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Functionalized Hollow Mesoporous Silica Nanoparticles for Tumor Vasculature Targeting and PET Image-Guided Drug Delivery

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Abstract

Aim—Development of multifunctional and well-dispersed hollow mesoporous silica nanoparticles (HMSNs) for tumor vasculature targeted drug delivery and positron emission tomography (PET) imaging.

Materials and Methods—Amine functionalized HMSNs (150–250 nm) were conjugated with a macrocyclic chelator, NOTA, PEGylated and loaded with anti-angiogenesis drug, Sunitinib. Cyclo(Arg-Gly-Asp-D-Tyr-Lys) (cRGDyK) peptide was attached to the nanoconjugate and radiolabeled with ⁶⁴Cu for PET imaging.

Results—⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK exhibited integrin specific uptake both in vitro and in vivo. PET results indicated ~ 8 % ID/g uptake of targeted nanoconjugates in U87MG tumors, which correlated well with ex vivo and histological analyses. Enhanced tumor targeted delivery of sunitinib was also observed.

Conclusions—We successfully developed tumor vasculature targeted HMSNs for PET imaging and image guided drug delivery.

Keywords

Cancer; Hollow mesoporous silica nanoparticles; Image-guided drug delivery; Positron emission tomography (PET); Theranostics

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SUPPORTING INFORMATION AVAILABLE

Results related to size-exclusion chromatography of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK, serum stability of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK, time-activity curves of tumor-to-muscle, tumor-to-blood and tumor-to-liver ratios upon intravenous injection of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK in U87MG tumor bearing mice and biodistribution studies of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK in U87MG tumor bearing mice are provided in the Supporting Information.

INTRODUCTION

Targeted drug delivery guided by *in vivo* PET imaging is a burgeoning area of clinical research, particularly for the treatment of cancer. [1–3] Personalized therapy is an ambitious vision for the future and the efficient use of PET-image guided drug delivery (IGDD) approach is an initial step towards its fulfillment. Nanosized platforms with their large surface-to-volume ratios and facile surface chemistry, offer myriad advantages such as targeted transportation of large payloads of drugs, reduced off-target toxicity and enhanced therapeutic efficacy.[4–9] Despite the excellent attributes, the multifunctionality of nanomaterials has been barely harnessed for simultaneous tumor detection, targeted drug delivery and monitoring. [10, 11]

Among various nanoplatfroms reported for tumor imaging and/or targeted drug delivery, functionalized silica nanoparticles have gained special attention due to their facile synthesis, uniform and controllable morphology and significant biocompatibility. [12–14] This class of nanoparticles has also received the United States Food and Drug Administration (US FDA) investigational new drug approval for in-human clinical trials. [15] In addition, mesoporous silica nanoplatfroms (MSNs) have a unique network of mesopores and nanochannels which facilitates loading of chemotherapeutic drugs and their controlled release. [16–20] Although MSNs are generally considered to be biocompatible with low cytotoxicity, there are concerns about their dosage-dependent toxic effects on biological systems. [21–23] Therefore, it is of great significance to increase the drug loading capacity of silica nanoplatfroms in order to achieve the required therapeutic effects using much lower amount of silica carriers to minimize toxicity.

Hollow mesoporous silica nanoparticles (HMSNs), with a large cavity inside each original mesoporous silica nanoparticle, provide a promising approach to address this challenge. [14, 24] The void cores of HMSN serve as reservoirs for the drug storage. At the same time, HMSNs can provide a higher outside shell surface area for conjugation with targeting ligands than with MSNs under the same amount of silica used, which may lead to enhanced drug delivery. [14] Despite the rapid advances in engineering of functionalized HMSNs over the last several years, [25–27] there are very few reports about the quantitative assessment of their *in vivo* biodistribution, tumor targeting and clearance studies. [14] Applying HMSNs to *in vivo* tumor vasculature targeted imaging and drug delivery is still considered as one of the major challenges in this field, possibly due to the lack of efficient *in vivo* targeting strategy and well-developed surface engineering techniques.

Altered integrin $\alpha_v\beta_3$ expression has been detected in carcinomas of breast, prostate, ovary, lung, and also in melanomas, and gliomas. [28, 29] The integrin $\alpha_v\beta_3$ expression has been correlated with an aggressive phenotype and metastatic dissemination. [30] It has been well established that the integrin $\alpha_v\beta_3$ is overexpressed on both tumor cells and neovasculature in various cancer models, [29, 31] and is therefore an interesting target to develop new imaging probes for early cancer detection and therapy. Synthetic peptides containing the arginine–glycine–aspartic acid (RGD) tripeptide sequence can specifically bind to integrin $\alpha_v\beta_3$ and significant progress has been made in development of radiolabeled cyclic RGD (cRGDyK)

peptide probes targeting integrin $\alpha_v\beta_3$ expression in various tumor models. [32, 33] In this context, targeting tumor vasculature by cRGDyK-conjugated HMSN may serve as a promising approach for delivering anticancer drugs or contrast agents for cancer diagnosis and therapy.

