenotype switch property was also characterized in inflamed RAW 264.7 macrophage cells.

2. Results and Discussions

2.1. MAP Preparation and pH-Responsive Membrane-Lytic Activity

As shown in Figure 1a, dextran was first acetalated by 2-methoxypropene to form AcDX, and then conjugated with a reversible addition-fragmentation chain-transfer (RAFT) polymerization agent, to synthesize pBMAMAA via a “grafting from” process. Each intermediate and the final product were characterized by nuclear magnetic resonance (NMR) spectroscopy, as shown in Figure 1b. The characteristic peaks of AcDX, which were attributed to the anomeric proton, appear from 4.6 to 5.6 ppm. The appearance of these peaks, compared with the starting material (dextran), confirms the successful acetalation. The following AcDX-Macro RAFT shows small peaks from 7.5 to 8.0 ppm (enlarged in Figure 1b), which was assigned to the benzyl ring of the RAFT agent. The final MAP polymer shows new peaks at 12.4 ppm (–COOH from methacrylic acid (MAA)) and 0.8–2 ppm (–CH\textsubscript{3} and –CH\textsubscript{2} from MAA and butyl methacrylate (BMA), partially overlapping with the –CH\textsubscript{3} from AcDX), which suggested the successful grafting of pBMAMAA.

Next, we investigated the interaction between MAP and plasma membrane by hemolysis assay, which is a well-established methodology for membrane interaction study.\textsuperscript{37–39} If the polymer has significant interaction and causes defect on the plasma membrane, hemoglobin will be released and detected subsequently by a microplate reader (different levels of hemolysis can be also visualized in Figure 1c, inset). As shown in Figure 1c, MAP showed significant hemolysis at late endosomal pH (5.5 and 5.0), but no hemolysis at physiological pH (7.4), which is similar to free pBMAMAA (Figure S1a, Supporting Information). This is because of the pH-dependent protonation of the pBMAMAA grafts. At pH 7.4, MAA residuals were deprotonated, thus negatively charged.\textsuperscript{26} As a result, the polymer was negatively charged and hydrophilic. The electrical repulsion between MAP and plasma membrane inhibited the mutual interaction. As the pH decreased, the MAA residuals became gradually protonated, and thus, more hydrophobic. The increased hydrophobicity enhanced the interaction with red blood cell (RBC) membranes, which caused significant hemolysis. The pH-dependent hemolysis profile indicates MAP can induce endosomal escape, but remains inert to the cell membranes in physiological conditions.

The concentration-dependent hemolysis was also investigated at the most potent pH (5.5) and physiological pH (7.4) conditions, as shown in Figure 1d. When the concentration reached 50 µg mL\textsuperscript{-1} and beyond, the hemolysis increased to 60–73%, which is comparable to pBMAMAA (Figure S1b, Supporting Information). As for pH 7.4, no hemolytic activity was observed over the studied concentration range. The concentration-dependent hemolysis results suggest that MAP induced membrane lysis at late endosomal pH, but remained inert to membrane over
a broad concentration range, thus enabling a broad therapeutic window.

2.2. Co-Loading of PDA and BUD in MAP

After MAP preparation, PDA and BUD were encapsulated in MAP by nanoprecipitation on a glass-capillary microfluidic device with two co-flow channels, as shown in Figure 2a. PDA NPs used here had a size of 136.7 nm measured by DLS. In the microfluidic setting, the inner fluid was the ethanol dispersion of PDA NPs, MAP, and BUD, while the outer fluid was Milli-Q water. Because MAP and BUD are poorly dissolved in Milli-Q water, the fast mixing of inner and outer fluids caused supersaturation, which initiated nanoprecipitation. PDA and BUD was thus encapsulated by nanoprecipitated MAP composites.

