Emergence of Gold-Mesoporous Silica Hybrid Nanotheranostics: Dox-Encoded, Folate Targeted Chemotherapy with Modulation of SERS Fingerprinting for Apoptosis Toward Tumor Eradication

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Strategically fabricated theranostic nanocarrier delivery system is an unmet need in personalized medicine. Herein, this study reports a versatile folate receptor (FR) targeted nanoenvelope delivery system (TNEDS) fabricated with gold core silica shell followed by chitosan–folic acid conjugate surface functionalization by for precise loading of doxorubicin (Dox), resembled as Au@SiO₂-Dox-CS-FA. TNEDS possesses up to 90% Dox loading efficiency and internalized through endocytosis pathway leading to pH and redox-sensitive release kinetics. The superior FR-targeted cytotoxicity is evaluated by the nanocarrier in comparison with US Food and Drug Administration (FDA)-approved liposomal Dox conjugate, Lipodox. Moreover, TNEDS exhibits theranostic features through caspase-mediated apoptosis and envisages high surface plasmon resonance enabling the nanoconstruct as a promising surface enhanced Raman scattering (SERS) nanotag. Minuscule changes in the biochemical components inside cells exerted by the TNEDS along with the Dox release are evaluated explicitly in a time-dependent fashion using bimodal SERS/fluorescence nanoprobe. Finally, TNEDS displays superior antitumor response in FR-positive ascites as well as solid tumor syngraft mouse models. Therefore, this futuristic TNEDS is expected to be a potential alternative as a clinically relevant theranostic nanomedicine to effectively combat neoplasia.

1. Introduction

Advances in nanotechnology have scrutinized an assortment of carriers for controlled delivery of therapeutic payloads against cancer[1] which still holds the second leading cause of modality worldwide. Nonspecific delivery of anticancer agents often results in damage of healthy organs, which significantly impedes cancer survival rates. Ongoing research on nanomedicine aims to enhance the therapeutic index of antineoplastic drugs by adjusting their pharmacokinetics and biodistribution. The idea that nanomedicine aims to enhance the therapeutic index of antineoplastic drugs by adjusting their pharmacokinetics and biodistribution to enhance conveyance to the site of action is clinically demonstrated. The enhanced permeation and retention effect of the tumor microenvironment provides easier access of chemotherapeutic drugs to tumor tissues. Doxorubicin (Dox) is a widely used antineoplastic agent, but its severe side effects[2] limit its clinical efficacy. Designed to
exploit the advantages of nanoparticles (NPs), liposomal Dox formulations were approved by the FDA\cite{3} but has a whimsical disadvantage of being nontargeted toward cancer cells\cite{4}. Among different nanocarriers, gold nanoparticle (AuNP)-based carriers are widely employed for cancer diagnosis and imaging, either alone or in conjunction with therapeutic motifs.\cite{5} Moreover, high localized surface plasmon resonance (LSPR) of AuNPs facilitates in diagnostic modality as a surface enhanced Raman scattering (SERS) substrate to provide structural information of many biomolecules within subcellular components without altering the biology of cells based on their unique vibrational Raman fingerprint. The LSPR spectral shifts induced by interparticle plasmonic coupling have attracted considerable interest in the fabrication of assembled plasmonic nanoparticles.\cite{6,7}

In SERS, hot spots created in the gaps between NPs or at the edges and tips of anisotropic NPs provide sufficiently intense electromagnetic fields to promote a tremendous increase in the Raman intensity of molecules located in these regions, making it possible to detect even minute changes. AuNPs with tunable size and shape act as excellent SERS substrates and were used in label-free immunoassays, biosensing, imaging of living cells, and microbes in ultralow level of limit of detection. Engineering plasmonic nanostructures confined within a defined architecture and surface caged with biomaterials often execute optical and structural properties enabling them for captivated biological applications. Moreover, SERS-encoded plasmonic NPs are capable of generating a stable and reproducible Raman spectral pattern and imaging in a particular clinical milieu.\cite{8} In the construction of nanocarriers, mesoporous silica nanoparticles (MSNs) have attracted much attention owing to their many unique features such as tunable pore morphologies, high surface area, large pore volume, facile functionalization of exterior surfaces, and biocompatibility. MSNs provided a high drug loading capability; therefore, encapsulation of AuNPs with silica layer (Au@SiO\textsubscript{2}) not only enables facile surface modification and stimuli responsive release but also enhance the LSPR of the construct to be an effective SERS substrate.\cite{9} Even though the clinically used NP formulations of Dox demonstrated effectiveness, the lack of cancer cell targeting moieties makes them amiable to normal cells. Capping of drug-loaded Au@SiO\textsubscript{2} NPs with biodegradable natural polymer chitosan will avert premature drug release and promote the biocompatibility. Folate receptor (FR) is known to be overexpressed in several human tumors, and the exploration of FR-mediated drug delivery has been referred to a molecular “Trojan horse” approach.\cite{10} Nontargeted chemotherapeutic strategies often caused greater side effects. On the other hand, a targeted nanocarrier system often allows the delivery of drugs at much lower doses, due to a combination of molecular targeting and increased circulation time preventing random exposure to normal tissues.\cite{11,12}

Considering the present necessity for the development of a clinically viable smart targeted nanovehicle delivery system (TNEDS), we aimed to build Au@SiO\textsubscript{2} NPs loaded with Dox that is enveloped by a layer of chitosan–folic acid covalent conjugate (CS-FA) Au@SiO\textsubscript{2}-Dox-CS-FA (Scheme 1).