Herein, we report the development of surface functionalized and well-dispersed HMSN-based nanoplatform that integrates molecular targeting, PET imaging and chemotherapy into a single system. Uniform HMSNs were synthesized adopting reported procedures, conjugated to p-SCN-Bn-NOTA and then PEGylated. cRGDyK peptide was attached onto the distal ends of the PEG arms and the nanoconjugate was radiolabeled with ^{64}Cu ($t_{1/2} = 12.7$ h) to form the ^{64}Cu -NOTA-HMSN-PEG-cRGDyK for *in vivo* PET imaging of integrin $\alpha_v\beta_3$ expression. As a proof of concept, a model hydrophobic anticancer drug [Sunitinib (SUN)] was loaded in the nanoplatform for targeted delivery to the cancerous lesions. Aside from its ability to deliver the anti-cancer drug more specifically at the tumor vasculature, the radiolabeled drug nanocarrier also offers the scope of using PET to non-invasively and quantitatively monitor its biodistribution, pharmacokinetics and tumor targeting efficacy.

MATERIALS AND METHODS

Materials

Cyclo-(Arg-Gly-Asp-D-Tyr-Lys) peptide (cRGDyK) was procured from Peptide International (Louisville, KY). PD-10 columns were purchased from GE Healthcare (Piscataway, NJ). Absolute ethanol, cyclohexane and sodium chloride (NaCl) were purchased from Fisher Scientific (Pittsburg, PA). Sunitinib was purchased from LC Laboratories (Woburn, MA). SCM-PEG_{5k}-Mal was obtained from Creative PEG works (Winston Salem, NC). p-SCN-Bn-NOTA was acquired from Macrocyclics, Inc. (Dallas, TX). NHS-Fluorescein, Chelex 100 resin (50–100 mesh), tetraethyl orthosilicate (TEOS), ammonia ($\text{NH}_3\cdot\text{H}_2\text{O}$), Igepal CO-520 (NP-5), triethylamine (TEA), (3-Aminopropyl)triethoxysilane (APS), dimethyl sulfoxide (DMSO), cetyltrimethylammonium chloride (CTAC, 25 wt%) and Kaiser test kit were purchased from Sigma-Aldrich (St. Louis, MO). Traut's Reagent (2-Iminothiolane. HCl) and PBS (1X) were purchased from Thermo Scientific (Rockford, IL). Water and all buffers were of Millipore grade and pretreated with Chelex 100 resin to ensure that the aqueous solution was free of heavy metals. All chemicals were used as received without further purification.

Characterization

Transmission electron microscopy (TEM) images were obtained on a FEI TecnaiTM T12 cryo-electron microscope operated at an accelerating voltage of 120 kV. The samples for TEM measurements were made by dropping dilute products onto carbon-coated copper grids. DLS and zeta potential analysis were performed on Nano-Zetasizer (Malvern Instruments Ltd.). Well-dispersed nanoparticle samples were directly used for the laser scattering experiments. Fluorescence images were acquired with a Nikon Eclipse Ti-E inverted microscope system (Nikon Instruments Inc.). High-performance liquid chromatography (HPLC) analysis was conducted in a Dionex Ultimate 3000 system using a

C-18 reversed phase column (Phenomenex Luna C18, 5 μ m, 10 $\times$ 250 mm). The HPLC analysis was monitored at 208 nm wavelength.

Synthesis of HMSN

HMSNs were synthesized following the literature protocol. [34] To synthesize uniform ~100 nm sized dense silica nanoparticles (dSiO₂), 35.7 mL of absolute ethanol was mixed with 5 mL water and 0.8 mL of ammonia and stirred at room temperature, followed by addition of 1 mL of TEOS. [35] The mixture was allowed to react at room temperature for 1 h and subsequently, washed with water and ethanol and suspended in 20 mL water.

In the second step, dSiO₂ was coated with mesoporous silica to form dSiO₂@MSN. CTAC (2 g) and TEA (20 mg) were dissolved in 20 mL of deionized water and added to 10 mL of dSiO₂ water solution. The mixture was stirred at room temperature for prolonged period, followed by the addition of 0.15 mL of TEOS. The mixture was stirred for 1 h at 80 °C. The last step involved etching of the dSiO₂ core from dSiO₂@MSN to form HMSN. 636 mg of Na₂CO₃ was added to the reaction mixture, which was stirred continuously at 50 °C for 30 min. Finally, CTAC was removed by NaCl: methanol extraction. [35]

Amine Modification of HMSN (HMSN-NH₂)

As prepared HMSNs were modified with –NH₂ groups to allow further surface functionalization. 1 mL of APS was added to HMSNs dispersed in 20 mL of absolute ethanol. The system was sealed stirred in a 86–90 °C water bath for 48 h. Afterward, the mixture was centrifuged and washed with ethanol for several times to completely remove the residual APS. The concentration of –NH₂ groups (nmol/mL) was measured using a Kaiser test kit.

Loading HMSN with SUN

To load HMSN with hydrophobic drug, SUN, HMSN (0.8 mg) was suspended in 1 mg/mL of SUN-DMSO solution. The mixture was kept under constant shaking for 24 h at room temperature. Subsequently, SUN loaded HMSN [HMSN(SUN)] was collected by centrifugation and washed with water for 3 times. UV-Visible spectrometry was used to quantify the unbound SUN in loading/washing solutions and the difference between this and SUN added was the amount of SUN loaded in HMSN. For this purpose, a calibration curve for SUN was obtained by using standard solutions of SUN in DMSO and the curve was fitted by linear regression. The drug encapsulation efficiency was calculated from the ratio of the drug amount incorporated into HMSNs to the total drug amount added.