To optimize the parameters on the microfluidic device, we first used MAP without BUD or PDA for the nanoprecipitation. At the flow rate of 2 mL h⁻¹ for inner and 20 mL h⁻¹ for outer fluids, the MAP NPs prepared had a uniform size of 150.7 nm with a polydispersity index (PDI) of 0.09 (Figure S2a, Supporting Information). The mono-dispersed MAP NPs were spherical with smooth surfaces, as shown in Figure S2b, Supporting Information. Then BUD was mixed with MAP in the inner fluid at 1 mg mL⁻¹ (5 wt% of MAP). The size of BUD loaded MAP NPs (BUD@MAP) slightly increased to 162.2 nm compared with MAP, with still a narrow size distribution (Figure 2e) and spherical morphology (Figure 2b and Figure S3a, Supporting Information). The PDA encapsulated by MAP (PDA@MAP, 182.0 nm, PDI = 0.10) also showed smooth surfaces and spherical morphology (Figure 2c and Figure S3b, Supporting Information), which is very different from bare PDA NPs that are not perfectly round with rough surfaces (Figure S4, Supporting Information). The increase in the hydrodynamic size from 136.7 to 182.0 nm, along with the changes in morphology, indicates the successful encapsulation of PDA in MAP. Unfortunately, the contrast of PDA and MAP were similar in TEM images, so it was difficult to evaluate the core-shell structure. Considering the metal-chelating property of PDA, we treated PDA NPs with copper sulfate solution, to increase the contrast differences. The high-magnification TEM image of Cu-PDA@MAP is shown in Figure S5, Supporting Information. The higher brightness of MAP compared with the Cu-stained PDA core confirmed the structure of PDA@MAP NPs.
Finally, the MAP NPs with both BUD and PDA encapsulated (BUDPDA@MAP, 190.1 nm, PDI = 0.11) were fabricated, as shown in Figure 2d,g and Figure S3c, Supporting Information. Fourier transform infrared spectroscopy (FTIR) confirmed the presence of both characteristic peaks from PDA and MAP in the BUDPDA@MAP spectrum (Figure S6, Supporting Information). The peak of BUD was hardly visible in BUDPDA@MAP, possibly due to the small amount incorporated. The loading degree (LD) of BUD and PDA is 1.9% and 18.9%, respectively (Figure 2h). This is similar to the LD of BUD or PDA separately in BUD@MAP (1.9%) or PDA@MAP (19.6%), which indicates the co-loading of two payloads using the nanoprecipitation microfluidic device is simple and orthogonal.

To investigate the pH-controlled drug release profile, free BUD and BUDPDA@MAP were incubated in HBSS–MES (pH 5.0) and phosphate buffered saline PBS (pH 7.4) buffers, mimicking the intracellular (late endosomal pH) and extracellular conditions, respectively. Free BUD showed identical burst release at both pH-values (Figure 2i,j). Within 1 h, more than 70% of BUD was released. On the contrary, BUDPDA@MAP only had a slight release (≈21%) at pH 7.4 over the 24 h incubation, suggesting that the MAP matrix can protect the loaded drugs from leakage. At pH 5.0, BUD was released quickly from BUDPDA@MAP. At 2 h, more than half (≈53%) was released and eventually achieved almost 100% after 24 h. The overall results demonstrate that the acid-triggered degradation of MAP can release the drugs in the acidic endosomal environment, but not in physiological conditions.

### 2.3. Cytotoxicity and Cellular Uptake Studies

Before moving on to the in vitro anti-inflammation studies, we first tested the cytotoxicity of the materials synthesized using RAW 264.7 macrophage cells, which are typical murine macrophages. The cells were cultured with MAP, or PDA@MAP for 6, 24, and 48 h, respectively, and the cell viability was evaluated by CellTiter-Glo luminescence assay. As shown in Figure 3a,b, both MAP and PDA@MAP had negligible cytotoxicity towards RAW 264.7 cells at all concentrations tested during 48 h incubation. We then tested the cytotoxicity in THP-1 cells (human monocytes, which are commonly used for macrophage differentiation). The results are similar to those of RAW 264.7 (Figure S7, Supporting Information). For MAP and PDA@MAP NPs, the percentage of viable cells were over 90% within the entire range of concentrations tested for all time points.

Then the cellular uptake of PDA and PDA@MAP was evaluated by confocal microscopy and flow cytometry. As shown in...
Figure 3. a,b) Cytotoxicity of MAP and PDA@MAP in RAW 264.7 cells after 6, 24, and 48 h incubation. The results were normalized to the untreated controls. Data are presented as the mean ± SD (n = 4). All the groups have no significant differences compared with negative control (p > 0.05). c) Confocal images of RAW 264.7 cells treated without particles (control), with FITC-labelled PDA, or FITC-labelled PDA encapsulated in MAP for 1 h. The endo/lyso-somes were stained with Lysotracker red, and the cell membranes were stained with CellMask deep red. Scale bar = 50 µm.

Figure 3c, the FITC-labelled PDA shows green fluorescence, while the cell membrane and endo/lyso-somes show red and yellow fluorescence (stained by CellMask and Lysotracker, respectively). The cells incubated with PDA NPs showed weak green fluorescence only in a few cells, while those incubated with PDA@MAP showed strong diffused green signal in almost all cells. This indicates the encapsulation in MAP significantly increased the particle uptake, possibly due to the endosomal escape and the subsequent cytoplasmic delivery of the NPs. Flow cytometry results also confirmed the trend observed in the confocal images, the mean fluorescence intensity (MFI) increased by 20 times after MAP encapsulation (Figure S8, Supporting Information). Then we quenched the fluorescence of the particles attached on the cell membranes by adding trypan blue (TB). Although the MFI slightly decreased after the TB treatment, the intensity of PDA@MAP is still much higher than that of PDA, suggesting the enhancement of MAP on PDA cell internalization.

