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RESEARCH PAPER

Synthesis of new biodegradable nanocarriers for SN38 delivery and synergistic phototherapy

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ABSTRACT

Objective (s): SN38 is the prominent and effective anticancer drug for treating various types of human cancers such as colorectal, ovarian and lung cancers. SN38 is highly toxic, and due to its poor solubility in aqueous media, and low stability and hydrolysis at physiological pH, it has not been used as an anti-cancer drug. To overcome these problems, SN38 was conjugated with new nanocarriers in order to efficiently deliver it into cancer cells.

Materials and Methods: We report the synthesis of nanocarriers based on covalent attachment of graphene oxide with β-cyclodextrin and coordinated with superparamagnetic iron oxide (SPION) nanoparticles for SN38 loading and delivery. Using SPION-functionalized graphene oxide provides magnetic properties and local hyperthermia due to laser irradiation at 808 nm. Structures were characterized by using FT-IR spectroscopy and the size and morphology of the nanoparticles were determined using Malvern Zetasizer and FE-SEM, respectively.

Results: The prepared nanoplatform was not significantly toxic to HT-29 cells. However, the developed graphene oxide-based nanocarrier containing SPION and SN38 was used to improve the chemotherapy through photothermal and photodynamic therapy. The optimal laser wavelength (808 nm) for PTT is consistent with that of IR-808 for PDT and the β-CD-GO-EDTA-Fe3O4-SN38 indicated synergistic effects of both drugs and SPION.

Conclusion: Biocompatible nanocarrier based on functionalized graphene oxide (β-CD-GO-EDTA-Fe_3O_4-SN38) demonstrated strong synergistic cytotoxic activity. The new formulation has a great potential as chemo-photothermal-photodynamic therapy agents in synergistic phototherapy.

Keywords : *Cyclodextrin, Drug Delivery, EDTA, Graphene oxide, SN38*

How to cite this article

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INTRODUCTION

SN38(7-ethyl-10-hydroxycamptothecin)is the metabolite of irinotecan (CPT-11) which is approximately 100-1000 times more potent in inhibiting cancer cells than the parent drug [1]. Only a small proportion (2-8%) of irinotecan is converted to SN38 by carboxylesterase in the liver and tumor. Thus, SN38 could be an excellent candidate for treating various types of human cancers including lung, colorectal, ovarian and breast [2, 3]. Despite the high potency of SN38, this drug has poor solubility in aqueous solution and low stability at physiological pH because of its ultra-flat aromatic structure [4], which render its use as an anti-cancer drug [5]. The aim of using nanocarriers for SN38 delivery is to overcome such problems. One of nanocarrier used for drug delivery purposes is graphene. The surface area of graphene and its derivatives are considerably higher than any other nanomaterial, and strong antibacterial effects and lower toxicity making them potential candidates for drug delivery and bioimaging systems [6, 7]. The surface of graphene