Thiolation of cRGDyK peptide to form cRGDyK-SH

In order to prepare thiolated cRGDyK peptide (cRGDyK-SH), 2 mg of cRGDyK was dissolved in 200 μ L of PBS and mixed with 6 mg of Traut's reagent dissolved in 100 μ L of PBS (molar ratio Traut's reagent: cRGDyK = 13.1). The pH of the resultant solution was adjusted to ~8 by addition of 0.1 M Na₂CO₃ buffer and it was allowed to react at room temperature under constant shaking for 2 h. The reaction mixture was purified using HPLC. Water (A) and acetonitrile (B) mixtures with 0.1% trifluoroacetic acid were used as the

mobile phase, and the gradient elution technique (B changes from 5% to 65% in 0.5 h) was adopted for the separation. Flow rate was maintained at 1 mL/min.

Synthesis of NOTA-HMSN-PEG-cRGDyK and NOTA-HMSN-Fluorescein-PEG-cRGDyK

To conjugate HMSN with NOTA, p-SCN-Bn-NOTA (~53 nmol) dissolved in DMSO and 2 μ L was used to react with 500 μ L HMSN-NH₂ solution (with ~100 nmol of -NH₂ groups) at pH 8.5 for 2 h to obtain NOTA-HMSN-NH₂. Subsequently, 4 mg (800 nmol) of SCM-PEG_{5k}-Mal was added and reacted for another 1 h, resulting in NOTA-HMSN-PEG-Mal. NOTA-HMSN-PEG-cRGDyK could be obtained by reacting cRGDyK-SH (5 nmol) with NOTA-HMSN-PEG-Mal (0.5 nmol) for 2 h. NOTA-HMSN-PEG-cRGDyK was purified from unreacted cRGDyK-SH using PD-10 column with PBS as the mobile phase.

For *in vitro* studies, NHS-fluorescein was conjugated with HMSN-NH₂ before conjugation of NOTA. The conjugation was carried out by reacting 2 nmol of fluorescein-NHS ester with HMSN-NH₂ for 2 h at room temperature (pH 8.5–9.0). Subsequently, NOTA conjugation, PEGylation and attachment of cRGDyK peptide was carried out as described above to form NOTA-HMSN-Fluorescein-PEG-cRGDyK.

Cellular uptake study

The cellular uptake behavior and intracellular distribution of NOTA-HMSN-Fluorescein-PEG-cRGDyK was analyzed using flow cytometry. For flow cytometry, U87MG cells were first harvested and suspended in cold PBS with 2 % bovine serum albumin at a concentration of 5×10^6 cells/mL, and then 500 μ L of cells were incubated with NOTA-HMSN-Fluorescein-PEG-cRGDyK (targeted) or NOTA-HMSN-Fluorescein-PEG (non-targeted) at two different concentrations (10 nM and 50 nM) for 30 min at room temperature. The cells were washed for three times with cold PBS and centrifuged for 5 min. Subsequently, the cells were analyzed using a BD FACSCalibur four-color analysis cytometer, which is equipped with 488 and 633 nm lasers (Becton-Dickinson, San Jose, CA) and FlowJo analysis software (Tree Star, Ashland, OR). Blocking experiment was also performed in cells incubated with the same amounts of NOTA-HMSN-Fluorescein-PEG-cRGDyK (10 nM or 50 nM), where 1 mg/mL of unconjugated cRGDyK was pre-added to evaluate the integrin $\alpha_v\beta_3$ specificity of NOTA-HMSN-Fluorescein-PEG-cRGDyK.

Animal model

All animal studies were conducted according to the University of Wisconsin Institutional Animal Care and Use Committee protocols. U87MG cells were used for tumor inoculation when they reached ~80% confluence. Four- to five-week-old female athymic nude mice were purchased from Harlan (Indianapolis, IN) and tumors were established by subcutaneously injecting 5×10^6 cells, suspended in 100 μ L of 1:1 mixture of DMEM medium and matrigel (BD Biosciences, Franklin lakes, NJ), into the front flanks of the mice. The tumor sizes were monitored every other day and *in vivo* experiments were carried out when the diameter of the tumors reached 6–8 mm (typically 3 weeks after inoculation).

⁶⁴Cu-labeling studies and serum stability of the radiolabeled agent

⁶⁴Cu-labeling was performed in a manner similar to our previous studies. [9] ⁶⁴CuCl₂ (~130 MBq) was diluted in 300 µL of 0.1 M sodium acetate buffer (pH 6.5) and added to NOTA-HMSN-PEG-cRGDyK (0.5 nmol). The reaction mixture was incubated at 37 °C for 30 min with constant shaking. ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK was purified on PD-10 columns with PBS as the mobile phase. The radioactivity fractions (typically between 3.0 and 4.0 mL) were collected for further *in vivo* experiments.

For serum stability studies, ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK was incubated in complete mouse serum at 37 °C for up to 24 h. Portions of the mixture were sampled at different time points and filtered through 100 kDa cut-off filters. The filtrates were collected, and the radioactivity was measured. The percentages of retained (i.e., intact) ⁶⁴Cu on the ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK were calculated using the equation [(total radioactivity - radioactivity in filtrate)/total radioactivity] × 100%.

Imaging and biodistribution studies

PET and PET/CT scans, image reconstruction and ROI analysis were performed using a microPET/microCT Inveon rodent model scanner (Siemens Medical Solutions USA, Inc.) as described previously. [10, 35] U87MG xenografted mice were injected with 5–10 MBq of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK (targeted) or ⁶⁴Cu-NOTA-HMSN-PEG (control) via the tail vein and static PET scans were performed at various time points, post-injection (p.i.). Another group of three tumor-bearing mice was co-injected with 10 mg/kg dose of cRGDyK peptide and ⁶⁴Cu-NOTA-HMSN-PEG-RGD in a blocking experiment to evaluate the integrin α_vβ₃ specificity of the radiolabeled nanoconjugate *in vivo*. Biodistribution studies were carried out after the last PET scans to validate the PET results. The radioactivity in the tissues was measured using a WIZARD² gamma-counter (Perkin Elmer) and presented as %ID/g.

