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Phenylboronic acid modification augments the lysosome escape and antitumor efficacy of a cylindrical polymer brush-based prodrug

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Abstract

Timely lysosome escape is very important for nanomedicines to avoid premature degradation. Herein, we report an exciting finding that phenylboronic acid (PBA) modification can greatly facilitate the lysosome escape of cylindrical polymer brushes (CPBs), and further promote their exocytosis and transcellular transfer. This fundamental finding for the first time reveals that PBA groups improve the tumor penetration of nanomaterials via an active transcytosis mechanism. We speculate that the mechanism of the PBA-enhanced lysosome escape is associated with the specific interactions of the PBA group with the lysosomal membrane proteins and heat shock proteins. The featured advantage of the PBA modification over the known lysosome escape strategies is that it does not cause significant adverse effects on the properties of the CPBs. Furthermore, doxorubicin was conjugated to the PBA-modified CPBs with drug loading content larger than 20%. This CPBs-based prodrug could eradicate the tumors established in mice by multiple intravenous administration.
Well-designed nano drug delivery systems can selectively accumulate in tumors via the enhanced permeability and retention (EPR) effect. However, they would be in risk of degradation after cellular internalization and subsequent lysosomal sequestration because of the hydrolytic enzymes and harsh acidic condition inside the lysosome. Thus, lysosomal entrapment is a major bottleneck for efficient delivery of therapeutic agents in subcellular level. It is crucial for nano carriers to take their cargoes out of lysosomes in time to avoid the drug degradation and inactivation.

Up to now, several approaches have been developed to facilitate the lysosome escape of nanomaterials. For example, by exerting the negative charge state of the lysosomal membrane, some positively charged materials have been used as drug carriers and achieve lysosome escape by destabilizing the lysosomal membrane through electrostatic interactions. Another approach involves the materials with exceptionally abundant basic groups, such as poly(ethylene imine) (PEI). They are used to rupture lysosomes via the so-called “proton sponge effect”. Moreover, membrane fusion is also an operational way for lysosome escape, which can be accomplished by using cationic liposomes or fusogenic peptides. The former fuses with the lysosome membrane via electrostatic interactions, and the fusogenic peptides achieve membrane fusion through conformational change in the acidic condition of lysosomes. The approach termed as photochemical internalization (PCI) achieves lysosome escape via photosensitizers that yield reactive oxygen species (ROS) under illumination and further induce lysosome rupture through the damage effect of ROS. Although the approaches mentioned above can achieve lysosome escape, they have respective
noticeable drawbacks. As well documented, after intravenous injection, the positively charged materials are prone to opsonization and hence have short blood circulation and little opportunity to reach tumors\textsuperscript{4,6,30}. The strategy of charge reversal can keep the materials neutral or anionic in blood circulation and trigger cationization at disease sites, however, sophisticated design and preparation are needed and the efficient charge reversal at the target sites is still a huge challenge\textsuperscript{31}. For the case of fusogenic peptides, their ability to escape from lysosomes would be diminished after conjugation to nanomaterials, and their high cost and quite complicated preparation may also limit greatly their applications\textsuperscript{32}. The PCI approach is greatly limited by the low tissue penetration depth of the exciting light and thus only applicable to superficial tumors, and introducing photosensitizers may cause additional toxicity\textsuperscript{33-35}. Accordingly, developing effective strategy for the lysosome escape of nanomaterials without adverse effects on other performances is very important for tumor therapy.

Herein, we present an exciting finding that phenylboronic acid (PBA) modification can greatly facilitate the lysosome escape of cylindrical polymer brushes (CPBs), and further promote their exocytosis and transcellular transfer. In published studies, it has been frequently observed that PBA groups can augment significantly the tumor targeting ability of nanomaterials through the specific interaction with the sialic acid (SA) residues overexpressed on various tumor cells and concomitantly enhance their tumor penetration\textsuperscript{36-38}. However, the mechanism for enhancing tumor penetration is still unclear. This work demonstrates that it is an active transcytosis process relying on the PBA-mediated cellular uptake, and PBA-enhanced lysosome escape and exocytosis.
Furthermore, using the PBA-modified CPBs as nano carriers, we incorporated doxorubicin (DOX) through pH-sensitive acylhydrazone linkage, and achieved high drug loading content larger than 20% and high tumor therapeutic efficacy. CPBs are a class of nanoscale one-dimensional polymers with wormlike morphology. This morphology together with their well-defined chemical structure and controllable size make them very suitable for delivering drugs, because the wormlike morphology is favorable to tumor penetration and well-controlled chemical structure and size provide reproducible pharmacokinetic and pharmacological profiles\textsuperscript{39,40}. The PBA-modified CPBs designed here also have several other advantages, including 1) the PBA groups can not only promote the lysosome escape but also augment the tumor targeting ability and tumor permeability of the CPBs as illustrated in Fig. 1a; 2) the zwitterionic poly(carboxybetaine) (PCB) block in the side chains provide high water solubility and high anti-biofouling ability\textsuperscript{41-43}; 3) the inner poly(glycidyl methacrylate) (PGMA) block in the side chains provide sufficient modification sites and high drug loading. These superiorities enable the prodrug to eradicate the tumors established in mice after multiple intravenous administration.
**Fig. 1 Biological functions and synthesis and of the CPBs.**

**a** Illustration of the tumor targeting and lysosome escape of BCPB-B-DOX. The abundant PBA groups of BCPB-B-DOX is conductive to tumor targeting and lysosome escape, and can greatly improve the antitumor efficacy of the prodrug. **b** Synthesis and drug loading of the CPBs.
Results

