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Protein-Gold Clusters-Capped Mesoporous Silica Nanoparticles for High Drug Loading, Autonomous Gemcitabine/Doxorubicin Co-Delivery, and In-vivo Tumor Imaging

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Abstract: Functional nanocarriers capable of transporting high drug contents without premature leakage and to controllably deliver several drugs are needed for better cancer treatments. To address this clinical need, gold cluster bovine serum albumin (AuNC@BSA) nanogates were engineered on mesoporous silica nanoparticles (MSN) for high drug loadings and co-delivery of two different anticancer drugs. The first drug, gemcitabine (GEM, 40 wt%), was loaded in positively-charged ammonium-functionalized MSN (MSN-NH₃⁺). The second drug, doxorubicin (DOX, 32 wt%), was bound with negatively-charged AuNC@BSA electrostatically-attached onto MSN-NH₃⁺, affording highly loaded pH-responsive MSN-AuNC@BSA nanocarriers. The co-delivery of DOX and GEM was achieved for the first time via an inorganic nanocarrier, possessing a zero-premature leakage behavior as well as drug loading capacities seven times higher than polymersome NPs. Besides, unlike the majority of strategies used to cap the pores of MSN, AuNC@BSA nanogates are biotools and were applied for targeted red nuclear staining and in-

vivo tumor imaging. The straightforward non-covalent combination of MSN and gold-protein cluster bioconjugates thus leads to a simple, yet multifunctional nanotheranostic for the next generation of cancer treatments.

1. Introduction

Multifunctional nanocarriers highly loaded with therapeutics and endowed with zero-premature leakage and controlled delivery features are expected to benefit millions of patients suffering from cancer worldwide.[1-3] Indeed, many cancer chemotherapeutic treatments still face low efficacies and failure despite tremendous financial investments of companies and governments. One of the main causes for this major societal concern is the lack of selectivity of anticancer drugs towards cancer tissues, leading to severe side effects on healthy tissues and eventually the death of patients.[4] This lack of spatial selectivity requires a high dose of drug to eradicate a tumor, which in turns limits the extent of the treatment that a patient can undergo before reaching a fatal dosage. Often, then, the treatment must be discontinued before the disappearance of the tumor, leaving the patient with little hope of remission. Additionally, cancer cells can become drug resistant after a prolonged treatment with a given drug.[5] As a result, the so-called nanomedicine has been an extensive research area aiming to design nanomaterials which could transport and deliver drugs, or other therapeutic agents,[6] selectively into tumors in order to decrease and preclude the side effects of chemotherapy, to decrease the dosage of drugs, and potentially transport several drugs to harness synergistic effects.[7, 8]

Drug delivery nanosystems capable of transporting multiple drugs are emerging for the treatment of cancers.[9-12] This strategy is based on the fact that chemotherapy was proved more effective for various pathologies when two drugs were combined.[13, 14] Combination chemotherapy using doxorubicin (DOX) and gemcitabine (GEM) simultaneously has produced better results than individual drugs,[15, 16] as the former causes both severe tissue necrosis and side effects,[17] and the latter has a short in-vivo half life (8-17 min).[18] Due to different mechanisms of toxicity, this drug combination produces synergistic cancer cell killing, but remains significantly toxic for healthy tissues, hence justifying the need for a multi-

drug delivery system.[19] DOX-loaded liposomes were clinically tested in combination with free GEM leading to promising results in patients.[20] Very few studies have reported the co-delivery of DOX and GEM in all areas of nanomaterial science. A polymeric carrier based on a copolymer of hydroxypropylmethacryl-amide encapsulated 6 weight percent (wt%) of each drug.[21] The system was applied in-vivo, but it should be noted that 25 % of the cargo was prematurely released after one day as the polymer entanglement could not prevent drug leakage. In 2014, vesicles of disulfide linked polyethyleneglycol-poly(lactic-acid) (PEG-PLA) loaded with DOX and GEM,[19] and a prodrug design of a biodegradable PEG-PLA covalently linking DOX and GEM,[22] were applied in-vitro. Both studies demonstrated the potential of their carriers, nonetheless, the leakage at neutral pH was severe (20 and 40 % of GEM and DOX loaded-cargos respectively).[22] To date, a robust carrier co-delivering DOX and GEM drugs in high content has not been achieved.

Among recent developments in the area of smart nanomaterials,[3, 23-25] mesoporous silica nanoparticles (MSN) have attracted much attention as one of the most promising nanoplatforms for nanomedicine.[26-34] MSN are biocompatible nanomaterials[35, 36] and possess distinctive characteristics such as their tunable porosity and particle size, versatile inner and outer surface chemistry, and capacity to transport and deliver various cargos make these scaffolds ideal drug delivery systems.[37, 38] Both molecular and supramolecular nanomachines have been designed with MSN to precisely control the release of guest molecules from their nanopores upon various stimuli including pH,[39, 40] one-[41, 42] and two-photon irradiation,[43-45] enzymes[46, 47] and redox processes.[48] Capping the pores of MSN was also carried out with inorganic particles,[49, 50] dendrimers,[51] and covalently-attached proteins.[52-54] However, few studies have described multi-drug delivery via MSN. Lin *et al.* demonstrated the co-delivery of insulin and cyclic adenosine monophosphate in a sequential manner.[55] Insulin molecules were grafted on the MSN surface capped with a glucose-responsive system, while the pores were loaded with the second cargo. Palanikumar and coworkers very recently demonstrated that the loading capacity of MSN could reach 80 wt% through higher drug concentrations in loading solutions,[56, 57] unlike the typical 10 wt% threshold of previous reports. Building on this discovery, they loaded

two[56] or three[57] different drugs and capped MSN with a glutathione-responsive polymer for co-delivery. The synergistic co-delivery of GEM and paclitaxel *in-vivo* via MSN for pancreatic cancer treatment was very recently reported as well.[58] MSN nanocarriers thus appear excellent candidates for the co-delivery of high contents of DOX and GEM drugs with zero premature release.

Gold nanoclusters (AuNCs) bioconjugated with proteins are highly attractive for bio-imaging as they are easily and reproducibly prepared, biocompatible, and have an intense red-NIR emission with high photo-stability, long fluorescence lifetime, and two photon excitation.[59] Commercially available bovine serum albumin (BSA) proteins were used to reduce gold salts and stabilize AuNCs composed of 25 gold atoms, affording highly biocompatible AuNC@BSA bioconjugates.[60, 61] The hydrodynamic diameter of AuNCs is typically below the kidney filtration threshold (~ 5.5 nm), which allows an efficient renal clearance after the intended biomedical application.[62] Controlling the size of AuNCs is also critical as it highly influences their fluorescence properties.[63] When compared to gold nanoparticles (AuNPs),[64] AuNCs-proteins have proved to be safer and more reliable imaging probes.[65] Interestingly, few reports have recently described the unique nuclei staining properties of AuNCs,[66, 67] which could allow the study of important biological processes such as mitosis.[67] This nuclei targeting is also the origin of several drug delivery studies,[68-71] such as DOX delivery via AuNC@BSA combined with two-photon imaging.[72] AuNCs were also covalently grafted on non-porous silica NPs for cytosol imaging and reactive oxygen species detection via the quenching of the gold fluorescence,[73] however, AuNCs were not combined with MSN.

