

Article

Surface-Directed Regulation of Intracellular Trafficking and Apoptotic Pathways by Fluorescent SBA-15 Nanocarriers Governs the Intracellular Fate of Emodin

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Abstract

Emodin (EO) is a naturally occurring anthraquinone with promising anticancer activity; however, its clinical application is limited by poor aqueous solubility and low bioavailability. Herein, we demonstrate that surface engineering of fluorescent SBA-15 nanocarriers governs the intracellular fate of EO by modulating drug confinement, release behavior, intracellular trafficking, and apoptotic responses. SBA-15 was functionalized with aminopropyl groups and a fluorescent moiety to generate traceable nanocarriers with distinct drug–carrier interactions. Physicochemical characterization (SAXS, BET, SEM/TEM, TGA, UV–Vis, and fluorescence spectroscopy) confirmed the preservation of the ordered mesoporous structure and efficient EO encapsulation (~35%). In PC3 prostate cancer cells, the functionalized system (SBA-15-M|EO) exhibited enhanced cytotoxicity ($IC_{50} = 18.2 \mu M$) compared to free EO and the non-functionalized carrier. Time-lapse fluorescence microscopy demonstrated efficient cellular uptake, perinuclear accumulation, and sustained intracellular release, whereas flow cytometry revealed distinct apoptotic responses. SBA-15|EO promoted rapid intracellular EO accumulation and extensive late apoptosis, while SBA-15-M|EO induced gradual intracellular accumulation accompanied by delayed but sustained apoptosis. Molecular docking further revealed favorable interactions of EO with the anti-apoptotic proteins BCL-2 and BCL-xL ($\Delta G_{bind} \approx -6.5 \text{ kcal mol}^{-1}$). These findings establish a direct relationship between nanocarrier surface chemistry, intracellular trafficking, and apoptotic fate, highlighting surface engineering as a strategy for controlling cellular responses beyond conventional drug delivery.

Keywords: emodin; SBA-15; mesoporous silica; surface functionalization; controlled release; anticancer activity; fluorescent nanocarriers

Academic Editor: James C. L. Chow

Received: 27 June 2026

Revised: 17 July 2026

Accepted: 22 July 2026

Published: 23 July 2026

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1. Introduction

Emodin (EO), a natural anthraquinone derivative present in numerous medicinal plants, has attracted considerable attention owing to its broad spectrum of biological activities, particularly its pronounced anticancer potential [1,2]. Previous studies have demonstrated that EO can induce apoptosis, inhibit cellular proliferation, and modulate multiple signaling pathways involved in tumor progression, including prostate cancer [3–5]. EO exerts its anticancer activity through multiple complementary pathways. It has been reported to induce mitochondrial apoptosis by increasing the Bax/BCL-2 ratio, promoting cytochrome C release, and activating caspases 9 and 3. In addition, EO suppresses several oncogenic signaling pathways, including PI3K/Akt, MAPK, and Notch, leading to the inhibition of cell proliferation and survival. Depending on the cellular context, EO may also modulate intracellular reactive oxygen species (ROS) homeostasis, thereby contributing to apoptosis and cell cycle arrest. These multimodal mechanisms make EO an attractive candidate for nanocarrier-mediated cancer therapy [6,7]. Nevertheless, current therapeutic strategies for prostate cancer remain challenged by treatment resistance, limited selectivity, and undesirable side effects [8,9]. In this context, EO has emerged as a promising bioactive molecule capable of suppressing prostate cancer (PC3) cell growth and interfering with pathways associated with tumor cell survival [10]. However, despite its pharmacological potential, the clinical translation of EO is severely hampered by poor aqueous solubility, limited bioavailability, and off-target toxicity toward healthy tissues [11].

Nanotechnology-based delivery systems have been extensively explored as a strategy to overcome these limitations by enhancing drug stability, improving intracellular accumulation, and enabling the controlled release of bioactive compounds [12,13]. Among the available nanocarriers, mesoporous silica nanoparticles (MSNs) have attracted particular interest owing to their high surface area, tunable pore architecture, chemical stability, excellent biocompatibility, and high drug-loading capacity [14]. SBA-15 is one of the most structurally well-defined mesoporous silica materials, characterized by an ordered hexagonal mesoporous architecture, a narrow pore-size distribution, and thick silica walls that provide exceptional structural robustness and loading capacity [15]. Beyond serving as an efficient drug reservoir, SBA-15-based nanocarriers can improve anticancer drug delivery by promoting passive accumulation within tumors through the enhanced permeability and retention (EPR) effect. Following accumulation in the tumor microenvironment, they are internalized by cancer cells through endocytosis pathways, enabling intracellular drug release. Moreover, surface functionalization, for example, with aminopropyl groups or polymers, further enhances their biomedical applicability by modulating drug-carrier interactions, colloidal stability, cellular uptake, and intracellular trafficking, thereby potentially improving therapeutic efficacy [16,17]. Consequently, SBA-15 has emerged as a versatile platform for the delivery of natural bioactive compounds, including anthraquinones such as EO, providing sustained drug release and offering opportunities to modulate intracellular drug fate through rational surface engineering. However, most previous studies have primarily focused on drug-loading efficiency, release kinetics, or *in vitro* cytotoxicity, whereas considerably less attention has been devoted to understanding how surface functionalization governs intracellular trafficking, intracellular drug fate, and the subsequent biological response. Moreover, studies specifically addressing emodin-loaded SBA-15 systems remain relatively scarce, and the mechanistic relationship between surface chemistry and apoptosis-related cellular behavior has not yet been systematically investigated [18,19].

While MSNs are traditionally viewed as passive drug reservoirs, increasing evidence suggests that nanocarrier surface chemistry plays a much broader role in determining biological performance [18]. Surface functionalization can alter drug confinement within

mesopores, regulate release kinetics, influence nanoparticle–cell interactions, and ultimately affect intracellular trafficking pathways [18]. Furthermore, the incorporation of fluorescent moieties enables the direct visualization of nanocarrier uptake and intracellular behavior, providing valuable mechanistic information regarding the relationship between nanocarrier design and cellular response [19]. Despite substantial progress in the development of MSN-based drug delivery systems, the extent to which surface functionalization governs intracellular drug fate and modulates downstream biological processes remains insufficiently understood.

Particularly important is the possibility that surface-engineered nanocarriers may regulate not only uptake kinetics and the amount of drug delivered to cells, but also the temporal profile of intracellular exposure. Such differences may directly influence reactive oxygen species (ROS) generation, apoptosis induction, and the overall kinetics of cell death. Consequently, understanding the relationship between nanocarrier surface properties, intracellular drug distribution, and cellular response is essential for the rational design of next-generation therapeutic platforms.

In the present study, we hypothesize that rational surface engineering of SBA-15 nanocarriers can regulate intracellular EO availability and thereby modulate ROS dynamics and apoptotic responses in cancer cells. To test this hypothesis, SBA-15 was functionalized with aminopropyl groups and subsequently modified with a fluorescent moiety to obtain traceable nanocarriers capable of establishing distinct drug–carrier interactions. Following EO loading, comprehensive physicochemical characterization and biological evaluation in PC3 prostate cancer cells were performed to establish direct relationships between surface functionalization, intracellular trafficking, release behavior, and biological response. In addition, molecular docking studies were carried out to investigate the interactions of EO with the apoptosis-related proteins B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra-large (Bcl-xL), providing complementary mechanistic insight into the observed cellular effects. Overall, this work explores how surface-directed engineering of mesoporous silica nanocarriers can be employed to modulate intracellular drug fate and apoptotic pathways, thereby contributing to the development of more effective nanomedicine platforms.

2. Materials and Methods

2.1. Synthesis of SBA-15

The mesoporous silica SBA-15 was synthesized according to previously reported procedures [20,21] with slight modifications. Briefly, Pluronic P123 (Sigma, St. Louis, MO, USA) (24.20 g) was dissolved in deionized water (180 mL) and 2 M HCl (697.17 mL) at 35 °C from Merck Group (Darmstadt, Germany) under continuous stirring until a clear solution was obtained. TEOS (tetraethyl orthosilicate, Sigma, St. Louis, MO, USA) was then added dropwise under vigorous stirring to avoid excessive foaming. The resulting mixture was stirred at 35 °C for 24 h to allow micelle formation and initial hydrolysis of the silica precursor. Subsequently, the reaction mixture was heated to 80 °C and maintained under stirring for an additional 24 h to promote condensation of the silica framework. The resulting solid product was collected by filtration, washed thoroughly with deionized water until no foaming was observed, and then washed with ethanol. The obtained material was air-dried and subsequently calcined in a muffle furnace at 500 °C for 24 h (heating rate 1 K/min) to remove the structure-directing agent.

2.2. Preparation of SBA-15-Based Nanomaterials

The SBA-15-based nanomaterials were prepared through a sequential post-synthetic modification strategy. A summary of the composition and designation of all SBA-15-based

nanomaterials investigated in this study is provided in Supplementary Table S1. SBA-15 served as the parent material for all subsequent modifications. First, SBA-15 was functionalized with 3-(triethoxysilyl)propylamine to obtain SBA-15-NH₂, which was subsequently modified with a fluorescent moiety to produce SBA-15-M. Finally, EO was loaded into both pristine SBA-15 and SBA-15-M, yielding the two final formulations, SBA-15|EO and SBA-15-M|EO, used for physicochemical characterization and biological evaluation.

Before each functionalization step, SBA-15 was dehydroxylated under high vacuum (10⁻² mmHg) at 150 °C for 7 h, allowed to cool to room temperature, and stored under dry nitrogen.

SBA-15~NH₂ (Aminopropyl-functionalized SBA-15): Functionalization of SBA-15 with (3-aminopropyl)triethoxysilane was performed following a published procedure [22]. Activated SBA-15 (2.00 g) was further dried at 150 °C for 6 h under vacuum and cooled to room temperature. (EtO)₃Si(CH₂)₃NH₂ (3 mL, 12.69 mmol) and anhydrous CH₂Cl₂ (30 mL) were added, and the suspension was shaken at 65 °C for 60 h. The solid was isolated by filtration and washed sequentially with toluene (3 × 10 mL) and n-pentane (3 × 10 mL). Successful amine functionalization (SBA-15~NH₂) was confirmed by a positive ninhydrin test.

SBA-15-M (Fluorophore-modified SBA-15): Modification of SBA-15~NH₂ with N-methylisatoic anhydride (M) was carried out according to literature methods [22,23]. Activated SBA-15 (1.21 g) was dried at 150 °C for 6 h under vacuum. After cooling, M (0.06 g, 0.29 mmol) and toluene (40.00 mL) were added. The suspension was shaken at 65 °C for 72 h. The product (SBA-15-M) was recovered by filtration and washed with toluene (3 × 10 mL) and n-pentane (3 × 10 mL). The material was dried under vacuum at room temperature for 16 h to obtain a free-flowing white powder.