2.4. ROS Scavenging Study

To evaluate the ROS scavenging capability, we first stimulated the RAW 264.7 cells with lipopolysaccharides (LPS) and interferon-gamma (IFN-γ), which were common reagents for inducing oxidative stress and inflammatory responses[4] (Figure 4a). Then the cells were treated with different NPs for 5 h and further analyzed with an oxidant-sensing fluorescent probe 2′,7′-dichlorofluorescin diacetate. Non-polar DCFH-DA can diffuse through cell membranes into cytoplasm where it gets hydrolyzed by esterases, and subsequently oxidized by ROS to become fluorescent.[40] The fluorescence intensity of all cells were then analyzed using flow cytometry and confocal microscopy.

As shown in Figure 4b, after LPS and IFN-γ stimulation (positive control in this study), the ROS level increased by 5.4 times, indicating the significant oxidative stress suffered from cells. Then the cells stimulated by LPS and IFN-γ but treated by different nanoparticles or drugs showed different levels of decrease in ROS. PDA NPs induced a decrease of ROS by 0.7 compared with positive control. PDA@MAP showed a further decrease of ROS by 2.0 times, which suggests the intracellular delivery of PDA by endosomolytic MAP facilitated the cytoplasmic ROS scavenge. Free anti-inflammatory drug, BUD, showed a ROS decrease by 1.8 times, possibly due to the alteration in the gene expression related to intracellular ROS metabolism. It has been found that glucocorticoids can up-regulate a series of antioxidant enzymes, to enhance the synthesis of glutathione for ROS neutralization.[34] The final formulation BUDPDA@MAP achieved the best ROS scavenging efficacy. The ROS level decreased to 2.7, only half of the positive control. This indicates both PDA and BUD in the final NP contribute to ROS down-regulation.

The intracellular ROS level was also visualized by confocal microscopy images (Figure 4c), which demonstrated consistent results compared with flow cytometry analysis in Figure 4b. The prevalent green fluorescence emitted by oxidized DCFH in positive control (cells stimulated by LPS and IFN-γ) confirms the high ROS level. Although PDA NPs did not show a significant dif-
Figure 4. a) Schematic showing the intracellular ROS study. b) Flow cytometry analysis of ROS level in RAW 264.7 cells stimulated by LPS and IFN-\(\gamma\) for 24 h, and then treated with different particles with equivalent amount of PDA at 20 \(\mu\)g mL\(^{-1}\) or BUD at 2 \(\mu\)g mL\(^{-1}\). The results were normalized to samples without DCFH-DA treatment. Data are presented as the mean ± SD (n = 4). (c) Confocal images of RAW 264.7 cells in the ROS study. Intracellular ROS level was indicated by the green fluorescence emitted by dichlorofluorescein. The plasma membranes were stained with CellMask deep red to outline the cells. Scale bar = 100 \(\mu\)m.

ference compared with the positive control, PDA@MAP showed better results of ROS scavenging, with less green cells. In the final formulation BUDPDA@MAP, almost no green fluorescence was observed, which indicates a great reduction in the cells’ ROS level.

2.5. Macrophage Phenotype Switch Study

The anti-inflammatory therapeutic efficacy was evaluated using RAW 264.7 cells in vitro. As shown in Figure 5a, the macrophages in resting state (M0) were first stimulated with LPS (100 ng mL\(^{-1}\)) and IFN-\(\gamma\) (2 ng mL\(^{-1}\)) for 1 day, and then treated by different NPs or drugs for another 2 days. The cells stimulated with LPS and IFN-\(\gamma\), but without any treatment developed into M1 phenotype (M1 control), while those M0 cells with anti-inflammatory cytokine stimulation (IL-4 and IL-13) for 3 consecutive days, developed into M2 phenotype (M2 control). M1 and M2 phenotypes have distinguishable markers on the cell’s surface and cytokine secretion profiles.\(^4\) Therefore, we evaluated the expression of CD80 (M1 marker) and CD206 (M2 marker) by immunostaining and subsequent analysis using flow cytometry, as well as the concentration of TNF-\(\alpha\) (M1 cytokines) and IL-10 (M2 cytokines) using enzyme-linked immunosorbent assay (ELISA), to identify the phenotypes after treatment.

