

Disulfidptosis Nanoinducer Interrupts Tumor Metabolic Privilege to Boost Sustained Immunotherapy

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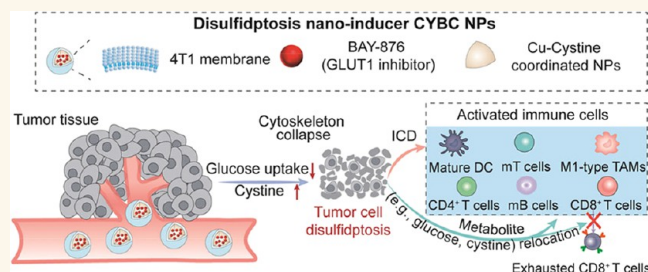
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ABSTRACT: Malignant tumor metabolic reprogramming drives proliferation and immune evasion by hijacking essential nutrients and shaping an immunosuppressive microenvironment. Although targeting tumor metabolism offers therapeutic promise, selectively modulating aberrant metabolic pathways without affecting normal cells remains a major challenge. Disulfidptosis, a recently identified form of metabolism-dependent regulated cell death, may offer an avenue for metabolic disruption; however, its immunomodulatory potential remains unexplored. Here, a disulfidptosis nanoinducer (CYBC NPs), which was constructed with cancer cell membrane-camouflaged nanoplateform coloaded with cystine and the GLUT1 inhibitor BAY-876, was designed to selectively induce disulfidptosis in triple-negative breast cancer cells. By concurrently blocking glucose uptake and supplementing cystine, CYBC NPs triggered disulfidptosis-mediated cytoskeletal collapse, relocated tumor metabolic fluxes, and induced immunogenic cell death. This metabolic perturbation promoted dendritic cell maturation, M1-like macrophage polarization, and cytotoxic T lymphocyte activation, thereby reversing ITME and suppressing tumor growth. Notably, CYBC NPs elicited robust, nonexhausted antitumor immunity and generated durable immune memory, effectively preventing tumor recurrence and metastasis. Together, our study demonstrated the implementation of disulfidptosis as a standalone immunotherapeutic strategy, offering a paradigm shift in metabolism-driven cancer immunotherapy.

KEYWORDS: disulfidptosis, metabolic regulation, immunosuppressive tumor microenvironment, T cell exhaustion, immune memory



INTRODUCTION

Emerging evidence reveals that metabolic dysregulation in tumors not only maintains their malignant phenotype but also underlies the formation of an immunosuppressive tumor microenvironment (ITME), thereby impeding the development of effective antitumor immunity.^{1,2} At the core of this metabolic-immune interplay lies intense nutrient competition between cancer cells and immune cells. Tumor cells exhibit an excessive uptake of key metabolites (e.g., glucose, lipid, and amino acids) through upregulated transporters and nutrient-sensing signaling pathways.³ This metabolic competition impairs the functionality of effector immune cells, notably, CD8⁺ T cells and natural killer (NK) cells, which rely on these essential substrates to sustain activation and proliferation. Concurrently, the accumulation of immunosuppressive metabolites (e.g., lactate, kynurenine, and adenosine) contributes to the reprogramming of tumor-associated macrophages (TAMs) toward an M2-like,⁴ tumor-promoting phenotype, the

expansion of myeloid-derived suppressor cells (MDSCs),⁵ and the upregulation of inhibitory immune checkpoints, such as PD-1 and PD-L1.⁶ Collectively, these events orchestrate a multilayered immunosuppressive network, particularly pronounced in immunologically “cold” tumors, contributing to poor responses to current immunotherapies.⁷

Given these findings, targeting tumor-intrinsic metabolic vulnerabilities has emerged as a promising approach to overcome immune resistance.^{8,9} However, most metabolic interventions predominantly focus on individual pathways (e.g., glycolysis,¹⁰ lipid synthesis,^{11,12} or amino acid metabo-

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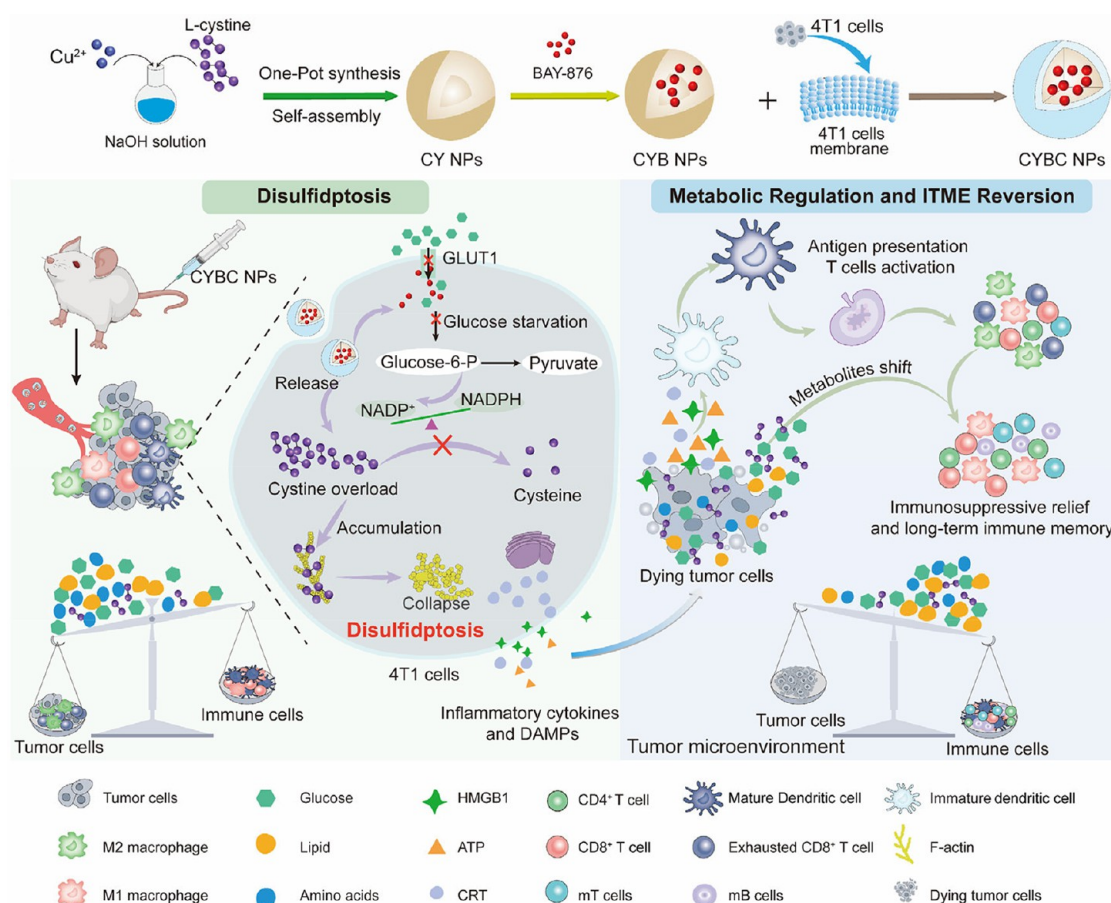
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Scheme 1. Schematic Illustration of CYBC NPs for Disulfidptosis-Driven Metabolic Intervention and Immune Reprogramming^a



^aSchematic illustration of fabrication of biomimetic CYBC NPs composed of copper-cystine cores coloaded with the GLUT1 inhibitor (BAY-876) and camouflaged with cancer cell membranes for homotypic targeting. Upon tumor accumulation, CYBC NPs simultaneously promote cystine supplementation and inhibit glucose uptake, triggering disulfidptosis in cancer cells; this process disrupts cytoskeletal integrity, induces ICD, and reprograms metabolic flux, collectively reversing the ITME and establishing durable antitumor immune memory.

lism^{13,14}), which are often thwarted due to the metabolic plasticity and compensatory mechanisms within tumor cells.^{15,16} In addition, nonselective disruption of core metabolic pathways risks off-target toxicity in normal proliferative tissues. These challenges highlight the need for tumor-selective strategies that combine metabolic intervention with immunomodulatory effects.

Disulfidptosis, a newly discovered form of metabolism-dependent regulated cell death, has garnered attention for its distinct mechanism. It is particularly inducible in cancer cells with an elevated expression of the cystine/glutamate antiporter SLC7A11 (also known as xCT). SLC7A11 mediates the import of extracellular cystine, which is subsequently reduced to cysteine, a key precursor for GSH synthesis, essential for detoxifying reactive oxygen species (ROS).^{17–19} This reduction requires nicotinamide adenine dinucleotide phosphate (NADPH), mainly generated via the pentose phosphate pathway during glucose metabolism.²⁰ Under glucose-depletion conditions, inadequate NADPH leads to excessive accumulation of intracellular cystine and formation of aberrant disulfide bonds within actin cytoskeletal proteins, resulting in cell structural collapse and cell death.^{21,22}

Given the high expression of SLC7A11 in various cancer cells, disulfidptosis emerges as a promising therapeutic

strategy.^{23–25} Current approaches to induce disulfidptosis include pharmacological inhibition of glucose transporters (e.g., using BAY-876, a GLUT1 inhibitor)²⁶ and enzymatic glucose depletion via glucose oxidase (GOx).²⁷ However, the application of disulfidptosis-based antitumor strategies is hindered by several factors: low cystine concentrations in the tumor microenvironment (TME),²⁸ which limit intracellular disulfide stress even when glucose metabolism is suppressed; instability and off-target toxicity of current inducers;^{29,30} and the complexity of compensatory redox and metabolic networks.³¹ In particular, prior studies that rely mainly on glucose deprivation and NADPH depletion fail to address this cystine limitation, resulting in suboptimal induction of disulfidptosis. We propose that synchronized blockade of glucose metabolism and exogenous cystine supplementation, delivered via a targeted nanocarrier, may overcome these limitations and selectively trigger disulfidptosis in tumor cells with minimal systemic toxicity. While prior studies have explored nanoparticle-mediated disulfidptosis, they have predominantly relied on combination strategies (e.g., coinduction of ferroptosis or pyroptosis).^{32–36} The potential of disulfidptosis as a standalone immunogenic trigger and its role in modulating metabolic flux to reprogram the ITME remain largely unexplored.

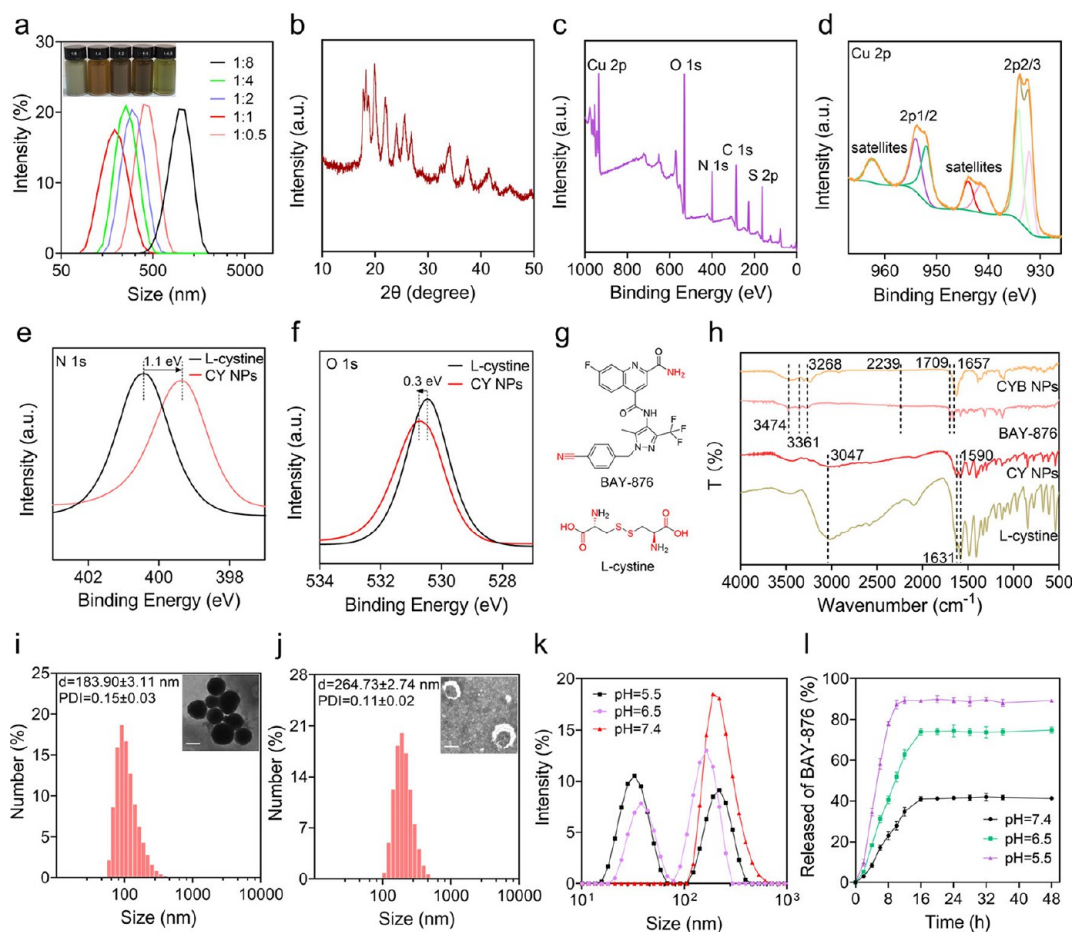


Figure 1. Preparation and characterization of CYBC NPs. (a) Size distribution of CY NPs prepared at varying molar ratios of Cu^{2+} to L-cystine. Inset: Corresponding color changes of the solution with different feed ratios. (b) XRD and (c) full XPS spectra of CY NPs. High-resolution spectrum of (d) Cu 2p, (e) N 1s, and (f) O 1s. (g) Chemical structure of BAY and L-cystine. (h) FTIR spectra of L-cystine, CY, BAY, and CYB NPs, indicating the coordination interactions among components. Size distribution of (i) CYB NPs and (j) CYBC NPs; insets: corresponding TEM images (scale bar: 200 nm). (k) Particle size changes of CYBC NPs at pH 7.4, 6.5, and 5.5 under 37 °C. (l) Cumulative release profiles of BAY from CYBC NPs in different pH buffers at 37 °C.

