The poor drug delivery to primary and metastatic tumors of breast cancer remains a great challenge for effective antimetastasis therapy. Herein, a tumor microenvironment-activated cabazitaxel micelles decorated with legumain-specific melittin (TCM-legM) are rationally designed for programed targeting of breast cancer metastasis. TCM-legM is quiescent in blood circulation, but can be specifically activated by the highly expressed legumain in tumor microenvironments to improve their specific targeting and deep penetrating to primary or metastatic tumors. Thereafter, the activated TCM-legM can be efficiently internalized by cancer cells and motivate the rapid pH-responsive drug release for antimetastasis therapy. In metastatic 4T1 breast cancer cells, TCM-legM presents significant inhibition on the proliferation, migration, and invasion activities. In vivo, TCM-legM can be effectively delivered to both primary and metastatic tumors of breast cancer with deep tumor penetration and efficient cellular internalization, thereby resulting in a notable reduction of tumor growth and producing a 93.4% suppression of lung metastasis. Taken together, the rationally designed TCM-legM can provide an intelligent drug delivery strategy to enhance the medical performance on treating breast cancer metastasis.

1. Introduction

Cancer metastasis is responsible for over 90% of lethality in breast cancer patients, which is extremely difficult to treat in the clinic. The metastasis is a complicated multistep process that refers to the spread of cancer cells from primary tumor to colonization at distant organs. A major issue for antimetastasis therapy is the poor efficiency of drug delivery to breast cancer metastasis where many current therapeutic agents cannot reach. The primary breast cancer are usually well-vascularized tumors with tortuous vasculature, elevated hydrostatic pressure, and complexed physical barriers of tumor stoma, causing the poor drug penetration in tumor mass. Conversely, the metastatic tumors are often small clusters of cancer cells with poor vasculature and high dispersion in invaded organs, which greatly restrict the drug accessibility to these metastatic foci. The distinct features of primary tumors and distant metastases exasperates the difficulty of metastasis-targeted drug delivery. Recently, it has been suggested that nanovectors represent the best efficacy of tumor-targeted drug delivery due to the enhanced permeability and retention (EPR) effects. However, these nanovectors are largely inadequate when improving drug penetration in tumor mass and enhancing their delivery to small-sized metastatic tumors. Therefore, it is highly desired to find novel drug delivery strategies that can efficiently target both primary tumors and distant metastases for antimetastasis therapy.

Ideally, the nanovectors should be endowed with specific targeting to primary tumor and metastatic foci, deep penetrating into these tumors and efficient internalization by cancer cells for antimetastasis therapy. Moreover, the therapeutic agents should also be intelligently released from nanovectors to target specific intracellular organelles for effective chemotherapy. Nevertheless, most of current nanovectors are partly considered in tumor targeted drug delivery, and are suboptimal for targeting cancer metastasis. For example, the decoration of nanoparticle with polyethylene glycol (PEG) or zwitterionic materials can extend the circulation time and improve the accumulation in tumor tissues, but also impede their internalization into cancer cells. Modifying nanoparticles with specific targeting ligands can enhance the uptake by cancer cells, but greatly impact the circulation time and passive targeting ability. The functionalization of nanoparticles with membrane-active peptides can afford effective intracellular delivery of various cargos in vitro, but are generally inapplicable for many in vivo...
studies due to their unavoidable nonspecific interactions.\cite{13} To circumvent these limitations, a rational design of programmed targeting strategy that can be specifically motivated by the abnormal microenvironments in primary or metastatic tumors, is particularly attractive for antimetastasis therapy.

In view of the specific microenvironments of breast cancer metastasis, the dysregulation of some specific enzymes and the acidic environments in endosome/lysosomes of cancer cells are striking characters that can be extensively detected in primary tumor, large metastatic tumors, or even small-sized metastatic lesions.\cite{5,6} For instance, legumain protease is highly expressed in tumor, matrix, and endothelial cells in the tumor microenvironment, but barely detected in normal tissues.\cite{14–16} In intracellular compartments, the pH value in early endosomes, late endosomes and lysosomes is around 6.3, 5.5, and 4.7, respectively.\cite{8,17,18} Accordingly, the nanovectors that can respond to the overexpressed legumain protease in tumor microenvironments and intracellular acidic environments would be preferred to improve the metastasis-targeting in a programmed manner.

