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DEVELOPMENT OF COPPER SULFIDE
NANOPARTICLES FOR PHOTOTHERMAL AND
CHEMO THERAPY OF CANCER CELLS

BY

YAJUAN LI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

IN

BIOMEDICAL AND PHARMACEUTICAL SCIENCE

UNIVERSITY OF RHODE ISLAND

2015

DOCTOR OF PHILOSOPHY DISSERTATION
OF
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UNIVERSITY OF RHODE ISLAND
2015

ABSTRACT

Cancer has become number one cause of death. Conventional treatment includes surgery, chemotherapy, radiation therapy, or combination. The combinatorial therapy in one system is highly efficient and economical. Herein, two drug delivery systems with chemo and photothermal therapy are developed in order to enhance the therapeutic efficacy in A549 human lung cancer cells. The first one is hollow copper sulfide nanoparticle carrying doxorubicin (PEG-HCuSNPs-DOX). The second one is mesoporous silica coated CuS nanoparticles (CuS NPs) loaded with doxorubicin (DOX) (PEG-CuS@MSNs-DOX).

Both nano-drug delivery systems are pH sensitive, laser responsive, and photothermal convertible. CuS NPs are the photothermal sensitizers in both drug delivery systems. However the drug loading efficiency is much higher in the PEG-CuS@MSNs-DOX, whereas its drug release rate is much slower. In addition, the anti-cancer efficacy of PEG-HCuSNPs-DOX is higher than that of PEG-CuS@MSNs-DOX.

Five chapters are prepared in this thesis. Each chapter includes an independent manuscript and separate abstract. Chapter 2 serves as preparation experiment for chapter 3. Chapter 4 is a review that expands the application of CuS NPs to transdermal delivery. Chapter 5 is a mini review on the *in vivo* application of CuS NPs

1. Cancer Photothermal Chemo Therapy Using Hollow Copper Sulfide Doxorubicin Nanoparticles

The design and synthesis of the multifunctional nanoparticles responsive to external stimuli provides potential applications in biomedical fields such as controlled drug delivery. Here, near infrared (NIR) laser-controlled fast and effective tumor cell killing is achieved based on the pH sensitive and NIR light responsive hollow copper sulfide nanoparticles chelated with doxorubicin molecules (HCuSNPs-DOX). Laser exposure at 900 nm and acidic environment facilitate the release of DOX from HCuSNPs-DOX. Spontaneously, the released DOX forms DOX/Cu²⁺ complex and generates cell-killing reactive oxygen species. Laser exposure to HCuSNPs-DOX also disrupts the integrity of the cell membrane instantly. The IC₅₀ of HCuSNPs-DOX with and without laser treatment was 4.0 and 7.6 µg/mL CuS, respectively. The approach developed here offers compelling chances for quick-responsive anticancer therapy.

2. Facile Direct Dry Grinding Synthesis of Monodisperse Lipophilic CuS Nanoparticles

Copper sulfide with near-infrared light absorption property is recently attracting broad interest as a photothermal carrier for smart cancer therapy. Lipophilic copper sulfide nanoparticle is preferred for high performance biomedical applications due to the high affinity with tissues. But it requires complex multi-step synthetic process under severe condition. Here, synthesis of hydrophobic copper sulfide possessing surface plasmon resonance was retained by direct dry grinding of copper(II) acetylacetonate with sulfur under ambient environment. The formed CuS nanoparticles were in uniform size of ~10 nm, and they were monodispersed in pure chloroform. Each covellite CuS nanocrystal surface was modified with oleylamine through hydrogen bonding between sulfur atoms and amine groups of oleylamine.

While those oleylamine capped CuS nanoparticles showed uniform morphological features, they demonstrated near-infrared light absorption for photothermal applications. The facile and mild synthetic methodology described here opened a powerful pathway for the design and preparation of photothermal lipophilic copper sulfide nanomaterials for smart cancer therapy.

3. Multifunctional Mesoporous Silica-Coated CuS Nanoparticles for Cancer Therapy: Synthesis, Characterization and *in vitro* Evaluation

Chemo therapeutic drug-caused side effects are commonly seen in clinical practice due to nonspecific toxicity and low therapeutic efficiency. Herein, we reported a cancer chemo-photothermal multifunctional drug delivery system. Polyethylene glycol decorated mesoporous silica nanoparticles entrapping CuS nanoparticles (PEG-CuS@MSNs) were successfully synthesized and characterized for the drug delivery application. Doxorubicin (DOX)-loaded PEG-CuS@MSNs showed laser stimulated and pH-responsive properties. *In vitro* cell experiments demonstrated that DOX-loaded PEG-CuS@MSNs combining laser exposure achieved the highest rate of death of A549 cells, in comparison to that of PEG-CuS@MSNs-DOX chemotherapy alone. These findings provided a promising drug delivery system for cancer combinatorial therapy, which could significantly reduce drug dose and improve patient compliance.

4. Laser ablation-enhanced transdermal drug delivery

Transdermal delivery offers an excellent route for drug and vaccine administration. Nonetheless, it presents a critical challenge due to the skin's lipid-rich outer stratum

corneum layer. Laser ablation perforates epidermis through selective photothermolysis, making skin more permeable to hydrophilic and macromolecular drugs such as peptides, proteins, and genes. This review summarizes recent applications to laser ablation-enhanced transdermal delivery. Needle- and pain-free transcutaneous drug delivery via laser ablation provides an alternative approach to achieve local or systemic therapeutics.

5. Cancer Photothermal Therapy and CuS Nanoparticles

This manuscript is being prepared according to the format of Lasers in Medical Science as a review article.

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PREFACE

This dissertation is written based on the University of Rhode Island “Guidelines for the Format of Theses and Dissertations” standards for Manuscript format. This dissertation is composed of four manuscripts that have been combined to satisfy the requirements of the department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island.

Manuscript 1: Cancer Photothermal Chemo Therapy Using Hollow Copper Sulfide Doxorubicin Nanoparticles

This manuscript is being prepared for submission to *ACS nano*.

Manuscript 2: Facile Direct Dry Grinding Synthesis of Monodisperse Lipophilic CuS Nanoparticles

This manuscript is being prepared for submission to *Materials Chemistry and Physics*.

Manuscript 3: Multifunctional Mesoporous Silica-Coated CuS Nanoparticles for Cancer Therapy: Synthesis, Characterization and *in vitro* Evaluation

This manuscript is being prepared for submission to *Journal of American Chemical Society*.

Manuscript 4: Yajuan Li, Liangran Guo, Wei Lu. Laser ablation-enhanced transdermal drug delivery. *Photonics and Lasers in Medicine*. 2012, 2(4): 315–322 (review)

This manuscript has been published as a review paper.

Manuscript 5: Cancer Photothermal Therapy and CuS Nanoparticles

This manuscript is being prepared for submission to *Lasers in Medical Science*.

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CHAPTER 1

This manuscript is being prepared for submission to *ACS nano*.

Cancer Photothermal Chemo Therapy Using Hollow Copper Sulfide Doxorubicin Nanoparticles

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KEYWORDS: hollow CuS nanoparticles (HCuSNPs); reactive oxygen species; photothermal therapy; chemotherapy

ABSTRACT: The design and synthesis of the multifunctional nanoparticles responsive to external stimuli provides potential applications in biomedical fields such as controlled drug delivery. Here, near infrared (NIR) laser-controlled fast and effective tumor cell killing is achieved through the pH sensitive and NIR light responsive hollow copper sulfide nanoparticles chelated with doxorubicin molecules (PEG-HCuSNPs-DOX). Laser exposure at 900 nm and acidic environment facilitate the release of DOX from PEG-HCuSNPs-DOX. Spontaneously, the released DOX forms DOX/ Cu^{2+} complex and generates cell-killing reactive oxygen species. Laser exposure to PEG-HCuSNPs-DOX also disrupts the integrity of the cell membrane instantaneously. The IC_{50} of PEG-HCuSNPs-DOX with and without laser treatment was 4.0 and 7.6 $\mu\text{g/mL}$ CuS, respectively. The approach developed here offers compelling chances for controlled anticancer therapy.

INTRODUCTION

Cancer has nowadays become one of the most deadly diseases in the world. As reported in 2013, the 5-year global cancer prevalence is estimated to be 28.8 million in a population of 49.2 million in 2008.¹ Doxorubicin (DOX), as an effective tumor chemotherapeutic drug, causes life-threatening dosage-dependent cardiac toxicity.² Efforts in drug delivery have been made to enhance efficacy, reduce dosage, and minimize side effects. The marketed DOX liposome injection Doxil^{®3} and various multifunctional nanoparticles with assorted composites were developed as reviewed by Prados⁴ and Hanušová.⁵

Copper sulfide nanoparticles are a new class of photothermal sensitizer providing an affordable counterpart for gold nanoparticles. The light absorption of the former is affected by the surrounding environment.^{6, 7} Originating from the $d-d^*$ transition of Cu^{2+} ions in copper sulfide, such nanoparticle exhibits stable light absorption towards near-infrared (NIR) light irradiation (650–900 nm),⁸ which can penetrate through normal tissues with minimal thermal injury.⁹ Instantaneously upon NIR light absorption, copper sulfide nanoparticles generated heat and photothermally ablated tumor *in vivo* after intratumor^{10, 11} or intravenous injection.¹² In our previous work, hollow copper sulfide nanoparticles (HCuSNPs) were applied for photothermal ablation-enhanced transdermal drug delivery.¹³ Once the HCuSNPs enriched on skin surface was irradiated by nanosecond-pulsed NIR laser, high heat energy was generated within very short time, which disrupted stratum corneum of the local skin and facilitated permeation of human growth hormone. Recently, copper sulfide nanoparticles were coated with DOX conjugated gelatin, achieving enzyme-responsive

drug release simultaneous photoacoustic imaging and photothermal therapy upon NIR laser.¹⁴ Unfortunately, the resultant nanocomposites required relatively long time and high power of NIR laser irradiation (> 5 min, 6 W/cm^2) to achieve desired therapeutic efficacy, which set barrier for practical applications in controlled cancer therapy.¹⁴

In this work, nanosecond-pulsed NIR laser controlled anticancer therapy is achieved by PEG-HCuSNPs loaded with DOX. The shell of PEG-HCuSNP consists of many 8-nm large polyethylene glycol (PEG) modified copper sulfide nanocrystals. DOX molecules are loaded on the PEG-HCuSNPs through chelation. The pH-sensitive nanocomposites tend to release a proportion of DOX after reaching the acidic tumor site or lysosomes in the cells. NIR laser irradiation of the PEG-HCuSNPs-DOX produces instantaneous heat and allows most of the DOX molecules to be released into surrounding environment. The chemotherapy drug molecules further interact with Cu^{2+} ions and generate reactive oxygen species (ROS), resulting in fast and highly efficient pH-sensitive, chemo and photothermal cell killing effect. The current work provides a facile and effective way for smart controlled antitumor therapy.

RESULTS AND DISCUSSION

The preparation of PEG-HCuSNPs-DOX was carried out as illustrated in Scheme 1.

Scheme 1. Schematic illustration of the preparation procedure of the PEG-HCuSNPs-DOX and NIR laser controlled drug release process.

The transmission electron microscopy (TEM) image of the as-prepared HCuSNPs demonstrated hollow structures with the average diameter of 75 ± 11 nm (Figure 1a). The shells were ~ 20 -nm thick, and consisted of 8-12-nm large nanoparticles. After surface modification with thiolated PEG, a thin layer (thickness ~ 4 nm) was clearly observed on the particle surfaces, while the initial structures of HCuSNPs did not change (Figure 1b). Loading of DOX did not significantly change the size and morphology of the nanoparticles (Figure 1c). Dynamic light scattering (DLS) analysis revealed that the hydrodynamic particle size of the PEG-HCuSNPs-DOX was 80 ± 10 nm, which agreed well with the TEM observations. As shown in the UV-Vis spectrum (Figure 1d), PEG-HCuSNPs exhibited strong absorbance peak centered at ~ 1050 nm, which was ascribed to the $d-d^*$ transition of copper sulfide.²⁰ Similar absorbance was found in the UV-Vis spectrum of PEG-HCuSNPs-DOX, but with a minor red shift of 5 nm due to the DOX chelation on HCuSNPs.¹⁵ When compared with the green

aqueous dispersion of PEG-HCuSNPs, the aqueous dispersion of PEG-HCuSNPs-DOX was more brownish (Figure 1e). The drug loading increased with the increase of time (Figure 1f). The PEG-HCuSNPs and PEG-HCuSNPs-DOX remained stable in DI water at room temperature for at least 3 months. The DOX loading efficiency of the PEG-HCuSNPs-DOX was optimized ~6.0 wt.%.

Figure 1. TEM images of HCuSNPs (a), PEG-HCuSNPs (b), PEG-HCuSNPs-DOX (c), Bars, 50 nm. UV-vis spectra (d), the inset is the enlarged UV-vis spectra. Photograph of the dispersions (e) containing PEG-HCuSNPs (left) and PEG-HCuSNPs-DOX (right). Loading efficiency change over 5 days (f). Samples were taken at time point 0, 0.42 , 1, 2, 3 and 5 day. Data were expressed as Mean $\pm$ SD. n=3.

The drug release profiles of HCuSNPs-DOX were studied in the media with respective pH of 7.4, 6.0 and 5.0 over a 4-h period. Without laser irradiation, the cumulative release of DOX was 16.6%, 23.0% and 33.9%, respectively (Figure 2). DOX release from PEG-HCuSNPs-DOX was pH dependent. On the other hand, NIR laser irradiation accelerated DOX release in various pH conditions. While the DOX released in 1 h was $3.8 \pm 2.2\%$ (pH 7.0), $12.6 \pm 0.9\%$ (pH 6.0), and $25.0 \pm 2.7\%$ (pH 5.0), the released amount increased to $27.9 \pm 0.9\%$, $37.8 \pm 2.0\%$, and $47.7 \pm 3.7\%$ after the first laser irradiation (2.0 W/cm^2 , 15 s, 900nm). The drug release slowed down when the laser was switched off. The laser-triggered release was well responsive

to the repeated laser irradiation. By the end of 4 h with 3 times of laser exposure, the cumulative release of DOX reached 48.1%, 58.7% and 75.6% at pH 7.4, 6.0 and 5.0, respectively. Significantly, in the pH 5.0 medium, the cumulative release of DOX with NIR laser treatment was 2.2 folds higher compared with that without laser irradiation. The dual-module release profile of PEG-HCuSNPs-DOX presented a spatiotemporal controlled release manner through which the drug can be specifically released at the tumor site either applying NIR laser or under the acidic environment of the tumor.¹⁶

Figure 2. DOX cumulative release from PEG-HCuSNPs-DOX under different pH buffers containing 10% BSA. The laser irradiation was administrated at time point 1, 2 and 3 h. (2 W/cm^2 , 15 s, red lines). Data were expressed as Mean $\pm$ SD. $n=3$.