Syntheses and characterizations of the drug-loaded polymer brushes. To make clear the effect of PBA modification on the lysosome escape of CPBs and achieve efficient tumor therapy, we meticulously designed the water-soluble PBA-modified CPBs (named BCPB-B) as well as PBA-free CPBs (named BCPB) as reference materials. Their chemical structures and synthesis routes can be found in Fig. 1b. The backbone (named PGA, Fig. 1b) of BCPB-B and BCPB was synthesized by polymerizing the monomer glycidyl methacrylate via reversible addition-fragmentation chain transfer (RAFT) polymerization (giving PGMA), followed by converting the epoxy groups to azido groups via the reaction with sodium azide (Supplementary Scheme 1). The side chain of BCPB-B is a triblock copolymer and synthesized by a three-step atom transfer radical polymerization (ATRP) with propargyl 2-bromoisobutyrate as an initiator (Supplementary Scheme 1). The inner block (the block adjacent to the backbone) of the side chain is designed to be PGMA, whose abundant epoxy side groups can be used for functionalizations. The second block is poly(2-tertbutoxy-N-(2-(methacryloyloxy)ethyl)-N,N-dimethyl-2-oxoethanaminium) (PCB-tBu). After the cleavage of the tert-butyl ester protecting groups, a zwitterionic poly(carboxybetaine) (PCB) block can be formed and impart high water solubility and anti-biofouling ability. The third block is poly(OEGMA-Bpin) (POBpin, Supplementary Scheme 1) and is used to provide PBA functional groups after removing the pinacol ester. The reference material BCPB has the side chains without the third block, which is the only difference comparing to BCPB-B. For both the two types of
CPBs, the side chains were grafted onto the backbones through the Cu(I)-
catalyzed alkyne-azide 1,3-dipolar cycloaddition (CuAAC). Antitumor agent DOX was
covaletly bound to the PGMA block of BCPB-B and BCPB through a pH-sensitive
acylhydrazone linkage to achieve the responsive drug release in the weak acid
environment of tumors. We take BCPB-B as an example to illustrate the drug loading
process. We first converted the epoxy groups in BCPB-B-EP (Fig. 1b) to azido groups
through the reaction with sodium azide, and then performed the CuAAC between the
azido groups and \(N\)-propynoyl-hydrazonecarboxylic acid tert-butyl ester (PHTE),
affording BCPB-B-Boc (Supplementary Scheme 1). After the cleavage of the tert-
butyloxy carbonyl (Boc) protecting groups by trifluoroacetic acid (TFA), acylhydrazine
side groups were formed in the inner block and used to link DOX via the reaction with
the carbonyl group in DOX. It is notable that in the process of the Boc cleavage, the
protecting groups of the tert-butyl ester in the second block and the pinacol ester in the
third block would also be completely cleaved, affording BCPB-B (Fig. 1b). The DOX-
loaded BCPB-B and BCPB are named BCPB-B-DOX and BCPB-DOX (Fig. 1b),
respectively.

The characterization data of BCPB-B-DOX and BCPB-DOX as well as their
precursors can be found in Supplementary Fig. 1-18. The \(^1\text{H} \) NMR spectrum of the
backbone PGA is compared with that of PGMA in Supplementary Fig. 1. As can be
seen, the proton signals of the glycidyl moieties are observed at 3.23, 2.84, and 2.64
ppm in the spectrum of PGMA, and disappear completely in the spectrum of PGA,
indicating the high conversion efficiency of the epoxy to azido group. The number
average molecular weight ($M_n$) and polydispersity indexes ($D$) of PGMA were measured to be $\sim 147,000$ and 1.13 by gel permeation chromatography (GPC), respectively (Supplementary Fig. 2). The narrowly distributed molecular weight of the backbone would provide the polymer brushes with narrowly distributed length. To minimize the confounding factors, we used the second-step ATRP product of the side chain of BCPB-B as the side chain of BCPB. The side chain of BCPB-B and its precursors were structurally characterized by $^1$H NMR spectra as shown in Supplementary Fig. 4-6. The polymerization degrees of the PGMA, PCB-tBu and POBpin blocks were estimated to be about 27, 55 and 12 by the $^1$H NMR spectra, respectively. The GPC data of the three-step ATRP products PGMA, PGMA-PCB-tBu and PGMA-PCB-tBu-POBpin are shown in Supplementary Fig. 7. Their $M_n$ and $D$ values are determined to be 11705/1.17, 18397/1.23, and 23418/1.28, respectively. After grafting the side chains onto the backbone, we obtained the polymer brushes BCPB-B-EP and BCPB-EP. The high grafting efficiencies are evidenced by the comparison of the Fourier transform infrared (FTIR) spectra of the backbone and the polymer brushes. As shown in Supplementary Fig. 18, a strong absorption band at 2100 cm$^{-1}$ assigned to the azido groups can be observed in the FTIR spectrum of PGA and disappears completely in the spectra of the polymer brushes, indicating that almost all the azido groups have reacted with the alkynyl end group of the side chain. We also checked the size distributions of BCPB-B-EP and BCPB-EP by GPC measurements, although the measurements can not provide accurate molecular weight information of the polymer brushes due to the huge structural difference between the brushes and the
linear polystyrene standards (Fig. 2a). Unimodal molecular weight distributions were observed for both BCPB-B-EP and BCPB-EP with $D$ values of about 1.29 and 1.26. The narrowly distributed sizes are greatly favorable for identifying the differences in biological properties caused by the chemical structure, since the interference resulting from different sizes can be essentially excluded. As stated above, DOX is conjugated to the polymer brushes through the pH-sensitive acylhydrazone linkage, affording BCPB-B-DOX and BCPB-DOX. The aromatic proton signals from the DOX moieties can be observed at 8.10-7.60 ppm in their $^1$H NMR spectra (Supplementary Fig. 16-17). The UV-vis absorption spectra of BCPB-B-DOX, BCPB-DOX and DOX are shown in Supplementary Fig. 19a. It can be seen that both BCPB-B-DOX and BCPB-DOX have the absorption characteristics of DOX. The DOX contents of BCPB-B-DOX and BCPB-DOX were determined by measuring the absorbance at 489 nm with a pre-established calibration curve. Thanks to the abundant reaction sites, BCPB-B-DOX and BCPB-DOX present high DOX contents of about 21% and 23%, respectively, and maintain desirable water solubility as shown by the photograph of their water solutions (Supplementary Fig. 19b).
Fig. 1 Characterizations and drug release behaviors of the polymer brushes. a GPC curves of BCPB-B and BCPB. b,c Typical AFM height images of BCPB-B (b) and BCBP (c) adsorbed on freshly cleaved mica from dilute water solutions. Scale bars = 200 nm. d,e In vitro DOX release profiles of BCPB-B-DOX (d) and BCPB-DOX (e) in PBS (0.01 M) with different pH values at 37 °C.

