Cancer therapeutic drugs face various transportation barriers in transit to the tumor site, making the delivery of effective drug concentrations problematic. Moreover, these drugs are very difficult to use due to their adverse off-target effects. Thus, it is very essential to develop a drug delivery system that can deliver drugs to achieve effective local concentrations without side effects on healthy tissues. Herein, the authors report a self-assembled nanodrug system in which hydrophobic antitumor drugs are packaged into nanoparticles to improve water solubility, tumor targeting ability, blood retention time, and chemotherapeutic effect. The nanodrugs are degraded into smaller ones when exposed to the tumor microenvironment, extravasated from leaky regions of the tumor vasculature, and displayed matrix metalloproteinase-2 (MMP-2)-induced degradation and antitumor property. To construct this unique system, an amphiphilic multifunctional molecule (Pep-Cy5) is synthesized by attaching a MMP-2-cleavable peptide to a hydrophobic near-infrared dye, Cy5. Two hydrophobic anticancer drugs are conjugated to Pep-Cy5 through hydrophobic interactions to form the self-assembled nanodrug system. The MMP-2-induced degradation and hydrophobic antitumor drug interchangeability features of this nanosystem enable the hydrophobic antitumor drugs to exhibit longer blood-retention times, improved intratumoral accumulation, fewer side effects, and higher anticancer efficacies compared with free drugs.
silica or mesoporous silica-modified nanoparticles. Because a major mechanism of multidrug resistance results from decreased drug accumulation in tumor cells due to the expression of one or more ATP-dependent efflux pumps (such as the MDR1 gene and its product P-glycoprotein), nanoparticles were selected as a drug delivery system to overcome drug resistance by neutralizing, evading, or exploiting various drug efflux pumps and other resistance mechanisms. Additionally, a nanoparticle that was modified with a specific tumor probe designed for molecular recognition may deliver the drug to the action site, improving the drug’s therapeutic efficacy. However, most nanodrug carriers have low drug-loading capacities and serious metabolic toxicities, which may cause damage to kidneys and other organs involved in metabolic and excretion processes.

Recently, a new approach that hydrophobic drugs are directly conjugated to hydrophilic drugs to construct a self-assembled nanodrug system was developed. For instance, Yan and his co-workers developed an anticancer nanodrug system through the self-assembly of an amphiphilic antitumor drug conjugate. The nanodrug exhibited a better plasma half-life compared with that of the free small molecule drugs. Cui and his co-workers reported a supra molecular strategy to directly assemble small hydrophobic drugs into well-defined nanostructures with a high and quantitative drug loading. However, this system demonstrated a limited drug selectivity. It required specific chemical groups to complete the conjugation process, to prevent the pharmacophore from being destroyed and to restore the drug to its original state to execute antitumor actions upon delivery to the tumor site.

We describe herein the design of a stimuli-responsive nanodrug self-delivery system, in which an amphiphilic molecule (Pep-Cy5) was synthesized by conjugating a matrix metalloproteinase (MMP)-2 enzyme-cleavable peptide to a hydrophobic near-infrared Cy5 dye. Pep-Cy5 would form a belt to assemble the nanodrug particles by “absorbing” hydrophobic antitumor molecules through hydrophobic or hydrogen bond interactions (Scheme 1). The MMP family, which consists of zinc-dependent endopeptidases predominantly secreted by tumor cells, regulates various cellular processes that are relevant in tumor biology. MMPs serve as prognostic indicators for tumor diagnosis because MMP expression and activation are dramatically increased in almost all human tumors. By taking advantage of this MMP-2 tumor-associated pathological feature, our self-assembled nanodrug system permits MMP-2-responsive co-delivery of a variety of hydrophobic antitumor drugs to the tumor site, and it enables simultaneous near-infrared fluorescent imaging of tumor tissues. The antitumor nanodrugs could be delivered to the tumor site through the enhanced permeation and retention (EPR) effect, thus reducing toxicities in normal tissues. However, the EPR effect would only provide a modest survival benefit due to the physiological barriers imposed by the abnormal tumor vasculature and the dense interstitial matrix throughout the tumor. To increase drug accumulation in tumor tissue, a tumor stimuli-responsive mechanism was incorporated into the nanodrug system’s design. To overcome each physiological barrier and facilitate intratumoral drug transport, the self-assembled nanodrugs would be degraded into smaller drugs by MMP-2 enzymes, highly expressed around the tumor tissues, by cleaving the peptide chain in the Pep-Cy5 molecule. The molecular structure of Cy5, GPLGVRGE-NH₂ peptide, and the MMP-2 induced peptide degraded process were presented in Figure S1 in the Supporting Information. All hydrophobic antitumor drugs would be adaptable to this system. Camptothecin (CP) and trans-Retinoic acid (RA) were selected as hydrophobic antitumor drugs for this study. CP is an inhibitor of nucleic acid synthesis and a strong inducer of DNA strand breaks in mammalian cells, while trans-RA modulates the transcriptional properties of the retinoic acid and retinoid X receptors. After cellular internalization of the degraded nanodrugs, all free drugs would rapidly kill the tumor cells.
2. Results and Discussion

