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Independent of EPR Effect: A Smart Delivery Nanosystem for Tracking and Treatment of Nonvascularized Intra-Abdominal Metastases

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Abstract: Nanoparticle-based delivery systems (NDS) have impacted the field of cancer therapy on account of the enhanced permeability and retention (EPR) effect that promotes passive accumulation in tumors through the tumor vasculature after intravenous (IV) administration. However, transplanted tumor xenografts on animal models used to justify the feasibility of EPR effect are quite different from clinical tumors in many aspects, a fact which becomes an impediment for NDS to succeed clinical trials. Particularly, early stage tumor metastases are usually non-vascularized and incapable of conforming the EPR effect after IV injection. Therefore, it is necessary to develop smart NDS to deliver drugs in an EPR-independent route. Herein, we report an NDS-based treatment approach for intra-abdominal metastases from ovarian carcinoma. Instead of IV injection, intraperitoneal (IP) injection was employed to directly apply the NDS to the metastatic lesions. The NDS was tailor-made with targeting groups to actively target the tumor nidus and redox-responsive drug release to reduce systematic toxicity. Comparing with IV administration, the IP injected NDS could be enriched in metastatic tumor more efficiently, leading to superior therapeutic outcome in vivo. This study provides a successful protocol of EPR-independent NDS-based cancer treatment, which may facilitate the clinical translation of nanoparticle-based cancer therapeutics.

1. Introduction

Along with the rapid development of nanotechnology, great hope has been placed on nanoparticle-based delivery system (NDS) to revolutionize the field of cancer therapy. First reported by H. Maeda in 1986,^[1] the enhanced permeability and retention (EPR) effect has been long regarded as the major underlying mechanism of NDS-based cancer therapy. More concretely, NDS can passively target the tumor through the loosely packed endothelial cells of the vasculature and be detained in tumors due to irregular lymphatic vessels.^[2] While a lot of studies have evidenced the superiority of NDS-formulated drugs over free drugs on mouse models, the amount of successful cases for clinical translation is nowhere near expectation. The failure of NDS in clinical translation is mainly owing to the deficiency of the investigated tumors on animal models. Firstly, in many key aspects such as tumor-to-body weight ratio and tumor microenvironment, the transplanted tumor models on rodents are widely different from the actual tumor on human patients.^[3] Secondly, the EPR tumor models are usually transplanted tumor xenografts, which can only represent a narrow range in a variety of tumors. Owing to the uncertainty of EPR effect on human patients, the field of nanomedicine has reached its bottleneck.^[4]

One important characteristic of the xenograft models is to generate well-vascularized tumors. The fenestrated tumor vasculature can act as the pipelines to allow the passive accumulation of NDS upon intravenous (IV) administration. For some specific tumors, however, the lack of angiogenesis may result in an impenetrable biological barrier for NDS. For instance, early-stage metastasis is one of the typical cases. Abdomen is a metastasis-prone site, and intra-abdominal metastasis (IAM) is particularly common for intra-abdominal malignancies.^[5] IAM usually progresses quickly with rapid deterioration, inducing severe suffering as well as high fatality. The standard treatment for IAM includes surgical cytoreduction and the following chemotherapy. However, IAM is randomly disseminated in several abdominal organs,^[6] making the complete elimination of all the nodules extremely difficult. Intraperitoneal (IP) chemoperfusion is a clinically proven approach to treat IAM, in which the chemotherapeutics are injected into the peritoneum.^[7] In this case, the chemotherapy is lack of specific tumor targeting capability. To ensure the intratumoral concentration of the anticancer drug above the effective standard, a considerable amount of the drug may be absorbed by the hepatic portal vein,^[8] causing serious side effects.^[9] Engineered NDS with targeting groups and release triggers can reduce the side effects and improve the treatment efficiency by active tumor targeting and controlled drug release pattern.^[10] Nevertheless, when using IV injection as an administration route, the EPR-dependent NDS is inapplicable to get through the peritoneal-plasma barrier in order to treat early-stage IAM lack of sufficient vascularization.^[11] Particle-based delivery systems have been applied for the treatment of intra-peritoneal metastases *via* IP administration.^[12] It was revealed that the particle size can significantly influence the biodistribution of the injected drug carriers, where particles with larger size are confined near the injection site, and those with smaller size can facilitate the dispersion in the peritoneal cavity.^[13] Therefore, it can be anticipated that nano-sized drug carriers are able to homogenously distribute in the cavity and wipe out the metastatic lesions.

