Novel tumor-targeting, self-assembling peptide nanofiber as a carrier for effective curcumin delivery

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Abstract: The poor aqueous solubility and low bioavailability of curcumin restrict its clinical application for cancer treatment. In this study, a novel tumor-targeting nanofiber carrier was developed to improve the solubility and tumor-targeting ability of curcumin using a self-assembled Nap-GFFYG-RGD peptide. The morphologies of the peptide nanofiber and the curcumin-encapsulated nanofiber were visualized by transmission electron microscopy. The tumor-targeting activity of the curcumin-encapsulated Nap-GFFYG-RGD peptide nanofiber (f-RGD-Cur) was studied in vitro and in vivo, using Nap-GFFYG-RGE peptide nanofiber (f-RGE-Cur) as the control. Curcumin was encapsulated into the peptide nanofiber, which had a diameter of approximately 10–20 nm. Curcumin showed sustained-release behavior from the nanofibers in vitro. f-RGD-Cur showed much higher cellular uptake in αvβ3 integrin-positive HepG2 liver carcinoma cells than did non-targeted f-RGE-Cur, thereby leading to significantly higher cytotoxicity. Ex vivo studies further demonstrated that curcumin could accumulate markedly in mouse tumors after administration of f-RGD-Cur via the tail vein. These results indicate that Nap-GFFYG-RGD peptide self-assembled nanofibers are a promising hydrophobic drug delivery system for targeted treatment of cancer.

Keywords: nanofiber, tumor-targeting, self-assembling, curcumin, drug delivery

Introduction

Because almost all chemotherapeutic antitumor agents have no tumor-targeting activity and cause serious adverse effects, their clinical application is restricted. Many nanomaterials have been proposed as drug carriers to overcome this barrier to the use of chemotherapeutic drugs.¹ The enhanced permeability and retention effect of nanomaterials can improve the accumulation of chemotherapeutic agents at tumor sites. For example, liposomes, carbon nanotubes, dendrimers, and micelles have been used as carriers for SN38, doxorubicin, paclitaxel, and cisplatin to improve drug concentrations in tumors and reduce adverse effects.²⁻⁵ Another advantage of using nanomaterials as drug carriers is the enhanced solubility of chemotherapeutic drugs. Many of these agents have poor aqueous solubility, leading to low bioavailability. For example, the aqueous solubility of paclitaxel is 30 µg/mL and for hydroxycamptothecin is only 3.9 µg/mL. The hydrophilic region of amphiphilic nanomaterials can solubilize drugs, and their hydrophilic region makes drugs soluble in an aqueous environment.⁶

Self-assembling peptide nanofibers have attracted considerable interest because of their good biocompatibility, easy modification, and design flexibility through a “bottom-up” approach. They have been widely used in three-dimensional cell culture,⁷ tissue engineering, and regenerative medicine research.⁸⁻⁹ Self-assembling peptide
nanofibers have also been used as drug delivery systems to enhance solubility of a hydrophobic drug, improve accumulation at the tumor site, and reduce adverse effects. Hydrophobic antitumor drugs such as paclitaxel, camptothecin, and ellipticine encapsulated into peptide nanofibers have shown improved antitumor effects. Some studies have demonstrated that the two-dimensional structure of peptide nanofibers is superior to the three-dimensional structure of nanoparticles for drug carriers. For example, Law et al found that peptide-based nanofibers have better biocompatibility, better tumor targeting within a shorter time frame, and more rapid elimination compared with spherical nanomaterials (poly(lactic-co-glycolic acid), gold, polystyrene, cadmium and selenium quantum dots), and carbon rods. The conjugation of tumor-targeting agents to nanomaterials is another strategy for improving tumor accumulation and bioavailability of drugs. Peptides, proteins, aptamers, vitamins, and polysaccharides have been developed as tumor-targeting agents. Among these molecules, the tripeptide motif, arginine-glycine-aspartic acid (RGD), has attracted great attention. RGD is the binding motif of integrin αvβ3, which is overexpressed on the angiogenic endothelium in malignant tumors and is vital for the adhesion, signaling, migration, and survival of integrin αvβ3 over-expressed cancer cells. It has been widely used for tumor diagnosis and targeting therapy. Liposomes, micelles, dendrimers and silica nanoparticles have been conjugated with RGD to introduce tumor-targeting activity. RGD has also been used in positron emission tomography, single photon emission computed tomography, magnetic resonance imaging, and optical imaging for diagnosis of cancer.