2.1. The Preparation of Pep-Cy5 and Self-Assembled Nanodrugs

The MMP-2-cleavable GPLGVRGE-NH$_2$ peptide was synthesized using the solid phase peptide synthesis technique,[38] and its N-terminal residue was coupled to the carboxyl moiety of Cy5. The chemical characterizations of the GPLGVRGE-NH$_2$ peptide and Pep-Cy5 were carried out by high performance liquid chromatography (HPLC) (Figures S2 and S3, Supporting Information) and mass spectrum (MS) analysis (Figures S4 and S5, Supporting Information). The two peaks at 783.3 and 1249.83 for the molecular weights observed by MS (Figures S3 and S5, Supporting Information) were similar to the theoretical molecular weights for the GPLGVRGE-NH$_2$ peptide (782.9) and the Pep-Cy5 molecule (1248.5), respectively. The HPLC peak results, listed in Tables S1 and S3 in the Supporting Information, exhibited 99.10% purity for GPLGVRGE-NH$_2$ and 95.84% purity for Pep-Cy5, which confirmed a successful and highly pure synthesis of the GPLGVRGE-NH$_2$ peptide and the Pep-Cy5 molecule. The data of ultraviolet–visible spectrophotometer (UV–vis) (Figure S6, Supporting Information), fluorescence spectroscopy (Figure S7, Supporting Information) and all the results listed above illustrate that the Pep-Cy5 was successfully synthesized.

The amphiphilicity property of Pep-Cy5 enables it to assemble hydrophobic antitumor drugs to form water-soluble nanoparticles. To synthesize the self-assembled nanoparticles, a dimethylsulfoxide (DMSO) solution containing the CP and RA mixture was added drop wise into the Cy5-Pep solution, then a stable yellow–green solution was obtained after 24 h water dialysis. The concentration of the CP, RA, and Pep-Cy5 in the nanoparticles are 0.46, 0.38, and 0.116 mg mL$^{-1}$ respectively. The drug-loading efficacy is 87.8%, which is tremendously high. Dynamic light scattering (DLS) (Figure 1b) and transmission electron microscopy (TEM) (Figure 1a) were selected to determine the size and morphologies of the nanodrugs. As can be seen from the DLS curve of the final nanodrug aqueous solution, the average diameter of the nanodrugs is ≈93 nm with a narrow size distribution. The TEM image exhibits that the self-assembled nanodrugs are spherical with an average size of 76 nm. The size measured by DLS is larger than the size measured by TEM, which is due to the fact that the solvation sphere around the nanoparticles is reflected only in the DLS value. The stability of the nanodrugs was evaluated by measuring the size of the nanodrugs aqueous solution with DLS at different time point of special intervals. The results showed that the nanodrugs stability was sufficient for extended storage (Figure 1c) investigated by measuring the critical aggregation concentration (CAC) of the nanoparticles. The most convenient way to measure the CAC value of nanodrug particles is by using pyrene as fluorescent probe. The CAC value is calculated according to the $I_3/I_1$ (emission wavelengths of 384 nm)/$I_1$ (emission wavelengths of 373 nm) ratio change of pyrene and was

![Image](https://www.afm-journal.de/doi/figure/10.1002/adfm.201601062)