In this study, we describe a theranostic nanocomposite combining for the first time MSN and AuNCs encapsulating an unprecedented high amount of DOX (32 wt%) and GEM (40 wt%) drugs for pH-triggered autonomous multi-drug delivery. Negatively-charged AuNCs@BSA electrostatically-attached on ammonium-functionalized MSN to effectively block the pores (unlike two previous systems with covalently-attached MSN-BSA with the multi-step methods[53, 54]), and provided a pH-responsiveness for cargo release and delivery. The interaction of DOX with BSA proteins was found to induce the formation of a highly loaded shell around GEM-loaded MSN NPs leading to exceptionally high payloads

(Figure 1). The uniqueness of the MSN-AuNC@BSA drug delivery system is summarized as follows: (1) The preparation of nanocarriers is straightforward as it requires non-covalent protein-MSN electrostatic interactions. (2) The system was however remarkably robust in blood serum with less than 3 % of drug leakage after one week thanks to the dense coating of BSA, which was also selected as it reduces the immunotoxicity of MSN.[74] (3) The dual loading of DOX and GEM had a total of 72 wt%, which is much higher than reported liposomes (7+3 wt%)[22] and polymers (6+6 wt%),[21] and forms an ideal drug combination for the treatment of ovarian and breast cancers.[15] (4) Unlike the loading of several drugs inside MSN, the spatial segregation of one drug into the pores of MSN, and a second drug inside the AuNC@BSA layer, differs substantially. It is also most-likely best suited for higher and more controlled cargo loadings, since for dual-loaded MSN, the first loading may interfere with or limit the second. (5) Dual drug release experiments and the DOX and GEM multi-drug delivery are reported for the first time via an inorganic carrier, with a nearly total cancer cell killing. (6) Unlike previous capping design onto MSN, our AuNC@BSA nanogates were applied not only to provide a novel and secondary mean of drug loading, but also for nuclear staining ($\lambda_{em\ MAX}= 645\text{ nm}$) and in-vivo tumor dual imaging with hoechst (HOE, $\lambda_{em\ MAX}= 461\text{ nm}$)-loaded MSN-AuNC@BSA suggesting a preferential tumor accumulation.

Figure 1. Self-assembly of fluorescent negatively charged AuNC@BSA with positively charged DOX-loaded MSN-NH₃⁺ leading to DOX-loaded MSN-AuNC@BSA carrier (A). Self-assembly of fluorescent negatively charged AuNC@BSA and DOX with positively charged GEM-loaded MSN-NH₃⁺ leading to MSN-AuNC@BSA carrier loaded with two drugs (B), and which could be disrupted by and acidic pH trigger (C). Applications of multifunctional dual drug-loaded MSN-AuNC@BSA nanocarriers (D).

2. Results and Discussion

2.1 Preparation of MSN-AuNC@BSA nanocarriers

MSN were prepared in a typical sol-gel process involving the hydrolysis and condensation of tetraethoxysilane (TEOS) in a basic aqueous solution of cetyltrimethylammonium bromide (CTAB) template at 80°C. Surface-functionalized amine-mesoporous NPs (MSN-NH₂) were then prepared by post-

grafting of aminopropyl-triethoxysilane (APTES) on MSN-CTAB, which were then extracted. This strategy allowed a controlled functionalization of the NP surface (see Scheme S1).[75] Transmission electron microscopy (TEM) micrograph depicted the 2D hexagonal array of the pores (Figure 2A), and NPs sizes close to 150 nm, as confirmed by scanning electron microscopy (SEM, see Figure S1). The organized long-ranged mesoporosity was evidenced by small-angle X-ray diffraction (XRD) patterns in MSN and MSN-NH₂ with the intense peak at 2 degrees, as the satellites between 4 and 5 degrees (Figure S2). The successful functionalization of MSN was validated by the Fourier transform infrared (FTIR) spectrum composed of the stretching vibration modes of aliphatic C-H bonds from 2950 to 2850 cm⁻¹ and the N-H bending at 1535 cm⁻¹ (Figure S3).

The protein-cluster bioconjugates were then prepared and characterized via various techniques. The synthesis of AuNC@BSA was carried out according to a reported method involving the reduction of gold (III) ions via BSA proteins in basic conditions for 12 h at 37 °C.[60] TEM micrograph displayed AuNC@BSA with an average diameter of 2.5 nm (Figs. S4-S5A), and dynamic light scattering (DLS) measurements provided slightly higher hydrodynamic diameter centred at 3.7 nm (Figure S5B). AuNC@BSA exhibited the absorption band of BSA ($\lambda_{\text{max}} = 390$ nm) arising from aromatic amino acids (tryptophan, tyrosine),[71] as well as the AuNCs plasmon band ($\lambda_{\text{max}} = 496$ nm, Fig S6). The strong broad emission of the clusters from the red to the NIR ($550 < \lambda_{\text{em}} < 800$ nm, $\lambda_{\text{max}} = 645$ nm) (Figure S6A).[60] The conjugation of BSA to the nanoclusters was confirmed FTIR analysis with the primary amine scissoring $\square$ $\square_{\text{N-H}}$ at 1538 cm⁻¹, the typical amide I $\square_{\text{C=O}}$ intense mode at 1659 cm⁻¹, as well as the $\square_{\text{N-H}}$ stretching mode at 3066 cm⁻¹ (Figure S7).[71]

