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Designing Mesoporous Silica Nanoparticles to Overcome Biological Barriers by Incorporating Targeting and Endosomal Escape

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ABSTRACT: The several biological barriers that nanoparticles might encounter when administered to a patient constitute the major bottleneck of nanoparticle-mediated tumor drug delivery, preventing their successful translation into the clinic and reducing their therapeutic profile. In this work, mesoporous silica nanoparticles have been employed as a platform to engineer a versatile nanomedicine able to address such barriers, achieving (a) excessive premature drug release control, (b) accumulation in tumor tissues, (c) selective internalization in tumoral cells, and (d) endosomal escape. The nanoparticles have been decorated with a self-immolative redox-responsive linker to prevent excessive premature release, to which a versatile and polyvalent peptide that is able to recognize tumoral cells and induce the delivery of the nanoparticles to the cytoplasm via endosomal escape has been grafted. The excellent biological performance of the carrier has been demonstrated using 2D and 3D in vitro cell cultures and a tumor-bearing chicken embryo model, demonstrating in all cases high biocompatibility and cytotoxic effect, efficient endosomal escape and tumor penetration, and accumulation in tumors grown on the chorioallantoic membrane of chicken embryos.

KEYWORDS: mesoporous silica nanoparticles, stimuli-responsive, drug delivery, redox-responsive, self-immolative, targeting, endosomal escape, chicken embryo model

INTRODUCTION

According to World Health Organization reports, cancer is currently a leading cause of death worldwide, with almost 10 million cancer deaths in 2018.1 The number of new cases is expected to reach 25 million over the next two decades.2 Among current cancer treatments, chemotherapy lacks tumor tissue selectivity, which leads to nonspecific drug distribution and subsequent toxicity to the patient. In this sense, nanoparticles have emerged as a compelling weapon to encapsulate cytotoxins and deliver them to cancerous cells, reducing those potential side effects.3–5 Among all the available nanocarriers, mesoporous silica nanoparticles (MSNs) have drawn attention owing to their exquisite properties, including a network of hollow cavities with the subsequent large surface areas (ca. 1000 m²/g) and pore volumes (ca. 1 cm³/g), tunable pore size and morphologies, facile surface modification, and biocompatibility.6–10 Their open porous structure suggests that the cargo molecules could easily diffuse out of the pores before reaching their target cells. This premature release can be diminished using stimuli-responsive gatekeepers. Those are structures that are able to open and close the pore gates in response to certain stimuli (internal or external).11–14 That responsive behavior can be accomplished using different types of gatekeepers, such as those based on self-immolative chemistry.15–17 These structures degrade in a domino-like fashion when a triggering moiety is removed from the backbone upon application of a very specific stimulus.18,19 In this sense, glutathione is overexpressed within the cytoplasm of cancer cells and can be employed to initiate the self-immolation of different redox-responsive structures.20–25

Although research on nanoparticles for drug delivery in cancer has been thoroughly investigated in the laboratory, few of them have reached the bed side. In fact, and despite all benefits that nanoparticles might offer, they have to face several biological barriers that might prevent their effective use for tumor drug delivery. Examples of these barriers include (1) lack of tumor tissue selectivity, (2) lack of selective cancer cell recognition, or (3) endolysosomal sequestration, among others.26,27

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Synthesis of the Redox-Responsive Self-Immolative Linker; bond. HAVAB was coupled to the PEG using carbodiimide chemistry, leading to the formation of an amide bond. overnight; (iii) DBTL, toluene, reflux, 4 h; (iv) pTsOH, dichloromethane:methanol (1:1), reflux, overnight. For more details, please see Scheme S2. The part in the right (green) is able to recognize cancer cells and can be tuned on-demand, increasing the versatility of the carrier. The part in the left (yellow) is able to promote endosomal escape via proton sponge effect thanks to the histidine residues. Upon application of a redox stimulus, the disulfide bond is cleaved, producing an electron pair that attacks the carbamate through cyclization, triggering the 1,6-self-immolation.

In this sense, it is well-known that the presence of fenestrations in the tumor blood vessels and the poor lymphatic drainage in the tissue promotes the passive accumulation of nanomedicines in the tumors. In consequence, the so-called enhanced permeability and retention (EPR) effect would provide an effective approximation to tackle the lack of tumor tissue selectivity.26 In addition, the overexpression of specific membrane receptors of tumoral cells enables their selective recognition by modifying the nanoparticles surface with molecules showing high affinity for those receptors. In this manner, nanoparticles would be selectively internalized, thereby addressing the lack of selectivity toward tumoral cells.29,30 Following internalization of nanocarriers, they might be sequestered within the acidic endolysosomes, which might lead to cargo degradation and reduced therapeutic effect.28 In this sense, these vesicles might be disrupted by modifying the particles with species with buffering capacity. These moieties would induce the influx of protons and chloride ions along with water molecules (proton sponge effect), causing the vesicle to swell and expand with the subsequent membrane rupture, thus avoiding the endolysosomal entrapment.31

To the best of our knowledge, this is the first time that the active recognition of particles and their subsequent endosomal escape have been demonstrated in 2D and 3D tumor models using such a simple molecule, paving the way for future nanomedicines that might improve the efficiency of cancer treatments.