Here, we engineered a biomimetic nanoplatform composed of copper-cystine nanoparticles coloaded with BAY-876 (BAY) and cloaked with cancer cell membranes (CYBC NPs) to induce tumor-selective disulfidptosis in triple-negative breast cancer (TNBC) cells (Scheme 1). Leveraging homotypic membrane targeting, CYBC NPs preferentially accumulate in tumor cells and trigger disulfidptosis by simultaneously elevating intracellular cystine levels and inhibiting glucose uptake.

Mechanistically, disulfidptosis-induced cytoskeletal collapse led to the release of damage-associated molecular patterns (DAMPs), which in turn induce immunogenic cell death (ICD). In parallel, transcriptome sequencing analysis revealed that reprogramming of intracellular metabolic fluxes facilitates the redistribution of tumor-sequestered nutrients (e.g., glucose, lipids, amino acids) to immune effector cells, thereby reversing metabolic suppression and transforming the ITME from a “cold” to “hot” phenotype. In vivo studies in 4T1 mouse models confirmed that CYBC NPs not only enhanced ICD and dendritic cell maturation but also induced TAM polarization toward the M1 phenotype, and most importantly, reallocated metabolic resources from tumor cells to infiltrating T cells to sustain their activated state and prevent exhaustion, resulting in superior in vivo antitumor efficacy. Furthermore,

CYBC treatment conferred durable antitumor immunity and long-term immune memory, effectively preventing tumor recurrence and metastasis.

This work represents the demonstration of disulfidptosis as a standalone immunotherapeutic mechanism. By integration of ICD induction, selective metabolic disruption, and ITME remodeling within a cystine-based nanoplatform, our strategy offers a promising paradigm for metabolism-driven immunotherapy.

RESULTS AND DISCUSSION

Preparation and Characterization of CYBC NPs.

Scheme 1 illustrates the synthesis procedure for CYBC. Cystine can coordinate with various metal ions to form nanoparticles due to its abundant ligand sites (e.g., thiol, amino, and carboxyl groups),³⁷ and we chose the copper ion (Cu^{2+}) as the coordinating metal because of its well-established ability to coordinate with thiol- and amino-containing ligands, enabling controlled nucleation and growth of nanoparticles with uniform size and morphology.³⁸ To synthesize Cu-cystine nanoparticles (CY NPs), an aqueous one-pot self-assembly approach was employed by mixing Cu^{2+} and L-cystine under mild conditions. The immediate formation of a brown colloidal suspension (Figure 1a) indicated the

spontaneous assembly of coordination complexes into nano-scale structures. The particle size of CY NPs was remarkably affected by the feeding ratio of Cu^{2+} to L-cystine (Figure 1a), with a 1:1 molar ratio yielding the smallest nanoparticles (180.67 ± 20.40 nm) and a low polydispersity index ($\text{PDI} = 0.22 \pm 0.01$; Figure S1).

To elucidate the structural characteristics and coordination mechanism, comprehensive physicochemical characterizations were conducted. The X-ray powder diffraction (XRD) analysis revealed intense and narrow peaks (Figure 1b), indicative of high crystallinity that was distinct from hexagonal cystine crystals,³⁹ presumably due to Cu^{2+} -induced alterations in molecular packing. The full X-ray photoelectron spectroscopy (XPS) spectrum confirmed the presence of C, N, O, S, and Cu in CY NPs (Figure 1c). In the Cu 2p XPS spectrum (Figure 1d), mixed $\text{Cu}^+/\text{Cu}^{2+}$ valence states were observed, with Cu^{2+} peaks at 934.3/953.9 eV and Cu^+ at 932.2/951.8 eV, suggesting partial reduction of Cu^{2+} during nanoparticle formation. In the S 2p spectrum, L-cystine showed a single $\text{S}_{2\text{p}_{3/2}}$ peak at 163.7 eV, whereas in CY NPs, this peak splits into two: 164.0 eV (uncoordinated S) and 163.1 eV (coordinated S) (Figure S2). Furthermore, the N 1s peak associated with the NH_2 group of L-cystine shifted from 400.4 to 399.3 eV upon coordination (Figure 1e), indicating the participation of NH_2 in Cu^{2+} binding. Similarly, a 0.3 eV upshift in the O 1s spectrum (Figure 1f) further confirmed Cu–O coordination in CY NPs. Collectively, these findings suggested that the formation of CY NPs was driven by the coordination of Cu^{2+} with the N, O, and S atoms of L-cystine.

Fourier-transform infrared (FTIR) spectroscopy provided additional evidence for the formation of Cu-cystine coordination complexes (Figure 1h). The stretching vibration peaks of the carbonyl group at 1631 and 1590 cm^{-1} were retained when cystine was ligated with copper, while the asymmetric stretching vibration peaks of the free amino group at 3047 cm^{-1} disappeared, and the characteristic sulfhydryl peaks were absent. These spectral changes confirmed the successful synthesis of the CY NPs.

The BAY was subsequently encapsulated in the CY NPs to generate CYB NPs. FTIR revealed that free amino peaks of BAY at 3474, 3361, and 3268 cm^{-1} were shifted, while the characteristic peaks of the carbon–nitrogen triple bond at 2239 cm^{-1} disappeared upon encapsulation (Figure 1g,h), and the symmetric telescopic vibration peaks of the amino group at 1700 and 1667 cm^{-1} were also altered, demonstrating the successful drug loading into the nanoparticle core.

Notably, the incorporation of BAY had a negligible effect on nanoparticle size (CYB) with 183.90 ± 3.11 nm, and the transmission electron microscopy (TEM) image confirmed a spherical and uniform morphology consistent with dynamic light scattering (DLS) data (Figure 1i). To enhance tumor targeting and prolong systemic circulation, a homologous cancer cell membrane coating was applied, resulting in the formation of CYBC NPs. Postcoating, the average diameter increased to 264.73 ± 2.74 nm, and the zeta potential shifted from -3.39 to -15.56 mV, aligning with the native cancer cell membrane potential (Figures 1j and S3). Furthermore, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S4) verified the successful membrane coating by comparing the protein profiles of CYBC NPs with native cancer cell membranes, revealing a high degree of protein retention. Additionally, CYBC NPs could remain stable in pH

7.4 PBS containing 10% serum for more than 96 h (Figure S5), suggesting their suitability for systemic drug delivery.

Quantitative UV–vis spectroscopic analysis via a standard curve calibration approach showed that the BAY encapsulation efficiency was about 90% (Figure S6a,b). Notably, CYBC NPs exhibited a pH-responsive change in size (Figure 1k), keeping stable under neutral conditions while disassembling in acidic environments. Thereafter, the drug release profiles of CYBC NPs were evaluated in PBS buffers at pH 5.5, 6.5, and 7.4, respectively (Figure 1l). Under pH 7.4, the cumulative drug release reached around 40% after 48 h of incubation, whereas acidic conditions (pH 6.5 and 5.5) triggered rapid release, reaching ~ 72 and $\sim 90\%$, respectively. The acid-sensitive cargo release pattern was ascribed to the protonation of amino groups in an acidic environment, which weakened coordination interactions and thus facilitated CYBC dissociation and drug liberation.⁴⁰

In Vitro Cytotoxicity, Metabolism Disruption, and Disulfidoptosis. To explore the therapeutic mechanism of CYBC NPs, we first identified a suitable in vitro tumor model based on SLC7A11 expression, a key transporter implicated in disulfidoptosis.⁴¹ Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was conducted to measure SLC7A11 mRNA levels in several murine cell lines, including triple-negative breast cancer cells (4T1), murine melanoma cells (B16), mouse leukemia cells of monocyte macrophage (RAW264.7), and normal fibroblast cells (L929). qRT-PCR analysis revealed significantly higher SLC7A11 expression in 4T1 cells compared to other cell lines (Figure S7). Therefore, 4T1 cells were chosen as the model for further study.

To determine its optimal therapeutic concentration, 4T1 cells were treated with varying doses of BAY (0–500 nM) for 24 h. A cell counting kit-8 (CCK-8) assay demonstrated a dose-dependent reduction in viability (Figure S8), with 100 nM BAY, achieving $\sim 50\%$ cell death. This concentration was selected for subsequent experiments, as it effectively reduced tumor cell viability while allowing for downstream mechanistic assays.

To evaluate the cellular uptake behavior of CYBC NPs, 4T1 cells were incubated with DIR-labeled CYBC (DIR-CYBC) for various time intervals (2, 4, 6, and 8 h), followed by flow cytometry analysis (Figure S9). The fluorescence intensity peaked after 4 h of incubation, confirming the rapid and efficient internalization of CYBC into 4T1, which can be attributed to the homologous targeting capability conferred by the cancer cell membrane coating. In contrast, minimal fluorescence signals were detected in L929 and B16 cells, suggesting negligible nonspecific uptake by nontarget cells (Figure S10). Besides, RAW264.7 cells exhibited a modest degree of uptake, likely due to the recognition of surface-associated proteins or other immunogenic components by macrophage receptors, leading to macrophage-mediated phagocytosis.⁴²

Prior to evaluating antitumor efficacy, we assessed the in vitro biocompatibility of four nanoparticle formulations: CY (Cu-Cystine NPs, $100\text{ }\mu\text{g/mL}$), BAY (BAY-876, 100 nM), CYB (CY loaded with 100 nM BAY, $100\text{ }\mu\text{g/mL}$), and CYBC (CYB nanoparticles camouflaged with a 4T1 cell membrane). Representative nontarget cell lines, including L929, B16, and RAW264.7, were incubated with the above formulations for 24 h (Figures S11–S13). CCK-8 assays revealed that the CYB nanoparticles induced substantial cytotoxicity, reducing cell viability by approximately 70% in both the L929 and B16 cells.

