Pulmonary Targeting Crosslinked Cyclodextrin Metal–Organic Frameworks for Lung Cancer Therapy

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Lung cancer is a serious threat to human health with the highest morbidity and mortality; metastatic lung cancer accounts for a majority of cancer-related deaths. Hence, there is considerable interest in developing efficient lung-targeted drug delivery systems to improve overall survival and quality of life of lung cancer patients. Based on the lung-targeting characteristics of cubic crosslinked cyclodextrin metal–organic framework (CDF) nanoparticles, this study shows the synthesis of a nanoplateform using RGD-functionalized CDF to co-deliver low-molecular-weight heparin (LMWH) and doxorubicin (DOX) for treatment of lung cancer. Rational design of the DOX-loaded RGD-CDF-LMWH nanoplatform (RCLD) is carried out. RCLD nanoparticles are efficiently targeted to lung tumors following intravenous administration; RCLD accumulation in the lung is 5.8 times greater than that in the liver. Moreover, RCLD inhibits migration and invasion of cancer cells in vitro and significantly diminishes lung tumor nodule count and area of spread in human A549 and murine B16F10 lung cancer models in vivo. Furthermore, the multiple antitumor activities of this novel RCLD nanoplatform, alongside its safety profile for normal tissues, strongly support its use for targeted treatment of lung cancer.

1. Introduction

Lung cancer is a major neoplastic disease showing the highest morbidity and mortality in the world,[1] and only has a poor five-year survival rate.[2] In recent years, major advances have been made in cancer therapy, partly due to the development of inhibitors of oncogenic drivers by bespoke molecular targeted agents. However, chemotherapy remains the mainstay of treatment for most cancers, especially in advanced stages of tumor progression. This partly depends on the general effectiveness of chemotherapy on most metastatic cancer deposits, since tumor metastasis is the main factor leading to treatment failure and death in cancer patients.[3] An important factor for the development of lung-targeting treatments is the prevalence of metastatic lung cancer in the progression of many cancer types. Indeed, metastatic lung lesions are reported to be associated with 20–54% of malignant tumor cases[4] and metastatic disease accounts for more than 90% of all cancer-related deaths.[3b,5] Lung metastasis treatment is an increasingly important clinical issue,[6] as traditional treatment methods are difficult to deliver to the lungs.[7] Therefore, improving the tumor selectivity of chemotherapy to enhance efficacy whilst minimizing systemic toxicity has long been a goal in drug targeting. In this regard, pulmonary targeted drug delivery systems that can concentrate drugs in the lungs, improve the efficacy

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of anticancer drugs, and reduce the occurrence of adverse reactions, have become the research focus for lung cancer treatment.[8]

Existing pulmonary targeting strategies include microspheres,[9] liposomes,[10] polymer nanoparticles,[11] polymer micelles,[12] solid lipid nanoparticles,[13] magnetic nanoparticles,[14] and metal nanoparticles.[15] Microparticles and liposomes with a particle size of 5–15 μm can achieve lung targeting through mechanical interception of the alveolar capillary barrier, but microparticle carriers can also block pulmonary capillaries, thereby causing chronic obstructive emphysema or pulmonary embolism.[16] Furthermore, endocytic particulate uptake and retention by pulmonary macrophages can result in the clearance of nanoparticles from lung tissues, thus reducing their tumor-targeting efficiency.[17] Also, the application of nanosized liposomes and polymer micelles is limited by their low drug loading, and high drug leakage and toxicity.[18] Therefore, there is an urgent need to develop safe and efficient lung-targeted drug delivery systems.

Suitable size, shape, and surface potential of carriers are useful for realizing pulmonary targeting.[19] For example, previous studies have reported that particles slightly larger than the capillary diameter are more easily entrapped in alveolar capillaries, thereby efficiently reaching lung targets.[20] Moreover, the zeta potential of nanocarriers affects their pharmacokinetic processes and biodistribution in the body. For instance, surface modification of nanoplatforms, especially in surface charge, is a common way to avoid opsonization.[21] The shape of carriers is also a key factor that influences the biological processes and functions of drug delivery systems. To date, most nanoplatforms for drug delivery are spherical, while the development of anisotropic particles for biomedicine has become popular only in recent years. For example, Zhang et al. reported that the shape of electrospun fiber rods had a great impact on their systemic circulation and biodistribution. In fact, compared with microspheres, fiber rods allowed an up to fourfold higher accumulation of doxorubicin (DOX) in tumors and therefore contributed to more efficient treatment of tumors and metastasis in the lungs.[22] In addition, a previous study demonstrated that cuboidal nanoparticles made contact with cancer cells more than rod-like nanoparticles.[23] Although few studies have dealt with the recognition of nanoplatforms with cuboidal morphology by endothelial cells and macrophages in vivo, emerging evidence has clearly proved that non-spherical particles possess diverse advantageous biological characteristics, such as an improved blood circulation time and organ distribution, and a more efficient ability to evade clearance by phagocytosis.[24]