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oxide (GO) contains multiple oxygen-containing functional groups such as hydroxyl, epoxy, carbonyl and carboxyl groups which could be readily modified for the covalent attachment of drug and bioactive molecules [8]. Other advantages of using GO is its unique intrinsic optical properties such as near infrared photoluminescence and optical absorption for live cell imaging and photothermal therapy, respectively [9]. α-, β- and γ-cyclodextrins (CD) are polysaccharides containing six, seven and eight glucose units, respectively. The external surface of cyclodextrins is hydrophilic while the internal core is hydrophobic. This property allows for the formation of guest-host relationships with small hydrophobic molecules. Functionalized graphene oxide with cyclodextrins improves both their physical and chemical properties improving the bioavailability and increasing the solubility of hydrophobic materials. Moreover, drug stability against heat, light, and oxygen and also protecting the cargo drug from degradation are other advantages of CD-functionalized GO [10]. Among cyclodextrins, β-CD is very interesting carbohydrate oligomer and is widely applied in various biological systems. In the current study, we synthesized a new class of β-CD functionalized graphene oxide conjugated with SN38 and Fe_3O_4 providing high chemo-photo therapy effect. Super paramagnetic iron oxide (SPION) due to its excellent magnetic properties, biocompatibility, and mobility in targeted area, has attracted great attention. Nano magnetic carriers are used for magnetic resonance imaging (MRI) [11], hyperthermia-induced cancer therapy and targeted drug delivery [12]. There are some reports on the noncovalent attachment of SN38 to graphene oxide. We recently reported the synthesis of non-covalent attachment of SN38 to CDs-GO and evaluated its biological activity. Liu et al. synthesized complex which contains SN38 grafted on PEGylated graphene through π,π stacking [13, 14]. However, no studies of the covalent binding of SN38 with graphene oxide has been reported so far. The goal of the current study was to develop GO-based nanocarrier to improve SN38 delivery to the cancer cells. A prodrug was obtained by covalent attachment to the active drug molecule (SN38) by ester bond. For this purpose, we used EDTA (ethylene diamine tetraacetic acid) linker for the conjugation of SN38 with β-CD-GO and coordination with Fe_3O_4. We hypothesized that this structural modification can improve the physicochemical, biopharmaceutical and pharmacokinetic properties of the drug [15]. Another advantage of this modification is the presence of graphene oxide and SPION nanoparticles as excellent therapeutic agents in tumors. Photodynamic therapy (PDT) is a clinical strategy that is based on the interaction of photosensitizing agent with reactive oxygen after irradiation for treating cancer. Photothermal therapy (PTT) is another type of phototherapy in which agents adsorb light and convert infrared light into heat quickly and efficiently to kill cancer cells [16]. The graphene oxide sheets produced large amount of reactive oxygen and used as topical hyperthermia in NIR irradiation at 808 nm while SPION nanoparticles are used as promising candidates for photothermal therapy (PTT) [17, 18].

MATERIALS AND METHODS

SN38 was purchased from Medkoo, USA. EDC(1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) was purchased from Fluka, Japan. DMAP (4-dimethylamino pyridine) was obtained from Aldrich, USA. EDTA (ethylenediaminetetraacetic acid) and DMSO (dimethyl-sulfoxide) were supplied from Merck, Japan. β-Cyclodextrin was obtained from Sigma-Aldrich, USA. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from Sigma-Aldrich, Germany.

Synthesis of GO and CD-GO

The preparation of GO was performed using a previously reported modified Hummer's method [19]. We designed a facile method for the synthesis of β-CD-GO by the esterification using EDC/DMAP as catalyst [14]. Briefly, GO (0.03 g) and EDC (0.15 g) were dispersed in water and stirred for 45 min and then, DMAP (0.03 g) and β-CD (0.3 g) were added. The mixture was stirred for 4 days. The product was washed and centrifuged and then freeze-dried.

Synthesis of superparamagnetic iron oxide nanoparticle (SPIONs)

SPION was prepared using chemical co-precipitation method [20]. Briefly, $FeCl_2.4H_2O$ (3.18 g) and $FeCl_3.6H_2O$ (7.57 g) were dissolved in 200 mL of deionized water. Thereafter, the solution was stirred under nitrogen gas for an hour at temperatures up to 80 °C. Then, 40 mL of aqueous ammonia was added slowly and continued for another 1 hr under nitrogen. The reaction was cooled down to room temperature. Then, the

product was washed once with ultrapure water and centrifuged at 9000 rpm for 10 min. Finally, the precipitated solid material was freeze-dried.

Covalent attachment of SN38 to cyclodextrin-graphene oxide complex (β-CD-GO-EDTA-SN38)

EDTA (25 mmol) and EDC (76 mmol) were dissolved in distilled water (10 mL). Then the reaction was stirred for 1 hr at room temperature. Thereafter, SN38 (25 mmol) was dissolved in DMSO (5 ml) and DMAP (25 mmol) was added to the mixture. Then, it was refluxed for 4 days at 25 °C. Afterward, EDC (76 mmol) and DMAP (25 mmol) were added and the mixture was stirred for another 1 h. Then, β-CD-GO (10 mg) was dispersed in 2 ml water with sonicated for 5 min using probe sonicator (Hielscher, Germany). The reaction was refluxed for another 4 days at 25 °C.