Histology

Frozen tissue slices of 7 µm thickness were fixed with cold acetone and stained for integrin α_vβ₃, through the use of a rat anti-mouse integrin β₃ antibody and a Cy3-labeled donkey anti-rat IgG. Fluorescence from fluorescein on the surface of NOTA-HMSN-Fluorescein-PEG-cRGDyK was used to monitor the distribution of this nanoparticle inside different tissues. All images were acquired with a Nikon Eclipse Ti microscope.

***In vivo* enhanced drug delivery**

SUN loaded HMSNs (0.4 mg) were conjugated with NOTA, PEGylated and cRGDyK, as described previously to form NOTA-HMSN(SUN)-PEG-cRGDyK. U87MG tumor bearing mice were then intravenously injected with NOTA-HMSN(SUN)-PEG-cRGDyK (targeted group: 10 mg HMSN/kg, 7.1 mg SUN/kg), and NOTA-HMSN(SUN)-PEG (non-targeted group: 10 mg HMSN/kg, 7.1mg SUN/kg). The mice were sacrificed 3 h p.i. for *ex vivo* optical imaging in the IVIS system (Excitation = 430 nm, Emission = 640 nm) for *in vivo* drug delivery efficacy studies.

RESULTS AND DISCUSSION

Synthesis and characterization

Uniform HMSNs with an average particle size of 150 nm were synthesized following the literature procedure with improved Na_2CO_3 -etching process. [34] Surface engineering plays a vital role in *in vivo* applications of nanoparticles, including HMSN. Figure 1 shows the major steps towards the synthesis of ^{64}Cu -NOTA-HMSN-PEG-cRGDyK. The detailed structural characterization of HMSN was reported in our recent paper. [37] The representative TEM image of amine functionalized HMSN (HMSN- NH_2) indicates well oriented mesoporous shell structures (Figure 2A). The HMSN- NH_2 were spherical with particle size in the range of 150–250 nm (Figure 2A), with uniform size distribution measured by dynamic light scattering. The pore size of HMSN- NH_2 was 2–3 nm as we reported previously. [35] No obvious changes in the morphology of HMSN- NH_2 were observed after surface modifications, as evidenced by TEM image and DLS evaluation of NOTA-HMSN-PEG-cRGDyK (Figure 2B). The successful surface modification at different steps, as confirmed by DLS and zeta potential measurements, are summarized in Figure 2C. The diameters of HMSNs based on DLS after step-by-step modification became larger to varied extents because of the presence of hydrated layers, PEG chains, and cRGDyK. The surface charge of HMSN- NH_2 (39.5 ± 0.6 mV) became negative (-11.8 ± 0.9 mV) on conjugation with NOTA, probably due to the presence of 3 negatively charged carboxylate groups in NOTA. On conjugation with Mal-PEG_{5k}- NH_2 , significant change in surface charge was observed (-3.6 ± 0.5 mV), indicating successful coating with the PEG layer. Hardly any change in surface charge was observed on conjugation with cRGDyK due to the much smaller size of the molecule compared to PEG. Taken together, these data confirmed the successful surface modification of HMSN- NH_2 to form NOTA-HMSN-PEG-cRGDyK. The final nanoconjugates could be well-dispersed in phosphate buffered saline (PBS) without any obvious aggregation for several weeks. The concentration of $-\text{NH}_2$ groups on HMSN was determined to be ~ 100 nmol of $-\text{NH}_2$ per nmol of HMSN, using Kaiser test kit. In all experiments, the concentration of HMSN was estimated based on the concentration of $-\text{NH}_2$ groups present on its surface.

In vitro integrin $\alpha_v\beta_3$ targeting

Before the *in vivo* tumor targeted imaging, U87MG human glioblastoma cells were used for flow cytometry studies to confirm the *in vitro* integrin $\alpha_v\beta_3$ targeting efficiency of cRGDyK conjugated HMSNs. Fluorescein (Excitation = 494 nm/Emission = 521 nm) conjugation to the surface of nanoparticles facilitated such investigation. The flow cytometry results (Figure 3A and B) indicated that incubation with NOTA-HMSN-Fluorescein-PEG-cRGDyK (targeted group) could significantly enhance their cellular uptake in U87MG cells, while treatment with NOTA-HMSN-Fluorescein-PEG (non-targeted group), or NOTA-HMSN-Fluorescein-PEG-cRGDyK with a blocking dose of cRGDyK (1 mg/mL, blocking group), only gave minimal fluorescence enhancement. Two different concentrations (10 nM and 50 nM) of the nanoconjugates were used for flow cytometry studies. At the concentration of 10 nM of nanoconjugate, the fluorescence due to targeted group was 10.2-fold higher than non-targeted group and 7.7-fold higher than blocking. At a higher concentration of 50 nM, the fluorescence enhancement in targeted group was 25.1-fold than of non-targeted group and

11.2-fold higher than blocking. Thus, the *in vitro* flow cytometry studies demonstrated that NOTA-HMSN-Fluorescein-PEG-cRGDyK exhibited strong and specific binding to integrin $\alpha_v\beta_3$ with low non-specific binding, which warranted further *in vivo* investigation of the nanoconjugates.