Herein, we describe a tumor microenvironment-activated cabazitaxel micelles functionalized with legumain-specific melittin (denoted as TCM-legM) for programmed targeting of breast cancer metastasis (Scheme 1). Cabazitaxel, a potent microtubule inhibitor, is selected as the anticancer drug for treating breast cancer metastasis.\cite{19} The legumain-specific pro-peptide of membrane-active melittin (named as legM) is linked to 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-methoxy (DMPE)-PEG to form the DMPE-PEG-legM conjugates. The methoxy poly(ethylene glycol)-block-poly (2-diisopropyl methacrylate) (PEG-PDPA) is used to trigger the pH-responsive drug release in intracellular acidic environments.\cite{20–22} As shown in Scheme 1, TCM-legM would be quiescent in blood circulation and can be specifically activated by the highly expressed legumain in tumor microenvironments to improve their targeting to primary or metastatic tumors. Then, the restored melittin in TCM-legM can promote the deep penetration in tumor and facilitate their internalization by cancer cells. After internalization, the pH-sensitivity of TCM-legM would motivate the on-demand drug release in endosomal/lysosomal acidic environments to achieve the antimetastatic effects. In this work, the rationality of this design for programmed targeting of breast cancer metastasis was validated by in vitro and in vivo evaluations.

2. Results and Discussion

2.1. Preparation and Characterization of TCM-legM

In this work, TCM-legM consisted of cabazitaxel, DMPE-PEG-legM, PEG-PDPA, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy (polyethylene glycol) (DSPE-PEG2000).
Meanwhile, a counterpart micelle formulation without DMPE-PEG-legM was prepared as control, which was named as TCM. In these micelles system, the PEGylated lipids were used to improve the stability of the micelles system and confer the surface modification of legM in TCM-legM. Melittin, a water-soluble membrane-active peptide containing 26 amino acids, is used as a potential drug delivery enhancer. To minimize the nonspecific interactions and realize the legumain-based tumor targeting, the primary amine in D-enantiomer of melittin (D-Melittin) was modified with specific legumain substrate (alanine–alanine–asparagines, AAN) to prepare the pro-peptide legM (Sequence: cgigavlk(NAA)vlttglpaliswik(NAA)rk(NAA)rqq), and then linked with DMPE-PEG5000-Maleimide to form the DMPE-PEG-legM conjugates (Figure S1, Supporting Information), thereby facilitating the surface modification of the micelles system. The pH-sensitive amphiphilic polymer of PEG-PDPA was synthesized by atom-transfer radical polymerization (ATRP) method as previously described and characterized (Figure S2, Supporting Information). The loading capacity of cabazitaxel was 6.37 ± 0.03% for TCM-legM and 8.60 ± 0.02% for TCM. The morphology was determined by field emission transmission electronic microscope (FE-TEM), which showed both TCM-legM and TCM were nanometer-sized spherical particles (Figure 1A). The dynamic light scattering (DLS) analysis showed the hydrodynamic diameter was 45.9 nm (polydispersity index, PDI, 0.236) for TCM-legM, and 44.8 nm (PDI, 0.215) for TCM (Figure S3, Supporting Information). The ζ potential values were −0.83 ± 0.11 mV for TCM-legM and −1.52 ± 0.47 mV for TCM. In addition, the mean diameter of TCM-legM and TCM was invariable within 24 h when they were incubated with phosphate buffered solution (PBS) at pH 7.4 (Figure S4, Supporting Information). The encapsulation efficiency of cabazitaxel was 95.6 ± 0.5% for TCM-legM and 94.6 ± 0.2% for TCM, indicating the high incorporation of cabazitaxel in these micelle systems. Moreover, the encapsulation efficiency values of TCM-legM and TCM was barely changed after their incubation with PBS (pH 7.4) or PBS (pH 7.4) with 10% fetal bovine serum (FBS) (Figure 1B), suggesting the good stability over time in the mimicked physiological environments. The high encapsulation efficiency of cabazitaxel in the micelles could be owing to hydrophobic interactions between the cabazitaxel and the lipid-coated core of the micelles system. The good stability of the micelles could provide an essential prerequisite for the enhanced targeting of breast cancer metastasis.