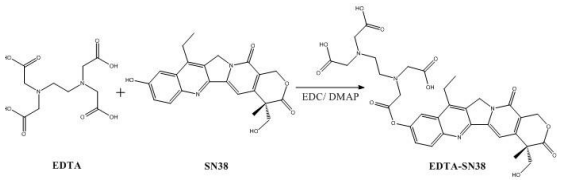

Fig 1. Covalent attachment of SN38 to EDTA

Conjugation of Fe_3O_4 (SPION) to β-CD-GO-EDTA-SN38

Fe_3O_4 (25 mmol), which was previously synthesized, was added to the aforementioned mixture of β-CD-GO-EDTA-SN38 and the reaction mixture was allowed to stir for overnight at room temperature.

After washing three times with 10 mL of deionized water and centrifugation at 1000 rpm for 5 min, the product was suspended in water. Then the product was dialyzed against deionized water for 24 h to remove the unconjugated SN38. The product was freeze-dried and stored for further use.

Physicochemical characterization

Fourier transform infrared spectra (FT-IR) was successfully used for the confirmation of the ester bond formation between β-CD-GO and EDTA and SN38. FTIR spectra (Shimadzu-Japan) were recorded in KBr disk from 4000 to 500 cm^{-1}. The concentration of SN38 was measured by a UV-Vis. spectrophotometer (Varian CARY 100 UV/Vis spectrophotometer, California, USA).

The structure and morphology of the products were characterized by using field emission

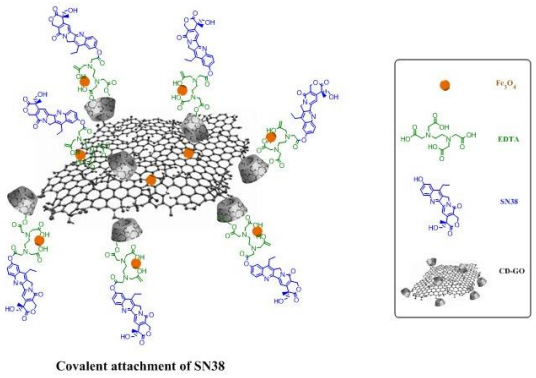

Fig 2. Schematic presentation of covalent conjugation of SN38 to the fabricated nanoplatform

electron microscope (FE-SEM), Mira III FEG, TESCAN-UK, Ltd.

To evaluate the hydrodynamic diameter and zeta potential of β-CD-GO and β-CD-GO-EDTA-Fe_3O_4-SN38 conjugate, we used Malvern ZetaSizer (Malvern Instruments, UK). The samples were prepared by dispersing the particles in deionized water (1 mg/mL) and each compound was tested in triplicate.

Drug content measurement

Drug content (DC%) is especially important for clinical studies and biological applications. We evaluated the loading efficiency by quantifying of UV absorbance of the formulations at 390 nm after dialyzing the product against deionized water for 24 h and freeze-dried and dissolved in DMSO using the mentioned equation:

$$DC\,(\%) = \frac{\text{mass of SN38 in the formulation}}{\text{Total mass of final formulation}} \times 100$$

The drug content was quantified by Beer-Lambert equation using extinction–coefficient (ε) of SN38 which is 25500 $L.mol^{-1}.cm^{-1}$

In vitro release assay

To examine the *in vitro* drug release of SN38 from β-CD-GO-EDTA-Fe_3O_4-SN38 conjugate in PBS (pH 7.4) and citrate buffers (pH 5.4), 1 mL of β-CD-GO-EDTA-Fe_3O_4-SN38 (0.25 mg/mL) was transferred into a dialysis bag and immersed in 200 mL of either PBS or citrate buffer with continuous and gentle stirring in a shaker incubator (60 rpm, 37 °C). At selected time intervals, aliquots (200 µL) of the aqueous solution were collected and the external media was immediately replaced with fresh release media. The concentration of

remaining SN38 in collected samples at various times was estimated by UV/Vis spectrophotometer at a wavelength of 390 nm. All experiments were measured in triplicate.