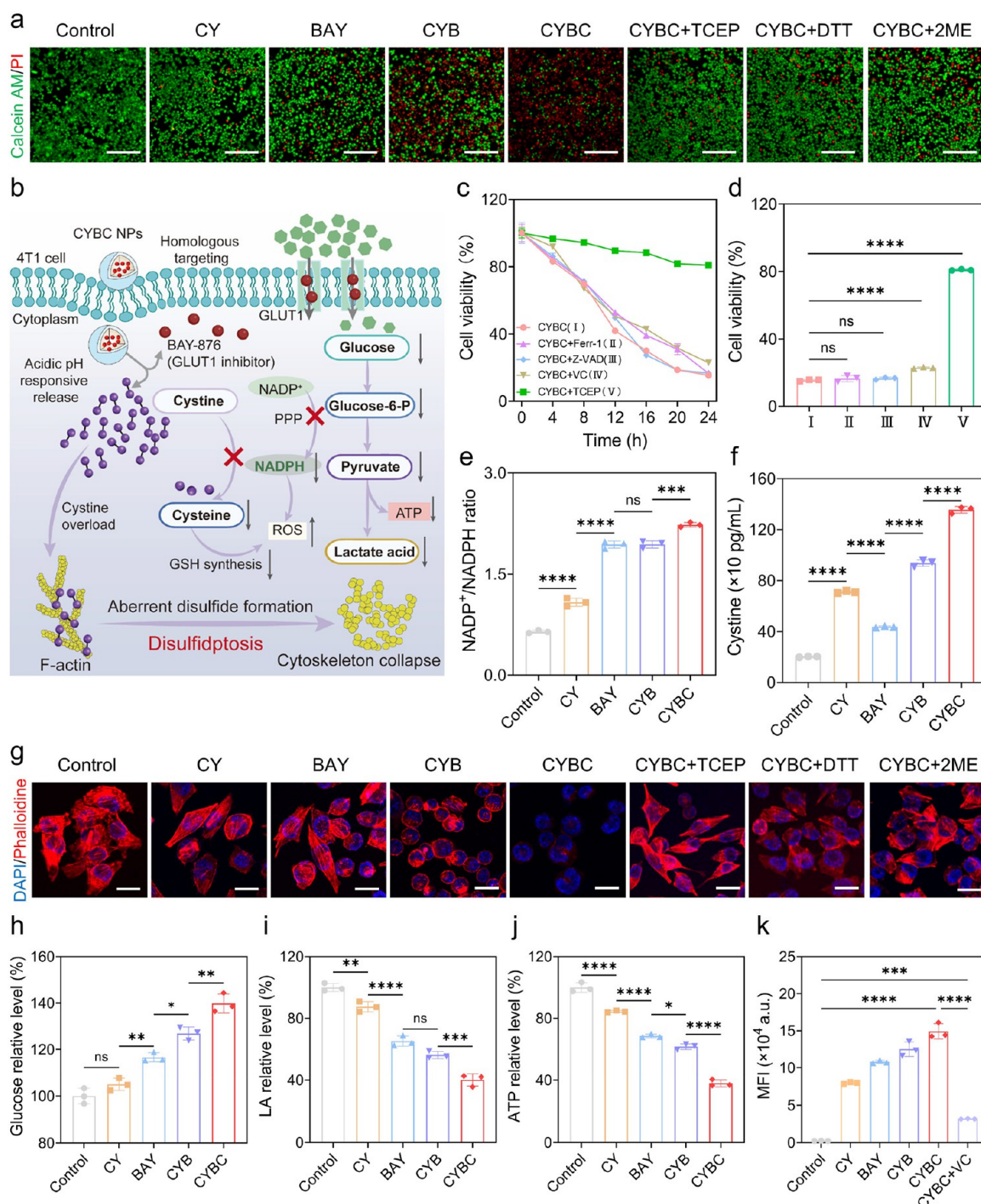


Figure 2. In vitro antitumor efficacy and disulfidptosis induction by CYBC NPs. (a) Fluorescence microscopy images of AM/PI-stained 4T1 cells following various treatments for 24 h. Scale bar: 200 μ m. (b) Schematic illustration of the CYBC-mediated disulfidptosis process. (c) Cell viability of 4T1 cells treated with CYBC (100 μ g/mL) in the presence of TCEP (1 mM), ferrostatin-1 (10 μ M), vitamin C (10 μ M), or Z-VAD (10 μ M) at indicated time points, and (d) statistical analysis at 24 h (I: CYBC NPs, II: CYBC+Ferr-1, III: CYBC+Z-VAD, IV: CYBC+VC, V: CYBC+TCEP). (e) Intracellular NADP⁺/NADPH ratio and (f) cystine concentration after various treatments. (g) Phalloidin staining of F-actin in 4T1 cells subjected to diverse treatments. Scale bar: 40 μ m. Quantification of extracellular (h) glucose, (i) lactate acid, and (j) intracellular ATP levels in 4T1 cells subjected to diverse treatments for 24 h. (k) ROS levels detected by flow cytometry following 6 h of nanoparticle exposure. For all figures, the data are presented as the mean $\pm$ SD. The one-way ANOVA, followed by Tukey's multiple comparisons, was employed to analyze statistical significance ($n = 3$, ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

In contrast, the CYBC formulation significantly mitigated the cytotoxicity, with only 16–25% reduction in viability across all tested nontarget cell lines. This improvement suggested that the membrane coating effectively masked the nanoparticle

surface, thereby reducing nonspecific cellular uptake and enhancing biocompatibility.

Next, the in vitro therapeutic effect of each formulation was evaluated in 4T1 cells (Figure S14). Treatment with CY or

BAY alone resulted in approximately 25 and 63% reductions in cell viability, respectively. The modest cytotoxicity of CY NPs likely reflected the limited therapeutic effect of cystine transport through SLC7A11 when acting alone. In contrast, CYB NPs, combining cystine overload with glucose uptake inhibition, showed an enhanced cytotoxicity. Notably, CYBC NPs treatment achieved approximately 80% reduction in tumor cell viability, representing a substantial enhancement over CYB. This superior efficacy was attributed to enhanced cellular internalization via cancer cell membrane-mediated homologous targeting, facilitating more efficient intracellular delivery of both active agents. These findings collectively highlight the potent antitumor potential of CYBC nanoparticles *in vitro*.

Given its pronounced cytotoxic effect, we investigated the underlying mechanism by which CYBC induced tumor cell death. 4T1 cells were treated with CY, BAY, CYB, CYBC, or CYBC in combination with representative thiol-reducing agents, including tris(2-chloroethyl) phosphate (TCEP), DL-dithiothreitol (DTT), and β -mercaptoethanol (2-ME). Cell viability was evaluated via calcein-AM/propidium iodide (AM/PI) dual staining, followed by confocal laser scanning microscopy (CLSM) (Figures 2a and S15). CYBC treatment induced extensive cell death, which was markedly attenuated by cotreatment with thiol-reducing agents. This finding suggested that CYBC-induced cytotoxicity is thiol-dependent and potentially linked to disulfide stress.

To further elucidate the mode of cell death (Figure 2b), a rescue assay was performed using pathway-specific inhibitors. 4T1 cells were pretreated for 1 h with specific inhibitors, including the ferroptosis inhibitor (ferrostatin-1, 10 μ M), apoptosis inhibitor (Z-VAD-FMK, 10 μ M), ROS scavenger (vitamin C (VC), 10 μ M), or thiol-reducing agent (TCEP, 1 mM), followed by incubation with CYBC (100 μ g/mL) for 24 h. Only TCEP significantly restored cell viability (from $\sim$ 15 to $\sim$ 80%), while the other inhibitors showed negligible effects (Figure 2c,d), excluding ferroptosis, apoptosis, and ROS-induced necrosis as dominant pathways. Moreover, the reliability of the inhibitors was validated by using sorafenib and cisplatin as positive controls (Figure S16).

To probe whether disulfidptosis was involved, we assessed the intracellular NADP⁺/NADPH ratio and cystine concentration. Cystine supplementation alone had a minimal impact on intracellular NADPH levels, indicating that elevated extracellular cystine did not disrupt redox homeostasis under normal metabolic conditions. In contrast, BAY treatment markedly reduced NADPH levels, consistent with its inhibition of glucose uptake and the consequent suppression of the pentose phosphate pathway (Figure 2e). Notably, BAY also caused a moderate increase in intracellular cystine compared with untreated 4T1 cells (Figure 2f), indicating that NADPH depletion impaired the reduction of cystine imported via the SLC7A11 antiporter to cysteine, thereby promoting cystine accumulation. However, this accumulation remained significantly lower than that observed in the CY, CYB, and CYBC groups (Figure 2f), underscoring that endogenous cystine levels are insufficient to induce robust disulfidptosis. In contrast, the CYBC group, which combined cystine supplementation with BAY-mediated GLUT1 inhibition, induced both a pronounced increase in intracellular cystine and a profound depletion of NADPH. This dual metabolic stress disrupted the redox equilibrium, leading to unreduced cystine overload and disulfide accumulation. The resulting

redox collapse triggered a self-amplifying disulfide stress cascade, ultimately driving potent disulfidptosis in tumor cells (Figure 2b).

Consistent with this hypothesis, we examined cytoskeletal integrity using phalloidin staining to visualize the F-actin structures. CY-treated cells exhibited minimal cytoskeletal changes, while BAY induced a partial disruption. Strikingly, CYB, and especially CYBC, treatment caused extensive cytoskeletal collapse, manifested as cell shrinkage and F-actin aggregation (Figures 2g and S17). Notably, this effect was substantially reversed upon cotreatment with thiol-reducing agents, consistent with previous reports that disulfidptosis was characterized by F-actin disassembly driven by disulfide bond accumulation.²²

To examine CYBC-induced metabolic stress, we measured extracellular glucose, intracellular ATP, and extracellular lactic acid (LA) levels. Among all treatment groups, CYBC-treated cells showed the highest extracellular glucose concentration ($\sim$ 1.4-fold increase vs control), indicating effective inhibition of glucose uptake (Figure 2h). Correspondingly, both intracellular ATP and extracellular LA levels were markedly reduced by 37.9 and 40%, respectively (Figure 2i,j), reflecting impaired glycolytic flux and the bioenergetic crisis within tumor cells.

Finally, we investigated redox imbalance by evaluating the intracellular ROS levels. Since glucose-derived NADPH is critical for maintaining redox homeostasis, its depletion following GLUT1 inhibition was expected to promote oxidative stress. Furthermore, as discussed above, impaired reduction of cystine to cysteine limits GSH biosynthesis, further amplifying ROS accumulation (Figure 2b). Cells treated with CYBC were stained with DCFH-DA, an ROS probe, and analyzed by CLSM. A strong green fluorescence signal, indicating ROS generation, was observed following CYBC treatment, and this signal was diminished by VC cotreatment. Flow cytometry analysis further corroborated these findings (Figures 2k and S18). Collectively, these data demonstrated that CYBC induced disulfidptosis by orchestrating a redox-metabolic collapse: enforced cystine overload, GLUT1-mediated glucose deprivation, NADPH exhaustion, cytoskeletal disruption, and ROS accumulation synergistically contribute to tumor cell death.

To assess whether CYBC-induced disulfidptosis depends on SLC7A11 expression, we employed B16 cells, which exhibit low endogenous SLC7A11 levels (Figure S7). By replacing the 4T1 membrane in CYBC NPs with B16 cell membranes, we constructed a homologous formulation (CYBC*). In this model, neither cystine (CY) and BAY-876 nor their combination (CYB) induced significant cytotoxicity. In contrast, CYBC* treatment reduced the cell viability to $\sim$ 20%, and this effect was reversed by disulfidptosis inhibitors, confirming a specific mechanism of action (Figure S19). Live/dead staining and F-actin imaging supported these results, revealing extensive cytoskeletal collapse only in the CYBC*-treated group (Figure S20a,b). Further analysis showed that intracellular cystine levels were more than 4-fold higher in CYBC*-treated cells compared to the uncoated CY group, indicating that membrane-mediated endocytosis bypasses the reliance on SLC7A11 transport (Figure S21). These findings demonstrated that CYBC-induced disulfidptosis was not strictly dependent on SLC7A11 expression and that codelivery of cystine and a glucose uptake inhibitor via membrane-coated