We examined the morphological structures of the two types of polymer brushes before and after drug loading by atomic force microscopy (AFM). As shown in Fig. 2b, c, both BCPB-B and BCPB have a typical one-dimensional wormlike morphology with an average length of ~90 nm and cross sectional diameter of ~18 nm. The various instantaneous configurations of the polymer brushes shown in the AFM images indicate their good flexibility. From the AFM images of BCPB-B-DOX and BCPB-DOX (Supplementary Fig. 19c, d), it can be seen that the morphologies of the polymer brushes do not change significantly after the drug loading.
In vitro drug release. We studied the in vitro DOX release profiles of BCPB-B-DOX and BCPB-DOX in different pH media simulating the physiological environments of blood circulation (pH \(\sim 7.4\)), tumors (pH 6.5−6.0), and lysosomes (pH 4.5−5.5), respectively. As shown in Fig. 2d, e, typical pH-dependent release behaviors are observed for both BCPB-B-DOX and BCPB-DOX. At pH 7.4, there are only 27.9 ± 1.1% and 27.3 ± 1.5% of DOX released within 156 h for BCPB-B-DOX and BCPB-DOX, respectively. As expected, with the decrease of pH, the drug release rates of the two samples increase remarkably. At pH 6.0, the cumulative release percentages within 156 h are 58.1 ± 3.2% for BCPB-B-DOX and 38.4 ± 2.7% for BCPB-DOX, and at pH 5.0, the values are 75.5 ± 5.6% and 43.5 ± 4.4%, respectively. It is notable that the drug release rate of BCPB-B-DOX is significantly higher than that of BCPB-DOX in the same conditions. We speculate that in BCPB-DOX, the strong dipole-dipole interactions between the zwitterionic PCB repeating units make the PCB segments packed closely, hindering greatly the DOX release from the inner block, by contrast, in BCPB-B-DOX, the third block may disrupt the closely packing of the zwitterionic PCB segments in some extent, leading to the faster drug release\(^{44}\). The pH-sensitive drug release behaviors of BCPB-B-DOX and BCPB-DOX are attributable to the acylhydrazone linkage between the DOX and polymer brush moieties, and are greatly beneficial for reducing unwanted side effects and enhancing therapy efficacy since the drug release in circulatory system is remarkably limited and tumor-specific release is basically achieved.
**In vitro cytotoxicity and cellular uptake.** To evaluate the pharmacological activity of the drug-loaded polymer brushes and the biosafety of the drug-free polymer brushes, the cytotoxicities of BCPB-B-DOX and BCPB-DOX against mouse colon carcinoma cells (CT26), human breast cancer cells (MCF-7) and human liver tumor cells (HepG2) were measured by MTT assay with BCPB-B and BCPB as negative control and DOX as positive control. For all the three cell lines, the blank brushes do not show significant cytotoxicity even at a high concentration of 200 μg/mL, indicating their good cytocompatibility (Fig. 3a, Supplementary Fig. 20). By contrast, each of the three DOX formulations of BCPB-B-DOX, BCPB-DOX and free DOX displays a dose-dependent cytotoxicity, and the former two show slightly lower anti-proliferation efficiency than free DOX, which can be ascribed to the sustained release behaviors of BCPB-B-DOX and BCPB-DOX (Fig. 3b, Supplementary Fig. 21). The IC50 values of the three DOX formulations against the three cell lines were calculated and compared in Fig. 3c. Notably, for each cell line, BCPB-B-DOX shows a much lower IC50 than BCPB-DOX, which is attributable to the PBA groups in BCPB-B-DOX that can promote the cellular uptake due to the targeting effect of the PBA groups on the SA-positive cells.
Fig. 2 In vitro cytotoxicity and cellular uptake of the polymer brushes. a,b MTT assays for blank brushes (a) and drug-loaded brushes (b) against CT26 cells after 24 h incubation. e IC50 calculated from MTT assay data. d,e CLSM images (d) and mean fluorescence intensity measured by flow cytometry (e) of the CT26 cells after 2 h incubation with the FITC-labeled BCPB-B-DOX and BCPB-DOX at 37 °C, respectively. Scale bars = 20 μm. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control.

We further studied the cellular uptake behaviors of BCPB-B-DOX and BCPB-DOX qualitatively and quantitatively by confocal laser scanning microscopy (CLSM) and flow cytometry after labeling them with fluorescein isothiocyanate (FITC) through the reaction of the residue acylhydrazine groups in the polymer brushes with the isothiocyanate group in the dye. As shown in Fig. 3d and Supplementary Fig. 22 after 2 h incubation, bright signals from FITC and DOX are observed in all the CT26, MCF-
7 and HepG2 cells incubated with BCPB-B-DOX and BCPB-DOX, respectively. The internalized samples are mainly distributed in the cytoplasm in a punctate pattern, indicating that endocytosis may play a major role in the cellular uptake of the samples. For all the three cell lines, it can be seen that the fluorescence signals from both FITC and DOX in the cells treated with BCPB-B-DOX are significantly stronger than those in the cells treated with BCPB-DOX, which is confirmed by the flow cytometry quantitative analyses. As shown in Fig. 3e and Supplementary Fig. 22 the mean fluorescence intensities in the CT26, MCF-7 and HepG2 cells incubated with BCPB-B-DOX are 1.67-fold, 2.67-fold, and 2.58-fold the intensities in the corresponding cells treated with BCPB-DOX, respectively, which can be attributed to the targeting effect of the PBA group. The effect degree of the PBA group on the cellular uptake is positively related with the SA expression level of the cells, hence the cellular uptake results are consistent well with the fact that MCF-7 cells have the highest SA level among the three cell lines.\textsuperscript{37,45} The higher cellular uptake of BCPB-B-DOX than BCPB-DOX also explains its lower IC50 in the three cell lines.