The primary design of TCM-legM is that the micelles would be responsively activated by the overexpressed legumain.
protease upon their arrival at primary or metastatic tumor microenvironments, thereby facilitating the specific accumulation in these tumor sites and further internalization by cancer cells. Although legumain is a highly conserved lysosomal protease in cancer cells or tumor associated macrophages, they can be moved to the cell surface on stress and secreted into extracellular media in tumor sites, enabling the activation of TCM-legM in tumor regions.[16,26,27] The activation is the cleavage of AAN peptide in legM by active legumain to expose the membrane-lytic peptide of melittin. To evaluate the legumain-responsiveness of TCM-legM, hemolysis assays were performed using erythrocytes in PBS at pH 7.4. By contrast, 1% triton X-100 was used as positive control and the PBS (pH 7.4) was performed as negative control (Figure S5, Supporting Information). Once the legM peptide was activated into melittin by legumain protease, hemolysis would be easily observed. As shown in Figure 1C, free melittin was used as inherent control, which displayed hemolysis in a concentration-dependent manner. When TCM-legM and TCM were incubated with PBS (pH 7.4) or PBS (pH 7.4)+10% FBS, the hemolysis was rarely detected. However, when TCM-legM was incubated with 50 ng mL\(^{-1}\) of active legumain in PBS (pH 7.4), hemolysis was readily visualized, suggesting the exposure of melittin by active legumain (Figure 1C). Of note, in the presence of legM, the hemolysis percentage of TCM-legM could reach 90% of free melittin at 40 µg mL\(^{-1}\), indicating the effective restoration of membrane-lytic activities of melittin (Figure 1D). In addition, when TCM-legM was incubated with active legumain in PBS (pH 7.4), the particle size and cabazitaxel encapsulation efficiency was barely changed within 24 h (Figure S6, Supporting Information), suggesting that the legumain treatment did not impact the stability of the micelles structure. Therefore, TCM-legM would be stable in blood circulation and be specifically activated to expose melittin by active legumain, indicating the specific responsiveness of TCM-legM to the highly expressed legumain in tumor microenvironments.

Moreover, the secondary design of TCM-legM is the pH-sensitive properties that could trigger the on-demand drug release in intracellular acidic environments. In TCM-legM, the hydrophobic PDPA segments of PEG-PDPA polymer would become hydrophilic in acidic endosomal/lysosomal environments, resulting in the collapse of micelle structure and rapid release of cabazitaxel. To verify the pH-sensitivity of TCM-legM, the morphology was visualized under FE-TEM and the drug release behavior was quantified. When TCM-legM and TCM were incubated with PBS at pH 5.5, the water-insoluble cabazitaxel would be readily precipitated, indicating the responsive drug release from these micelles. Then, the drug precipitation was removed by centrifugation and the supernatant was examined by FE-TEM measurements. Compared with the typical images of TCM and TCM-legM at pH 7.4, the spherical particles of these micelles were rarely observed (Figure 1A), indicating the dissociation of the micelle structure. Moreover, considering the different pH values in blood, tumor regions, or various intracellular compartments of cancer cells,[11] TCM and TCM-legM were respectively incubated in PBS at pH 7.4, 6.8, 6.3, 5.5, and 4.7 at 37 °C (Figure 1E). The released drug amount from TCM-legM was minimal at pH 7.4 and pH 6.8, moderately increased to 29.3%, and markedly increased to 51.3% at pH 5.5 and 67.7% at pH 4.7, respectively, which effectively verified the on-demand drug release in intracellular acidic environments. In addition, the drug release from TCM-legM at each pH value was comparable to that from TCM, suggesting that the incorporation of legM in TCM-legM did not impact its pH-responsive drug release behavior. Thus, the pH-responsive properties of TCM-legM could be advantageous for cabazitaxel to exert the pharmacological activities.

2.2. In Vitro Therapeutic Efficacy on Metastatic 4T1 Cells

To evaluate the therapeutic effects of TCM-legM on breast cancer metastasis, the highly metastatic 4T1 breast cancer cells were used for the detections.[25,28] For cellular uptake measurements, the hydrophobic Nile red labeled TCM and TCM-legM were incubated with 4T1 cells for 4 h and then observed using laser confocal scanning microscopy (LCM, Leica-SP8 STED). As control, cells were stained with Hoechst 33342 (Blue, Beyotime) and LysoTracker Green DND-26 (Green, Molecular probe) for the observation. As shown in Figure 2A, a few yellow spots were detected in TCM and TCM-legM groups, indicating the efficient uptake of these micelles in 4T1 cells and their colocalization with lysosomes. In view of the pH-responsive properties of TCM and TCM-legM, the localization in lysosomes could be beneficial for motivating the pH-stimuli responsive drug release behavior for anticancer therapy. Thereafter, the cellular uptake of TCM and TCM-legM were quantified using the FACSCalibur system (BD, USA). At 4.0 h of incubation, TCM-legM had a 1.67-fold higher signals than that of TCM (Figure 2B). It has been reported that melittin could interact with specific endocytic receptors of cancer cells and facilitate the internalization by cancer cells.[29] Thus, the involved legM peptide in TCM-legM could be responsible for the significant enhancement of cellular uptake by 4T1 cells.