SBA-15|EO (Emodin-loaded SBA-15): Activated SBA-15 (150 mg) was dried at 150 °C for 6 h under vacuum and cooled to room temperature. EO (90 mg) was dissolved in toluene (5.00 mL) and added to the silica. The mixture was shaken at room temperature for 48 h. The solid was collected by filtration, washed with toluene (4 × 10 mL), and dried under vacuum at room temperature for 16 h to yield an orange, free-flowing powder.

SBA-15-M|EO (Emodin-loaded fluorescent SBA-15): Activated SBA-15-M (150 mg) was similarly dried at 150 °C for 6 h, cooled, and suspended in a solution of EO (90 mg) in toluene (5.00 mL). The mixture was shaken at room temperature for 48 h. After filtration and washing with toluene (4 × 10 mL), the product (S15-M|EO) was dried under vacuum at room temperature for 16 h to obtain an orange free-flowing powder.

2.3. Physicochemical Characterization of SBA-15 Derivatives

For BET surface area analysis, the samples were dried at 120 °C for 24 h in a drying oven and subsequently degassed under high vacuum for an additional 4 h. Nitrogen adsorption–desorption isotherms were recorded using a Sorptomatic 1900 analyzer (Carlo Erba Instruments). Fluorescence spectra were obtained on a FluoroMax-2 spectrofluorometer (Jobin Yvon Inc., NJ, USA) using Suprasil® precision quartz cuvettes (Hellma® Analytics, Müllheim, Germany). For each measurement, an excitation wavelength specific to the fluorophore was selected, followed by recording of the corresponding emission spectrum over an appropriate wavelength range. Powder X-ray diffraction (XRD) patterns were collected using a Bruker D8 diffractometer. Thermogravimetric (TGA) and differential thermal analysis (DTA) were performed simultaneously using a Netzsch STA 449 instrument under synthetic air (flow rate 20 mL/min) with a heating rate of 10 K/min. Scanning electron microscopy (SEM) images and morphological characterization were carried out on an ESEM XL30 microscope operated under high vacuum at accelerating voltages of 1–2 kV. Transmission electron microscopy (TEM) was conducted on a Zeiss EM 900 microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany), and images were cap-

tured using an SSCCD SM-1k-120 camera (TRS, Moorenweis, Germany). For TEM sample preparation, 20 µg of each nanomaterial was dispersed in 1 mL of methanol, sonicated briefly, and vortexed three times. A 10 µL aliquot of the dispersion was dropped onto a Formvar-coated copper grid (PLANO, Wetzlar, Germany), allowed to adsorb for 1 min, blotted with filter paper, and stained with 5 µL of a 1% aqueous uranyl acetate solution for 1 min before drying. UV–Vis absorption spectra were recorded on a Jasco V-560 spectrophotometer over the 200–700 nm range, with a spectral bandwidth of 1.0 nm and a scanning speed of 200 nm/min. The low infrared spectrum was recorded by a Bruker Vertex 70 with a Diamond ATR unit (range of 4000 to 200 cm⁻¹). The electrokinetic properties of the synthesized nanostructures were evaluated using a Zetasizer Ultra (Malvern Panalytical Ltd., Malvern, UK) equipped with a diffusion barrier cell. Zeta potential measurements were performed by electrophoretic light scattering employing the patented mixed-mode measurement-phase analysis light scattering (M3-PALS) technology. Samples were dispersed in acetate buffer (pH 5.0) or phosphate-buffered saline (pH 7.4) and analyzed at 25 °C using disposable folded capillary cells.

2.4. *In Vitro* Studies

2.4.1. Drug Preparation

Stock suspensions of all mesoporous silica nanomaterials (MSN) were prepared at a concentration of 4 mg/mL in Dulbecco's phosphate-buffered saline (DPBS). Immediately before use, the suspensions were diluted with cell culture medium to the desired working concentrations. EO was used as a reference compound. A stock solution of EO (20 mM) was prepared in dimethyl sulfoxide (DMSO) and subsequently diluted with culture medium to obtain the required working concentrations. In all experiments, RPMI-1640 medium without phenol red, supplemented with 10% fetal calf serum (FCS), was used as the nutrient medium (both purchased from PAA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was dissolved at 0.5 mg/mL in RPMI-1640 medium. DPBS (HyClone), Trypsin-EDTA 1× (Biochrom-Merck), and DMSO (Duchefa Biochemie) were used as received. Crystal violet (CV), trypan blue, paraformaldehyde, and MTT were obtained from Sigma-Aldrich. The PC3 cell lines were purchased from ATCC. Cells were routinely cultured in RPMI-1640 medium supplemented with 10% FCS and maintained at 37 °C in a humidified incubator with 5% CO₂. Unless otherwise stated, the concentrations of the mesoporous silica nanomaterials used in the individual cellular assays are specified in the corresponding experimental subsections. Initial screening was performed at 100 and 150 µg/mL, dose–response experiments at 12.5–200 µg/mL, whereas mechanistic studies (cell cycle, apoptosis, and fluorescence microscopy) were conducted at the respective MC₅₀ values determined in the cytotoxicity assay.

2.4.2. Cell Culture

PC3 human prostate cancer cells were cultured as adherent monolayers in complete RPMI-1640 nutrient medium. Cells were maintained at 37 °C in a humidified incubator with 5% CO₂.

2.4.3. Determination of Target Cell Survival

An initial screening was performed on PC3 (5000 cells/well) by exposing them to each MSN sample at concentrations of 100 and 150 µg/mL, while EO was tested at 20 and 60 µM. Based on the screening results, a detailed cytotoxicity study was conducted on both cell lines using MSN concentrations ranging from 12.5 to 200 µg/mL and EO as a reference compound at 3.125–150 µM. Cells were incubated with the tested materials for 48 h. All experiments were performed in triplicate. Cell viability was assessed using MTT and crystal violet (CV) assays according to established procedures [24]. For the MTT assay, cells

were incubated with MTT staining solution (3.5 mg MTT dissolved in 7.0 mL medium per 96-well plate) at 37 °C for 15 min. After removal of the staining solution, DMSO was added to solubilize the formazan crystals, and absorbance was recorded at 570 nm with background correction at 670 nm using a SpectraMax M5 microplate reader (Molecular Devices). For the CV assay, DMSO was removed, cells were washed with DPBS, and fixed with 4% paraformaldehyde (15 min, room temperature). After fixation, cells were stained with 1% crystal violet solution, and absorbance was measured using the same microplate reader system. Cell survival was expressed as a percentage of untreated control cells, which was set to 100%. For each well, the absorbance at 570 nm (after blank subtraction) of treated cells was divided by the absorbance of the corresponding control well (cells grown only in nutrient medium), yielding the percentage of viable cells.

2.4.4. Cell Cycle Analysis

Cells were treated with the IC₅₀/MC₅₀ concentrations of the respective MSN samples for 48 h. After incubation, the culture medium from each well was collected, and the cells were washed, trypsinized, and washed again with DPBS. The cell pellets were then slowly resuspended in cold 70% ethanol while vortexing to ensure proper fixation. Fixed cells were stored briefly at 4 °C, then centrifuged and resuspended in 1 mL of DAPI staining solution (1% *v/v* Triton X-100 and 1 µg/mL DAPI in DPBS). Samples were incubated for 10 min in the dark. Following staining, cell cycle distribution was analyzed by flow cytometry.

2.4.5. Annexin-FITC/PI

Cells were treated with the IC₅₀/MC₅₀ concentrations of the respective MSN samples for 48 h. Following treatment, the medium was collected, and the cells were washed, trypsinized, and transferred into fresh tubes. Each sample was incubated with 100 µL of a freshly prepared working solution containing Annexin V-FITC and propidium iodide (PI) in Annexin binding buffer. The staining was performed for 15 min at room temperature in the dark, after which additional binding buffer was added, and the samples were immediately analyzed on a FACS Aria III flow cytometer. For proper interpretation of apoptotic profiles, control samples were included in each experiment. A sample treated with hydrogen peroxide served as a positive control for apoptosis, an unstained cell suspension was used to define background fluorescence, and a fully stained control enabled accurate compensation and gating during flow cytometric analysis.

2.4.6. Fluorescence Microscopy

Fluorescence microscopy was employed to evaluate both intracellular uptake and the time-dependent behavior of mesoporous silica nanomaterials (MSNs) in PC3 prostate cancer cells. Cells were seeded in six-well plates at an appropriate density to ensure proper adhesion and optimal growth before treatment. After overnight attachment, the cells were exposed to MSNs at concentrations corresponding to their respective MC₅₀ values, while inactive materials were applied at the highest non-toxic concentration determined in preliminary assays. Microscopic imaging was performed under physiological conditions (37 °C, 5% CO₂). The fluorescence microscopy setup and imaging conditions were based on the experimental configuration described previously [25,26]. For static fluorescence analysis, cells were incubated with the tested materials for 48 h, after which fluorescence micrographs were acquired to assess intracellular localization and distribution of the nanomaterials.

For dynamic analysis, time-resolved fluorescence microscopy was performed to monitor intracellular drug delivery and nanoparticle behavior in real time. Prior to imaging, cells were exposed to MSNs|EO at MC₅₀ concentrations and EO at IC₅₀ for 48 h to

optimize imaging conditions. Subsequently, fluorescence measurements were conducted continuously for 18 h immediately after treatment, with images recorded at 1 h intervals at predefined positions. This approach enabled detailed monitoring of nanoparticle internalization, intracellular trafficking, and temporal changes in fluorescence intensity, providing insights into release kinetics and cellular responses. The acquired image sequences were processed and compiled into videos (Supplementary Videos S1–S3) to visualize the kinetics of cellular uptake, intracellular distribution, and drug release behavior.