The major emphasis on theranostic efficiency of TNEDS has been exploited by (i) impressing biocompatibility with efficient Dox loading and tumor niche-specific release enabled targeted cytotoxicity by FR-mediated endocytosis; (ii) the significant Raman spectral enhancement of the TNEDS utilized as an SERS nanotag for quantifying Dox loading and tracing of intracellular Dox release by Raman imaging; (iii) evaluation of apoptotic cell death by TNEDS using SERS/fluorescence bimodality; (iv) detailed investigation with tumor challenged mouse models has been highlighted in terms of therapeutic potential of Au@SiO\textsubscript{2}-Dox-CS-FA and compared clinically used Dox and Lipodox. Even though further investigations are warranted to establish the current TNEDS in clinical milieu, the contemporary studies presented here emphasized the superiority of Au@SiO\textsubscript{2}-Dox-CS-FA as a futuristic multimodal theranostic nanoprobe toward efficient cancer management.

2. Results and Discussion

2.1. Preparation and Characterization of TNEDS

The successful preparation of Au@SiO\textsubscript{2}-Dox-CS-FA in a stepwise manner (Scheme 1; Figure S1, Supporting Information) was effectively monitored by the evaluation of physicochemical parameters by UV–vis spectrophotometry, high-resolution transmission electron microscopy (HRTEM), dynamic light scattering, and confocal Raman spectroscopy. The presence of a silica layer on the AuNP core was indicated by the redshifted (531–534 nm) plasmon maxima of the nanoconstruct. Dox loaded on Au@SiO\textsubscript{2}, further shielded by CS-FA, has been well studied by UV–vis in which absorbance maxima showed a slight blueshift with peak broadening (Figure 1a; Figure S2a, Supporting Information). HR-TEM analysis further confirmed the stepwise formation of the nanoconstruct where the average size of AuNPs (40–45 nm) increased to 105 nm upon silica coating. Additional surface functionalization with CS-FA increased the size to 125 nm of the nanoconstruct (Figure 1b; Figure S2b, Supporting Information). Morphological analysis revealed the presence of clusters of AuNPs confined within the silica shell along with single AuNPs. A nontargeted construct, Au@SiO\textsubscript{2}-Dox-CS, was also prepared by using CS instead of CS-FA (Section SI-1, Supporting Information). Dox impregnation into the SiO\textsubscript{2} pores was evaluated by the drug-loading efficiency (DLE), drug-loading content (DLC), and actual amount of drug in NPs. Au@SiO\textsubscript{2}-Dox-CS-FA showed a high DLE of 90% with around 93.75 mg Dox per gram of the construct and 9.3% DLC whereas Au@SiO\textsubscript{2}-Dox-CS demonstrated 81% DLE, 8.5% DLC, and 85 mg Dox per gram of the construct, respectively. The successful functionalization of CS-FA with Dox-loaded silica nanoparticle was initially confirmed by UV–vis and later with Fourier transform infrared (FTIR) analysis (Section SI-2 and Figure S3 of the Supporting Information). The FT-IR spectrum of CS was characterized with the bands around 3396, 1655, 1378, 1075, and at 611 cm\textsuperscript{-1} whereas the CS-FA spectrum shows the appearance of −CONH amide band at 1645 cm\textsuperscript{-1} and the N–H bending in the second amine
at 1590 cm$^{-1}$ which indicated the amide coupling between FA and CS. Furthermore, the peak at 3300 cm$^{-1}$ of CS-FA becomes wider, indicating the extended hydrogen bonding between the FA and CS. Elemental characterization with Au@SiO$_2$ revealed the presence of Au and Si (Figure 1c), and the average hydrodynamic size (Figure 1d) of around 125 nm was in agreement with TEM analysis. Due to the existence of silica coating on the exterior surface of AuNPs, a $\zeta$ potential value of -26.2 was observed, but it has been changed to +25.6 upon CS-FA coating indicating the presence of an amino group on the surface of NPs (Figure 1e; Table S1a, Supporting Information). Finally, Dox loading was confirmed by SERS spectral analysis, where signature Raman peaks of Dox appeared from the nanoconstruct (Figure 1f; Table S1b, Supporting Information). The presence of silica envelope and surface modifications will not affect the robust SERS intensity of the analyte.\textsuperscript{15} In UV-vis, the presence of drug loading was confirmed by the blueshift of the absorption maxima of 521 nm as a broad peak. More precisely, the presence of Dox within Au@SiO$_2$-Dox-CS-FA was established by SERS single spectral analysis, which was in agreement with the SERS spectra of bare Dox incubated with AuNPs. Dox showed characteristic Raman signature peaks at 455 cm$^{-1}$ assigned to the C$\equiv$O in plane deformation, peaks between 1247 and 1318 cm$^{-1}$ correspond to the in-plane bending motions from C$\equiv$O, C$\equiv$O$\equiv$H, and C-H, respectively, and peak at 1438 cm$^{-1}$ attributed to the presence of skeletal ring vibrations of Dox.\textsuperscript{16} The SERS signal of Dox is originated from the hot spot generated by the cluster of AuNPs capped with the silica shell. The close attachment of AuNPs within

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**Scheme 1.** a) Various steps involved in the fabrication of Au@SiO$_2$-Dox-CS-FA starting from AuNPs. b) Chemical structure of chitosan–folic acid (CS-FA) and Dox. c) Biological evaluation after i.p. administration of TNEDS on tumor-bearing mice.
the silica layer leads to strong interparticle plasmonic coupling, as evidenced by the noticeable redshift of plasmon resonance of the nanoparticles.