⁶⁴Cu labeling and stability evaluation

NOTA-HMSN-PEG-cRGDyK was labeled with ⁶⁴Cu for *in vivo* studies. The radiolabeled nanoconjugates were purified using PD-10 columns with PBS as the mobile phase. The radioactivity fractions (typically elute between 3 and 4mL) were collected for further *in vivo* experiments, and atypical size exclusion column chromatography profile can be seen in Figure S1. After passing 6mL of PBS, the unreacted ⁶⁴Cu started eluting from the column. The decay-corrected radiochemical yield was >50% for both conjugates, with radiochemical purity of >95%. The specific activity of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK was ~120 MBq/nmol, assuming complete recovery of NOTA-HMSN-PEG-cRGDyK after size-exclusion chromatography. The whole procedure of ⁶⁴Cu labeling and purification of the HMSN nanoconjugates could be completed within 60 min.

Before *in vivo* investigation in mice, serum stability studies were carried out to assess the stability of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK. High serum stability is the prerequisite in order to use radiolabeled agents for *in vivo* applications. If the radiolabeled complexes are not stable in serum, transchelation of ⁶⁴Cu in serum protein might occur *in vivo*, resulting in accumulation of the radioactivity in non-targeted organs. It was found that >90% of ⁶⁴Cu remained within the NOTA-HMSN-PEG-cRGDyK conjugates over a 24 h incubation period (Figure S2), indicating high stability of the ⁶⁴Cu-NOTA complex.

***In vivo* tumor targeting and PET imaging**

The time-points of 0.5, 3, 6 and 18 h p.i. were chosen for serial PET scans. The coronal slices that contain the U87MG tumors are shown in Figure 4A. In addition, representative microPET, microCT and fused images of a mouse at 3 h p.i. of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK are shown in Figure 4B for direct visual comparison. Quantitative data obtained from region-of-interest (ROI) analysis of the PET images are shown in Figure 5 and Figure S3. Quantitative data is presented as percentage injected dose per gram (%ID/g) of tissue. Accumulation of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK in the tumor occurred very quickly, which could be clearly visible at 0.5 h p.i. (7.2 ± 0.6 %ID/g) and peaked at around 3 h p.i. (8.1 ± 0.4 %ID/g), as shown in Figure 4A and Figure 5A. In control experiments, when ⁶⁴Cu-NOTA-HMSN-PEG (without cRGDyK conjugation) was intravenously administered in U87MG tumor bearing mice, the tumor uptake was found to be 1.5–2 times lower than that of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK at all-time points examined (n = 3; Figure 5B and Figure 5D). This indicates that the enhanced tumor uptake of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK is dependent on both active targeting of integrin $\alpha_v\beta_3$ expression by cRGDyK peptide as well as passive targeting due to enhanced permeability and retention (EPR) effect. To further support integrin $\alpha_v\beta_3$ specificity of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK *in vivo*, blocking studies were performed. It was found that administration of a blocking dose of cRGDyK 1 h before ⁶⁴Cu-NOTA-HMSN-PEG-RGD injection, could significantly reduce the tumor uptake to 4.2 ± 0.6 %ID/g at 3 h p.i. (n = 3, Figure 4A, Figure

5C, and Figure S3), demonstrating integrin $\alpha_v\beta_3$ specificity of ^{64}Cu -NOTA-HMSN-PEG-cRGDyK *in vivo*.

The liver uptake of ^{64}Cu -NOTA-HMSN-PEG-cRGDyK was found to be 23.2 ± 4.1 %ID/g at 0.5 h p.i. and decreased gradually to 11.1 ± 2.1 %ID/g at 18 h p.i. ($n = 3$; Figure 5A), which is expected for intravenously injected nanomaterials. [37] It may be noted that the liver uptake and radioactivity in blood were not significantly affected by the blocking dose of cRGDyK, as shown in Figure 4A, Figure 5C and Figure S3. Figure 5D summarizes the U87MG tumor uptake of ^{64}Cu -NOTA-HMSN-PEG-cRGDyK in all the groups (targeted, non-targeted and blocking) over time, where ^{64}Cu -NOTA-HMSN-PEG-cRGDyK shows significantly higher tumor uptake throughout the study period ($P < 0.05$ in all cases, $n = 3$). Administration of a blocking dose of cRGDyK did not change the *in vivo* kinetics of the radiolabeled nanoconjugates in U87MG tumor bearing mice, which indicated that integrin $\alpha_v\beta_3$ binding enhances tumor uptake of ^{64}Cu -NOTA-HMSN-PEG-cRGDyK.

Ex vivo biodistribution studies

After the last PET scans at 18 h p.i., the mice were euthanized. The tissues were collected for biodistribution studies to further validate the *in vivo* PET data (Figure S4). The uptake of ^{64}Cu -NOTA-HMSN-PEG-cRGDyK in the tumor was lower than that in the liver but higher than all other organs examined, indicating good tumor-targeting capability. The tumor/muscle ratio was 7.7 ± 0.4 at 18 h p.i. ($n = 3$). Clearly, co-injection of excess cRGDyK significantly reduced the tumor uptake of ^{64}Cu -NOTA-HMSN-PEG-cRGDyK (2.5 ± 0.5 %ID/g with blocking versus 3.9 ± 0.6 %ID/g without blocking at 18 hp.i.). Uptake in other organs was not significantly affected by co-injection of excess cRGDyK. The blockage of radiotracer uptake observed in the tumor xenografts strongly suggests that the tumor localization of the radiotracer is indeed receptor mediated. Overall, the quantification results obtained from biodistribution studies corroborated well with PET scans, confirming that quantitative ROI analysis of non-invasive microPET scans truly reflected the distribution of the radiolabeled nanoconjugates *in vivo*.