### RESULTS AND DISCUSSION

**Targeted Redox-Responsive Mesoporous Silica Nanoparticles.** The synthesis of MSNs was carried out using a modified Stöber method, employing TEOS as silica source and CTAB as structure directing agent, to obtain colloidally stable and homogeneous spherical nanoparticles presenting hexagonally ordered cylindrical mesopores (see Supporting Information). Then, the particles were endowed with stimuli-responsiveness behavior by grafting a redox-responsive self-immolative molecule acting as linker and gatekeeper. This linker would be cleaved thanks to the difference in the concentration of glutathione in cancer cells (2–10 mM in the cytoplasm vs 2–20 μM in the extracellular fluids).32,33 A comprehensible representation of the synthesis of the linker is depicted in Scheme 1B. As shown there, the redox-responsive self-immolative linker was synthesized from compounds 2 and 3. Compound 2 was a protected analogue of...
a standard self-immolative monomer, which was synthesized from commercially available 4-aminobenzyl alcohol. Compound 3 was synthesized by reacting 2-mercaptoethanol with 2,2′-dipyridildisulfide to introduce the disulfide bond functionality responsive to redox species. Afterward, compounds 2 and 3 were reacted in the presence of a tin catalyst, and the benzyl alcohol was finally deprotected under acidic conditions to accomplish self-immolative compound 5.

The redox-responsive linker was then grafted to the particles via silylation and subsequent condensation on the particles surface to yield MSN-S. The grafting process was optimized in terms of (1) the most adequate solvent for the silylation, (2) the most convenient mixture of solvents for the grafting to the nanoparticles, and (3) the best MSNs:Linker ratio. Overall, the silylation was found to be most effective in DCM, as demonstrated by the changes in the chemical environment of the benzylic alcohol of 5 (reduction of signal 1 along with appearance of signal 4) in the ¹H NMR spectrum (Figure S10). Then, a mixture of DCM:AcN was observed to provide the best results for the grafting, as demonstrated by the higher weight loss observed in the TG analysis (Figure S11). Finally, the higher amount of additional organic matter observed by TG analysis confirmed 1:7 as the best ratio for the functionalization (Figure S13).

MSN-S were PEGylated (MSN-SP) via thiol exchange with the redox-responsive linker. In this regard, increased colloidal stability was observed as the different functionalization steps were carried out (Figure 1A), as demonstrated by the decreasing values of the polydispersity index (PDI) width. The successful outcome of the different reaction steps was confirmed by the weight loss observed (ca. 24% for MSN-S and ca. 26.5% for MSN-SP) in the TG analysis (Figure S14) as well as the changes in the FTIR spectrum, namely, the bands from the carbamate group of 5 and the C−H groups of the PEG chains) in the FTIR spectrum (Figure S15). The success of each functionalization reaction was also confirmed by the variations in the zeta potential, from −34.3 mV (MSNs) to −37.5 mV (MSN-SP). Besides, the presence of organic matter was confirmed through TEM microscopy upon treatment of MSN-SP with phosphotungstic acid (Figure 1B). Additional physicochemical characterization can be found in the Supporting Information.

The redox-responsiveness and subsequent self-immolation of the linker were evaluated using a solution containing dithiothreitol (DTT). A comprehensible representation of the self-immolative process is depicted in Scheme 1D. Briefly, the presence of redox species triggers the cleavage of the disulfide bond of either MSN-S or MSN-SP, generating a free electron pair. Such electron pair undergoes cyclization onto the

Figure 1. Representative characterization of the mesoporous silica nanoparticles developed in this work. (A) DLS measurements of MSNs, MSN-S, MSN-SP, and MSN-SP-PEPT. The dispersibility of the material increased after each of the functionalization steps, as confirmed by the decreasing values of the PDI width. This value is obtained from the width of the distribution at half its maximum height value. Hence, the lower the value, the more monodispersed the sample and smaller the amount of aggregates in the sample. (B) TEM micrographs of MSNs vs MSN-SP. The samples were stained with phosphotungstic acid, and the blurrer surface of MSN-SP indicated that the surface of the nanoparticles contained organic matter. (C) Release experiment of MSN-SP. The experiment was carried out using Transwells, at 37 °C and under orbital stirring. DOX was employed as cargo. One group was treated with DTT in PBS 1× as redox stimulus, while the other received only PBS 1x. The group treated with 10 mM DTT PBS 1× showed a 2-fold DOX release compared with the control group (PBS 1×) at 24 h and released additional ca. 40% after 48 h, compared with the control group that did not receive any stimuli.