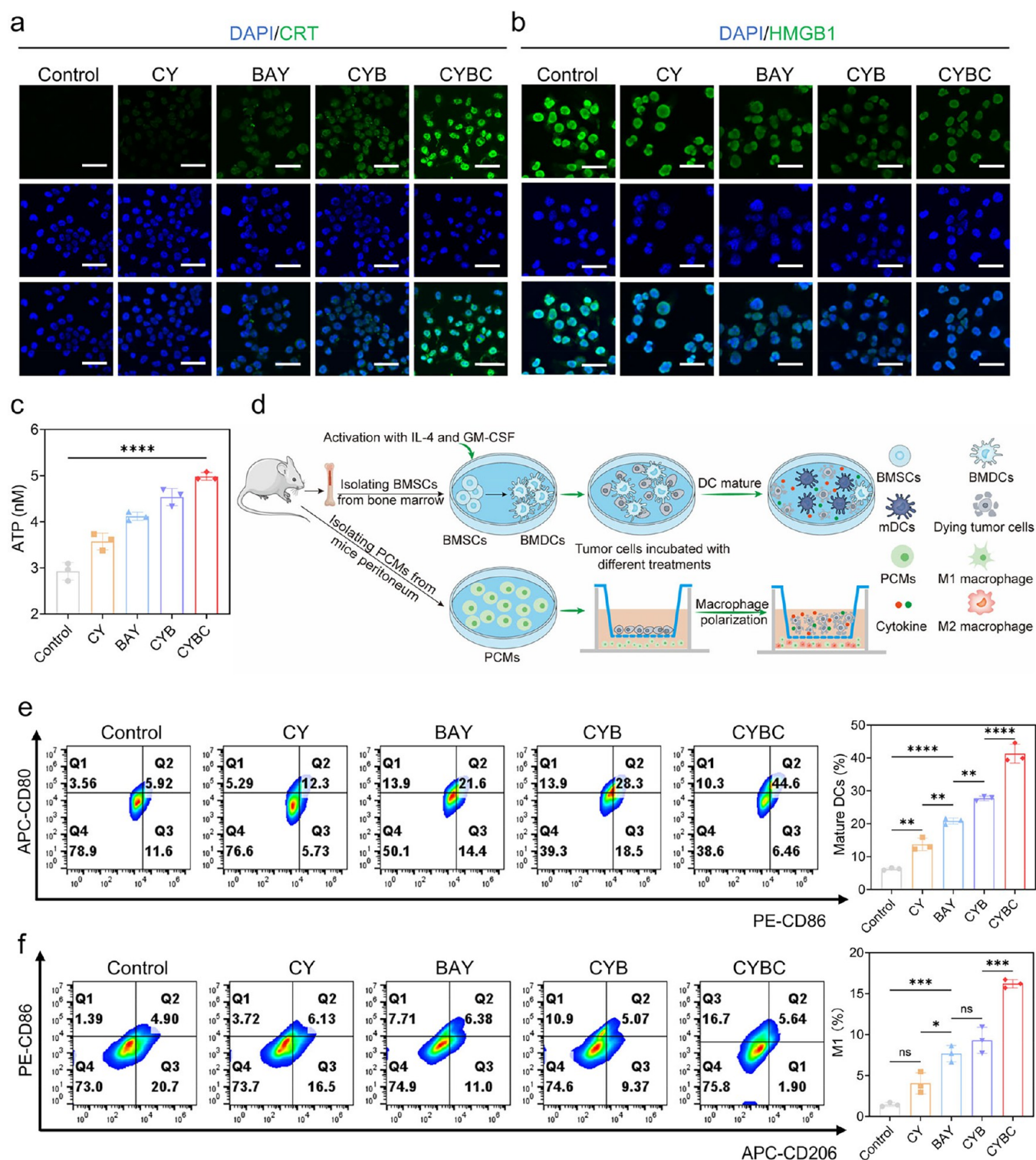


Figure 3. CYBC-induced ICD and macrophage polarization in vitro. Immunofluorescence images of (a) CRT and (b) HMGB1 of 4T1 cells after various treatments. Scale bar: 50 μ m. (c) Quantification of extracellular ATP levels in 4T1 cells under diverse treatment conditions. (d) Schematic diagram of the in vitro assay for immune cell responses. A coculture and Transwell system were established to investigate the BMDC maturation and macrophage polarization. (e) Flow cytometry analysis of BMDCs stained with anti-CD11c, CD80, and CD86; percentages of mature DCs are indicated. (f) Flow cytometry analysis of macrophages stained with anti-CD86 and CD206; percentages of M1 macrophages are indicated. For all figures, the data are presented as the mean $\pm$ SD. The one-way ANOVA, followed by Tukey's multiple comparisons, was employed to analyze statistical significance ($n = 3$, ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$).

nanoparticles provides an effective strategy to overcome transporter limitations and broaden therapeutic applicability.

Immune Activation Induced by CYBC Nanoparticles In Vitro. Given that cytoskeletal integrity is essential for

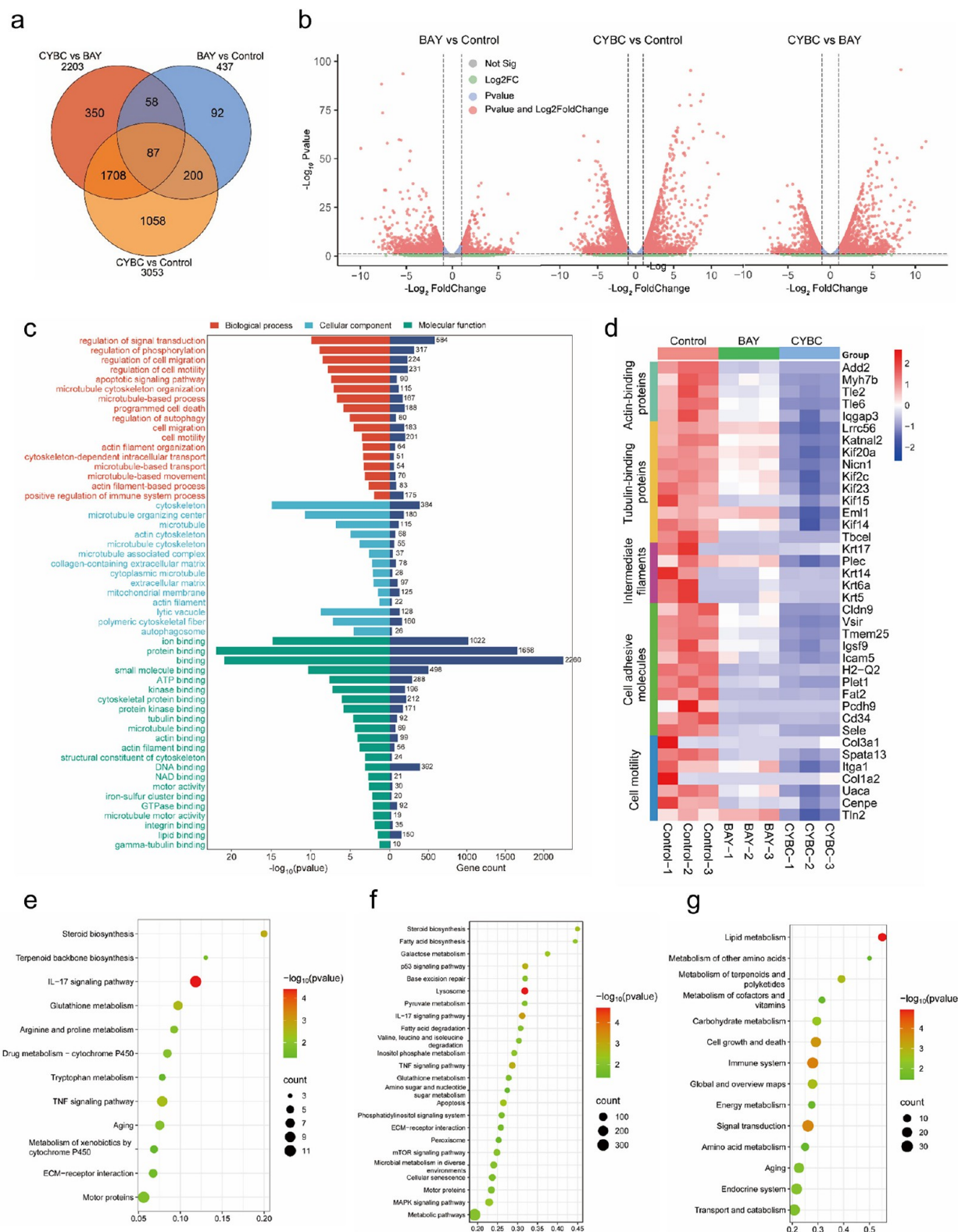


Figure 4. Overview of 4T1 gene transcriptome sequencing outcomes. (a) Venn diagram indicating the number significant differentially expressed genes (DEGs) between the three comparisons. (b) Volcano plot highlighting significantly upregulated (in red) and downregulated (in green) genes. (c) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis comparing CYBC and control. (d) Heat map showcasing specific gene expression levels. Gene ontology (GO) enrichment analysis comparing (e) BAY and control, (f) CYBC and control, and (g) CYBC and BAY.

cellular homeostasis⁴³ and that its disruption can lead to membrane rupture and leakage of intracellular DAMPs,⁴⁴ we hypothesized that the CYBC NPs, which were previously

demonstrated to induce disulfidptosis and significant cytoskeletal collapse, might also initiate ICD in tumor cells. Immunofluorescence analysis revealed that CYBC treatment

elicited the most pronounced CRT surface exposure and HMGB1 release among all groups (Figure 3a,b). In addition, extracellular ATP levels increased by 1.7-fold following CYBC treatment compared with the control (Figure 3c), providing compelling evidence for ICD induction.

Simultaneously, these released DAMPs serve as endogenous adjuvants, promoting the maturation of bone marrow-derived dendritic cells (BMDCs) and the activation of CTLs (CD8⁺ T and CD4⁺ cells) through enhanced antigen presentation.⁴⁵ To verify whether tumor cells undergoing ICD after CYBC treatment could stimulate DC maturation, we established a tumor-immune cell coculture system (Figure 3d). First, mouse bone marrow mesenchymal stem cells (BMSCs) were isolated and differentiated into immature DCs using IL-4 (10 ng/mL) and GM-CSF (20 ng/mL) for 5 days.

Morphological changes were further validated by phase-contrast microscopy: BMSCs exhibited a round or oval morphology in suspension, whereas BMDCs adhered to the culture surface, displayed increased cell volume, and developed dendritic pseudopods (Figure S22a, b). Flow cytometry further confirmed efficient differentiation with ~78% CD11c⁺ DCs generated (Figure S22c). Subsequently, 4T1 cells were treated with CYBC or control formulations for 12 h, and their culture supernatants were collected and added to the BMDC culture. Flow cytometry showed that BMDCs exposed to CYBC-conditioned media exhibited a 7.5-fold increase in the expression of maturation markers CD80 and CD86 compared with untreated controls (Figure 3e). This rate also exceeded that observed in the CYB group by 1.6-fold, highlighting the critical role of homologous targeting and metabolic interference in ICD enhancement. ELISA assays further confirmed this trend, as CYBC-stimulated DCs secreted the highest levels of proinflammatory cytokines, including TNF- α (691.8 pg/mL), IL-6 (91.6 pg/mL), and IFN- γ (826 pg/mL) (Figure S22d–f), thereby validating the establishment of an immunostimulatory microenvironment.

In addition to dendritic cell activation, TAMs play a dual role in shaping the ITME. It is typically dominated by immunosuppressive M2-like TAMs, whose polarization is driven in part by high lactate concentrations, resulting from aerobic glycolysis (the Warburg effect).⁴⁶ Reprogramming TAMs from a protumorigenic M2 phenotype to an antitumorigenic M1 phenotype has emerged as a promising immunotherapeutic strategy. To assess the impact of CYBC on TAM polarization, peritoneal cavity-derived macrophages (PCMs) were isolated from mice and cocultured with treated 4T1 cells in a Transwell system. Flow cytometry analysis revealed the increased expression of the M1 marker CD86, with percentages of 3.74, 7.71, 10.9, and 16.7% for CY, BAY, CYB, and CYBC, respectively. Concurrently, the percentage of CD206⁺ M2 macrophages decreased to 1.9% in the CYBC group (Figures 3f and S22g). ELISA results also showed that TNF- α and IL-6, key cytokines secreted by M1 macrophages, were most abundant in the CYBC group (Figure S22h,i), suggesting effective TAM repolarization.

Overall, the combination of ICD biomarker expression, extracellular ATP release, BMDCs maturation, proinflammatory cytokine secretion, and TAM repolarization collectively demonstrated that CYBC treatment not only induces a robust ICD response in vitro but also effectively remodels the immune microenvironment, promoting antitumor immunity. These findings position CYBC as a promising immunomodulatory nanotherapeutic for cancer therapy.

RNA-Sequencing Analysis of Anticancer Mechanism In Vitro. To investigate the antitumor mechanism of CYBC NPs, transcriptome sequencing was performed on 4T1 cells treated with PBS (control), BAY, or CYBC. Principal component analysis (PCA), sample correlation, and clustering analysis confirmed strong correlations among the three experimental groups (Figure S23a–c). A Venn diagram illustrated the overlap in gene expression profiles among the treatment groups (Figure 4a). In comparison to the control group, 437 genes were differentially expressed in the BAY group, while 3053 genes were affected in the CYBC group. Differentially expressed genes were identified using a threshold of $\log_2$ fold change > 1 . The volcano plot revealed 204 upregulated and 809 downregulated genes in the BAY group. In contrast, the CYBC group exhibited 941 upregulated and 1173 downregulated genes (Figure 4b).

Next, Gene Ontology (GO) analysis categorized the results into three major domains: biological process (BP), cellular component (CC), and molecular function (MF).⁴⁷ In the CYBC group, there was notable enrichment in pathways related to cell migration, the microtubule cytoskeleton, actin filament organization, programmed cell death, and immune system processes (Figure 4c), whereas the BAY group predominantly affected cell migration, intermediate filament organization, and glucose metabolic processes (Figure S23d).