2.5. Molecular Docking Simulation

In addition to the experimental analyses, molecular docking studies were performed to evaluate interactions between acid–base species of EO and the anti-apoptotic proteins BCL-2 and BCL-xL, which play a crucial role in the survival of PC3 cells. Docking simulations were conducted using AutoDock Tools 1.5.7 in combination with AutoDock 4.2.6 [27], applying the Lamarckian genetic algorithm (LGA) [28] to estimate the binding affinity and preferred binding orientations of emodin within the hydrophobic binding groove of these proteins. The algorithm parameters were set to 250,000 energy evaluations and 27,000 generations, with mutation and crossover rates of 0.02 and 0.8, respectively. To ensure reproducibility of the computational results, 10 independent docking runs ($N = 10$) were performed for each emodin protonation state using the Lamarckian Genetic Algorithm. The resulting binding poses were clustered based on a root mean square deviation (RMSD) tolerance of 2.0 Å. The most populated cluster with the lowest mean binding energy was selected as the representative binding mode (consensus pose). The molecular structures of the examined acid–base forms of EO were optimized using the Gaussian16 software package [29], applying the B3LYP–D3BJ functional in combination with the 6-311++G(d,p) basis set for all atoms [30,31], while solvation effects were included using the CPCM implicit solvation model for water [32]. Ligand flexibility was allowed, while the receptor backbone was kept rigid, and Kollman partial charges together with polar hydrogens were assigned using AutoDockTools [33].

The three-dimensional crystal structures of human BCL-2 in complex with venetoclax (PDB ID: 6O0K) [34] and BCL-xL in complex with ABT-737 (PDB ID: 2YXJ) were retrieved from the RCSB Protein Data Bank. Before docking, co-crystallized ligands and water molecules were removed from the protein structures using Discovery Studio Visualizer, after which the structures were energy-minimized and prepared for docking. The docking search space was defined to encompass the canonical BH3-binding groove of both proteins, corresponding to the experimentally validated binding site of small-molecule inhibitors. This computational procedure is consistent with our previously reported protocols for the investigation of ligand–protein interactions and antioxidant mechanisms in bioactive compounds [35–37].

Because emodin exists as several protonation species depending on the pH of the medium, its interaction with the target proteins was evaluated under physiologically relevant conditions (pH 7.4). The relative abundance of the individual acid–base forms was determined from the reported pK_a values, and these molar fractions were taken into account in the binding analysis. To obtain a physiologically meaningful estimate of ligand affinity, the effective binding free energy (ΔG_{bind}^{eff}) was calculated as the mole-fraction-weighted average of the binding free energies of the individual protonation states:

$$\Delta G_{bind}^{eff} = f(EO) \times \Delta G_{bind}^{EO} + f(EO^-) \times \Delta G_{bind}^{EO^-} + f(EO^{2-}) \times \Delta G_{bind}^{EO^{2-}} + f(EO^{3-}) \times \Delta G_{bind}^{EO^{3-}} \quad (1)$$

where f represents the mole fraction of each protonation state at pH 7.4, while the corresponding ΔG_{bind} values were obtained from molecular docking calculations. Similarly, the

effective inhibition constant (k_{bind}^{eff}) were calculated from the binding free energies using the following relation:

$$k_{bind}^{eff} = e^{\frac{\Delta G_{bind}^{eff}}{RT}} \quad (2)$$

where R is the universal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T is the absolute temperature (298.15 K), and ΔG_{bind}^{eff} is expressed in J mol^{-1} . By incorporating the pH-dependent distribution of emodin species into the calculations, this approach provides a more realistic description of its binding behavior and inhibitory potential under physiological conditions than considering a single protonation state alone.

3. Results and Discussion

3.1. Preparation and Characterization of Materials

The mesoporous silica SBA-15 was successfully synthesized using the triblock copolymer Pluronic P123 as a structure-directing agent under strongly acidic conditions, which promotes the formation of a highly ordered hexagonal pore arrangement. The cooperative self-assembly between silica precursors and surfactant micelles leads to the formation of uniform cylindrical pores, while subsequent hydrothermal treatment ensures structural condensation and stability. Post-synthetic functionalization with (3-aminopropyl)triethoxysilane introduced reactive amino groups (SBA-15-NH₂), providing anchoring sites for further surface modification. The successful grafting of the fluorophore N-methylisatoic anhydride (SBA-15-M) allowed the preparation of fluorescently traceable nanomaterials, while maintaining the structural integrity of the SBA-15 framework.

The FTIR spectrum of SBA-15 showed a broad band in the region of $3000\text{--}3500 \text{ cm}^{-1}$, assigned to O–H stretching vibrations of surface silanol groups and adsorbed water, together with an H–O–H bending band at approximately 1600 cm^{-1} . The characteristic silica-framework absorptions were observed at approximately $1067, 954, 805, \text{ and } 451 \text{ cm}^{-1}$, corresponding to asymmetric Si–O–Si stretching, Si–OH stretching, symmetric Si–O–Si stretching, and Si–O–Si bending vibrations, respectively. The spectrum of SBA-15-NH₂ retained the principal silica bands at approximately $1060, 950, 801, \text{ and } 453 \text{ cm}^{-1}$. An additional absorption near 2964 cm^{-1} , attributed to C–H stretching of the grafted propyl chain, supported the successful introduction of aminopropyl groups onto the mesoporous silica surface. A contribution in the region around 1600 cm^{-1} may include N–H deformation, although it overlaps with the bending vibration of adsorbed water. For SBA-15-M, bands associated with the organic surface layer were observed at approximately $2780\text{--}2980 \text{ cm}^{-1}$, in addition to the silica absorptions at approximately $1064, 953, 796 \text{ and } 453 \text{ cm}^{-1}$. These changes are consistent with the modification of the surface amino groups by the MIA-derived functionality, while retention of the Si–O–Si bands confirms preservation of the mesoporous silica framework. After immobilization of EO into MSNs, the SBA-15-M|EO spectrum has distinct contributions at approximately $1627 \text{ and } 1477 \text{ cm}^{-1}$, assigned to vibrations of the conjugated aromatic system of emodin. Similar bands were present in SBA-15|EO, together with a broad O–H absorption around 3300 cm^{-1} . Nevertheless, the C=O bands and the retained silica-framework vibrations support EO incorporation into the M-functionalized nanostructure without disruption of the inorganic matrix.

After filtration of the EO-loaded materials, the non-adsorbed emodin was recovered by washing with toluene and quantified by HPLC analysis. This approach enabled the precise determination of the loading efficiency. The obtained results revealed that SBA-15-based nanomaterials contain approximately 35% of EO (Table 1), which is significantly higher than previously reported spherical mesoporous systems ($\sim 18\%$), indicating the su-

terior loading capacity of SBA-15 due to its larger pore diameter and higher internal surface accessibility.

Table 1. Results of characterization by SAXS, BET, HPLC, and TGA.

Material	Dimension	Lattice Constant	Crystallite Size	Spec. Surf. Area	Spec. Pore Vol.	EO [%]	
	[nm]	[Å]	[nm]	[m ² /g]	[cm ³ /g]	HPLC	TGA
SBA-15-M	700 ± 130 × 420 ± 90	74	101	247	0.17	—	—
SBA-15 EO	740 ± 60 × 450 ± 100	87	92	211	0.15	34.8	33.3
SBA-15-M EO	630 ± 90 × 370 ± 90	84	98	56	0.05	35.1	34.4

Thermogravimetric analysis (TGA) was performed to evaluate the thermal behavior and loading efficiency of SBA-15-based nanomaterials. All samples exhibited an initial mass loss below 120 °C (approximately 1–3%), attributed to the removal of physically adsorbed water, which is characteristic of mesoporous silica materials (Figures 1 and S6).

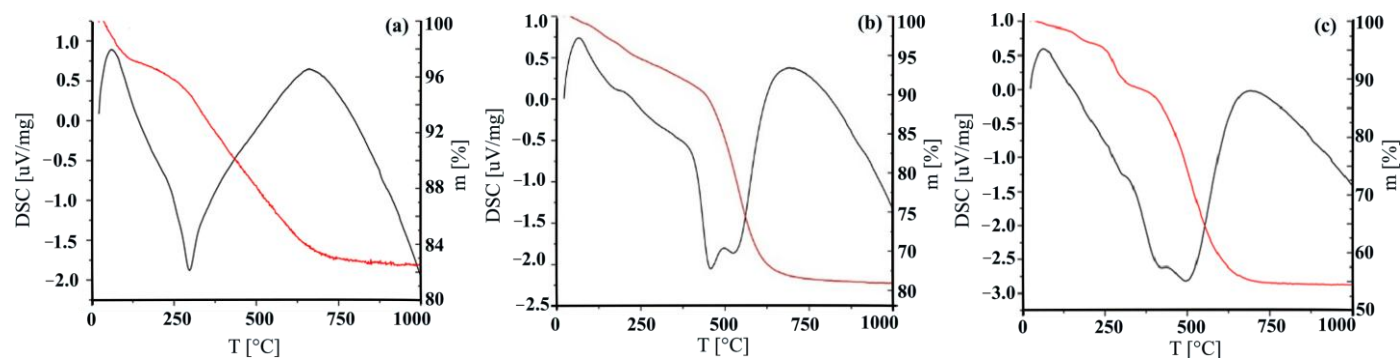


Figure 1. Thermogravimetric (TGA) and Differential Scanning Calorimetry (DSC) profiles of SBA-15-based MSN samples: (a) SBA-15-M, (b) SBA-15|EO, and (c) SBA-15-M|EO. The red curve represents TGA (mass loss), while the black curve corresponds to DSC signals.

The SBA-15-M sample showed a relatively low and gradual mass loss over a broad temperature range (approximately 120–600 °C), indicating the presence of a limited amount of surface-bound organic moieties. This confirms successful surface modification without significant organic content. In contrast, EO-loaded samples (SBA-15|EO and SBA-15-M|EO) exhibited substantially higher mass loss, particularly within the temperature range of approximately 250–600 °C, corresponding to the thermal decomposition of emodin molecules confined within the mesoporous structure. Notably, the SBA-15-M|EO sample demonstrated the highest total mass loss, indicating significantly improved loading capacity due to surface functionalization. Additionally, a slight shift and broadening of the decomposition region suggest stronger interactions between emodin and the functionalized silica surface. At higher temperatures (above ~700 °C), a minor additional mass loss was observed for all samples, which can be attributed to the condensation of residual silanol groups and further stabilization of the silica framework. The DSC curves support these findings, showing thermal events associated with dehydration at low temperatures and pronounced exothermic effects in the 250–550 °C region, corresponding to the decomposition of organic components. Differences in peak shape and intensity between SBA-15|EO and SBA-15-M|EO further indicate variations in interaction strength and the distribution of emodin within the mesoporous matrix. The combined TGA and DSC results confirm successful loading of emodin and demonstrate that surface modification significantly enhances the loading capacity and interaction of EO with the SBA-15 framework.