**Figure 1.** Characterization of self-assembled nanodrugs. a) TEM image of self-assembled nanodrugs. b) DLS curve of self-assembled nanodrugs shows the average size of the nanodrugs is 93 nm with 0.170 polydispersity index (PDI). Inset: Digital photograph of the self-assembled nanodrugs solution. c) The time-dependent stability of self-assembled nanodrugs in water. d) Relationship between the fluorescence intensity ratio ($I_3/I_1$) and CP concentration in water. The CAC value is ≈13 µg mL$^{-1}$. 

plotted in Figure 1d versus the CP concentration. The emission intensity ratio of $I_3/I_1$ is highly sensitive to the vicinal polarity of the pyrene in the surrounding medium. As shown in Figure 1d, the $I_3/I_1$ value is unchanged when the concentration of the CP is low. The ratio of $I_3/I_1$ increases dramatically when it reaches a specific CP concentration, which is the characteristic level of pyrene in hydrophobic environment. According to the inflexion of the curve, the CAC value of the nanodrugs is $\approx 13 \mu\text{g mL}^{-1}$ for CP.

The time-dependent stability of the nanodrugs was investigated using phosphate buffered saline (PBS) alone or supplemented with different fetal bovine serum (FBS) concentrations (1%, 3%, 5%, 10%). The time-dependent changes to nanodrug hydrodynamic diameters were measured by DLS, which showed strong stabilities in all solutions tested (Figure S8, Supporting Information). The release behavior of self-assembled nanodrugs was also determined by measuring the dialysis solution concentration of the nanodrugs in PBS alone, in PBS containing the different concentrations of FBS and in 100 ng mL$^{-1}$ human recombinant MMP-2 solution at pH 7.4, 37 °C. As can be seen in Figure 2, the released RA and CP is low in PBS and in PBS containing 1% FBS, 3% FBS, 5% FBS, and 10% FBS, indicating strong nanodrug stability under physiological conditions. However, in the MMP-2 environment, the Cy5-Pep belt was cleaved by the MMP-2 enzyme, which accelerated the degradation of the nanodrugs and the release of RA and CP molecules into the MMP-2 solution.

2.2. In Vitro Studies of Self-Assembled Nanodrugs

The Cy5 Near-Infrared Dye in the self-assembled nanodrugs allows them to simultaneously be used as probes for cell imaging and as tools to quantify the relative amounts of anti-tumor drugs in cells. Confocal laser scanning microscopy was utilized to provide visual evidence for the theranostic potential of self-assembled nanodrugs to distinguish the tumor cells from normal cells, which have clear differences in MMP-2 expression levels. To simulate the typical expression level of MMP-2 around tumor cells in vivo, an appropriate amount of MMP-2 over-expressing NCI-H460 cells was cultured with the self-assembled nanodrugs for 4 h, then stained the nuclei with Hoechst 33342 for 15 min before observation with Leica TCS SP8. Strong red signals were observed in both the cytoplasms and the nuclei of NCI-H460 cells, shown in the merged image of Figure 3. To confirm the peptide cleavage-responsiveness to MMP-2, confocal microscopy was performed in the presence of 1,10-phenanthroline monohydrate (PAM), an MMP-2 inhibitor. Compared with the aforementioned experimental group, the self-assembled nanodrug treatment led to a weaker red fluorescence. The quantity of nanoparticles in the cells was lower than in the cells from the experimental group, and the nanoparticles largely distributed to the cell surface or to the cytoplasm. Exposure to the MMP-2 inhibitor, which inhibits MMP-2 expression,[39] was the primary cause of this result. The low MMP-2 expression prevents the nanoparticles from being degraded into smaller particles. The larger nanoparticle size makes crossing into the cell and into the nucleus difficult. Nanoparticles must be small enough to fit through the nucleopore to enter the nucleus. The specificity of the Cy5-Pep assembled nanodrug for the MMP-2 over-expressing cells is promising toward potential diagnostic applications. Figure 3a1,c1 shows increasing red fluorescence intensities in cells overtime, which is attributed to the increasing cellular uptake of the self-assembled nanodrugs by NCI-H460 cells.