The MSN-AuNC@BSA nanocarrier was then designed and fully characterized. The design principle of the system is illustrated in Figure 1B and Scheme S2. Negatively charged AuNCs@BSA were attached to the surface of positively charged MSN-NH₃⁺ by simple mixing in a neutral aqueous solution saturated in cargo molecules. Hence, guest molecules were trapped both within the porous channels of MSN as well as in the capping layer of AuNC@BSA. Several lines of evidence unambiguously showed the successful design of the MSN-AuNC@BSA nanocarriers. Firstly, TEM images of functionalized NPs displayed the

protein coating of MSN-NH₂ (Figs. 2B) and of DOX-loaded NPs (Figure S8). Secondly, the DLS hydrodynamic diameter increase is shown in Figure 2C. Thirdly, nitrogen-sorption measurements performed before and after the formation of the AuNC@BSA shell around cargo-free MSN showed the disappearance of the type IV isotherm with a sharp drop of the Brunauer-Emmet-Teller (BET) surface area, from 863 to 162 m².g⁻¹ (Figure 2D). The diameter of the pores of silica NPs was centered at 2.3 nm (Figure S9),^[76] which is lower than that of the gold-protein bioconjugates. Fourthly, the coloration of capped NPs was obvious (Figure 2E) and the inductively coupled plasma-optical emission spectroscopy (ICP-OES) quantification confirmed the presence of gold atoms (2.10 ± 0.23 wt% of gold, Figure 2F), which allows the calculations of a BSA content of about 16 wt% (7.6 wt% of gold are present in AuNC@BSA⁴⁰). Fifthly, the FTIR spectrum of MSN-AuNC@BSA displayed the presence of the carbonyl (1659 cm⁻¹) and the N-H amide I (1538 cm⁻¹) vibration modes of BSA (Figure S7).^[71] Note that, the decrease of intensity of the N-H bending (1384 cm⁻¹) from the amino-propyl groups of MSN-NH₂ is consistent with the electrostatic complexation of protein with NPs. Sixthly, the apparent zeta potential measurements validated the complexation of the gold-protein bioconjugates (-30 mV) with MSN-NH₃⁺ (+25), as shown by the resulting negative charge (-4 to -18 mV) of cargo-free and cargo-loaded MSN-AuNC@BSA (see Figure 2G).

Figure 2. TEM micrographs (A-B), DLS analyses (C), nitrogen sorption isotherms (D) of MSN-NH₂ (blue) and MSN-AuNC@BSA (red). Photographs of MSN-NH₂ and MSN-AuNC@BSA NPs powders (E). ICP-OES quantification of the gold content of MSN-AuNC@BSA, allowing the BSA content calculation (F). Zeta potentials at pH 7 confirming the Au@BSA functionalization of cargo-free and cargo-loaded MSN-NH₂ (protonated at pH 7) (G).

2.2 Preparation of Dual-Loaded MSN-AuNC@BSA

The loading of various cargos in the MSN-AuNC@BSA nanocarrier was then investigated. In order to find out if the AuNC@BSA shell could act as a reservoir for DOX drugs, we investigated two methods for the loading of cargos into MSN. The MSN-drug aqueous mixture could be either centrifuged or not before the AuNC@BSA addition. In the first scenario, only DOX-loaded MSN are reacting with gold-proteins bioconjugates, which would minimize the amount of DOX potentially penetrating the AuNC@BSA shell, affording MSN-DOX-AuNC@BSA. The second scenario, however, would maximize the DOX loading both in the MSN pores and in the gold-proteins shell (MSN-DOX-AuNC@BSA-DOX, see Figure 1A). In all cases, unbound AuNC@BSA were removed by selective centrifugation; cargo-loaded MSN-AuNC@BSA could easily be precipitated and unbound cluster-protein conjugates were removed with the supernatant. Photographs presented in Figure 3A and B respectively display the successful increase of DOX payloads when the MSN pores and the AuNC@BSA shell are combined as drug reservoirs. It can

also be seen that dispersed NPs (photographs on the left) are highly stable (for one month) and do not leak, as shown by the localized red color after centrifugation (photographs on the right). UV-visible spectroscopy clearly displayed the absorption band of DOX guests inside MSN-DOX-AuNC@BSA-DOX (Figure 3C), which possessed a much higher payload than MSN-DOX-AuNC@BSA (38 vs 12 wt%, calculated via analyses of drug supernatants: $[m_{\text{DRUG LOADED}}/(m_{\text{NPs}})]*100$). As a control, we mixed BSA and DOX overnight in neutral aqueous medium, centrifuged and washed the resulting sample, and then analyzed it by TEM. We observed the formation of polydisperse 200 to 400 nm objects (see Figure S10), and DLS depicted a broad size distribution centered at 365 nm (Figure S11). The presence of a high content of DOX (29 wt%) was also monitored via uv-visible spectroscopy ($\lambda_{\text{max}} = 494$ nm, Figure 3D), along with the BSA aromatic band ($\lambda_{\text{max}} = 390$ nm). Thus, MSN-DOX-AuNC@BSA-DOX has a fraction of drug content into the MSN pores and second fraction is contained in the cluster-protein shell. The drugs contained in MSN are trapped inside the pores with (e.g. DOX) or without electrostatic binding with silanolates,²⁴ while the drugs contained in the AuNC@BSA shell interact electrostatically with the BSA proteins. Yao *et al.* also proposed that the lipid-binding domain of BSA could form hydrophobic interactions with DOX.[77] Note that this strategy could be extended to other cargos, such as rhodamine B (RhoB) dyes (Figures S12-13), since many dyes, fatty acids, and drugs strongly bind BSA proteins.[78-80] Besides, we decided to take advantage of this unique feature to load different drugs into MSN-AuNC@BSA for multiple-cargo delivery. As depicted in Figure 1B, two types of cargo molecules could thus be encapsulated via a similar approach, loading first one cargo in the MSN pores, and then the second in the AuNC@BSA shell. We thus prepared MSN-GEM-AuNC@BSA-DOX nanocarriers with 40 wt% of GEM and 32 wt% of DOX, as well as HOE dyes-loaded NPs, MSN-HOE-AuNC@BSA-DOX with 45 wt% of HOE and 35 wt% of DOX (see Table S1). The loading of HOE/DOX is delineated alongside with GEM/DOX as it will be used as control to confirm the dual delivery of cargos (GEM cannot be visualized in cells). In-vivo studies described hereafter also necessitated the use of HOE to avoid the overlap of the DOX and AuNCs red fluorescence in order to track the nanoclusters in animal model for imaging purposes. The construction of dual loaded nanocarriers was supported by uv-visible (Figure S14) and

fluorescence spectroscopies (Figure S15-17), DLS (Figure S18), TEM (Figure S19), and zeta potential (Figures 2G and S20) characterizations. Zeta potentials were consistently negative for MSN-AuNC@BSA (-7 mV, as opposed to positively-charged MSN-NH₂), MSN-Cargo-AuNC@BSA (-5 to -8 mV), and MSN-Cargo-AuNC@BSA-Cargo (-24 to -39, see Figure S20). The zeta potential data thus strongly implies that the outer surface of Cargo-MSN-DOX-AuNC@BSA consists of the negatively-charged protein, positively-charged DOX being inside the AuNC@BSA shell. Note that given the extensively washings of NPs after loading, the DOX surface physisorption is insignificant. This is supported by the nearly-zero drug leakage of NPs described hereafter.

Figure 3. Photographs of DOX-loaded MSN-AuNC@BSA NPs with cargos solely in the pores (A) and in the pores and in the shell (B). Photographs support the high DOX loading capacities and the absence of leakage from NPs dispersions then centrifuged 5 minutes at 14 krpm (red arrows). Uv-visible spectra demonstrating the DOX loading in MSN-AuNC@BSA (B) and BSA (C).