As reported, DOX formed water soluble complexes with Cu^{2+} below pH 8. Specifically, within pH range of 4.2 to 5.8, the drug and Cu^{2+} formed incomplete 2:1 DOX/ Cu^{2+} complex; at pH 5.8, 2:1 ratio complex formed exclusively regardless of

excessive copper; above 5.8, both DOX/Cu²⁺ complexes with ratio of 1:1 and 2:1 existed; and at pH 7.3, only 1:1 complex was obtained.¹⁷ To test whether the laser-treated PEG-HCuSNPs-DOX released free drug only or both free drug and copper ion, or even further to form DOX/Cu²⁺ complex, we used the UV-vis, circular dichroism (CD), and fluorescence spectra to characterize free DOX, DOX/Cu²⁺ complex and the supernatant of PEG-HCuSNPs-DOX with or without NIR laser irradiation at pH 5 and 6. UV and CD spectra of samples at pH 7.4 were not able to be analysed due to the limitation of sampling quantity and the instrument sensitivity, however, inductively coupled plasma mass spectrometry (ICP-MS, data not shown) and fluorescence data clearly indicating the existence of copper and doxorubicin. At pH 5, the band intensity of DOX near 478 and 498 nm decrease upon the partial formation of DOX/Cu²⁺ 2:1 complex. In addition, the absorbance peak near 535 nm increased (Figure 3a). The released samples with or without laser treatment all showed similar absorbance curves to the complex rather than free DOX, indicating formation of the DOX/Cu²⁺ complex. When pH increased to 6, both 2:1 and 1:1 complex exist. As shown in Figure 3a, the absorbance of the DOX/Cu²⁺ complex at 480 nm continued to decrease and the broad band was centered at 506 nm. The band at 550 nm increased significantly. The released samples showed similar changes.

In comparison with free DOX, the CD spectrum of DOX/Cu²⁺ at pH 5 and 6 showed negative and positive bands at 490 and 550 nm respectively, which were typical of DOX/Cu 2:1 complexes (Figure 3b). The same trend was shown in the supernatant from laser treated PEG-HCuSNPs-DOX at pH 5 and 6. Combining the

UV results, it could be concluded that the DOX/Cu²⁺ 2:1 complex forms after laser treatment at pH 5 and 6.

Fluorescent spectra of the DOX and Cu²⁺ mixture demonstrated the reduced fluorescent intensity at both pH 5 and 6, indicating the formation of DOX/Cu²⁺ complex, the fluorescence of which was quenched (Figure 3c). It should be noticed that as pH increased from 5 to 6 and 7.4, the intensity decreased, revealing that more free DOX were chelated by Cu²⁺. Addition of excess ethylenediaminetetraacetic acid (EDTA) with 1 h incubation at 60 °C recovered the fluorescence intensity since EDTA competed for the ligation of Cu²⁺.¹⁸ The same trend was shown in the supernatant from laser treated PEG-HCuSNPs-DOX at pH 5, 6 and 7.4. In addition, the released amount of DOX was pH-dependent. At pH 5, the laser treated PEG-HCuSNPs-DOX sample showed the highest absorbance of DOX among the different buffer solution groups.

Figure 3. UV-vis (a) and CD (b) spectra of 100 μM DOX, 100 μM DOX/ Cu^{2+} , 100 μM DOX released from PEG-HCuSNPs-DOX and 100 μM DOX released from HCuSNPs-DOX with laser treatment in the medium of pH 5, 6 acetic buffer solution. Fluorescence (c) spectra of 100 μM DOX, 100 μM DOX/ Cu^{2+} , 100 μM DOX/ Cu^{2+} +EDTA and DOX released from PEG-HCuSNPs-DOX containing 100 μM DOX in the medium of pH 5, 6 acetic buffer solution and pH 7.4 phosphate buffer solution respectively. Released samples were incubated with excessive EDTA for 1 h at 60 $^{\circ}\text{C}$ to recover the fluorescence of DOX.

To ensure the successful uptake of PEG-HCuSNPs-DOX into cells, uptake and elimination studies were performed. The result showed that the intracellular amount of DOX reached plateau at 2 h following incubation with either free DOX or PEG-HCuSNPs-DOX (Figure 4). The uptake of DOX in PEG-HCuSNPs-DOX was 1.23 fold as high as that of free DOX ($p < 0.05$). There was no significant difference in elimination profile between PEG-HCuSNPs-DOX and free DOX following 4-h uptake.

Figure 4. Intercellular uptake and eliminate of DOX or PEG-HCU-SNPs-DOX after the 4-h uptake. Mean $\pm$ SD, n= 3. (* $p < 0.05$)

To investigate the mechanism of drug release *in vitro*, cell lysosome was stained with Lysotracker Blue to track the intracellular distribution of the drug. As shown in Figure 5a, most of the fluorescence of DOX colocalized with that of Lysotracker, and achieved high intensity adjacent to the nuclei, suggesting intracellular uptake of DOX through the process of endocytosis of the PEG-HCU-SNPs-DOX. Upon laser exposure, the fluorescence of DOX spread throughout the cells except nuclei, indicating laser-induced endolysosomal escape of the nanoparticles and drug release into cytoplasm (Figure 5b). This was attributed to photothermally induced disruption of endolysosomal membranes mediated by HCU-SNPs.¹⁹

Figure 5. Fluorescent microscopy images of A549 cells incubated for 4 h with PEG-HCuSNPs-DOX (a) and PEG-HCuSNPs-DOX plus laser (2.0 W/cm^2 , 15 s) (b) at the end of incubation. Lysosome fluorescence (pseudo-green); DOX fluorescence (red); overlap (yellow). Scale bar: $50 \mu\text{m}$.

DOX induced the generation of reactive oxygen species (ROS), thus causing the oxidation of lipid, protein, and DNA in cancer cells^{20–25}. More interestingly, transition metal ion has been indicated to be a critical cofactor facilitating this process.¹⁵ To prove that DOX/ Cu^{2+} complex released from PEG-HCuSNPs-DOX could assist ROS generation and therefore induce therapeutic effects on cancer cells, ROS level was tested via fluorescence imaging and quantification. As shown in the fluorescent microscope images (Figure 6a), only weak signal of ROS species was detected from free DOX, PEG-HCuSNPs or PEG-HCuSNPs with NIR laser exposure. However, existence of ROS was clearly shown by the green fluorescence in the cases of PEG-HCuSNPs-DOX and DOX/ Cu^{2+} complex with NIR laser irradiation (Figure 6a). The intensity of reactive oxygen species signal increased after NIR laser irradiation (Figure 6b), because the HCuSNPs released Cu^{2+} ions¹³ and facilitated DOX

molecules to chelate with Cu^{2+} . As comparisons, the generation of reactive oxygen species by hollow gold nanoparticles (HAuNPs) and DOX modified gold nanoparticles (HAuNPs-DOX) was studied under the identical condition. However, no such fluorescence was observed from either sample despite of the NIR laser irradiation (Figure 6b). Therefore, the cancer killing effect of PEG-HCuSNPs-DOX could be attributed to a combination of DOX release, photothermal effect, and copper induced ROS generation following NIR laser irradiation. The One-way analysis of variance (One-way ANOVA) results were shown in **Table 1.** and **Table 2.**

Figure 6. (a) Fluorescence microscopy images of A549 cells incubated for 2 h with DOX, DOX/Cu²⁺, PEG-HCuSNPs or PEG-HCuSNPs-DOX and then treated with the NIR laser (2.0 W/cm², 15 s). The cells were sustained with fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Green fluorescence indicated the existence of ROS. Scale bar: 50 μ m. (b) Normalized ROS intensity before and after treatments. (* $P < 0.05$ when compared with free DOX. Mean $\pm$ SD, n=3).

Table 1. ANOVA Result

ROS Level					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16.696	9	1.855	19.704	.000
Within Groups	2.824	30	.094		
Total	19.521	39			

Table 2. Multiple Comparisons

Dependent Variable: ROS Level

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
DOX	DOX/CU2+	-1.0705 [*]	.2170	.001	-1.811	-.330
	PEG-HCuSNPS	-.3350	.2170	.863	-1.075	.405
	PEG-HCuSNPs-DOX + Laser	-1.1725 [*]	.2170	.000	-1.913	-.432
	DOX + Laser	-.2250	.2170	.987	-.965	.515
	DOX/CU2+ + Laser	-1.3233 [*]	.2170	.000	-2.063	-.583
	PEG-HCuSNPs + Laser	-.7375	.2170	.050	-1.478	.003
	PEG-HCuSNPs-DOX + Laser	-1.9825 [*]	.2170	.000	-2.723	-1.242
	HAuNP-DOX	-.0270	.2170	1.000	-.767	.713
	HAuNP-DOX + Laser	-.0083	.2170	1.000	-.748	.732
DOX/CU2+	DOX	1.0705 [*]	.2170	.001	.330	1.811
	PEG-HCuSNPS	.7355	.2170	.052	-.005	1.476
	PEG-HCuSNPs-DOX + Laser	-.1020	.2170	1.000	-.842	.638
	DOX + Laser	.8455 [*]	.2170	.016	.105	1.586
	DOX/CU2+ + Laser	-.2528	.2170	.972	-.993	.487
	PEG-HCuSNPs + Laser	.3330	.2170	.867	-.407	1.073
	PEG-HCuSNPs-DOX + Laser	-.9120 [*]	.2170	.007	-1.652	-.172
	HAuNP-DOX	1.0435 [*]	.2170	.001	.303	1.784
	HAuNP-DOX + Laser	1.0622 [*]	.2170	.001	.322	1.802
PEG-HCuSNPS	DOX	.3350	.2170	.863	-.405	1.075
	DOX/CU2+	-.7355	.2170	.052	-1.476	.005
	PEG-HCuSNPs-DOX + Laser	-.8375 [*]	.2170	.017	-1.578	-.097
	DOX + Laser	.1100	.2170	1.000	-.630	.850
	DOX/CU2+ + Laser	-.9883 [*]	.2170	.003	-1.728	-.248

	PEG-HCuSNPs + Laser	-.4025	.2170	.697	-1.143	.338
	PEG-HCuSNPs-DOX + Laser	-1.6475 ⁺	.2170	.000	-2.388	-.907
	HAuNP-DOX	.3080	.2170	.911	-.432	1.048
	HAuNP-DOX + Laser	.3267	.2170	.879	-.413	1.067
PEG-HCuSNPs-DOX + Laser	DOX	1.1725 ⁺	.2170	.000	.432	1.913
	DOX/CU2+	.1020	.2170	1.000	-.638	.842
	PEG-HCuSNPs	.8375 ⁺	.2170	.017	.097	1.578
	DOX + Laser	.9475 ⁺	.2170	.005	.207	1.688
	DOX/CU2+ + Laser	-.1508	.2170	.999	-.891	.589
	PEG-HCuSNPs + Laser	.4350	.2170	.602	-.305	1.175
	PEG-HCuSNPs-DOX + Laser	-.8100 ⁺	.2170	.023	-1.550	-.070
	HAuNP-DOX	1.1455 ⁺	.2170	.000	.405	1.886
	HAuNP-DOX + Laser	1.1642 ⁺	.2170	.000	.424	1.904
DOX + Laser	DOX	.2250	.2170	.987	-.515	.965
	DOX/CU2+	-.8455 ⁺	.2170	.016	-1.586	-.105
	PEG-HCuSNPs	-.1100	.2170	1.000	-.850	.630
	PEG-HCuSNPs-DOX + Laser	-.9475 ⁺	.2170	.005	-1.688	-.207
	DOX/CU2+ + Laser	-1.0983 ⁺	.2170	.001	-1.838	-.358
	PEG-HCuSNPs + Laser	-.5125	.2170	.382	-1.253	.228
	PEG-HCuSNPs-DOX + Laser	-1.7575 ⁺	.2170	.000	-2.498	-1.017
	HAuNP-DOX	.1980	.2170	.995	-.542	.938
	HAuNP-DOX + Laser	.2167	.2170	.990	-.523	.957
DOX/CU2+ + Laser	DOX	1.3233 ⁺	.2170	.000	.583	2.063
	DOX/CU2+	.2528	.2170	.972	-.487	.993
	PEG-HCuSNPs	.9883 ⁺	.2170	.003	.248	1.728
	PEG-HCuSNPs-DOX + Laser	.1508	.2170	.999	-.589	.891
	DOX + Laser	1.0983 ⁺	.2170	.001	.358	1.838
	PEG-HCuSNPs + Laser	.5858	.2170	.219	-.154	1.326
	PEG-HCuSNPs-DOX + Laser	-.6592	.2170	.113	-1.399	.081
	HAuNP-DOX	1.2963 ⁺	.2170	.000	.556	2.036