Metal–organic frameworks (MOFs) have gained increased attention owing to their tunable structures and chemical versatility. MOF-based cancer theranostic platforms have been described in recent years.[25] In particular, cyclodextrin (CD)-MOFs are green and renewable framework materials[26] with significant potential for use in drug delivery and biomedicine owing to their high biocompatibility, porosity, and diverse functionalities.[16b,27] For instance, we and others have previously shown that CD-MOF crystals can improve the stability, solubility, and bioavailability of sucralose[28] and some drugs.[29] Specifically, the hydroxyl groups present in the CD torus can serve as reactive functional groups for the modification of CD-MOFs and broaden their applications. For example, researchers have tried to crosslink the hydroxy groups in γ-CD of CD-MOFs to prepare cubic organic cages. For example, CD-MOF crosslinking by glycidyl ethers allowed to retain the shape and size of the MOF crystals.[30] Crosslinked CD-MOFs could also be obtained via boronate esterification of the hydroxy groups of γ-CD within CD-MOFs.[31] In another case, the γ-CDs of CD-MOF crystals were reacted with diphenyl carbonate to obtain the species of crosslinked CD-MOF by our team.[32] Following this approach, crosslinked CD-MOFs displaying glucose (GSH)-triggered drug release were prepared using a biodegradable disulfide bond-bearing linker.[33] Furthermore, in order to enhance the water stability of CD-MOFs, we grafted cholesterol over the surface of CD-MOFs to form a protective hydrophobic layer. Notably, cholesterol-shielded CD-MOFs significantly increased cellular penetration capability and blood circulation half-life of DOX.[34] Recently, we functionalized cubic crosslinked γ-CD-MOFs with GRGDS pentapeptides to construct efficient artificial platelets for bleeding control.[35] Our results proved that the unique cuboidal shape of CD-MOFs plays an important role in achieving hemostasis and in vivo targeting of artificial platelets. Porous γ-CD-MOFs were also utilized as templates to synthesize ultrathin silver nanoparticles (Ag NPs) by our group. After further modification with GRGDS, Ag NP-loaded CD-MOFs exhibited an efficient and synergistic hemostatic and antibacterial effect.[36] In summary, it is clear that the use of CD-MOFs can be extended to more advanced applications via functionalization. Therefore, cuboidal γ-CD-MOFs possess the potential to overcome the limitations of currently available nanoparticles for lung cancer treatment. In fact, very recently we confirmed that the crosslinked γ-CD-MOF (referred to as CDF) nanoparticles possess a strong lung-targeting ability, as they were mainly distributed in lung tissues after intravenous injection (Figure S1, Supporting Information). In conclusion, CDF offers a new carrier for the development of lung cancer targeted drug delivery systems because of its lung-targeting characteristics.

It is generally accepted that integrins play a significant role in tumor cell invasion and metastasis. Indeed, tumor cell expression of αvβ3, αvβ5, α5β1, and α6β4 correlates with metastatic progression in melanoma, breast carcinoma, and prostate, pancreatic, and lung cancer.[37] In particular, the expression of the αvβ3 integrin on tumor cells, together with its binding to fibronectin, vitronectin, and osteopontin in the extracellular matrix, is considered critical in determining the sites of metastasis. In fact, fibrinogen bridges between αvβ3 integrins on tumor cells and αIIbβ3 integrins on platelets promote tumor cell arrest in the vasculature and metastasis to various tissues, including bone marrow and lung. Furthermore, a relationship between integrins and the coagulation factor thrombin has been described in several types of tumor cells, including melanoma and lung cancer cells.[38] The integrins that are commonly found on tumor cells and associated vasculature, namely αvβ3, αvβ5, and αIIbβ3, recognize the RGD motif within extracellular matrix proteins. For this reason, the inclusion of short peptides that incorporate the RGD sequence has been widely adopted in the development of targeted delivery devices.[39] For instance, Huang et al. modified paclitaxel nanocrystals with RGD peptide for tumor targeting, suggesting the superiority.
of RGD-modified PEGylated paclitaxel nanocrystals as a lung cancer-targeted delivery system. Moreover, RGD-modified paclitaxel- and cisplatin-loaded lipid-polymer nanoparticles showed good antitumor efficiency in vivo, as they inhibited the tumor size of mice from 1486 to 263 mm$^3$. Another report proved that RGD-conjugated, DOX, and mitomycin C co-loaded polymer-lipid nanoparticles markedly inhibited the progression of lung metastasis. Zou et al. developed cyclic RGD peptide-directed and disulfide-crosslinked polymersomal doxorubicin (cRGD-PS-DOX) as a targeted chemotherapeutic agent for human non-small cell lung cancer. Notably, cRGD-PS-DOX effectively suppressed the growth of A549 lung tumors in both subcutaneous and orthotopic models at a DOX dose of 12 mg kg$^{-1}$. In the present study, in order to improve the targeted chemotherapy of lung cancer, we prepared a novel lung cancer targeting nanoplatform using GRGDS functionalized CDF to facilitate targeted tumor delivery of low-molecular-weight heparin (LMWH) and DOX (Figure 1). Rational design of the RGD-CDF-LMWH-DOX (RCLD) complex considered that 1) RGD displays a relatively high specific affinity for integrins expressed on lung tumor cells and tumor neovasculature, 2) CDF strongly targets lung tissues, 3) LMWH inhibits the adhesion between platelets and tumor cells, and numerous studies have demonstrated the favorable antitumor and anti-metastatic effects of LMWH in combination with chemotherapy, and 4) DOX is an established chemotherapeutic agent for the treatment of advanced cancers, especially lung metastases. We predicted that RCLD nanoparticles would target and concentrate in the tumor site following intravenous injection, exerting inhibition on growth, and metastasis of lung cancer.