Additional quantitative analysis of the TGA data was performed to estimate the efficiency of amino-group grafting and subsequent fluorophore immobilization. After correction for the intrinsic mass loss of SBA-15, the amount of grafted aminopropyl groups was estimated to be $1.03 \text{ mmol g}^{-1} \text{ SiO}_2$ ($0.88 \text{ mmol g}^{-1} \text{ SBA-15-NH}_2$). Using the additional mass loss observed after reaction with *N*-methylisatoic anhydride (M), the fluorophore loading was estimated to be $0.36 \text{ mmol g}^{-1} \text{ SiO}_2$ ($0.30 \text{ mmol g}^{-1} \text{ SBA-15-M}$), corresponding to an approximate conversion of 35.4% of the available surface amino groups. These values were obtained from TGA-derived mass losses using a semi-quantitative approach and therefore should be regarded as approximate estimates of the surface functionalization efficiency rather than absolute values.

The morphology and structural characteristics of the synthesized SBA-15-based materials were examined by SEM and TEM (Figure 2). SEM micrographs (Figure 2a) reveal elongated, rod-like particles, characteristic of SBA-15 materials. All samples (SBA-15-M, SBA-15|EO, and SBA-15-M|EO) exhibit similar overall morphology, indicating that surface functionalization and EO loading do not induce significant changes in particle shape.

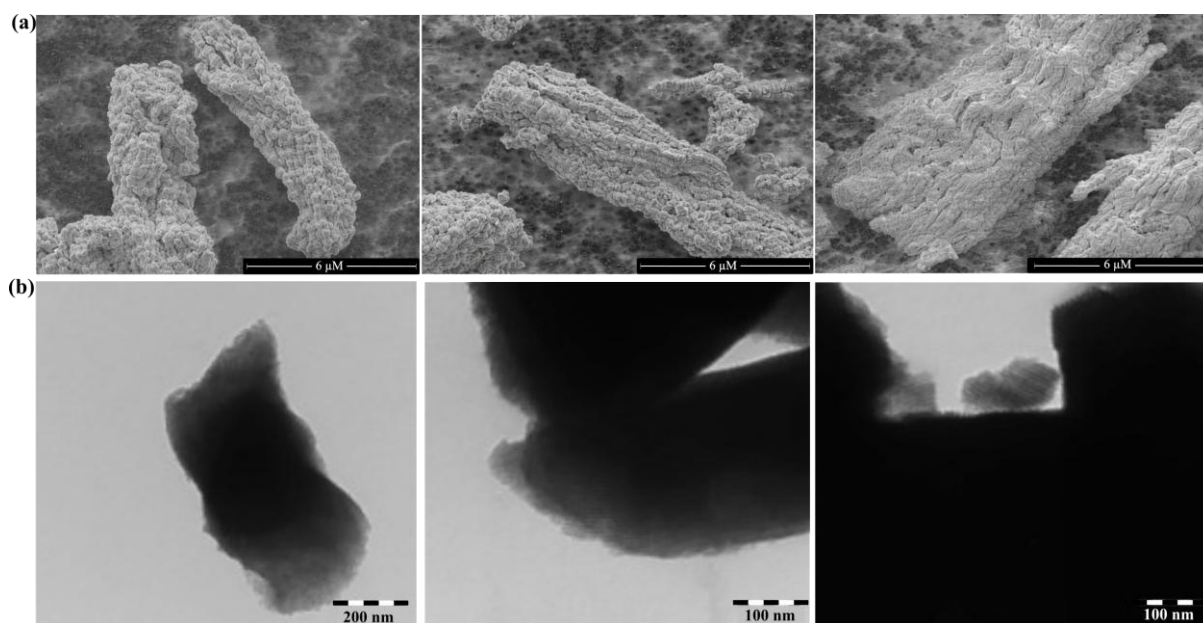


Figure 2. SEM (a) and TEM (b) images of (left) SBA-15-M, (middle) SBA-15|EO, and (right) SBA-15-M|EO.

Slight differences in surface texture can be observed among the samples. The SBA-15-M, SBA-15|EO, and SBA-15-M|EO materials show a somewhat more pronounced roughness, while EO-loaded samples (SBA-15|EO and SBA-15-M|EO) appear more compact, which may be attributed to the presence of EO on the surface and/or within the pores. However, these differences are subtle and do not affect the overall morphology. Particle size analysis (Table 1) indicates that all materials fall within the submicrometer range: SBA-15-M ($700 \pm 130 \times 420 \pm 90 \text{ nm}$), SBA-15|EO ($740 \pm 60 \times 450 \pm 100 \text{ nm}$), and SBA-15-M|EO ($630 \pm 90 \times 370 \pm 90 \text{ nm}$). These results confirm a relatively uniform size distribution and suggest that the modification and loading processes do not lead to significant aggregation or fragmentation. TEM images (Figure 2b) confirm the presence of ordered mesoporous channels typical of SBA-15. In all samples, a parallel arrangement of pores is observed, indicating that the structural organization of the silica framework is preserved after functionalization and EO loading. In EO-loaded samples, a slight decrease in pore contrast is observed compared to SBA-15, which may indicate the presence of EO within

the mesoporous channels. This effect is more pronounced in SBA-15-M|EO, suggesting more efficient loading in the functionalized material.

Small-angle X-ray scattering (SAXS) analysis was employed to evaluate the structural ordering and the periodicity of SBA-15-based nanomaterials after surface modification and EO loading (Figure 3, Table 1). All samples exhibit a well-defined and intense diffraction peak in the low-angle region ($2\theta \approx 1^\circ$), corresponding to the (100) reflection of a two-dimensional hexagonal (p6mm) mesostructure, confirming the preservation of long-range order typical for SBA-15 materials.

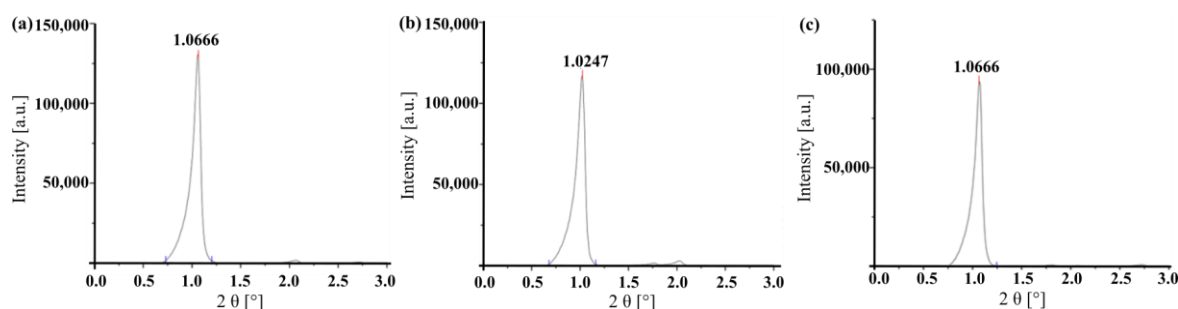


Figure 3. Diffractograms of nanomaterials based on (a) SBA-15-M, (b) SBA-15|EO, and (c) SBA-15-M|EO.

For the SBA-15-M sample, the main diffraction peak appears at $2\theta = 1.0666^\circ$, corresponding to a lattice constant of $74 \text{ \AA}$ and a crystallite size of 101 nm . Upon EO loading (SBA-15|EO), the peak shifts slightly toward lower angles ($2\theta = 1.0247^\circ$), accompanied by an increase in the lattice constant to $87 \text{ \AA}$ and a decrease in crystallite size to 92 nm . This expansion of the lattice parameter indicates pore filling and structural expansion due to the incorporation of EO molecules within the mesoporous channels. In the case of SBA-15-M|EO, the peak position returns to $2\theta = 1.0666^\circ$, with a slightly reduced lattice constant of $84 \text{ \AA}$ and an increased crystallite size of 98 nm . These results suggest partial structural reorganization and stabilization of the mesostructure after combined functionalization and EO loading. Importantly, all samples retain a sharp and intense (100) reflection without significant peak broadening or disappearance, indicating that the hexagonally ordered mesoporous framework remains intact throughout the modification processes. The observed variations in lattice constant ($74\text{--}87 \text{ \AA}$) and crystallite size ($92\text{--}101 \text{ nm}$) are relatively minor and confirm that EO incorporation occurs primarily within the pores without causing structural collapse or disruption of the silica framework.

Nitrogen adsorption–desorption (BET) analysis was performed to evaluate the textural properties of the SBA-15-based nanomaterials and to assess the impact of surface modification and EO loading (Table 1). All samples exhibited characteristics typical of mesoporous materials, with high surface area and defined pore structure, although significant differences were observed after modification and loading. The SBA-15-M sample shows a relatively high specific surface area of $247 \text{ m}^2/\text{g}$ and a pore volume of $0.17 \text{ cm}^3/\text{g}$, indicating well-preserved mesoporosity after surface functionalization. Upon EO loading (SBA-15|EO), both parameters decrease moderately to $211 \text{ m}^2/\text{g}$ and $0.15 \text{ cm}^3/\text{g}$, respectively. This reduction suggests partial filling of the mesopores by EO molecules, which limits nitrogen accessibility while still maintaining a substantial fraction of the porous structure. A much more pronounced decrease is observed for the S15-M|EO sample, where the specific surface area drops to $56 \text{ m}^2/\text{g}$ and the pore volume to $0.05 \text{ cm}^3/\text{g}$. This significant reduction indicates extensive pore occupation and possible partial pore blocking, resulting from the combined effect of surface modification and EO incorporation. The drastic decrease in accessible surface area strongly supports the successful loading of EO within the mesoporous network. Importantly, despite the reduction in textural param-

ters, the preservation of mesostructural ordering observed by SAXS confirms that these changes are primarily due to pore filling rather than structural collapse. The correlation between decreasing surface area and pore volume with increasing EO content (34.8–35.1% by HPLC and 33.3–34.4% by TGA) further supports efficient encapsulation of EO within the SBA-15 framework.