In this study, we report the design, synthesis, and characterization of a novel peptide nanofiber, self-assembled from Nap-GFFYG-RGD, as a tumor-targeting drug carrier. In order to study the RGD tumor-targeting activity, nanofibers self-assembled from Nap-GFFYG-RGE were also synthesized as the control. Curcumin, a hydrophobic antitumor agent, was used as the model drug. The nanofibers were characterized with respect to their morphology, drug encapsulation efficiency, and in vitro drug release. In vitro cytotoxicity was analyzed in human hepatocellular liver carcinoma (HepG2) and mouse embryonic fibroblast (NIH3T3) cell lines. Cellular uptake was investigated in HepG2, human embryonic kidney (293T), and human breast cancer (MCF-7) cell lines. Ex vivo biodistribution on HepG2 xenografts in nude mice was also studied to confirm the tumor-targeting activity of the nanofibers.

Materials and methods
Curcumin (diferuloyl methane) and peptides were purchased from Sigma-Aldrich Corporation (Carlsbad, CA, USA). 2-chlorotrityl chloride resin and Fmoc-amino acids were obtained from GL Biochem (Shanghai, People’s Republic of China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide, and other chemical reagents and solvents were sourced from Alfa (Shenzhen, People’s Republic of China). A dialysis membrane (molecular weight cut-off 1,000 Da) was purchased from BBI Inc (Shanghai, People’s Republic of China). All cell culture media and supplies were purchased from Gibco Corporation (Grand Island, NY, USA).

NIH3T3, HepG2, 293T, and MCF-7 cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained at 37°C with 5% CO2. Other reagents were commercially available and used as received.

1H nuclear magnetic resonance (NMR, Varian Unity Plus 400 mHz, Varian Instruments, Palo Alto, CA, USA) was used to characterize the peptides. Electrospray ionization tandem mass spectrometric analyses were performed on an LCQ AD system (Thermo Finnigan, Silicon Valley, CA, USA). Transmission electron microscopy was performed on a Tecnai G2 F20 system (FEI Co, Columbia, MD, USA) operating at 200 kV. The crude peptide was purified on a high-pressure liquid chromatography system (LumTech, Moorestown, Germany) using a C18 reversed phase column with MeOH (0.1% trifluoroacetic acid) and water (0.1% trifluoroacetic acid) as the eluents.

Preparation and characterization of peptides
The peptides, ie, Nap-GFFYG-RGD (p-RGD) and Nap-GFFYG-RGE (p-RGE), were prepared by solid phase peptide synthesis using 2-chlorotrityl chloride resin. The first Fmoc-protected amino acid was loaded onto the resin at the C-terminal. The Fmoc group of the amino acid was deprotected with 20% piperidine in anhydrous N,N,N′,N′-tetramethylformamide. The next Fmoc-protected amino acid was activated by N,N′-dicyclohexylcarbodiimide and coupled to the free amino group using O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethylethylendiamine hexafluorophosphate as the coupling reagent. The growth of the peptide chain was allowed to proceed according to the standard Fmoc solid phase peptide synthesis protocol. After the last coupling step, the peptide derivatives were cleaved from the resin with a mixture of 1% trifluoroacetic acid and...
99% dichloromethane for 10 minutes, then dried via rotary evaporation followed by diethylether precipitation. The crude products were purified by reverse phase high-pressure liquid chromatography. 1H NMR spectroscopy and high-resolution mass spectrometry were used to characterize the peptides.

**Preparation of f-RGD-Cur and f-RGE-Cur**

Fifty milligrams of curcumin were dissolved in 5 mL of anhydrous dimethyl sulfoxide and stirred at 40°C. p-RGD and p-RGE were dissolved in phosphate-buffered saline (pH 7.4) and the concentrations were adjusted to 3 mg/mL. Next, 500 µL of p-RGD and p-RGE solutions were heated to boiling point and cooled to 40°C, and 22 µL of curcumin solution at 40°C was then added to the p-RGD and p-RGE solutions. The reaction mixtures were incubated in a 40°C water bath for 2 hours. Curcumin-encapsulated Nap-GFFYG-RGD peptide nanofiber (f-RGD-Cur) and curcumin-encapsulated Nap-GFFYG-RGE peptide nanofiber (f-RGE-Cur) were then formed by incubation at room temperature for 2 hours. Unassembled curcumin was removed by centrifugation for 20 minutes at a speed of 1,500×g. The morphologies of the nanofibers were observed by transmission electron microscopy.