	HAuNP-DOX + Laser	1.3150 ⁺	.2170	.000	.575	2.055
PEG-HCuSNPs + Laser	DOX	.7375	.2170	.051	-.003	1.478
	DOX/CU2+	-.3330	.2170	.867	-1.073	.407
	PEG-HCuSNPs	.4025	.2170	.697	-.338	1.143
	PEG-HCuSNPs-DOX					
	+ Laser	-.4350	.2170	.602	-1.175	.305
	DOX + Laser	.5125	.2170	.382	-.228	1.253
	DOX/CU2+ + Laser	-.5858	.2170	.219	-1.326	.154
	PEG-HCuSNPs-DOX					
	+ Laser	-1.2450 ⁺	.2170	.000	-1.985	-.505
	HAuNP-DOX	.7105	.2170	.068	-.030	1.451
	HAuNP-DOX + Laser	.7292	.2170	.056	-.011	1.469
PEG-HCuSNPs-DOX + Laser	DOX	1.9825 ⁺	.2170	.000	1.242	2.723
	DOX/CU2+	.9120 ⁺	.2170	.007	.172	1.652
	PEG-HCuSNPs	1.6475 ⁺	.2170	.000	.907	2.388
	PEG-HCuSNPs-DOX					
	+ Laser	.8100 ⁺	.2170	.023	.070	1.550
	DOX + Laser	1.7575 ⁺	.2170	.000	1.017	2.498
	DOX/CU2+ + Laser	.6592	.2170	.113	-.081	1.399
	PEG-HCuSNPs + Laser	1.2450 ⁺	.2170	.000	.505	1.985
	HAuNP-DOX	1.9555 ⁺	.2170	.000	1.215	2.696
	HAuNP-DOX + Laser	1.9742 ⁺	.2170	.000	1.234	2.714
HAuNP-DOX	DOX	.0270	.2170	1.000	-.713	.767
	DOX/CU2+	-1.0435 ⁺	.2170	.001	-1.784	-.303
	PEG-HCuSNPs	-.3080	.2170	.911	-1.048	.432
	PEG-HCuSNPs-DOX					
	+ Laser	-1.1455 ⁺	.2170	.000	-1.886	-.405
	DOX + Laser	-.1980	.2170	.995	-.938	.542
	DOX/CU2+ + Laser	-1.2963 ⁺	.2170	.000	-2.036	-.556
	PEG-HCuSNPs + Laser	-.7105	.2170	.068	-1.451	.030
	PEG-HCuSNPs-DOX					
	+ Laser	-1.9555 ⁺	.2170	.000	-2.696	-1.215
	HAuNP-DOX + Laser	.0187	.2170	1.000	-.721	.759
HAuNP-DOX + Laser	DOX	.0083	.2170	1.000	-.732	.748
	DOX/CU2+	-1.0622 ⁺	.2170	.001	-1.802	-.322
	PEG-HCuSNPs	-.3267	.2170	.879	-1.067	.413

PEG-HCuSNPs-DOX + Laser	-1.1642*	.2170	.000	-1.904	-.424
DOX + Laser	-.2167	.2170	.990	-.957	.523
DOX/CU2+ + Laser	-1.3150*	.2170	.000	-2.055	-.575
PEG-HCuSNPs + Laser	-.7292	.2170	.056	-1.469	.011
PEG-HCuSNPs-DOX + Laser	-1.9742*	.2170	.000	-2.714	-1.234
HAuNP-DOX	-.0187	.2170	1.000	-.759	.721

*. The mean difference is significant at the 0.05 level.

The total effect of PEG-HCuSNPs-DOX on cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Figure 7a, NIR laser exposed PEG-HCuSNPs-DOX exhibited the highest anti-cancer effect within the concentration of 0.002–0.78 $\mu\text{g/mL}$ DOX 24 h after treatment. The IC_{50} of PEG-HCuSNPs-DOX combining NIR laser treatment was 0.11 $\mu\text{g/mL}$ DOX. The IC_{50} of PEG-HCuSNPs-DOX without NIR laser treatment was 0.21 $\mu\text{g/mL}$ DOX. Comparatively, the IC_{50} of PEG-HCuSNPs with and without laser and was 0.24 and 2.07 $\mu\text{g/mL}$ in DOX, respectively. The administration of NIR laser drastically decreased the viability for cells treated with PEG-HCuSNPs and PEG-HCuSNPs-DOX when compared with the non-laser groups at comparative concentration. Besides, the IC_{50} of HAuNPs-DOX on A549 cells with laser (2.0 W/cm^2 , 15 s) dose was 2.2 $\mu\text{g/mL}$ DOX, while the IC_{50} of PEG-HCuSNPs-DOX with the same laser dose was 0.11 $\mu\text{g/mL}$ DOX, significantly less than the former treatment.

Further, we used propidium iodide (PI) staining to test whether the nanoparticles could bring quick damage to cell membrane upon NIR laser administration because PI

is a fluorescence marker permeable to porous cell membrane and staining nuclei selectively. For the PEG-HCuSNPs-DOX group, the ratio of the PI stained cells to the total cells immediately after the NIR laser irradiation was much higher than that of PBS control, PBS with laser treatment or even free DOX under identical experimental condition (Figure 7b). In addition, the HAuNPs-DOX with laser treatment didn't show significant difference from the PBS control group. It should be noticed that the laser induced cell death by PI staining at 4 h after laser treatment was less effective than that from the cell viability assay at 24 h after treatment. This result indicated that laser-induced instantaneous ablation to the cell membrane was only one among various modalities that PEG-HCuSNPs-DOX caused cancer cell death. Other modalities also played important role in causing cell death progressively and accumulatively, including the DOX or DOX/Cu²⁺ complex induced toxicity and ROS species.

Figure 7. (a) and (b) 24-h viability of A549 cells exposed to different concentration of nanoparticles or DOX with or without NIR laser irradiation (2.0 W/cm^2 , 15 sec) by MTT assay. Data are plotted as the percentage of viable cells compared to untreated controls. Each value represents mean $\pm$ SD (n = 3). (c) Percentage of PI stained A549 cells exposed to different treatment of 0.5 µg/mL DOX equivalent after 4-h incubation followed successively by NIR laser irradiation (2 W/cm^2 , 15 s). The control group was treated with PBS. (* $P < 0.05$ as compared with control. Percentage = PI stained cells/total cells counted $\times 100\%$, n=3)

SUMMARY AND CONCLUSIONS

In summary, NIR laser controlled anticancer nanocomposite was achieved by modification of PEG-HCuSNPs with DOX through chelating interaction. The nanocomposite was capable of pH-sensitive drug release, photothermal conversion, and generating ROS in response to NIR laser irradiation. With the NIR laser responsive multi-functions, highly controlled and effective cancer killing performance was achieved. The current work sheds a considerable light on the smart antitumor materials by NIR laser control for transdermal cancer therapy.

METHODS

Chemicals. Doxorubicin hydrochloride and Dulbecco's Modified Eagle Medium (DMEM) were purchased from AK scientific and Lonza, respectively. Lysotraker®Blue DND-22 and propidium iodide were supplied from Life Technologies. A549 cell line was obtained from American Type Culture Collection (ATCC). All the other chemicals were bought from Sigma-Aldrich without further purification. The water was purified by using a Milli-Q Synthesis system (Millipore) with the resistivity higher than 18.2 MΩ·cm.

Materials Preparations. HCuSNPs was synthesized according to the previous reports.^{6,13} Briefly, 0.24 g poly(vinylpyrrolidone) and 25 mL sodium hydroxide aqueous solution (pH 9.0) were successively added into 25 mL 0.05 mM copper chloride aqueous solution, and the mixture was stirred vigorously at room temperature. Subsequently, 2.0 mL 0.10 M hydrazine hydrate solution was added, and suspension was formed after reaction for 5 min. Then, 0.266 mmol sodium sulfide was added to the suspension and stirred at 60 °C for 1 h. The formed HCuSNPs was collected by

centrifuging at 11,000 rpm for 10 min, washing three times with pure water and suspending in 5 mL pure water.

To prepare PEG-HCuSNPs-DOX, 20 μ L thiolated PEG was added to 5 mL aqueous dispersion of 18 mg HCuSNPs and stirred overnight at 1000 rpm. The resultant PEG-HCuSNPs was washed three times by centrifuging at 12 000 rpm for 10 min for three times. Then, 1 mL 1 mg/mL Doxorubicin hydrochloride aqueous solution was mixed with the above dispersion containing PEG-HCuSNPs and stirred at 1000 rpm for 24 h. After washing with water for three times, PEG-HCuSNPs-DOX was obtained.

Characterizations. To prepare samples for transmission electron microscope (TEM) observations, the corresponding materials were ultrasonicated in pure water until homogeneous suspension was formed, and the resulting suspension was then dropped onto a nickel micro grid, followed by drying in air. TEM observations were respectively performed on a JEOL 2100EX microscope operating at an accelerating voltage of 100 kV. DLS analysis was proceeded with the Malvern® nanoseries Nano-ZS90 nanoparticle size analyzer. UV–visible–NIR absorbance spectra were obtained with a Beckmann Coulter DU800 UV–visible–NIR spectrophotometer with a quartz cuvette of 1.0 cm optical path length in the transmission mode employing pure water as the reference standard. Circular Dichroism spectra were acquired by Jasco J-810 Circular Dichroism spectropolarimeter (163~900 nm). Fluorescent spectra were measured by SpectraMax Multi-Mode Microplate Readers.

The DOX loading efficiency was calculated from the weight of DOX component detached from the PEG-HCuSNPs-DOX. The washed PEG-HCuSNPs-DOX was mixed with 0.5 mL of 10 mM EDTA and 5 μ L of 2 M HCl and incubated at 60 °C for 1 h. Then, the dispersion was centrifuged at 12000 rpm for 10 minutes. The supernatant was collected and analyzed by fluorescent spectroscopy to determine the weight of DOX extracted. The DOX loading efficiency was calculated *via* the following equation:

$$E_{DOX} = \frac{W_{DOX}}{W_{PEG-HCuSNPs}} \times 100\% \quad (1)$$

where E_{DOX} represents the DOX loading efficiency, W_{DOX} stands for the weight of DOX extracted from the nanoparticles, and the $W_{PEG-HCuSNPs-DOX}$ shows the weight of PEG-HCuSNPs added.

Cell Culture. A549 cell line was cultured in DMEM media, supplemented with 10% fetal bovine serum (FBS), L-glucose, glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin.

In Vitro Release. *In vitro* release of DOX from the as-prepared nanoparticles by adding 10 mg PEG-HCuSNPs-DOX in a dialysis tube (1000 Da cut off; Sigma-Aldrich), placing the tube in 100 mL of phosphate-buffered saline (PBS, pH 7.4, 10 mM) containing 10% BSA, and shaking constantly at 150 rpm at 37°C. The NIR laser was administered as needed after taking out the sample from the dialysis tube at 2.0 W/cm² for 15 s at a time interval of 1 h. The *in vitro* DOX release was continued for 4 h. The release medium was replaced with a fresh one at a determined

interval to maintain sink conditions. The amount of released DOX was monitored by fluorescent measurement. The fluorescent emission peak at $\lambda = 590$ nm under excitation light ($\lambda = 485$ nm) of each solution was recorded to determine the released DOX amount. As a comparison, *in vitro* release was proceeded identically without NIR irradiation. All measurements were performed in triplicate.

MTT Assay. Cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) method. Briefly, A549 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 24 h prior to use. The cells were respectively incubated with blank PEG-HCuSNPs, PEG-HCuSNPs-DOX, and free DOX at equivalent drug concentrations ranging from 50 nM to 15 000 nM for 4 h. After replacing the culture medium with FBS-free DMEM and irradiation with NIR laser at 2 W/cm^2 for 15 s, the FBS-free DMEM was substituted with DMEM containing 10% FBS and the cells were further incubated for 24 h. Then, MTT tetrazolium salt (0.25 mg/ml) was added to each well and incubated in CO_2 incubator at 37°C for 4 h. Finally, 150 μL DMSO was added to each well dissolve formazan crystals and the UV-vis absorbance peak at 570 nm was monitored by a plate reader.²⁶

ROS Assay A549 cell line was cultured in the coverglass chambers at a concentration of 20,000 per well, and free DOX, DOX/ Cu^{2+} complex (DOX: $\text{Cu}^{2+} = 1:1$), PEG-HCuSNPs, and PEG-HCuSNPs-DOX were respectively added to each well. The final concentration of DOX was 0.5 μM , and the amount of PEG-HCuSNPs was kept the same in the wells. After incubating for 4 h, the drug-containing medium was replaced with new one and irradiated with NIR laser (900 nm, 2 W/cm^2 , 15 s). Then,

the medium was substituted with 1 mM DFCH-DA medium and incubated for 30 min. After washing with PBS for 3 times, the wells were observe by using the fluorescent microscope.

To determine the ROS level in A549 cells, A549 cell line was cultured in the 96-well plate at a concentration of 10,000 per well. Then, the cells were treated identically by replacing the cell culture medium with DMEM, incubated for 3 h, changing the culture medium with pre-warmed freshmedium, adding samples, incubated for 4h, replacing medium with DMEM, exposed with corresponding light irradiations, substituting the medium with 1 mM DFCH-DA medium, incubated for 30 min, washing with PBS for 3 times, adding 100 μ L cell lysis buffer. Finally, the treated specimens were subjected to fluorescent test (excitation λ = 480 nm, emission λ = 530 nm) to measure the protein content. As a comparison, DMEM containing 5 mM NAC was applied as the cell culture medium and tested through the same procedure.

PI exclusion assay. A549 cell viability was determined by staining the cells with propidium iodide according to the manufacturer's instructions. In a typical procedure, the A549 cells were seeded into 96-well culture plates at a concentration of 1.5×10^5 cells per well and incubated for 24 h to allow cell attachment. The cells were respectively administered with free DOX, PEG-HCuSNPs-DOX, and PEG-HCuSNPs at equivalent drug concentrations (5 μ M) for 4 h. The washing procedure is the same as MTT assay. After NIR laser irradiation, the cells were collected by trypsinization using a 0.125% trypsin solution. The cells collected were washed twice with PBS (pH = 7.4) and re-suspended in 100 μ L PBS at a density of 1×10^5 cell mL^{-1} . Subsequently, 5 μ L of propidium iodide solution (100 $\mu\text{g mL}^{-1}$) and 400 μ L of PBS solution were

successively added to the cells. The cells were further incubated for 5 min in darkness, and the total number of the cells stained was counted with a cellometer (Nexcelom vision).

Intracellular Uptake. For the PEG-HCuSNPs-DOX uptake study, 12-well plates were seeded with A549 cells at 2×10^5 per well, and the cells were allowed to attach for 24 h. The medium was replaced with 1 mL of medium containing PEG-HCuSNPs-DOX or DOX solution (final Dox concentration 5 μ M), and the samples were incubated for a determined time period (0.5 h and 2 h). The cells were washed three times with PBS buffer to remove the excess drug component, and then the cells were lysed in 100 μ L of cell lysis buffer for 10 min. 10 μ L portion of the resultant cell lysate was used to quantify the protein concentration through the BCA protein assay. The remained cell lysate dispersion was mixed with 0.2 mL of acidified methanol solution (0.1 M HCl in 90% methanol) and centrifuged at 16800 rpm for 10 min. The supernatant was extracted and subjected for fluorescent spectroscopy to analyze DOX level. The fluorescent emission peak at $\lambda = 591$ nm was detected under excitation $\lambda = 485$ nm. The data were normalized to per milligram cell protein.