As shown in Figure 5b–e, all treated groups showed alleviation of inflammation to different extents. PDA and PDA@MAP reduced inflammation effectively by downregulation of CD80 and TNF-\(\alpha\), but had little effect on the expression of CD206 and IL-10 secretion. This indicates PDA and PDA@MAP fa-
Figure 5. a) Schematic representation of the macrophages stimulation and treatment. b,d) Concentration of TNF-α and IL-10 in the macrophage culture medium after stimulation and treatment, quantified by ELISA. c,e) Flow cytometry analysis of macrophage markers CD80 and CD206 expression after immunostaining. The fold of change in MFI was plotted compared with non-stained samples. Data are presented as the mean ± SD (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001.

ciliated the conversion of pro-inflammatory macrophages (M1, CD80^{high}, CD206^{low}) to resting states (M0, CD80^{low}, CD206^{low}), possibly due to the clearance of ROS. However, both PDA and PDA@MAP did not promote the conversion of M2 phenotype (CD80^{low}, CD206^{high}). BUD and BUD@MAP also downregulated CD80 and TNF-α, though not as effective as PDA. Unlike PDA, the elevation of CD206 and IL-10 treated with BUD was significant, indicating M2 conversion. The final formulation, BUDPDA@MAP, combined the ROS scavenging effects from PDA and the M2-induction capability of BUD. Compared with M2 control that were stimulated with anti-inflammatory cytokines, the cells treated with BUDPDA@MAP had similar TNF-α and IL-10 profiles, indicating the successful macrophage phenotype conversion.

The co-delivery of PDA and BUD in BUDPDA@MAP, targets both oxidative stress and inflammation. According to a recent study, PDA actively reduced the down-regulation of Toll-like receptor 4 expression (for sensing LPS) and NF-κB signaling pathway by scavenging ROS and activating HO-1-related antioxidant signaling pathway.[41] This indicates PDA mainly settled down the inflammation due to its anti-oxidative properties, but with very little effect in the M2 phenotype macrophage induction. BUD, a glucocorticoid drug, complemented PDA in the anti-inflammation therapy, by reprogramming macrophages to M2 phenotype. BUD can regulate the expression of critical inflammatory-related genes, such as those responsible for IL-10 production, ROS metabolism, and NF-κB deactivation.[33,34] By modulating the transcriptional response, BUD converts the pro-inflammatory M1 phenotypes to anti-inflammatory M2, which in turn promotes the inflammation resolution. The complementary function is achieved by combining both PDA and BUD anti-oxidative and anti-inflammatory therapeutic efficacies, respectively, followed by the intracellular delivery of MAP, thus enabling an efficient therapeutic response.

3. Conclusion

In summary, a ROS-scavenging co-loaded PDA and BUD nanocomposite was developed for anti-inflammatory therapy, using a microfluidic technique. The NPs, characterized by DLS and TEM, showed spherical morphology and monodispersed size, due to the precise control of the nanoprecipitation on a glass-capillary microfluidic device. MAP facilitated the endosomal escape and the intracellular delivery of PDA, thus enhancing the uptake and ROS scavenging efficacy. The co-delivery of BUD with PDA by MAP reduced the expression of pro-inflammatory cell makers and induced the secretion of immune-suppressive
cytokines IL-10, thus modulating the polarity of macrophages from pro-inflammatory to anti-inflammatory. The simple, but advanced preparation of NPs by the microfluidic device, along with the endosomal escape capability and pH-controlled drug release profile of the nanocomposites makes the nanosystem developed here a promising candidate for ROS scavenging and macrophage phenotype switch related applications.

4. Experimental Section

Materials for Map and NP Synthesis: Dextran (40 kDa), pyridinium-p-toluenesulfonate (PTPS), 2-methoxypropene, triethylamine (TEA), cyano-4-(phenylcarboxonothiyl)thiopentanoic acid (CPADB), N,N′,N′,N′-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), 2,2′-azobisis(2-methylpropionitrile) (AIBN) solution (0.2 M in toluene), MAA, BMA, ethyl acetate, dichloromethane (DCM), dimethyl acetamide (DMAc), anhydrous dimethyl sulfoxide (DMSO), 2-propanol, acetonitrile (ACN), acetic acid, 2-(N-morpholino)ethanesulfonic acid (MES) and dopamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC) isomer I was purchased from Life Technologies Gibco, USA. CellTiter-HBSS, Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), and Versene solution were purchased from Life Technologies Gibco, USA. CellTiter-Glo assay was purchased from Promega Corporation, USA. 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 10× PBS, non-essential amino acids (NEAA), l-glutamine 200 mM, penicillin (100 IU mL−1), streptomycin (100 mg mL−1), and trypsin (2.5%) were purchased from HyClone, GE Healthcare Lifesciences (Logan, UT, USA). Triton X-100 was purchased from Merck Millipore (Darmstadt, Germany). CellMask DeepRed, LysoTracker, and trypan-blue were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Mitotracker DeepRed, Ethidium homodimer, and DNA ladder were purchased from BioLegend (San Diego, CA, USA).