2.3 Stability and pH-Triggered Release Studies

The stability of MSN-AuNC@BSA nanocarriers encapsulating various cargos was first studied. Both MSN-DOX-AuNC@BSA and MSN-DOX-AuNC@BSA-DOX nanocarriers were remarkably stable in

neutral deionized water and blood serum for 2 to 3 weeks with less than 0.17 wt% of drug leakage ($< 1\%$ of loaded cargos) (Figure S21-24). Similarly, GEM/DOX and HOE/DOX dual-loaded nanocarriers showed less than 2 % of loaded cargos leaking out in aqueous solution after two weeks (Figure S23), 1 to 3 % and 1 to 7 % after 7 and 15 days in blood serum (Figure 4 and S24). The slight leakage increase in the presence of serum results from the fact that this solution contains more molecules susceptible to interact with the electrostatically-assembled MSN-AuNC@BSA particles. Hence, the nearly zero leakages observed after few days in serum for dual-loaded NPs strongly suggest that both nanocarriers would remain stable in-vivo during the time necessary for their biomedical applications. At acidic pH, however, AuNCs@BSA become protonated (see zeta potentials in Figure S25) and thus detach from the MSN-NH₃⁺ ($\zeta = +25$ mV, Figure 2G) surface leading to the release of cargos (Figure 1C). This pH-responsiveness was then first demonstrated in MSN-DOX-AuNC@BSA and MSN-HOE-AuNC@BSA controls (Figure S26-27) displaying the progressive release of DOX and HOE at pH 5 (Figure 5A). Secondly, the dual cargo release experiments were performed via MSN-GEM-AuNC@BSA-DOX and MSN-HOE-AuNC@BSA-DOX nanocarriers (Figure 5B-E). The release of GEM/DOX and HOE/DOX combinations was triggered at pH 5 from the MSN-AuNC@BSA nanoplatform (see Figure 5B and D respectively). Uv-visible spectroscopic monitorings of the release of drugs unequivocally demonstrated the dual-release capability of the design carrier (Figure 5C,F). Note that, the nanocarriers did not open from pH 5.5 to 6.5 (data not shown) as the pK_A of BSA is around 4.8[77] and the charge of AuNC@BSA reaches zero only at pH 5 (Figure S25).

Figure 4. Stability study of cargo-loaded MSN-AuNC@BSA NPs in physiological serum. The cargos leakages are remarkably low in the designed carrier. Given the loading capacities of MSN-GEM-AuNC@BSA-DOX (40/32 wt%) and MSN-HOE-AuNC@BSA-DOX (45/35 wt%), the passive leakages correspond to less than 3 % of loaded cargos after one week, and to less than 6 % after two weeks.

Figure 5. Releases profile of single-loaded MSN-AuNC@BSA nanocarriers upon acidic stimulus (A). Release profile of dual-loaded MSN-AuNC@BSA with DOX with Gem drugs upon acidic stimulus (B), along with uv-visible spectroscopic monitoring data (C). Release profile of dual-loaded MSN-AuNC@BSA with DOX with Hoe cargos upon acidic stimulus (D), along with uv-visible spectroscopic monitoring data (E). In-vitro studies of cargo-free, DOX-loaded, and DOX-GEM-loaded MSN-AuNC@BSA NPs (from left to right) on lung cancer cells (F).

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2.5 Cell Studies of MSN-AuNC@BSA: DOX and GEM co-delivery and imaging

In vitro studies of MSN-AuNC@BSA nanomaterials were then performed on cancer cells. MSN-AuNC@BSA nanovehicles were biocompatible at least up to $100 \mu\text{g.mL}^{-1}$ in A549 lung cancer cells (Figure 5F). The internalization of NPs into cells and detachment AuNC@BSA was demonstrated in by confocal laser scanning microscopy (CLSM) images (Figure 6A). Cargo-free MSN-AuNC@BSA clearly induced the unique nuclear red staining properties of AuNCs, which likely occurred as NPs encountered lower pH inside cancer cells. Note that, the same conclusions of biocompatibility and nuclear staining were observed on HeLa cancer cells (Figures S28-29), and that covalently-labelled fluorescein-NPs were also the endocytosed in PANC-1 cells (Figure S30-31). The nuclear targeting feature of AuNC@BSA is uncommon, and given the interactions between BSA and many drugs,[78, 79] they could serve for nuclear-targeted drug delivery of specific therapeutics which would otherwise not reach this target. Therefore, AuNC@BSA not only deliver a high content of DOX around MSN but also provide imaging multifunctionality to the drug delivery system.

Drug delivery experiments were then carried out in A549 cancer cells. MSN-DOX-AuNC@BSA-DOX were efficiently killing cancer cells, with nearly no cell survival at $100 \mu\text{g.mL}^{-1}$. Investigations of the first co-delivery of GEM and DOX drugs via MSN then lead to potent killings $100 \mu\text{g.mL}^{-1}$ as well (Figure 5F), while at $25 \mu\text{g.mL}^{-1}$ the DOX/GEM dual drug delivery caused a lower cell survival than DOX-loaded NPs thanks to the growth inhibitory effects of GEM. We additionally performed a control experiment to confirm the dual cargo delivery capability of our carrier by incubating MSN-HOE-AuNC@BSA-DOX nanocarriers. As shown by Figure 6B, the co-delivery of DOX and HOE induces an intense nuclear staining in red and in blue, which supports the DOX and GEM co-delivery results.

Figure 6. CLSM images of A549 cells after incubation with cargo-free MSN-AuNC@BSA (A), and MSN-HOE-AuNC@BSA-DOX NPs (B), demonstrating the nuclear staining of AuNC@BSA and delivery of HOE dyes in single and double loaded nanocarriers. The blue emission (470 nm) corresponds to HOE dyes which stain cell nuclei. The red emission (600 nm) corresponds either to AuNC@BSA (A) or to AuNC@BSA and DOX nuclear stainings (B). Scale bars 50 μ m.

2.6 In-vivo preliminary study of MSN-AuNC@BSA for tumor imaging

In-vivo biodistribution and imaging studies were then conducted as proof of principle of the MSN-AuNC@BSA carrier multifunctionality. Human pancreatic cancer cells (MiaPaca-2) were subcutaneously injected into the right lateral back of the nude mice. Three weeks later, mice were injected via tail vein with Hoe-loaded MSN-AuNC@BSA suspended in PBS solution or PBS only as control. To track the AuNCs and Hoe in the mouse body, we took advantage of the red fluorescence of the clusters and the blue fluorescence of Hoe. Since the diameter of the nanocarriers was approximately 150 nm, we expected them to accumulate in tumors due to the enhanced permeability and retention effect. *In-vivo* imaging was performed via red and blue channels which displayed the bioaccumulation of Hoe and AuNC mainly in the tumor, and partly in the liver and in kidneys (see Figure 7). Afterwards, all mice were euthanized, all organs were collected and fluorescence analyses were carried via the Maestro 2 imaging system. Once

again, the fluorescence of AuNCs and Hoe was mainly detected in the tumor, and also in the liver and in kidneys (Figure 8), which clearly demonstrated the potential of the MSN-AuNC@BSA for tumor imaging.