Intracellular Elimination. For the PEG-HCuSNPs-DOX elimination study, 12-well plates were seeded with A549 cells at 2×10^5 per well, and the cells were allowed to attach for 24 h. The medium was replaced with 1 mL medium containing PEG-HCuSNPs-DOX or Dox solution (final DOX concentration 5 μ M) and incubated for 4 h. The cells were washed three times to remove the free DOX or PEG-HCuSNPs-DOX with PBS buffer and continued to incubate with fresh medium.

Intracellular DOX level was determined after being washed for 12 h, 24 h and 48 h *via* the same method as described in the intracellular uptake assay section.

Lysotracking. The lysotracking study was carried out by using Lysotraker®Blue DND-22. Generally, 8-well cover slide chambers were seeded with A549 cells at 2×10^5 per well, and the cells were left for 24 h to achieve attachment. The cells were incubated for 4 h with 0.5 $\mu\text{g/mL}$ PEG-HCuSNPs-DOX or PEG-HCuSNPs-DOX plus laser (2.0 W/cm^2 , 15 s) at the end of incubation. Then the initial medium was removed from the chamber, and the pre-warmed probe-containing medium (37°C) was added and incubated for 30 min. The cells were washed with phenol red-free DMEM for 3 times, and characterized with fluorescent spectrosopes by monitoring the emission peak at $\lambda = 424 \text{ nm}$ under excitation light of $\lambda = 373 \text{ nm}$.

All data were expressed as mean SD and IC_{50} values were calculated by using nonlinear regression analysis. The statistical significance was determined using a t test. A p value less than 0.05 (*i.e.*, $p < 0.05$) was considered to indicate statistical significance for all comparisons.

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CHAPTER 2

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Facile Direct Dry Grinding Synthesis of Monodisperse Lipophilic CuS Nanoparticles

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Abstract

Copper sulfide with near-infrared light absorption property is recently attracting broad interest as a photothermal carrier for smart cancer therapy. Lipophilic copper sulfide nanoparticle is preferred for high performance biomedical applications due to the high affinity with tissues. But it requires complex multi-step synthetic process under severe condition. Here, synthesis of hydrophobic copper sulfide possessing surface plasmon resonance was retained by direct dry grinding of copper(II) acetylacetonate with sulfur under ambient environment. The formed CuS nanoparticles were in uniform size of ~10 nm, and they were monodispersed in pure chloroform. Each covellite CuS nanocrystal surface was modified with oleylamine through hydrogen bonding between sulfur atoms and amine groups of oleylamine. While these oleylamine capped CuS nanoparticles showed uniform morphological features, they demonstrated near-infrared light absorption for photothermal applications. The facile and mild synthetic methodology described here opened a powerful pathway for the design and preparation of photothermal lipophilic copper sulfide nanomaterials for smart cancer therapy.

KEYWORDS: Copper sulfide; oleylamine; nanoparticle; lipophilicity; photothermal therapy.

1. INTRODUCTION

Copper sulfide (CuS) nanocrystals with determined vacancies have recently demonstrated to be capable of absorbing near-infrared (NIR) light irradiation (650–900 nm) [1] and instantaneously converting into local heat. This unique property has attracted broad interest for a variety of scientific and technological applications such as solar cells, electroconducting electrodes, sensors, and clinics.[2–4] Notably, the NIR light is able to penetrate through normal tissues with minimal thermal injury.[5] The photothermal conversion effect of the CuS nanoparticles is independent of the surrounding environment.[6,7] These features are especially useful for controlled drug delivery and photothermal cancer therapy.[8–10]

For the synthesis of CuS nanoparticles with desired nanostructures, a series of approaches have been developed, such as hydrothermal [11], solvothermal method [12], solid-state reaction [13], microemulsion[14], and reflux condensation [15] have been developed. In order to endow the CuS nanoparticles with NIR absorption, the as-prepared nanoparticles are usually further oxidized to produce vacancies in the crystalline structures.[16] One of the most commonly used method is based on the reaction of water soluble copper (II) salt and sodium sulfide as the precursor at 90 °C through wet chemistry. The formed citric acid capped CuS nanoparticles were applied as a photothermal coupling agent for PTA of cancer cells *in vitro* and *in vivo* under the laser irradiation (808 nm, 16 W/cm² and 24 W/cm²).[1] Alternatively, spherical copper (I) oxide nanoparticle aggregation was used as an sacrificial template and it was hydrothermally treated in the presence of polyvinylpyrrolidone (PVP) as a capping agent. Through Kirkendall effect, vacancies

were introduced to CuS, forming hollow CuS nanospheres with surface plasmonic performance.[6] In addition, the controllable hydrothermal approach was employed to develop hydrophilic flower-like CuS superstructures with the assistance of PVP (K30, 0.2 g/mL) at 180 °C for 48 h. The resulting nanostructured CuS was used for ablation of cancer cells upon 980 nm laser irradiation.[8] Unfortunately, these methodologies generally involved complicated processes as well as excess toxic reagents, which caused severe environmental pollution.

Recently, lipophilic nanomaterials have been developed for their drug delivery into hydrophobic tissues such as brain and vascular tissues. To retain CuS nanoparticles dispersible in organic phase, hot injection [17], cation exchange, [18] and solventless approach[19] have been reported. Among them, hot injection method based on high temperature reaction of copper (II) acetylacetonate and elemental sulfur or sulfur provider (*e.g.*, dodecanethiol) has been widely used. However, the lipophilic CuS nanoparticles synthesized by these methods are not able to absorb NIR light. Thus, they require additional complex oxidization treatment to show photothermal performance.

In this paper, lipophilic CuS nanoparticles were synthesized by directly grinding copper (II) acetylacetonate with sulfur in oleylamine at room temperature. Within a few minutes of grinding in the ambient environment, the CuS nanoparticles were attained in high yield. The resulting CuS nanoparticles were in uniform particle size of ~10 nm. Each nanoparticle had fine CuS nanocrystal core, which was capped with oleylamine through hydrogen bonding between sulfur atom and amine group of oleylamine. These nanoparticles were readily dispersible in chloroform without

aggregation. While these CuS nanoparticle showed almost identical features as those synthesized by the traditional solution based solvothermal approach, the current CuS nanoparticles demonstrated distinguished absorption of NIR light, capable of photothermal applications. Compared with the traditional solvothermal method, this synthetic approach did not need excess toxic chemicals. And this process can scale up easily. The unique facile synthetic method presented here sheds a considerable light on the synthesis high performance lipophilic CuS nanoparticles for smart photothermal therapy.

2. EXPERIMENTAL

Materials. Chloroform (>99%), cyclohexane (>99%) and ethanol (>99%) were purchased from Fisher Scientific. Oleylamine, Sulfur, and copper(II) acetylacetonate were bought from Sigma-Aldrich. All chemicals were used as received.

Synthesis of CuS Nanoparticles. To dry grinding synthesis of CuS nanoparticles, 0.131 g of copper (II) acetylacetonate, 0.016 g sulfur and 2 mL oleylamine were thoroughly mixed by grinding for 5 min using a mortar and a pestle. During the grinding process, the mixture gradually became brown translucent liquid. Then, the liquid was transferred into a round bottom flask and stirred at 70°C for 30 min, upon which the mixture color further turned from brown to green. Subsequently, the resulting mixture was dispersed in 20 mL chloroform and centrifuge for 30 min at 11,000 rpm. The collected precipitation was dispersed in 10 mL chloroform, and 50 mL ethanol was added to precipitate the formed nanoparticles. These nanoparticles

were collected by centrifugation and washed by excess ethanol repeatedly to remove the remaining surfactant. After vacuum drying at room temperature, lipophilic CuS nanoparticles were obtained.

As a comparison, CuS nanoparticle were prepared by traditional solution based hot injection approach. 0.131 g copper (II) acetylacetonate was dissolved in a mixture of 1 mL oleylamine and 3 mL chloroform, and 0.016 g sulfur was dissolved in 3 mL oleylamine. The sulfur solution was dispersed in 10 mL cyclohexane and stirred at 70 °C for 10 min. After slowly injecting the copper (II) solution into the cyclohexane solution and stirring at 1,000 rpm at 70 °C for 30 min, the mixture solution gradually transformed from brown to green. The powder collected by centrifugation at 11,000 rpm for 30 min was then dispersed in 10 mL chloroform and mixed with 50 mL ethanol to purify the resultant CuS nanoparticle. These purified nanoparticles were further washed with ethanol for several cycles to exclude the excess surfactant and dried in vacuum oven overnight.

Transmission electron microscope (TEM). To prepare samples for transmission electron microscope (TEM) observations, the corresponding materials were suspended in chloroform and then dropped onto a carbon coated nickel micro grid, followed by drying in air in fume hood. TEM observations were performed on a JEOL 2100EX microscope operating at an accelerating voltage of 100 kV.

Fourier transform infrared (FT-IR) spectra were measured on a Nicolet Nexus 670 spectrometer using KBr pellets.

Powder X-ray diffraction (XRD) patterns of the synthesized nanoparticles were recorded on Rigaku Ultima IV multipurpose X-ray diffractometer with a CuK_α ($\lambda = 0.15405 \text{ nm}$) radiation source. The X-ray tube current was 100 mA with a tube voltage of 40 kV. Each sample was scanned at a scan rate of 0.5° with resolution of $\sim 0.02^\circ$ from 2θ of 20° to 70° .

Dynamic light scattering (DLS). DLS analysis was proceeded with the Malvern® nanoseries Nano-ZS90 nanoparticle size analyzer using a 1.0 cm path length 4-way glass cuvette.

UV–Vis–NIR Spectroscopy. Extinction spectra of all nanoparticles were recorded with a PerkinElmer Lambda 1050 UV–visible–NIR spectrophotometer with a quartz cuvette of 1.0 cm optical path length in the transmission mode employing pure chloroform as the reference standard.

X-ray Photoelectron Spectroscopy (XPS). A Measurement was carried out on a PHI 5500 system and $\text{Al K}\alpha$ radiation. Multipak versions 6.1 as well as XPS Peak 4.0 software were utilized for analysis and curve fitting respectively. A combination of Lorentzian and Gaussian functions was used for the least squares curve fitting.

3. RESULTS AND DISCUSSION

The TEM image of the CuS nanoparticles synthesized by the dry grinding process is shown in Fig. 1a. Many nanoparticles were clearly observed. These metallic nanoparticles were mainly in cubic geometry, and they were monodispersed. Some

minor aggregation was caused by the evaporation of chloroform component during the TEM sample preparation process, which was a common situation. Calculated based on at least 300 particles, the average size for these CuS nanoparticles was ~ 10 nm. This result matched well with the hydrodynamic particle diameter of the DLS analysis (Fig. 2), indicating excess surfactant was cleared and monodisperse fine nanoparticles remained. As a comparison, CuS nanoparticles were prepared through the previously reported solution based technique. As shown in TEM (Fig. 1b), the formed CuS nanoparticle size was ~ 9 nm on average, and they were similar as the above nanoparticles obtained by the dry grinding process. The solution based approach derived nanoparticles were more spherical, because the liquid environment inhibited directional crystal growth of the nanocrystals. Therefore, the dry grinding synthesis approach achieved the fine nanocrystals, which was almost identical to the traditional solution based method.

The XRD pattern of the powder sample prepared by the dry grinding method presented clear peaks at 29.3° , 31.8° , 47.9° , 52.7° , and 59.3° (Fig. 3a), which were in fair agreement with (102), (103), (110), (108), and (116) plane of covellite phase CuS (powder diffraction file or PDF# 06-0464). The broad peaks inferred the nanoscale nature of the sample.[20] The crystal size calculated based on (110) plane was ~ 7.73 nm. This size was relatively smaller than the particle size measured in TEM images (10 nm), because a minor amorphous oleylamine layer was modified on the nanocrystal surface. These characteristic peaks were identical to those prepared through the solution based method. Therefore, the current CuS nanoparticles prepared through dry grinding process formed high quality fine covellite CuS nanoparticles.

XPS spectra of the as-prepared CuS nanoparticles were summarized in Fig. 4. The Cu 2p XPS spectrum exhibited $2p_{3/2}$ peak at 932.0 eV and $2p_{1/2}$ peak at 952.2 eV, which were typical peaks for Cu(II) in copper sulfide.[21] The C 1s peak was resolved as two peaks located at 284.6 eV and 285.7 eV, which respectively corresponded to the hydrocarbon (C-C, C-H) in oleylamine and the C-N bond in oleylamine.[22] The S 2p peak of the CuS nanoparticles consisted of two distinct peaks. The one at 161.5 eV originated from typical sulfide bond, and the doublets at 162 eV and 163.5 eV demonstrated the formation of S-H bonds.[23] These peaks matched well with the XPS spectra obtained from the CuS nanoparticle fabricated via the hot-injection method (Fig. 5), supporting that the current CuS nanoparticles were capped with oleylamine. Hydrophilic Sulfur atoms in CuS was qualified electron acceptors.[16] Although it hardly interacted with the hydrophobic alkyl terminals of the oleylamine, it readily accepted electron from the amine group in oleylamine, forming S-H bonds.

In the FTIR spectrum of the resultant CuS nanoparticles (Fig 6), the broad band at $\sim 3450\text{ cm}^{-1}$ was assigned to N-H stretching vibration of the amine group in oleylamine,[24] the two bands at 2922 cm^{-1} and 2852 cm^{-1} were assigned to the asymmetric (ν_{as}) and symmetric (ν_s) stretching vibrations of methylene ($\text{CH}_2=\text{CH}$) in the alkyl chain of oleylamine, the bands centered at 1634 cm^{-1} was attributed to N-H bending vibration.[25-27] All of these characteristic bands were in fair agreement with the FTIR spectrum of pure oleylamine. Hence, oleylamine was capping on the CuS nanoparticles.

Interestingly, the dry grinding synthesized CuS nanoparticles demonstrated broad NIR absorption peaks centered at ~ 1100 nm (Fig. 7), which was very close to the traditional CuS nanoparticles prepared by solution based technique. This strong absorption suggested that the current CuS nanoparticles possessed localized surface plasmon resonances for photothermal ablation applications.