Then, the cytotoxicity of TCM-legM, TCM, and free cabazitaxel was investigated in 4T1 cells. By contrast, blank micelles of TCM (denoted as bTM) and TCM-legM (denoted as bTM-legM) were performed as control. In Figure 2C, free cabazitaxel, TCM, and TCM-legM showed significant inhibition on the viability of 4T1 cells in a concentration-dependent manner, but bTM exhibited negligible inhibition on cell viability (Figure 2C). Interestingly, bTM-legM had little effects on the viability of 4T1 cells at the equivalent concentration less than 0.4 µg mL\(^{-1}\), but presented moderate inhibitory effects thereafter (Figure 2C). The inhibitory effects of bTM-legM could be largely owing to the membrane lytic activities of activated legM peptide after endocytosis. In addition, when TCM-legM was incubated with 50 ng mL\(^{-1}\) of active legumain at 4 µg mL\(^{-1}\) of cabazitaxel in the cell viability assays, no significance was detected between TCM-legM and TCM-legM + legumain, indicating that the in vitro activation of TCM-legM by legumain did not impact the cytotoxicity measurements (Figure S7, Supporting Information). As a result, the considerable inhibition of TCM-legM on 4T1 cells demonstrated great potential for effective anticancer therapy.

Moreover, transwell-mediated assays were performed to assess the inhibitory effects of TCM-legM on the migration and invasion activities of metastatic 4T1 cells (Figure 3). The cell migration and invasion are two key steps of cancer
metastasis.\cite{22,25,30} In Figure 2C, at 40 ng mL\(^{-1}\) of cabazitaxel or equivalent concentration, each formulation showed high cell viability and would not impact the assays. TCM-legM, TCM, and cabazitaxel showed considerable inhibition on the migration and invasion activities of 4T1 cells (Figure 3), but blank micelles of bTM and bTM-legM had no inhibitory effects (Figures S8 and S9). Compared to the negative control, the TCM-legM treatment resulted in an 88.2% inhibition of cell migration and 92.4% suppression of cell invasion, which was significantly higher than that of free cabazitaxel and TCM (Figure 3B,C). As a result, TCM-legM had considerable inhibition on the migration and invasion of 4T1 cells, demonstrating great potential for antimetastatic efficacy.

2.3. In Vivo Specific Targeting to Breast Cancer Metastasis

The specific targeting of TCM-legM to breast cancer metastasis was determined in the orthotopic metastatic breast cancer model. TCM and TCM-legM were fluorescently labeled with hydrophobic near-infrared dye of IR-780 for in vivo imaging detection (Spectrum, Perkin–Elmer). In Figure 4A, the fluorescence signals of TCM and TCM-legM were obviously observed in the tumor sites at predetermined time points. The fluorescence intensity was gradually increased with time up to 12 h, and remained at a high level even at 24 h of injection, indicating the high accumulation and retention of TCM and TCM-legM in the primary tumors of breast cancer (Figure 4A). At 4.0 h, the fluorescence signals of various major organs from TCM and TCM-legM treated groups were recorded. The ex vivo images showed both TCM and TCM-legM were mainly located in liver, tumor, and lung (Figure 4B). Typically in tumor, the fluorescence intensity of TCM-legM was 1.7-fold higher than that of TCM (Figure 4C). The enhanced tumor accumulation of TCM-legM could be attributed to the good stability in blood circulation, the involvement of legumain-sensitive legM peptide, and their specific targeting to the legumain-overexpressed tumor regions.

Then, the deep penetration of TCM-legM in tumor mass was determined by photocoacoustic imaging analysis (Vevo 2100 LAZR, VisualSonic FUJIFILM). In the captured images, TCM and TCM-legM were denoted as green signals. In Figure 4D, the green signals of TCM and TCM-legM could be obviously observed in tumor tissues. TCM could penetrate the tumor
mass with the depth of 3 mm, but were negligibly detected in the deep inner sides. However, for TCM-legM, the signals could be readily detected in the surface and inner regions of tumor tissues with stronger intensity over TCM, which effectively verified the superior tumor penetrating ability of TCM-legM. The deep penetration of nanoparticle in solid tumors could be an essential prerequisite for effective chemotherapy,\cite{11,31,32} which significantly depended on the particle size, shape, and surface modification of the micelles system. Moreover, the deep penetration in solid tumors could also be achieved by modulating these parameters in respond to the abnormal tumor-microenvironments or physical stimuli.\cite{33} Accumulating evidences indicated that the smaller-sized nanoparticles showed superior tumor penetration capability, especially when the particle size was less than 50 nm.\cite{9,34} Both TCM and TCM-legM showed the hydrodynamic particle size around 45 nm, demonstrating significant potential for its deep penetrating in tumor mass. As evidenced by TCM in Figure 4D, the small particle size of nanoparticles alone might be partial for its deep penetrating in solid tumors. In comparison with TCM, the involved legM peptide in TCM-legM that could respond to the highly expressed legumain in tumor region could play an essential role for the enhanced tumor accumulation and deep penetration in tumor mass. Therefore, the superior penetration of TCM-legM in tumor could be owing to the synergistic effects of the small particle size and the incorporation of legM peptide.