Key distinctions between the BAY group and the CYBC group were primarily in processes related to “cell death”, “metabolic process”, “cell migration”, “MAPK cascade”, and “cytoskeleton” (Figure S23e). For CC-enriched genes, the CYBC group showed significant enrichment in pathways related to the “cytoskeleton”, microtubules”, and “lytic vacuoles”, suggesting that cytoskeletal collapse triggers cell death and promotes lysosomal phagocytosis of dead cells.

To further explore the impact of these treatments on cytoskeletal integrity, we categorized cytoskeleton-related genes into five clusters: actin-binding proteins, tubulin-binding proteins, intermediate filaments, cell adhesion molecules, and cell motility factors (Figure 4d).

The CYBC group exhibited a stronger disruption of cytoskeletal structures than the BAY group, suggesting that the supplementation of cystine amplified disulfidptosis. Additionally, metabolism-related gene expression analysis revealed that the CYBC group significantly downregulated genes involved in carbohydrates, lipids, and amino acid metabolism, whereas the BAY group did not completely inhibit these metabolic pathways (Figure S23f). These results indicated that CYBC profoundly disrupted tumor cell metabolism, potentially facilitating nutrient redistribution from tumor cells to surrounding immune or stromal cells.

Specifically, genes involved in NADPH metabolism, such as *Idh1*, *Cyp2s1*, *Slc2a8*, and *Vdr*, were downregulated in the CYBC group; however, these genes were not significantly altered in the BAY group. This supports the hypothesis that glucose uptake inhibition, when combined with massive cystine uptake, synergistically enhanced disulfidptosis and impaired redox homeostasis. Beyond the functional annotation of differentially expressed genes, pathway enrichment analysis provided deeper insights into the regulatory mechanisms underlying these changes. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (Figure 4e–g) revealed that genes in the CYBC group were predominantly enriched in pathways related to lipid, amino acid, terpenoid, polyketide, and carbohydrate metabolism, cell growth and death, immune

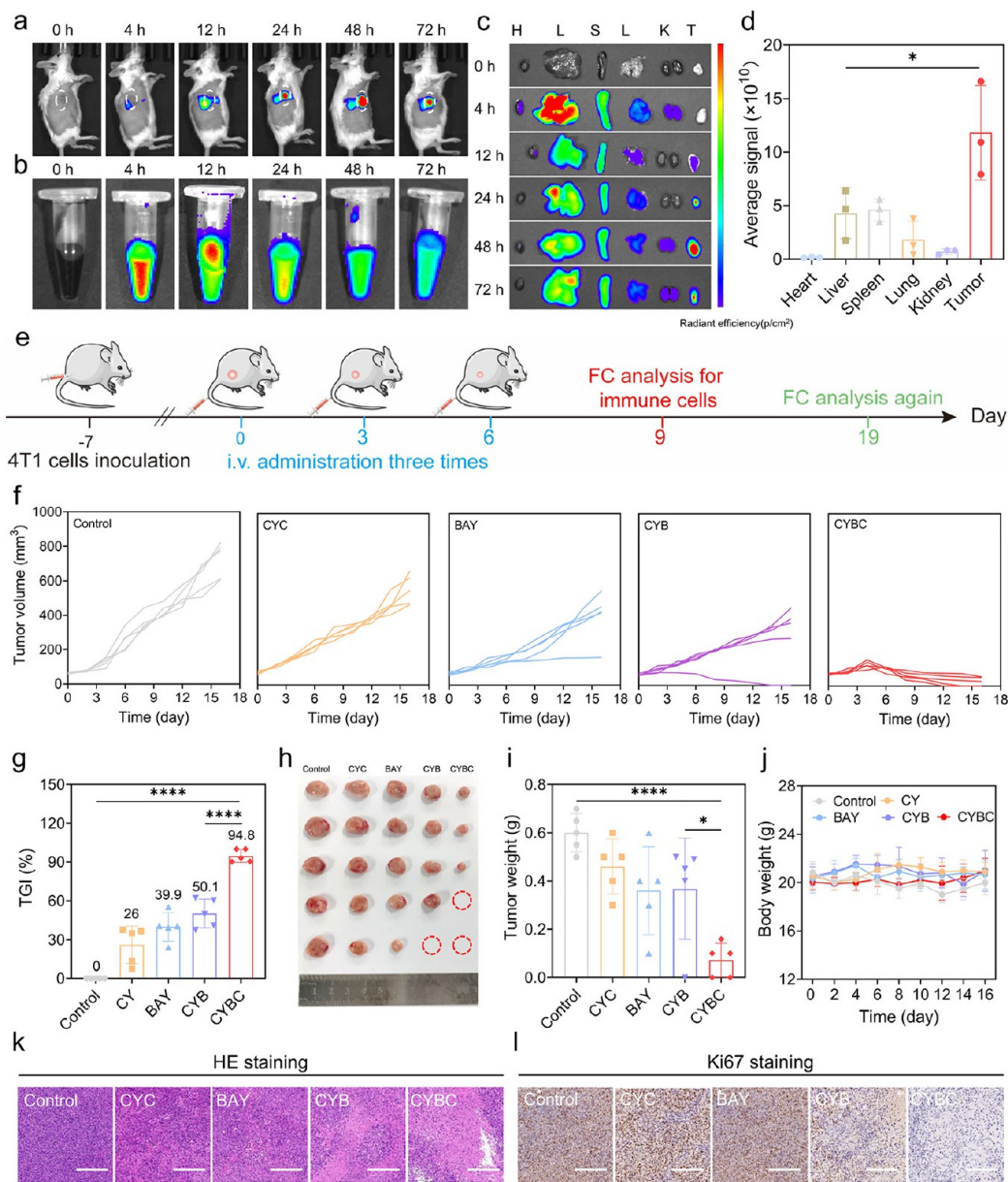


Figure 5. In vivo antitumor efficacy of CYBC NPs. (a) In vivo fluorescence images of 4T1 tumor-bearing mice after administration of DIR-CYBC at different monitoring times and (b) ex vivo fluorescence images of tumor (T) and major organs, including heart (H), liver (L), spleen (S), lung (L), and kidney (K) collected at different time postinjection. (c) Quantitative fluorescence analysis in major organs and tumors at 48 h postinjection. (d) Fluorescence images of blood samples at corresponding time points. (e) Schematic illustration of the experimental regimen. (f) Tumor growth curves of individual mice after different treatments. (g) Tumor growth inhibition (TGI) percentage. (h) Photographs of excised tumors. (i) Weights of excised tumors. (j) Changes in body weights of mice in each group over a 19-day treatment period. (k) H&E and (l) Ki67 immunohistochemistry staining of tumors. Scale bar: 200 μm . For all figures, the data are presented as the mean $\pm$ SD. The one-way ANOVA, followed by Tukey's multiple comparisons, was employed to analyze statistical significance ($n = 5$, $*p < 0.05$, $****p < 0.0001$).

regulation, energy metabolism, signal transduction, aging, endocrine regulation, and intracellular transport and catabolism. Furthermore, galactose metabolism, along with amino sugar and nucleotide sugar metabolism, processes essential for tumor growth, immune modulation, and signal transduction^{48,49} were also affected, suggesting that metabolic disruption may contribute to the observed antitumor effects.

To validate the GO analysis and evaluate the inhibitory effect of CYBC NPs on 4T1 cell migration, a wound-healing assay was performed. Compared to the control group, BAY treatment reduced cell migration by approximately 1.5-fold,

whereas CYBC treatment resulted in a nearly 4-fold reduction (Figure S24a,b). These results were consistent with the GO analysis, which indicated the suppression of migration-related pathways.

In Vivo Antitumor Efficacy and Biosafety Evaluation of CYBC NPs. To investigate the therapeutic potential of CYBC NPs in vivo, we first evaluated their biodistribution using fluorescence imaging. The lipophilic dye DIR was encapsulated in CYBC NPs to enable the fluorescence-based tracking of their in vivo distribution. As shown in Figure 5a, a strong fluorescence signal was observed at the tumor site 24 h

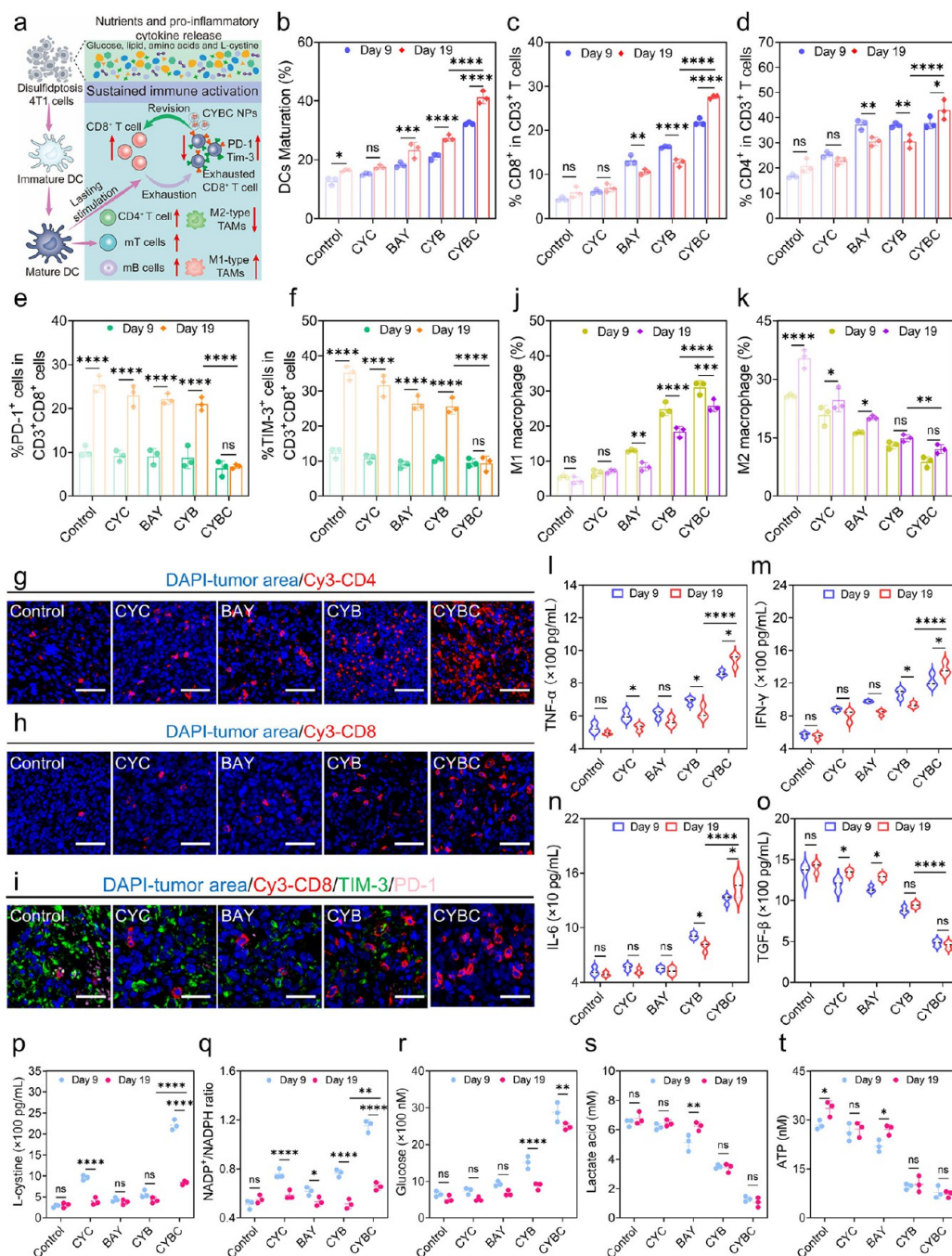


Figure 6. In vivo immune modulation and tumor microenvironment remodeling by CYBC NPs. (a) Pattern diagram for remodeling of the immunosuppressive TME. (b) Flow cytometry quantitative data of CD80⁺ and CD86⁺ DCs in the spleen. Quantification of (c) CD8⁺ T cells, (d) CD4⁺ T cells, and exhausted T cells expressing (e) PD-1⁺ and (f) Tim-3⁺. Representative immunofluorescence images of (g) CD4⁺ and (h) CD8⁺ tumor-infiltrating T cells at day 19 with different treatments (blue: DAPI; red: Cy3-CD4/CD8; scale bar: 50 μ m). (i) Representative immunofluorescence images of the CD8⁺ T-cell exhaustion status in the tumor microenvironment (blue: DAPI; red: Cy3-CD8; green: PE-Tim-3; pink: Cy5-PD-1; scale bar = 50 μ m). Quantification of (j) M1 and (k) M2 macrophages in tumor tissues after receiving different treatments at days 9 and 19. Cytokine levels (l) TNF- α , (m) IFN- γ , (n) IL-6, and (o) TGF- β in the harvested tumors at days 9 and 19. (p) Intratumoral L-cystine concentration. (q) NADP⁺/NADPH ratio change in the harvested tumors. The concentration of (r) glucose, (s) lactate acid, and (t) ATP in tumors as indicators of metabolic status. For all figures, the data are presented as the mean $\pm$ SD. The two-way ANOVA, followed by Tukey's multiple comparisons, was employed to analyze statistical significance ($n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

after intravenous administration, indicating efficient tumor accumulation. Remarkably, while the fluorescence intensity gradually attenuated over time, it remained clearly detectable even at 72 h postinjection, suggesting prolonged tumor retention of CYBC NPs. Furthermore, ex vivo fluorescence

imaging of major organs and tumors at 48 h postinjection (Figure 5b,c) revealed significantly higher fluorescence intensity in the excised tumors compared to other major organs. Hemodynamic profiling revealed that the plasma concentration of CYBC NPs peaked at 4 h postinjection,

followed by a gradual decline (Figure Sd). The above results indicated that the membrane-coated nanoparticles leverage the unique “self-recognition” properties of cancer cell membranes, enhancing the accumulation of nanoparticles through membrane–membrane interactions, adhesion molecules, and shared surface antigens.⁵⁰ This approach enhances the specificity of tumor delivery while minimizing off-target effects and systemic toxicity.