**Transmission electron microscopy**

Solutions of f-RGD, f-RGE, f-RGD-Cur, and f-RGE-Cur were prepared as described above. Next, 10 µL samples of each were placed on a carbon-coated copper grid and incubated for 30 seconds to allow the peptide nanostructures to adhere to the substrate, then rinsed twice with ultrapure water. The samples were then stained with a saturated uranyl acetate solution and incubation for the specified time at 37°C in a humidified incubator, 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline) were added to each well. Four hours later, the medium containing MTT was removed and 150 µL of dimethyl sulfoxide were added to dissolve the formazan crystals. The optical density of the solution was measured at 570 nm on a microplate reader (Varioskan Flash, Thermo Fisher Scientific, South Logan, UT, USA). The effect of each agent on growth inhibition was determined as a percentage of the viability of cells treated with nanofiber without curcumin, for which the viability was taken as 100%. The cell viability was calculated from the equation:

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\text{Cell viability} (%) = \frac{\text{Average OD of test group}}{\text{Average OD of control group}} \times 100%.
\]

**Ex vivo distribution**

BALB/c mice (male, aged 4–6 weeks, weight 18–22 g) were purchased from the Laboratory Animal Center of The Academy of Military Medical Sciences (Beijing, People’s Republic of China). All animal procedures were performed according to the guidelines of the Regulations for the Administration of Affairs Concerning Experimental Animals (Tianjin, People’s Republic of China, revised in June 2004) and adhered to the Guiding Principles in the Care and Use of Animals of the American Physiological Society. HepG2 cells (1 × 10⁶) suspended in phosphate-buffered saline were subcutaneously seeded into 24-well plates at a density of 3 × 10⁶ cells/well in 500 µL of Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum. After incubating for 24 hours, the culture medium in each well was replaced with 500 µL of fresh medium containing 10 µg/mL of free curcumin or equivalent curcumin-containing f-RGD-Cur or f-RGE-Cur. After incubating for 4 hours, the cells were rinsed three times with 500 µL of phosphate-buffered saline to remove the free curcumin, f-RGD-Cur, or f-RGE-Cur. Cellular uptake was visualized on an inverted fluorescence microscope (DMI6000B, Leica, Wetzlar, Germany).

**In vitro cytotoxicity**

The cytotoxicity of free curcumin, f-RGD-Cur, and f-RGE-Cur was evaluated using the MTT assay with NIH3T3 and HepG2 cells. The cells were seeded into 96-well plates at a density of 1 × 10⁴ cells/well in 100 µL of culture medium and incubated for 24 hours. Next, 100 µL of culture medium containing different concentrations of free curcumin, f-RGD-Cur, or f-RGE-Cur were added to 96-well plates for 24 hours. Each treatment was repeated in eight wells. After incubation for the specified time at 37°C in a humidified incubator, 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline) were added to each well. Four hours later, the medium containing MTT was removed and 150 µL of dimethyl sulfoxide were added to dissolve the formazan crystals. The optical density of the solution was measured at 570 nm on a microplate reader (Varioskan Flash, Thermo Fisher Scientific, South Logan, UT, USA). The effect of each agent on growth inhibition was determined as a percentage of the viability of cells treated with nanofiber without curcumin, for which the viability was taken as 100%. Cell viability was calculated from the equation:

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\text{Cell viability} (%) = \frac{\text{Average OD of test group}}{\text{Average OD of control group}} \times 100%.
\]
Liu et al injected into the right flank to produce liver cancer xenografts. About 2 weeks after inoculation, when the tumors had reached a volume of approximately 300 mm$^3$, the mice were randomized into three groups (n=5 each), and 200 µL (10 µg/mL) of free curcumin or equivalent curcumin-containing f-RGD-Cur or f-RGE-Cur were injected via the tail vein. Four hours following injection, the mice were sacrificed, and the heart, liver, spleen, lung, kidneys, and tumor were isolated under white light and immediately scanned using a Kodak IS in vivo FX imaging system (Kodak, New Haven, CT, USA). The fluorescence images were acquired using a 464 nm excitation filter and 535 nm emission filter. The relative fluorescence intensity per unit area was calculated using Kodak 5.1 software.

Statistical analysis
All statistical analysis was performed using Statistical Package for the Social Sciences version 16 software (SPSS Inc, Chicago, IL, USA). The statistical significance of differences was determined by one-way analysis of variance. All statistical tests were two-sided, and $P$-values less than 0.05 were considered to be statistically significant. The results are shown as mean ± standard deviation.