Histology

To further validate that tumor uptake of ^{64}Cu -NOTA-HMSN-Fluorescein-PEG-cRGDyK is integrin $\alpha_v\beta_3$ specific and nanoconjugates were indeed delivered to the tumor, three U87MG tumor-bearing mice were each injected with a larger dose of NOTA-HMSN-Fluorescein-PEG-cRGDyK (5 nmol/kg of mouse body weight) and euthanized at 3 h p.i. As clear from Figure 6 accumulation of NOTA-HMSN-Fluorescein-PEG-cRGDyK (green fluorescence) co-localized with the expression of integrin $\alpha_v\beta_3$ receptors in tumor tissues (red fluorescence). The histology data demonstrated that at 3 h p.i., NOTA-HMSN-Fluorescein-PEG-cRGDyK distribution in the U87MG tumor was primarily based on tumor vasculature targeting with little extravasation (indicated by the good overlay of red and green fluorescence signals, which represented integrin $\alpha_v\beta_3$ and NOTA-HMSN-Fluorescein-PEG-cRGDyK, respectively). Visible green fluorescence signals from the liver and spleen indicated significant uptake of NOTA-HMSN-Fluorescein-PEG-cRGDyK in these three organs. Though kidney shows visible red signal which is indicative of integrin $\alpha_v\beta_3$ expression, green signal due to uptake of NOTA-HMSN-Fluorescein-PEG-cRGDyK was

not observed. This can be explained by the fact that intravenously injected nanoparticles are rapidly cleared by reticuloendothelial system (liver and spleen) and renal clearance is generally very low. [14, 39] No observable green fluorescence was detected in muscle, which is consistent with the PET imaging results.

Enhanced tumor targeted drug delivery *in vivo*

The hollow cavity inside HMSN was utilized for enhanced loading of a model hydrophobic drug (SUN). Appreciably high drug loading capacity, up to 430.5 mg per gram of HMSNs could be achieved. The enhancement in drug loading capacity of HMSN over mesoporous silica nanoparticles (MSNs) (synthesized adopting the reported procedure [10]) was determined by loading the same drug in MSN under similar conditions. The drug loading capacity of MSN (138.2 mg/g) was much lower than what was observed with HMSN (430.5 mg/g), establishing the superiority of HMSN over MSN as a drug carrier nanopatform. The SUN-encapsulation efficiency of HMSN (0.8 mg) was determined to be 29.8 %. The successful loading of SUN in HMSN was further confirmed by the UV-Visible absorbance spectra of HMSN(SUN), which exhibited the characteristic absorption peak at around 440 nm (Figure 7A). The drug release profiles were observed in PBS and in DMSO (Figure 7B). It can be seen from the figure that there was minimal release of SUN in aqueous PBS medium (pH 7.4) even after 1 week of incubation. On the contrary, in DMSO medium, >85% of the drug was released from HMSN within just 1 h (Figure 7B). This suggests that the loaded drug would not be released in the blood stream and non-targeted organs during the course of delivery to the cancerous lesion, thereby minimizing the toxicity and potential side effects.

As a proof-of-concept, we further demonstrated the feasibility of enhanced image-guided, tumor targeted drug delivery after intravenous injection of drug loaded nanoconjugates in U87MG tumor-bearing mice. After intravenous injection of NOTA-HMSN(SUN)-PEG-cRGDyK (targeted) and NOTA-HMSN(SUN)-PEG (non-targeted), the mice were euthanized after 3 h and the major organs were collected and imaged in the IVIS Spectrum system (Excitation=430 nm; Emission= 640 nm) to detect the presence of SUN, as shown in Figure 7C. It is important to note that due to different absorption/scattering behavior of SUN in various tissues, optical signal intensities from different organs may not accurately reflect the absolute uptake level of injected NOTA-HMSN(SUN)-PEG-cRGDyK or NOTA-HMSN(SUN)-PEG. For example, although liver is the dominant organ for accumulation of nanoconjugates, as evidenced in our PET imaging and biodistribution studies (Figure 4 and Figure 5), only weak optical signal could be observed based on *ex vivo* optical imaging because of its dark color and strong absorbance of visible SUN fluorescence (Figure 7C). In contrast, due to the much lighter color of tumor tissue, dominant optical signal from SUN could be observed in mice injected with NOTA-HMSN(SUN)-PEG-cRGDyK, which is significantly stronger than the control group without cRGDyK conjugation [NOTA-HMSN(SUN)-PEG].

It is pertinent to point out that among members of the integrin family, a prominent role in angiogenesis and metastatic dissemination is played by both $\alpha_v\beta_3$ as well as $\alpha_v\beta_5$. [40] In the present study, characterization of $\alpha_v\beta_5$ was not carried out because human glioblastoma

(U87MG) tumor shows high expression of integrin $\alpha_v\beta_3$ and undetectable levels of integrin $\alpha_v\beta_5$ expression. [40] Also, the selectivity of cRGDyK (which was used for conjugation with HMSN in the present study) is 60–100 fold higher for integrin $\alpha_v\beta_3$ than integrin $\alpha_v\beta_5$. [41, 42] Though both cyclic RGD peptides cyclo(Arg-Gly-Asp-D-Phe-Lys) [c(RGDfK)] and cyclo (Arg-Gly-Asp-D-Tyr-Lys) [c(RGDyK)] have frequently been used for radiolabeling, c(RGDyK) is often considered to be superior to c(RGDfK) as it exhibits better *in vivo* pharmacokinetics due to rapid renal excretion. [43] Also, c(RGDfK) is not specific for integrin $\alpha_v\beta_3$ and binds almost equally to both integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ expressions. [44] Therefore, c(RGDyK) peptide was aptly chosen as the ligand in order to specifically target integrin $\alpha_v\beta_3$ expression in U87MG tumor bearing mice.