Moreover, the in vivo internalization by cancer cells was measured by LCSM (FV1000, Olympus, Japan). The IR-780 loaded TCM and TCM-legM were depicted as red fluorescence signals. As control, the tumor sections were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue, Beyotime) and phalloidin-fluorescein isothiocyanate (FITC) (green, Beyotime) for the visualization. The red fluorescent signals of TCM and TCM-legM could be extensively observed in the tumor regions and the intensity of fluorescence signals from TCM-legM were much stronger than that from TCM (Figure 5A). Meanwhile, in the enlarged images in Figure 5B, the red fluorescence signals of TCM-legM could be largely detected in cancer cells with stronger intensity over TCM, indicating the efficient internalization by cancer cells in tumor tissues. Compelling evidences suggested the highly abnormal expression of legumain protease in tumor regions,\cite{14,16,27} which could motivate the exposure of melittin in TCM-legM. Moreover, melittin could interact with the specific highly expressed receptors to enhance the endocytosis by cancer cells. Accordingly, the exposed melittin in TCM-legM by active legumain in tumor microenvironments could account for the enhanced internalization by cancer cells.

The metastatic tumors in distant organs that occurred with small size, high multiplicity, and dispersion were the lethal part of breast cancer.\cite{1,2} The specific targeting to distant metastases was a substantial prerequisite for treating breast cancer metastasis.\cite{22,32} To evaluate the specific metastasis targeting of TCM-legM, a 4T1-induced lung metastatic breast cancer model was used for the detection. The IR-780 labeled TCM and TCM-legM were injected to the lung metastatic model via tail vein. After 4 h, the lung tissues were removed, sectioned, and stained with DAPI (blue, Beyotime) and phalloidin-FITC (green, Beyotime) for the observations. In Figure 5C, the red fluorescence signals

Figure 3. Inhibition of TCM-legM on the migration and invasion of 4T1 cells. A) Typical images of migrated or invaded cells across the transwell membrane, where cells were denoted as cell clusters of violet regions; B) the percentage of migrated cells compared to the negative control, *p < 0.05; C) the percentage of invaded cells compared to the negative control, *p < 0.05.
of TCM and TCM-legM could be considerably detected in the metastatic tumor sites despite the small size of metastatic lesions in lung (less than 1.0 mm). TCM was mainly distributed in the outer side of metastatic foci, but rarely detected in the inner sides. However, in TCM-legM treated group, the red fluorescence signals could be observed in the whole metastatic lesions, wherever in the exterior or interior regions. Accordingly, both TCM and TCM-legM showed effective targeting to the metastatic sites, while TCM-legM presented a superior tumor penetrating ability in the metastatic lesions. Since the tumor EPR effects were mainly limited to tumors larger than 4.6 mm in diameter,[2] the specific metastasis targeting of TCM-legM could be owing to the long circulation and small particle size of the micelles system. Moreover, for TCM-legM, the restoration of melittin in metastatic regions could enhance the specific accumulation and deep penetration in metastatic tumors, thereby providing an essential requirement for antimetastasis therapy.

2.4. In Vivo Therapeutic Effects

The effectiveness of TCM-legM on treating breast cancer metastasis was evaluated in an orthotopic metastatic breast cancer model. Every 3 d, mice were respectively treated with saline control, bTM, bTM-legM, cabazitaxel, TCM, and TCM-legM at 5 mg kg$^{-1}$ of cabazitaxel or equivalent dose by tail vein injection. The body weight of tumor bearing from each group was rarely changed during the treatment (Figure S10, Supporting Information). Similar with the saline control, the tumor volume in bTM and bTM-legM treated groups was progressively increased with time with no difference among them (Figure 6A). However, when mice were treated with cabazitaxel, TCM and TCM-legM, the tumor growth was greatly depressed. Compared to the saline group, the tumor volume was obviously reduced to 48.3% for cabazitaxel, 39.0% for TCM and 24.8% for TCM-legM, respectively. At the end, the tumor tissues were collected, imaged and weighed to evaluate the
inhibitory rate on tumor growth (Figure 6B; Figure S11, Supporting Information). Compared to the control group, TCM-legM treatment produced a 74.4% reduction of tumor growth, which was significant higher than that of free cabazitaxel (36.4%) and TCM (56.8%). By contrast, blank micelles of bTM-legM showed little effects on the tumor growth, which could be attributed to the lower dose of active melittin involved in the micelles system. Moreover, the treatment efficacy of TCM-legM in tumor was also evaluated by the terminal deoxy-nucleotidyl transferase dUTP nick end labeling (TUNEL) assays, which was denoted as green fluorescence signals. In Figure 7A, the TCM-legM treatment showed the highest level of cell apoptosis in tumor tissues. Therefore, TCM-legM displayed a significant inhibition on tumor growth, which is more effective than TCM.