To verify the cytotoxicity of the self-assembled nanodrugs, proliferation was evaluated by measuring the reduction of methyl thiazolyl tetrazolium (MTT) in MMP-2-treated NCI-H460 and A549 cells co-treated with free CP, RA, or self-assembled nanodrugs and comparing them to MMP-2 inhibitor-treated cells that were treated with self-assembled nanodrugs. As shown in Figure 4a, the nanodrugs cytotoxicity to MMP-2 over-expression NCI-H460 cancer cells was higher than that of cells treated with MMP-2 inhibitor, probably due to the degradation of the spherical nanoparticles by MMP-2. Once the nanoparticles were degraded to small ones, the small size and surface area of nanoparticles reduce the number of binding and contact points compared with larger nanoparticles, which can increase tumor drug accumulation and affect active targeting strategies. The low cytotoxicities of free CP and RA may be explained by their hydrophobic properties, which would prevent their uptake by tumor cells. For $40 \times 10^{-6}$ M dose self-assembled nanodrugs in MMP-2 condition, the synergistic ratio (CD1) of CP and RA is 0.233, much better than 0.81 of the CP/RA mixture, suggesting better synergistic action.
The proliferation inhibition efficacy of the self-assembled nanodrugs increased linearly with the self-assembled nanodrug concentration when the concentration was higher than the CAC value. This result implies that the MMP-2 degradable nanodrugs promote their cellular uptake, and degradation of the self-assembled nanoparticles near the tumor cells induces MMP-2-dependent toxicity. A similar observation was also made using the A549 lung cancer cell line (Figure 4b).

In addition to the MTT assay, flow cytometry with Annexin V-FITC/propidium iodide double-staining was used to determine the degree of apoptosis induced by antitumor drugs. After a 24 h incubation period at 37 °C, NCI-H460 cells were incubated with CP, RA, CP/RA mixture or self-assembled nanodrugs for another 18 h. Cells incubated with self-assembled nanodrugs were treated with MMP-2 and compared with cells treated with the MMP-2 inhibitor. The untreated cells were used as the control group. After staining the cells with Alexa Fluor 488 annexin V and propidium iodide (PI), flow cytometry was performed. Maximal apoptosis and necrosis were observed in cells treated with MMP-2 and self-assembled nanodrugs. The early apoptosis and late apoptosis subpopulations were 75.3% and 8.51%, respectively. The cell apoptosis level decreased to 60.8% (44.2% and 16.6% for early and late apoptosis, respectively) in the cell group treated with PAM and self-assembled nanoparticles. These results show that in the presence of MMP-2, the self-assembled nanoparticles were degraded into smaller ones, increasing the cellular uptake of the antitumor drugs. In the presence of the MMP-2 inhibitor, the larger nanoparticle size reduced the cellular uptake and decreased the cytotoxicity of the self-assembled nanoparticles. The smaller nanoparticles were internalized into tumor cells more quickly and more efficiently than the larger nanoparticles.[40] However, the cytotoxicity of the nanoparticles in PAM-treated cells was still higher than that associated with the free CP, RA, and CP/RA mixture antitumor drugs.

Physicochemical parameters are critical for determining particle distribution and intracellular trafficking in biological environments. Nanoparticles smaller than 5 nm can be rapidly cleared by the kidney or through extravasation,[22] while spleen filtration accounts for the retention of particles that are larger than 200 nm, due to the 200–500 nm size range of the interendothelial cell spaces.[41] To escape clearance by the mononuclear phagocyte system and by hepatic filtration, the ideal nanoparticle for drug-delivery is less than 100 nm, and the nanoparticles smaller than 50 nm would passively enter the solid tumor mass through its leaky vasculature. Moreover, the tumor retention time would be extended due to impaired lymphatic flow, and nanoparticles averaging ≈100 nm generally prove long-lasting in the circulation.[42,43] To confirm these hypotheses, the pharmacokinetics of the self-assembled nanodrugs were investigated by injecting the self-assembled nanodrugs into Sprague–Dawley rats and comparing them with rats injected with CP or RA. Drug metabolism was analyzed by measuring the plasma drug concentrations in the rats. As shown in Figure 5a, the self-assembled nanodrugs were retained in the bloodstream at a higher concentration than free CP or RA 12 h after intravenous administration by tail vein injection at a dose of 7.73 mg kg⁻¹. Therefore, increase of drug concentration in tumor tissues is anticipated.
For in vivo biodistribution imaging studies, athymic nude mice were injected with 10 mg kg\(^{-1}\) of self-assembled nanodrugs through the tail vein. In vivo imaging of tumor-bearing mice, injected with self-assembled nanodrugs, was performed over a time-course from 1 to 24 h. As shown in Figure 5c, a strong signal was observed predominantly from the spleen and liver, and a weak signal was observed at the tumor site at 30 min. No significant uptake was observed in other organs. The maximal NCI-H460 tumor uptake of the self-assembled nanodrugs occurred \(\approx 1\) h after the injection, and retention was evident after 24 h, though the signal at the tumor site was weak. No signal was observed in the spleen 24 h after injection.
Importantly, the ROI analysis of the fluorescent signal at the tumor site revealed a high signal to noise ratio over the background. These data indicated that tumor imaging with Cy5-Pep-assisted self-assembled nanodrugs was successful. The self-assembled nanodrugs showed a longer retention time at the tumor site, which also confirmed the enhancement of drug accumulation in tumor tissues. The in vivo near infrared fluorescence imaging of inhibitor pretreated NCI-H460 tumor-bearing nude mice were presented in Figure S9 in the Supporting Information. At corresponding time point, the tumor fluorescence intensity is weaker than that of tumor site without inhibitor, suggesting nanodrugs were responsive to MMPs.