2. Results and Discussion

2.1. Synthesis and Characterization of RGD-CDF-LMWH Nanoparticles

Nanoscale monodispersed cubic γCD-MOFs were prepared using GRGDS functionalized CDF to facilitate targeted tumor delivery of low-molecular-weight heparin (LMWH) and DOX (Figure 1). Rational design of the RGD-CDF-LMWH-DOX (RCLD) complex considered that 1) RGD displays a relatively high specific affinity for integrins expressed on lung tumor cells and tumor neovasculature, 2) CDF strongly targets lung tissues, 3) LMWH inhibits the adhesion between platelets and tumor cells, and numerous studies have demonstrated the favorable antitumor and anti-metastatic effects of LMWH in combination with chemotherapy, and 4) DOX is an established chemotherapeutic agent for the treatment of advanced cancers, especially lung metastases. We predicted that RCLD nanoparticles would target and concentrate in the tumor site following intravenous injection, exerting inhibition on growth, and metastasis of lung cancer.
by toluidine blue assay and spectrophotometry (Figure S4, Supporting Information). Moreover, SEM results revealed that RGD-CDF-LMWH nanoparticles had a cubic morphology (Figure 2B). Finally, dynamic light scattering (DLS) analysis showed that the mean size of RGD-CDF-LMWH nanoparticles was approximately 150 nm and their zeta potential was approximately $-25$ mV (Figure S5 and Table S1, Supporting Information). To investigate their biocompatibility, the
cytotoxicity of CDF-LMWH and RGD-CDF-LMWH nanoparticles against B16F10 melanoma cells, A549 lung cancer cells, and WI26-VA4 normal alveolar epithelial cells was determined using a Cell Counting Kit-8 (CCK-8) cell viability assay. The results showed that CDF-LMWH and RGD-CDF-LMWH nanoparticles at a concentration of up to 133 µg mL⁻¹ exerted no significant effects on the viability of B16F10, A549, and WI26-VA4 cells (Figure 2C, Figure S6 and S7, Supporting Information). The effects of RGD-CDF-LMWH nanoparticles on red blood cells were also evaluated. Similarly, RGD-CDF-LMWH or CDF-LMWH up to a concentration of 600 µg mL⁻¹ had no measurable influence on the integrity of red blood cell membranes or on hemolysis (Figure 2D,E). Indeed, the hemolysis ratio of RGD-CDF-LMWH was less than 1% (Figure 2F), and an intravenous injectable formulation is generally considered safe when triggering a hemolysis ratio of less than 5%.

2.2. Cytotoxicity of DOX Loaded RGD-CDF-LMWH

DOX loaded CDF-LMWH (CLD) and RGD-CDF-LMWH (RCLD) nanoparticles were prepared by the impregnation method. The DOX-loading capacity of these nanoplatforms was determined to be 14 ± 1% by quantification of the characteristic fluorescence emission of this anthracycline (Figure S9, Supporting Information). Next, the DOX release behavior of RCLD was investigated in phosphate-buffered saline (PBS) supplemented with different concentrations of GSH. Interestingly, RCLD released only a small portion of DOX over an incubation of 72 h in PBS at pH 7.4, while releasing about 50% of DOX in presence of 1 mM GSH. Further, the cumulative release of DOX was increased to 80% through incubation with 10 mM GSH (Figure 3A). The results suggested that DOX was released from RCLD in a GSH-sensitive manner. In addition, both CLD and RCLD were investigated for their inhibitory effects on the migration and invasion of B16F10 melanoma cells. A representative experiment showed that RCLD released DOX at a higher rate compared to CLD (Figure 3B, C, E, F). The results indicated that RCLD exhibited a higher inhibitory effect on migration and invasion compared to CLD (Figure 3B, C, E, F).