Results and discussion
Preparation and characterization of f-RGD-Cur and f-RGE-Cur
Recent studies have indicated that the shape of nanostructures may greatly affect their in vivo biodistribution, and some flexible filament nanostructures have shown longer blood circulation and better antitumor effects than spherical nanostructures.\textsuperscript{14,30,31} The p-RGD and p-RGE peptides were synthesized (Figures 1A and S1–S4) and used to form nanofiber skeletons by self-assembly via electrostatic and hydrophobic interactions. The peptides formed single-layer nanofibers (approximately 10–20 nm in width) that could be visualized by transmission electron microscopy (Figure 2A and B).

![Figure 1](image-url) (A) Molecular structures of Nap-GFFYG-RGD and Nap-GFFYG-RGE. (B) Schematic illustration for formation of Nap-GFFYG-RGD peptide nanofibers and encapsulation of curcumin.
Curcumin was encapsulated into the nanofibers without any obvious effects on the shape of the nanofibers (Figure 2C and D). This result is consistent with the report of Zhang et al.\(^{11}\) Figure 1B shows the Nap-GFFYG-RGD peptide self-assembling into nanofibers and drug encapsulation.

**Encapsulation efficiency and in vitro release of curcumin**

As a natural medicine used in Asia, curcumin extracted from different plants has been shown to have potential antitumor activity. It can suppress the expression of epidermal growth factor receptor and estrogen receptors, which are cancer-associated growth factor receptors.\(^{32}\) Curcumin has been widely used in antitumor research, but its poor solubility in water results in poor bioavailability and restricts its clinical application.\(^{33,34}\) Amphiphilic nanomaterials have been used to improve the solubility of curcumin.\(^{35,36}\) Zhang et al found that the hydrophobic drug, paclitaxel, could be encapsulated into the hydrophobic core formed by self-assembled peptide nanofibers.\(^{11}\) Pochan et al also reported that curcumin could interact with hydrophobic peptide moieties and encapsulate into self-assembled peptide hydrogels.\(^{37}\) Figure 3A shows the encapsulation efficiency of curcumin with RGD and RGE peptide nanofibers. The amount of curcumin solubilized in the nanofibers increased with the peptide concentration from 0.5 mg/mL to 5 mg/mL. When the concentration of curcumin was 0.5 mM and the peptide concentration was 5 mg/mL, the efficiency of encapsulation of curcumin into the nanofibers reached about 32%.

Controlled and sustained drug release is a desirable feature of drug carriers. In order to verify the sustained drug release characteristics of curcumin-loaded peptide nanofibers, the in vitro release of f-RGD-Cur and f-RGE-Cur was studied using a dialysis method. The drug release profile was performed under simulated physiologic conditions (pH 7.4). As shown in Figure 3B, both f-RGD-Cur and f-RGE-Cur showed a sustained drug release profile, with total curcumin release from the nanofibers being about 51.6% and 60.9%, respectively, while treatment with free curcumin led to release of more than 95% of the drug.

**In vitro cellular uptake**

One of the advantages of curcumin is its intrinsic fluorescence, which can be detected directly to quantify its cellular uptake and intracellular trafficking. We used fluorescence microscopy to compare the fluorescence intensity of free curcumin, f-RGD-Cur, and f-RGE-Cur in normal human 293T cells, human MCF-7 cancer cells with low expression of αvβ3, and human HepG2 cancer cells with high expression of αvβ3. To better compare their internalization, parameters such as laser power, offset, sensitivity, and gain constant were...
Figure 4 Uptake of various curcumin formulations by 293T (A–C), MCF-7 (D–F), and HepG2 (G–I) cells. The cells were incubated with free curcumin, f-RGD-Cur, or f-RGE-Cur at an equivalent curcumin concentration of 10 µg/mL for 4 hours. Scale bar, 30 µm. Uptake of f-RGD-Cur was greater by HepG2 cells overexpressing integrin αvβ3 than by αvβ3-negative MCF-7 cells and 293T cells.