**Lysosome escape.** Timely lysosome escape is very important for preserving the pharmacological activity of the endocytosed nanomadicines. We investigated the lysosome escape behaviors of the FITC-labeled BCPB-B and BCPB by fluorescence co-localization of the labeled brushes and LysoTracker red in CT26 cells via CLSM imaging and by Pearson co-localization analyses to quantify the escape rate (Fig. 4a, b). The Pearson’s correlation coefficients between the green signals from the FITC-labeled CPBs and the red signals from LysoTracker as a function of incubation time are shown
in Fig. 4b. It can be seen that for both BCPB-B and BCPB, the coefficients at 4 h post-treatment are larger than those at 2 h, probably because at 2 h, the CPBs have not been adequately internalized by the cells yet. When the incubation time is prolonged from 4 h to 8 h, for BCPB, the coefficient only decreases slightly from 0.93 to 0.85, by contrast, for BCPB-B, the coefficient decreases dramatically from 0.82 to 0.37, revealing that the lysosome escape rate of BCPB-B is much higher than that of BCPB in the same condition. To confirm the higher lysosome escape ability of BCPB-B, we further examined the lysosome escape behaviors of BCPB-B and BCPB in MCF-7 and HepG2 cell lines and obtained similar results (Supplementary Fig. 23). It is worth noting that for all the three cell lines, at each time point, the Pearson’s correlation coefficient of BCPB-B is lower than that of BCPB, probably because the lysosome escape of BCPB-B occurs very soon after entering lysosomes.

To explore the mechanism of the PBA-enhanced lysosome escape, we conjugated alkynyl-bearing Fe₃O₄ nanoparticles (the diameter is ~10 nm) to BCPB-B-N₃ and BCPB-N₃ via CuAAC. After incubated with CT26, MCF-7 and HepG2 cells for 12 h, respectively, the Fe₃O₄-modified BCPB-B and BCPB together with the adsorbed proteins were isolated via magnetic separation technique. The proteins adsorbed specifically by the Fe₃O₄-modified BCPB-B and BCPB were collected and identified by GeneOntology (GO) pathway Cellular Component (CC) analysis (Fig. 4c, Supplementary Fig. 24, Supplementary Table 1, 2). It is found that in all three cell lines, comparing to BCPB, BCPB-B uniquely adsorbs lysosomal membrane proteins and heat shock proteins (HSPs). The unique protein adsorption behavior of BCPB-B is
ascribable to the specific interactions of the PBA groups in BCPB-B with the glycosyl and basic groups, such as amino or guanidine group, in the proteins. We surmise that the lysosomal membrane proteins adsorbed by BCPB-B may play a mediation role in the lysosome escape of BCPB-B. In addition, it is notable that among the HSPs adsorbed by BCPB-B, Hsp90 is a crucial regulator of vesicular transport of cellular cargo\textsuperscript{46}, and Hsp70 is a survival protein directly related to endo-lysosomal compartment and maintains the integrity of lysosome by preventing membrane permeabilization\textsuperscript{47,48}. Accordingly, we speculated that the PBA-enhanced lysosome escape may also be associated with the adsorption of the HSPs.

Fig. 3 Lysosome escape behaviors of BCPB-B and BCPB. a Co-localization observation by CLSM of the FITC-labeled brushes (green) and LysoTracker (red) in

\[ \text{Co-localization coefficient} = \frac{\text{Intensity overlap}}{\text{Total intensity of both components}} \]
Penetration in multicellular spheroids (MCs). High tumor permeability of nanomedicines enables them to penetrate in avascular regions and hence can improve significantly their treatment effect. We studied the permeabilities of BCPB-B and BCPB in MCs to simulate and evaluate their tumor penetration. Two different MCs prepared from HepG2 and CT26 cells were used. To clearly compare the permeabilities of BCPB-B and BCPB, we labeled them with two different dyes FITC and rhodamine B isothiocyanate (RBITC), respectively, treated MCs with the FITC-labeled BCPB-B and RBITC-labeled BCPB together for different periods, and observed the incubated MCs by CLSM. As shown in Fig. 5a and Supplementary Fig. 25, both the labeled BCPB-B and BCPB exhibit time-dependent penetration behaviors in HepG2 MCs. After 6 h incubation, the FITC-labeled BCPB-B already penetrated to the center of the MCs, whereas, the RBITC-labeled BCPB penetrated very little. As time goes by, more BCPB-B and BCPB penetrated into the MCs. At 24 h post-treatment, bright green signals from BCPB-B are observed in the center of the MCs, whereas, the penetration depth of BCPB is only about 50 μm. The different permeabilities of BCPB-B and BCPB are also clearly reflected by the fluorescence plate quantification data of the MCs (Fig. 5a). The relatively higher permeability of BCPB-B was confirmed in CT26 MCs (Supplementary Fig. 26 and 27). Due to the lower SA level of CT26 cells than HepG2
cells, the penetration depth of BCPB-B in CT26 MCs is lower than that in HepG2 MCs, nevertheless, the penetration of BCPB in CT26 MCs is comparable to that in HepG2 MCs, which further proves the role of the PBA group in the penetration of BCPB-B.

**Fig. 4 Penetration behaviors of BCPB-B and BCPB in MCs.**

a) CLSM images of the optical slices through the centers of HepG2 MCs incubated jointly with the FITC-labeled BCPB-B and RBITC-labeled BCPB for different periods (left) and the corresponding fluorescence plate quantification data of the MCs (right). Scale bars = 100 μm.

b) Transcellular transfer study of the FITC-labeled BCPB-B and BCPB in CT26 cells. The left diagram illustrates the general experimental procedures. The cells on coverslips (I) were coincubated with the FITC-labeled CPBs for 4 h, washed with PBS...
and imaged by CLSM. Thereafter, the coverslips (I) were coincubated with coverslips (II) bearing fresh cells in fresh culture medium for 12 h. After repeating the above process, coverslips (III) were obtained. The right picture shows the CLSM images of the cells on the coverslips (I), (II) and (III), respectively. Scale bars = 20 μm.