The evolution of the surface charge during the sequential functionalization of SBA-15 was investigated by zeta potential measurements performed at tumor microenvironment simulated pH (5.0) and physiological pH (7.4). The SBA-15 mesoporous silica exhibited negative zeta potentials of -9.22 mV at pH 5.0 and -15.12 mV at pH 7.4. The more negative surface charge observed at physiological pH reflects the progressive deprotonation of surface silanol groups, leading to an increased density of negatively charged siloxide species on the silica surface. Following grafting with aminopropyl groups, the SBA-15-NH₂ nanostructure exhibited a pronounced shift in the zeta potential toward neutral values. At pH 5.0, the zeta potential increased to $+0.82$ mV, demonstrating efficient protonation of surface amino groups and partial compensation of the negative charge originating from silanol groups. At pH 7.4, the average zeta potential remained close to neutrality ($+0.88$ mV), although a bimodal distribution with both positive and negative populations was observed. This behavior is attributed to the coexistence of deprotonated silanol groups and partially protonated aminopropyl functionalities, indicating a chemically heterogeneous surface after APTES grafting. Subsequent grafting of methyl isatoic anhydride produced the SBA-15-M nanostructure, whose zeta potential shifted slightly toward negative values. The average zeta potentials of -1.96 mV at pH 5.0 and -4.65 mV at pH 7.4 indicate partial consumption of the surface amino groups during the coupling reaction, thereby reducing their contribution to the overall positive surface charge. Nevertheless, the material remained substantially less negative than unmodified SBA-15, confirming that the MIA-functionalized surface retained a different electrochemical character from the parent silica. After loading with emodin, the SBA-15-M|EO nanostructure exhibited zeta potentials of $+0.25$ mV at pH 5.0 and -8.44 mV at pH 7.4. Compared with SBA-15-M, emodin loading induced only a moderate increase in the negative surface charge at physiological pH, suggesting that the adsorbed drug contributes to the electrokinetic behavior without completely masking the MIA-derived surface functionality. In contrast, the SBA-15|EO material exhibited zeta potentials of $+0.93$ mV at pH 5.0 and -16.54 mV at pH 7.4. The substantially more negative zeta potential of SBA-15|EO at pH 7.4 indicates that emodin adsorption on unmodified mesoporous silica is dominated by the negatively charged silica surface. Conversely, the considerably less negative value observed for SBA-15-M|EO demonstrates that M-functionalization effectively modifies the surface electrostatic environment and partially compensates the negative charge of the silica framework even after drug loading. These findings indicate that M-derived surface functionalities remain exposed following emodin immobilization and continue to influence the electrokinetic properties of the nanostructure. The gradual evolution of the zeta potential from SBA-15 to SBA-15-NH₂, SBA-15-M, and finally SBA-15-M|EO provides strong evidence for the successful stepwise surface modification of the mesoporous silica nanostructures. Although all materials exhibited relatively low absolute zeta potential values ($|\zeta| < 20$ mV), such behavior is expected because the measurements were performed in acetate buffer or phosphate-buffered media of relatively high ionic strength, which compresses the electrical double layer and decreases the magnitude of the measured zeta potential. The observed systematic changes therefore primarily reflect alterations in surface chemistry rather than differences in colloidal stability.

Fluorescence spectroscopy was employed to evaluate the optical properties of SBA-15-based nanomaterials and to confirm the successful incorporation of fluorescent moieties and EO (Figure 4). As expected, the pristine SBA-15 matrix does not exhibit intrinsic

fluorescence, indicating that all observed signals originate exclusively from incorporated species.

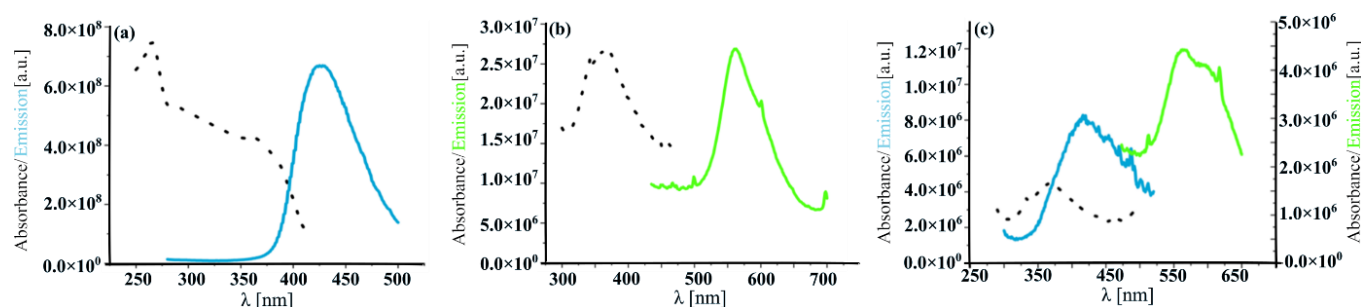


Figure 4. Fluorescence spectra of nanomaterials based on SBA-15 (a) SBA-15-M, (b) SBA-15|EO, and (c) SBA-15-M|EO.

For the SBA-15 -M sample (Figure 4a), a pronounced emission band is observed in the region around 420–450 nm upon excitation in the UV region, confirming the presence of the grafted fluorescent moiety. The well-defined emission peak, together with the corresponding absorption band in the UV region, indicates successful covalent attachment and preservation of the photophysical properties of the modifier.

In the case of SBA-15|EO (Figure 4b), a strong emission band appears in the visible region, centered approximately at 550–580 nm. The position and shape of the emission band are consistent with the intrinsic fluorescence of EO, suggesting that incorporation into the mesoporous structure does not significantly alter its electronic structure. The slight broadening of the emission band may be attributed to heterogeneous microenvironments within the pores.

The dual-functionalized sample SBA-15-M|EO (Figure 4c) exhibits two distinct emission features: one in the blue region (~400–450 nm) corresponding to the grafted fluorophore, and another in the green region (~550–600 nm) associated with EO. The presence of both emission bands clearly confirms the simultaneous incorporation of both components. Importantly, no significant spectral overlap or quenching is observed, indicating that the two species retain their individual fluorescence behavior and can be detected independently.

The coexistence of two well-resolved emission bands suggests minimal electronic interaction between the grafted fluorophore and EO, which implies that EO is predominantly confined within the pores, while the fluorescent groups remain anchored to the silica surface. This spatial separation is crucial for applications involving simultaneous tracking and delivery, as it enables independent monitoring of both components.

3.2. Cytotoxic Studies

The cytotoxic effects of EO and the prepared mesoporous silica nanomaterials were evaluated in PC3 human prostate cancer cells using MTT and crystal violet (CV) assays after 48 h of treatment (Table 2). The unloaded carriers, SBA-15 and SBA-15-M, did not exhibit cytotoxicity at concentrations up to 120 µg/mL in either assay ($MC_{50} > 120$ µg/mL), confirming that both the silica matrix and its surface modification are biologically inert under the tested conditions and suitable as drug delivery platforms.

Table 2. In vitro cytotoxicity of emodin (EO) and EO-loaded mesoporous silica nanomaterials against PC3 prostate cancer cells (CV and MTT assay, 48 h). Values are presented as mean $\pm$ standard deviation obtained from three independent experiments ($n = 3$).

Material	CV Assay		MTT Assay	
	MC ₅₀ ($\mu\text{g/mL}$)	IC ₅₀ (μM) *	MC ₅₀ ($\mu\text{g/mL}$)	IC ₅₀ (μM) *
SBA-15	>120	–	>120	–
SBA-15–M	>120	–	>120	–
SBA-15 EO	91.2 $\pm$ 6.3	117.4 $\pm$ 8.1	78.6 $\pm$ 7.0	101.2 $\pm$ 9.1
SBA-15–M EO	50.2 $\pm$ 5.9	65.2 $\pm$ 7.6	25.6 $\pm$ 7.5	18.2 $\pm$ 5.3
Compound	MC ₅₀ ($\mu\text{g/mL}$)	IC ₅₀ (μM)	MC ₅₀ ($\mu\text{g/mL}$)	IC ₅₀ (μM)
EO	8.1 $\pm$ 1.0	29.9 $\pm$ 3.6	6.5 $\pm$ 2.3	23.9 $\pm$ 8.5

* μM calculated EO concentration based on the HPLC results.

In contrast, EO-loaded materials showed pronounced cytotoxic effects. The SBA-15–M|EO system demonstrated the highest activity, with IC₅₀ values of 18.2 $\pm$ 5.3 μM (MTT) and 65.2 $\pm$ 7.6 μM (CV), along with significantly lower MC₅₀ values (25.6 $\pm$ 7.5 $\mu\text{g/mL}$ in MTT and 50.2 $\pm$ 5.9 $\mu\text{g/mL}$ in CV) compared to SBA-15|EO. The SBA-15|EO material exhibited moderate activity, with IC₅₀ values of 101.2 $\pm$ 9.1 μM (MTT) and 117.4 $\pm$ 8.1 μM (CV). Free EO showed IC₅₀ values of 23.9 $\pm$ 8.5 μM (MTT) and 29.9 $\pm$ 3.6 μM (CV), indicating that encapsulation within S15–M significantly enhances cytotoxic potency, particularly in the MTT assay.

The observed differences between SBA-15|EO and SBA-15–M|EO can be attributed to the effect of surface functionalization on drug loading, release behavior, and cellular internalization. The modified surface in SBA-15–M likely promotes more efficient EO delivery and/or controlled release, resulting in enhanced biological activity. In contrast, the lower activity of SBA-15|EO suggests less efficient interaction with cellular targets. Overall, the results confirm that while the silica carrier itself is non-toxic, its functionalization plays a crucial role in modulating the therapeutic performance of EO, highlighting the importance of rational nanocarrier design.

Fluorescence microscopy was used to evaluate the cellular internalization and intracellular distribution of the fluorescently labeled nanomaterials in PC3 cells after 48 h of treatment (Figure 5). The experiments were performed at concentrations corresponding to the MC₅₀ values for the cytotoxic nanomaterials and at 120 $\mu\text{g/mL}$ for the non-toxic ones. As shown in Figure 5, the modified carrier SBA-15–M exhibited a pronounced blue fluorescence signal, confirming successful cellular uptake and accumulation within PC3 cells. The fluorescence signal was primarily localized in the perinuclear region, indicating potential involvement of intracellular trafficking pathways.

In the case of EO-loaded systems, both SBA-15|EO and SBA-15 –M|EO displayed a strong green fluorescence signal originating from emodin, which was distributed throughout the cytoplasm. This observation confirms efficient intracellular release of EO from the mesoporous carriers. Notably, SBA-15–M|EO showed a more intense and homogeneous green signal compared to SBA-15|EO, suggesting improved delivery and/or release efficiency of EO from the functionalized carrier. The overlap of blue (carrier) and green (EO) fluorescence further supports successful co-localization and intracellular transport of the nanomaterial–drug system.