Abbreviations: f-RGD-Cur, curcumin-encapsulated Nap-GFFYG-RGD peptide nanofiber; f-RGE-Cur, curcumin-encapsulated Nap-GFFYG-RGE peptide nanofiber.

harmonized during the cell imaging procedure. As shown in Figure 4, very similar and low fluorescence intensity was observed in normal human 293T cells incubated with free curcumin, f-RGD-Cur, or f-RGE-Cur (Figure 4A–C). For MCF-7 cells, the fluorescence intensity was slightly higher when they were incubated with f-RGD-Cur rather than with free curcumin or f-RGE-Cur (Figure 4D–F). In HepG2 cells, the fluorescence intensity following incubation with f-RGD-Cur was much higher than following incubation with free curcumin and f-RGE-Cur (Figure 4G–I). The cells incubated with free curcumin showed the lowest fluorescence intensity, and the fluorescence intensity was HepG2 > MCF-7 > 293T cells when incubated with f-RGD-Cur (Figure 4B, E, and H). These results indicate that cellular uptake of f-RGD-Cur was higher in cancer cells overexpressing integrin αvβ3 than in αvβ3-negative cancer cells and normal cells and that the RGD-containing peptide self-assembled nanofibers possessed in vitro cancer cell-targeting activity.

In vitro cytotoxicity

The cytotoxicity of free curcumin, p-RGD, p-RGE, f-RGD-Cur, and f-RGE-Cur was evaluated by MTT assay in the HepG2 cell line overexpressing integrin αvβ3, using the normal NIH3T3 cell line with low expression of integrin αvβ3 as the control. The peptide nanofibers showed good biocompatibility, and the viability of both normal cells and tumor cells was greater than 80% when the peptide concentration was less than 180 µg/mL (Figure 5A and C). f-RGD-Cur showed lower cytotoxicity than f-RGE-Cur in normal NIH3T3 cells (Figure 5B), but higher cytotoxicity in HepG2 tumor cells (Figure 5D). This may be caused by the active targeting ability of RGD, which could enable f-RGD-Cur to kill tumor cells more efficiently and thus protect normal tissues. Free curcumin showed higher cytotoxicity than f-RGD-Cur and f-RGE-Cur. The phenomenon of free drug showing higher cytotoxicity in normal cells than in cancer cells and lower cell viability than nanoparticle-encapsulated drugs has also been reported by Xu et al and Su et al, and may be caused by the controlled and sustained release of active drug from f-RGD-Cur and f-RGE-Cur. From the in vitro release results, we found that free curcumin had higher drug release than f-RGD-Cur and f-RGE-Cur, and this may be the reason for the higher cytotoxicity of free curcumin.

Ex vivo distribution

Because of the high proteolytic activity and low pH in the gastrointestinal tract, oral delivery of intact peptide nanofibers to the target site is difficult. The low permeability of peptides at the intestinal barrier is another factor adding
to their low bioavailability. Gastrointestinal delivery of peptide nanofibers is not feasible. Upon intravenous administration, nanostructures will undergo adsorption or opsonization by serum proteins, which results in a change in the physicochemical properties of the nanostructures. This may affect their half-life in blood and their biodistribution. Therefore, some agents show good targeting ability in vitro but no effect in vivo. Ex vivo optical imaging may have some diagnostic value because it is not subject to interference from overlying tissues, eg, skin and blood vessels, and thus closely resembles intraoperative procedures in surgical oncology as opposed to whole-body imaging. To determine whether curcumin accumulates efficiently at tumor sites after injection of f-RGD-Cur, ex vivo imaging of all major organs was performed 4 hours post-injection using a tumor xenograft assay. Representative color-coded fluorescence images of the excised tumors and tissues (heart, liver, spleen, lung, and kidney) obtained 4 hours post-treatment are shown in Figure 6A. These images show that the tumor group treated with f-RGD-Cur had the strongest curcumin fluorescence intensity, followed by tumors in the f-RGE-Cur group. Minimal curcumin fluorescence intensity was observed for tumors in the free curcumin group. The total fluorescence intensity in the free curcumin group was lower than in the f-RGD-Cur and f-RGE-Cur groups. As shown in Figure 6B, the fluorescence intensity in the f-RGD-Cur group was about 4-fold higher than that in the free curcumin group ($P < 0.01$). In addition, the fluorescence intensity in the f-RGD-Cur group was also significantly higher than in the f-RGE-Cur group ($P < 0.05$). Our results indicate that these peptide nanofibers can deliver curcumin into tumors, perhaps as a result of the enhanced permeability and retention effect. The RGD motif may enable RGD-containing peptide nanofibers to accumulate more effectively in tumors by active tumor-targeting. Further in...
activity. Nanofibers may provide a useful platform for developing f-RGE-Cur to tumor cells and lower cytotoxicity to normal tumor cells and xenografts overexpressing integrin nanofibers, f-RGD-Cur showed highly selective targeting of the agent. This nanofiber efficiently encapsulated the hydro peptide together with an RGD peptide as the tumor-targeting peptide nanofiber, f-RGD-Cur, formed by the Nap-GFFYG-RGE peptide nanofiber; f-RGE-Cur, curcumin-encapsulated Nap-GFFYG-RGE peptide nanofiber.