[Image of Figure 1 showing DLS measurements, TEM micrographs, and release experiment results]
carbamate, consequently initiating the 1,6-self-immolation of the linker.18,35,36 Both MSN-S and MSN-SP were incubated with the reducing agent, showing appreciable changes in their C,N,S composition that were ascribed to the self-immolation of the linker (see Supporting Information). Finally, MSN-SP was loaded with doxorubicin (DOX) to evaluate the responsiveness to redox stimuli. The experiment showed that the redox-triggered material released a 2-fold amount of cargo at 24 h and ca. 40% of additional drug release at 48 h, compared with the control group (Figure 1C), confirming the redox-responsiveness of the material and the feasibility of using a self-immolative construct to hamper excessive drug release.

One of the key parts of this work was the design of a multifunctional molecule that is able to mediate the active recognition of the redox-responsive particles by cancer cells and to promote their subsequent endosomal escape into the cytoplasm (Scheme 1C). HAVAB was produced by employing solid-phase peptide synthesis. Then, it was attached to the particles using carbodiimide chemistry between the C-terminal of the peptide and the amino groups of the PEG chain (see Supporting Information), yielding MSN-SP-PEPT. These nanoparticles exhibited the lowest PDI values and, consequently, the highest colloidal stability (Figure 1A). The rationale behind the increasing colloidal stability as the different functionalization steps were carried out relies on different factors. First, MSN-S was produced by attaching a carbamate-containing self-immolative linker, showing a lower PDI width than MSNs. This is in agreement with our previous results, which showed that functionalizing the surface of pristine MSNs or different hydrophobic mesoporous carbon nanoparticles with a carbamate-based self-immolative polymer resulted in increased colloidal stability.18,47 Then, MSN-S was functionalized with PEG, displaying lower PDI width than MSN-S. This would be a consequence of the hydration layer generated around the PEGylated nanoparticles, which has been shown to increase the colloidal stability of different mesoporous silica nanoparticles.37,46 Finally, the attachment of HAVAB, a readily water-soluble peptidic molecule, produced MSN-SP-PEPT, which showed the lowest PDI width, highlighting its colloidal stability and demonstrating its suitability to be employed as drug delivery system via systemic administration.

**2D Biological Evaluation: Cellular Uptake.** Because biotin receptors are overexpressed in many tumor cells, its conjugation to different types of nanoparticles has yielded nanomaterials with application in imaging, sensing, and drug delivery.59 Indeed, its conjugation to MSNs has been demonstrated to improve their internalization in biotin-receptor-positive cells.37,40–42 In this sense, the presence of the biotin targeting moiety promoted the preferential accumulation of MSN-SP-PEPT in biotin-receptor-positive cells rather than in healthy cells (see Supporting Information). Then, the effect of HAVAB on the cellular uptake was further evaluated by flow cytometry, using fluorescein-labeled nanoparticles and analyzing the fluorescence intensity inside the cells. To that aim, MSN-SP-PEPT and MSN-S were placed with HeLa cells. Besides, nanoparticles functionalized with biotin but without the amino acids, denoted as MSN-SP-Biotin (MSNs + linker + PEG + Biotin), was also included (Figure 2A). MSN-SP-PEPT displayed the performance, showing greater internalization for all the concentrations studied in two independent experiments (75 and 37.5 μg/mL; 18 and 9 μg/mL), highlighting the remarkable influence of HAVAB on the cellular uptake. The noticeable difference between MSN-SP-Biotin and MSN-SP-PEPT might rely on the effect of pH on the histidine residues, as they progressively protonate as pH acidifies.43 Then, the remarkable cellular uptake of MSN-SP-PEPT might be explained as a consequence of two possible phenomena, which might reinforce the active binding of biotin: (1) the partial protonation would lead to local positive charge effects, and the material would interact more tightly with the cell membrane, which is negatively charged; or (2) it has been demonstrated that peptides or proteins that present histidine residues may lead to membrane fusion upon protonation,44–47 which is a phenomenon known to mediate the internalization of some cell-penetrating peptides.58