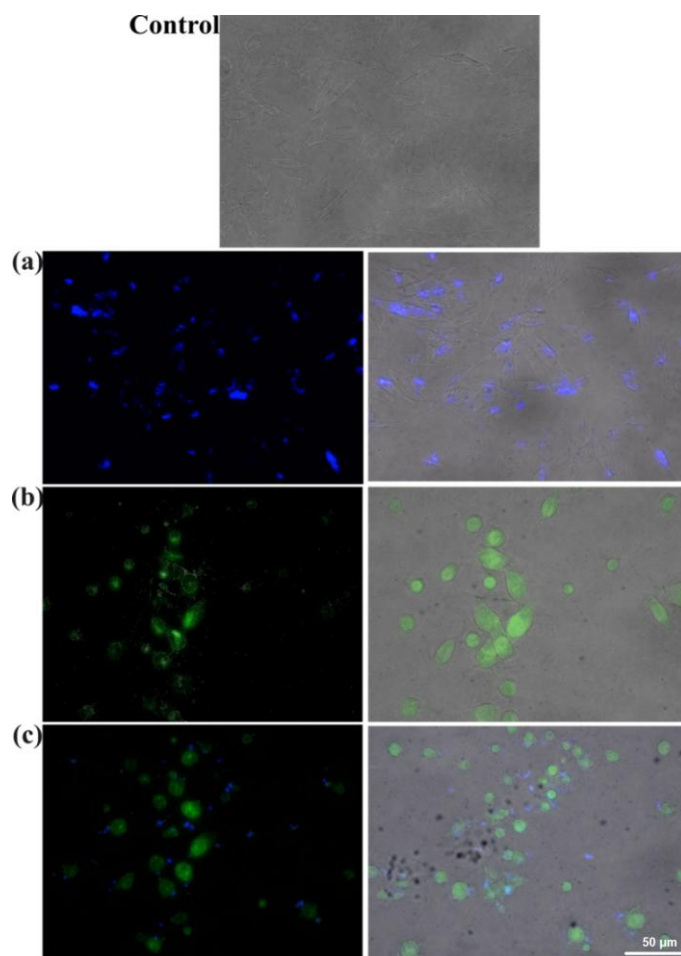


Figure 5. Fluorescence microphotographs of untreated (**left**) and treated (**right**) PC3 cells (48 h) with nanomaterials based on SBA-15 (**a**) SBA-15-M, (**b**) SBA-15|EO, and (**c**) SBA-15-M|EO.

3.3. Mechanisms of Actions

The biological effects of EO and EO-loaded nanomaterials on PC3 cells were further evaluated by flow cytometric analysis of the cell cycle and apoptosis (Figure 6). Cell cycle distribution, assessed by DAPI staining, revealed noticeable differences between free EO and EO-loaded systems. In control cells, the majority of the population was located in the G1/G0 phase, indicating normal proliferative activity. Treatment with free EO did not induce significant alterations in cell cycle distribution, suggesting a limited effect on cell cycle progression under the applied conditions. In contrast, EO-loaded nanomaterials caused more pronounced changes. Both SBA-15|EO and SBA-15-M|EO slightly increased the Sub-G1 population, indicative of DNA fragmentation and apoptotic processes. This increase was more prominent in SBA-15|EO, confirming a stronger induction of cell death. Additionally, a slight redistribution of cells toward the S phase was observed, suggesting replication stress and impaired DNA synthesis.

Apoptosis was further evaluated using Annexin V/propidium iodide staining (Figure 7). As expected, control cells were predominantly viable (Ann-/PI-, ~88%), confirming the absence of significant basal apoptosis. Treatment with free EO resulted in a modest shift toward apoptotic populations, with a slight increase in late apoptotic cells (Ann+/PI+, ~14%) and a minor fraction of early apoptotic cells (Ann+/PI-, ~4%), indicating limited cytotoxic activity under the applied conditions. In contrast, the SBA-15|EO formulation induced a pronounced transition toward late apoptosis, with the vast majority of cells (~98%) located in the Ann+/PI+ quadrant. This finding clearly demonstrates a strong pro-apoptotic effect associated with EO delivery via the

unmodified SBA-15 carrier, suggesting rapid intracellular release and accumulation of the active compound. Interestingly, the SBA-15-M|EO system exhibited a substantially different apoptotic profile. A large proportion of cells remained viable (~81%), accompanied by a moderate increase in late apoptotic cells (~17%) and a minimal early apoptotic fraction (~2%). Compared to SBA-15|EO, this indicates a significantly attenuated cytotoxic response. These differences can be attributed to variations in EO release behavior and nanoparticle–cell interactions. The unmodified carrier (SBA-15|EO) likely enables rapid EO release, leading to high intracellular concentrations and extensive cell death. In contrast, surface modification (SBA-15-M|EO) appears to regulate EO availability, resulting in a more controlled and less aggressive biological response while still retaining pro-apoptotic activity.

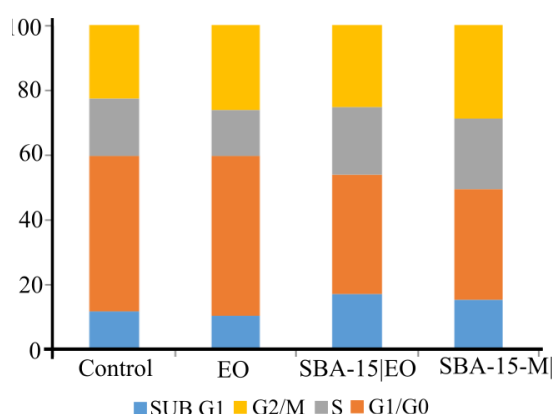


Figure 6. PC3 cells treated with nanomaterials based on SBA-15, SBA-15-M, SBA-15|EO, and SBA-15-M|EO, and analyzed on the cell cycle using DAPI.

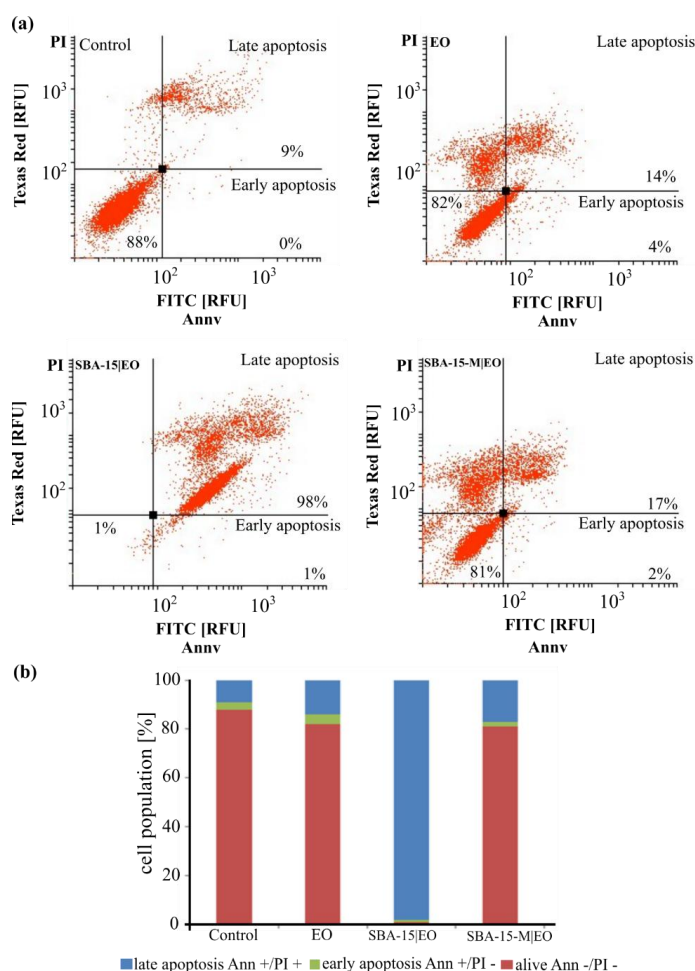


Figure 7. Representative Annexin V/PI flow cytometry plots (a) and quantitative distribution of viable, early apoptotic, and late apoptotic PC3 cells after treatment with EO and MSN-based formulations (b).

These results demonstrate that EO encapsulation significantly enhances its biological activity compared to the free compound, while surface functionalization plays a critical role in modulating the intensity and mechanism of the cytotoxic response. The ability to tune between rapid and controlled apoptosis highlights the importance of rational nanocarrier design for optimizing therapeutic performance.

To further elucidate the mechanism of cytotoxic activity, intracellular reactive oxygen species (ROS) levels were assessed using the DHR123 assay (Figure 8). Compared to the control, all EO-treated samples suppressed ROS generation. In accordance with this, EO-loaded systems (SBA-15|EO and SBA-15-M|EO) also exhibited significant suppression of ROS generation. These results suggest that ROS generation is unlikely to be the primary mechanism responsible for apoptosis induction.

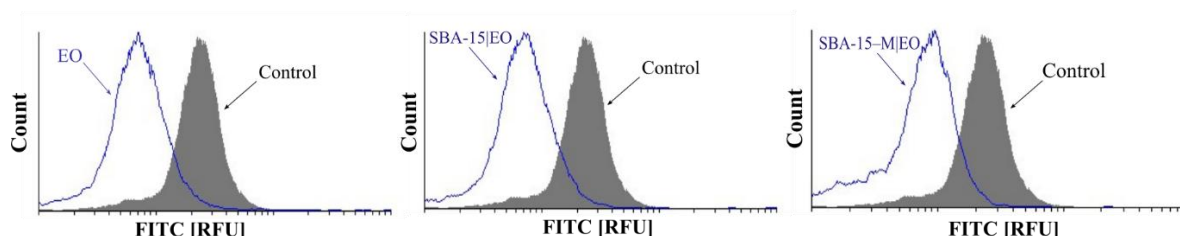


Figure 8. Representative flow cytometry histograms showing intracellular ROS levels in PC3 cells after treatment with EO, SBA-15|EO, and SBA-15-M|EO, measured by DHR123 fluorescence (FITC channel). Control cells are shown in gray.

3.4. Time-Dependent Intracellular Behavior and Cell Death Kinetics

Time-dependent fluorescence microscopy provided visual insight into the intracellular behavior of EO-loaded nanomaterials (Supplementary Videos S1–S3). Representative micrographs illustrate the progressive changes in fluorescence intensity and cellular morphology at defined time intervals (0–18 h). In the case of SBA-15|EO (Figure 9a), a rapid increase in intracellular green fluorescence was observed, indicating efficient cellular uptake followed by fast intracellular release of EO. This was accompanied by a noticeable reduction in cell density and pronounced morphological alterations over time, suggesting rapid induction of cytotoxic effects. In contrast, the SBA-15-M|EO system (Figure 9b) exhibited a markedly different intracellular profile. In addition to the green fluorescence of EO, the presence of the blue signal corresponding to the SBA-15-M functionalized carrier (M) enabled visualization of nanoparticle distribution within the cells. The fluorescence intensity increased more gradually, indicating slower intracellular accumulation and a more controlled release of EO. Morphological changes were less pronounced at earlier time points, further supporting a delayed onset of cytotoxicity compared to SBA-15|EO.