2.3. In Vivo Tumor Curative Effect Evaluation

The therapeutic efficacies of the self-assembled nanodrugs were investigated in vivo by comparing them with the PBS control, free CP, RA, and a CP/RA mixture of antitumor drugs. The self-assembled nanodrugs and the free CP, RA, and CP/RA mixture were intravenously injected into NCI-H460 tumor-bearing mice, and phosphate-buffered saline was injected via the tail vein as the control. Figure 6c shows a graphical representation of tumor inhibition by PBS, free CP, RA, the CP/RA mixture, and self-assembled nanodrugs. The tumor volumes and body weights of tumor-bearing mice were monitored every three days for 21 d, and the results are shown in Figure 6a,b. At 14 d, the tumor volumes of self-assembled nanodrugs treated mice were significantly smaller than those treated with PBS, CP, RA, or the CP/RA mixture. The tumor volumes of the CP/RA mixture treated mice were also smaller than the ones treated with CP or RA, but they were not as small as those treated with self-assembled nanodrugs. At 21 d, the tumors exhibited a noticeable response to the self-assembled nanodrugs. The tumor inhibition rate of the self-assembled nanodrugs was 69.9% compared with the inhibition rates of
41.2%, 53.1%, and 59.3% for RA, CP, and the CP/RA mixture, respectively. Tumor inhibition values larger than 40% were regarded as effective treatments, and these observations were consistent with the in vitro experiments.

As shown in Figure 6b, a small decline in body weight occurred during the first week of treatment. However, no significant body weight changes were observed in mice treated with CP, RA, or the CP/RA mixture during the subsequent two weeks, indicating that all doses used in this study were within a safe range. A small increase in weight was observed in mice treated with the self-assembled nanodrugs. Compared with the PBS-treated control group, the percent weight loss was 26.83%, 22.76%, 30.89%, and 10.16% for mice treated with CP, RA, the CP/RA mixture, and the self-assembled nanodrugs, respectively. The weight loss percentage of the tumor-bearing mice treated with self-assembled nanodrugs was less than that of mice treated with CP, RA, or the CP/RA mixture, suggesting fewer self-assembled nanodrug-associated side effects for tumor therapy. The schedule used in this study allowed an early evaluation of drug therapeutic efficacies by measuring their abilities to inhibit tumor growth. However, tumor regression did not persist in any of the tumor models tested. Regrowth of the residual tumor was observed 10–14 d after the end of drug treatment. To address this problem, a longer drug treatment time will need to be tested.

The antitumor efficacy and the potential toxicity of the self-assembled nanodrugs were evaluated by hematoxylin and eosin (H&E)-staining. As shown in Figure 7, there are clear differences in tumor tissue morphology between the PBS and self-assembled nanodrug treatment groups. The large nuclei and spindle shapes evident in the PBS-treated tumor tissues indicated a rapid growth trend for the tumor. However, large necrotic areas were observed in the self-assembled nanodrug-treated tumor tissues, and the average tumor cell numbers were significantly lower in each microscopic field. Nuclear shrinkage fragmentation was also observed. The results indicate that the number of tumor cells decreased in the mice treated with the self-assembled nanodrugs.