In order to address abovementioned issue, herein, we report a tailor-made NDS (Figure 1) for the treatment of IAM. A hollow polymer-silica nanohybrid (HPSN) with the modification of folic acid (FA) was used as a nanocarrier for triggered delivery of anticancer drug doxorubicin (Dox). β -Cyclodextrin (β -CD) as a gatekeeper was introduced onto the surface of HPSN through a disulfide bond to achieve redox-responsive release of the loaded Dox using intracellular glutathione (GSH) in cancer cells as the stimulus.^[14] In order to steer by the peritoneal-plasma barrier, IP injection was employed as an important part of the NDS-treatment package. Once the developed NDS is IP-injected to the mice with IAM, the NDS can widely spread in the peritoneal cavity, get in direct touch with IAM, actively target the tumor, and

release the loaded Dox under relatively high concentration of GSH in cancer cells, thus reducing the systematic toxicity of the IP chemotherapy.^[15] Experiments on animal models with ovarian IAM demonstrated that, comparing with EPR-dependent strategy of IV injection, the EPR-independent strategy of IP administration showed significantly better curative effect to the IAM and lower toxicity to the subjected mice. This research provides a successful example to utilize NDS for the treatment of a tumor model close to actual cases, which would promote the clinical translation of nanomedicine.

2. Results and discussion

2.1. Design, synthesis and characterization of NDS

2.1.1. Design strategy of NDS

We synthesized unfunctionalized HPSN as an NDS via a sol-gel method by the self-assembly of pluronic copolymer F108 (PEO₁₃₂-PPO₅₀-PEO₁₃₂, PEO represents polyethylene oxide and PPO represents polypropylene oxide) and silica precursors.^[16] The hybrid nanoparticles possess a micelle-like structure in which the amphiphilic copolymer provides the PPO blocks as the hydrophobic core and PEO blocks as the hydrophilic ligands.^[17] The silica species in the interface of PPO and PEO blocks could serve as the cross-linker to enhance the stability of the copolymer micelles against the dilution.^[18] Cyclohexane was employed as a pore-expanding agent to obtain a hollow cavity for improved drug-loading capacity. As a volatile solvent, cyclohexane can be removed from the as-prepared sample by rotary evaporation at 50 °C under vacuum.^[16] In order to modify the NDS with targeting groups, FA was modified to the terminal hydroxyl groups of F108 with a modification ratio of 85% (FA-F108, Scheme S1). The obtained FA-F108 was mixed with F108 in a ratio of 5% as the co-surfactant to produce FA-modified HPSN (FA-HPSN). The schematic illustration for the synthesis of the Dox-loaded NDS is shown in Figure 1a. Surface thiol groups (FA-HPSN-SH) were introduced by the addition of 3-mercaptopropyltriethoxysilane after the formation of FA-HPSN. Adamantane (Ada) was then modified onto the surface (FA-HPSN-SS-Ada) bridged by the disulfide bond using the thiol group as the modification site. After the loading of Dox, Ada was capped by β -CD as the gatekeeper to render the final NDS (Dox@FA-HPSN-SS-CD) with responsive release capability, since the disulfide bond could be broken by GSH as a stimulus.

2.1.2. Characterization of NDS

The TEM images of FA-HPSN-SH, FA-HPSN-SS-Ada and Dox@FA-HPSN-SS-CD are shown in Figure 1b-d, respectively. The obtained hybrid drug carriers are hollow nanoparticles with spherical morphology (inserts of Figure 1b-d). The average diameter of the silica shell for

FA-HPSN-SH is 19.4 nm. Its average hydrated size measured by dynamic light scattering (DLS) is 38.2 ± 1.1 nm (Figure 1e), which is very similar to HPSN without FA-F108 (Figure S1a). Larger hydrated size than the particle size determined by TEM should be due to the PEG chain outside the nanoparticles.^[16] When using higher FA-F108 doping ratio of more than 5%, some vesicle-like nanoparticles were observed in the product (encircled in Figure S1c,d), and the hydrated size increased to 51 and 59 nm under the doping ratio of 10 and 15%, respectively. Hence, the nanoparticles with FA-F108 doping ratio of 5% were adopted as the drug carrier. Ada was linked to the thiol groups of FA-HPSN-SH to produce FA-HPSN-SS-Ada, followed by loading with Dox and capping with the β -CD gatekeeper to afford Dox@FA-HPSN-SS-CD. Comparing with FA-HPSN-SH, the hydrated sizes of FA-HPSN-SS-Ada and Dox@FA-HPSN-SS-CD were slightly changed (Figure 1f,g), and the zeta potentials before and after the modifications were almost neutral (-3.2 to -3.8 mV, Figure S2).