2.2. Stimuli Responsive Drug Releases

The release kinetics of Dox from Au@SiO₂-Dox-CS-FA, Lipodox, and doxorubicin hydrochloride was evaluated under parameters of pH and reducing atmosphere at ambient temperature initially by UV–vis spectroscopy. It was found that doxorubicin hydrochloride showed a burst release within 2–4 h regardless of the change in pH and reducing agents (Figure S4a, Supporting Information). Lipodox displayed minimal response toward stimuli in which an acidic pH with a reducing atmosphere caused a rapid Dox release whereas at neutral pH the release was prolonged up to 7–8 h (Figure S4b, Supporting Information). Au@SiO₂-Dox-CS-FA

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Figure 1. a) UV–vis spectra, b) TEM images and c) Energy dispersive X-ray spectroscopy (EDAX) spectrum of TNEDS from different stages. d) Hydrodynamic size distribution of Au@SiO₂-Dox-CS-FA determined by dynamic light scattering (DLS), e) ζ potential changes, and f) SERS spectral pattern analysis of Au@SiO₂-Dox-CS-FA compared with pure Dox spectra (Au@Dox). Results are plotted as the mean ± standard deviation (SD), n = 4.
exhibited an optimal Dox release kinetics (Figure 2a) which is largely dependent on pH and, to a lesser extent, toward reducing atmosphere. A cumulative Dox release kinetics was observed with a pH of 5 wherein almost 96% and 89% was released within 9 h in the presence and absence of reducing agents. The Dox release was further evaluated in the SERS...
2.4. SERS Imaging of Live Cells Using Au@SiO₂-CS-FA

As a new insight, we explored the efficiency of Au@SiO₂-CS-FA as an SERS nanotag on HeLa cells (Figure 2c–f) with the aid of a confocal Raman microscope (WI-Tec, Inc., Germany). HeLa cells upon treatment with Au@SiO₂-CS-FA for 1 h revealed a well-defined Raman image (Figure 2d) with a proper clustering pattern (Figure 2e). 3D representations of the images clearly indicated the enhanced cellular information in contrast with the surroundings (Figure 2f,g). Condor images and histogram of the relative intensity of the two most intense peaks from the NP-treated cells deciphered the significant signal enhancement (Figure 2 h,i). Raman spectra (Figure 2j; Figure S5a–g, Supporting Information) abstracted at various regions of the Raman image exhibited characteristic fingerprint SERS peaks from subcellular locations such as cell membrane, cytoplasm, nucleus, and other cellular organelles (Table S2, Supporting Information). SERS imaging of live cells is a new arena involving intensive study utilizing the high SPR of NPs and among which silica-coated AuNPs are reported to be excellent SERS substrates with uniform hot spot generation.[21] The hypercluster analysis from the strongest Raman bands of the HeLa cells clearly provided label-free methods for the visualization of intracellular components and processes based on strong Raman signals.[22] SERS spectra obtained from the nuclear regions are mainly characterized with regions including 1487, 1510, and 1534 cm⁻¹ which corresponds to nucleic acids whereas the spectra obtained from the cytoplasmic region bears signatures from ribose vibration of RNA (917 cm⁻¹), carotenoids (1006 cm⁻¹), C–C stretching of lipids, and carbohydrates (1060 cm⁻¹), C=C stretching of proteins (1615 cm⁻¹), and many more.[23] The distinct between 3D spectra and cluster analysis obtained using Au@SiO₂-CS-FA could be a promising step to understand the physicochemical processes in the local cellular environment in real time, in order to benefit for specific intracellular targeting.
2.5. Evaluation of Cytotoxicity and FR Inhibition