Synthesis of Membrane-Active Polymer: To prepare MAP, AcDX was first prepared, as reported elsewhere. Briefly, dextran (1.0 g) was dissolved in 10 mL of anhydrous DMSO. PPTS (15.6 mg) and 2-methoxypropene (3.4 mL) were added and the reaction was allowed for 4 h under the argon atmosphere before quenching by TEA (1 mL). The AcDX was obtained by precipitation in MilliQ water (100 mL). After centrifugation (15 min, 16,110 g), the pellet was redissolved in ethanol (20 mL), and precipitated in water again. The purification process was repeated twice, and the residual water was removed by freeze-drying for 48 h. The purified AcDX was characterized by 1H-NMR with an Avance III 400 MHz NMR spectrometer (Bruker, Switzerland).

Then a RAFT agent CPADB was conjugated on AcDX, following the protocol described elsewhere. AcDX (100 mg) was dissolved in DCM (1.6 mL), and then CPADB (10 mg) was added and stirred until dissolved. DCC (20 mg) and DMAP (1 mg) were separately dissolved in additional 0.2 mL of DCM. DCC solution was added to the polymer solution, followed by DMAP solution. The reaction proceeded overnight at room temperature. The reaction mixture was dialyzed against acetone for 2 days using dialysis bag (MWCO 3.5 kDa), and the solvent was replaced twice a day. After dialysis, the pink polymer solution was dried by rotary evaporator to obtain the AcDX macroRAFT, which was characterized by 1H-NMR spectroscopy. By the peak integration of benzyl rings (5 protons) from 7.4 to 7.9 ppm and the anomeric proton of AcDX from 4.7–5.2 ppm, it is possible to deduce the amount of RAFT agent on AcDX. On average, there are ≈3.6 CPADB per AcDX chain.

The MAP polymer was obtained by RAFT polymerization of MAA and BMA using AcDX macroRAFT agent ([CPADB]/[AIBN]/[MAA]/[BMA] = 1:0.3:140:60). The AcDX macroRAFT (100 mg, equivalent to 0.015 mmol CPADB) was dissolved in DMAc and IPA mixture solution (25 mL, 1:1 v/v). After AcDX macroRAFT was completely dissolved, MAA (176.6 µL, 2.08 mmol) and BMA (142.0 µL, 0.89 mmol) were added, followed by the addition of AIBN solution (22.3 µL, 0.2 M in toluene, 4.5 µmol). The flask was sealed and purged with nitrogen for 40 min, and then immersed in an oil bath at 70 °C for 16 h. The MAP polymer was purified by precipitation in cold diethyl ether and separated by centrifugation at 3214 g for 5 min. The collected pellet was redissolved in methanol and precipitated again in diethyl ether. The precipitation was repeated three times to remove unreacted monomers and other impurities. The final polymer pellet was dried in a vacuum oven at 40 °C for 1 day. The MAP was subsequently characterized by 1H-NMR.

Synthesis of PBMAAAM: A RAFT agent CPAAD (10 mg, 0.036 mmol) was dissolved in 5 mL 2-propanol in a round-bottom flask, and two monomers, MAA (425 µL, 5.0 mmol) and BMA (342 µL, 2.1 mmol), were added directly into the solution, followed by the addition of AIBN solution (54 µL, 0.2 M in toluene). The solution was purged with nitrogen for 20 min, and then the reaction was initiated by immersing the flask in an oil bath at 70 °C. After 16 h, the reaction was quenched, and the polymer was purified by precipitation in cold diethyl ether. The polymer precipitants were separated by centrifugation at 3214 g for 5 min. After discarding the supernatants, the precipitants were re-dissolved in methanol, and then precipitated in cold diethyl ether again. The precipitation was repeated 3 times to remove the residual monomers and other impurities. The final polymer products were dried in a vacuum oven, and characterized by 1H-NMR before use. The salt form of the polymers for hemolysis study were obtained by dissolving the polymer in 1 mM of sodium bicarbonate solution, following by overnight dialysis (MWCO 3.5 kDa) against Milli-Q water to remove excess of sodium bicarbonate. The final solution after dialysis was freeze-dried, yielding light pink fine powders.

Hemolysis Assay: The pH responsive cell membrane permeability properties were investigated, using human blood hemolysis assay. Briefly, the RBCs (1 mL) at the cell density of 5 × 10⁸ cells per mL was washed by 150 mM of sodium chloride solution and centrifuged at 835 g, 4 min for 3 times to remove protective reagents and cell debris. MAP and PBMAAAM were prepared at the concentration 10 mg mL⁻¹ in Milli-Q water, and diluted in citric acid buffer solution (pH 4.5, 5.0, 5.5, and 6.0, respectively) or PBS 1× (pH 7.4) to specific concentrations. Then, the purified blood (5 µL, 2 × 10⁸ cells) was added into each sample solution (200 µL) in triplicates and incubated in a 37 °C incubator with gentle shaking for 1 h. After that, all samples were centrifuged at 1485 g for 4 min, and the supernatant (100 µL) was transferred to a 96-well plate. The UV absorbance at 540 nm was recorded using a Varioskan LUX multimode microplate reader (Thermo Scientific, USA), to identify the hemoglobin concentration. RBCs incubated with buffers only were used as negative controls, while RBCs incubated with Milli-Q water were used as positive controls. The positive control was to lyse all RBCs since the water causes RBC swollen and breaks the cell membranes afterwards. The positive control was defined as 100% hemolysis, and the RBC lysis of negative control was defined as 0%. The final hemolysis for a specific polymer sample was calculated as:

\[
\text{Relative Hemolysis} = \frac{\text{Abs (positive control)} - \text{Abs (negative control)}}{\text{Abs (positive control)}} \times 100
\]