Figure 7. Representative images of nude mice with MiaPaca-2 cells injected in the tail vein with saline (control group) (A), and Hoe-loaded MSN-AuNC@BSA (B).

Figure 8. Representative images of tumor tissues after saline (control group) (A) and Hoe-loaded MSN-AuNC@BSA injections (B). L: Liver; Lu: Lung; K: kidney; T: tumor; S: spleen.

3. Conclusions

We have described the first co-delivery of the gemcitabine and doxorubicin anticancer drugs via an inorganic nanocarrier. Our design involved the mesoporous silica nanoparticles encapsulating a high

content of gemcitabine (40 wt%) and electrostatically-gated with a gold cluster-protein shell acting as a reservoir for doxorubicin (32 wt%). Unlike the few reports on liposome and polymeric nanocarriers, our vehicle was remarkably robust since less than four percent of loaded drugs leaked out after one week in blood serum, and the drug payloads were four times higher. This smart nanocarrier autonomously opened at lysosomal pH to deliver the drug combination in cancer cells with a nearly total cell killing. Besides, conversely to most MSN drug delivery systems, these uniform nanocarriers can be simply prepared via mixing, harnessing diagnostic and therapeutic features of the MSN and AuNCs in a multifunctional theranostic nanoplatform. Furthermore, the strong fluorescence of the AuNC@BSA was found almost exclusively in the tumor, and slightly in the liver and kidneys, with the same trend with Hoe cargos. The designed nanocarrier thus combined the unique features of mesoporous silica and AuNCs for the straightforward preparation of a theranostic nanomedical device endowed with tumor diagnosis, especially relevant for the treatment of ovarian and breast cancers via combination therapy.

Conflict of interest

Authors declare to have no conflict of interest.

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References

- [1] J.A. Barreto, W. O'Malley, M. Kubeil, B. Graham, H. Stephan, L. Spiccia, Nanomaterials: Applications in cancer imaging and therapy, *Adv. Mater.* 23 (2011) H18-H40.
- [2] Y. Wang, M.S. Shim, N.S. Levinson, H.-W. Sung, Y. Xia, Stimuli-responsive materials for controlled release of theranostic agents, *Adv. Funct. Mater.* 24 (2014) 4206-4220.
- [3] J.G. Croissant, X. Cattoën, M. Wong Chi Man, J.-O. Durand, N.M. Khashab, Syntheses and applications of periodic mesoporous organosilica nanoparticles, *Nanoscale* 7 (2015) 20318-20334.
- [4] V.P. Chauhan, R.K. Jain, Strategies for advancing cancer nanomedicine, *Nat. Mater.* 12 (2013) 958-962.
- [5] A.L. Harris, D. Hochhauser, Mechanisms of multidrug resistance in cancer treatment, *Acta Oncologica* 31 (1992) 205-213.

- [6] J. Croissant, M. Maynadier, O. Mongin, V. Hugues, M. Blanchard-Desce, A. Chaix, X. Cattoën, M. Wong Chi Man, A. Gallud, M. Gary-Bobo, M. Garcia, L. Raehm, J.-O. Durand, Enhanced two-photon fluorescence imaging and therapy of cancer cells via gold@bridged silsesquioxane nanoparticles, *Small* 11 (2015) 295–299.
- [7] K.H. Bae, H.J. Chung, T.G. Park, Nanomaterials for cancer therapy and imaging, *Mol. Cells* 31 (2011) 295–302.
- [8] A. Khadair, D. Chen, Y. Patil, L.N. Ma, Q.P. Dou, M.P.V. Shekhar, J. Panyam, Nano particle-mediated combination chemotherapy and photodynamic therapy overcomes tumor drug resistance, *J. Control. Rel.* 141 (2010) 137–144.
- [9] Q. He, Y. Gao, L. Zhang, Z. Zhang, F. Gao, X. Ji, Y. Li, J. Shi, A pH-responsive mesoporous silica nanoparticles-based multi-drug delivery system for overcoming multi-drug resistance, *Biomaterials* 32 (2011) 7711–7720.
- [10] H.-C. Shin, A.W. Alani, D.A. Rao, N.C. Rockich, G.S. Kwon, Multi-drug loaded polymeric micelles for simultaneous delivery of poorly soluble anticancer drugs, *J. Control. Rel.* 140 (2009) 294–300.
- [11] H. Gong, L. Cheng, J. Xiang, H. Xu, L. Feng, X. Shi, Z. Liu, Near-infrared absorbing polymeric nanoparticles as a versatile drug carrier for cancer combination therapy, *Adv. Funct. Mater.* 23 (2013) 6059–6067.
- [12] T. Jiang, R. Mo, A. Bellotti, J. Zhou, Z. Gu, Gel–liposome-mediated co-delivery of anticancer membrane-associated proteins and small-molecule drugs for enhanced therapeutic efficacy, *Adv. Funct. Mater.* 24 (2014) 2295–2304.
- [13] J.S. Kwon, C. McGahan, U. Dehaeck, J. Santos, K. Swenerton, M.S. Carey, The significance of combination chemotherapy in epithelial ovarian cancer, *International journal of gynecological cancer* 24 (2014) 226–232.
- [14] M. Fassnacht, M. Terzolo, B. Allolio, E. Baudin, H. Haak, A. Berruti, S. Welin, C. Schade-Brittinger, A. Lacroix, B. Jarzab, Combination chemotherapy in advanced adrenocortical carcinoma, *N. Engl. J. Med.* 366 (2012) 2189–2197.
- [15] G. Perez-Manga, A. Lluch, E. Alba, J. Moreno-Nogueira, M. Palomero, J. Garcia-Conde, D. Khayat, N. Rivelles, Gemcitabine in combination with doxorubicin in advanced breast cancer: Final results of a phase ii pharmacokinetic trial, *Journal of clinical oncology* 18 (2000) 2545–2552.
- [16] T. Yang, C. Wang, R. Hsieh, J. Chen, M. Fung, Gemcitabine and doxorubicin for the treatment of patients with advanced hepatocellular carcinoma: A phase i–ii trial, *Annals of oncology* 13 (2002) 1771–1778.
- [17] F. Koinis, A. Polyzos, A. Christopoulou, Z. Zafeiriou, C. Emmanouilidis, E. Papadimitraki, A. Kalykaki, K. Kalbakis, G. Samonis, V. Georgoulas, Salvage chemotherapy with docetaxel and pegylated liposomal doxorubicin in pretreated patients with platinum- and taxane-sensitive ovarian cancer: A multicenter phase ii trial of the hellenic oncology research group (horg), *Cancer chemotherapy and pharmacology* 73 (2014) 819–825.
- [18] L.H. Reddy, P. Couvreur, Novel approaches to deliver gemcitabine to cancers, *Curr. Pharm. Des.* 14 (2008) 1124–1137.
- [19] R. Nahire, M.K. Halder, S. Paul, A.H. Ambre, V. Meghnani, B. Layek, K.S. Katti, K.N. Gange, J. Singh, K. Sarkar, Multifunctional polymersomes for cytosolic delivery of gemcitabine and doxorubicin to cancer cells, *Biomaterials* 35 (2014) 6482–6497.
- [20] V. Adamo, V. Lorusso, R. Rossello, B. Adamo, G. Ferraro, D. Lorusso, G. Condemi, D. Priolo, L. Di Lullo, A. Paglia, S. Pisconti, G. Scambia, G. Ferrandina, Pegylated liposomal doxorubicin and gemcitabine in the front-line treatment of recurrent/metastatic breast cancer: A multicentre phase ii study, *Br. J. Cancer* 98 (2008) 1916–1921.
- [21] T. Lammers, V. Subr, K. Ulbrich, P. Peschke, P.E. Huber, W.E. Hennink, G. Storm, Simultaneous delivery of doxorubicin and gemcitabine to tumors in vivo using prototypic polymeric drug carriers, *Biomaterials* 30 (2009) 3466–3475.