2.2. *In vitro* drug release and cell targeting experiments

2.2.1. Loading and stimuli-responsive release of Dox

Thermal gravimetric analysis results of FA-HPSN-HS, FA-HPSN-SS-Ada and FA-HPSN-SS-CD reveal a modification rate of 420 β -CD molecules on a single nanoparticle (Figure S3). The surface coverage rate of FA-HPSN-SS-CD by β -CD was calculated to be 66%, based on the diameter of FA-HPSN-SS-CD (19.4 nm) and outer diameter of β -CD (1.54 nm).^[19] Upon the addition of GSH, the disulfide bond would be cleaved to thiol groups, leading to the removal of the β -CD gatekeeper and the release of the loaded Dox (Figure 2a). *In vitro* drug release assays were carried out to investigate the redox-responsive release behavior of Dox@FA-HPSN-SS-CD. The release rate of loaded Dox was dependent on both GSH concentrations and pH values (Figure 2b). To mimic the intracellular environment of cancerous cell, an acidic phosphate-buffered saline (PBS) solution with pH = 5.5 was used as the medium to study the influence of GSH. The results show that only a small amount of Dox (> 8%) was released in the absence of GSH after 10 h, demonstrating that the β -CD gatekeeper could efficiently entrap the drug inside the NDS. As GSH can result in the removal of β -CD by the cleavage of disulfide bond, when raising the GSH concentration, the release rate of Dox was significantly accelerated. A cumulative release amount of 75% at 10 h was obtained when 10 mM GSH was added. As the solubility of Dox depends on the pH value, the Dox release speed was increased by 2.5 times when the pH value was decreased from 7.4 to 5.5.^[20]

2.2.2. *In vitro* cell targeting of NDS to SKOV-3

In order to verify that the introduced FA group is capable of rendering targeting ability to the NDS, confocal microscopy and flow cytometry were carried out using FA receptor-bearing ovarian cancer cells SKOV-3. The nanoparticles (HPSN-SS-CD) obtained without the doping of FA-F108 were used as a negative control. Both HPSN-SS-CD and FA-HPSN-SS-CD were labelled with tetramethylrhodamine isothiocyanate (TRITC) and then incubated with the cells to compare their cellular internalization capability. As observed from the confocal images, the intracellular fluorescence of SKOV-3 incubated with TRITC labelled FA-HPSN-SS-CD was apparently stronger than that of TRITC labelled HPSN-SS-CD (Figure 2c). In addition, the flow cytometric analysis shows that the median TRITC fluorescence intensity of the cell incubated with FA-HPSN-SS-CD after 1, 4 and 24 h was 2.5, 3.2 and 3.7 times higher than its FA-free analogue, respectively. Upon the incubation with the FA-modified NDS, the percentage of the cells with fluorescence intensity above 10^3 also overwhelmed that of the cells incubated with FA-free NDS, further demonstrating the receptor-mediated endocytosis of FA-HPSN-SS-CD by SKOV-3 cells (Figure 2d). Confocal z-scan results further verified the internalization of the nanocarriers into the cytoplasm of SKOV-3 cells (Figure S4). The better cellular uptake efficiency reveals that the FA-modified NDS is adequate for tumor targeting. The *in vitro* cancericidal effect was then performed (Figure 2e). The SKOV-3 cells were incubated with Dox-loaded NDS or free Dox for 6 h, followed by treating with fresh Dulbecco's Modified Eagle's Medium for another 24 h. Comparing with the Dox loaded in NDS, free Dox shows the lowest half maximal inhibitory concentration (IC_{50}) of $0.9 \mu\text{g mL}^{-1}$, which may be due to faster diffusion rate of free drug than the loaded drug. For the Dox loaded in NDS, Dox@FA-HPSN-SS-CD exhibits obviously higher cytotoxicity ($IC_{50} = 1.3 \mu\text{g mL}^{-1}$) than Dox@HPSN-SS-CD ($IC_{50} = 2.8 \mu\text{g mL}^{-1}$). On the other hand, when the FA receptors on the cells were pretreated by the FA incubation, the obtained cell viability and IC_{50} of Dox@FA-HPSN-SS-CD and Dox@HPSN-SS-CD were very similar ($2.7 \mu\text{g mL}^{-1}$, Figure 2f), indicating that the receptor-mediated endocytosis plays an important role for the uptake of NDS. Human hepatocyte cell LO2 with a low level of intracellular GSH was also employed for the cell viability assay (Figure S5). Owing to the delayed release rate under low concentration of GSH in LO2 cells, Dox@FA-HPSN-SS-CD shows an IC_{50} of $4.1 \mu\text{g mL}^{-1}$, which is 1.5-folds higher than the IC_{50} value for SKOV-3 with the FA pretreatment.