Cytotoxicity of the prepared nanoconstructs along with free Dox and Lipodox was evaluated on FR-positive and negative cell lines. Au@SiO₂-Dox-CS-FA displayed preferential cytotoxicity toward human cervical (HeLa) and ovarian cancer (SKOV3) cells that overexpress FR, but was less toxic toward human lung adenocarcinoma (A549) and murine fibroblasts (3T3L1) with minimal FR expression (Table S3, Supporting Information). Cytotoxicity of Au@SiO₂-Dox-CS-FA toward HeLa cells was less pronounced than Dox and Lipodox but was efficient than Au@SiO₂-Dox-CS (Figures S7a and S8a,b of the Supporting Information). In the case of SKOV3 cells, Au@SiO₂-Dox-CS-FA demonstrated an enhanced cytotoxic potential than Lipodox and Au@SiO₂-Dox-CS, but less efficient than Dox (Figures S7b and S8c,d of the Supporting Information). The TNEDS demonstrated least toxicity on A549 (Figures S7c and S8e,f of the Supporting Information) and 3T3L1 (Figures S7d and S8g,h of the Supporting Information) cells. Furthermore, both the carriers Au@SiO₂ (Table S4, Supporting Information) and Au@SiO₂-CS-FA (Table S5, Supporting Information) were observed to be devoid of any significant toxicity toward either FR-positive or FR-negative cells. The FR-targeted cytotoxicity demonstrated by Au@SiO₂-Dox-CS-FA was further confirmed after preincubation with folic acid wherein a dramatic decrease in the cytotoxicity toward HeLa and SKOV3 cells (Figure S7f,c, Supporting Information) and no significant alteration in FR-negative cell lines A549 and 3T3L1 (Figure S7g,h, Supporting Information) were observed, validating the FR-targeting capacity of Au@SiO₂-Dox-CS-FA. As an alternative, the targeted cytotoxic behavior displayed by Au@SiO₂-Dox-CS-FA was confirmed by 5-bromo-2'-deoxyuridine (BrDU) assay, wherein all the agents demonstrated a similar cytotoxicity profile as observed with 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay (Figure S9a-d, Supporting Information). The indiscriminate toxicity exhibited by Dox toward normal cells is well documented whereas the liposomal Dox–NP formulations such as Doxil and Lipodox are relatively safer toward normal cells[24] and are more effective than Dox for in vivo applications, yet they are still not effective toward targeted delivery approaches.

2.6. Investigation of Cellular Uptake Pathways and Intracellular Dox Release by Fluorescence Modality

Cellular uptake and intracellular release of Dox from Au@SiO₂-Dox-CS-FA were compared with naked Dox, Lipodox, and Au@SiO₂-Dox-CS on HeLa and A549 cells by fluorescence measurements. Among them, Dox uptake was maximum in Au@SiO₂-Dox-CS-FA and minimum in Au@SiO₂-Dox-CS on HeLa cells (Figure 3a,b). However, in the case of FR-negative A549 cells, TNEDS displayed minimal Dox uptake wherein the naked Dox displayed the maximal effect (Figure 3b: Figure S10, Supporting Information). Dox was found co-localized with Hoechst indicating the nuclear transport. The FR-mediated cellular uptake of Au@SiO₂-Dox-CS-FA was further confirmed by flow cytometry wherein HeLa cells displayed an enhanced Dox internalization but A549 cells were minimally responsive. Further, preincubation of cells with external supply of folic acid has no effect on A549 cells whereas HeLa cells were severely affected (Figure S11a,b, Supporting Information) with significantly (p < 0.01) reduced Dox uptake. Since positively charged nanoconstructs are reported to follow receptor-mediated uptake pathways, the cellular uptake mechanism of Au@SiO₂-Dox-CS-FA was investigated fluorimetrically after preincubation of cells with specific inhibitors and folic acid (Figure 3c). Cellular endocytosis is used for uptake nutrients and growth factors, and plays as a master regulator of the signaling circuitry. In order to examine the mode of cellular uptake mechanism of our TNEDS, the Dox concentration was estimated in FR over expressed cells preincubated with specific endocytosis inhibitors. Estimation of Dox uptake displayed a significant (p < 0.001) reduction upon treatment with genistein (100 µg mL⁻¹), chlorpromazine (20 µg mL⁻¹), and folic acid (2 × 10⁻³ m) but was not affected with 5-(N-ethyl-N-isopropyl)-amiloride (10 µg mL⁻¹) upon comparison with the respective controls. Moreover, cellular uptake was visualized using a fluorescent microscope which again confirmed the highly FR-selective and efficient cellular Dox uptake by Au@SiO₂-Dox-CS-FA (Figure 3d). The released Dox was perfectly localized in the nuclear region for effective therapeutic utility within 4 h of administration. Functional biomolecules on the surface, size, and morphology of nanoconstruct play a predominant role in determining the probable mechanism of internalization, which are, namely, clathrin, caveolae, micropinocytosis, and phagocytosis.[25] Endocytosis pathway determination using specific inhibitors revealed that FR-mediated caveolae- and clathrin-dependent pathways play a prominent role in the cellular uptake of Au@SiO₂-Dox-CS-FA whereas micropinocytosis has a little effect. Cationic AuNPs around 100 nm are reported to follow receptor-mediated endocytosis mainly through caveolae, which are flask shaped imaginations of the plasma membrane coated by caveolin-1 and, to a lesser extent, by clathrin-dependent pathways.[26]