The present study demonstrated the proof-of-principle for active tumor vasculature targeting using cRGDyK conjugated HMSN. Integrin $\alpha_v\beta_3$ was chosen as the vascular target, which is almost exclusively expressed on proliferating tumor endothelial cells. [45] Angiogenesis plays a critical role in cancer progression and integrin $\alpha_v\beta_3$ expression has significant prognostic value and potential usefulness as a target for specific anti-angiogenic therapy. Since nanoparticles generally suffer from poor extravasation in the tumor tissue, [14] tumor vasculature targeting was adopted where extravasation of nanoparticles would not be required. It is also pertinent to point out that the model anticancer drug (SUN) used in the present study is a widely used angiogenesis inhibitor that targets vascular endothelial growth factor receptor (VEGFR) which is expressed at the tumor vasculature. [46] Therefore, extravasation of nanoparticles is not of much concern in this study as the chemotherapeutic drug needs to be delivered at the tumor vasculature for specific anti-angiogenic therapy.

The delivery of anticancer therapeutics into cancer cells by employing cRGDyK-conjugated HMSN dispersed in aqueous medium can overcome common issues of conventional systemic drug supply such as limited stability, rapid metabolism of the drug during transit, undesired side effects, and the lack of selectivity towards specific cells types. [4, 47] This is particularly important in the delivery of hydrophobic anticancer drugs, such as SUN, where low solubility of the drugs in aqueous media might hamper their ability to be administered through normal intravenous route. The major advantages of using the engineered HMSN based nanoplatfroms for such applications include: (a) the ease of nanoparticle functionalization for conjugation with suitable targeting vectors such as cRGDyK, (b) the ability to deliver higher concentration of contrast agents for every targeted binding event to achieve higher detection sensitivity, (c) improved treatment effects when used as drug carriers by protecting entrapped drugs from degradation and release during transit, (d) enhancing tumor uptake through the enhanced permeability and retention (EPR) effect as well as receptor-mediated endocytosis, thereby achieving increased exposure of the tumor to therapeutic drugs. These desirable features make cRGDyK conjugated HMSN a highly attractive nanoplatfrom for future cancer targeted imaging and therapy.

CONCLUSIONS

In summary, we have successfully synthesized functionalized HMSN-based nanoconjugates which can not only be used for PET imaging of integrin $\alpha_v\beta_3$ expression but also for tumor vasculature targeted delivery of chemotherapeutic drugs to the cancerous lesions. Uniform

and size-controllable HMSNs were synthesized with a modified hard-templating method, which were subsequently subjected to a generally applicable surface engineering process, including amine group functionalization, NOTA linkage, PEGylation, cRGDyK conjugation and radiolabeling, forming well-dispersed ^{64}Cu -NOTA-HMSN-PEG-cRGDyK nanoconjugates. The specific binding of the nanoconjugates to the integrin $\alpha_v\beta_3$ receptors was established by *in vitro* flow cytometry studies, *in vivo* PET imaging and histological examination of the tissue samples. Tumor-targeting ability of the cRGDyK-conjugated nanoconstructs was significantly enhanced in integrin $\alpha_v\beta_3$ -overexpressing U87MG tumor models by integrin $\alpha_v\beta_3$ mediated active targeting as well as EPR effect. A model hydrophobic anticancer drug (SUN) was loaded in the void space inside HMSNs with high loading capacity (>400 mg/g) and enhanced *in vivo* drug delivery could be demonstrated in U87MG tumor-bearing mice.

FUTURE PERSPECTIVES

The use of drug loaded-target specific nanocarriers in clinics in the future would allow physicians to predict the therapeutic effects, based on drug accumulation in the tumor site and also monitor the cancer progression in individual patients by PET imaging, thereby paving the way for personalized medical treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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EXECUTIVE SUMMARY

- Introduction: Nanoparticle mediated tumor vasculature targeting, though a promising strategy for cancer diagnosis, therapy and treatment monitoring, remains vastly unexplored.
- Methods: In this study, multifunctional hollow mesoporous silica nanoparticles (HMSNs) were successfully synthesized for integrin $\alpha_v\beta_3$ targeted PET imaging and image guided drug delivery in human glioblastoma (U87MG) xenografted mice.
- Surface engineering of HMSNs with chelator NOTA, PEG and the targeting peptide cRGDyK, yielded ^{64}Cu -NOTA-HMSN-PEG-cRGDyK nanoconjugates after radiolabeling which were then subjected to systematic *in vitro* and *in vivo* studies.
- In vitro Targeting: *In vitro* flow cytometry experiments depicted 10– 25 fold higher uptake of FITC labeled HMSN-PEG-cRGDyK in HUVECs when compared to non-targeted nanoconjugates (FITC labeled HMSN-PEG).
- In vivo PET Imaging: *In vivo* PET imaging exhibited enhanced and integrin $\alpha_v\beta_3$ specific uptake in U87MG tumors for the targeted cohort (^{64}Cu -NOTA-HMSN-PEG-cRGDyK), compared to the non-targeted (^{64}Cu -NOTA-HMSN-PEG) cohort.
- In vivo results corroborated well with *ex vivo* biodistribution and histology studies.
- Enhanced Drug Loading and Image Guided Drug Delivery: As a proof-of-concept, anti-angiogenesis drug, sunitinib was also loaded into the nanoconjugates with high loading capacity (>400 mg/g) and targeted *in vivo* drug delivery was demonstrated in U87MG tumor-bearing mice.