2.3. Biodistribution and IAM targeting of NDS

2.3.1. *In vivo* biodistribution of NDS

Mice with IAM as animal models were built to study the *in vivo* performance of the NDS. As a special tumor model, the intra-abdominal metastases are in many ways different from the subcutaneous transplants, and more similar to the actual case. The IP injection of SKOV-3 cells was adopted to simulate the seeding and spreading process of ovarian cancer, and the obtained lesions randomly root at the intra-abdominal organs. In contrast, traditional tumor xenografts only employ subcutaneous implantation of a certain cell line to build a solid tumor mass at a designed position. The pathogenesis, tumor morphology, and surrounding biological environment differ a lot from the practical tumor.

Fluorescent dye Cy5.5 was covalently labelled on FA-HPSN-SS-CD for tracing their biodistribution. The two administration routes, IP and IV injections, were parallelly conducted for comparison. Invasive abdomen imaging was performed by laparotomizing the subjected mice at designed time, and *ex vivo* imaging of digestive tract and main organs (heart, liver, spleen, lung and kidney) were then carried out (Figures S6-S8). The red fluorescence (RFL) from Cy5.5 outlines the biodistribution of FA-HPSN-SS-CD after the injection. We previously reported HPSN without FA and β -CD modifications as drug carriers and investigated their biodistribution.^[16] For FA-HPSN-SS-CD with similar size and structure, the metabolic pathway upon IV injection was very similar to HPSN. The imaging results show that the NDS mainly concentrated in liver, gallbladder and intestine, and the RFL intensity collected from blood decreased sharply in the first 24 h (Figure S9), indicating the NDS was cleared quickly through the hepatobiliary route and excreted along with feces.^[18a] After IP injection with FA-HPSN-SS-CD, the RFL was observed from the lining of the abdomen and the mesenterium (Figure S7, indicated by arrow) of mice in the first 24 h post injection. Small molecule compounds would be predominantly absorbed by the portal vein system and partially drained by splanchnic veins after the IP administration in order to pass the peritoneal-plasma barrier. As for the present nanocarrier, the bioimaging of the blood harvested from the IP injected mice showed significant RFL signal, indicating that the nanocarrier could also be absorbed by the capillary vessels from the portal system of peritoneum for getting into the blood circulation.^[21] Similar to the IV administration, the IP injected NDS could also be excreted *via* hepatobiliary pathway, while the retention time in blood was prolonged due to the delay by the peritoneal absorption process (Figure S6).

2.3.2. Distribution of IAM in intraperitoneal organs

As IAM can scatter among the peritoneum and guts, the metastases cannot be simply recognized by naked eye. In order to discriminate the IAM from normal tissue, SKOV-3-Luc cells were used to build the animal model. After IP injection of luciferin, the green bioluminescence (GBL)

generated from the luciferase-expressing tumor cells could be utilized to visualize the IAM and quantify their sizes. The GBL signal could explicitly indicate the location of the IAM and outline their shapes. It can be concluded from Figures S6-S8 that the IAM mainly sprouted at the pancreas, and a minor portion distributed over the surface of intestine and mesenterium. As observed from Figure S8, some of the metastases also randomly rooted on the intra-abdominal organs such as liver (4, 24, 72, and 120 h IP, as well as 4 and 120 h IV), spleen (24 and 120 h IP, as well as 24 h IV) and kidney (24 h IP). Except for the IAM on pancreas, the sizes of all other metastases were less than 2 mm, which were too immature to be well-vascularized. Although the overall size of the metastases on pancreas was big enough, they could possibly be the aggregates of smaller nodes, and the biological barrier in pancreas could greatly inhibit the infiltration of nanoparticle carriers from blood vessels to pancreatic tumor.^[22] Therefore, even if the IV administrated HPSN is capable of targeting subcutaneously inoculated tumor via the EPR effect,^[16] such method is unsuitable for the IAM without vascularization (Figure 3a). Immunofluorescence staining of FITC-anti-CD-31 was employed to visualize well-developed endothelial cells of blood vessels from the subcutaneous xenograft of SKOV-3 (Figure 3b). By contrast, the IAM shows almost no green fluorescence, confirming the non-vascularization nature of the early-stage metastases.