2.7. Cellular Internalization and Dox Release by SERS Imaging

Again, we have explored the cellular internalization and Dox release kinetics from TNEDS by Raman spectral and imaging analysis. Bright field (Figure 4a) and Raman mapping for Dox localization were observed within 1 h (Figure 4b) and 3 h (Figure 4c) of incubation of Au@SiO₂-Dox-CS-FA in HeLa cells. Condor images and histogram (Figure S12a-c, Supporting Information) from HeLa cells showed the enhanced Raman signal. Raman spectra (Figure 4d; Figure S13a-d, Supporting Information) abstracted from nucleus and cytoplasmic regions after 1 h of treatment clearly demonstrated the signature Dox peak around 455 cm⁻¹ only from the cytoplasmic area. However, after 3 h, the integrated Dox peak from the nucleus and the absence from the cytoplasmic region were detected (Table S6, Supporting Information). The ability of TNEDS to retain and enhance Dox signature enabled real-time monitoring for intracellular Dox release kinetics. SERS technique has recently been used for
Figure 3. a) Evaluation of the intracellular uptake and distribution of Dox in HeLa cells after 4 h of administration ($1 \times 10^{-6}$ M) using fluorescence microscopy. The legend [1] represents Au@SiO$_2$-Dox-CS-FA; [2] Au@SiO$_2$-Dox-CS, [3] Dox, and [4] Lipodox. b) Quantitation of Dox uptake fluorimetrically. c) Estimation of the effect of inhibitors on the uptake of Au@SiO$_2$-Dox-CS-FA on HeLa cells by fluorimetry. d) Fluorescence images of Au@SiO$_2$-Dox-CS-FA uptake on HeLa cells with inhibitors represented by the following legends: [1], vehicle control; [2], folic acid; [3], genistein; [4], chlorpromazine; and [5], amiloride. The first panel represents bright field; the second panel DAPI; the third panel propidium iodide (PI), and the fourth panel indicates the merged images. Scale bar: 50 µm. Data are the mean ± SD of three independent experiments; ***$p < 0.001$; ns: not significant relative to control.
label-free in situ monitoring of drug release from a variety of carrier constructs. The Dox Raman peaks from the cells effectively enabled tracking of the intracellular drug distribution pathway\textsuperscript{[27]} in a noninvasive manner. Raman imaging and corresponding spectral analysis of Dox demonstrated a time-dependent localization with an early distribution in the
cytoplasm and later migration toward the nucleus. Disappearance of the Dox peak at 455 cm$^{-1}$ from the cytoplasm and appearance of the same in the nuclear region over time clearly defined the Dox distribution (Figure 4e).

2.8. Evaluation of Apoptotic Events by SERS Fingerprinting

Raman spectral analysis from HeLa cells in a time-resolved manner after the treatment of Au@SiO$_2$-Dox-CS-Fa revealed characteristic biochemical changes (Figure 4e). The subcellular SERS spectral patterns are well characterized with the dominant Dox signatures with unique Raman peaks corresponding to the nuclear damage due to apoptosis (Table S7, Supporting Information). Furthermore, monitoring of SERS spectra from the cytoplasmic region over a time period of 1–4 h showed the disappearance of Dox peak over time (Figure S14, Supporting Information). The subcellular SERS spectral patterns are well characterized with the dominant Dox signatures with unique Raman peaks corresponding to the nuclear damage due to apoptosis (Table S7, Supporting Information). Although the initiation of apoptosis by Dox from Au@SiO$_2$-Dox-CS-Fa has been established with SERS spectral analysis, a time-dependent uptake study was performed by fluorescence imaging (Figure 4f) in order to confirm the apoptotic event by the TNEDS. Interestingly, Dox was distributed in the cytoplasmic area within 2 h and slowly migrated into nucleus after 3 h. Nuclear accumulation was observed after 4 h with no cytoplasmic fluorescence that rationalizes the SERS studies. In addition to the effective monitoring of drug release, time-resolved SERS spectra have been well explored to monitor cellular changes in response to chemotherapeutic agents demonstrating a more sensitive and faster detection modality than conventional assays.[28] The disappearance of cellular peaks at 846 cm$^{-1}$ and decrease in the intensity of peaks at 1250–1350 and 1450–1650 cm$^{-1}$ were assigned to the nucleic acids, O–P–O backbone of DNA, and protein degradation upon treatment of Au@SiO$_2$-Dox-CS-Fa which are consistent with the expected Dox-induced biochemical changes in the nucleus. We have previously reported the effective real-time monitoring of DNA backbone breakage and the corresponding nuclear degradation upon treatment with a Dox-loaded nanoprobe using differential SERS spectral analysis.[30] Thus, it is evident that apoptotic DNA fragmentation induced by Au@SiO$_2$-Dox-CS-Fa can be effectively monitored through SERS, which enabled label-free monitoring and could further potentiate the therapeutic utility.

2.9. Investigation of Cell Death Mechanism

In order to validate the apoptosis-mediated growth inhibition by TNEDS reflected by molecular changes in SERS fingerprinting, various apoptosis assays were conducted with HeLa, SKOV3, and A549 cells. Morphological observation revealed salient features of apoptosis such as distorted shape, membrane blebbing, and decreased cell number relative to control groups for HeLa (Figure 5a, upper panel) and SKOV3 cells (Figure S15a (upper panel), Supporting Information).