Moreover, the inhibition of TCM-legM on lung metastasis was evaluated. The lung is a particular organ that breast cancer cells are disseminated.\citep{2,25,28} At the end time point of treatment, the lungs from each group were carefully collected to record the visually detected metastatic nodules. In Figure 6C, the metastatic nodules could be extensively detected in saline control, bTM, and bTM-legM treated group, indicating the invalidity of blank micelles on preventing lung metastasis. Compared with the saline control, the lung metastasis was moderately reduced by 55.2% and 77.8% in free cabazitaxel and TCM treated group. In particular, in TCM-legM group, the average number of metastatic nodules was 2.8 ± 1.3, which was only 14.8% and 29.8% of cabazitaxel and TCM treated group, respectively. Accordingly, the TCM-legM treatment resulted in a 93.4% inhibition of lung metastasis, which was significantly higher than that of cabazitaxel and TCM (Figure 6D). Moreover, histological studies of the lung tissues were performed using hematoxylin and eosin (H&E) staining assay (Figure 6E; Figure S12, Supporting Information). The metastatic lesions, which was denoted as darkly stained nuclei, was barely detected in TCM-legM treated group, but obviously observed in other groups. Meanwhile, the TUNEL assay of lung tissues suggested the highest level of cell apoptosis in metastatic lung tissues in TCM-legM treated group (Figure 7B). Therefore, the incidence of lung metastasis was effectively suppressed by the programed targeting TCM-legM system.
The in vivo therapeutic evaluations showed both TCM-legM and TCM had considerable reduction of primary tumor burden and notable prevention of lung metastasis. Compared with TCM, the TCM-legM treatment showed a significant improvement of the therapeutic effects, which could be largely resulted from the programmed targeting of primary and metastatic tumors. The involved legM peptide in TCM-legM remains inactivated in blood circulation, but could be specifically recognized and activated by the highly expressed legumain proteases in primary and metastatic tumor microenvironments, thereby ensuring the specific targeting to breast cancer metastasis. Moreover, the exposed melittin of TCM-legM in tumor regions could significantly improve the specific accumulation in primary and metastatic tumors, facilitate the deep penetration in
this tumor mass and promote their internalization into cancer cells. Furthermore, the internalized TCM-legM could trigger the pH-responsive drug release behavior in intracellular acidic environments, thereby facilitating cabazitaxel to exert the pharmacological effects. Therefore, the rationally designed TCM-legM system with programmed targeting capability demonstrated great potential for treating breast cancer metastasis.

### 3. Conclusions

In summary, we rationally designed the TCM-legM with specific responsiveness to the highly expressed legumain in tumor region and mildly acidic pH-stimuli in intracellular endosomes/lysosomes for programmed targeting of breast cancer metastasis. In metastatic 4T1 breast cancer cells, TCM-legM could be largely internalized and showed considerable inhibition on the proliferation, migration, and invasion activities. In vivo, TCM-legM could be specifically delivered to the sites of primary and metastatic tumors with deep penetrating ability and efficiently internalized by cancer cells, thereby leading to a considerable reduction of primary tumor growth and producing a 93.4% prevention on the incidence of lung metastasis. Therefore, the rational design of TCM-legM can provide an intelligent drug delivery strategy for programmed targeting of breast cancer metastasis.

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**Figure 7.** The apoptosis assay of A) primary tumor and B) metastatic lung tissues from various formulations treated groups. The apoptosis was denoted as green fluorescence signals and the nuclei was depicted as blue signals, scale bar = 50 µm.
4. Experimental Sections

**Materials:** Cabazitaxel was supported by Shanghai Chemleader Biomedical Co., Ltd (Shanghai, China). DSPE-PEG2000 was supplied by Lipoid Gmbh (Ludwigshafen, Germany). The pH-sensitive amphiphilic block polymer of PEG-PDPA was synthesized by ATRP method as previously described and characterized (Figure S2, Supporting Information).[20,23,35] Free melittin peptide (26 amino acids, CGIGAVLKVLTGTPALISW1KRRQQ) and IR-780 iodide were purchased from Sigma-Aldrich (Shanghai, China). The legM peptide (Sequence: CIGAVLKVLTGLPSW1KRRQQ) was provide by GL Biochem (Shanghai) Ltd (Shanghai, China). DMPE-PEG5000Maleimide was provided by Xi’an ruixi Biological Technology Co. Ltd. DMPE-PEG-legM was synthesized by conjugating legM to DMPE-PEG-Maleimide (Figure S1, Supporting Information). Nile red was provided by J&K scientific Co. Ltd (Shanghai, China).