Usually, it requires a liquid environment, high temperature, and N_2 protection to achieve fine CuS nanoparticles. The liquid environment allows oleylamine to form micelles to direct the nucleation as well as growth of nanocrystals and prohibit nanoparticle agglomeration. Meanwhile, high temperature accelerates the reaction and inert environment prevents over oxidation of CuS to damage NIR absorbance (peak absorbance < 1150 nm).[16] In the current study, it was proved that such conditions were not mandatory for the synthesis of monodispersive fine CuS nanoparticles. The grinding process realized fully contact of copper (II) salt with oleylamine for complexation. The copper(II) salt complexed oleylamine consisted of hydrophilic salt terminal and long alkyl chain groups, which still formed micelle structure and control the crystal growth. Moreover, the existence of oxygen in the reaction process induced the formation of vacancy in CuS crystals, resulting in NIR absorption. Although further work is needed to clarify the actual reaction mechanism of the dry grinding synthesis approach, this method attained monodisperse fine CuS nanoparticles with surface plasmonic performance in a facile and mild process.

Fig. 1. TEM micrograph for CuS nanoparticles synthesized by the dry grinding approach (a) and the traditional hot-injection method (b). Bars: 20 nm.

a

b

Fig. 2. Dynamic light scattering (DLS) analysis of the oleylamine coated CuS nanoparticle deprived by the dry grinding approach (a) and the traditional hot-injection method (b).

Fig. 3. XRD spectra of the CuS nanoparticles synthesized through the dry grinding approach (a) and the traditional hot-injection method (b).

b

c

Fig. 4. X-ray Photoelectron Spectroscopy (XPS) spectra of CuS synthesized by the dry grinding approach. (a) C 1s, (b), Cu 2p, and (c) S 2p regions.

Fig. 5. XPS spectra of CuS synthesized by the traditional hot-injection approach. (a) C 1s, (b) Cu 2p, and (c) S 2p regions.

Fig. 6. FT-IR spectra of CuS synthesized by the dry grinding approach and the traditional hot-injection method.

Fig. 7. Visible-NIR spectra of CuS nanoparticle (1 mM) dispersion in chloroform synthesized by the dry grinding approach and the traditional hot-injection method.

4. CONCLUSIONS

Synthesis of monodisperse CuS nanocrystals was achieved by a facile one step dry grinding process. The nanoparticles were composed of covellite phase CuS, and the particle size was finely controlled as ~10 nm. The CuS nanoparticle surface was capped with oleylamine by hydrogen bonding between sulfur atoms with amine group of oleylamine. While the resultant CuS nanoparticles were highly comparable with those prepared through traditional solvothermal method, the current approach was carried out at ambient condition and decreased use of toxic solvents. This

environmental benign opened a facile pathway for the large-scale production of photothermal nanocrystals for applications smart drug delivery.

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CHAPTER 3

This manuscript is being prepared for submission to *Journal of American Chemical Society*.

Multifunctional Mesoporous Silica-Coated CuS Nanoparticles for Cancer Therapy: Synthesis, Characterization and *in vitro* Evaluation

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ABSTRACT: Chemo therapeutic drug-caused side effects are commonly seen in clinical practice due to nonspecific toxicity and low therapeutic efficiency. Herein, we reported a cancer chemo-photothermal multifunctional drug delivery system. Polyethylene glycol decorated mesoporous silica nanoparticles entrapping CuS nanoparticles (PEG-CuS@MSNs) were successfully synthesized and characterized for the drug delivery application. Doxorubicin (DOX)-loaded PEG-CuS@MSNs showed laser stimulated and pH-responsive properties. *In vitro* cell experiments demonstrated that DOX-loaded PEG-CuS@MSNs combining laser exposure achieved the highest rate of death of A549 cells, in comparison to that of PEG-CuS@MSNs-DOX alone. These findings provided a promising drug delivery system for cancer combinatorial therapy, which could significantly reduce drug dose and improve patient compliance.

KEYWORDS: magnetism, mesoporous silica nanoparticles, photothermal therapy, doxorubicin

INTRODUCTION

Cancer chemotherapy is often frightening because of not only the side effects but also the high possibility of recurrence rate. In clinical practice, chemotherapy usually accompanies with surgery, radiotherapy, *etc.* However, the 5-year relative survival rate is still low, 68% for all cancers diagnosed between 2003 and 2009 in the United States.^[1] Various methods have been developed in addition to chemotherapy, such as nano technique, targeted delivery, photothermal therapy, photodynamic therapy, *etc.*^[2] One highlighted trend is the application of nanotechnology, which delivers drug more precisely at cancer cells and brings less damage to normal cells, thus diminishing side effects. Beside, photothermal therapy can ablate cancer cells. Combination of chemo and photothermal therapy with targeting feature into a nano delivery system would be a practical and efficient solution worth trying.

Copper sulfide nanoparticles (CuS NPs) are a new class of photothermal sensitizer. Their light absorption is not affected by the surrounding environment.^[3, 4] They exhibit stable light absorption towards near-infrared (NIR) light irradiation (650–900 nm),^[5] which will bring minimal thermal injury to normal tissues.^[6] Immediately upon NIR light absorption, CuS NPs generate heat and photothermally ablate tumor *in vivo* after intratumor^[7, 8] or intravenous injection.^[9]

Although CuS NPs are promising, when applying to drug delivery, the nanoparticle itself has limitation as a platform.^[10] Since the surface is only one layer to attach the chemicals, the loading efficiency is limited. To address this problem, mesoporous silica is chosen considering its large pore size and high surface area. Moreover, the technique of synthesizing a layer of mesoporous silica on the surface of

inorganic template is relatively mature. There have been many reports on the preparation of nanocrystals coated with mesoporous shells such as gold nanorods,^[11] iron oxide,^[12] manganese oxide nanoparticle^[13], graphene nanosheet,^[14] etc. However, studies on CuS NPs coated with mesoporous silica have not been reported.

In this study, a chemo photothermal drug system was formulated to increase drug loading efficiency. Specifically, mesoporous silica spheres containing CuS NPs in the core and DOX loaded in the silica channels (PEG-CuS@MSNs-DOX) were prepared. Furthermore, these mesoporous silica spheres were applied to photothermal therapy. A procedure for the synthesis of the CuS@MSNs-DOX is shown in Scheme 1.

Scheme 1. Schematic illustration of the synthesis process of PEG-CuS@MSNs-DOX.

EXPERIMENTAL SECTION

2.1. Chemicals. Doxorubicin hydrochloride and Dulbecco's Modified Eagle Medium (DMEM) were purchased from AK scientific and Lonza, respectively. All the other chemicals were bought from Sigma-Aldrich without further purification. The water was purified by using a Milli-Q Synthesis system (Millipore) with the resistivity higher than 18.2 M Ω ·cm.

CuS nanoparticle preparation. Copper acetylacetonate (0.131 g) and 0.032 g sulfur (0.032 g, 1 mmol) and oleylamine (4.0 mL) were thoroughly ground and mixed for 5 min using a mortar and a pestle. During the grinding process, the mixture became a brown, translucent liquid. Then, the liquid was transferred into a round bottom flask and heated up to 70°C with magnetic stirring for 1 h. The color of the mixture then turned from brown to green. The product was thereafter suspended in 20 mL chloroform and transferred into a centrifuge tube and centrifuged for 30 min at 11,000 rpm. The supernatant was decanted and the precipitated pellet was collected. This pellet was suspended with 10 mL of chloroform. Subsequently, 30 ml ethanol was added to precipitate the nanoparticles. After centrifugation, the nanoparticles were separated from the supernatant. Then wash the nanoparticles twice with ethanol to remove the excess oleylamine. The final CuS NPs capped with surfactant were dried at room temperature in a high vacuum. The as-prepared nanoparticles are re-dispersible in chloroform.

Synthesis of CuS nanoparticles embedded in mesoporous silica spheres (CuS@MSNs). 7.5 mg CuS nanoparticles in chloroform (0.2 mL) were added to a 1.5

mL centrifuge tube, and total volume was complemented by chloroform to 0.5 mL. The resulting CuS NPs suspension (0.5 mL) was added into 5 mL 0.02 g/mL cetyltrimethylammonium bromide (CTAB) aqueous solution and stirred vigorously for 30 min. The mixture gradually turned to turbid green color, indicating the formation of an oil-in-water microemulsion. Then, the solution was heated up to 60 °C for 10 min under stirring to evaporate the chloroform, resulting in a transparent green CTAB stabilized CuS NPs suspension. The resultant suspension was added to a mixture of 45 mL of water and 0.3 mL of 1 M NaOH solution and the mixture was heated up to 70°C under stirring. Then, 3 mL of ethylacetate and 0.15 mL of tetraethylorthosilicate (TMOS) were added to the reaction solution in sequence. After 10 min, 50 µL of APTES was added and the solution was stirred for 1 h. The precipitate was separated by centrifugation and washed with ethanol for 3 times and suspended in 20 mL ethanol.

PEGylation of MSNs. 50 mg of methoxy poly (ethylene glycol) succinimidyl glutarate (Mw 2000) dissolved in 20 ml ethanol was added to the as-synthesized particles followed by stirring at 40°C for 3 h. The unreacted PEG was removed by ethanol and water. The PEGylated particles were dispersed in phosphate buffer solution (PBS, 10 mM, pH 7.4).

Loading of doxorubicin. The loading of doxorubicin for *in vitro* experiment were performed as follows: PEGylated MSN solution (5 mL, 4 mg/mL) and doxorubicin ethanol solution (5 mL of 2 mg/mL) were mixed by stirring at 300 rpm for 24 h. Then,

the nanoparticles were isolated by centrifugation and vacuum dried overnight. Fully-dried sample was suspended into 10 mL of 100 mM PBS buffer (pH 7.0).

Characterizations. To prepare samples for transmission electron microscope (TEM) observations, the corresponding materials were ultrasonicated in pure water until homogeneous suspension was formed, and the resulting suspension was then dropped onto a nickel micro grid, followed by drying in air. TEM observations were respectively performed on a JEOL 2100EX microscope operating at an accelerating voltage of 100 kV. DLS analysis was proceeded with the Malvern® nanoseries Nano-ZS90 nanoparticle size analyzer. UV–visible–NIR absorbance spectra were obtained with a PerkinElmer Lambda 1050 UV–visible–NIR spectrophotometer with a quartz cuvette of 1.0 cm optical path length in the transmission mode employing pure water as the reference standard. FT-IR spectra were obtained with Thermos Scientific Nicolet 380 FT-IR Spectrometer. X-ray diffraction (XRD) patterns of the produced nanoparticles were recorded on a Rigaku Ultima IV multipurpose X-ray diffraction system. Fluorescent spectra were measured by SpectraMax Multi-Mode Microplate Readers.

***In Vitro* Release.** *In vitro* release of DOX from the as-prepared nanoparticles by adding 10 mg PEG-CuS@MSNs-DOX in phosphate-buffered saline (PBS, pH 7.4, 10 mM), and stirred constantly at 500 rpm. The NIR laser was administered as needed at 900 nm with power density of 2 W/cm² for 2 min at time points of 1, 4, 8, 12 and 24 h. The samples were collected before and after laser irradiation by centrifuge at 15,000 rpm for 10 min followed by filtration with 0.22 µm filter. The release medium was replaced with a fresh one each time to maintain sink conditions. The amount of

released DOX was monitored by fluorescent measurement. The fluorescent emission peak at $\lambda = 590$ nm under excitation light ($\lambda = 485$ nm) of each solution was recorded to determine the released DOX amount. As a comparison, *in vitro* release was preceded identically without NIR irradiation. All measurements were performed in triplicate.

Cell Culture. A549 cell line was cultured in DMEM media, supplemented with 10% fetal bovine serum (FBS), L-glucose, glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin.

Cell uptake of PEG-CuS@MSNs-DOX. 8-well cover slide chambers were seeded with A549 cells at 2×10^5 per well, and the cells were allowed to attach for 24 h. The medium was replaced with 1 mL medium containing PEG-CuS@MSNs-DOX or Dox solution (final DOX concentration 5 μ M) and incubated for 4 h. The cells were washed three times with PBS to remove the drug. Cell nuclei were stained with Hoechst 33258. The cellular fluorescence were obtained as described in the preceding paragraph.

Cytotoxicity. Cytotoxicity was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) method. Briefly, A549 cells were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated for 24 h prior to use. The cells were respectively incubated with PEG-CuS@MSNs-DOX, PEG-CuS@MSNs, and free DOX at equivalent drug concentrations ranging from 50 nM to 15000 nM for 4 h. Then the culture medium containing particles was replaced with fresh medium and continued incubation for 20 h. Then, MTT tetrazolium salt

(0.25 mg/ml) was added to each well and incubated in CO₂ incubator at 37°C for 4 h. Finally, 150 µL DMSO was added to each well dissolve formazan crystals and the UV–vis absorbance peak at 570 nm was monitored by a plate reader.

***In vitro* efficiency test** was carried out *via* trypan blue exclusive assay. A549 cells were seeded in 12-well plates at a density of 5×10^4 cells/well and incubated for 24 h prior to use. 20 µL of PBS control or 20 µL of 2 µg/mL (in Cu²⁺) nanoparticles dispersed in PBS was added into designated wells of the 12 well plate. Then the plate was incubated for 4 h. After irradiation with laser (900 nm, 2W/cm²) for 1 min, the sample was stained by 0.4 mL trypan blue for 3 min. The stained sample was washed twice with PBS, and mixed with 0.4 mL PBS. The *in vitro* efficiency was observed by inverted microscope, and images were taken with a Cannon digital camera.

All data were expressed as mean SD. The statistical significance was determined using a t test. A p value less than 0.05 (*i.e.*, $p < 0.05$) was considered to indicate statistical significance for all comparisons.

RESULTS AND DISCUSSION

Oleylamine coated CuS NPs was synthesized with a newly developed facile non-solvent method. The TEM image suggested that CuS NPs were in flake shape (Figure 1a) with an average size of 12 nm. The lateral view of some particles demonstrates thin and rod-like structure, which agreed well with the CuS NPs prepared by the complicated multi-step techniques.^[15] It was considered that several CuS nanoflakes stacked and were wrapped together into one silica nanoparticle during the sol–gel reaction (Figure 1b). The average size of CuS@MSN was ~40 nm, meeting the practical requirement for drug delivery.^[16] Figure 1c showed the

PEG-CuS@MSNs nanoparticles (right) turned to purple after loading DOX (left). The loading efficiency was as high as 18.4%.