**In vitro transcellular transfer.** We surmise that the higher permeability of BCPB-B in MCs may be associated with the PBA-enhanced lysosome escape. After escaping from lysosomes, BCPB-B may further achieve efficient transcytosis by repeating exocytosis, PBA-augmented cellular uptake and PBA-accelerated lysosome escape. To verify this hypothesis, we studied the transcellular transfer of BCPB-B and BCPB among different batches of CT26 cells by CLSM observations, respectively. As illustrated by the left diagram in Fig. 5b, the cells on coverslips (I) were first coincubated with the FITC-labeled CPBs for 4 h, and then washed with PBS and observed representatively by CLSM. Thereafter, the coverslips (I) were further coincubated with coverslips (II) bearing fresh cells in fresh culture medium for 12 h. After repeating the above process, we also obtained coverslips (III). In doing so, the transfer of BCPB-B and BCPB among the cells on the coverslips (I), (II) and (III) can be compared clearly. From the typical CLSM images shown in Fig. 5b, it can be seen that the signals from BCPB-B in the cells of the coverslips (I), (II) and (III) are significantly higher than the signals from BCPB in the cells of corresponding coverslips, indicating that for BCPB-B, the processes of cellular uptake, exocytosis and subsequent transcellular transfer are faster than the case of BCPB. The faster cellular uptake of BCPB-B is attributable to the targeting effect of the PBA groups, and the faster exocytosis should result from the...
PBA-enhanced lysosome escape, which jointly facilitated the transcellular transport and also the penetration of BCPB-B in MCs. Similar results were obtained by using CT26 and MCF-7 cells (Supplementary Fig. 28).

**In vivo near infrared fluorescence (NIRF) Imaging.** To gain insight into the nano-bio interactions in a living body, we traced the in vivo behaviors of BCPB-B-DOX and BCPB-DOX by NIRF imaging after labeling them with a near infrared dye NIR-797, and injecting the labeled samples into subcutaneous hepatic H22 tumor-bearing mice via tail vein. As shown in Fig. 6a, the signals from BCPB-B-DOX and BCPB-DOX can be observed in the tumors, livers and intestines during 1-12 h postinjection (p.i.). As time elapsed, the signals in tumors increase and those in normal tissues decrease. At 96 h p.i., the signal intensities of BCPB-B-DOX and BCPB-DOX in tumors reach their respective maxima, and at each test time point, the signal intensity of BCPB-B-DOX in tumors is higher than that of BCPB-DOX (Fig. 6b). After finishing the in vivo imaging at 168 h p.i., we excised and imaged the tumors and main organs for semiquantitative biodistributions (Fig. 6c, d). Notably, the signal intensity of BCPB-B-DOX in tumors is about 2.36-fold that of BCPB-DOX, indicating the higher tumor targeting ability of BCPB-B-DOX than BCPB-DOX, which should be contributed by the PBA-SA specific recognition.
Fig. 5 Biodistributions of BCPB-B-DOX and BCPB-DOX in tumor-bearing mice.

a,b NIRF images of the H22 tumor-bearing mice (a) and mean fluorescence intensities of the tumors (b) at different time points after tail-vein injection of the NIR-797-labeled BCPB-B-DOX and BCPB-DOX, respectively. The tumor region is circled by a red dotted line. c,d NIRF images (c) and mean fluorescence intensities (d) of the tumors and organs excised at 168 h after injecting the labeled BCPB-B-DOX and BCPB-DOX.

e DOX concentrations in plasma versus time after tail-vein injection of BCPB-B-DOX
and BCPB-DOX. f, g DOX concentrations in different tissues at different time points after tail-vein injection of BCPB-B-DOX (f), and BCPB-DOX (g), data are presented as mean ± SD (n = 3). h AUC of DOX accumulation in tumors in BCPB-B-DOX and BCPB-DOX groups. i CLSM images of the frozen sections of the tumors from the mice at 96 h after tail-vein injection of the FITC-labeled BCPB-B and BCPB, respectively. Scale bars = 100 μm.

**In vivo drug delivery.** To understand the different properties of BCPB-B and BCPB in drug delivery, we analyzed quantitatively the DOX distributions in subcutaneous H22 tumor-bearing mice as a function of time after tail-vein injection of BCPB-B-DOX and BCPB-DOX at a dose of 4 mg/kg DOX equivalent. The DOX concentrations in the blood, heart, liver, spleen, lung, kidney and tumor were determined by fluorescence spectroscopy after DOX extraction from the tissue homogenates, respectively. The evolution with time of the DOX concentrations in blood is shown in Fig. 6e. For both BCPB-B-DOX and BCPB-DOX, the concentration-time profiles fit well into the two-compartment model and their elimination half-lives are calculated to be about 29.7 h and 30.6 h, respectively. As shown in Fig. 6f, g, all the DOX contents in the test organs and tumor tissues experience a process of growing and then decreasing over time. The livers and spleens exhibit relatively higher uptake among all the test samples due to the capture of mononuclear phagocytic system (MPS). For BCPB-B-DOX, the maximal DOX concentrations in the heart, liver, spleen, lung, and kidney are 7.79 ± 1.81%, 17.59 ± 7.64%, 14.1 ± 1.07%, 7.16 ± 1.70% and 5.92 ± 2.53% of the injected dose per gram of wet tissues (% ID/g), respectively, and for BCPB-DOX, the values are 4.41 ± 0.40%,
16.73 ± 1.68%, 8.59 ± 2.03%, 3.94 ± 0.83% and 6.45 ± 1.39% ID/g, respectively. In tumors, the DOX concentrations of BCPB-B-DOX and BCPB-DOX groups reach their maxima of 9.38 ± 1.28% and 5.75 ± 1.91% ID/g at 12 h p.i., respectively. Over the 48 h monitoring duration, the tumor area-under-curve (AUC) of BCPB-B-DOX group reaches 298.3% ID·h/g, which is 1.78-fold that of BCPB-DOX group (167.2% ID·h/g tumor), confirming the higher tumor targeting ability of BCPB-B-DOX than BCPB-DOX (Fig. 6h).

**In vivo tumor penetration.** We further checked the in vivo tumor penetration of BCPB-B and BCPB. After labeled with FITC, the two samples were injected intravenously into subcutaneous H22 tumor-bearing mice. At 96 h p.i., the tumors were excised, sectioned with cryostat, and stained immunochemically with anti-CD31. As observed by CLSM (Fig. 6i), the green signals of BCPB-B almost do not overlap and keep a distance with the red signals of the vessels, suggesting that most of BCPB-B have extravasated from the tumor vessels and penetrated further in the tissues. By contrast, most BCPB still stay around the tumor vessels at the moment, confirming the higher tumor permeability of BCPB-B than BCPB.