2.3.3. Comparing *in vivo* tumor targeting efficiency between IP and IV administration

The co-localization between GBL from the metastases and RFL from the NDS was used to determine whether the NDS was internalized by the tumor or not. The tumor uptake of NDS by the two administration routes, IP and IV, was thereby compared. Figure 3c shows the typical bioimaging of mice at 24 and 72 h post administration of FA-HPSN-SS-CD. As shown in the images, significant overlapping of GBL and RFL was observed for the IP injection group. Strong RFL could be detected at from most regions with BL. The co-localization area shown in the black-and-white image matches well with the shape of BL, which is a clear proof that the FA-HPSN-SS-CD nanocarrier could actively target the IAM after IP injection. It is noteworthy that the significant RFL detected from the gallbladder (encircled in Figure 3c) should be attributed to the hepatobiliary excretion of the IP injected nanocarrier rather than FA-mediated targeting. Moreover, the statistical result of Pearson correlation coefficients also reveals high correlation between GBL and RFL (Figures 3d and S6-S8). By contrast, no co-localization of GBL and RFL could be found from the IV injection group, suggesting that EPR effect completely failed in the present metastatic tumor model. Not surprisingly, the calculated Pearson correlation coefficients for IV group indicate almost no correlation between GBL and RFL. Thus, the co-localization results confirm that IP administrated NDS could successfully

overcome the biological barrier of IAM. From the confocal microscopic images of the IAM section, the fluorescence of Cy5.5 was detected from both the surface and interior of the tumor tissue at 24 h post IP injection, revealing good permeability of the NDS. The surface of the metastases showed more fluorescence than their inner parts, indicating that the infiltration of the nanocarrier to the metastases was time-dependent. No fluorescence was observed from the metastasis section of the IV injected mice, which was in line with the bioimaging results (Figure 3e).

2.4. In vivo chemotherapy using NDS as drug carriers

2.4.1. Comparing curative effect of IP and IV administrated NDS

The in vivo chemotherapy assessment further demonstrated the feasibility of the IP injection as an administration route for the treatment of IAM. Upon the IP injection, the NDS can actively target the metastatic nodules, internalized by the tumor cells through FA receptor-mediated endocytosis, and release the drug under intracellular environment with high-concentration of GSH (Figure 4a). The GBL from SKOV-3-Luc cells could be utilized to estimate the progression of metastases. Four groups of mice were used to demonstrate the advantages of IP administrated drug-loading NDS for the treatment of IAM. The blank control group of mice was IP injected with saline. The mice in the treatment group were IP injected with 0.2 mL solution containing 10 mg mL⁻¹ Dox@FA-HPSN-SS-CD (equivalent of 0.93 mg mL⁻¹ Dox). The mice IV injected with same amount of Dox@FA-HPSN-SS-CD and IP injected with 0.2 mL of free Dox solution (0.93 mg mL⁻¹) were used as two negative control groups. It was clearly observed that, for the treatment group with IP injected NDS, the GBL decreased explicitly as compared with other three groups (Figure 4b). Statistical data reveal that both the luminescent area (Figure 4c) and intensity (Figure 4d) shrank remarkably. At the 10th day, the luminescence area and intensity for the IP injected group was only 16% and 5% when compared with the blank control, demonstrating excellent therapeutic outcome of the IP administrated NDS for IAM. For the IV injected group, although the average values of luminescence area and intensity are 10-15% lower than that of the blank control, no statistical difference could be obtained at either the 4th day or 10th day. The representative tumor tissues from the IAM of the mice stained by hemotoxylin and eosin (H&E) also show similar results, where massive cell necrosis and apoptosis could only be found from the group with IP administrated Dox@FA-HPSN-SS-CD (Figure 4e).