Synthesis of Polydopamine Nanoparticles: PDA-NPs were synthesized using the co-flow microfluidic device, as reported in detail elsewhere. Briefly, the inner fluid (dopamine hydrochloride aqueous solution at 200 mg mL⁻¹) was pumped into the inner tapered glass capillary at a flow rate of 1 mL h⁻¹. The first outer fluid (0.1 M of NaOH solution) was pumped into the first outer capillary at a flow rate of 30 mL h⁻¹. PDA NPs formed immediately by self-polymerization reaction, and particle formation reaction was terminated by the second outer fluid (1 M of HCl)
solution, 2.5 mL h⁻¹) after the mixing of the dopamine and NaOH. The obtained PDA NPs were then collected, and purified by centrifugation at 16,110 g for 10 min, and washed twice with Milli-Q water before further use. For the synthesis of FITC-labelled PDA NPs, FITC was added into the inner fluid at a concentration of 0.2 mg mL⁻¹, and the rest of the procedure was the same as the method mentioned above.

**Synthesis of PDA@MAP, BUD@MAP, and BUDPDA@MAP by Microfluidics:** The co-flow microfluidic device was fabricated according to a previously reported method.[46] Briefly, the inner capillary (120 µm in diameter) was inserted inside the outer capillary (1.2 mm in diameter), as shown in Figure 2a. Both capillaries were connected to blunt needles with polyethylene tubes for the injection of inner and outer fluids. The transparent epoxy resin was used to seal the device as required. The MAP encapsulating PDA NPs with or without BUD were prepared on the microfluidic device described above. The inner fluid was 20 mg mL⁻¹ of MAP and 2 mg mL⁻¹ of PDA NPs in ethanol, and the outer fluid was Milli-Q water (pH 7.4). The flow rate of the inner and the outer fluids was 2 and 20 mL h⁻¹, respectively. To produce BUD encapsulated NPs (BUD@MAP and BUDPDA@MAP), BUD (1 mg mL⁻¹) was dissolved in the inner fluid, and the rest of the procedure was as the same as the method mentioned above. The produced particles were collected by centrifugation at 16,110 g for 10 min and washed twice with Milli-Q water.

The particle size and PDI were measured using DLS (Malvern Zetasizer Nano-ZS, UK). The morphology of the different NPs was evaluated by TEM (Tecnai F12, FEI Company, USA). The chemical composition was confirmed by FTIR on a Bruker Vertex 70 spectrometer. The samples were mixed with KBr (FTIR grade, Sigma-Aldrich, USA) and pressed into pellets. The pellets consisted of 1 mg of sample and 200 mg of KBr. The FTIR spectra were recorded in the range of 4000–400 cm⁻¹ with a resolution of 4 cm⁻¹ using OPUS 8.1 software.

For the preparation of FITC-labelled PDA@MAP NPs, FITC labelled PDA NPs was used in the inner fluid, and the rest of the procedure was the same as the method mentioned above.

**Determination of LD:** The LD of BUD in BUD@MAP and BUDPDA@MAP was determined by stirring and dissolving the particles in a mixture of ethanol:ACN (1:1 v/v) to release all the loaded BUD. The amount of BUD was quantified by Agilent 1100 high performance liquid chromatography (HPLC) (Agilent Technologies, USA). The mobile phase was comprised of acetic acid (0.5% v/v, pH 3) and ACN (62:38 v/v) at a flow rate of 1.3 mL min⁻¹ at 25 °C, and the detection wavelength was 244 nm. A Discovery C18 column (4.6 × 150 mm, 5 µm, Supelco Analytical, USA) was used as stationary phase and the injection volume of the samples was 20 µL. The LD for the drug loaded particles was calculated as [mass of loaded drug/mass of drug loaded samples] × 100%. The LD of PDA in PDA@MAP and BUDPDA@MAP was determined by stirring the particles in ethanol to dissolve the matrix MAP and the loaded BUD. The undissolved PDA NPs were collected by centrifugation at 16,110 g for 10 min and washed twice with ethanol. The LD for the PDA loaded particles was calculated as [(mass of loaded PDA/mass of PDA loaded samples) × 100%]. Triplicate samples were used for LD determination.