**In vivo antitumor effect.** Since BCPB-B exhibits excellent properties in cellular uptake, lysosome escape, tumor accumulation and penetration, the high antitumor effectiveness of BCPB-B-DOX is anticipated. Using subcutaneous H22 tumor-bearing mice as the model animals, we compared the antitumor performance of BCPB-B-DOX, BCPB-DOX and DOX at a dose of 4 mg/kg DOX equivalent. The mouse groups treated with BCPB-B, BCPB and normal saline were used as negative control. Each sample
was injected as a solution in 0.2 ml of saline via tail vein. The study schedule is presented in Fig. 7a. The tumor volume and body weight were measured every other day (Fig. 7b and Supplementary Fig. 29). During the 15-day experiments, the tumors of the negative control groups grew fastest among all the test groups, and BCPB-B and BCPB did not show any antitumor activity. For the three DOX formulations, both BCPB-B-DOX and BCPB-DOX showed much higher tumor suppression effect than free DOX, and BCPB-B-DOX was better than BCPB-DOX. From the 9th day p.i., the relative tumor volumes of the BCPB-B-DOX and BCPB-DOX groups exhibited a statistically significant difference ($P < 0.05$). The tumor growth inhibitions (TGIs) on the 15th day p.i. were calculated to be 88%, 82% and 40% for BCPB-B-DOX, BCPB-DOX and DOX groups, respectively (see Supporting Information for the calculation method). Based on the body weight evolution with time of all the test groups (Supplementary Fig. 29), the DOX formulations and the blank brushes did not impose significant toxicity on the experimental animals, indicating the good biocompatibility of the polymer brushes and the well-tolerated dose level of DOX. The survival situation of all the test groups was examined and shown in Fig. 7c. As can be seen, all the mice in the BCPB-B, BCPB and normal saline groups died within 49 days after the treatments, and all the mice in the DOX group died within 53 days. By contrast, only 37% and 53% of the mice in the BCPB-B-DOX and BCPB-DOX groups died over the 60-day monitoring duration, respectively.

Encouraged by the high antitumor effect of BCPB-B-DOX, we further performed multiple-dose experiments (dosing every other day at 4 mg/kg DOX equivalent, Fig.
After five doses of treatments, all the tumors of BCPB-B-DOX group disappeared on the 13th day, while the TGIs of BCPB-DOX and DOX groups were 94.6% and 46.9%, respectively. Due to the lack of tumor-selective accumulation, free DOX still showed poor antitumor effect even after the five-dose treatments. The distinct tumor suppression effects of the different formulations are evidenced intuitively by the representative photographs of the excised sarcomas (Fig. 7e). Furthermore, as shown in Fig. 7f, the body weight evolution with time indicates that BCPB-B-DOX and BCPB-DOX did not cause significant toxicity to the mice since their body weights were comparable with the saline-treated mice at all the test time points, whereas, the average body weight of the DOX group began to decline sharply from the 5th day p.i., which should result from the even distribution of DOX in bodies that cause significant unwanted side effects.

**Fig. 6 In vivo antitumor effect.** a Illustration of antitumor study schedule with one dose treatment of 4 mg/kg DOX equivalent. b, c Relative tumor volume (b) and survival
rate (c) of the H22 tumor-bearing mice after one dose treatment with different protocols indicated. d Illustration of antitumor study schedule with multiple intravenous administration with each dose of 4 mg/kg DOX equivalent. e Relative tumor volume of the H22 tumor-bearing mice treated by five doses with different protocols indicated, and photographs of the sarcomas excised from the mice on the 13th day after the first treatment. f Body weight change of the H22 tumor-bearing mice treated by five doses with different protocols indicated. Data are presented as mean ± SD (n = 8).

Discussion

Timely lysosome escape is of paramount importance for the endocytosed nanomedicines to avoid premature degradation. The existing strategies for lysosome escape mainly include lysosomal membrane destabilization induced by cationic materials, lysosome rupture caused by the “proton sponge effect”, membrane fusion with the cationic liposomes or fusogenic peptides, and membrane damage evoked by ROS. There are noticeable deficiencies with these strategies. Briefly, the positively charged materials widely used for lysosome escape are subject to opsonization and short blood circulation, fusogenic peptides generally suffer function reduction after conjugation to nanomaterials and are also limited by their high cost and complicated preparation, and ROS-induced membrane damage is overshadowed by the low tissue penetration depth of the exciting light. Therefore, developing novel effective lysosome escape strategy is urgently needed.
In this work, we demonstrated clearly that PBA modification could promote greatly the lysosome escape of CPBs, which further facilitated their exocytosis and transcytosis. Such phenomena have never been observed before and support the mechanism that the PBA group improves the tumor permeability of nanomaterials via an active transcytosis process. Through analyzing the proteins adsorbed by BCPB-B and BCPB after incubation with tumor cells, we speculate that the specific interactions of the PBA group with the lysosomal membrane proteins and hot shock proteins may play important role in the lysosome escape of BCPB-B. Different from the known strategies for lysosome escape, the strategy of PBA modification does not give rise to significant adverse effects on the properties of nanomaterials, by contraries, it enhances remarkably their tumor targeting ability and tumor permeability, which is the featured advantage of this strategy.

In conclusion, we synthesized water soluble PBA-modified CPBs and found that PBA modification could greatly facilitate the lysosome escape of the CPBs, and further promote their exocytosis and transcellular transfer, imparting higher tumor penetration than the PBA-free CPBs. We speculate that the mechanism of the PBA-enhanced lysosome escape is associated with the specific interactions of the PBA group with the lysosomal membrane proteins and hot shock proteins. DOX was conjugated to the CPBs via pH-sensitive acylhydrazone linkage with drug loading larger than 20%. By using the PBA-modified prodrug, the H22 tumor-bearing mice were thoroughly cured after multiple intravenous administration. This work provided a novel strategy for
facilitating the lysosome escape of nanomaterials and a practical design for the CPBs with high water solubility, high drug loading, and high tumor therapy efficacy.

Methods

Synthesis of backbone PGA. Backbone PGA was synthesized following published procedures\textsuperscript{39}. \textsuperscript{1}H NMR (400 MHz, D\textsubscript{2}O) \(\delta\) (ppm): 0.76-0.93 (d, 3H), 1.76 (m, 2H), 3.29 (s, 2H), 3.88 (m, 3H), 5.50 (s, 1H).