2.10. Therapeutic Efficacy on Murine Models

The excellent in vitro targeted cytotoxicity urged us to investigate the in vivo antitumor therapeutic potential of the TNEDS. Evaluation of various parameters on DLA ascites tumor-bearing mice on days 16 and 23 of administration revealed a significant reduction in the tumor volume ($p < 0.001$) in both groups administered with Au@SiO$_2$-Dox-CS-Fa, Au@SiO$_2$-Dox-CS, Dox, and Lipodox (Figure 6a), except Au@SiO$_2$-CS-Fa, which did not elicit any response. Among the various treatment options, Au@SiO$_2$-Dox-CS-Fa generated the best overall response (Figure S16a, Supporting Information). Detailed investigation with tumor cell count, percentage of viable cells, body weight change, and mean survival at the end of the experimental period (Tables S9 and S10, Supporting Information) revealed the superior therapeutic efficacy of Au@SiO$_2$-Dox-CS-Fa over other treatment options. The Kaplan–Meier survival analysis (Figure 6b,c) revealed that Au@SiO$_2$-Dox-CS-Fa treatment prolonged the survival probability significantly ($p < 0.001$). There was a significant reduction in the tumor burden by both intraperitoneal (i.p.) and intratumoral (i.t.) administration of Au@SiO$_2$-Dox-CS-Fa in EAC-induced solid-tumor mice syngraft where i.p. (Figure 6d; Table S11, Supporting Information) mode was observed to be less effective than i.t. mode (Figure 6e; Table S12, Supporting Information). Although...
Lipodox was observed to be more effective than naked Dox, the nontargeted construct Au@SiO₂-Dox-CS produced better therapeutic efficacy than Dox with i.t. administration. The nanoconstructs demonstrated a maximum reduction in tumor volume and a significant \( p < 0.001 \) increase in life span (Figure S16b, Supporting Information). Furthermore, the

Figure 5. Induction of apoptosis in a) HeLa and b) A549 cells by Au@SiO₂-Dox-CS-FA \( (1 \times 10^{-6} \text{m}) \) for 24 h, and the cells were evaluated using phase contrast microscopy (upper panel), acridine orange-ethidium bromide (middle panel), and Hoechst staining (lower panel). The left panel represents the control and the right panel indicates the treatment groups. Evaluation of apoptosis by TUNEL staining assay in c) HeLa and d) A549 cells; the left row represents the control and the right row indicates the treatment groups. Scale bar: 50 \( \mu \text{m} \). e) Caspase profiling of cell lines treated with Au@SiO₂-Dox-CS-FA. Data are the mean \( \pm \) SD of three independent experiments; ***\( p < 0.001 \), **\( p < 0.01 \); ns: not significant relative to control.
Kaplan–Meier analysis (Figure 6f,g) exposed the prolonged survival of tumor-bearing mice with Au@SiO2-Dox-CS-FA administration up to a level for making it a therapeutically viable construct. Representative images of expunged tumors with different treatments (Figure 6h) revealed the greater tumor volume reduction upon Au@SiO2-Dox-CS-FA
treatment. This superior therapeutic effect could be due to the optimal drug loading and targeting efficiency of the TNEDS for more intensive localized applications without causing any nonspecific toxicity, especially in the case of easily accessible solid malignancies and potentiates the future preclinical investigations. The efficiency of tumor targeting by Au@SiO2-FL-CS-FA was notable, particularly considering that the mice used in this study were fed with a regular diet rather than a folate-free diet, which is usually necessary to increase the detection sensitivity of the FR-targeted delivery systems.[31]

3. Conclusions

In summary, the novel TNEDS explored in this study not only demonstrated excellent in vitro FR-targeted cytotoxicity but also presented to be an appalling biocompatible targeted nanocarrier delivery construct. High Dox loading with tumor niche specific release kinetics enabled the TNEDS to execute superior salutary therapeutic effects. In diagnostic modality Au@SiO2 core transformed TNEDS to be an effective SERS nanoprobe for Raman imaging to monitor the cellular uptake, release of the impregnated Dox, and dynamic visualization of biochemical changes at molecular level during apoptosis. Finally, the TNEDS was explored in mouse models that explicitly showed greater therapeutic efficiency superior to the clinically used Dox and Lipodox. Albeit further investigations are justified, the outcome exhibited by the TNEDS definitely holds an aspiration for future preclinical and clinical applications in oncology.

4. Experimental Section

Materials: Gold (III) chloride hydrate, trisodium citrate dehydrate, tetraethyl orthosilicate (TEOS), aqueous ammonia (NH₂OH, 25–28%), hydrochloric acid (HCl, 36–38%), fluorescein isothiocyanate (FITC), (3-aminopropyl) triethoxysilane (APTES), MT, and doxorubicin hydrochloride were purchased from Sigma Aldrich. Acetic acid (CH₃COOH, 99.5% purity), chitosan, and 85% deacetylated power were purchased from Alfa aesar. Ethyl alcohol (99.9% purity) was purchased from Changshu Yangyuan Chemical, China. Lipodox was generously gifted by Sun Pharma Advanced Research Company Ltd, India.

TEOS-Mediated Synthesis of Au@SiO2 Nanoparticles: The synthesis of spherical citrate-stabilized gold nanoparticles of 40 nm was done using the citrate reduction method.[32] Mono-dispersed Au@SiO2 nanoparticles with 40 nm gold cores and uniform thicknesses of silica shell were successfully synthesized via the modified Stober method[33] using vigorous shaking in alcohol solutions. Briefly, 50 mL of citrate stabilized gold nanoparticle was centrifuged using 8000 rpm for 30 min, and the pellets were dissolved in 5 mL of Milli-Q water. The solution was diluted with 15 mL of ethanol solution and adjusted the pH to 10 using ammonia solutions. To this solution, 48 µL of 1:45 3-isocyanato propyl trimethoxy silane (NCO)-TEOS:TEOS was added and shaken for 24 h. After 24 h, the solution was centrifuged two or three times with 10 000 rpm for 30 min to remove uncoated silica on AuNPs.