Figure 3. DOX release behavior and inhibitory effects of RCLD on migration and invasion of B16F10 melanoma cells. A) Cumulative release of DOX from RCLD in PBS or upon treatment with 1 mM and 10 mM GSH at different time points (n = 3). B) Cell viability of RCLD, CLD, and free DOX treated B16F10 melanoma cells in vitro (n = 6). C, E) Images and wound healing rates of B16F10 melanoma cells after incubation for 24 h with PBS, LMWH, DOX, CLD, or RCLD (n = 3). D), F) Images and invasion rates of B16F10 melanoma cells after incubation for 48 h with PBS, LMWH, DOX, CLD, or RCLD (n = 3). **p < 0.01, ***p < 0.001.
and RCLD nanoparticles exerted a cytotoxic effect on B16F10 cells (Figure 3B). In cell culture, the conjugation of RGD peptide onto CDF does not appear to influence tumor cell targeting of CDF in vitro, although RGD-binding integrins such as αvβ3 and α2b are expressed on melanoma cells, facilitating their adhesion to the pulmonary microvasculature as well as promoting the development of lung metastases. However, in our experience, tumor cells grown in 2D culture can lack expression of integrins, possibly due to their redundancy in an environment with limited integrin-dependent cell–cell contact. Finally, RCLD and CLD showed similar cytotoxicity to that of free DOX up to 10 µg mL⁻¹, indicating that, at these concentrations, RCLD and CLD nanoparticles released sufficient DOX to produce a cytotoxic response (Figure 3B).

2.3. RCLD Nanoparticles Inhibit Cell Migration and Invasion In Vitro

Cell migration in the extracellular matrix is a multistep process involving dynamic changes in cytoskeleton, cell-substrate adhesion, and extracellular matrix components. Wound healing and transwell assays are commonly used to quantify the effects of inhibitors on cell migration and invasion, two indicators of metastatic potential. Therefore, we used these assays to investigate whether B16F10 cell migration was inhibited after treatment with RCLD nanoparticles, free LMWH, or free DOX. Notably, the gap-healing rate of B16F10 melanoma cells in the presence of free LMWH and free DOX was significantly lower than that of the PBS-treated control, indicating that both compounds inhibited B16F10 cell motility. In this study, CLD and RCLD nanoparticles displayed the most pronounced inhibitory effect on B16F10 cell motility (Figure 3C,E), consistent with the additive effect of LMWH and DOX. Similarly, the transwell assay showed that the number of cells that penetrated the transwell membrane was significantly decreased by free LMWH (with a relative invasion rate of 71%). Importantly, both CLD and RCLD treatments triggered a strong inhibition of cell invasion, reducing the invasion rate to 13.6% and 7.4%, respectively (Figure 3D,F), as a result of the additive effect of DOX and LMWH. In this cell-based assay, LMWH directly affected tumor cell motility. These results are consistent with previous studies showing that LMWH directly inhibits the motility of A549 lung cancer cells. Such inhibition was proposed to occur through restraint of the actin cytoskeleton reorganization via suppression of the PI3K-AKT-mTOR signaling pathway.

2.4. RCLD Nanoparticles Target Lung Tumors

Lung cancer targeting by RCLD was investigated in a mouse B16F10 metastatic lung cancer model. CLD and RCLD nanoparticles, fluorescently labeled with Cy5, were injected into mice through the tail vein, and their biodistribution was measured after 3 h using an in vivo imaging system (IVIS). The results showed that both CLD and RCLD accumulated in the lungs, indicating that CDF exhibits per se lung-targeting capability (Figure 4A,B). Importantly, the measured fluorescence intensity

![Figure 4](image-url)
of RCLD accumulated in lung tumors was almost twice that of CLD (Figure 4A,B), indicating that RCLD possessed more efficient lung cancer-targeting ability owing to the synergistic effect of the CDF carrier and the RGD peptide. Furthermore, targeted accumulation of RCLD in lung tissues was 5.8 times greater than in the liver, as measured by relative fluorescence intensity (Figure 4C,D).

The RCLD drug nanocarriers were constructed from CDF nanoparticles as described in Figure 1. The fate of administered CDF nanoparticles showed they were distributed mainly in the lungs after intravenous injection. Specifically, the fluorescence intensity for Cy5-labelled CDF in mouse lungs was 31.7 times greater than that in the liver (Figure S1, Supporting Information). The relative distribution of systemically administered particles in lung compared to the liver is a measure of the lung-targeting coefficient, which is usually less than 10.[51] For example, the highest uptake of discoidal polymeric particles in the lungs was 8.7-fold higher than that in the liver.[51a] Moreover, the lung-targeting efficiency of tilmicosin microspheres was only 4.6.[51b] In comparison, the highest lung-targeting coefficients of cuboidal CDFs in mice, rats, and rabbits were as high as 31.7, 10.1, and 129.0, respectively (Figure S1, Supporting Information). For our Cy5-labelled CDF nanoparticles, 65% of the injected nanoparticles were concentrated in lung tissues 2 h after tail vein injection in mice. Previously, a type of nano MOF was reported to target lung tissues based on its pH-responsiveness and reversible aggregation behavior.[52] The report describes that after intravenous administration into neutral-pH blood, MIL MOF nanoparticles formed micro-sized particles that were retained within lung capillaries. Subsequently, owing to its charged species and protein adsorption on the surface, the aggregated MOFs progressively disaggregated to restitute the initial MOF nanoparticles.[52] The CDF carrier was prepared by crosslinking nanoscale CD-MOF, and our preliminary research found that the hydrodynamic diameter and zeta potential of our CDF carrier were pH dependent, with acidic pH increasing particle diffusion and negative surface charge (Figure S11, Supporting Information). Conversely, neutral and alkaline pH increased the hydrodynamic diameter and reduced the zeta potential of the carrier, leading to particle aggregation. Therefore, CDF nanoparticles may undergo rapid aggregation/disaggregation in serum to achieve lung targeting. In addition, the porous supramolecular structure of CDF can also interact with biological macromolecules, including surface formation of protein corona.[53] In addition, the presence of CDF surface-bound RGD and LMWH could facilitate the capture of CDF by lung tumor cells through RGD integrin receptor interactions and elements of the LMWH-thrombin-coagulant cascade, respectively. Furthermore, CDF possesses a unique cubic shape, a morphology that can be specifically recognized by the pulmonary endothelium and macrophages.