2.4.2. Advantages of IP administrated NDS over free drug

As a clinically applied treatment approach, IP injected free Dox also works for IAM. However, given the same dose and administration frequency, the NDS-formulated Dox reveals better

therapeutic efficacy. This improvement could be ascribed to longer retention time in the peritoneum of the NDS. The pharmacokinetics of free Dox and Dox@FA-HPSN-SS-CD inside the peritoneum after IP injection were semi-quantitatively studied by the *in vivo* bioimaging of fluorescence from Dox (Figure S10). The fluorescence of free Dox was already feeble at 24 h, and almost extinct at 48 h, indicating that this small-molecule drug was quickly absorbed by the peritoneum.^[9] By contrast, Dox@FA-HPSN-SS-CD with a nano-formulation could retain inside the peritoneum for longer than 48 h, which was in good agreement with the bioimaging results of Cy5.5-labelled NDS. In addition, free Dox is lack of targeting capability, while the NDS with active targeting groups and responsive release triggers could improve the bioavailability of Dox and simultaneously reduce the systematic toxicity. The histopathological analysis was studied by H&E staining of the main organs of mice IP injected with saline, Dox (10 mg kg⁻¹ d⁻¹), and Dox@FA-HPSN-SS-CD with the same exposure dose of free Dox. The obtained images (Figure S11) of heart, liver, spleen, lung and kidney indicate significant toxicity of IP injected free Dox. For example, karyolysis and karyopyknosis were observed in myocardial cells, accompanied with myocardial fibrosis. The liver tissues show swelled and necrotic hepatocyte, amyloidosis as well as the appearance of eosinophilic bodies. The shrinkage of lymphoid follicle along with the formation of cavities was found in the spleen. In addition, the kidney also characterizes by increased glomerulus volume and swelled renal tubular epithelial cells. By contrast, when IP injected to the mice, the Dox-loading nanocarriers merely lead to a fraction of degenerated liver cells, demonstrating that the systemic toxicity of IP chemotherapy could be decreased by formulating the drug in the nanocarriers. The body weights of the mice in each group were recorded and shown in Figure S12. Under the same dose, the weights of mice IP injected with free Dox were ~11% less than that of the IP injected Dox@FA-HPSN-SS-CD group at the 10th day of treatment, implying that the NDS could relieve the side effect of the loaded Dox.

3. Conclusions

In this study, we chose IAM as the tumor model, and designed a smart NDS to address the limitations in current EPR-based antitumor approach. In our strategy, IP injection was employed as an important part of the treatment package. The NDS bearing targeting groups and stimulus-responsive release trigger was developed. As revealed by *in vivo* bioimaging, the NDS could successfully seek out the disseminated IAM in the peritoneum. The implementation of IP chemotherapy using Dox-encapsulated NDS also exhibited excellent therapeutic outcome, which was much better than free Dox due to longer intraperitoneal retention time and site-

specific release pattern of the NDS. In stark contrast, when the NDS was IV administrated as in the conventional EPR strategy, it cannot steer by the biological barrier of the IAM, and also failed to suppress the progression of IAM in the chemotherapy. This work would provide an NDS-based strategy with potential clinical value in the combat against intraperitoneal metastases, thereby pushing the nanomedicine one more step forward to clinical applications.

4. Experimental

Synthesis of FA-HPSN-SH: FA-HPSN-SH was synthesized using a modified protocol according to our previous reported HPSN.^[16] F108 (0.225 g), FA-F108 (0.025 g) and KCl (0.112 g) were mixed with HCl solution (7.5 mL, 2M). The mixture was stirred for 1 h to form a transparent solution. Then, cyclohexane (240 μ L) was added under sonication and stirred for another 1 h. Tetraethyl orthosilicate (268 μ L) was then added. After 2 h, (3-mercaptopropyl)trimethoxysilane (20 μ L) was added. The mixture was kept stirring for another 2 h. Afterwards, dimethyldimethoxysilane (80 μ L) was added. The reaction was continued for another 24 h, and the product was dialyzed 4 times in a membrane with molecular weight cut-off (MWCO) of 25,000 in deionized water. Cyclohexane was removed at 50 °C under vacuum. The solution was filtered by a 0.2 μ M membrane filter before further application or characterizations. The concentration of the obtained FA-HPSN-SH was determined by freeze-drying the solution in a weighed plastic centrifuge tube.