- [22] D. Liu, Y. Chen, X. Feng, M. Deng, G. Xie, J. Wang, L. Zhang, Q. Liu, P. Yuan, Micellar nanoparticles loaded with gemcitabine and doxorubicin showed synergistic effect, *Colloids and Surfaces B: Biointerfaces* 113 (2014) 158-168.
- [23] B. Li, M. Chrzanowski, Y. Zhang, S. Ma, Applications of metal-organic frameworks featuring multi-functional sites, *Coord. Chem. Rev.* 307 (2016) 106-129.
- [24] V. Malgras, Q. Ji, Y. Kamachi, T. Mori, F.-K. Shieh, K.C. Wu, K. Ariga, Y. Yamauchi, Templated synthesis for nanoarchitected porous materials, *Bull. Chem. Soc. Jpn.* 88 (2015) 1171-1200.
- [25] Y. Fatieiev, J. Croissant, S. Alsaïari, B.A. Moosa, D.H. Anjum, N.M. Khashab, Photoresponsive bridged silsesquioxane nanoparticles with tunable morphology for light-triggered plasmid DNA delivery, *ACS Appl. Mater. Interfaces* 7 (2015) 24993-24997.
- [26] B. Rühle, P. Saint-Cricq, J.I. Zink, Externally controlled nanomachines on mesoporous silica nanoparticles for biomedical applications, *ChemPhysChem* (2016) DOI: 10.1002/cphc.201501167.
- [27] K.K. Coti, M.E. Belowich, M. Liong, M.W. Ambrogio, Y.A. Lau, H.A. Khatib, J.I. Zink, N.M. Khashab, J.F. Stoddart, Mechanised nanoparticles for drug delivery, *Nanoscale* 1 (2009) 16-39.
- [28] Z. Li, J.C. Barnes, A. Bosoy, J.F. Stoddart, J.I. Zink, Mesoporous silica nanoparticles in biomedical applications, *Chem. Soc. Rev.* 41 (2012) 2590-2605.
- [29] C. Argyo, V. Weiss, C. Bräuchle, T. Bein, Multifunctional mesoporous silica nanoparticles as a universal platform for drug delivery, *Chem. Mater.* 26 (2013) 435-451.
- [30] J. Croissant, X. Cattoën, M. Wong Chi Man, P. Dieudonné, C. Charnay, L. Raehm, J.-O. Durand, One-pot construction of multipodal hybrid periodic mesoporous organosilica nanoparticles with crystal-like architectures, *Adv. Mater. (Weinheim, Ger.)* 27 (2015) 145-149.
- [31] J. Croissant, X. Cattoën, M. Wong Chi Man, A. Gallud, L. Raehm, M. Maynadier, J.-O. Durand, Biodegradable ethylene-bis(propyl)disulfide-based periodic mesoporous organosilica nanorods and nanospheres for efficient in-vitro drug delivery, *Adv. Mater. (Weinheim, Ger.)* 26 (2014) 6174-6180.
- [32] J. Croissant, D. Salles, M. Maynadier, O. Mongin, V. Hugues, M. Blanchard-Desce, X. Cattoën, M. Wong Chi Man, A. Gallud, M. Garcia, M. Gary-Bobo, L. Raehm, J.-O. Durand, Mixed periodic mesoporous organosilica nanoparticles and core-shell systems, application to in vitro two-photon imaging, therapy and drug delivery, *Chem. Mater.* 26 (2014) 7214-7220.
- [33] C. Mauriello-Jimenez, J.G. Croissant, M. Maynadier, X. Cattoën, M.W.C. Man, J. Vergnaud, V. Chaleix, S. Vincent, M. Garcia, M. Gary-Bobo, Porphyrin-functionalized mesoporous organosilica nanoparticles for two-photon imaging of cancer cells and drug delivery, *Journal of Materials Chemistry B* (2015).
- [34] L. He, Y. Huang, H. Zhu, G. Pang, W. Zheng, Y.-S. Wong, T. Chen, Cancer-targeted monodisperse mesoporous silica nanoparticles as carrier of ruthenium polypyridyl complexes to enhance theranostic effects, *Adv. Funct. Mater.* 24 (2014) 2754-2763.
- [35] F. Tang, L. Li, D. Chen, Mesoporous silica nanoparticles: Synthesis, biocompatibility and drug delivery, *Adv. Mater.* 24 (2012) 1504-1534.
- [36] D. Tarn, C.E. Ashley, M. Xue, E.C. Carnes, J.I. Zink, C.J. Brinker, Mesoporous silica nanoparticle nanocarriers: Biofunctionality and biocompatibility, *Acc. Chem. Res.* 46 (2013) 792-801.
- [37] C.T. Kresge, M.E. Leonowicz, W.J. Roth, J.C. Vartuli, J.S. Beck, Ordered mesoporous molecular-sieves synthesized by a liquid-crystal template mechanism, *Nature* 359 (1992) 710-712.
- [38] Q. Zhang, X. Wang, P.-Z. Li, K.T. Nguyen, X.-J. Wang, Z. Luo, H. Zhang, N.S. Tan, Y. Zhao, Biocompatible, uniform, and redispersible mesoporous silica nanoparticles for cancer-targeted drug delivery in vivo, *Adv. Funct. Mater.* 24 (2014) 2450-2461.
- [39] H. Meng, M. Xue, T. Xia, Y.-L. Zhao, F. Tamanoi, J.F. Stoddart, J.I. Zink, A.E. Nel, Autonomous in vitro anticancer drug release from mesoporous silica nanoparticles by ph-sensitive nanovalves, *J. Am. Chem. Soc.* 132 (2010) 12690-12697.
- [40] C. Théron, A. Gallud, C. Carcel, M. Gary-Bobo, M. Maynadier, M. Garcia, J. Lu, F. Tamanoi, J.I. Zink, M. Wong Chi Man, Hybrid mesoporous silica nanoparticles with ph-operated and complementary h-bonding caps as an autonomous drug-delivery system, *Chem. Eur. J.* 20 (2014) 9372-9380.