Building on these promising biodistribution results, we next assessed the therapeutic efficacy of CYBC NPs in a 4T1 tumor-bearing BALB/c mouse model, following the treatment schedule outlined in Figure 5e. The mice were randomly divided into the following five groups: (1) Control (saline injection), (2) CYC (cancer cell membrane-coated CY NPs), (3) BAY, (4) CYB, and (5) CYBC. To enhance tumor accumulation of CY NPs and to better evaluate the specific contribution of cystine within the therapeutic strategy, we employed cancer cell membrane coating to construct CYC NPs.

Tumor growth curves and tumor growth inhibition (TGI) values (Figure 5f,g) demonstrated that monotherapy with either CYC or BAY led to modest tumor growth inhibition rates of 26.0 and 39.9%, respectively, relative to those in the control group. In contrast, CYB NPs resulted in a more pronounced tumor suppression rate of 50.1%. Strikingly, CYBC achieved a TGI of 94.8%, demonstrating superior therapeutic efficacy that can be attributed to the integration of multimodal metabolic modulation and homologous membrane-mediated tumor targeting. Representative images of harvested tumors (Figure 5h) and tumor weight records (Figure 5i) at 19 days post-treatment visually corroborated the quantitative data. Several tumors in the CYBC group exhibited near-complete regression, with some achieving complete ablation.

Hematoxylin and eosin (H&E) staining of tumor sections from the CYBC-treated group revealed widespread necrotic regions (Figure 5k). Concurrently, immunohistochemical staining for Ki67, a well-established marker of cellular proliferation, showed markedly reduced expression in the CYBC group, suggesting effective suppression of tumor cell proliferation (Figure 5l), further supporting the potent therapeutic activity of CYBC.

Importantly, body weights remained stable across all treatment groups throughout the study (Figure 5j), indicating minimal systemic toxicity. Comprehensive biosafety evaluations were also performed. Hematological parameters across all treatment groups remained within the normal ranges (Figure S25). Hemolysis assays confirmed excellent blood compatibility with hemolysis rates below 5% even at 500 $\mu\text{g/mL}$ (Figure S26). The potential off-target induction of disulfidptosis in normal tissues is critical for assessing the biosafety of our nanoplatform. H&E staining of major organs (heart, liver, spleen, lungs, and kidneys) revealed no evident tissue damage or pathological abnormalities in any treatment group (Figure S27). Collectively, these results indicated that CYBC NPs exhibited favorable biocompatibility and biosafety in vivo.

Mechanistic Insights into CYBC-Induced Antitumor Immunity. Given the superior in vivo antitumor efficacy of CYBC nanoparticles, we next sought to elucidate the underlying mechanisms, particularly their immunomodulatory effects (Figure 6a). As hallmarks of ICD, CRT exposure and HMGB1 release were first examined. Immunofluorescence staining revealed that CYBC-treated tumors exhibited

prominent CRT exposure on the cell membrane and HMGB1 translocation from the nucleus into the extracellular space (Figure S28), confirming effective induction of ICD in vivo and the consequent release of DAMPs. These immunogenic signals facilitate tumor antigen presentation and bridge innate and adaptive immune responses.⁵¹

To investigate the initiation of adaptive immunity, we assessed DC maturation by quantifying CD86⁺CD80⁺ populations in splenic DCs. CYBC treatment markedly enhanced DC maturation, with proportions rising from 12.8% at the baseline to 32.8% on day 9 and 43.5% on day 19 (Figures 6b and S29). These values were markedly higher than those in other treatment groups, suggesting that CYBC promoted efficient in situ ICD, likely due to CYBC-induced disulfidptosis, which enhanced antigen release and DC priming.

Effective tumor inhibition requires not only initial T-cell priming but also sustained effector function within the metabolically hostile TME.⁵² Subsequent evaluation of T-cell infiltration revealed that CYBC treatment led to sustained increases in CD4⁺ and CD8⁺ T-cell populations within tumor tissues (Figures 6c,d and S30). Notably, the proportion of CD8⁺ T cells in the CYBC group expanded from 22.8 to 27.8% between days 9 and 19, reflecting persistent effector activity. In contrast, although BAY and CYB treatments initially activated T cells, their efficacy diminished over time by day 19, coinciding with upregulation of T-cell exhaustion markers PD-1 and TIM-3. In CYBC-treated tumors, however, CD8⁺ T cells maintained low levels of PD-1 and TIM-3 expression by day 19 (Figures 6e,f and S31), suggesting effective prevention of T-cell exhaustion. These findings were consistent with the immunofluorescence staining (Figure 6g–i), which further demonstrated that CYBC not only enhanced T-cell infiltration but also preserved the effector phenotype under chronic antigenic stimulation.

Macrophage plasticity was also significantly impacted by CYBC. At day 9, CYBC treatment induced a dramatic phenotypic shift: M1 macrophages increased from 5.27 to 32.2%, while M2 macrophages decreased from 26.3 to 9.96% (Figures 6j,k and S32). Although a partial reversion toward the M2 phenotype was observed by day 19, the early shift toward a proinflammatory state underscored CYBC's capacity to reshape the TME through macrophage repolarization.

To further confirm systemic immune potentiation, we quantified the serum cytokines. CYBC treatment resulted in robust and sustained upregulation of proinflammatory cytokines, including TNF- α , IL-6, and IFN- γ , throughout the treatment period (Figure 6l–o). Meanwhile, immunosuppressive cytokine TGF- β remained suppressed. This cytokine profile suggested that CYBC induced a shift from an immunosuppressive to an immunostimulatory systemic environment, thereby synergizing with local TME remodeling to support antitumor immunity. Metabolic constraints within the TME, particularly glucose depletion and oxidative stress, are major contributors to T-cell exhaustion and immune escape. To assess how CYBC impacted this metabolic landscape, we quantified key tumor metabolites, including L-cystine, the NADP⁺/NADPH ratio, glucose, ATP, and lactate (Figure 6r–t). CYBC treatment led to pronounced accumulation of cystine and NADP⁺, alongside increased glucose and decreased ATP and lactate levels, indicative of glycolysis suppression and enhanced oxidative stress. These findings confirmed the successful induction of disulfidptosis and metabolic collapse

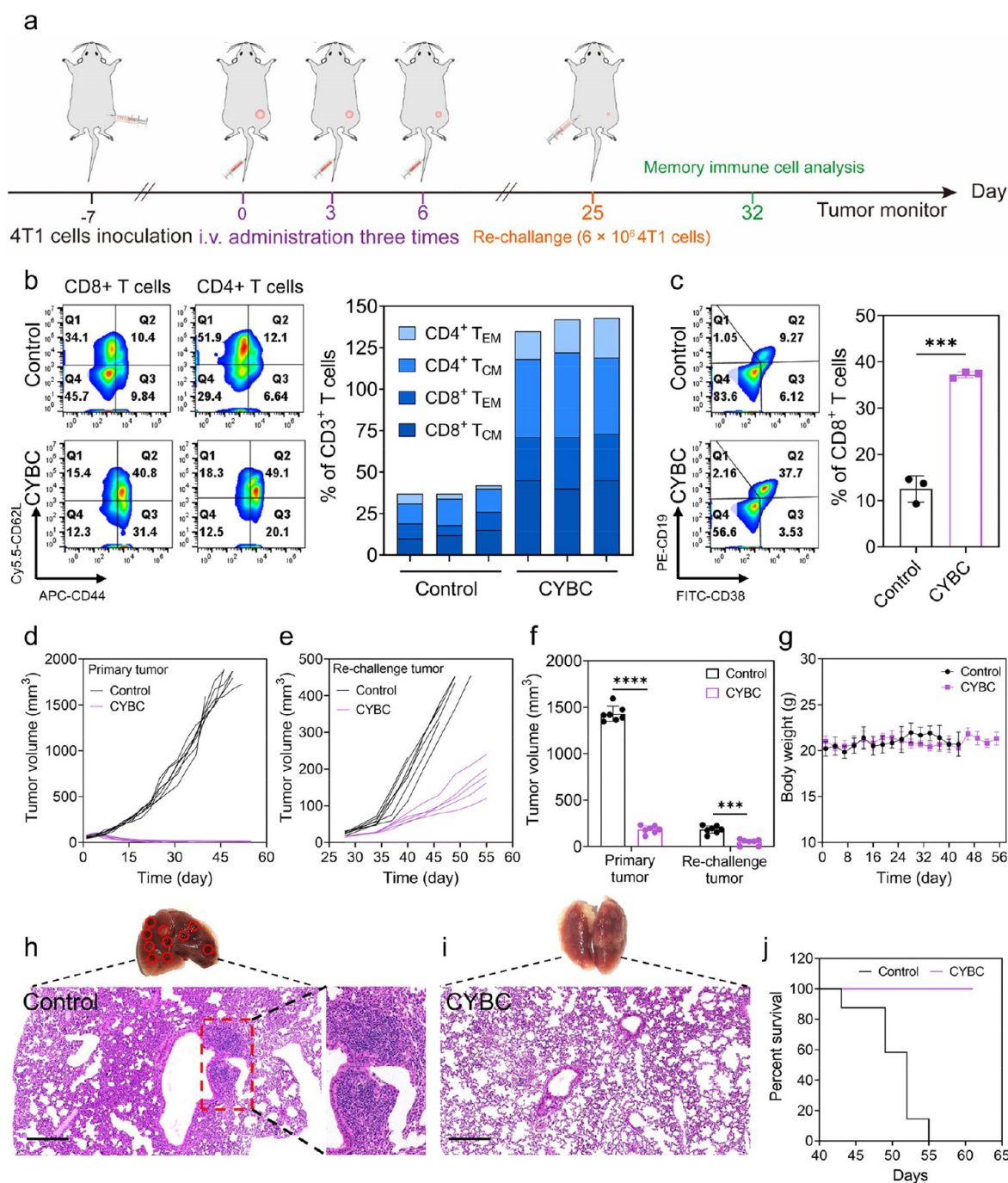


Figure 7. Long-term immunological memory induced by CYBC NPs. (a) Schematic illustration of the experiment design to assess the immunological memory response triggered by CYBC NPs. (b) Representative flow cytometry images and quantitative analysis of memory T cells (T_{EM} and T_{CM}) and (c) memory B cells in the splenic lymphocytes. The tumor volume of (d) primary and (e) rechallenged tumor, and (f) tumor volume analysis at day 40. (g) Body weight during the treatment. Representative images of lungs with metastatic nodules at day 55 in (h) control and (i) CYBC-treated groups. Scale bar: 200 μ m (j) Kaplan–Meier survival curves of mice ($n = 7$). For all figures, unless otherwise specified, the data are presented as the mean $\pm$ SD. The unpaired two-tailed Student's t -test comparisons were employed to analyze statistical significance ($n = 3$ *** $p < 0.001$, **** $p < 0.0001$).

in tumor cells. Notably, although CYC transiently elevated cystine levels on day 9, they returned to baseline by day 19, likely due to insufficient glucose inhibition and ongoing tumor metabolic activity.