**2D Biological Evaluation: Endosomal Escape.** Histidine was used owing to the buffering capacity of the imidazole ring at the acid pH of endosomes, which can lead to the escape of the nanoparticles from the endosomes via proton sponge effect.49–51 The endosomal escape capabilities were evaluated loading the particles (MSNs or MSN-SP-PEPT) with calcine (Figure 2B). The calcine method is a standardized approach to evaluate the endosomal escape, owing to its membrane-impermeable nature.52 Its fluorescence is self-quenched at high concentrations, such as when it is trapped in the endolysosomes, and it can be detected again when calcine leaves the vesicles, as a consequence of their disruption.53–55 As shown in Figure 2B, MSNs loaded with calcine produced low green fluorescence as a result of the nanoparticles being trapped in the endolysosomes. However, cells incubated in the
The presence of MSN-SP-PEPT loaded with calcein displayed strong green fluorescence with a slight diffusion pattern. This was associated with successful escape from the endosomes, verifying the endosomal escape features of this nanocarrier.

**2D Biological Evaluation: Cytotoxicity.** The particles (MSN-SP-PEPT or MSN) were loaded with doxorubicin and placed with A549 cells to evaluate their cytotoxicity on tumoral cells. The amount of DOX loaded in the nanoparticles was analyzed by thermogravimetric analysis and was found to be ca. 4%. As shown in Figure 2C, drug-containing MSN-SP-PEPT significantly inhibited the cell viability (ca. 60% at 24 h and ca. 70% after 48 h). The much higher cytotoxic effect of this carrier compared with the control group was ascribed to the enhanced uptake of the particles bearing HAVAB. This effect was observed to be independent of the tumoral cell line employed because MSN-SP-PEPT could also inhibit the viability of HeLa cells (see Supporting Information), confirming the versatility of the produced nanocarrier in this work.

**3D Biological Evaluation: Endosomal Escape and Penetration in Tumor Spheroids.** The endosomal escape features of the system were also analyzed using tumor spheroids grown from A549 cells (Figure 3A). This in vitro 3D tumor model constitutes a powerful tool to evaluate the biological performance of nanoparticles as it shows several features of in vivo tumors. Calcein-loaded nanoparticles (MSNs or MSN-SP-PEPT) were used. The results were analogous to those in the 2D tumoral model. Although MSNs remained trapped in the endosomes, as demonstrated by the negligible green fluorescence detected, a strong fluorescence was observed for MSN-SP-PEPT, clearly indicating the achievement of the endosomal escape in the 3D tumor model and confirming the versatility of the nanocarrier.

The distribution of particles in the spheroids was also evaluated. As observed in Figure 3B, MSN-SP-PEPT accumulated much more than bare MSNs in tumoral spheroids, highlighting again the positive effect of HAVAB. The depth reached by the nanoparticles, analyzed employing ImageJ, demonstrated that MSN-SP-PEPT could penetrate deeper than MSNs.
deeper in the spheroid (250 μm for MSN-SP-PEPT vs 200 μm for MSNs). OVCAR8 spheroids were also incubated with such nanoparticles, obtaining analogous results (see Supporting Information). The rationale behind this higher penetration might rely on the endosomal escape. On the one hand, it is known that endolysosomal sequestration reduces the rate of exocytosis of nanoparticles.\(^{60,61}\) Besides, Lu et al. demonstrated that nanocarriers penetrate deeper in tumor spheroids via transcytosis if the nanoparticles have undergone exocytosis from the outer cells of the spheroid.\(^{61}\) In addition, Cui et al. established the endolysosomal entrapment of nanoparticles as a limiting factor for achieving transcytosis.\(^{62}\) In consequence, MSN-SP-PEPT were preferentially internalized owing to the overexpressed biotin receptors to then escape from the endosomes. This would lead to the nanoparticles being exocytosed toward inner areas of the AS49 tumor spheroids, to then undergo further cellular uptake through transcytosis, thus improving the final tumor penetration.

**3D Biological Evaluation: Cytotoxicity.** The particles (MSNs or MSN-SP-PEPT) were loaded with doxorubicin to further evaluate their cytotoxic effect on AS49 tumor spheroids. The cytotoxicity was studied by analyzing the spheroid volume at different time points. In this sense, the spheroid images shown in Figure 3C were numerically represented in Figure 3D to facilitate the understanding of the trends observed in Figure 3C.\(^{63}\) The spheroids were unaffected by unloaded MSN-SP-PEPT, confirming again the high biocompatibility of the carrier and laying the foundations for future analysis using animals. Moreover, MSN-SP-PEPT loaded with DOX reduced the volume of the spheroids by ca. 30% after just 48 h and ca. 40% after a week, corroborating their capability to penetrate in the tumoral spheroids and deliver cytotoxic compounds to the cancerous cells. Furthermore, drug-loaded MSN-SP-PEPT led to greater cytotoxic effect than drug-loaded MSNs, in agreement with all the previous results. Analogous results were obtained when a lower particles concentration was employed (see Supporting Information).