DLS measurement presented a particle diameter of 58 nm. This was close to the TEM result, but it was slightly larger (Figure 2). Because DLS measurement acquired hydrodynamic data, and the swollen state of the nanoparticle inevitably became bigger than the value at dry shrunk state.^[17]

Zeta potential of CuS@MSN was -36.2 mV. Although the initial nanoparticle surfaces are negatively charged, the coating of PEG on the surface brought the zeta potential to nearly neutral, ~ 0.5 mV, which was preferable for drug delivery to the negatively charged cell membranes.^[18]

Figure 1. TEM image of oleylamine-coated CuS NPs (a), and CuS@MSNs (b). The red circle, a nanoparticle showing the panel of CuS NPs. The yellow circle, a nanoparticle showing the lateral of CuS NPs stacks. A photograph (c) showed PEG-CuS@MSNs-DOX (left) and PEG-CuS@MSNs (right).

Figure 2. Hydrodynamic particle diameter of CuS@MSNs by DLS analysis.

The XRD patterns of CuS@MSNs were exhibited in Figure 3. The typical peak of SiO₂ was found at $2\theta = 21.7^\circ$ (Figure 3). It also exhibits the CuS (Powder diffraction file or PDF#65-3588) characters with 2θ at 29.3° (1 0 2), 31.8° (1 0 3), 32.9° (1 0 6), 48.0° (1 1 0), 52.7° (1 0 8), 59.3° (1 1 6), 69.4° (1 0 11).

Figure 3. XRD pattern of CuS@MSNs.

The UV-vis-NIR spectra of CuS NPs, and PEG-CuS@MSNs and PEG-CuS@MSNs-DOX all showed strong absorbance at the NIR region between 800 to 1400 nm, which is within photothermal treatment range (Figure 4). However, a closer look at the spectra of the silica nanoparticles revealed that there was a slight shift in the spectra, which might due to the change in the local refractive index of the surrounding medium.^[19] In addition, PEG-CuS@MSNs-DOX also exhibited the typical absorbance band of Dox. It should be notified a red shift from 490 nm to 516 nm due to the influence of copper. (There is a detailed discussion on the interaction between DOX and copper ion in Chapter 1.)

Figure 4. UV-vis-NIR spectra of oleylamine coated CuS NPs (a), PEG-CuS@MSNs (b) and PEG-CuS@MSNs-DOX (c). Concentration: 1.5 mM in copper.

The drug release behavior of PEG-CuS@MSNs-DOX was studied in the pH of 7.4, 6.0, and 5.0 buffer solutions over a 24-h period (Figure 5). It can be seen that DOX release was pH dependent. At pH 5, the 24 h cumulative release of DOX for PEG-CuS@MSNs-DOX was 17.9%. At pH 6.0, the released ratio decreased to 12.4%, and dropped down to 5.7% when pH increased to 7.4. In addition, the release results also indicated that NIR laser irradiation accelerated DOX release in various pH conditions. Each time upon laser irradiation for 2 min at 2 W/cm^2 , released DOX is enhanced 1-2 % immediately. The laser-triggered release was well responsive by repeating the laser irradiation. The drug release slowed down when the laser was switched off. Compared with PEG-HCuSNPs-DOX, the percentage release triggered

by laser was much lower. Since DOX was loaded in the channels of MSNs, limited proportion of the DOX were drove out of the channel by Cu NPs explosion upon laser irradiation. But for PEG-HCuSNPs-DOX, laser irradiation brought devastating damage to each nanoparticle, thus the DOX adsorbed or chelated to the surface was released significantly. Back to the PEG-CuS@MSNs-DOX, in addition to pH sensitive and laser triggered release, the release showed sustained release profile, as over a period of 24 h, less than 20% of DOX was released. The triple-module release profile, namely pH sensitive, laser controlled and sustained release, presented a controlled release manner through which the drug could be specifically and extended released at the tumor site either by applying NIR laser or replying on the acidic environment of the tumor.

Figure 5. Cumulative release profile of PEG-CuS@MSNs-DOX at different pH buffers with 1 min of 2 W/cm² NIR laser irradiation at 1 , 2, 4, 8, 12 and 24 h. Data are expressed as mean $\pm$ SD (n = 3).

PEG-CuS@MSNs-DOX was internalized into A549 cells after 2 h incubation. PEG-CuS@MSNs-DOX showed strong red fluorescence signal from DOX, despite the quenching effect of DOX bond to PEG-CuS@MSNs. The fluorescence of DOX was limited in spots scattered throughout the cytoplasm, indicating a distribution in endolysosomal vehicles. (Figure 6) In comparison, free dox diffused in both cytoplasm and nuclei after 2 h incubation.

Figure 6. Fluorescence images of A549 cells after 2 hour incubation with DOX (a) and PEG-CuS@MSNs-DOX (b), respectively. The cell nuclei were stained with Hoechst 33258 (blue, the left column). Red, DOX, in the middle column. The overlay, in the right column. Scale bar: 50 μm .

Cytotoxicity was tested by the methyl thiazolyl tetrazolium (MTT) assay in A549 cells derived from human lung carcinoma cell line (Figure 7). The results showed that the cellular viability was estimated to be higher than 71% after 24 h incubation in the presence of the PEG-CuS@MSNs or PEG-CuS@MSNs-DOX with Cu concentrations of 0–10 $\mu\text{g/mL}$, indicating a relatively low cytotoxicity within this concentration range (Figure 7). The IC_{50} of free DOX was about 0.12 $\mu\text{g/mL}$ (data not shown) while the IC_{50} of PEG-CuS@MSNs-DOX was significantly higher, 15 $\mu\text{g/mL}$, because of the slow release profile of PEG-CuS@MSNs-DOX. PEG-CuS@MSNs-DOX exhibited enhanced toxicity ($\text{IC}_{50} = 59 \mu\text{g/mL}$ of Cu^{2+}) than PEG-CuS@MSNs did ($\text{IC}_{50} = 90 \mu\text{g/mL}$ of Cu^{2+}).

Figure 7. Cell viability of A549 cells when incubated 4 h with (a) PEG-CuS@MSNs or PEG-CuS@MSNs-DOX, with different dosages (n = 4).

The therapeutic efficacy test *in vitro* was explored on A549 cells incubated in 24-well plate. It was observed efficient photothermal ablation of the A549 cells only after 1 min irradiation of the 900 nm laser in the presence of the nanoparticles. Also, in the trypan blue assay as shown in Figure 7, few cells were dead either after laser exposure alone (7b) or after treated with different nanoparticles without laser exposure (Figure 7c and 7e). However, almost all the cells were dead after laser irradiation in all nanoparticle groups (Figure 7d 7f and 7g). This was attributed to the efficient intracellular uptake of PEG-CuS@MSNs-DOX after 2 h incubation.

Figure 8. Optical images of A549 cells obtained after the cell viability (trypan blue) test. (a) and (b), cells treated with PBS alone and PBS plus NIR laser (900 nm, 2 W/cm², 1 min), respectively. (c) and (d), cells treated with PEG-CuS@MSNs alone and PEG-CuS@MSNs plus NIR laser, respectively. (e) and (f), cells treated with PEG-CuS@MSNs-DOX alone and PEG-CuS@MSNs-DOX plus NIR laser, respectively. Scale Bar: 50 μ m. (g) Quantification of cell viability of (a-f).

CONCLUSIONS

In summary, monodisperse CuS nanocrystals coated in uniform pore-sized mesoporous silica nano spheres with an average particle size of 40 nm were successfully synthesized. Mesoporous silica spheres adsorbed doxorubicin and enabled high drug loading capacity. The release rate of doxorubicin was faithfully controlled by pH, laser exposure and the surface property of mesoporous silica. They showed photothermal effects on cancer cells upon laser exposure. These mesoporous silica nanoparticles provide a facile pathway for versatile biomedical applications.

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CHAPTER 4

This manuscript has been published as a review paper on Photonics and Lasers in
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Laser ablation-enhanced transdermal drug delivery

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Abstract:

Transdermal delivery offers an excellent route for drug and vaccine administration. Nonetheless, it presents a critical challenge due to the skin's lipid-rich outer stratum corneum layer. Laser ablation perforates epidermis through selective photothermolysis, making skin more permeable to hydrophilic and macromolecular drugs such as peptides, proteins, and genes. This review summarizes recent applications to laser ablation-enhanced transdermal delivery. Needle- and pain-free transcutaneous drug delivery via laser ablation provides an alternative approach to achieve local or systemic therapeutics.

Keywords: Laser ablation, Transdermal, Drug delivery, Microporation, Nanoparticle

1 Introduction

The development of transdermal drug delivery systems (TDDS) is attractive because skin is the largest organ. TDDS have the distinct advantages over oral administration or injections since they directly deliver drugs into the skin or even the systemic circulation, avoiding first-pass clearance of liver thus enhancing bioavailability. TDDS provide sustained and steady-state pharmacokinetics, therefore decreasing administration frequency and improving the patient compliance. Further, TDDS avoid the limitation of injections such as pain, accidental needle-sticks, and possible side effects due to transiently high plasma drug concentration [1-3].

However, the skin presents a natural barrier to protect our body from the rough environment. It forms multilayers in the epidermis, which include stratum corneum (SC), stratum lucidum, stratum granulosum, stratum spinosum, stratum basale from topical toward dermis. The SC is the outmost layer and consists of dead keratinocytes or corneocytes intercalated with lipids [4]. This 10- to 20- μm thick layer is the formidable barrier preventing most drug molecules from permeation. Only lipophilic drug with molecular weight (MW) less than 500 Daltons is able to penetrate the skin barrier, such as clonidine, fentanyl, and lidocaine [3, 5].

A variety of methods have been tried to enhance the permeability of the SC. Chemical enhancers promote the drug penetration through the SC by disrupting the highly ordered bilayer structures of the intracellular lipids in the SC [3]. Conventional chemical enhancers such as Azone (1-dodecylazacycloheptan-2-one) as well as newly developed biochemical enhancers like peptides are of interest [6, 7]. However,

chemical enhancement has been shown little impact on delivery of hydrophilic drugs and macromolecules and irritation to living cells in the deeper skin [3]. On the other hand, physical enhancement techniques including mechanical and thermal approaches have been used to make micrometer dimensions of disruptions to SC structures. These micro-scale disruptions create channels of sufficient dimensions for passage of macromolecules. The thermal ablation activated by microheaters,[8] radio-frequency [9-11], superheated steam ejectate [12] or laser [13-16] is non-invasive technique to selectively remove small portions of the SC. These perforations are temporary, since the layers of the SC are continually replaced through the natural process of desquamation [8]. Some of the physical enhancement technologies have been applied in clinical trials for TDDS such as BA058 transdermal microneedle patch [17], transdermal basal insulin patch with microporation [18], teriparatide acetate TDDS transdermal [19], and electroacupuncture for opioid detoxification [20].

Laser ablation enhancement belongs to a physical approach that utilizes laser to perforate or remove the SC barrier in order to enhance the drug penetration. Water and pigments in the skin absorb the laser light energy and transform it into heat to achieve theromolysis of the skin. The heating duration must be controlled within microseconds in order to avoid heat propagation to deeper tissues [21]. The laser ablation approach enables precise control of depth of skin permeation, having the potentials for percutaneous delivery of biomacromolecules such as peptides, proteins, vaccines, DNAs [15]. In this review, we will focus on recent progresses of laser ablation enhanced TDDS.

2 Direct laser ablation enhancement

Although many types of laser with a broad wavelength range (193 – 10,600 nm) are available in clinical practice such as ruby laser, neodymium:yttrium-aluminum-garnet (Nd:YAG) laser, alexandrite laser, CO₂ laser and erbium:yttrium-aluminum-garnet (Er:YAG) laser (**Table 1**), only a few are applied to transdermal delivery so far. Pulsed CO₂ and Er:YAG laser are in common use for SC ablation [22]. The ruby laser (694 nm) and the alexandrite laser (755 nm) belong to near-infrared (NIR) laser (650 – 900 nm). The NIR light causes little tissue absorption or minimal thermal effect [23], which is not sufficient to remove the SC. By contrast, the wavelengths of the CO₂ and Er:YAG laser are 10,600 nm and 2,940 nm, respectively. Both lasers directly induce heating and microporation of the skin through water excitation and explosive evaporation from the epidermis. Between these two laser types, the wavelength of the mid-infrared Er:YAG light matches a principal absorption wavelength for water molecules [13]. Compared with the CO₂ laser, the Er:YAG laser is about 15 times better absorbed in skin [22]. Therefore, the Er:YAG laser has a much higher ablation efficacy and a lower ablation threshold [24]. The Er:YAG laser shows the reduced thermal damage even in deeper crater holes in comparison with the pulsed CO₂ laser [22, 24]. These favorable properties make the Er:YAG laser an ideal light source not only for skin surgery but also for enhanced transdermal drug delivery. A comparison of three sources of laser, the ruby, CO₂ and Er:YAG laser, on the skin permeability for 5-Fluorouracil (5-FU) showed that the ruby laser only moderately enhanced the drug flux [25]. The Er:YAG laser with fluence at 0.8 – 1.4 J/cm² enhanced the flux of 5-FU

by 53 – 133 times than untreated skin. The CO₂ laser increased penetration of 5-FU by 36 – 41 times under the fluences of 4.0 and 7.0 J/cm² with certain thermal effects [25].

Laser-induced thermal ablation heats the skin to hundreds of degrees for very short periods of time (micro- to milli-seconds) to disrupt the SC [3]. The extent of structure alteration of the SC is proportional to the temperature locally elevated, i.e. (i) disordering of SC lipid structure by temperature between 100°C and 150°C, (ii) disruption of SC keratin network structure by temperature between 150°C and 250°C, and (iii) decomposition and vaporization of keratin to create micron-scale holes in the SC by temperature above 300°C [21]. Correspondingly, skin permeability was increased from a few fold to three orders of magnitude [21]. For thermal ablation-enhanced TDDS, high energy of laser with pulse duration less than microseconds is required because it generates limited or negligible heat transfer to surrounding tissue [13-16]. The microsecond-pulsed laser steepens the temperature gradient across the SC. The skin surface is extremely hot but not the viable epidermis and deeper skin tissues [12]. This technique referred to as “cold ablation”, thereby, largely eliminates side effects and vastly improves safety.