To address toxicity, tissue sections from nanodrug-treated mice organs were examined to evaluate the self-assembled nanodrug potential toxicities. As shown in Figure 7, the organ morphologies of the treated mice were similar to the organs from the healthy mice. Myocardial fibers showed no degeneration or necrosis change. The hepatic lobule structure were clear, and no obvious fat vacuoles were found in hepatic cells. The structure of alveolar is normal, and no interstitial fibrosis was observed. The glomerulus and tiny tubules of the kidney was normal, and the hyaline change or necrotic area did not appear. Together, these results confirm the superior antitumor efficacy of the nanodrugs. Importantly, there was no significant toxicity observed in self-assembled nanodrug-treated mice compared with the control group.

Figure 6. In vivo antitumor activities of self-assembled nanodrugs after intravenous tail vein injections of tumor-bearing nude mice: a) Tumor volume changes within 21 d. Data represent the mean ± standard deviation (n = 6). b) Body weight changes within 21 d. Data represent the mean ± standard deviation (n = 6). c) Digital photographs of mice treated with different kinds of antitumor drugs monitored during the 21-d evaluation period.
To overcome the poor biocompatibilities and severe side effects of hydrophobic antitumor drugs and to increase intratumoral accumulation, we established a simple small-molecule self-assemble method using an amphiphilic molecule (Pep-Cy5) to promote tumor imaging to simultaneously be performed. Surprisingly, we found that the limitations of traditional chemotherapeutic drugs can easily be overcome using the self-assembled nanodrug approach. Compared with free CP, RA, and a CP/RA mixture, the self-assembled nanodrugs exhibit a superior tumor inhibitory efficacy that benefits from its optimal physicochemical characteristics. The self-assembled nanodrugs at the tumor site. These outcomes may result from the small (average 93 nm) nanodrug diameter, which may reduce drug elimination through the reticuloendothelial system. The nanodrug’s ability to degrade MMP-2 promoted its cellular uptake. The in vivo nanodrug toxicities were evaluated by monitoring the mental states, diets, and activities of the mice. The results indicated that the mice treated with self-assembled nanodrugs had less weight loss and exhibited better mental states compared with the mice treated with free CP, RA, or the CP/RA mixture. This result was also confirmed by an immunohistochemical analysis of the organs coming from the drug-treated mice. The structures of the organs from the self-assembled nanodrug-treated mice were normal, and no pathological changes was observed in the self-assembled drug treated mice. This new MMP-2-responsive self-assemble strategy offers a new approach to improving the biological compatibilities and antitumor efficacies of chemotherapy drugs while reducing adverse effects. This strategy would be particularly beneficial with well-known curative drugs, whose use is limited by severe side effects. We believe that this Pep-Cy5-assisted self-assemble nanodrug system have the great potential in clinical translation.

3. Conclusion

To overcome the poor biocompatibilities and severe side effects of hydrophobic antitumor drugs and to increase intratumoral accumulation, we established a simple small-molecule self-assemble method using an amphiphilic molecule (Pep-Cy5) to promote the self-assemble process. Through hydrophobic interactions, any hydrophobic drug can self-assemble into nanoparticles, and the amphiphilic properties of the Pep-Cy5 molecular belt increases its water-solubility. The Cy5 near-infrared dye allows tumor imaging to simultaneously be performed. Surprisingly, we found that the limitations of traditional chemotherapeutic drugs can easily be overcome using the self-assembled nanodrug approach. Compared with free CP, RA, and a CP/RA mixture, the self-assembled nanodrugs exhibit a superior tumor inhibitory efficacy that benefits from its optimal physicochemical characteristics. The self-assembled nanodrugs exhibit longer blood retention times and higher tumor accumulations than free CP and RA. The signal at the tumor site lasts up to 24 h, demonstrating the long retention time of the nanodrugs at the tumor site. These outcomes may result from the small (average 93 nm) nanodrug diameter, which may reduce drug elimination through the reticuloendothelial system. The nanodrug’s ability to degrade MMP-2 promoted its cellular uptake. The in vivo nanodrug toxicities were evaluated by monitoring the mental states, diets, and activities of the mice. The results indicated that the mice treated with self-assembled nanodrugs had less weight loss and exhibited better mental states compared with the mice treated with free CP, RA, or the CP/RA mixture. This result was also confirmed by an immunohistochemical analysis of the organs coming from the drug-treated mice. The structures of the organs from the self-assembled nanodrug-treated mice were normal, and no pathological changes was observed in the self-assembled drug treated mice. This new MMP-2-responsive self-assemble strategy offers a new approach to improving the biological compatibilities and antitumor efficacies of chemotherapy drugs while reducing adverse effects. This strategy would be particularly beneficial with well-known curative drugs, whose use is limited by severe side effects. We believe that this Pep-Cy5-assisted self-assemble nanodrug system have the great potential in clinical translation.