2.5. RCLD Exerts Significant Antitumor Activity Against Lung Cancer

The therapeutic effects of RCLD on lung cancer in vivo were investigated using an A549 human lung cancer model. All treatments were dosed at three-day intervals on five occasions from day 7 after intravenous injection of A549 cells (Figure 5B). Lung cancer progressed rapidly in PBS-treated mice (Figure 5A). On the other hand, LMWH alone produced an anti-metastatic effect consistent with the known effects of this anticoagulant.[54] DOX (at 2.5 mg kg\(^{-1}\)) facilitated the same anti-metastatic effect at a fivefold lower dose of DOX than DOX (2.5 mg kg\(^{-1}\)) alone. While both treated groups displayed a similar reduction in the number of metastatic nodules (Figure 5A,C), hematoxylin and eosin (H&E) staining showed that the mean size of metastatic nodules was markedly smaller in the RCLD 0.5 treated group than in the DOX-treated group (Figure 5D). Further, treatment with CLD 1 and RCLD 1 (DOX at 1.0 mg kg\(^{-1}\)) produced a significant increase in antitumor activity compared to either DOX (at 2.5 mg kg\(^{-1}\)) or LMWH alone (Figure 5C), with almost undetectable metastatic nodules in RCLD 1 treated mice (Figure 5A). Systemic toxicity, as defined by mouse body weight loss, was observed with DOX (at 2.5 mg kg\(^{-1}\)) administration but not RCLD or CLD (Figure 5E).

Moreover, to explore the effect of RCLD on a lung tumor originating from a cancer distant to the thoracic cavity, a B16F10 mouse melanoma pulmonary metastatic model was used. All treatments were dosed at three-day intervals on five occasions from day 3 after inoculation of B16F10 melanoma cells (Figure 6B). As shown in Figure 6A, the dark melanin pigment of B16F10 melanoma cells could be observed abundantly in the excised lungs of PBS-treated mice. Similar to the A549 lung cancer model (Figure 5), whilst both LMWH alone and DOX alone produced an anti-metastatic effect (Figure 6C), CLD 1 and RCLD 1 (DOX at 1.0 mg kg\(^{-1}\)) treatment resulted in the most effective inhibition of pulmonary metastasis via co-delivery of therapeutic DOX and LMWH, with almost invisible metastatic nodules in the RCLD 1 group (Figure 6A,D).

2.6. Safety Evaluation of RCLD as Lung-Targeting Nanoparticles

Our in vivo data further demonstrated the significant effects of RCLD as a lung-targeted nanoplasform with potent antitumor activity. However, it was necessary to evaluate its safety profile. In this respect, none of the mice showed signs of pulmonary distress after administration of RCLD. We investigated the effect of RCLD on major organs following anti-cancer treatment of lung cancer-bearing mice. After sacrifice, mouse heart, liver, spleen, and kidney were harvested, sliced, and stained with H&E for histopathologic analysis. Mice treated with DOX alone showed histopathologic changes of the cardiac tissue (Figure 7A). Moreover, serum biochemical analysis showed that DOX significantly increased the levels of alanine aminotransferase (ALT), aspartate transaminase (AST), and creatine kinase (CK), indicative of liver and heart toxicity (Figure 7B). Both liver and especially heart damage are consequences of DOX chemotherapy in the clinic.[56,55]
Figure 5. Antitumor activity of RCLD nanoparticles against A549 lung cancer. A) Photos of lungs excised from mice bearing A549 lung cancer after the indicated treatments. B) Schematic of treatment dosing regimens. C) Number of lung surface metastatic nodules in A549 lung cancer model mice \((n = 5)\). D) H&E histology staining of A549 lung tumors following treatments. Scale bar, 200 \(\mu\)m. E) Effect of treatments on mice body weight. \(*p < 0.05, ***p < 0.001. The abbreviation “ns” denotes no significant difference between relevant treatment groups.