These observations are consistent with the time-dependent cell death kinetics (Table 3). Free EO induced the most rapid cytotoxic response, with the first detectable cell death and complete cell death occurring within 1 h, consistent with its direct availability to the cells following administration. In comparison, SBA-15|EO exhibited a rapid but slightly delayed cytotoxic response (3–6 h), whereas SBA-15-M|EO showed a more gradual onset and completion of cell death (4–8 h). The observed differences between the two nanocarrier systems can be attributed to the influence of surface functionalization on EO release kinetics and nanoparticle–cell interactions, which modulate the intracellular availability of EO and consequently affect the temporal progression of cell death.

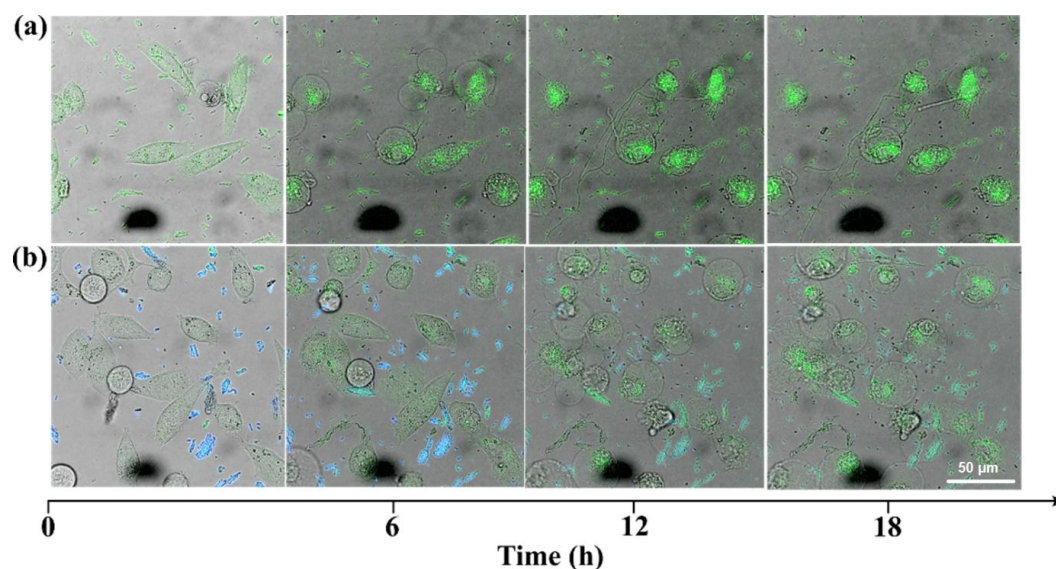


Figure 9. Time-dependent fluorescence microscopy images showing intracellular behavior of EO-loaded nanomaterials in PC3 cells at 0, 6, 12, and 18 h. (a) SBA-15|EO system, where green fluorescence represents EO, illustrating rapid cellular uptake and progressive intracellular accumulation accompanied by pronounced morphological changes and reduction in cell density over time. (b) SBA-15-M|EO system, where green fluorescence corresponds to EO and blue fluorescence to the functionalized carrier (M), demonstrating a more gradual increase in intracellular fluorescence and a controlled distribution of the nanomaterial.

Table 3. Time-dependent cell death kinetics of EO and EO-loaded nanomaterials in PC3 cells.

Material	PC3	
	t [h]	
	Time to First Cell Death	Time to Complete Cell Death
Control	17	23
EO	1	1
SBA-15-M	19	x
SBA-15 EO	3	6
SBA-15-M EO	4	8

Importantly, the unloaded SBA-15-M carrier did not induce detectable cell death in PC3 cells within the observed timeframe, confirming its biocompatibility and supporting its role as a delivery platform rather than a cytotoxic agent. Morphological alterations, including decreased cell density, cell shrinkage, and loss of normal cellular architecture, were clearly observed in EO-loaded systems, while control and SBA-15-M-treated cells retained typical morphology.

Overall, these findings are in good agreement with the cytotoxicity and flow cytometry results, confirming that nanocarrier-mediated delivery enhances intracellular availability of EO while modulating the temporal progression of cell death. The comparative analysis of fluorescence signals further highlights the critical role of surface functionalization in governing intracellular trafficking, release behavior, and the subsequent biological response. These results demonstrate that surface engineering of SBA-15 nanostructures regulates the intracellular fate and release profile of EO, thereby modulating its biological activity and apoptotic response in PC3 cells.

3.5. Molecular Docking Study of EO with BCL-2 and BCL-xL Proteins

The acid–base behavior and protonation states of EO have been thoroughly analyzed and discussed in our previous study. Briefly, due to its pronounced acid–base character and the reported pK_a values ($pK_{a1} = 6.2$; $pK_{a2} = 8.3$; $pK_{a3} = 12.7$), EO exists in multiple protonation forms whose distribution is strongly pH-dependent. At physiological pH (7.4), the monodeprotonated form (EO^-) predominates, while the neutral and doubly deprotonated species are present in smaller fractions, and the fully deprotonated form can be considered negligible. A detailed discussion of these equilibria and their implications for molecular interactions is provided in our previous work [30].

Considering this distribution, the docking analysis was performed for the relevant protonation states in order to provide a more realistic description of EO binding to the anti-apoptotic proteins BCL-2 and BCL-xL. Given its predominance at physiological pH, EO^- is expected to play the principal role in mediating biological activity, while EO^{2-} may additionally contribute due to its distinct electronic structure and interaction capabilities. In agreement with the relative abundance of protonation forms, the calculated effective binding energies (BCL-2- EO_{eff} : $\Delta G_{bind} = -6.24$ kcal mol $^{-1}$, $K_i = 26.8$ μ M; BCL-xL- EO_{eff} : $\Delta G_{bind} = -5.47$ kcal mol $^{-1}$, $K_i = 97.9$ μ M) indicate that the overall interaction is largely governed by the monodeprotonated species, with a secondary contribution from the dianionic form. The individual docking results reveal that both EO^- and EO^{2-} establish favorable interactions within the binding pockets of BCL-2 and BCL-xL, with differences in binding affinities reflecting variations in charge distribution and their ability to engage in electrostatic and hydrogen-bonding interactions (Table 4).

Table 4. Important thermodynamic parameters (kcal mol $^{-1}$) for the most stable conformation of investigated acid–base species in the active site of BCL-2 and BCL-xL determined after molecular docking simulation: inhibition constants (K_i , μ M) and binding free energy (ΔG_{bind} , kcal mol $^{-1}$) values obtained from various energy components such as total internal energy (ΔG_{total}), torsional free energy (ΔG_{tor}), unbound system's energy (ΔG_{unb}), electrostatic energy (ΔG_{elec}), and the sum of dispersion and repulsion (ΔG_{vdw}), hydrogen bond (ΔG_{hbond}), and desolvation (ΔG_{desolv}).

Complex	ΔG_{bind}	K_i (μ M)	ΔG_{inter}	$\Delta G_{vdw+hbond+desolv}$	ΔG_{elec}	ΔG_{total}	ΔG_{tor}	ΔG_{unb}
BCL-2- EO^-	-6.59	14.8	-7.18	-7.01	-0.18	-1.22	0.60	-1.22
BCL-2- EO^{2-}	-6.55	15.7	-6.85	-6.70	-0.15	-0.61	0.30	-0.61
BCL-2- EO_{eff}	-6.24	26.8	–	–	–	–	–	–
BCL-xL- EO^-	-5.74	61.9	-6.34	-6.26	-0.08	-1.31	0.60	-1.31
BCL-xL- EO^{2-}	-6.06	36.3	-6.36	-6.22	-0.14	-0.60	0.30	-0.60
BCL-xL- EO_{eff}	-5.47	97.9	–	–	–	–	–	–

For the BCL-2 system, EO^- ($\Delta G_{bind} = -6.59$ kcal mol $^{-1}$) and EO^{2-} ($\Delta G_{bind} = -6.55$ kcal mol $^{-1}$) exhibit comparable binding affinities, indicating that both species can effectively stabilize the complex. However, due to its significantly higher population, EO^- is expected to dominate the overall inhibitory effect. In contrast, for BCL-xL, the dianionic form EO^{2-} ($\Delta G_{bind} = -6.06$ kcal mol $^{-1}$) shows somewhat stronger binding compared to EO^- ($\Delta G_{bind} = -5.74$ kcal mol $^{-1}$), although its lower abundance limits its overall contribution under physiological conditions. Overall, the results suggest that the biological activity of EO toward BCL-2 family proteins arises from a combined effect of multiple protonation states, with EO^- acting as the primary contributor due to its predominance in solution. At the same time, the presence of EO^{2-} introduces an additional, structure-dependent contribution, particularly relevant for BCL-xL interactions. This interplay between protonation equilibria and binding affinity highlights the importance of considering acid–base speciation when interpreting docking results in biologically relevant environments.

Structural analysis of the complexes shows that both protonation states, EO^- and EO^{2-} , adopt a similar orientation within the active site of the BCL-2 protein (Figure 10).

The planar aromatic scaffold of the ligand is positioned in proximity to residues A:MET115 and A:LEU137, enabling stabilizing π -alkyl interactions that play a key role in anchoring the molecule within the binding pocket. These interactions significantly contribute to hydrophobic stabilization and ensure proper alignment of the ligand for the formation of additional noncovalent contacts. In both EO forms, hydrogen bonds are primarily established with A:ASP111 and A:TYR108 through the phenolic hydroxyl groups, forming a localized hydrogen-bonding network. These interactions reduce the conformational flexibility of the ligand and enhance the stability of the complex, contributing favorably to the enthalpic component of binding.