In physically enhanced TDDS, the controllable depth and wound area of skin perforation by the laser ablation should be well considered. Based on the clinical data from microneedle and thermal ablation-enhanced transdermal delivery, micron-scale defects in the SC are well tolerated by patients as long as no significant damage to living cells in the viable epidermis and dermis [3]. To solve this issue, a laser microporation technology called P.L.E.A.S.E.[®] (Precise Laser Epidermal System;

Pantec Biosolutions) has been developed by using a diode-pumped fractional Er:YAG laser (**Fig. 1A**) [14, 15]. Instead of conventional Er:YAG in clinics that ablates a 7-mm spot on the skin, P.L.E.A.S.E.[®] generates a matrix of identical micropores with 100 – 150 μm wide of each (**Fig. 1B**). Since the concentrated laser beam are divided into microbeams, P.L.E.A.S.E.[®] efficiently and fractionally ablates skin with less damages (**Fig. 1C**) [14]. In addition, the pulse duration of the fractional laser from P.L.E.A.S.E.[®] is shorter than conventional Er:YAG laser to assure the localization of heat transfer to the skin surface without allowing heat to propagate to the viable tissues below. This technology is patient-friendly since it is programmed to precisely control the number of micropores in unit area and depth of micropores based on the laser fluence [15].

3 Photothermal nanoparticle-mediated laser ablation enhancement

The development of nanotechnology brings a breakthrough to the limited application of NIR laser in TDDS. Gold nanostructures such as nanoshells [26], nanorods [27], nanocages [28, 29], and hollow nanospheres [30] possess unique optical properties due to strong and tunable surface plasmon resonance (SPR). They can be synthesized to specifically absorb NIR light and convert photo energy into thermal energy to raise the temperature of surrounding tissue [26, 31]. Nanoparticles with the property of photothermal coupling effect are called photothermal nanoparticles. Gold photothermal nanoparticles can be applied to photothermal ablation therapy of tumor cells [32-35], as well as the NIR laser-controlled drug release [36-40]. The absorbance of NIR light is desirable because it causes minimal thermal injury to normal tissues with optimal light penetration [23, 41]. Recently, a surfactant/protein/gold nanorod

complex has been applied to transdermal delivery of proteins [42]. The solid-in-oil dispersion system has been formulated through incorporation of gold nanorods as the photothermal ablation enhancer to disrupt the skin barrier. This approach effectively enhances the protein permeation through the skin *in vitro* and induces an immune response *in vivo* [42]. In this application, instead of pulsed laser, a xenon lamp that required high light power (6 W/cm^2) and long duration of light exposure (20 min) has been used to ablate the stratum corneum [42]. Therefore, the heat propagation to the deeper tissue could be a major concern.

Semiconductor CuS nanoparticles (CuSNPs) are a new class of photothermal nanoparticles that provide an alternative to gold analogs. Compared to gold, CuS is much less expensive [43]. Irradiated with NIR laser, CuSNPs generate heat for photothermal destruction of tumor cells [43-46]. Hollow CuSNPs (HCuSNPs) have been utilized for photothermal ablation-enhanced transdermal drug delivery [47]. A nanosecond-pulsed Nd:YAG laser in tandem with Ti:Sapphire laser (900 nm) has been used to induce rapid heating of the nanoparticles and instantaneous heat conduction. Such type of laser with nanosecond pulse duration provides focused thermal ablation of the SC and minimizes skin heat accumulation. The average temperature of the irradiated skin area only increases to $\sim 40 - 50^\circ\text{C}$. The depth of skin perforation can be precisely controlled by adjusting the laser power. The skin disruption by HCuSNPs-mediated photothermal ablation significantly increases the permeability of macromolecule drugs, providing effective percutaneous delivery [47].

4 Drugs applied to laser ablation-enhanced transdermal delivery

In comparison with chemical enhancers that only improve the transdermal delivery of small molecules, laser ablation enhancement makes micrometer dimensions of disruptions to the SC structures suitable for the passage of both small and macromolecules such as 5-FU [25, 48], lidocaine [14], diclofenac [16], human growth hormone (hGH) [47], antithymocyte globulin (ATG) [15], ovalbumin (OVA) [42], polypeptides [49], fluorescein isothiocyanate(FITC)-labeled dextran (FD) [13], nalbuphine [50], vitamin C [51], 5-aminolevulinic acid (ALA) [52], genes [53], and stem cells [54] (**Table 2**).

Dextran, a hydrophilic macromolecular model drug, was used to evaluate the skin permeation. By using a laser with fluence above 1.7 J/cm^2 , the transdermal transport of FDs with molecular weight ranging from 4.4 kDa to 77 kDa was significantly enhanced. The possible mechanism could be ablation of the SC layer, photomechanical stress on intercellular regions, and alterations of the morphology and arrangement of corneocytes by the Er:YAG laser. Further, the transdermal delivery of hexameric insulin was higher than that of 38-kDa FD, suggesting the potential of laser ablative transdermal delivery of Insulin [13].

ATG and Basiliximab, two marketed antibodies for the induction of immunosuppression, were studied with fractional Er: YAG laser [15]. The result showed that the increase of pore numbers and laser fluence promoted the transdermal permeation of the antibodies. Total delivery of ATG at 24 h after laser treatment (900 pores, at a fluence of 45.3 J/cm^2) increased 82.8-fold over the control (untreated skin).

Increasing laser fluence from 22.65 to 135.9 J/cm² enhanced total ATG delivery from 1.70 ± 0.65 to 8.70 ± 1.55 µg/cm², respectively. Similar penetration enhancement was observed in Basiliximab. Moreover, the *in vitro* and *in vivo* result was well correlated in a mouse model [15].

Topical delivery of DNA and RNA were also enhanced by laser ablation [53, 55]. With Er:YAG treatment, *in vitro* permeation of antisense oligonucleotides (ASOs) increased 3 – 30-folds, depending on the laser fluence and the molecular weight of ASO. *In vivo* results showed an enhanced expression of plasmid DNA in the epidermis and subcutis [53]. Besides, it was also found that the delivery rate of siRNA was raised by several times by the laser application [55].

Laser-enhanced transcutaneous protein delivery provided a non-invasive immunization method [15, 56, 57]. The laser induced microporation allowed high levels of antigen uptake. Further, transdermal delivery of vaccine targets the potent epidermal Langerhans and dermal dendritic cells that generate a strong immune response at much lower doses than hypodermic injection [58]. Transcutaneous application of OVA via laser-generated micropores using the P.L.E.A.S.E.[®] device induced equal or higher immune responses compared to immunization by s.c. injection [57]. In addition, targeting different layers of the skin had the potential to bias different T cell polarization patterns [57]. The laser ablation enhancement followed by transcutaneous immunization of lysozyme with 129 amino acids (14,307 Da) induced antigen-specific IgG in the serum by 3-fold compared to the control without laser treatment [56].

In addition to deliver drug compounds, laser ablation-enhanced transdermal delivery of adipose-derived stem cells (ADSC) were explored for wound healings [54]. After fractional Er:YAG laser treatment, bromodeoxyuridine (BrdU)-labeled ADSC was applied to the laser treated areas. After 4 and 48 hours, 12% and 5.5% of the stem cells were found in the pretreated tissue, respectively [54]. This encouraging result furthered the studies to optimize the technology for future clinical applications.

Because of high photothermal conversion effect, the gold nanoparticles were utilized to achieve thermal ablation of skin to enhance transdermal delivery of OVA [42]. In this study, a solid-in-oil dispersion was formulated to incorporate both the gold nanorods and the drug. Therefore, the nanodispersion exerted two modules upon NIR light irradiation, i.e. thermal ablation of the SC by the gold nanorods and enhancement of skin permeation of OVA. *In vivo* experiment showed significant increase of immune response for the gold nanorod-OVA solid-in-oil dispersion with NIR light treatment than other groups [42]. Another study investigated the use of HCuSNPs as photothermal ablation enhancers [47]. The permeability of human growth hormone (hGH) in skin applied with HCuSNPs plus NIR laser was increased by 3 orders of magnitude in comparison with that of the intact skin. *In vivo* study showed that transdermal delivery of hGH using the HCuSNP-mediated photothermal ablation technique reached an average bioavailability of 83% relative to that of the subcutaneous injection. The peak drug concentration through transdermal delivery was only one-third of that via subcutaneous delivery [47]. This was clinical beneficial because it reduced the risk of side effect related to high concentrations and controlled the drug concentration in a relatively stable level.

5 Conclusion

In conclusion, this review has discussed recent progresses of laser ablation technology to enhance transdermal drug delivery. The success of delivery relies on locally thermal ablation of the SC. By adjusting the laser fluence and exposure time, the depth of the microporation can be controlled without harming the deeper living tissues such as the dermis. The microchannels allow skin permeation of hydrophilic and macromolecular compounds. Particular interest has been shown in the development of the photothermal nanoparticles that mediate photothermal ablation of skin and deliver drug in a single setting. As a clean, needle-free and non-invasive approach, laser ablation enhancement technology shows great potential for future market.

Table 1. Light sources for thermal ablation-enhanced transdermal drug delivery

Light Source	Wavelength	Pulse Duration	Role of the light in TDDS	Other Characteristics
Traditional Er:YAG laser	2,940 nm	250 – 400 μ s	Epidermal ablation; Dermal removal [25, 49]	One beam; Spot ablation
Fractional Er:YAG laser	2,940 nm	10 – 300 μ s	Fractional Epidermal removal [15]	Microbeams; Shorter pulse; Less damage to epidermis; Fractional photothermolysis
Short pulse CO ₂ laser	10,600 nm	50 ms	Epidermal removal; Dermal thermal injury [25]	Ablation; Vaporization
Nd:YAG in tandem with Ti:sapphire laser	690 – 950 nm	15 ns	Photothermal ablation [47]	Surface plasmon resonance by HCuSNPs
Xenon bulb	750 – 1,000 nm	Continuous light	Photothermal ablation [42]	Surface plasmon resonance by gold nanorods

Table 2. Drugs/compounds used for transdermal delivery by laser ablation

Drug/Compound	Indication/Purpose	Molecule Weight (Da)	Enhanced Fold of Permeability	Laser source
5-FU	Antitumor	130	133	Er:YAG [25, 48]
Imiquimod	Immune response modifier	240.3	127	Fractional Er:YAG [49]
Lidocaine	local anesthetic	234.3	13	Fractional Er: YAG [14]
Diclofenac	Non-steroidal anti-inflammatory drug (NSAID)	296.15	118.9	Fractional Er: YAG [16]
Vitamin C	Model hydrophilic drug	176	260	Er:YAG [51]
Methotrexate (MTX)	Psoriasis or rheumatoid arthritis		80	Er:YAG [59]
hGH	Growth hormone deficiency	22 k	>1000	Nd: YAG tandem with Ti: sapphire [47]
Antithymocyte/ Basiliximab	Immunosuppressive antibodies	155 k/144 k	145/ N/A	Fractional Er: YAG [15]
OVA	Antigen	44 k	~8	Xenon light [42]
Beta-galactosidase (bGal)	Antigen	465 k	Significant elevation	Fractional Er:YAG [57]
Recombinant Phl p 5	grass pollen allergen	38 k		
Equine heart cytochrome c	Model proteins	12.4 k	From 0 to >0	Fractional Er:YAG [60]
Urinary follicle stimulating hormone		30 k		
FITC-labeled bovine serum albumin		70 k		
Peptides	Model peptides	716 – 2864	10 – 140	Er:YAG [56]
Dextran	Model hydrophilic	4.4 k	50	Er:YAG

	macromolecule	10 – 150 k	From 0 to >0	[13]
Insulin (hexameric)	Diabetes mellitus	36 k	>100	
Nalbuphine	analgesic /Hydrophilic model drug	357	194	Er:YAG [50]
Indomethacin	NSAID/Lipophilic model drug	357	30	
ALA	Anti-tumor/photosensitizer	131	260	Fractional Er:YAG [52]
Antisense oligonucleotides	Test model	5 k – 8 k	~29	Er:YAG [53]
Plasmid DNA	Express green fluorescent protein	4.7 k base pairs	160 base pair	
RNA	Small interfering RNA	9266	10	Er:YAG [55]
Adipose-derived stem cells	wound healing	--	5.5 – 12 % of penetration	Fractional Er:YAG [54]

Figures

Figure 1. P.L.E.A.S.E® technology. (A), The photograph of the hand-held device. (B), Formation of a micropore array in the skin surface using the P.L.E.A.S.E® device. (C), Hematoxylin and Eosin staining of micropores created in porcine ear skin after laser microporation using the P.L.E.A.S.E® device at fluences of 4.53 J/cm², 22.65 J/cm² or 135.9 J/cm² (from left to right). From reference.[15] Copyright Elsevier. Reprinted with permission.

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CHAPTER 5

This manuscript is being prepared for submission to *Lasers in Medical Science*.

Cancer Photothermal Therapy and CuS Nanoparticles

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Cancer is a disease when abnormal cells lose control in the body. It is caused by genome damage or the disruption of cellular metabolic processes. It can be inherited or induced by external factors. Cancer can form solid tumors on tissues or grow in the blood. Solid tumors include cancers of the brain, lung, breast, colon and other tissues.

Current first-line treatments include surgery, chemo or radiation therapy. Surgery is to remove a cancerous tumor. About 60% of cancer patients undergo surgery. The limitation of surgery is that some cancerous cells may not be removed. So it is often followed by a combination of other therapy to completely kill all cancer cells. Chemotherapy is to use drugs to fight cancerous cells. However, the unselective damage of normal cells will also cause significant side effects. Besides, the cancer cells can develop drug resistance [1]. Radiation therapy is to use ionizing radiation, (X-rays, gamma rays or electrons) to damage the DNA of cancerous cells. It is often used to synergize chemotherapy or surgery. However, it can damage healthy tissues close to the cancer cells or in the path of the radiation beam.