Conjugation of Folic Acid with Chitosan: A solution of folic acid and 1-ethyl-3-(3-dimethylaminopropyl) carbo diimide (EDC) in 20 mL of anhydrous dimethyl sulfoxide (DMSO) was prepared and stirred at room temperature until EDC and folic acid were well dissolved.[34] The mixture was then added slowly to 0.5% (w/v) chitosan in the aqueous solution of acetic acid and stirred at room temperature in the dark for 16 h to let folic acid conjugate onto chitosan molecules (Figure S1, Supporting Information). The solution was adjusted to pH 9.0 by adding NaOH aqueous solution (1.0 m) and centrifuged at 2500 rpm. The precipitate was dialyzed first against phosphate buffered saline (PBS, pH 7.4) for 3 d and then against water for 4 d. NMR spectra were recorded on the Bruker Advance 500 NMR spectrometer, and chemical shifts are expressed in parts per million.

Synthesis of Chitosan–Folic Acid (CS-FA)-Coated Au@SiO2 Nanoparticle: Chitosan–folic acid conjugate (25 mg) was dissolved in 5 mL of 3% acetic acid, and the suspension was stirred at 600 rpm for 24 h to form CS-FA solution (0.5% w/v). About 5 mL of Au@SiO2 nanoparticles was centrifuged and made a solution in 5 mL of ethyl alcohol, and the dispersion was adjusted to pH 3.5–4.5 by adding acetic acid. Subsequently, 200 µL of APTES was added to modify Au@SiO2 nanoparticle and was reacted with silanol groups on the SiO2 surface to form Si–O–Si bonds. Then CS-FA solution was added to accomplish cross-linking of nanoparticle and allowed to stir at room temperature for 24 h. The CS-FA-coated Au@SiO2 (Au@SiO2-CS-FA) was collected by centrifugation at 10 000 rpm, followed by washing with excessive distilled water and ethyl alcohol solution.

Doxorubicin Loading and Redox and pH-Responsive Dox Release Kinetics of Au@SiO2-Dox-CS-FA NPs: The detailed procedure and the DLE, DLC,[35] and actual amount of drug in nanoparticle calculated are shown in Section SI-2 of the Supporting Information. The release of Dox from Au@SiO2-Dox-CS-FA, Lipodox, and doxorubicin hydrochloride under various pH and redox conditions was evaluated as reported elsewhere[36] and detailed in Section SI-3 of the Supporting Information.

Culture and Maintenance of Cell lines: Human cancer cell lines HeLa (cervical cancer), MCF-7 (breast cancer), and A549 (lung adenocarcinoma) were obtained from American Type Culture Collection (Manassas, USA). SKOV3 (ovarian cancer) cells were generously provided by the Rajiv Gandhi Centre for Biotechnology (Thiruvananthapuram, India). The fibroblast-like murine preadipocyte cell line 3T3L1 was gifted from the Inter-University Centre for Genomics and Gene Technology, University of Kerala (Thiruvananthapuram, India). A375 (malignant melanoma) cells were obtained from NCCS (Pune, India). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 5% CO₂ at 37 °C. Source and maintenance of murine cancer cell lines are described in Section SI-4 of the Supporting Information. Animal experiments were performed according to the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines and was approved by the Institutional Animal Ethics Committee (IAEC) of the Regional Cancer Centre (Accreditation number: 657/Go/Re/02/CPCSEA), Trivandrum, India.

Cytotoxicity Assays: The growth inhibitory capacity was initially evaluated on cancer and normal cell lines by MTT assay as previously reported.[17] The absorbance was measured at 570 nm using a microplate spectrophotometer (BioTek, Power Wave XS) after incubation for 12, 24, and 48 h with test compounds. The cytotoxicity
was further confirmed with BrdU assay kit (colorimetric – 1164729001, Roche Diagnostics, IN, USA), and the experiments were carried out with the instructions given in the kit and the measurements were made at 450/690 nm. Evaluation of the effect of folic acid on the growth inhibitory capacity of the constructs was also carried out by MTT assay after the addition of 2 × 10^{-3} \text{M} folic acid to the medium for 2 h as reported.[31]

**Western Blot Analysis and Hemolysis assay:** The expression status of folate receptors in the cell lines was evaluated using western blot analysis using FOLR2 (folate receptor b) primary antibodies and β-actin as the reference control. The resulting bands were then quantitated using Image J software (version 1.48, NIH, USA), normalized with β-actin and the experimental procedure was detailed in Section SI-5 of the Supporting Information. Furthermore, the biocompatibility of the constructs against red blood cells was evaluated using hemolysis assay using 2% Triton X-100 and PBS as positive and negative controls, respectively, and was detailed in Section SI-6 of the Supporting Information.