Figure 6. Anticancer activity of RCLD nanoparticles against B16F10 melanoma lung metastases. A) Photos of lungs excised from mice bearing B16F10 lung cancer after the indicated treatments. B) Schematic of treatment dosing regimens. C) B16F10 lung metastasis surface area \((n = 5)\). D) H&E histology staining of B16F10 lung tumors following treatments. Scale bar, 200 \(\mu\)m. \(*p < 0.01, ***p < 0.001. The abbreviation “ns” denotes no significant difference between relevant treatment groups.
In contrast, neither RCLD nor CLD treated mice showed any indicators of normal tissue damage or adverse hematologic effects (Figure 7A, B, and Figure S13, Supporting Information). Therefore, this preliminary safety investigation confirmed that RLCD nanoparticles are biocompatible and expected to be translated into clinical applications.
3. Conclusion

In conclusion, our results support the use of RCLD as a systemically acceptable nanoparticle for the pulmonary targeted treatment of lung cancer. This study found that biocompatible CDF nanoparticles possess a lung-targeting ability. In addition, CDF surface functionalization with RGD and LMWH not only conferred antitumor activities but also rationally supported the recognition of cellular elements in the tumor microenvironment. The obtained RGD-CDF-LMWH nanoparticles not only target to lung tissues by leveraging the lung-targeting property of the CDF carrier but also specifically targeted lung cancer cells through the RGD motif. Via DOX loading, RGD-CDF-LMWH was converted to a multifunctional antitumor nanoparticle, RCLD. The designed RCLD nanoplatform could efficiently deliver antitumor drugs to lung cancer sites. In particular, RCLD inhibited the migration and invasion of cancer cells in vitro and significantly diminished the lung tumor nodules in A549 and B16F10 tumor models in vivo. Importantly, RCLD nanoparticles allowed to achieve similar effects with a five-times lower dose of DOX, thereby reducing the cardiac and hepatic toxicity of DOX. The anti-tumor activity of RCLD was rationalized to involve a contribution from CDF-conjugated RGD and LMWH together with DOX loading. Finally, RCLD nanoparticles displayed good biological safety in vivo. Therefore, the designed RCLD nanoparticles could serve as a promising nanoplatform for the targeted treatment of lung cancer. It is likely that CDF carriers can efficiently target lung tissues directly through a pH-responsive aggregation/dissaggregation behavior and supramolecular recognition. The ability of CDF to carry out targeted delivery of DOX, whilst protecting normal tissues from toxicity, is a powerful feature of this nanoplatform, which will continue to explore as a potential clinical approach to lung cancer treatment.

4. Experimental Section

Materials: γCD was purchased from MaxDragon Biochem Ltd. (Guangzhou, Guangdong, China). Low-molecular-weight heparin (LMWH, Enoxaparin sodium) was kindly gifted by Changzhou Qianhong Bio-pharma Co., Ltd. (Changzhou, Jiangsu, China). The GRGDS peptide was prepared by China Peptides Co., Ltd. (Shanghai, China). DOX, N-hydroxysuccinimide (NHS), N,N′-disuccinimidyl carbonate (DSC), 4-dimethylaminopyridine (DMAP), triethylamine, N,N-dimethylformamide (DMF), acetonitrile, and formamide were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was purchased from Tokyo Chemical Industry Co., Ltd. (Shanghai, China). CYS was purchased from Aladdin Reagent Database Inc. (Shanghai, China). Bovine serum albumin (BSA) was purchased from Beijing Zhongshuo Xianxin Biological Technology Co., Ltd. (Beijing, China). Low-molecular-weight heparin (LMWH-CYS) was synthesized by coupling the carboxy group of LMWH (MW 4500 Da, 0.71 mmol) with the primary amino group of CYS (24.8 mmol) in sodium carbonate buffer (pH 11). The resulting solution was incubated for 15 min to activate the carboxyl group of LMWH. Next, 5.6 g of CYS (24.8 mmol) was added and the reaction mixture was incubated for 48 h at room temperature under stirring. The resulting solution was first dialyzed with 0.1 M NaCl for 12 h and then dialyzed with distilled water for 48 h to remove unreacted CYS, EDC, and NHS agents. Finally, the liquid obtained was filtered through a 0.22 µm filter membrane and lyophilized.

Synthesis of CDF-LMWH and RGD-CDF-LMWH Nanoparticles: The hydroxy groups of CDF and RGD-CDF were first activated for further reaction. Then, 34.13 mg (0.133 mol) of DSC was dissolved in 3 mL of acetonitrile by sonication, 28 µL (0.2 mol) of triethylamine was added, and finally 96.33 mg (0.067 mol) of CDF or RGD-CDF nanoparticles were added. The reaction mixture was incubated for 12 h at room temperature under stirring in the dark.