- [41] J. Croissant, J.I. Zink, Nanovalve-controlled cargo release activated by plasmonic heating, *J. Am. Chem. Soc.* 134 (2012) 7628-7631.
- [42] L. Zhao, J. Peng, Q. Huang, C. Li, M. Chen, Y. Sun, Q. Lin, L. Zhu, F. Li, Near-infrared photoregulated drug release in living tumor tissue via yolk-shell upconversion nanocages, *Adv. Funct. Mater.* 24 (2014) 363-371.
- [43] J. Croissant, A. Chaix, O. Mongin, M. Wang, S. Clément, L. Raehm, J.-O. Durand, V. Hugues, M. Blanchard-Desce, M. Maynadier, A. Gallud, M. Gary-Bobo, M. Garcia, J. Lu, F. Tamanoi, D.P. Ferris, D. Tarn, J.I. Zink, Two-photon-triggered drug delivery via fluorescent nanovalves, *Small* 10 (2014) 1752-1755.
- [44] J. Croissant, M. Maynadier, A. Gallud, H. Peindy N'Dongo, J.L. Nyalosaso, G. Derrien, C. Charnay, J.-O. Durand, L. Raehm, F. Serein-Spirau, N. Cheminet, T. Jarrosson, O. Mongin, M. Blanchard-Desce, M. Gary-Bobo, M. Garcia, J. Lu, F. Tamanoi, D. Tarn, T.M. Guardado-Alvarez, J.I. Zink, Two-photon-triggered drug delivery in cancer cells using nanoimpellers, *Angew. Chem. Int. Ed.* 125 (2013) 14058-14062.
- [45] J.G. Croissant, C. Qi, O. Mongin, V. Hugues, M. Blanchard-Desce, L. Raehm, X. Cattoën, M.W.C. Man, M. Maynadier, M. Gary-Bobo, Disulfide-gated mesoporous silica nanoparticles designed for two-photon-triggered drug release and imaging, *Journal of Materials Chemistry B* 3 (2015) 6456-6461.
- [46] C. Park, H. Kim, S. Kim, C. Kim, Enzyme responsive nanocontainers with cyclodextrin gatekeepers and synergistic effects in release of guests, *J. Am. Chem. Soc.* 131 (2009) 16614-16615.
- [47] K. Patel, S. Angelos, W.R. Dichtel, A. Coskun, Y.-W. Yang, J.I. Zink, J.F. Stoddart, Enzyme-responsive snap-top covered silica nanocontainers, *J. Am. Chem. Soc.* 130 (2008) 2382-2383.
- [48] J.G. Croissant, C. Mauriello-Jimenez, M. Maynadier, X. Cattoën, M.W.C. Man, L. Raehm, O. Mongin, M. Blanchard-Desce, M. Garcia, M. Gary-Bobo, Synthesis of disulfide-based biodegradable bridged silsesquioxane nanoparticles for two-photon imaging and therapy of cancer cells, *Chem. Commun.* 51 (2015) 12324-12327.
- [49] S. Giri, B.G. Trewyn, M.P. Stellmaker, V.S.Y. Lin, Stimuli-responsive controlled-release delivery system based on mesoporous silica nanorods capped with magnetic nanoparticles, *Angew. Chem. Int. Ed.* 44 (2005) 5038-5044.
- [50] B.G. Trewyn, S. Giri, I.I. Slowing, V.S.Y. Lin, Mesoporous silica nanoparticle based controlled release, drug delivery, and biosensor systems, *Chem. Commun.* 31 (2007) 3236-3245.
- [51] D.R. Radu, C.-Y. Lai, K. Jeftinija, E.W. Rowe, S. Jeftinija, V.S.Y. Lin, A polyamidoamine dendrimer-capped mesoporous silica nanosphere-based gene transfection reagent, *J. Am. Chem. Soc.* 126 (2004) 13216-13217.
- [52] Z. Luo, K. Cai, Y. Hu, L. Zhao, P. Liu, L. Duan, W. Yang, Mesoporous silica nanoparticles end-capped with collagen: Redox-responsive nanoreservoirs for targeted drug delivery, *Angewandte Chemie-International Edition* 50 (2011) 640-643.
- [53] Y. Liu, X. Ding, J. Li, Z. Luo, Y. Hu, J. Liu, L. Dai, J. Zhou, C. Hou, K. Cai, Enzyme responsive drug delivery system based on mesoporous silica nanoparticles for tumor therapy in vivo, *Nanotechnology* 26 (2015) 145102.
- [54] Z. Luo, K. Cai, Y. Hu, B. Zhang, D. Xu, Cell-specific intracellular anticancer drug delivery from mesoporous silica nanoparticles with pH sensitivity, *Advanced healthcare materials* 1 (2012) 321-325.
- [55] Y.N. Zhao, B.G. Trewyn, Slowing, II, V.S.Y. Lin, Mesoporous silica nanoparticle-based double drug delivery system for glucose-responsive controlled release of insulin and cyclic amp, *J. Am. Chem. Soc.* 131 (2009) 8398-8399.
- [56] L. Palanikumar, E.S. Choi, J.Y. Cheon, S.H. Joo, J.H. Ryu, Noncovalent polymer-gatekeeper in mesoporous silica nanoparticles as a targeted drug delivery platform, *Adv. Funct. Mater.* 25 (2015) 957-965.
- [57] L. Palanikumar, H.Y. Kim, J.Y. Oh, A.P. Thomas, E.S. Choi, M.T. Jeena, S.H. Joo, J.-H. Ryu, Noncovalent surface locking of mesoporous silica nanoparticles for exceptionally high hydrophobic drug loading and enhanced colloidal stability, *Biomacromolecules* (2015).