Modification of disulfide bond-linked Ada to FA-HPSN-SH to form FA-HPSN-SS-Ada: FA-HPSN-SH (5 mL, 15 mg mL⁻¹) was mixed with ethanol solution (5 mL) containing disulfide bond-linked Ada (0.5 mg mL⁻¹, see Supporting Information for the synthesis details of compound **1**). After stirring for 24 h, the mixture was dialyzed in water/ethanol solution (400 mL 1:1 (v/v)) for 12 h for 3 times, and then dialyzed in deionized water (500 mL) for 5 times to obtain an aqueous solution of FA-HPSN-SS-Ada.

Loading of Dox into FA-HPSN-SS-Ada followed by capping with β -CD to form Dox@FA-HPSN-SS-CD: Anticancer drug Dox was dissolved in aqueous solution (8 mL) with FA-HPSN-SS-Ada, and then PBS (10X, 1 mL, pH = 8) was added. The solution was stirred overnight. Aqueous solution (1 mL) of β -CD was added to cap the Ada unit on Dox@FA-HPSN-SS-Ada to form Dox@FA-HPSN-SS-CD. The final concentration of Dox, FA-HPSN-SS-Ada and β -CD in the mixed solution was 2 mg mL⁻¹, 10 mg mL⁻¹, and 1 mg mL⁻¹, respectively. Afterwards, the solution was dialyzed for 5 times inside a membrane with MWCO of 25,000 in PBS (500 mL, pH = 7) to remove excessive Dox and β -CD. The final loading capacity of Dox in FA-HPSN-SS-CD was determined as 9.3% using fluorescence spectrophotometry.

Preparation of FA-HPSN-SS-CD, HPSN-SS-CD, and Dox@HPSN-SS-CD: FA-HPSN-SS-CD was prepared with the same protocol as Dox@FA-HPSN-SS-CD without the Dox loading. HPSN-SS-CD and Dox@HPSN-SS-CD were prepared using F108 (0.25 g) as the surfactant instead of the mixture of F108 and FA-F108. Other procedure was the same as the preparation of FA-HPSN-SS-CD and Dox@FA-HPSN-SS-CD.

Ovarian cancer peritoneal metastasis model: To simulate peritoneal metastasis status of ovarian cancer, SKOV-3/Luc cells (1×10^5) were injected intraperitoneally into female BALB/c mice (4-week-old). The imaging and therapy experiments were performed 3 weeks after the injection.

Bioimaging: Aqueous dispersion (0.2 mL) of Cy5.5-labeled nanocarriers with a concentration of 10 mg mL^{-1} was intraperitoneally or intravenously injected into mouse models expressing the ovarian cancer peritoneal metastasis. At designed time, relevant substrate luciferin (0.2 mL, 15 mg mL^{-1}) was administrated intraperitoneally to the mice. After 20 min, the mice were sacrificed and laparotomized, and then the GBL (full-wavelength) and fluorescence imaging were carried out. Fluorescent signals from Cy5.5-labelled nanocarriers were collected with a continue-wavelength 635 nm laser (MW-GX-635, Changchun Laser Optoelectronics Technology Co., Ltd.) as the excitation source and Andor DU897 EMCCD (with a bandpass filter from 695 to 770 nm) as the collector.

In vivo therapy experiments: The ovarian cancer peritoneal metastasis models were randomly separated into 4 groups ($n = 8$). For intraperitoneal injection group, aqueous dispersion (0.2 mL) of Dox@FA-HPSN-SS-CD with a concentration of 10 mg mL^{-1} was intraperitoneally injected to each mouse. For intravenous injection group, aqueous dispersion (0.2 mL) of Dox@FA-HPSN-SS-CD with a concentration of 10 mg mL^{-1} was intravenously injected to each mouse. For free Dox group, Dox in PBS solution (0.2 mL, 0.93 mg mL^{-1}) was intraperitoneally injected to each mouse. For the control group, saline (0.2 mL) was intraperitoneally injected to each mouse. At the 4th day, mouse models were sacrificed and subjected for GBL imaging as described above. The injection was repeated at the 5th day to the surviving mice, and at the 10th day, the mice were all sacrificed for GBL imaging. The integral intensity of GBL was recorded to compile statistics.

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Figure 1. Characterization and drug release process of the hybrid nanocarrier. (a) Schematic illustration for the synthesis of Dox@FA-HPSN-SS-CD. TEM images of (b) FA-HPSN-SH, (c) FA-HPSN-SS-Ada and (d) Dox@FA-HPSN-SS-CD. Hydrated size of (e) FA-HPSN-SH, (f) FA-HPSN-SS-Ada and (g) Dox@FA-HPSN-SS-CD measured by DLS.