Figure 1.

A schematic illustration of the synthesis of ⁶⁴Cu-NOTA-HMSN-PEG-cRGDyK. Uniform HMSN nanoparticles (A) were first modified with NH₂ groups to form HMSN-NH₂ (B), which was then subjected to NOTA and Fluorescein conjugation to form NOTA-HMSN-Fluorescein (C). Subsequently the nanoconjugate was PEGylated with SCM-PEG_{5k}-Mal to form NOTA-HMSN-Fluorescein-PEG (D). The maleimide groups on the PEG were further used for conjugation with thiolated cRGDyK (cRGDyK-SH) to yield NOTA-HMSN-Fluorescein-PEG-cRGDyK (E), which was then labeled with ⁶⁴Cu to form the ⁶⁴Cu-NOTA-HMSN-Fluorescein-PEG-cRGDyK nanoconjugate (F). For drug delivery studies, a model hydrophobic anticancer drug (SUN) was loaded in HMSN-NH₂ (G) and the conjugation steps (A–E) were carried out to form ⁶⁴Cu-NOTA-HMSN(SUN)-PEG-cRGDyK. It must be noted that this Figure only provides the schematic depiction of the different reaction steps

involved in preparation of the radiolabeled nanoconjugates and it does not accurately represent the structure of the nanoconjugates.

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Figure 2. Structural characterization of HMSNs. TEM images of (A) HMSN-NH₂ and (B) NOTA-HMSN-PEG-cRGDyK nanoconjugates. Insets show uniform DLS size distribution of the nanoparticles. (C) Schematic showing step-by-step surface modification of HMSN along with the DLS diameter and zeta potential of the nanoparticles at each stage. Scale bar = 0.5 μ m.

Figure 3.

Flow cytometry analysis of U87MG human glioblastoma cells treated with NOTA-HMSN-Fluorescein-PEG (non-targeted), NOTA-HMSN-Fluorescein-PEG-cRGDyK (targeted) or NOTA-HMSN-Fluorescein-PEG-cRGDyK with a blocking dose of cRGDyK for 30 min at 37 °C. The cells were incubated with (A) 10 nM and (B) 50 nM of nanoconjugates.

Figure 4.

Positron emission tomography imaging of ^{64}Cu -labeled nanocarriers in U87MG tumor-bearing mice. (A) Serial coronal PET images of U87MG tumor-bearing mice at various time points p.i. of ^{64}Cu -NOTA-HMSN-PEG-cRGDyK (targeted), ^{64}Cu -NOTA-HMSN-PEG (non-targeted) or ^{64}Cu -NOTA-HMSN-PEG-RGD with a blocking dose of cRGDyK (blocking). (B) Representative PET/CT images of a U87MG tumor bearing mouse at 3 h p.i. of ^{64}Cu -NOTA-HMSN-PEG-cRGDyK.

Figure 5. Region-of-interest analysis and biodistribution studies. (A) Time-activity curves of the U87MG tumor, liver, blood, and muscle upon intravenous injection of ^{64}Cu -NOTA-HMSN-PEG-cRGDyK (targeted; n = 3). (B) Time-activity curves of the U87MG tumor, liver, blood, and muscle upon intravenous injection of ^{64}Cu -NOTA-HMSN-PEG (non-targeted; n = 3). (C) Time-activity curves of the U87MG tumor, liver, blood, and muscle upon intravenous injection of ^{64}Cu -NOTA-HMSN-PEG-cRGDyK with a blocking dose of cRGDyK (blocking; n = 3). (D) Comparison of U87MG tumor uptake upon intravenous injection of ^{64}Cu -NOTA-HMSN-PEG-cRGDyK (targeted), ^{64}Cu -NOTA-HMSN-PEG (non-targeted) and ^{64}Cu -NOTA-HMSN-PEG-cRGDyK with a blocking dose of cRGDyK (blocking). The difference in tumor uptake in the two groups was statistically significant (P < 0.05).

Figure 6.

Ex vivo histological analysis at 3 h p.i. of NOTA-HMSN-Fluorescein-PEG-cRGDyK. The integrin $\alpha_v\beta_3$ expression is shown by staining in red, using an anti-mouse integrin β_3 primary antibody, while the green fluorescence from NOTA-HMSN-Fluorescein-PEG-cRGDyK was used to indicate the location of the nanoconjugate. Merged images are also shown. Scale bar = 100 μm .

Figure 7.

In vivo enhanced drug delivery. (A) UV-vis spectra of HMSN and HMSN(SUN) in aqueous solution and SUN dissolved in DMSO. (B) Release profiles of HMSN(SUN) in PBS and in DMSO. (C) *Ex vivo* optical imaging of SUN after intravenous injection of NOTA-HMSN(SUN)-PEG (non-targeted) or NOTA-HMSN(SUN)-PEG-cRGDyK(targeted) in U87MG tumor-bearing mice. HMSN dose was 10 mg/kg, while the SUN dose was 7 mg/kg.