4. Experimental Section

Materials: CP and trans-RA were obtained from Aladdin Reagent Co. Ltd. (Shanghai, China). MMP-2 was purchased from ACRO BIOSYSTEMS Shanghai, China. 1, 10-phenanthroline monohydrate (MMP inhibitor) was provided by Aladdin Reagent Co. Ltd. (Shanghai, China). The GPLVGRGE-NH2 and Pep-Cy5 peptides were synthesized by SciLight Biotechnology Co., (Beijing, China). Anhydrous DMSO was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Alexa Fluor 488 annexin V and PI were purchased from life technologies Co. MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Invitrogen Corporation (Carlsbad, CA, USA). NCI-H460 and A549 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Cell culture products and reagents, unless otherwise indicated, were purchased from Gibco. The chemicals listed above were used without any further purification. Ultrapure water (18.2 MΩ cm, Millipore Co., USA) was used in all preparations.

Characterization: DLS measurements were performed using a NICOMP 380 ZLS Zeta Potential/Particle Sizer (PSS Nipyc, Santa Barbara, CA, USA). TEM studies were performed with a JEOL 2010 instrument operated at 200 kV. A small drop of the sample solution (0.5 mg mL⁻¹) was spread onto the carbon-coated copper grid. The grid was then immersed into liquid nitrogen and freeze-dried in a vacuum at −50 °C before obtaining measurements. Ultraviolet-visible (UV–vis) absorption measurements of the sample solutions were obtained at room temperature using a Varian Cary 50 UV–vis spectrophotometer. Fluorescent spectra were recorded using a Hitachi FL-4600 spectrofluorometer. The MS system was performed in positive ion mode. The parameters in the source were set as follows: capillary voltage, 350 V; source temperature, 100 °C; desolvation temperature, 350 °C; collision energy 4.0 eV; cone gas flow, 50.0 L h⁻¹; desolvation gas flow, 600.0 L h⁻¹. Data were collected in centroid mode from mass-to-charge ratio (m/z) 50 to 1000 at scan time of 0.25 s with an interval of 30 s. MS analysis was performed using Masslynx 4.1 software (Waters MS Technologies, Manchester, UK). The high performance liquid chromatography analysis was performed on a Surveyor Plus HPLC system. The cellular uptake of self-assembled nanodrugs was observed using a confocal laser scanning microscope (CLSM) (Leica TCS SP8, Germany).

Synthesis of Self-Assembly Nanodrugs: 5 mg CP, 5 mg RA, and 0.5 mg Pep-Cy5 were dissolved in 2 mL of DMSO and stirred at room temperature for 5 min. The solution was then slowly added to 5 mL of deionized water and gently stirred for 30 min. The solution was dialyzed against deionized water for 24 h (molecular weight cutoff (MWCO) = 3000 g mol⁻¹). The self-assembly nanodrug concentration in this paper is presented as the CP concentration unless otherwise indicated. The concentration of CP and RA was measured by HPLC.