**Tumor-Bearing Chicken Embryo Model.** The chicken embryo model allows the growth of human-like tumors, providing valuable information about the biodistribution and cytotoxic effect of nanoparticles.\(^{64,65}\) The tumors were grown within 3–4 days by placing the tumoral cells in a Teflon ring on the chorioallantoic membrane (CAM) of the chicken embryo. Afterward, rhodamine B-labeled MSN-SP-PEPT were injected into the AS49 tumor-bearing chicken embryos through a vein that was far from the tumor and allowed to accumulate in the tumoral mass for 48 h (Figure 4). The red fluorescence of the rhodamine B-labeled nanoparticles could be observed in the tumoral mass 48 h after their administration, confirming their ability to accumulate in the tumor via EPR effect (Figure 4A). Indeed, many marketed nanomedicines rely on this principle.\(^{66}\)

The embryos were subsequently dissected and imaged (Figure 4B). Overall, MSN-SP-PEPT accumulated preferentially in the tumoral mass, and reduced accumulation was noted only in the kidneys and the liver, indicating the low clearance from the bloodstream. In addition, no significant organ damage was observed. These observations further confirmed the high colloidal stability of MSN-SP-PEPT, given that it could circulate for up to 72 h in the embryos without leading to aggregates, further emphasizing the biocompatibility of this drug delivery system.

Finally, MSN-SP-PEPT was loaded with DOX and a preliminary cytotoxicity study was performed. The drug-loaded nanoparticles were injected into the bloodstream of the AS49 tumor-bearing chicken embryos through an injection in a vein, which were sacrificed after 72 h. As observed in Figure 4C, the DOX-loaded nanocarrier could lessen the tumor weight by ca. 50% after only 72 h. In this sense, it was found that at least 25 μg of free DOX were needed to obtain comparable results (data not shown), highlighting the suitability of MSN-SP-PEPT to be employed as a nanomedicine in the treatment of tumors.
CONCLUSIONS

In this research, redox-responsive mesoporous nanoparticles showing remarkable targeting and endosomal escape features was designed and produced. The particles were effective in controlling premature drug release thanks to the redox-responsive self-immolative molecule. A simple and versatile molecule capable of targeting tumoral cells and to induce the escape from the endosomes was here synthesized for the first time and further grafted to the particles. The biological capabilities of this nanocarrier was extensively studied using 2D and 3D tumoral models, demonstrating remarkable cellular uptake and endosomal escape capabilities as well as excellent cytotoxic profile. In addition, the presence of HAVAB significantly enhanced the penetration of the particles in tumor spheroids. Finally, the particles were shown to passively accumulate in tumors of tumor-bearing chicken embryos. In addition, the here produced particles were shown to significantly reduce the weight of the tumor in a short space of time, confirming the potentiality of the engineered nanoparticle to addresses essential problems in current nanomedicine.

MATERIALS AND METHODS

The majority of the reagents employed in this work were bought from Sigma-Aldrich Inc.: ammonium nitrate; tetraethyl orthosilicate (TEOS); fluorescein isothiocyanate isomer I (FITC); cetyltrimethylammonium bromide (CTAB); 3-(aminopropyl)triethoxysilane (APTES); rhodamine B isothiocyanate (RhB); phenyl chloroformate; 4-sminobenzyl alcohol; dibutyltin dilaurate (DBTL); N,N-diisopropyl-2-mercaptoethanol; 2,2′-dipyrididilisulphide; imidazole; 3-(triethoxysilyl)propyl isocyanate; p-toluenesulfonic acid (TsOH); tert-butylmethylsiloxyl chloride (TBDMSCI); His-PEG5000NH2 (PEG-SH); Tris(2′,2′-bipyridyl)dichlororuthenium (II) hexa drate (Ru); dithiothreitol (DTT); 2-chlorotriethyl chloride resin; Fmoc-S-Ava—OH (Ava); Fmoc-His(Trr)—OH (His); 1-hydroxybenzotriazole hydrate (HOBt); N,N,N′,N′-Tetramethyl-O-(1H-benzo[1,2,3] 4-[[tert-butyl(dimethyl)silyl)oxy]methyl]phenyl)carbamate (1). Compound 1 was produced following a modified method published previously. In brief, 6 g (48.7 mmol) of 4-aminobenzyl alcohol was dissolved in 240 mL of THF/saturated aqueous sodium bicarbonate/water (2:2:1). Then, 7 mL (5.8 mmol) of phenyl chloroformate was dropwisely added, and the reaction mixture was stirred at room temperature upon completion. Then, ethyl chloride was employed to extract compound 1, and the organic phase was washed several times with sodium bicarbonate, dried over sodium sulfate, and finally removed in vacuo. Finally, chloroform was used to recrystallize the crude, yielding compound 1. The product was characterized by 1H NMR.