The above results suggested that CYBC NPs supported T-cell functionality, likely via two coordinated mechanisms. First, by inhibiting glucose uptake in tumor cells, CYBC redistributed glucose toward immune effector cells, sustaining their

glycolytic metabolism and cytotoxic activity. Second, cystine supplementation following cytoskeletal collapse promoted glutathione biosynthesis in T cells, thereby maintaining redox homeostasis, preserving mitochondrial integrity, and preventing metabolic exhaustion. This dual mechanism was critical for preserving long-term T-cell effector function within the nutrient-deprived tumor microenvironment. In addition to T-cell activation, CYBC NPs significantly reprogrammed the

tumor microenvironment. It repolarized TAMs from an immunosuppressive M2 phenotype toward a proinflammatory M1 phenotype and reshaped the systemic cytokine profile to favor immune activation. Elevated levels of IFN- γ , TNF- α , and IL-6, together with reduced levels of TGF- β , indicated broad immunostimulatory remodeling of both local and systemic immune landscapes.

Although direct evidence of glucose and cystine transfer to T cells was not obtained in this study, the combination of transcriptomic, metabolic, and phenotypic data strongly suggests a redistribution of metabolic resources within the TME following CYBC treatment. Suppression of tumor glycolysis, increased intratumoral glucose levels, and enhanced T-cell activation with reduced exhaustion markers (PD-1, TIM-3) together support the notion that CYBC relieves metabolic competition and promotes T-cell functional recovery.

Long-Term Immunotherapeutic Effects of CYBC NPs against Tumor Recurrence and Metastasis. TNBC is associated with a high risk of metastasis and recurrence.⁵³ Conventional therapeutic regimens often fail to induce durable immune responses, underscoring the urgent need for innovative strategies capable of both eliminating primary tumors and preventing disease relapse. In this context, we explored the long-term immunotherapeutic efficacy of CYBC NPs, which were previously shown to induce disulfidptosis-mediated tumor cell death and potent immune activation. To assess whether CYBC NPs could establish immunological memory and suppress metastatic dissemination, a dual-tumor model was developed, as illustrated in Figure 7a. Initially, 1×10^6 4T1 cells were subcutaneously injected into the right flank of BALB/c mice to establish primary tumors. Mice received three intravenous administrations of the corresponding formulations, after which a secondary tumor challenge was performed by injecting 2×10^6 4T1 cells into the contralateral (left) flank on day 25. On day 32, splenic immune profiling was performed via flow cytometry to assess the populations of memory T cells (mT cells), including central memory T cells (T_{CM} : CD3⁺CD8⁺CD44⁺CD62L⁺) and effector memory T cells (T_{EM} : CD3⁺CD8⁺CD44⁺CD62L⁻), which are critical for long-term immune surveillance. Concurrently, the progression of both primary and secondary tumors, along with the survival of the animals, was monitored.

As shown in Figure 7b, the proportions of T_{CM} and T_{EM} in the CYBC group were increased considerably compared with the control group, in both the CD8⁺ and CD4⁺ T cells. In detail, the CD8⁺ T_{EM} cells increased from 9.84 to 31.4%, and the CD4⁺ T_{EM} cells increased from 6.84% to 20.1%. Similarly, the proportion of CD8⁺ and CD4⁺ T_{CM} rose from 10.4 to 40.8% and from 12.1 to 49.1%, respectively, indicating robust immune memory generation. Moreover, memory B cells (mB cells) were markedly activated in the CYBC-treated group, showing a nearly 3-fold increase compared to that of controls (Figure 7c), suggesting enhanced humoral responses and possible facilitation of antigen presentation.

The therapeutic benefits extended to tumor suppression, as well. As depicted in Figure 7d–f, primary tumor growth was substantially inhibited in the CYBC group throughout the initial treatment phase. Upon rechallenge, tumors in the control group exhibited rapid progression, whereas tumor growth in the CYBC group remained minimal. Notably, two mice in the CYBC group showed complete resistance to secondary tumor inoculation, suggesting effective immuno-

logical memory and tumor-specific adaptive immunity. The body weight of the mice remained unchanged throughout the long-term treatment, which further illustrated the good biocompatibility of CYBC NPs (Figure 7g).

Given that our transcriptomic analysis revealed a down-regulation of metastasis-related genes during disulfidptosis, we further evaluated the antimetastatic potential of CYBC NPs. Lung tissues collected at the study end point showed a dramatic reduction in metastatic lesions in the CYBC-treated group (Figure 7h,i), reinforcing the hypothesis that disulfidptosis impaired the metastatic capability of tumor cells. Importantly, CYBC treatment led to a significant survival advantage, with all mice in the CYBC group surviving beyond 60 days compared to complete mortality in the control group by day 55 (Figure 7j).

Together, these results provided compelling evidence that CYBC NPs not only eradicated primary tumors through metabolic reprogramming and immune activation but also conferred robust systemic immunity capable of a vaccine-like ability to prevent tumor recurrence and metastasis.

CONCLUSIONS

In summary, our findings establish CYBC as a versatile nanoinducer capable of orchestrating metabolic collapse in tumor cells while simultaneously reprogramming the immune system for durable cancer control. The synergy among disulfidptosis induction, immune activation, and immune memory formation represents a promising strategy to overcome the metabolic and immunosuppressive barriers of the tumor microenvironment.

Notably, this work presents several conceptual and translational advances. First, by codelivering exogenous cystine and a GLUT1 inhibitor via a biomimetic nanoplateform, it overcomes the intrinsic limitation of low cystine availability in the tumor microenvironment, maximizing the induction of disulfidptosis. Second, this approach enables cystine delivery via endocytosis, bypassing the requirement for SLC7A11 expression and thus broadening the therapeutic applicability to SLC7A11-low tumors. Third, it demonstrates for the first time that disulfidptosis can function as a standalone immunogenic modality capable of triggering ICD and promoting immune metabolic reprogramming. This results in sustained T-cell activation and alleviation of immune exhaustion, effectively converting immunologically “cold” tumors into “hot” ones.

Collectively, this study uncovers the previously unexplored immunometabolic effects of disulfidptosis and provides new perspectives for developing metabolism-targeted immunotherapies against tumors.

METHODS AND MATERIALS

Chemicals and Reagents. Cupric chloride (CuCl_2) and BAY-876 (BAY) were purchased from Bidepharm (Shanghai China). 2,7-Dichlorofluorescein diacetate (DCFH-DA), tris(2-chloroethyl) phosphate (TCEP), DL-dithiothreitol (DTT), β -mercaptoethanol (2-ME), and TRIzol reagent were purchased from Sigma-Aldrich (Shanghai, China). L-Cystine, calcein acetoxymethyl ester (Calcein-AM), propidium iodide (PI), ATP assay kit, glucose assay kit, NADP⁺/NADPH assay kit, ActinTracker Red-Rhodamine, and red blood cell lysis buffer were purchased from Beyotime (Shanghai, China). Collagenase IV, DNase, DIR, and 4',6-diamidino-2 phenylindole (DAPI) were purchased from Solarbio (Beijing, China). HMGB1 and CRT were from HUABIO (Hangzhou, China). Steady Pure Quick RNA Extraction Kit, Evo M-MLV RT Mix Kit, and SYBR Green Premix Pro Taq HS qPCR Kit (Rox Plus) were obtained from

Accurate Biology (Hunan, China). The cystine ELISA kit was purchased from RuiDaHengHui Science (Beijing, China). The FITC-anti-CD11c antibody, PE-anti-CD86 antibody, APC-anti-CD80 antibody, PerCP/cyanine 5.5-anti-CD45 antibody, FITC-anti-CD3 antibody, APC-anti-CD8a antibody, PE-anti-CD8a antibody, PerCP/cyanine 5.5-anti-CD11b antibody, PE-anti-CD86 antibody, FITC-anti-CD206 antibody, anti-PE-CD4 antibody, FITC-anti-CD38 antibody, anti-PE-CD19 antibody, anti-CD44 antibody, anti-CD62L antibody, and ELISA kits of IFN- γ , TNF- α , IL-6, and TGF- β were purchased from Elabscience (Wuhan, China). The PE-anti-Tim-3 antibody was purchased from Thermo Fisher (Massachusetts, USA). The APC-anti-PD-1 antibody was purchased from Biolegend (Santiago de Chile, USA).

Cell Culture. 4T1 cells (mouse triple-negative breast cancer cells) and L929 cells (mouse fibroblast cells) were cultured in a high-glucose DMEM medium. B16 cells (mouse melanoma cells) were cultured in RPMI 1640 medium. RAW264.7 cells (mouse leukemia cells of monocyte macrophages) were cultured in RAW 264.7 cell-specific medium. All media were supplemented with 10% fetal bovine serum (FBS) and a 100 μ g/mL double antibiotic solution (penicillin–streptomycin mixture). Cells were incubated at 37 °C with 5% CO₂ (Thermo Forma 311, Thermo Scientific, USA) and passaged as required. Primary immune cells were cultured in RPMI-1640 and used for 4 days.

Animals. Female BALB/c mice (6–8 weeks old, 20 $\pm$ 2 g) were purchased from Chengdu Dashuo Laboratory Animal Co. All animal studies were carried out in accordance with the Guidelines for Animal Experimentation of Southwest Jiaotong University, and the Animal Ethics Committee of the institution approved the protocol.

Preparation of 4T1 Membranes. 4T1 cells were seeded into 10 cm culture dishes and grown until they reached full confluency. Cells were collected using a cell scraper and resuspended in hypotonic lysing buffer, containing a membrane protein extraction reagent and phenylmethanesulfonyl fluoride (PMSF). Then, the suspension was incubated on ice for 1 h, followed by ultrasonic disruption on ice. Cell debris was removed via centrifugation at 1000g for 10 min at 4 °C. The supernatant was then centrifuged at 15,000g for 1 h at 4 °C to isolate membrane fragments. The pellet was passed 10 times through a 200 nm polycarbonate membrane using a mini-extruder (Avanti) to ensure uniformity. The protocol for extracting the B16 cell membrane was similar to that for the 4T1 membrane.

Preparation and Characterization of CYBC NPs. To obtain the CYBC NPs, we first prepared CY NPs via a facile one-pot self-assembly method. 0.5 mmol of L-cystine was predissolved in 10 mL of 0.2 M NaOH solution, and 0.5 mmol of CuCl₂ was dissolved in 10 mL of double-distilled water (ddH₂O). The CuCl₂ solution was slowly added dropwise into the cystine solution under constant stirring at room temperature, forming a brown colloidal suspension. After 30 min of reaction, the suspension was centrifuged at 1000g for 10 min. The precipitate was washed 3 times alternately with ddH₂O and methanol, followed by freeze-drying to obtain CY NPs. Next, CY NPs (100 μ g) were mixed with 100 nM BAY (dissolved in DMSO) and sonicated at RT for 10 min to form drug-loaded CYB NPs. The mixture was centrifuged at 10,000g for 10 min, and the standard curve of BAY at 320 nm was detected. The supernatant was collected for UV analysis (320 nm) to determine the encapsulation efficiency (EE %):

$$EE(\%) = \frac{\text{Total amount of BAY} - \text{Amount of free BAY}}{\text{Total amount of BAY}} \times 100\%$$

The CYB NPs were then incubated with isolated 4T1 cell membranes and extruded 11 times through a mini-extruder using a 200 nm membrane. Excess membranes were removed by centrifugation. The resulting CYBC NPs were resuspended in PBS (pH 7.4, 20 mM) and characterized for hydrodynamic size and zeta potential using a dynamic light scattering (DLS) analyzer (Zetasizer, Malvern, UK). The morphology of the particles was examined by using transmission electron microscopy (TEM, JEM-2010HT). Samples for TEM were prepared by placing a droplet of CYBC

suspension onto copper grids, followed by 60 s of adsorption, negative staining with 2% phosphotungstic acid for 30 s, and air drying.

SDS-PAGE. The protein expression profiles of CYB, 4T1 cell lysate, 4T1 cell membrane, and CYBC NPs were measured by SDS-PAGE. Samples were mixed with loading buffer and boiled for 20 min. Proteins were separated on 12% polyacrylamide gels using the Mini-PROTEAN Tetra System (Bio-Rad, USA), followed by Coomassie Brilliant Blue staining overnight and imaging.