Figure 7. a,b,c,g,h,i) H&E-stained tissue sections from mice treated with self-assembled nanodrugs for 21 d postinjection and d,e,f,i,k,l) mice injected with PBS. a,d) Tumor, b,e) heart, c,f) liver, g,j) spleen, h,k) kidney, and i,l) lung. Magnification: x 100.
CAC Measurement: Pyrene was used as a fluorescence probe to determine the CAC value. 1 µL of pyrene acetone solution (6 × 10^{-6} mol L^{-1}) was added to 1 mL of each self-assembled nanodrug aqueous solution; although the nanodrug solution concentrations varied, the pyrene concentration was kept at 6 × 10^{-7} mol L^{-1} in each sample. The fluorescence emission spectra for all samples were recorded on a Hitachi FL-4600 spectrophotometer with a 336 nm excitation wavelength and an 8 nm slit width. The emission intensity ratio values of I_{635}/I_{384} (emission wavelengths of 384 nm/373 nm) were calculated, and the fitted curve of the intensity ratio values versus the logarithm of the nanodrug concentration (CP or RA concentration) was made.

In Vitro Drug Release From Self-Assembled Nanoparticles: A dialysis membrane was chosen to release the profiles of CP and RA from self-assembled nanodrugs. Approximately 3 mL self-assembled nanodrug solution (0.5 mg mL^{-1}) was transferred into a dialysis tube with 3500 MWCO. The tube was then inserted into a tube containing 60 mL of PBS (pH = 7.4) supplemented with 1%, 3%, 5%, or 10% FBS. For the MPP-2 group, 12 µL active MPP-2 (50 µg mL^{-1}) was added into the dialysis tube together with the self-assembled nanodrugs. All samples were incubated at 37 °C with continuous shaking at 200 rpm. 1 mL of the external buffer was removed and replenished with an equal volume of fresh media at predetermined time intervals. The amount of CP or RA release was measured by HPLC.

Cell Culture: NCI-H460 cells and A549 cells were acquired from the Cell Bank of the Chinese Academy of Sciences. The NCI-H460 cells were maintained at 37 °C with 5% CO2 in RPMI-1640 medium, supplemented with 10% FBS, 100 U mL^{-1} penicillin, and 0.1 mg mL^{-1} streptomycin. A549 cells were incubated in DMEM (Dulbecco’s modified Eagle medium) medium with 10% FBS at 37 °C with 5% CO2. The cells were cultured until 75% confluence was reached, and experiments were performed.

Cellular Uptake and Distribution of Self-Assembled Nanodrugs by NCI-H460 Cells: A CLSM was utilized to detect the cellular uptake and distribution of self-assembled nanodrugs in NCI-H460 cells. The cells were seeded onto 14 mm glass cover slips at 2.0 × 10^5 cells per well in 150 µL of culture medium. After 24 h incubation, the medium was replaced with 150 µL of culture medium. After 24 h incubation, the cells were harvested and washed twice with ice-cold PBS and stained with Alexa Fluor® 488 annexin V and PI according to the manufacturer’s instructions.

In Vivo Anticancer Activity: In vivo anticancer activity experiments were performed using NCI-H460 tumor-bearing mice. When the tumor size reached ~200 mm^3, the NCI-H460 tumor-bearing mice were randomly divided into five groups, and the mice in the different treatment groups were intravenously administered PBS, CP (3.83 mg kg^{-1}), RA (3.3 mg kg^{-1}), a CP/RA mixture (3.3 mg kg^{-1} CP and 3.83 mg kg^{-1} RA), and self-assembled nanodrugs (3.88 mg kg^{-1}) by tail vein injection once every 3 d for 21 d. Consider the self-assembled nanodrugs as a molecule, which is composed by CP, RA, and Pep-Cy5. The molecular weight of the molecular is about 356.3, which is calculated according to their final molar ratio of 14:14:1. In order to compare the anticancer activity, the dose for CP, RA, CP/RA mixture, and nanodrugs were given in equal molar ratio (~11 µmol kg^{-1}). The vehicle used for CP, RA, and CP/ RA mixture is 0.9% physiological saline. Each mouse was marked and followed individually throughout the whole experiment. The tumor size (length and width) and the body weight of the mouse were measured before every injection. Tumor volume (V) was calculated as follows: V (mm^3) = 1/2 × length (mm) × width (mm^2). Mice were killed 3 d after the last injection on day 21, and the organs and tumors of the mice were cut into small pieces for fixation in 10% paraformaldehyde for 7 d. The tissues were then sectioned for histopathological analysis by H&E staining.

Statistical Analysis: Data are presented as the mean ± SD unless otherwise indicated. Statistical significance was determined using a two-tailed student’s t-test (P < 0.05) unless otherwise indicated.
Supporting Information

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

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