Pheny1(4-[[tert-butyl(dimethyl)silyl)oxy]methyl]phenyl)carbamate (2). Compound 2 was synthesized using a published method with minor modifications. In brief, 1 g (4.2 mmol) of compound 1 and 0.31 g (4.52 mmol) of imidazole (0.31 g, 4.52 mmol) were dissolved in 20 mL of DCM. Then, 0.75 g (5 mmol) of TBDMS-Cl were dropwisely added, and the reaction was stirred overnight at room temperature. Then, the imidazole salts generated were removed by filtration. Afterward, the solvent was removed in vacuo, and the crude was purified on a silica column (DCM), leading to compound 2. The product was characterized by 1H NMR.

2-(Pyridin-2-ylsulfanylethyl)ethan-1-ol (3). Compound 3 was produced according following a published method. In brief, 2.42 g (10.98 mmol) of 2,2′-dipyridylsulphide and 0.26 mL (4.53 mmol) of acetic acid were dissolved in 40 mL of methanol. Then, 0.6 mL (8.52 mmol) of 2-mercaptoethanol in 10 mL of methanol was added dropwisely, and the reaction was stirred for 48 h at room temperature. Afterward, the crude obtained after removing the solvent was purified on a silica column (heptane/ethyl acetate, 1:1) to yield compound 3. The product was characterized by 1H NMR.

2-(Pyridin-2-ylsulfanylethyl)ethan-1-ol (4-[[tert-butyl(dimethyl)silyl)oxy]methyl]phenyl)carbamate (4). First, 1.9 g (5.3 mmol) of compound 2 and 1.1 g (5.9 mmol) of compound 3 were dissolved in 40 mL of toluene. Then, 0.63 mL of DBTL were added, and the reaction was heated at 110 °C for 4 h. Afterward, the solvent was evaporated to yield a crude that was purified on a silica column (heptane/ethyl acetate, 4:1), leading to compound 4. The product was characterized by 1H NMR.

2-(Pyridin-2-ylsulfanylethyl)ethan-1-ol (4-[[tert-butyl(dimethyl)silyl)oxy]methyl]phenyl)carbamate (5). First, 2 g (4.44 mmol) of compound 4 was dissolved in 50 mL of methanol and DCM (1:1). Then, 84.4 mg of TsOH in 15 mL of methanol and DCM (1:1) was added, and the reaction was refluxed overnight. Afterward, the solvent was removed, and the crude was purified on a silica column (heptane/ethyl acetate, 1:2) to lead to compound 5. 1H NMR was employed to characterize the final product.

MSNs Functionalized with the Redox-Responsive Linker (MSN-S). Initially, 148.5 μL (0.6 mmol) of 3-(triethoxysilyl)propyl isocyanate was dissolved in 0.5 mL of dry DCM (vial 1). Separately, 0.24 g (0.72 mmol) of compound 2 was added at 50 mL of methanol and DCM (1:1). Then, 2-(Pyridin-2-ylsulfanylethyl)ethan-1-ol (4-[[tert-butyl(dimethyl)silyl)oxy]methyl]phenyl)carbamate (4). Afterward, the solution was evaporated to yield a crude that was purified on a silica column (heptane/ethyl acetate, 1:1) to yield compound 4. The product was characterized by 1H NMR.

MSNs Functionalized with the Redox-Responsive Linker (MSN-SP). To PEGylate the MSNs Functionalized with the Redox-Responsive Linker (MSN-SP). To PEGylate the as-produced redox-responsive particles, 45 mg of MSN-S was dispersed in 15 mL of methanol. Then, 15 mg (0.004 mmol) of PEG-SH in 1 mL of methanol was slowly incorporated into the mixture containing MSN-S, and the reaction was stirred at room temperature overnight. Finally, the nanoparticles were collected by centrifugation and washed with methanol to yield MSN-SP, which was analyzed by FTIR spectroscopy, TG, DLS, and zeta potential, and TEM. 9662
Biotinylated Histidine-Rich Peptide (HAVAB). The biotinylated peptide (6, HAVAB, from Histidine–Alanine/Valeric Acid–Biotin) was produced through solid-phase peptide synthesis, using Fmoc-protected amino acids (Figure S3). Amide bonds were formed by adding 3.5 mL of DMF containing 300 mg (0.792 mg) of HBTU, 107.66 mg (0.792 mmol) of HOBT, and 0.275 mL (1.58 mmol) of DIPEA to the resin (200 mg, 0.264 mmol). Then, the mixture was shaken overnight. After that, the resin was extensively rinsed with DMF to get rid of byproducts and unbounded amino acids. Then, 2 mL of piperidine (20% in DMF) was added to cleave the Fmoc-protecting group. Afterward, the resin was thoroughly rinsed with DMF, and the addition of the next amino acid was carried out as mentioned above. After incorporating the last amino acid, 270 mg (0.792 mmol) of NHS-Biotin and 0.275 mL (1.58 mmol) of DIPHA were dissolved in 6.5 mL of DMF, and the mixture was added to the resin and shaken in a 360° vertical rotator overnight.