Drug Release from CYBC NPs. 2.0 mL of CYBC NPs (50 mg/mL) was sealed in a dialysis bag (MWCO: 1000 Da) and immersed in 8 mL of PBS buffer (7.4, 6.5, or 5.5) under gentle shaking (100 rpm) at 37 °C. At predefined intervals, 2 mL of PBS medium was withdrawn and replenished with an equal volume of fresh buffer. The collected samples were lyophilized, redissolved in 200 μ L of PBS, and analyzed by UV–vis spectrophotometry at 320 nm to quantify the released BAY using a standard curve.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). qRT-PCR was performed to investigate the mRNA levels of SLC7A11. Following treatments, cells were rinsed with sterile PBS. Subsequently, the total RNA was extracted using a Steady Pure Quick RNA Extraction Kit. cDNA was generated using an Evo M-MLV RT Mix Kit, following the instructions provided by the manufacturer. After that, real-time PCR was conducted using the SYBR Green qPCR Kit on a Step One Plus Real-Time PCR System (Thermo Fisher Scientific). Each sample was tested in triplicate, and the gene expression was normalized to the internal housekeeping gene. Primer sequences are listed in Table S1.

In Vitro Cytotoxicity Assay. Cytotoxicity was evaluated using CCK-8. 4T1, L929, B16, and RAW264.7 cells were seeded in 96-well plates and incubated for 12 h. Cells were then treated with various formulations (CY, BAY, CYB, and CYBC) for 24 h. For BAY dose–response, concentrations ranged from 0 to 500 nM. To verify the death pathway, 4T1 cells were seeded in 96-well plates 1 day before treatment, and the culture medium was replaced with 100 μ L of fresh DMEM medium or that containing 100 μ g/mL CYBC NPs at specific times. TCEP, Ferr-1, Z-VAD, and vitamin C (VC) were used as the inhibitors of disulfidptosis, ferroptosis, apoptosis, and reactive oxygen species, respectively. After treatment, the medium was replaced with 100 μ L of fresh medium containing 10 μ L of the CCK-8 solution. Plates were incubated at 37 °C for 30 min, and the absorbance was measured at 450 nm using a microplate reader.

$$\text{Cellviability}(\%) = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100\%$$

Following 24 h of treatment with PBS, CY, BAY, CYB, CYBC, or CYBC + reducing agents (e.g., TCEP, DTT, and 2-ME), cells were stained with 2 μ M Calcein-AM and 2 μ M propidium iodide (PI) for 30 min. Fluorescence was observed under CLSM.

Glucose, Lactate, and ATP Measurements. 4T1 cells were seeded into 6-well plates and treated with CY, BAY, CYB, or CYBC for 24 or 48 h. Culture supernatants were collected for analysis using Lactate Assay and Glucose Assay Kits (according to manufacturer's protocols). ATP levels were measured using an ATP Assay Kit. After treatment, cells were digested, washed with PBS, and lysed for intracellular ATP quantification.

NADP⁺/NADPH Detection (WST-8 Method). To assess the redox state, the ratio and concentration of NADP⁺/NADPH were measured by using a colorimetric assay. After 24 h of treatment, 4T1 cells were lysed in a NADP⁺/NADPH extraction buffer. NADP⁺ was decomposed by heating, while total NADP (NADP⁺ + NADPH) was measured in parallel. Samples were incubated with the developer solution and analyzed at 450 nm. A standard NADPH curve was used for quantification, allowing calculation of the NADP⁺/NADPH ratio.

Intracellular Cystine Quantification. To measure cystine levels, 4T1 cells were seeded into 12-well plates and treated with PBS, CY, BAY, CYB, or CYBC for 24 h. Cells were lysed, and 10 μ L of each lysate was mixed with 90 μ L of the extraction reagent. The reaction was carried out according to the kit protocol, and the absorbance was measured at 450 nm.

Intracellular ROS Detection. For intracellular ROS detection, 4T1 cells were cultured in 12-well plates and treated with PBS, CY, BAY, CYB, CYBC, or CYBC + VC for 6 h. Cells were then incubated with 5 μ M DCFH-DA (ROS probe) in DMEM for 20 min at 37 °C. After fixation with 4% paraformaldehyde for 30 min, ROS levels were visualized using CLSM. For quantitative analysis, cells were detached with TrypLE Express, resuspended in FACS buffer (PBS + 2% FBS + 0.5 mg/mL heparin), and analyzed by flow cytometry (Accuri C6 PLUS, BD).

Cellular Uptake. To study cellular uptake, cells were incubated with DIR-labeled CYBC NPs for various time points (2, 4, 6, and 8 h). Cells were then digested with TrypLE Express for 5 min, resuspended in a FACS buffer, and analyzed via flow cytometry.

Actin Cytoskeleton Visualization. To assess cytoskeletal integrity, 4T1 cells were cultured on coverslips and treated with PBS, CY, BAY, CYB, CYBC, or CYBC mixed with a thiol-reducing agent (TECP, DTT, and 2-ME). After 24 h, the cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with ActinTracker Red. Nonspecific binding was blocked using 5% BSA. Nuclei were counterstained with DAPI, and samples were imaged via CLSM.

Immunofluorescence Staining. After different treatments, cells were fixed with 4% paraformaldehyde (15 min), permeabilized with 0.5% Triton X-100 (10 min), and blocked with 5% BSA (30 min). Samples were incubated overnight at 4 °C with primary antibodies against HMGB1 or calreticulin (CRT), followed by 1 h of incubation with fluorescently labeled secondary antibodies in the dark. Nuclei were stained with DAPI. Imaging was performed by using CLSM.

Isolation of Murine Dendritic Cells (DCs). Bone marrow was harvested from the femurs and tibias of female BALB/c mice (6–8 weeks old) and flushed with RPMI-1640 medium. Cells were filtered through a 70 μ m strainer and cultured in RPMI-1640 medium containing 10% FBS, 10 ng/mL IL-4, and 20 ng/mL GM-CSF. At days 3 and 6, 50% of the medium was replaced with fresh cytokine-supplemented medium. On day 7, CD11c expression was analyzed by flow cytometry to confirm DC maturation.

In Vitro DC Maturation Assay. 4T1 cells were seeded in 12 culture dishes and incubated overnight and then treated with PBS, CY, BAY, CYB, or CYBC and maintained for another 24 h. After treatment, the cell culture supernatants were collected and applied to immature DCs, followed by incubation for an additional 24 h. Subsequently, DCs were harvested, stained with fluorescence-conjugated antibodies against CD11c, CD80, and CD86, and analyzed by flow cytometry to evaluate their maturation status.

Isolation of Primary Macrophages (PCMs). The BALB/c mice were injected intraperitoneally with 1 mL of 5% starch solution daily for 3 days to promote macrophage aggregation. After 12 h of fasting, mice were euthanized, and peritoneal lavage was collected using cold RPMI-1640. Following centrifugation (300g, 5 min) and red blood cell lysis, the remaining cells were resuspended in RPMI-1640 with 10% FBS and cultured as PCMs.

Macrophage Polarization Observation. 4T1 cells were seeded in the apical chamber (1.0×10^6 cells/well) of a transwell insert and treated with PBS, CY, BAY, CYB, or CYBC. After 4 h, the medium was replaced, and PCMs were seeded in the lower chamber. Following 24 h coculture, PCMs were collected, stained with polarization markers (CD11b, CD86, CD206), and analyzed by flow cytometry.

Enzyme-Linked Immunosorbent Assay (ELISA). Tumor tissues were homogenized by using a Precellys 24 Dual homogenizer in cold lysis buffer with protease/phosphatase inhibitors. After centrifugation, cytokine levels (TNF- α , IFN- γ , IL-6) in the supernatants were quantified using commercial ELISA kits (Elabscience), following the manufacturer's protocols.

Transcriptomic Analysis. 4T1 cells were treated with BAY (100 nM) or CYBC (100 μ g/mL) for 24 h. Total RNA was extracted using TRIzol, and libraries were prepared and sequenced using an Illumina NovaSeq 6000 platform (Biozon Biotechnology, Shanghai). Differential gene expression analysis focused on metabolic and cytoskeletal pathways.

Wound-Healing Migration Assay. Cells were plated on 6-well dishes and grown to 90% confluence in 2 mL of growth medium. The cells were damaged using a 2 mm wide tip and were then treated with different nanoparticles. They were allowed to migrate, and the images were captured using an inverted microscope (10 $\times$ magnification).

In Vivo Antitumor Efficacy. The subcutaneous tumor model was established by injecting 100 μ L of PBS-resuspended 4T1 cells into the right flank of 6–8-week-old female BALB/c mice. When tumors reached ~ 100 mm³, mice were randomized into five groups: (1) PBS, (2) CYC, (3) BAY, (4) CYB, and (5) CYBC. Treatments were administered intravenously every 2 days. Tumor volumes and mouse body weights were monitored every 2 days, as well. Tumor volume was calculated as

$$V = \frac{L \times S^2}{2}$$

where L is the longest and S is the shortest tumor diameter.

Tumor growth inhibition (TGI%) was calculated as

$$\text{TGI} = \left(1 - \frac{\frac{T_e}{T_s} \times \frac{C_e}{C_s}}{1 - \frac{C_e}{C_s}} \right) \times 100\%$$

where T_e , T_s , C_e , and C_s represent the average tumor volumes of treated mice at the end point, treated mice at the starting point, control mice at the end point, and control mice at the starting point, respectively.

In the survival experiment, mice were euthanized when their tumor volumes reached 2000 mm³. The major organs (heart, liver, spleen, lung, and kidney) and tumors were sliced and stained with H&E.

Immune Profiling Post-Therapy. At day 9 or 19 post-treatment, the tumor-bearing mice were humanely euthanized, and their tumors and spleens were excised and sliced into small fragments. These fragments were then enzymatically digested using collagenase IV (1 mg/mL) and DNase (0.1 mg/mL) for a duration of 1 h at 37 °C to dissociate the cells into a single-cell suspension. Subsequently, the suspensions were passed through 70 μ m nylon strainers to eliminate any residual debris and cell clumps. The filtered cells were then stained following the established protocol (Table S2) and were analyzed using a flow cytometry system. All flow cytometry data were processed and analyzed by utilizing FlowJo v10 software; the gating strategies are described in Figure S33.

Tumor Rechallenge Study. Mice cured by CYBC therapy ($n = 7$) were rechallenged with 2 $\times$ the initial dose of 4T1 cells on day 30. Naïve mice injected with the same number of cells served as the controls. Tumor growth was monitored every other day. Mice were euthanized when tumors reached 2000 mm³.

Histological Analysis. Tumors and major organs (heart, liver, spleen, lung, and kidney) were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into 10 μ m slices. Sections were deparaffinized and rehydrated, and antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) at 95 °C for 15 min. Hematoxylin and eosin staining was performed using an H&E kit (Servicebio, China). Slides were imaged and analyzed using ImageJ Fiji software.

Hemolysis Assay. Red blood cells (RBCs) were isolated from mouse blood by centrifugation (3000 rpm, 15 min), washed with PBS, and diluted to 2%. RBC suspensions were incubated with CYBC NPs (50–500 μ g/mL) for 30 min and then centrifuged at 10,000 rpm for 10 min. Absorbance at 570 nm was recorded. Hemolysis was calculated as

$$\begin{aligned} \text{Hemolysis}(\%) &= \frac{\text{Absorbance of sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control} - \text{Absorbance of negative control}} \\ &\times 100\% \end{aligned}$$

Blood Biochemistry Analysis. After 21 days of treatment, blood samples ($n = 3/\text{group}$) were collected and analyzed for hematological and biochemical parameters, including WBC, RBC, HGB, HCT,

MCV, MCH, MCHC, RDW, PLT, MPV, ALT, AST, ALP, ALB, and UREA (Chengdu Lilai Biotechnology Co., Ltd.).

Statistical Analysis. All measurements contained three or more independent replicates from separate experiments. The exact sample size and statistical test for each experiment are described in the relevant figure legends. All statistical analysis results are presented as mean value $\pm$ standard deviation (SD). All statistical data were processed in GraphPad Prism (version 9.0 for Windows) by unpaired two-tailed Student's *t*-test, one-way ANOVA, or two-way ANOVA. The findings were deemed statistically significant when the *p*-value was less than 0.05 (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.5c08432>.

Additional size distributions, UV–vis absorption spectra, qPCR, cell viability data, CLSM images, intracellular ROS data, RNA-seq results and analysis, wound-healing assay, hemocompatibility assessment, H&E-stained images, representative flow cytometry plots, and representative gating strategies (PDF)

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Conceptualization: Y.X., Y.W., M.W., and S.F. Methodology: Y.X., Y.W. Investigation: Y.X., X.M., J.Q., Z.H. and H.Z. Visualization: Y.X. Supervision: Y.W. and S.F. Writing—original draft: Y.X. Writing—review and editing: Y.W. and S.F. Funding acquisition: Y.W. and S.F.

Notes

The authors declare no competing financial interest.

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