**In Vitro Drug Release:** The pH-responsive drug release profile of BUDPDA@MAP was studied in sink conditions and compared with free BUD in both PBS (pH 7.4) and HEPES/MES pH 5.0 buffer solutions, in which the physiological pH and late endosomal pH, respectively, were represented. The release medium was stirred at 150 rpm at 37 °C. At predetermined time intervals, the drug concentrations in the supernatant were quantified by HPLC as mentioned above. Triplicate samples were used for release study.

**Cell Culture:** RAW 264.7 murine macrophage cells (passage #4-10) were cultured in DMEM with 4.5 g L⁻¹ glucose, supplemented with 10% of FBS, 1% of l-glutamine, 1% of NEAA, penicillin (100 IU mL⁻¹), and streptomycin (100 µg mL⁻¹). The cells were cultured in the 5% CO₂-incubator at 37 °C, and 95% relative humidity. The culture media were changed every other day. Prior to each test, the cells were passaged and incubated for 5 min using 0.25% (v/v) trypsin EDTA/PBS.

THP-1 human monocytes leukemia cells (passage #14-16) were cultured in RPMI 1640, supplemented with 10% of FBS, 1% of l-glutamine, 1% of NEAA, penicillin (100 IU mL⁻¹), and streptomycin (100 µg mL⁻¹). The cell suspension was maintained in the 5% CO₂-incubator at 37 °C, and 95% relative humidity. The culture media were changed every 2–3 days.

**Cytotoxicity Studies:** The in vitro cytotoxicity was performed using a CellTiter-Glo luminescent cell viability assay (Promega Corp., WI, USA). The cells were seeded overnight on a 96-well plate at density of 1 × 10⁴ cells per well in cell culture medium. MAP from 100 to 1000 µg mL⁻¹, PDA from 50 to 500 µg mL⁻¹, or PDA@MAP at the concentrations based on the amount of PDA encapsulated MAP were tested. Triton X-100 solution (1% v/v in cell culture medium), and cell culture medium without any tested materials were used as negative and positive controls, respectively. After 6, 24, and 48 h, the cell viability was detected from the luminescent intensity, in which represent the amount of ATP produced by the viable cells. The assay was carried out using Varioskan LUX multimode microplate reader. All samples were tested in four replicates. The final cytotoxicity was normalized to controls without any tested materials.

**Nanoparticle Cellular Uptake Study:** For confocal imaging of nanoparticle cellular uptake, RAW 264.7 cells were seeded in Lab-Tek chambered borosilicate coverglass (8-chamber, ThermoFisher Scientific, USA) at a density of 1.5 × 10⁴ cells per well in cell culture medium. Then FITC-labelled PDA (50 µg mL⁻¹) or FITC-labelled PDA@MAP (equivalent to FITC-labelled PDA NPs 50 µg mL⁻¹) were added to the cells, and incubated for 1 h. The cells without any particle incubation were used as control. After incubation, the particles were gently removed and the cells were washed with PBS for three times. The cells were then incubated with CellMask DeepRed for 5 min at room temperature, followed by washing with PBS for three times. Finally, the cells were imaged by a Leica TCS SPS II HCS-A confocal microscope (Leica Microsystems, Germany). The images acquired were processed by Fiji 1.51 software.

For flow cytometry analysis of cellular uptake, RAW 264.7 cells were seeded at the density of 75,000 cells per well in a 24-well plate in cell culture medium. Then, cells were incubated with FITC-labelled PDA (50 µg mL⁻¹) or FITC-labelled PDA@MAP (equivalent to FITC-labelled PDA NPs 50 µg mL⁻¹). After incubation, the particles were removed, and the cells were washed twice with PBS before detachment by trypsin. The detached cells were centrifuged at 317 g, washed again with PBS, and analyzed on a BD LSR-II Cell Analyzer flow cytometer (USA). For those cell samples treated with fluorescent PDA or PDA@MAP, after flow cytometry analysis, trypan blue was added and incubated for 15 min to quench the fluorescence on the cell surface. After trypan blue incubation, the cells were washed by PBS, and analyzed again by flow cytometer. The MFI of each sample (in triplicates) was calculated by Flowjo software and normalized according to the negative control (cells without particle treatment).