Synthesis of side chain PGMA. PGMA was synthesized by the ATRP of glycidyl methacrylate (GMA) with propargyl 2-bromoisobutyrate propargyl 2-bromoisobutyrate (PBIB)\textsuperscript{49} as the initiator. PBIB (88 mg, 0.43 mmol), GMA (10 g, 70.30 mmol), CuBr (62 mg, 0.43 mmol), PMDETA (74 mg, 0.43 mmol) and anisole (10 mL) were placed in a 25 mL Schlenk flask followed by freeze-pump-thaw for three cycles in liquid N\textsubscript{2}. The resulting solution was stirred at room temperature for 2 h. After diluted with DCM, the resulting solution was filtered through a column of neutral Al\textsubscript{2}O\textsubscript{3} to remove copper salts. The product was collected and purified by precipitating from DCM to n-hexane 3 times, and dried under vacuum. Yield: 1.50 g (monomer conversion: 16.5%). The polymerization degree and absolute molecular weight were calculated to be about 27 and 4043 by \textsuperscript{1}H NMR, respectively. \textsuperscript{1}H NMR (400 MHz, chloroform-\textit{d}) \(\delta\) (ppm): 0.94-1.25 (d, 3H), 1.90-2.03 (m, 2H), 2.64 -2.84 (d, 2H), 3.24 (s, 1H), 3.80 (s, 1H), 4.31 (s,1H).

Synthesis of PGMA-PCB-tBu. PGMA (400 mg, 0.10 mmol), 2-tert-butoxy-N-(2-(methacryloyloxy)ethyl)-N,N-dimethyl-2-oxoethanaminium (CB-tBu, 4.15 g, 11.80
mmol), DMF (10 mL), CuBr (14 mg, 0.10 mmol), and \( N,N,N',N',N''- \)pentamethyldiethylenetriamine (PMDETA, 17 mg, 0.10 mmol) were placed in a 25 mL Schlenk flask followed by freeze-pump-thaw for three cycles in liquid N\(_2\). The resulting solution was stirred at room temperature for 24 h. Thereafter, the product was collected and purified by precipitating from MeOH to the mixture of diethyl ether and acetone (1:1, v/v) 3 times, and dried under vacuum. Yield: 2.10 g (monomer conversion: 46.7%). The polymerization degree and absolute molecular weight were calculated to be about 55 and 23418 by \(^1\)H NMR, respectively. \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) δ (ppm): 0.81-1.10 (m, 3H), 1.50 (s, 9H), 1.82-1.90 (d, 2H), 2.66-2.81 (d, 2H), 3.45 (s, 6H), 4.30 (s, 4H), 4.67 (s, 2H, in PCB-tBu).

**Synthesis of PGMA-PCB-tBu-POBpin.** PGMA-PCB-tBu (1.50 g, 0.064 mmol), OEGMA-Bpin\(^{36}\) (487 mg, 0.84 mmol), DMF (4 mL), MeOH (4 mL), Cu (0) wire (l = 10 cm, d = 1 mm), and PMDETA (11 mg, 0.064 mmol) were placed in a 25 mL Schlenk flask followed by freeze-pump-thaw for three cycles in liquid N\(_2\). The resulting mixture was stirred at 50°C for 24 h. Thereafter, the crude product was purified by precipitating from MeOH to the mixture of diethyl ether and acetone (1:1, v/v) 3 times, dialyzing against deionized water and lyophilization successively. Yield: 1.42 g (monomer conversion: 91.2%). The polymerization degree and absolute molecular weight were calculated to be about 12 and 30521 by \(^1\)H NMR, respectively. \(^1\)H NMR (500 MHz, methanol-\(d_4\)) δ (ppm): 1.15-1.25 (m, 3H), 1.34 (s, 12H), 1.57 (s, 9H), 2.02 (m, 2H), 3.46 (s, 24H), 3.56 (s, 6H), 4.20 (s, 2H), 4.50 (s, 2H), 7.13-7.70 (m, 4H).
**Synthesis of BCPB-EP.** PGMA-PCB-tBu (843 mg, 0.036 mmol), backbone PGA (3.34 mg, 0.018 mmol), CuSO$_4$·5H$_2$O (0.45 mg, 0.0018 mmol), PMDETA (3 mg, 0.018 mmol), DMF (4 mL), and MeOH (2 mL) was placed in a 10 mL Schlenk flask. After degassing, a solution of ascorbic acid (6.3 mg, 0.036 mmol) in 1 mL of DMF was added followed by 3 freeze-pump-thaw cycles. The resulting mixture was stirred at 40°C for 48 h under dark. Thereafter, the crude product was purified by precipitating from MeOH to acetone 3 times, dialyzing against deionized water and lyophilization successively. Yield: 683 mg (80.7% conversion rate). $^1$H NMR (500 MHz, DMSO-d$_6$) δ (ppm): 0.83 (m, 3H), 1.49 (s, 9H), 1.85-1.99 (m, 2H), 2.64-2.81 (d, 2H), 3.53 (s, 6H), 3.92 (s, 2H), 4.31 (s, 2H), 4.67 (s, 2H), 5.57 (s, 1H), 7.23 (s, 1H).

**Synthesis of BCPB-B-EP.** PGMA-PCB-tBu-POBpin (1g, 0.036 mmol), backbone PGA (3.34 mg, 0.018 mmol), CuSO$_4$·5H$_2$O (0.45 mg, 0.0018 mmol), PMDETA (3 mg, 0.018 mmol), DMF (4 mL), and MeOH (2 mL) were placed in a 10 mL Schlenk flask. After degassing, a solution of ascorbic acid (6.3 mg, 0.036 mmol) in 1 mL of DMF was added followed by 3 freeze-pump-thaw cycles. The resulting mixture was stirred at 40°C for 48 h under dark. Thereafter, the crude product was purified by precipitating from MeOH to acetone 3 times, dialyzing against deionized water and lyophilization successively. Yield: 738 mg (73.6% conversion rate). $^1$H NMR (400 MHz, methanol-d$_4$) δ (ppm): 0.82 (m, 3H), 1.29 (s, 12H), 1.48 (s, 9H), 1.85-2.0 (m, 2H), 2.67-2.81 (d, 2H), 3.34 (m, 30H), 3.73 (s, 2H), 4.32 (s, 2H), 4.71 (s, 2H), 5.32 (s, 1H), 7.89 (s, 1H).