**Apoptotic Assays:** Morphological assessment of apoptosis was conducted in HeLa, SKOV3, and A549 cells treated with Au@SiO\textsubscript{2}-Dox-CS-FA (1 × 10^{-3} \text{M}) for 24 h. Initially, cells were observed for any visible gross morphological changes under phase contrast objective (Olympus 1 × 51, Singapore) to view the apoptotic or nonapoptotic cells. Acridine orange–ethidium bromide dual staining was performed as described earlier,[37] and the cells were observed under an FITC filter (Olympus 1 × 51, Singapore). Observation of nuclei for any apoptosis related changes was done with Hoechst 33342 staining using a 4′,6-diamidino-2-phenylindole (DAPI) filter (Olympus 1 × 51, Singapore) as described before. TUNEL assay (DeadEnd Fluorometric TUNEL system, Promega, Madison, WI, USA) was used to detect the incorporation of the fluorescein-12-dUTP in the fragmented DNA of apoptotic cells, using the terminal-deoxynucleotidyl-transferase recombinant enzyme as per the manufacturer's instructions using propidium iodide as counterstain. Caspase profiling was determined by using ApoAlert Caspase Profiling kit (Clontech, CA, USA) as per the manufacturer's protocol. Cells were treated with Au@SiO\textsubscript{2}-Dox-CS-FA (1 × 10^{-3} \text{M}) for 24 h, and samples were transferred to 96-well plates for fluorometric reading (λ\textsubscript{ex} = 380 nm; λ\textsubscript{em} = 460 nm), and signals were recorded by spectrofluorimetry (FLX800, BioTek).

**Live Cell Raman Imaging:** Evaluating the efficacy of Au@SiO\textsubscript{2}-CS-FA as a Raman substrate was done with the aid of a confocal Raman microscope (Wi-Tec, Inc., Germany) with a laser beam directed to the sample through 20× objective with a Peltier cooled CCD detector. For cellular imaging, 20 μL (1 × 10^{-3} \text{M}) of Au@SiO\textsubscript{2}-CS-FA was added to HeLa cells and was incubated at 37 °C for 1 h. SERS mapping was recorded by focusing the laser beam on the cell surface selected at a position z = 0 μm using 0.5 as the integration time, 150 × 150 as points per line, and 50 × 50 μm mapping area along the X and Y directions. The Raman and SERS cell maps were acquired over a motorized scan stage. The chemical images were computed from the 2D collection of Raman/SERS spectra by integrating the intensity of a specific band over a defined wave number range after baseline subtraction. Raman images were subsequently subjected to cluster mapping and later 3D plots were made out of it. A minimum of three independent measurements were made for each sample.

**Cellular Internalization Study:** Cellular uptake and release of Dox or FITC were tested with various constructs with fluorescent microscopy, Raman microscopy, fluorimeter, and flow cytometer. HeLa and A549 cells were treated with (1 × 10^{-6} \text{M}) Dox, Lipodox, Au@SiO\textsubscript{2}-Dox-CS, and Au@SiO\textsubscript{2}-Dox-CS-FA for 4 h (Section SI-7, Supporting Information).

**Nanoparticle Uptake Pathway Determination:** Pharmacological inhibitors were employed to determine the cellular uptake pathways responsible for cellular uptake and transport of positively charged Au@SiO\textsubscript{2}-Dox-CS-FA nanoparticles, and the experiments were performed as previously described.[37] HeLa cell monolayers were incubated with previously optimized doses of endocytosis inhibitors, and the detailed procedures are given in Section SI-8 of the Supporting Information.

**Evaluation of Dox Uptake and Apoptosis Using SERS:** Since Au@SiO\textsubscript{2}-Dox-CS-FA could generate sufficiently higher intensity Dox fingerprints and enabled cellular imaging in the SERS platform, it was used for tracing Dox release using a confocal Raman microscope, and the cellular imaging was performed as explained above. The specific Raman band area of Dox was selected to create an SERS maps after 1 and 3 h of Au@SiO\textsubscript{2}-Dox-CS-FA treatment. The intensity of the SERS spectra was measured at different spots belonging to the nuclear and cytoplasmic regions, and the spectra were averaged to create every single curve.[38] The cells were maintained for up to 6 h, and SERS spectra were extracted from the nuclear area for every 30 min for identification of change in spectral pattern over time with Dox exposure.

**Tumor Reduction Studies on Ascites and Solid-Tumor Mice:** Female BALB/c mice were maintained in well-ventilated cages with free access to normal mouse food and water. Temperature (25 ± 2 °C) and humidity (50% ± 5%) were regulated and the illumination cycle was set to 12 h light/dark. Experimental design with DLA ascites tumor was illustrated (figure S17, Supporting Information). Tumor volume, mean survival time, and percentage of increment in life span were calculated as previously reported (Section SI-9a, Supporting Information). Experiments with EAC–solid-tumor mice synergia were performed by two different routes: i.p. and i.t. injections, daily for 14 consecutive days, starting on day 9 after tumor inoculation (days 9–22 as shown in Figure S17 (Group 3), Supporting Information). Animals were sacrificed on day 23 to determine tumor volume and overall survival (n = 6 per subgroup).

The radii of the developing tumors were measured every third day from day 7 to day 31 using Vernier calipers, and the tumor volume was estimated using the following formula: \( V = \frac{4}{3} \pi r_1^2 r_2 \), where \( r_1 \) and \( r_2 \) represent the radii from two[37,39] and was described in Section SI-9b of the Supporting Information.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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