**Measurement of Intracellular ROS:** RAW 264.7 cells at the density of 75,000 cells per well was cultured in a 24-well plate in cell culture medium. The cells were stimulated using LPS (100 ng mL⁻¹ in PBS) and IFN-γ (2 ng mL⁻¹ in 0.1% of BSA) prepared in cell culture medium. After 24 h, the stimulants were removed and replaced with the new fresh stimulants at the same concentrations containing samples including free BUD (2 µg mL⁻¹), PDA NPs (20 µg mL⁻¹), MAP (100 µg mL⁻¹), PDA@MAP (equivalent to PDA NPs 20 µg mL⁻¹), and BUDPDA@MAP (equivalent to 2 µg mL⁻¹ of BUD and 20 µg mL⁻¹ of PDA NPs). The cells treated with only LPS and IFN-γ for 24 h were used as positive control. After 5 h, the cells were washed with PBS and incubated with 40 µM of DCFH-DA dissolved in PBS containing 0.1% of DMSO in dark condition for 30 min. After that, the cells were washed once and detached with Versene (0.2 mL per well) for 15 min at 37 °C. The suspended cells were mixed with PBS-EDTA (0.2 mL) and collected in each glass tube, and centrifuged at 317 g for 5 min. The cell pellets were collected and washed twice with PBS before analyzing on the flow cytometer (BD LSR-II Cell Analyzer). The MFI of each sample (four replicates) was calculated by Flowjo software and normalized according to the negative control (cells without particle treatment).

For confocal imaging of the intracellular ROS, cells were seeded in Lab-Tek chambered borosilicate coverglass at a cell density of 1.5 × 10⁴ cells per well in cell culture medium. After attachment, the cells were stimulated using LPS (100 ng mL⁻¹ in PBS) and IFN-γ (2 ng mL⁻¹) similarly to the samples for flow cytometry analysis. After 24 h stimulation, the cells
were treated with nanoparticles for 5 h stated above, followed by DCFH-DA staining. Finally, the cells were washed and stained with CellMask DeepRed, before imaging by a Leica TCS SP5 II HCS-A confocal microscope. The images acquired were processed by Fiji 1.5.1 software.

In Vitro Anti-Inflammatory Therapeutic Efficacy Evaluation: RAW 264.7 cells were cultured in 24-well plate in cell culture medium at the density of 50000 cells per well. The cells were incubated overnight and stimulated by 100 ng mL\(^{-1}\) of LPS and 2 ng mL\(^{-1}\) of IFN-\(\gamma\)-prepared in cell culture medium for 1 day. Different particles including PDA NPs (20 \(\mu\)g mL\(^{-1}\)), PDA@MAP (equal to 20 \(\mu\)g mL\(^{-1}\) of PDA), BUD@MAP (equal to 2 \(\mu\)g mL\(^{-1}\) of BUD), and BUDPDA@MAP (equal to 20 \(\mu\)g mL\(^{-1}\) of PDA and 2 \(\mu\)g mL\(^{-1}\) of BUD), and free drug BUD (2 \(\mu\)g mL\(^{-1}\)), were added into the cells in the present of 20 ng mL\(^{-1}\) LPS and 0.4 ng mL\(^{-1}\) IFN-\(\gamma\) during the treatment for 2 days. The cells, in which stimulated by LPS and IFN-\(\gamma\) at the same concentrations according to the treated groups, were used as the M1 phenotype control, and the cells, in which stimulated by anti-inflammatory cytokine IL-4 (20 ng mL\(^{-1}\)) and IL-13 (20 ng mL\(^{-1}\)), was used as M2 phenotype control. After 3 days, the culture supernatants were collected and frozen at \(-20^\circ\)C for ELISA analysis, and the expression of CD80 and CD206 on the cell surfaces was detected by immunostaining with the CD80 and CD206 antibodies. The cells were washed with PBS twice and detached by Versene (200 \(\mu\)L per well) for 15 min at 37 \(^\circ\)C. Then, 200 \(\mu\)L of PBS-EDTA was added into each well, and the cells were transferred to each glass tube. The cells were centrifuged at 317 \(g\) for 5 min and washed with PBS twice, followed by immunostaining the cell pellets with APC anti-CD80 and PE anti-CD206 at the concentration of 1 \(\mu\)g mL\(^{-1}\) in PBS at 4 \(^\circ\)C for 30 min. After that, the cells were washed again with PBS twice, and subsequently analyzed by flow cytometry. In each group, cells without antibody staining were used as the negative control. The fold of change of MFI in each sample (in triplicates) was calculated as [MFI of antibody-stained sample/MFI of non-stained negative control]. All flow cytometry data were processed by FlowJo software.

The culture supernatants were frozen at \(-20^\circ\)C until analysis with pre-coated ELISA kits (PeproTech, Stockholm, Sweden), according to the manufacturer’s protocol. Secreted TNF-\(\alpha\) and IL-10 were used as M1 and M2 markers, respectively. Triplicate samples were used for ELISA analysis.

Statistical Analysis: The sample size and pre-processing method varied in each experiment, so the detailed description was included in each experimental section separately. The experimental data were presented as mean \(\pm\) SD. For the significance analysis, all data were analyzed by one-way ANOVA, followed by Tukey’s post-test with OriginPro 2018. The levels of significant differences were set at probabilities of *\(p < 0.05\), **\(p < 0.01\), and ***\(p < 0.001\).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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