**Synthesis of BCPB-N$_3$ and BCPB-B-N$_3$.** BCPB-EP (400 mg, containing 4.66 mmol epoxy groups), NaN$_3$ (909 mg, 14 mmol), and ammonium chloride (748 mg, 14 mmol)
were dispersed in DMF (25 mL) and stirred at 50°C for 48 h. After removal of the white precipitate by filtration, the crude product was purified by precipitating from MeOH to cold diethyl ether 3 times, dialyzing against deionized water and lyophilization successively to give BCPB-N$_3$. Yield: 509 mg (96.8% conversion rate). BCPB-B-N$_3$ was obtained by the same procedures as stated above. $^1$H NMR (500 MHz, methanol-$d_4$) $\delta$ (ppm): 0.74-1.18 (m, 3H), 1.60 (s, 9H), 1.98 (m, 2H), 3.29 (s, 6H), 3.95 (m, 4H), 4.40 (s, 2H). BCPB-B-N$_3$ was obtained following the same procedures as stated above. $^1$H NMR (500 MHz, methanol-$d_4$) $\delta$ (ppm): 1.13 (m, 3H), 1.28 (s, 12H), 1.49 (s, 9H), 2.01 (m, 2H), 3.41-3.45 (m, 30H), 4.00 (s, 2H), 4.13 (m, 2H), 4.43 (s, 2H), 7.71-8.55 (m, 4H).

**Synthesis of BCPB-Boc and BCPB-B-Boc.** BCPB-N$_3$ (509 mg, containing 4.51 mmol azide groups), PHTE (957 mg, 45.1 mmol, synthesized following published procedures$^{36}$), CuSO$_4$·5H$_2$O (11 mg, 0.045 mmol), PMDETA (50 mg, 0.29 mmol), DMF (4 mL), and MeOH (3mL) were placed in a 10 mL Schlenk flask. After degassing, a solution of ascorbic acid (158 mg, 0.09 mmol) in 2 mL of DMF was added followed by 3 freeze-pump-thaw cycles. The resulting mixture was stirred at 40°C for 48 h under dark. Thereafter, the crude product was purified by precipitating from MeOH to diethyl ether 3 times, dialyzing against deionized water and lyophilization successively to give BCPB-Boc. Yield: 590 mg (97.5% conversion rate). $^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm): 0.76 (m, 3H), 1.17 (s, 9H), 1.50 (s, 9H), 1.99 (m, 2H), 2.40 (s, 2H), 2.60 (s, 2H), 3.37 (s, 6H), 3.97 (s, 4H), 4.41 (s, 2H), 6.91 (s, 1H), 7.22 (s, 1H), 7.78 (s, 1H). BCPB-B-Boc was obtained following the same procedures as stated above. $^1$H NMR (500 MHz,
methanol-$d_4$ δ (ppm): 1.13 (m, 3H), 1.29 (s, 12H), 1.49 (s, 3H), 1.65 (s, 9H), 2.04 (m, 2H), 3.40 (s, 6H), 4.14 (m, 4H), 4.42 (s, 2H), 8.08 (s, 1H).

**Synthesis of BCPB and BCPB-B.** BCPB-Boc (500 mg, containing 3.72 mmol Boc groups) was dissolved in 10 mL of TFA and stirred at room temperature for 2 h. After removal of the solvent under reduced pressure, the crude product was purified by precipitating from methanol to diethyl ether 3 times, and dried under vacuum to give BCPB.

$^1$H NMR (400 MHz, D$_2$O) δ (ppm): 0.77-1.20 (m, 3H), 2.41 (m, 2H), 3.31 (s, 6H), 3.97 (m, 4H), 4.43 (s, 2H), 7.27 (s, 1H), 7.50 (s, 1H). BCPB-B was obtained following the same procedures as stated above. (400 MHz, D$_2$O) δ (ppm): 1.16 (m, 3H), 2.02 (m, 2H), 3.46 (m, 30H), 4.29 (m, 6H), 7.35-7.72 (m, 4H).

**Synthesis of BCPB-DOX and BCPB-B-DOX.** BCPB (20 mg, 0.15 mmol) and DOX·HCl (87 mg, 0.034 mmol) were dissolved in 4 mL of trifluoroethanol containing a drop of TFA. The reaction mixture was stirred at room temperature under dark for 48 h. Thereafter, the crude product was purified by a Sephadex LH-20 column with trifluoroethanol as an eluant. $^1$H NMR (500 MHz, methanol-$d_4$) δ (ppm): 1.11 (m, 3H), 1.29 (s, 3H), 2.02 (m, 2H), 2.85-2.98 (m, 9H), 3.43 (s, 6H), 3.87 (s, 2H), 4.29-4.41 (m, 4H), 5.14-5.45 (m, 3H), 7.62-8.04 (m, 4H). BCPB-B-DOX was obtained following the same procedures as stated above. $^1$H NMR (500 MHz, methanol-$d_4$) δ (ppm): 1.11 (m, 3H), 1.29 (s, 3H), 1.98 (m, 2H), 2.91 (m, 9H), 3.39 (s, 30H), 3.87 (s, 2H), 4.04 (m, 2H), 4.41 (m, 2H), 5.33 (m, 3H), 7.64 (m, 4H). The DOX contents were determined by measuring the absorbance at 489 nm in water using UV-vis spectroscopy with a pre-
established calibration curve. The drug loading contents of BCPB-B-DOX and BCPB-
DOX are about 21% and 23%, respectively.

**Statistical analysis.** Statistical results were calculated from at least three independent
experiments and expressed as mean ± standard deviation. P values are included to
show the correlation using the Student’s t-test. *p < 0.05 was considered statistically
significant, **p < 0.01, ***p < 0.001, and ****p < 0.0001 were considered highly
statistically significant.

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**Author contributions**

R.W. and W.W. conceived and designed the research. R.W., C.Y. and C.L. performed the experiments. Y.S., P.X, J.L. and S.Y. provided helpful suggestions. W.W. and X.J. analyzed data, edited and revised the paper. W.W. supervised the whole project and designed the outline.

**Competing financial interests:** The authors declare no competing financial interests.
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