Once all the components of compound 6 were incorporated, the resin was thoroughly rinsed first with DMF and then with DCM. Then, 1.2 mL of a cleavage mixture (TFA (95%), TIPS (2.5%) and water (2.5%)) was added to the resin and kindly shaken for 2 h, to release the peptide from the resin. Afterward, the solution was filtered and collected. Then, a small amount of cleavage mixture was used to rinse the resin, and both solutions were mixed. Such solution was precipitated into cold ether and centrifuged. Then, the crude was purified via molecular exclusion chromatography, using Sephadex G-25 as stationary phase and water as mobile phase. Compound 6 was characterized by 1H NMR and mass spectrometry.

Targeted Mesoporous Silica Nanoparticles (MSN-SP-PEPT).

Thirty milligrams (0.04 mmol) of compound 6, 21.7 mg (0.11 mmol) of DCC, and 12.1 mg (0.11 mmol) of NHS were dissolved in 1.2 mL of dry DMF. The mixture was stirred for 1 h at room temperature for acid activation. Afterward, 35 mg of MSN-SP were dispersed in 3.5 mL of dry DMF, and the solution that contained the acid-activated peptide was added dropwise. The reaction was stirred at room temperature for overnight. Afterward, the particles were centrifuged, washed with DMF, ethanol, water and methanol, leading to MSN-SP-PEPT. The as-synthesized nanoparticles were characterized in terms of FTIR spectroscopy, TGA analysis, zeta potential, and DLS.

Release Experiments from MSN-SP. The response of the particles to redox stimuli was studied using DOX-loaded MSN-SP. For that purpose, MSN-SP were dispersed in a saturated solution of DOX in methanol and stirred overnight. Then, the particles were collected by centrifugation and washed until no drug could be observed in the supernatant.

The release experiment was performed in vial using Transwells (3 per condition). PBS 1X solutions in the presence or absence of DTT (10 mM) were used to replicate the cytoplasm of cancer cells. Briefly, 10 mg of particles were dispersed in 1.8 mL of the corresponding solution to then transfer 0.5 mL of the corresponding dispersion into each Transwell. The samples were kept with orbital stirring at 37 °C during the whole experiment. The media were removed every time point and studied using a fluorescence microscope (Unicam UV-500 UV−Vis).

Internalization Studies in Spheroids. The studies of internalization were performed employing OVCAR8 or A549 tumor spheroids. For that purpose, rhodamine B-labeled nanoparticles (MSNs or MSN-SP-PEPT) were incubated with the cellular spheroids for 24 h. Then, the wells were washed with PBS 1X to remove noninternalized nanoparticles and the samples were transferred to an Eppendorf tube. A formaldehyde solution (4%) at 4 °C was employed to fix the spheroids overnight. After that, the spheroids were rinsed with PBS 1X and treated with cold methanol for 0.5 h. Finally, the samples were kept in PBS 1X in the fridge for confocal solution of calcine (CAL) in methanol, and the loading protocol was carried out as described in the previous section.

Two-Dimensional Cell Viability. Cell viability was checked through the Alamar Blue method. For that purpose, the Alamar Blue solution (AbD Serotec) at 10% (v/v) was added to the cell culture at the studied time points, according to manufacturer protocols. After 4 h, aliquots of each well (200 μL) were placed in 96-well plates, and the absorbance intensity was recorded (λex=570 nm; λem=600 nm) using a spectrophotometer (Unicam UV-500 UV−Vis).

2D Internalization Studies. Cells were seeded following the above-described protocol for the fluorescence microscopy studies. Rhodamine B-labeled nanoparticles were employed.