- [58] H. Meng, M. Wang, H. Liu, X. Liu, A. Situ, B. Wu, Z. Ji, C.H. Chang, A.E. Nel, Use of a lipid-coated mesoporous silica nanoparticle platform for synergistic gemcitabine and paclitaxel delivery to human pancreatic cancer in mice, *ACS Nano* 9 (2015) 3540-3557.
- [59] L. Zhang, E. Wang, Metal nanoclusters: New fluorescent probes for sensors and bioimaging, *Nano Today* 9 (2014) 132-157.
- [60] J. Xie, Y. Zheng, J.Y. Ying, Protein-directed synthesis of highly fluorescent gold nanoclusters, *J. Am. Chem. Soc.* 131 (2009) 888-+.
- [61] N. Fernandez-Iglesias, J. Bettmer, Synthesis, purification and mass spectrometric characterisation of a fluorescent au-9@bsa nanocluster and its enzymatic digestion by trypsin, *Nanoscale* 6 (2014) 716-721.
- [62] X.-D. Zhang, D. Wu, X. Shen, P.-X. Liu, F.-Y. Fan, S.-J. Fan, In vivo renal clearance, biodistribution, toxicity of gold nanoclusters, *Biomaterials* 33 (2012) 4628-4638.
- [63] X. Le Guevel, B. Hoetzer, G. Jung, K. Hollemeyer, V. Trouillet, M. Schneider, Formation of fluorescent metal (au, ag) nanoclusters capped in bovine serum albumin followed by fluorescence and spectroscopy, *J. Phys. Chem. C* 115 (2011) 10955-10963.
- [64] A.M. Alkilany, P.K. Nagaria, C.R. Hexel, T.J. Shaw, C.J. Murphy, M.D. Wyatt, Cellular uptake and cytotoxicity of gold nanorods: Molecular origin of cytotoxicity and surface effects, *Small* 5 (2009) 701-708.
- [65] L.-Y. Chen, C.-W. Wang, Z. Yuan, H.-T. Chang, Fluorescent gold nanoclusters: Recent advances in sensing and imaging, *Anal. Chem.* 87 (2015) 216-229.
- [66] V. Venkatesh, A. Shukla, S. Sivakumar, S. Verma, Purine-stabilized green fluorescent gold nanoclusters for cell nuclei imaging applications, *Acs Applied Materials & Interfaces* 6 (2014) 2185-2191.
- [67] J.-Y. Zhao, R. Cui, Z.-L. Zhang, M. Zhang, Z.-X. Xie, D.-W. Pang, Cytotoxicity of nucleus-targeting fluorescent gold nanoclusters, *Nanoscale* 6 (2014) 13126-13134.
- [68] X. Zhang, F.-G. Wu, P. Liu, H.-Y. Wang, N. Gu, Z. Chen, Synthesis of ultrastable and multifunctional gold nanoclusters with enhanced fluorescence and potential anticancer drug delivery application, *J. Colloid Interface Sci.* (2015).
- [69] N. Li, Y. Chen, Y.-M. Zhang, Y. Yang, Y. Su, J.-T. Chen, Y. Liu, Polysaccharide-gold nanocluster supramolecular conjugates as a versatile platform for the targeted delivery of anticancer drugs, *Scientific reports* 4 (2014).
- [70] T. Chen, S. Xu, T. Zhao, L. Zhu, D. Wei, Y. Li, H. Zhang, C. Zhao, Gold nanocluster-conjugated amphiphilic block copolymer for tumor-targeted drug delivery, *ACS Appl. Mater. Interfaces* 4 (2012) 5766-5774.
- [71] H. Chen, B. Li, X. Ren, S. Li, Y. Ma, S. Cui, Y. Gu, Multifunctional near-infrared-emitting nano-conjugates based on gold clusters for tumor imaging and therapy, *Biomaterials* 33 (2012) 8461-8476.
- [72] R. Khandelia, S. Bhandari, U.N. Pan, S.S. Ghosh, A. Chattopadhyay, Gold nanocluster embedded albumin nanoparticles for two-photon imaging of cancer cells accompanying drug delivery, *Small* (2015).
- [73] T. Chen, Y. Hu, Y. Cen, X. Chu, Y. Lu, A dual-emission fluorescent nanocomplex of gold-cluster-decorated silica particles for live cell imaging of highly reactive oxygen species, *J. Am. Chem. Soc.* 135 (2013) 11595-11602.
- [74] Z. Luo, Y. Hu, R. Xin, B. Zhang, J. Li, X. Ding, Y. Hou, L. Yang, K. Cai, Surface functionalized mesoporous silica nanoparticles with natural proteins for reduced immunotoxicity, *Journal of Biomedical Materials Research Part A* 102 (2014) 3781-3794.
- [75] S. Dib, M. Boufatit, S. Chelouaou, F. Sadi-Hassaine, J. Croissant, J. Long, L. Raehm, C. Charnay, J.O. Durand, Versatile heavy metals removal via magnetic mesoporous nanocontainers, *RSC Adv.* 4 (2014) 24838-24841.
- [76] K.C.F. Leung, T.D. Nguyen, J.F. Stoddart, J.I. Zink, Supramolecular nanovalves controlled by proton abstraction and competitive binding, *Chem. Mater.* 18 (2006) 5919-5928.
- [77] W. Deng, J. Li, P. Yao, F. He, C. Huang, Green preparation process, characterization and antitumor effects of doxorubicin-bsa-dextran nanoparticles, *Macromol. Biosci.* 10 (2010) 1224-1234.

- [78] P. Bourassa, C. Kanakis, P. Tarantilis, M. Pollissiou, H. Tajmir-Riahi, Resveratrol, genistein, and curcumin bind bovine serum albumin†, *The Journal of Physical Chemistry B* 114 (2010) 3348-3354.
- [79] H. Shen, Z. Gu, K. Jian, J. Qi, In vitro study on the binding of gemcitabine to bovine serum albumin, *J. Pharm. Biomed. Anal.* 75 (2013) 86-93.
- [80] L.A. Sklar, B.S. Hudson, R.D. Simoni, Conjugated polyene fatty acids as fluorescent probes: Binding to bovine serum albumin, *Biochemistry* 16 (1977) 5100-5108.

Graphical abstract

Protein-Gold Clusters-Capped Mesoporous Silica Nanoparticles for High Drug Loading, Autonomous Gemcitabine/Doxorubicin Co-Delivery, and In-vivo Tumor Imaging

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ABSTRACT: Multi-drug delivery of doxorubicin and gemcitabine has produced enhanced cancer treatments, but the side effects of this drug combination remain severe. Hence, an effective multi-drug delivery system is urgently needed. In this contribution, we designed protein-gold cluster-coated mesoporous silica nanoparticles for an exceptionally high total drug loading capacity of 62 weight percent. The system displayed zero premature release and autonomous pH-triggered delivery.

KEYWORDS: mesoporous silica nanoparticles, gold nanoclusters, bovine serum albumin, multi-drug delivery, cancer