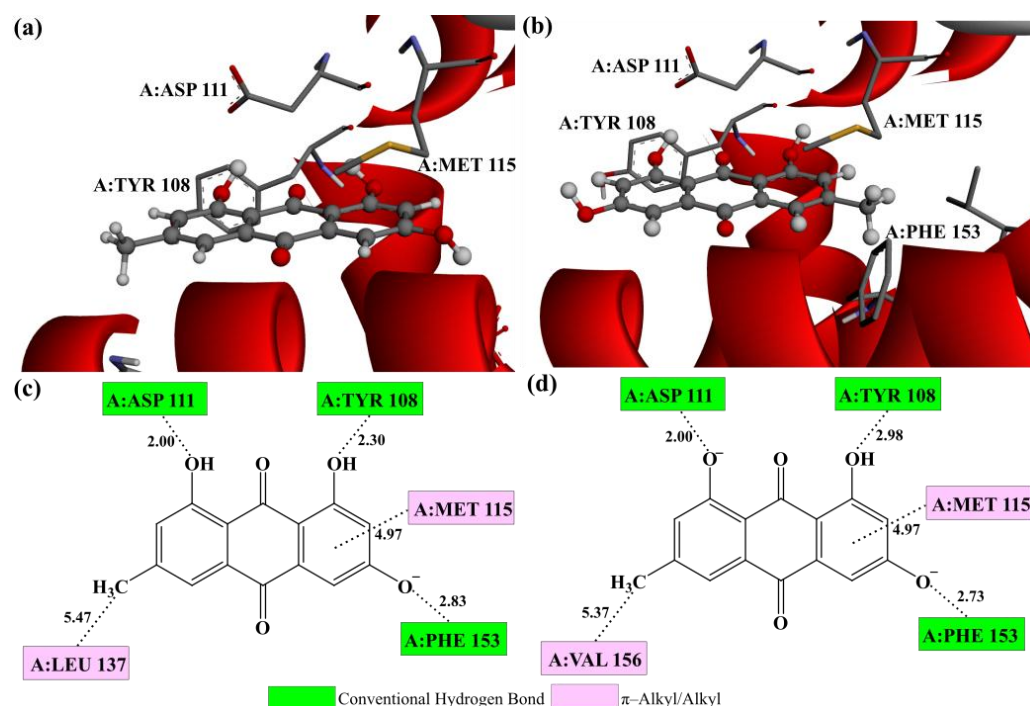


Figure 10. Three-dimensional (up) and 2D (down) representations of the investigated acid-base species of EO^- (a,c) and EO^{2-} (b,d), within the active site of the BCL-2 protein, are presented. The red α -helices correspond to the helical regions of the BCL-2 protein, which play a crucial role in maintaining the protein's structural integrity and overall stability. The green loops and β -turns represent flexible regions of the protein, essential for facilitating conformational changes required for effective ligand binding. Numerical values, expressed in Ångström (Å), denote the interatomic distances, while different colors in the representations correspond to various types of intermolecular interactions, as detailed in the accompanying legend.

Structural analysis of the complexes indicates that both protonation states, EO^- and EO^{2-} , adopt a similar orientation within the active site of the BCL-xL protein (Figure 11). The planar aromatic scaffold of the ligand is positioned in proximity to residue A:TYR101, enabling stabilizing π - π interactions that contribute to anchoring the ligand within the binding pocket. These interactions enhance hydrophobic stabilization and ensure favorable surface complementarity between the ligand and the protein. In both EO forms, hydrogen bonds are primarily established with A:TYR101 through the phenolic hydroxyl and carbonyl groups, forming a stable hydrogen-bonding motif that plays a key role in ligand stabilization. Additionally, interactions with A:ARG100, A:ALA142, and A:ARG139 contribute to the stability of the complex, mainly through π -alkyl interactions that further reinforce binding and restrict ligand mobility within the active site.

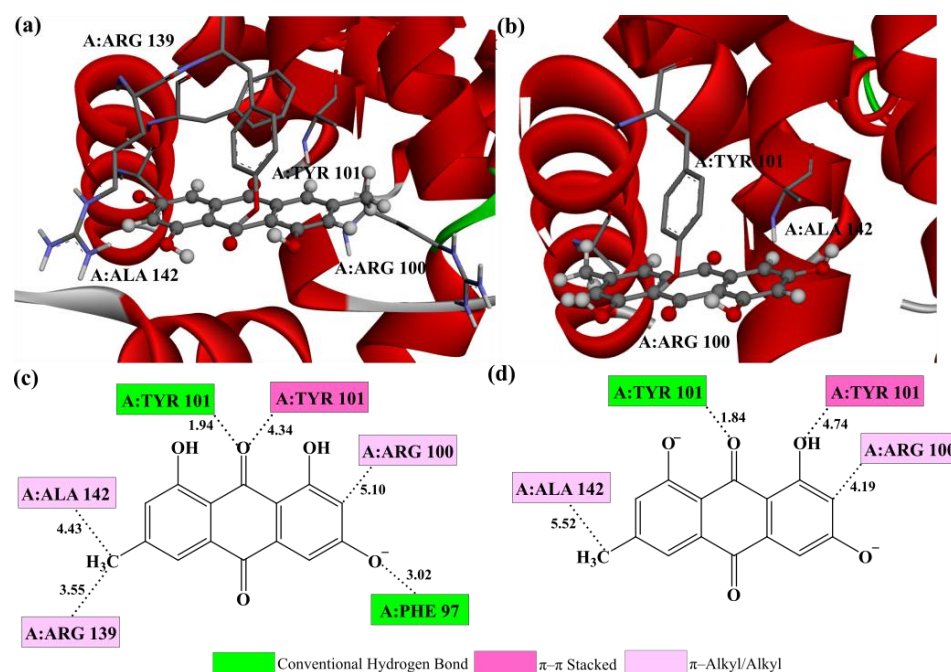


Figure 11. Three-dimensional (up) and 2D (down) representations of the investigated acid–base species of EO^- (a,c) and EO^{2-} (b,d), within the active site of the BCL-xL protein, are presented. The red α -helices correspond to the helical regions of the BCL-xL protein, which play a crucial role in maintaining the protein’s structural integrity and overall stability. The green loops and β -turns represent flexible regions of the protein, essential for facilitating conformational changes required for effective ligand binding. Numerical values, expressed in Ångström (Å), denote the interatomic distances, while different colors in the representations correspond to various types of intermolecular interactions, as detailed in the accompanying legend.

4. Conclusions

This study demonstrates that surface-engineered SBA-15 mesoporous silica nanocarriers represent an effective platform for regulating the intracellular fate of emodin (EO) in PC3 prostate cancer cells. Successful synthesis and fluorescent surface functionalization enabled the development of traceable nanocarriers while preserving the structural integrity and ordered mesoporosity of the SBA-15 framework. Complementary physicochemical characterization confirmed high EO loading (~35%) and efficient confinement of the drug within the mesoporous structure.

Biological evaluation revealed that nanocarrier surface chemistry plays a decisive role in determining the cellular response to EO delivery. Distinct surface modifications produced markedly different intracellular behaviors, release profiles, and biological outcomes. The SBA-15|EO system promoted rapid intracellular EO accumulation and extensive late apoptosis, whereas the functionalized SBA-15-M|EO system induced more gradual intracellular accumulation accompanied by sustained apoptotic progression. Both EO-loaded systems suppressed intracellular ROS levels, indicating that apoptosis induction was largely independent of ROS generation under the investigated conditions. These findings demonstrate that surface functionalization does not merely influence delivery efficiency but actively governs the temporal evolution of cellular responses following nanocarrier internalization.

Time-resolved fluorescence microscopy established a direct link between intracellular trafficking, release behavior, and cytotoxic activity, while flow cytometry confirmed significant differences in apoptosis induction and cell-cycle perturbation between the investigated formulations. Together, these results indicate that nanocarrier-mediated mod-

ulation of intracellular EO availability can influence both the kinetics and mechanism of cell death.

Molecular docking studies further revealed favorable interactions of EO with the anti-apoptotic proteins BCL-2 and BCL-xL, suggesting a potential contribution of mitochondrial apoptotic signaling to the observed biological effects. Although additional mechanistic studies are required to fully elucidate these pathways, the computational findings are consistent with the experimentally observed apoptotic responses.

Overall, this work establishes a direct structure–function relationship between surface functionalization, intracellular trafficking, ROS dynamics, and apoptotic progression. More importantly, it demonstrates that rational surface engineering of mesoporous silica nanocarriers can be used not only to improve drug delivery but also to regulate intracellular drug fate and thereby control the dynamics of cancer cell death. These findings provide new insights into the design of next-generation nanomedicine platforms in which surface properties actively direct biological outcomes following cellular internalization.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/nano16150905/s1>. Table S1. Composition and designation of the SBA-15-based nanomaterials investigated in this study. Figure S1. FTIR spectrum of the pristine SBA-15 mesoporous silica. Figure S2. FTIR spectrum of the pristine SBA-15-NH₂ mesoporous silica. Figure S3. FTIR spectrum of the pristine SBA-15-M mesoporous silica. Figure S4. FTIR spectrum of the pristine SBA-15|EO mesoporous silica. Figure S5. FTIR spectrum of the pristine SBA-15-M|EO mesoporous silica. Figure S6. Thermogravimetric (TGA) and Differential Scanning Calorimetry (DSC) profiles of SBA-15-based MSN samples: (a) SBA-15 (left), (b) SBA-NH₂ (right). The red curve represents TGA (mass loss), while the black curve corresponds to DSC signals. Video S1. Time-lapse fluorescence microscopy of SBA-15M uptake in PC3 cells over 18 h. Blue fluorescence corresponds to the functionalized SBA-15M carrier and demonstrates rapid cellular uptake, progressive intracellular accumulation, pronounced morphological changes, and a reduction in cell density over time. Video S2. Time-lapse fluorescence microscopy of EO-loaded SBA-15 nanomaterials in PC3 cells over 18 h. Green fluorescence corresponds to emodin (EO), illustrating rapid cellular uptake, progressive intracellular accumulation, pronounced morphological changes, and a reduction in cell density over time. Video S3. Time-lapse fluorescence microscopy of EO-loaded SBA-15-M nanomaterials in PC3 cells over 18 h. Green fluorescence corresponds to emodin (EO), whereas blue fluorescence corresponds to the functionalized SBA-15-M carrier, demonstrating gradual intracellular accumulation and a more controlled intracellular distribution of the nanomaterial.

Author Contributions: All authors contributed substantially to the work. Conceptualization, Ž.M. and G.N.K.; methodology, Ž.M., P.J., P.E., S.Č., E.F. and G.N.K.; investigation, P.J., P.E., N.Đ.P., E.F., Ž.M. and G.N.K.; resources, L.A.W., Ž.M. and G.N.K.; formal analysis, P.J., P.E., S.Č., Ž.M. and G.N.K.; writing—original draft preparation, P.J., Ž.M. and G.N.K.; writing—review and editing, P.J., P.E., N.Đ.P., S.Č., E.F., L.A.W., Ž.M. and G.N.K.; visualization, P.J., S.Č. and Ž.M.; supervision, L.A.W. and G.N.K.; project administration, Ž.M. and G.N.K.; funding acquisition, L.A.W. and G.N.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding

Acknowledgments: This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Agreement No. 451-03-33/2026-03/200378) and the German Academic Exchange Service DAAD PPP (Project Number: 57656312).

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Conflicts of Interest: The authors declare no conflicts of interest.

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