**Figure 6** Ex vivo fluorescence images of tissues and tumors. Tissues were isolated from animals treated with 200 µL (10 µg/mL) of free curcumin or equivalent curcumin containing f-RGD-Cur or f-RGE-Cur 4 hours after intravenous administration (n=5). (A) Representative images from one of the experiments for the organs. The fluorescence intensity reflects the concentration of curcumin. f-RGD-Cur showed the highest accumulation in tumors, followed by f-RGE-Cur, and free curcumin had the lowest distribution in tumors. (B) Relative fluorescence intensity of curcumin in the excised tumors. *P<0.05 for f-RGD-Cur compared with the free curcumin group, indicating a very significant difference in curcumin concentration between the two groups; **P<0.01 for f-RGD-Cur versus the f-RGE-Cur group, indicating a significant difference in curcumin concentration between the two groups. Abbreviations: f-RGD-Cur, curcumin-encapsulated Nap-GFFYG-RGD peptide nanofiber; f-RGE-Cur, curcumin-encapsulated Nap-GFFYG-RGE peptide nanofiber.

Conclusion

In this study, we have identified a new tumor-targeting peptide nanofiber, f-RGD-Cur, formed by the Nap-GFFYG peptide together with an RGD peptide as the tumor-targeting agent. This nanofiber efficiently encapsulated the hydrophobic drug, curcumin, and showed sustained drug release. Compared with free curcumin and non-targeting f-RGE-Cur nanofibers, f-RGD-Cur showed highly selective targeting of tumor cells and xenografts overexpressing integrin αvβ3. f-RGD-Cur also demonstrated higher cytotoxicity than f-RGE-Cur to tumor cells and lower cytotoxicity to normal cells. These results suggest that RGD-containing peptide nanofibers may provide a useful platform for developing hydrophobic drug delivery carriers with tumor-targeting activity.

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Disclosure

The authors report no conflicts of interest in this work.

References


Supplementary materials

Figure S1 \textit{1}H NMR spectrum of Nap-GFFG-rgD.

\textbf{Notes}: Nap-GFFG-rgD: \textit{1}H NMR (400 MHz, DMSO-d6) \(\delta\) 8.20 (s, 3H); 8.02–8.05 (m, 5H); 7.80–7.84 (m, 3H); 7.74 (s, 1H); 7.40–7.47 (m, 4H); 7.15–7.21 (m, 11H); 7.04 (d, 2H); 6.64 (d, 2H); 4.40–4.49 (m, 4H); 4.30–4.35 (m, 1H); 3.69–3.78 (m, 6H); 3.59–3.66 (m, 4H); 3.08–3.10 (m, 2H); 2.90–2.99 (m, 3H); 2.56–2.75 (m, 6H); 2.33 (s, 1H); 1.72 (m, 1H); 1.50–1.53 (m, 3H). HR-MS: calc. \(M^+ = 1,085.4607\), obsvd \((M+H)^+ = 1,086.4686\).

Figure S2 HR-MS of Nap-FFG.

\textbf{Notes}: Nap-GFFG-RGE: \textit{1}H NMR (400 MHz, DMSO-d6) \(\delta\) 8.22 (s, 3H); 8.02–8.09 (m, 6H); 7.83–7.85 (m, 3H); 7.74 (s, 1H); 7.40–7.47 (m, 4H); 7.15–7.18 (m, 11H); 7.03 (d, 2H); 6.65 (d, 2H); 4.35–4.50 (m, 4H); 4.25–4.32 (m, 4H); 3.70–3.76 (m, 7H); 3.58–3.62 (m, 8H); 3.09 (s, 3H); 2.90–2.94 (m, 4H); 2.65–2.78 (m, 5H); 2.30–2.35 (m, 3H); 1.40–1.52 (m, 3H); 1.243 (s, 1H). HR-MS: calc. \(M^+ = 1,099.4763\), obsvd \((M+H)^+ = 1,100.4829\).
Figure S3 ¹H NMR spectrum of Nap-GFFYG-RGE.

Figure S4 HR-MS of Nap-FFG.