The metastatic 4T1 breast cancer cells were supported by Shanghai Cell Bank, Chinese Academy of Sciences (CAS). Cells were incubated in RPMI 1640 culture media supplemented with 10% FBS (Gibco), 100 U mL$^{-1}$ penicillin G sodium and 100 µg mL$^{-1}$ streptomycin sulfate (Invitrogen, USA). The cell culture was performed at 37 °C and 5% CO$_2$ in a humidified incubator.

Female nude mice (18-22g, BALB/c) were purchased from Shanghai Experimental Animal Center, CAS (Shanghai, China). The animals were kept in the animal care facility for 3 d, and then used for in vivo evaluations. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Institute of Materia Medica, CAS. The orthotopic metastatic breast cancer model was induced by injecting 4T1 cells to the mammary pad of nude mice at 5 × 10^5 cells per mouse. For developing the lung metastatic breast cancer model, 4T1 cells were given to these animals at 2 × 10^6 cells per mouse by tail vein injection.

**Preparation and Characterization of TCM-legM:** TCM-legM was composed of cabazitaxel, DMPE-PEG-legM, PEG-PDPA, and DSPE-PEG (1:4:8:2, weight ratio), and fabricated by a self-assembly technique. In brief, these ingredients were dissolved in methanol and evaporated into dryness under reduced pressure to form thin film in a round flask. Then, the film was dispersed into double-distilled water and filtered through a filter unit (MillexGP, 0.22 µm, Millipore). By contrast, a counterpart formulation of TCM that consisted of cabazitaxel, PEG-PDPA, and DSPE-PEG (1:8:2, weight ratio) was prepared in the similar procedure. The terminal concentration of cabazitaxel in TCM-legM and TCM was about 1.0 mg mL$^{-1}$. The counterpart blank micelles of bTM-legM and bTM were prepared as control.

The particle size distribution and zeta potential of these micelles were determined using a Nano ZS 90 Zetasizer (Malvern, UK). Then, TCM-legM and TCM were negatively stained with 3% phosphotungstic acid solution (pH 5.0) for observation under FE-TEM (Tecnai G2 F20 S-Twin, FEI). The encapsulation efficiency of cabazitaxel in these micelles was quantified by high-performance liquid chromatography (HPLC) analysis. The unentrapped drug was separated from the micelles by ultracentrifugation at 15000 × g for 30 min. The drug content was determined with a HPLC system (Waters 2695-2489, USA) equipped with an Agilent ZORBAX SB-C$_{18}$ column (5 µm, 4.6 × 250 mm). The mobile phase was acetonitrile-water (70:30, v/v) with the flow rate of 1.0 mL min$^{-1}$ and detection wavelength of 230 nm. To evaluate the stability over time, TCM and TCM-legM was incubated with PBS (pH 7.4) and PBS (pH 7.4)+10% FBS for 24 h. The particle size of these samples was monitored by DLS method. Meanwhile, the encapsulation efficiency of cabazitaxel in these micelles at certain time intervals of incubation was determined by HPLC analysis.

To determine the specific legumain-responsiveness of TCM-legM, a hemolysis assay was used for the evaluation. Melittin was a membrane-lytic peptide that could disrupt the cell membrane of erythrocyte to induce obvious hemolysis.[36] Once the legM was activated into melittin, hemolysis would be readily visualized. TCM and TCM-legM was respectively mixed with erythrocyte and incubated with PBS (pH 7.4), PBS (pH 7.4)+10% FBS, and PBS + active legumain (50 ng mL$^{-1}$) at 37 °C for 5 h. By contrast, the untreated erythrocyte in PBS (pH 7.4) was denoted as negative control, and erythrocyte treated with 1% Triton X-100 was performed as positive control. Afterward, the cell suspension was centrifuged, and the supernatants were analyzed using a microplate reader (Enspire, Perkin-Elmer, Singapore). The absorbance from 1% Triton X-100 treated group was set as 100%.

To evaluate the pH-responsive capability, TCM-legM and TCM was incubated with PBS at pH 5.5. The water-insoluble cabazitaxel would be largely precipitated when it was released from the micelles system. After 30 min of incubation, samples were centrifuged at 5000 × g for 5 min, and the supernatant was measured under FE-TEM to detect the existence of typical micelles structure. Then, to evaluate the pH-responsive drug release profiles, TCM-legM and TCM were mixed with a series of PBS at pH 7.4, 6.8, 6.3, 5.5, and 4.7, respectively, and then incubated at 37 °C for 30 min. Afterward, these samples were centrifuged at 5000 × g for 5 min and supernatant was analyzed by aforementioned HPLC method.