Figure 2. *In vitro* controlled release and cell targeting experiments. (a) Scheme of GSH triggered release by redox-responsive cleavage of the disulfide bond. (b) Dox release profiles from Dox@FA-HPSN-SS-CD in PBS under different GSH concentrations and different pH values. (c) Confocal images of SKOV-3 cells incubated with TRITC labelled FA-HPSN-SS-CD (left column) or HPSN-SS-CD (right column), the scale bars are 20 μ m. (d) Flow cytometric results of SKOV-3 cells incubated with TRITC labelled HPSN-SS-CD (blue) or FA-HPSN-SS-CD (red) for 1, 4, and 24 h. The numbers indicate the median fluorescence intensity of TRITC for each sample. *In vitro* cell viability of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for (e) SKOV-3 cells incubated with free Dox, Dox@HPSN-SS-CD or Dox@FA-HPSN-SS-CD, and (f) SKOV-3 cells incubated with free Dox, Dox@HPSN-SS-CD or Dox@FA-HPSN-SS-CD after the FA pretreatment.

Figure 3. Comparing the bioavailability of IP and IV administrated NDS by *in vivo* bioimaging. (a) Scheme of why intravenously administrated nanoparticles can work for mature tumor with vascularization by EPR effect but cannot cross the biological barrier to reach the early stage metastases. (b) Confocal images of tumor section from a subcutaneous xenograft (upper) and intraperitoneal metastases (lower) derived from SKOV-3 cells (scale bar = 100 μ M). The nuclei

and vessels were stained by 4',6-diamidino-2-phenylindole (DAPI) and FITC-anti-CD31, respectively. (c) *Ex vivo* imaging of digestive tract and main organs of IAM-bearing Balb/c mice after the injection of Cy5.5-labelled FA-HPSN-SS-CD for 24 and 72 h through IP or IV administration route (scale bar = 2 cm). The green color indicates the GBL of luciferin (full wavelength received), and the red color in the second column is from the RFL of Cy5.5 ($\lambda_{\text{Ex}} = 635 \text{ nm}$, $\lambda_{\text{Em}} = 695\text{-}770 \text{ nm}$). The regions with GBL signals are framed and enlarged, and the co-localized regions of GBL and RFL are shown in the black-and-white images. The dash circles indicate the gallbladders of the IP injected group. (d) Statistical data of Pearson correlation coefficient of abdomen, digestive tract and organs were analyzed to quantify the overlapping level of GBL and RFL. (e) Confocal images of the metastasis tissue section from mice administrated with Cy5.5 labelled FA-HPSN-SS-CD after 24 h ($\lambda_{\text{Ex}} = 633 \text{ nm}$, $\lambda_{\text{Em}} = 660\text{-}745 \text{ nm}$, scale bar = 20 μM).

Figure 4. Chemotherapy results of mice with IAM. (a) Tumoricidal mechanism for the NDS after IP administration to mice with IAM. When injected into the peritoneum, the NDS could get direct contact with the tumor cells, internalized by receptor-mediated endocytosis, and release the loaded Dox under the intracellular GSH to kill the cancer cells. (b) *Ex vivo* imaging of digestive tract and main organs of mice (scale bar = 2 cm). The framed regions are uniformly enlarged by 4 times and shown in the right side. Statistical data of (c) integrated luminescent

area and (d) integrated luminescence intensity of the GBL from the images of each group (n = 4) to semi-quantify the therapeutic effect (*: $P < 0.05$ vs the control group, **: $P < 0.01$ vs the control group, +: $P < 0.05$ vs IP injected Dox@FA-HPSN-SS-CD group, ++: $P < 0.01$ vs IP injected Dox@FA-HPSN-SS-CD group). (e) Histopathology of tumor tissues by H&E staining after 10 days of treatment (scale bar = 200 μ M).

As the common tumor models, xenografts are quite different from clinical tumors. Herein, intra-abdominal metastases are employed as tumor models, and a tailor-made delivery system is intraperitoneally administrated for the tumor treatment. Comparing with traditional intravenous injection, the intraperitoneal administration leads to superior therapeutic outcome.

Keyword: enhanced permeability and retention, intra-abdominal metastases, intraperitoneal injection, nanocarrier, stimuli-responsive release

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Independent of EPR Effect: A Smart Delivery Nanosystem for Tracking and Treatment of Nonvascularized Intra-Abdominal Metastases