Preparation of DOX Loaded CDF-LMWH and RGD-CDF-LMWH Nanoparticles: First, 273 mg of DOX was dissolved in 3 mL of purified water; then, 68 mg of CDF-LMWH or RGD-CDF-LMWH nanoparticles were added. The mixture was incubated in the dark for 24 h at 37 °C. Following this reaction, the products were washed with water to remove free DOX. The DOX loaded CDF-LMWH and RGD-CDF-LMWH (abbreviated as CLD and RCLD, respectively) were obtained after lyophilization. The content of DOX loaded into RGD-CDF-LMWH was measured using a fluorospectrophotometer (Hitachi, F-4600, Techcomp) at excitation wavelength (Ex) = 471 nm and emission wavelength (Em) = 598 nm.

Drug Release In Vitro: RCLD (5 mL, at an equivalent dose of DOX of 750 µg) was dispersed into dialysis bags (with a 14-kDa molecular weight cutoff). Each dialysis bag was immersed in PBS (pH 7.4, 100 mL) with different GSH concentrations (0, 1, and 10 mM) and incubated in a shaking bed at 75 rpm and 37 °C. Aliquots (5 mL) of the release media were collected at predetermined time points (0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, and 72 h), while the same volume of medium was replenished. The amount of released DOX was determined using a fluorospectrophotometer at Ex = 471 nm and Em = 598 nm.

Cell Viability Assay: Cell viability was evaluated in B16F10 cells using the CCK-8 assay. B16F10 cells were grown in Dulbecco’s modified Eagle medium (DMEM, with phenol red and L-glutamine) supplemented with 10% fetal bovine serum (FBS). Cells were seeded onto 96-well plates at a density of 5000 cells well⁻¹ and maintained in a humidified incubator with 95% air and 5% CO₂ at 37 °C. After incubation overnight, a series of nanoparticle solutions (100 µL) were added to the medium and incubated for 12 h. Next, CCK-8 solution (20 µL) was added to each well and incubated for 4 h, and the absorbance was measured at 450 nm (reference wavelength of 630 nm) using a microplate reader (Multiskan GO, Thermo Fisher Scientific). Six replicate wells were used for the control and test concentrations. Non-treated cells were used as a blank control, and cell viability (%) was calculated using Equation (1).

$$\text{Cell viability} (%) = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100 \quad (1)$$

Effect of RCLD Nanoparticle Components on Erythrocyte Integrity: The extent of red blood cell hemolysis in presence of RGD-CDF-LMWH nanoparticles was examined by spectrophotometry. Freshly collected citrated blood from C57BL/6 mice was centrifuged at 2500 rpm for

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10 min to remove the serum. Then, red blood cells were isolated after washing three times with normal saline solution and diluted to 2% (v/v) in normal saline. CDF-LMW and RGD-CDF-LMW nanoparticles were dispersed in saline solution at concentrations ranging from 20 to 600 µg mL⁻¹. Saline solution and ultrapure water were used as negative and positive controls, respectively. Then, 1.5 mL of the diluted suspension of mouse red blood cells was mixed with 1.5 mL of the above samples. The mixtures were incubated at 37 °C for 2 h and centrifuged for 10 min at 3000 rpm. The absorbance (540 nm) of the resulting supernatant was measured using a spectrophotometer (UH4300, Hitachi, Tokyo, Japan). All hemolysis experiments were performed in triplicates. The hemolysis ratio (%) was calculated using Equation (2):

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\text{Hemolysis ratio (\%)} = \frac{A_{\text{sample}} - A_{\text{negative control}}}{A_{\text{positive control}} - A_{\text{negative control}}} \times 100
\] (2)

Compounds inducing a hemolysis ratio less than 5% are regarded as nontoxic. The morphology of precipitated erythrocytes was also observed under light microscopy to further check the effect of treatments on erythrocyte integrity.

In vitro Cell migration and Invasion assays: The effects of drugs and nanoparticles on B16F10 cell migration and invasion were investigated using wound healing and transwell invasion assays, respectively. For the wound healing assay, B16F10 cells were seeded into six-well plates. When monolayer cells had grown to near confluence (90%), a scratch (cell-free) wound was created with a 200-µL sterile pipette tip and the cells were washed once with PBS. Subsequently, the cells were treated with PBS, free DOX (0.5 µg mL⁻¹), free LMWH (20 µg mL⁻¹), CLD, or RCLD (at an equivalent DOX concentration of 0.5 µg mL⁻¹), and incubated for 24 h. Images were obtained at 0 h and 24 h using an inverted optical microscope (10 ×, Leica DMi1, Wetzlar, Germany), and the distance between the gaps was measured. The wound healing rate was calculated according to Equation (3):

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\text{Wound healing rate (\%)} = \frac{\text{Distance}_{\text{at 24h}}}{\text{Distance}_{\text{at 0h}}} \times 100
\] (3)