**Cellular Uptake:** The uptake of TCM-legM and TCM in 4T1 cells were visualized under LCM (Leica SP8 STED, Germany). TCM-legM and TCM were labeled with Nile red by physical entrapment for the detection. The sterile round glass coverslips were put into the well of a 24-well plate and cells were seeded at 1 × 10^4 cells per well. After 24 h, the fluorescent TCM or TCM-legM was added to each well and incubated for 4 h. Then, cells were stained with LysoTracker Green DND-26 (Molecular Probe, USA) and Hoechst 33342 (Blue, Beyotime) for visualization under LCM. Moreover, the cellular uptake of TCM-legM and TCM were quantified by flow cytometry analysis. Cells were seeded into 12-well culture plate at 1 × 10^6 per well and cultured overnight, and then incubated with fluorescent TCM or TCM-legM at 20 µg mL$^{-1}$ of cabazitaxel. After 4 h, cells were harvested and the mean fluorescence intensity was determined by flow cytometer analysis (FACSCalibur system, BD, USA).

**Cytotoxicity:** The cytotoxicity of TCM-legM in metastatic 4T1 cells was measured by sulforhodamine B (SRB, Sigma) method. Cells were seeded to 96-well plate at 3 × 10^3 cells per well and cultured overnight. Various formulations of cabazitaxel, TCM, TCM-legM, bTM, and bTM-legM were added at series concentrations ranging from 4 ng mL$^{-1}$ to 40 µg mL$^{-1}$ of
cabazitaxel or equivalent concentration. After 24 h of incubation, the cell viability was quantified by SRB method (Enspire, Perkin–Elmer, Singapore).

**Inhibitory Effects on Cell Migration and Invasion Activities:** The inhibition of TCM-legM on cell migration and invasion was determined by transwell mediated assays. For cell migration assay, the 4T1 cells in 200 µL of serum-free media were seeded into the top chamber of inserts (24-well, pore size, 8 µm, Costar) at 2 × 10⁵ cells per well. For cell invasion assay, 20 µL of Matrigel (BD, USA) was added to the inserts and stationed for 30 min. Then, cells in 200 µL of serum-free media were added to the inserts at 2.5 × 10⁵ cells per well. Afterward, 600 µL of culture media with 10% FBS was added to the well of 24-plate. Various formulations were added to the upper chambers of inserts and lower well of 24-well plate at 40 ng mL⁻¹ of cabazitaxel or equivalent concentration. The untreated cells were performed as negative control. After 24 h, the migrated or invaded cells across the membrane were stained with crystal violet, imaged and counted under a microscope (IX81, Olympus, Japan) to assess the inhibition of cell migration and invasion.

**In Vivo Specific Targeting of TCM-legM to Primary Breast Cancer:** The specific targeting of TCM-legM to the primary tumor was measured in an orthotopic breast cancer model. For the in vivo detection, TCM and TCM-legM were labeled with hydrophobic IR-780 via tail vein. After 4.0 h, the tumor tissues from each group were removed, frozen in cryoembedding media and sectioned. The sections were stained with DAPI (blue, Sigma) and phalloidin-FITC (green, Beyotime) for observation under LCSM. The metastatic lesions were depicted as white circles.

**In Vivo Therapeutic Effects of TCM-legM on Breast Cancer Metastasis:** The effectiveness of TCM-legM on breast cancer metastasis was measured in an orthotopic metastatic breast cancer model. When the tumor volumes reached 100–150 mm³, the tumor bearing mice were randomly divided into six groups, and respectively treated with saline control, blank micelles of bTM, bTM-legM, cabazitaxel solution, TCM, and TCM-legM at 5.0 mg kg⁻¹ of cabazitaxel or equivalent dose. The treatment was performed by tail vein injection every 3 d. Cabazitaxel was dissolved in the mixed solution of HS-15/dimethyloctamide (1:1, v/v) (10 mg mL⁻¹) and diluted with water to form the cabazitaxel solution (1.0 mg mL⁻¹). The body weight and tumor size were monitored every 3 d after the first treatment. At the end, the tumor tissues from each group were carefully collected, weighed, and photographed. The inhibition of tumor growth was expressed as the relative tumor weight from each group compared to the saline control. Meanwhile, the lung tissues were carefully removed and the visually detected metastatic nodules in lung were counted to calculate the inhibition of lung metastasis. Afterward, the lungs were performed histological examinations by H&E staining. Moreover, the apoptosis in primary tumor and metastatic lungs were examined using the TUNEL assay kit (Roche, USA). As control, the nuclei were stained with DAPI for visualization under inverted fluorescence microscope (Nikon Eclipse Ti-SR, Japan). The apoptosis was depicted as green fluorescence signals.

**Statistical Analysis:** Data were presented as mean ± standard deviation. For a two-group comparison, statistical analysis was performed using a two-tailed Student's t test. The difference could be significant when the p value was less than 0.05.

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

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**Conflict of Interest**
The authors declare no conflict of interest.

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cabazitaxel, cancer metastasis, micelles, programed targeting, tumor microenvironment

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