Photothermal therapy (PTT) is a therapeutic method in which electromagnetic radiation is converted to heat to treat of various medical conditions including cancer. The electromagnetic radiation resources include near infrared or visible light, radiofrequency waves and microwaves. They can enhance the temperature in a targeting region to destroy the cells[2].

PTT possesses a lot of advantages over other therapeutic methods in the treatment of cancer. First, in comparison with the most common open surgery for tumor removal, photothermal therapy only requires minimal invasion. It can accurately locate the

tumor and reduce the harm to health tissue. Therefore, the average hospital stay time can be significantly reduced. Second, when compared with commonly applied chemotherapy, PTT largely reduces the side effects since it can be guided to focus on and ablate the tumor, whereas chemo drugs do not differentiate the normal cells and cancer cell. Third, PTT also shows advantages over radiotherapy, which has already less the damage to healthy cells due to the precise delivery of high-energy radiation to one particular location. Radiation nevertheless presents short-term risks such as skin rash or problems with tissues or organs near radiation pass and long-term side effects such as infertility or even secondary cancer due to radiation exposure.

The heat generated by laser has been used frequently in clinic practice. For example, in dentistry, laser is used for photobiomodulation in treatment of recurrent aphthous stomatitis and traumatic ulcers to accelerate healing process [3]. It is also used in thermal therapy to heat malignant tissue and tumor. Other examples include laser coagulation to seal blood vessels and stop bleeding, laser welding to join tissues and blood vessels, laser shock waves to remove urinary, kidney and biliary stones [4].

The detailed strategy and outcome of PTT on tumor treatment is largely depending on the properties of tumor, such as location, size, surface characteristic, water content, etc. Generally, laser can only reach less than a few (3–4) centimeters under skin [5]. For superficial tumors, external heating could be achieved by superficial applicators. For tumors deep inside the body but not close to a body cavity, a fiber need be inserted into the center of the target tumor [6]. Moreover, it is important to keep well-localized heating high enough in the tumor but not harm the surrounding normal tissue. To

achieve this, additional use of real time imaging techniques such as MRI and ultrasound imaging are required.

PTT has been applied to various solid tumors, including penile cancer, bladder tumors, renal tumor, melanoma, cervical cancer, breast cancer and so on. For selected cases of penile primary cancer in early stage, Nd:YAG or CO₂ lasers therapy was found effective [7,8]. For bladder and renal cancer, lasers is feasible for resection, coagulation, and enucleation of non-muscle invasive bladder tumors, but it should only be used in clinical trial setting or for patients who are not applicable to conventional treatment either because of co-morbidities or other complications [9]. For cutaneous melanoma, multiple small (<1 cm) lesions respond well to the CO₂ laser. CO₂ laser is recommended or palliative treatment of locoregional recurrence in a limb [10]. Local laser ablation can also be used on precancerous dysplasia to prevent cervical cancers [11].

There are also a lot of clinical trials for PTT on various carcinomas. For brain tumors, patients with recurrence of glioblastoma who had previous received total resection, chemotherapy and radiation therapy and ineligible for a secondary surgery was proposed for MIR guided laser-induced thermal therapy (LITT) salvage therapy [12]. Patients with resistant metastatic intracranial tumors who had previously undergone chemotherapy, whole-brain radiation therapy, and radiosurgery were given real-time magnetic resonance-guided laser-induced thermal therapy. The procedure was safely carried out with minimal invasion in one day [13]. Other MRI-guided laser interstitial thermal therapy was investigated on liver metastasis and prostate cancer [14–16]. A minimal invasion method, percutaneous laser ablation, by inserting optical

fibers into the cancer through 21-gauge needles, is safe and effective for cirrhotic patients with hepatocellular carcinoma when resection or liver transplantation is not possible [17]. In addition, tumor caused thrombus and mucostis are also eligible for laser therapy [18,19].

Normally, tumor larger than 3 cm is not proper for laser ablation. However, a drug might change this situation. Sorafenib, a multikinase inhibitor to reduce intratumoral blood flow, might enhance the effectiveness of laser ablation on hepatocellular carcinoma larger than 4 cm by decreasing cancer microvessel density and thus enlarge laser-induced coagulation necrosis area [20].

Recently PTT has attracted new interest because of the arising of photothermal nanoparticles, especially gold colloidal. Gold nanoparticles have superior light absorption the excited electrons on the surface can produce strong localized heat thus damage the cancer cell. Gold nanoparticles have involved in clinical trials. The first example, which has phase II result, is gold nanoparticles with silica-iron oxide shell for PTT treatment of atherosclerosis. The gold nanoparticles were integrated in stem cells grown on a bioengineered patch. Then the patch was implanted onto the artery through the minimally invasive cardiac surgery. Under near-infrared laser irradiation, the nanoparticles in the patch can burn the plaque. The dense calcium area, fibrous and fibro-fatty tissue with fulminant necrosis significantly decreased due to thermolysis of the nanoparticles after 12 months [21]. In addition, Tumor necrosis factor (TNF) – bound colloidal gold (Aurimmune®) was under phase I clinical trial. Patients with advanced solid organ malignancies or primary and metastatic cancer undergoing surgical resection received colloidal gold-bound TNF intravenously 12-78 hours prior

to surgery. The antitumor effect and biodistribution is still under evaluation [22,23]. Another gold nanoparticle based laser photothermal therapy (Aurolase®) is also under clinical trial for patients with refractory and/or recurrent tumors of the head and neck and subjects with primary and/or metastatic lung tumors. The gold-silica (Auroshell®) nanoparticles accumulated at the tumor respond to the interstitial illuminations of an 808-nm laser [24,25].

Besides gold nanoparticles, other types of nanoparticles are also widely explored. For example, graphene, two-dimensional (2D) crystal of sp^2 -hybridized carbon atom arranged in six-membered rings with high optical absorption in the NIR region are utilized for PTT to ablating tumor [26,27]. Semiconductor nanocrystals such as CdS, CdSe, CdTe, have been intensively used for fluorescence bioimaging due to the size and shape-dependent quantum confinement effect [28]. They can efficiently emit fluorescence when excited by visible or infrared light. Among those nanostructures, some of them were highlighted as photothermal agents due to the high converting efficiency of heat. Typical examples include nanostructures of MoS_2 , CuS, CuSe, CuTe, etc [29,30]. Inorganic nanoparticles is hard to degrade in vivo and might possess potential long- term toxicity. As a promising substitute, organic nanostructures are explored for photothermal therapy based on the discovery of NIR-absorbing organic nanomaterials. For instance, indocyanine green (ICG) has been approved by FDA. Other NIR dyes include heptamethine indocyanine dye- IR780, IR783, IR808, IR825, PcBu4, porphyrins [31–36]. In order to prepare more stable formulation with various sophisticated purpose, the NIR dyes incorporated into micelles, liposomes or even proteins, have been used for photothermal tumor ablation [37–39]. Conjugated

polymers such as polyaniline, polypyrrole and PEDOT: PSS-PEG with extended π -electrons also show high NIR absorbance as PTT agents, and have been found to be robust photothermal agents [40–42]. In addition, metallic nanoparticles, mesoporous silica nanoparticles and rare earth doped nanoparticles were also explored for photothermal therapy of cancer [43,44]. It is not possible to highlight just one nanoparticle since their parameters are so different. However, they should meet the general requirement for in vivo application—deep tissue penetration ability for the corresponding wavelength, significant photothermal conversion efficiency and decent biocompatibility.

PTT is effective for recurrent tumors not sensitive to chemo drug or radiation. However, PTT may not be effective against all — particularly cancers that have already metastasized all over the body. Chemo drugs, in contrast, are able to distribute throughout the body to destroy cancer cells that have spread, although chemo drugs might cause huge side effects. The combination of PTT and chemotherapy might conquer the limitation of PTT and chemotherapy. Doxorubicin (DOX) is a chemo drug used for various cancers, such as blood cancers, many solid tumors and soft tissue sarcomas. It works by intercalating DNA and increasing free radical. DOX has a ‘lifetime maximum dose’ due to its life-threatening dosage-dependent cardiac toxicity [45]. In addition to DOX, other chemo drugs were tested for synergistic enhanced photothermal therapy effect. For instance, small molecule chemo drugs like camptothecin, a DNA enzyme topoisomerase I inhibitor, was loaded into hollow copper sulfide nanoparticles for synergistic PTT therapy of cancer cells in vivo [46]. Hydrophobic curcumin (regulation multiple cell signaling pathways of tumor cells)

was combined in Ag/Au nanogels for PTT [47]. Rotenone, a mitochondrial complex I inhibitor, [48] and cisplatin (binding to and causing crosslinking of DNA) and so far were also explored [49]. Besides, oligodeoxynucleotides containing cytosine-guanine (CpG) motifs (a small single strand DNA functions as immunoadjuvant), single and double strand DNA [50], tumor necrosis factor (TNF), antibody anti-VEGFR2, etc were all investigated [51].

The CuS nanoparticles are considered as potent counterpart to gold nanoparticles, since they are economically efficient, not affected by the surrounding (The PTT of CuS nanoparticles originated from d-d* electron transition instead of surface plasmon resonance effect), low long-term toxicity [52]. Various Cu_{2-x}S nano composites (e.g. chelator-free [(64)Cu]CuS nanoparticles, hollow nanoshell, core-shell nanoparticle [46,53,54] have been extensively explored as a transformer of laser to thermal heat either accountable for therapeutic effects or as vehicles for drug delivery.

Table 1 shows several representative examples of CuS nanoparticles in PTT therapy. They are still undergoing bench-side research. However, they are very promising for the clinical application. As shown in the table, some CuS structures are used for synergistic chemo or immune therapy. Some of them possess very high (56.7%) photothermal conversion efficiency. Most of them were tested in animal models with subcutaneous inoculation of tumor cells. These mouse xenograft models are convenient for intratumoral injection of nanoparticles and laser irradiation spot location.

In my work, doxorubicin was selected as a model drug and hollow CuS nanoparticle and oleylamine-coated CuS were selected as photothermal sensitizers.

Doxorubicin utilized could easily form complex with copper through hydrogen bond to load DOX. Due to the synergistic effect of photothermal therapy, only a small amount of DOX is required, thus the risk of causing heart disease is expected to be significantly decreased. The CuS nanoparticles synthesized exhibit remarkable photothermal effect. Specifically, hollow CuS NPs have large surface volume and considerable number of mesoporous pores beneficial for the drug loading. Unlike gold nanostructures such as gold nanorods, they are photo-stable and more biocompatible. Oleylamine stabilized CuS are readily dispersible in chloroform without aggregation and have facile preparation method.

In conclusion, among various photothermal nanoparticles, CuS nanoparticles are promising photothermal sensitizers in cancer PTT therapy low toxicity, low cost, high efficiency. CuS nanoparticles have great potential for clinical application as gold analogs.

Table 1. Selective publications on *in vivo* CuS nanoparticles cancer photothermal therapy

Nanoparticle Structure	Drug	Animal model	Administration Route	Laser Administration
CuS	--	--	--	$\lambda = 808 \text{ nm}$ [55]
Cysteine-coated CuS nanoparticles	--	Severe-combined immunodeficient mice (SCID) bearing s.c. xenografts of K7M2 osteosarcoma cells	Intratumoral injections	$\lambda = 980 \text{ nm}$ 0.72 W cm^{-2} 10 min [56]
Hydrophilic flower-like CuS superstructures	--	SCID bearing mice s.c. xenografts of PC-3 human prostate cancer cells	Intratumoral injections	$\lambda = 980 \text{ nm}$ 0.51 W cm^{-2} 5 min[57]
Folate receptor-targeted CuS (FA-CuS) NP	--	Nude mice bearing s.c. folate receptor-positive KB cancer cells	i.v. injection	$\lambda = 808 \text{ nm}$ 1.5 W cm^{-2} 3 min [58]
Chelator-free [^{64}Cu]CuS nanoparticles (NPs)	--	Nude mice inoculated s.c. with U87 human primary glioblastomacells	Intratumoral injections	$\lambda = 808 \text{ nm}$ 16 W cm^{-2} 5 min [53]
Hollow CuS nanoparticles	Camptothecin	Kunming mice inoculated with H22 mouse hepatocellular carcinoma cells on the mammary fat pads.	i.v. injection	$\lambda = 980 \text{ nm}$ 0.7 W cm^{-2} 5 min [46]
Chitosan-coated hollow copper sulfide nanoparticles	Oligodeoxynucleotides containing cytosine-guanine	EMT6 murine mammary carcinoma-bearing mice	Intratumoral injections	$\lambda = 900 \text{ nm}$ 2.0 W cm^{-2} 40 s [59]
$\text{Cu}_9\text{S}_5@\text{mSiO}_2$ core-Shell nanocomposites	Doxorubicin	Nude mice of HCT 116 human colon carcinoma model.	Intratumoral injections	$\lambda = 980 \text{ nm}$ 0.72 W cm^{-2} 10 min [60]

CuS nanoparticles-decorated graphene oxide functionalized with polyethylene glycol	Doxorubicin	Nude mice bearing Hela cervical cancer cells	Intratumoral injections	$\lambda = 980 \text{ nm}$ 1 W cm^{-2} 5 min [61]
Cu ₇ S ₄ Hollow Structure	--	SCID mice inoculated s.c. with hepatocarcinoma cell lines Hep3B cell	Intratumoral injections	$\lambda = 980 \text{ nm}$ 0.72 W cm^{-2} 10 min [62]
CuS NPs stabilized with drug-conjugated gelatin	Doxrubicin	--	--	$\lambda = 808 \text{ nm}$ 6 W cm^{-2} 10 min [63]
CuS self-assembly Shuttle-like bundles	--	--	--	[64]
Cu _{7.2} S ₄ nanocrystals	--	SCID bearing s.c. xenografts of K7M2 osteosarcoma cells	Intratumoral injections	$\lambda = 980 \text{ nm}$ 0.72 W cm^{-2} 7 min [65]
Gadolinium chelate functionalized CuS NPs	--	--	--	$\lambda = 808 \text{ nm}$ 6 W cm^{-2} 5 min [66]
Fe ₃ O ₄ @Cu _{2-x} S Core-Shell Nanoparticles	--	Nude mice bearing Hela cervical cancer cells	Intratumoral injections	$\lambda = 980 \text{ nm}$ 0.6 W cm^{-2} 10 min [55]

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