For the transwell invasion assay, 1 × 10⁵ B16F10 cells were suspended in 0.5% serum medium and plated in the top chamber of a Transwell insert (24-well insert, pore size, 8 µm, Corning, New York, USA) precoated with 60 µL of Matrigel. In this assay, 100 µL of serum-free medium diluted with PBS, free DOX (0.5 µg mL⁻¹), free LMWH (20 µg mL⁻¹), CLD, or RCLD (at an equivalent DOX concentration of 0.5 µg mL⁻¹) was added to the upper chamber, while the lower chambers were filled with 600 µL of culture medium containing 20% FBS as a chemoattractant. After 48 h of incubation, the remaining cells in the upper chambers were removed with a cotton swab, and cells that had migrated to the lower chamber were fixed in 4% paraformaldehyde solution for 10 min and stained with crystal violet. Finally, cells in each lower chamber were viewed under an inverted microscope in five predetermined fields. Then, the crystal violet-stained cells were eluted with 33% acetic acid and crystal violet absorbance was measured at 570 nm using a microplate reader (Multiskan GO). The relative invasion rate was calculated using Equation (4):

\[
\text{Relative invasion rate (\%)} = \frac{A_{\text{sample}}}{A_{\text{PBS control}}} \times 100
\] (4)

Biodistribution of CLD and RCLD Nanoparticles in Normal Tissues and Lung Tumors: The CLD and RCLD nanoparticles were first labeled with Cy5 fluorescent probes via ester linkage according to our previous report. Briefly, 2.4 mg of Cy5 dye was dissolved in 5 mL of DMF, 230 mg of CLD or RCLD was added, and the reaction was stirred for 12 h at 37 °C in the dark. A metastatic lung cancer mouse model was used to study the biological distribution and lung cancer targeting of CLD and RCLD nanoparticles. In brief, a total of 2 × 10⁶ B16F10 murine melanoma cells in 100 µL of PBS were injected into C57BL/6 mice via the tail vein to establish a lung metastatic mouse model of melanoma. On day 15 following intravenous injection of B16F10 cells, the mice were injected with PBS, Cy5-labelled CLD, or Cy5-labelled RCLD nanoparticles via the tail vein. The mice were anesthetized and monitored 3 h after administration of Cy5-labelled nanoparticles by IVIS (Spectrum, PerkinElmer, Waltham, MA, USA). Then, the mice were sacrificed, and the lungs, livers, spleens, hearts, and kidneys were collected and subjected to ex vivo imaging to investigate the biodistribution of RCLD nanoparticles.

Measurement of the Effects of RCLD on Lung Cancer In Vivo: Two lung cancer mouse models were used to investigate the inhibitory effects of RCLD nanoparticles on lung tumors in vivo. First, a human lung cancer model was prepared by intravenous injection of A549 lung cancer cells (1 × 10⁵ cells). On day 7 following intravenous injection of tumor cells, the mice were randomly divided into six groups and intravenously treated with PBS, free DOX (2.5 mg kg⁻¹), free LMWH, CLD (with DOX at 1 mg kg⁻¹), or RCLD (with DOX at 0.5 or 1.0 mg kg⁻¹) every three days for a total of five times. At the same time, the weight change of each mouse was monitored daily. On day 33, the mice were sacrificed, and the lungs and major organs were collected, and lung metastatic nodules on the lung whole surface were counted. Moreover, the lungs, hearts, livers, spleens, and kidneys were H&E-stained and subjected to histopathological examination.

A B16F10 murine melanoma lung metastasis model was established in C57BL/6 mice as described above. On day 3 following intravenous injection of tumor cells, the mice were randomly divided into six groups and treated with PBS, free DOX (2.5 mg kg⁻¹), free LMWH, CLD (with DOX at 1.0 mg kg⁻¹), or RCLD (with DOX at 0.5 or 1.0 mg kg⁻¹) every three days for a total of five times. At day 16, the mice were sacrificed and the area of pulmonary metastatic nodules on the lung surface was calculated. Moreover, the lungs, hearts, livers, spleens, kidneys were H&E-stained and subjected to histopathological examination.

Measurement of the Effects of RCLD on Blood Cell Count and Serum Enzymes: To evaluate the effects of RCLD nanoparticle components on the blood cell count and the levels of serum enzymes in mice, male C57BL/6 mice were treated intravenously with PBS, DOX (2.5 mg kg⁻¹), LMWH, CLD (with DOX at 1.0 mg kg⁻¹), or RCLD (with DOX at 1.0 or 0.5 mg kg⁻¹) every three days for a total of five times. 24 h after the final treatment, whole blood was collected in EDTAK anticoagulative tubes for platelet, white and red blood cell examination (BC-2800Vet, Mindray, China), and serum was obtained for determination of ALT, AST and CK levels (Chemray 800, Rayto, China).

Statistical analysis: All data were presented as mean ± standard deviation (SD) of at least three independent experiments, and one-way ANOVA followed by Tukey’s multiple comparisons test was conducted for statistical analysis.

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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