MC3T3-E1 or HeLa cells were seeded in each well of a 6-well plate for the flow cytometry studies. After 24 h, different concentrations of fluorescein-labeled nanoparticles were added to the cells. After 2 h, cells were washed with PBS and incubated with trypsin−EDTA at 37 °C for cell detachment. After 5 min, culture medium was added, and the cells were collected by centrifugation (10 min, 1500 rpm). The cells were resuspended in fresh medium and the fluorescence from the surface of the cells was quenched using trypan blue (0.4%) to avoid the detection of fluorescence from noninternalized particles. Flow cytometry was carried out at an excitation wavelength of 488 nm and measured at 530 nm. The trigger was set for the green fluorescence channel. The data acquisition and analysis conditions were established using positive and negative controls with the CellQuest Program of Becton−Dickinson. The conditions were kept constant during all the experiments. Three replicas per condition were employed. At least 10,000 cells of each sample were analyzed for statistical significance.

OVCAR8 or A549 cells were placed with different concentrations of rhodamine B-labeled nanoparticles (MSNs or MSN-SP-PEPT) for confocal laser scanning microscopy. After 2 h, noninternalized nanoparticles were removed by rinsing each well with iced PBS.

3D Cellular Experiments. Three-Dimensional Cell Cultures.

Spheroids were grown in a 96 U-shaped-well plate. To that aim, OVCAR8 or A549 cells (10,000 cells) were added to a well containing 200 μL of either DMEM or RPMI medium, respectively. Cellular spheroids were grown for a week in a humidified CO2 atmosphere at 37 °C.

Spheroid Viability. The effect of the particles on the cellular viability was assessed using A549 spheroids. Different concentrations of DOX-loaded and drug-free nanoparticles were incubated with the spheroids for 24 h. After that, noninternalized nanoparticles were removed by rinsing the wells with PBS, and 200 μL of the appropriate culture medium were added. The spheroids were then maintained for a week in a humidified atmosphere at 37 °C and imaged at different time points. The viability of the spheroids was studied through the variation in the spheroid volume, using the formula:

\[ V = 0.5 \times (\text{short}\, d) \times \text{long}\, d \times \pi \]

where “V” indicates the volume of the spheroid and “short\, d” and “long\, d” indicate the short and long diameters of the spheroid, respectively. The values for each diameter were determined analyzing each image with the software ImageJ.

Internalization Studies in Spheroids. The studies of internalization were performed employing OVCAR8 or A549 tumor spheroids. For that purpose, rhodamine B-labeled nanoparticles (MSNs or MSN-SP-PEPT) were incubated with the cellular spheroids for 24 h. Then, the wells were washed with PBS 1X to remove noninternalized nanoparticles and the samples were transferred to an Eppendorf tube. A formaldehyde solution (4%) at 4 °C was employed to fix the spheroids overnight. After that, the spheroids were rinsed with iced PBS 1X and treated with cold methanol for 0.5 h. Finally, the samples were kept in PBS 1X in the fridge for confocal experiments, MSNs and MSN-SP-PEPT were dispersed in a saturated...
microscopy. The confocal microscope generated multiple two-dimensional images along the z-axis, from the top to the bottom, that were then stacked using the software ImageJ to accomplish the 2D projection of the 3D spheroids.

**Chicken Embryo Experiments. Tumor Formation.** Freshly fertilized chicken eggs were kept for 10 days in a humidified atmosphere (60%) at 37°C. On day 10, a small portion of the eggshell was cut out, and a Teflon ring was then placed onto the CAM membrane. After that, 100,000 A549 cells were carefully injected into the ring, and the window was closed to grow the tumor for 3 days.

**Biodistribution Studies.** Once the tumor mass was completely developed, a small window was opened in a different place, and rhodamine B-labeled MSN-SP-PEPT were administered following the protocol described above at a concentration of 100 μg/mL. The embryos (n = 5) were incubated for 72 h and subsequently dissected to measure the weight of the tumors. A control group without nanoparticles (n = 5) was also included.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.0c21507.

Summary of the materials produced in this work; Characterization of MSNs and functionalized MSNs (TGA analysis, XRD, FTIR, N2 adsorption–desorption isotherms, DLS measurements and zeta potential); Schematic representation of the synthesis of the redox-responsive linker with 1H NMR characterization; Optimization of the grafting protocol (1H NMR, TGA and FTIR); Validation of the redox-responsiveness (elemental analysis, FTIR); Additional release experiment; Schematic synthesis of HAVAB with 1H NMR characterization; Additional release experiment; Characterization of MSNs and functionalized MSNs; Additional FRET experiment; Characterization of MSNs and functionalized MSNs; Additional FRET experiment; Characterization of MSNs and functionalized MSNs; Additional FRET experiment.

Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acsami.0c21507

**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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**Notes**

The authors declare no competing financial interest.

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