In vitro cytotoxicity assay

To evaluate the cytotoxicity of the synthesized SN38-loaded formulation (covalently attached SN38 to β-CD-GO-EDTA-Fe_3O_4-SN38), it was incubated with HT-29 cell line. Briefly, HT-29 human adenocarcinoma cells (10000 cells per well) were seeded in 96-well culture plates. 24 h later, cells were exposed to 100 µL of fresh solution of various concentrations of either nanocarrier (15.6–250 µg/mL), or nanocarrier conjugated with SN38 (SN38 concentration of 1–20 µg/mL) for 24 h at 37 °C. Subsequently, the medium was replaced with 20 µL MTT solution (0.5 mg/mL in PBS).

After 4 hr of incubation, the MTT solution was removed and DMSO (200 mL) was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (TECAN Infinite M200, Switzerland).

Synergistic activity of β-CD-GO-EDTA-Fe_3O_4-SN38

We also evaluated the combined chemo-photothermal-photodynamic effect of the β-CD-GO-EDTA-Fe_3O_4-SN38 formulation. To determine the cell growth inhibition by the MTT assay, HT-29 cells were seeded in 24-well plates at a density of 1000 cells per well, and were incubated with either covalently attached SN38 formulation or free SN38 for 2 hr at various concentrations of 5 and 10 µg/mL, followed by exposure to laser irradiation at 808 nm 2 w cm^{-2}) for 5 min. The optimal laser wavelength (808 nm) for PTT is constant with that of IR-808 for PDT [18].

Statistical analysis

Statistical significance was determined by One-way ANOVA (analysis of variance) test using Prism 6 software and the P-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Synthesis and characterization of the β-CD-GO-EDTA-Fe_3O_4-N38 formulation

Although SN38-GO conjugate was previously used in another study [14] but there was no detailed study on covalent attachment of SN38 to GO. In the current study, we benefited from the use of composite consisted of graphene oxide, β-cyclodextrin, and Fe_3O_4 as drug carrier and use of SN38 as a hydrophobic drug attached to GO-CD through EDTA as a linker. The FTIR spectra are useful in depicting the interaction between β-CD-GO and drug molecule. Infrared spectra of the complexes β-CD-GO-Fe_3O_4-SN38, β-CD-GO-EDTA-SN38 and β-CD-GO-EDTA-Fe_3O_4-SN38 were analyzed using Shimadzu Fourier transform infrared spectrophotometer (Japan), scanning between 450 and 4000 cm^{-1}.

The FTIR spectra of all composites showed obvious characteristic absorption peaks corresponding to β-CD and GO including O–H (3200–3700 cm^{-1}), C–O in epoxy groups (1050 and 1220 cm^{-1}), COOH (1730 cm^{-1}), C=O (1380 cm^{-1}), C–H asymmetric stretching vibrations (2925 cm^{-1}) and (C=C) sp^2 carbon skeletal network (1400–1600 cm^{-1}), glycosidic C–O–C (1030 cm^{-1}) and R-1, 4-bond skeleton vibration of CD (942 cm^{-1}).

The FTIR spectrum of SN38 has been reported before in other studies [21, 22]. SN38 showed sharp absorption peak at 3550 cm^{-1} corresponding to the O–H stretching vibrations of phenolic hydroxyl and the strong peak at 1732 cm^{-1} and 1168 cm^{-1} corresponding to stretching vibrations of COOH and C–O, respectively and multiple absorption bands between 1400 and 1600 cm^{-1} was attributed to aromatic C=C stretching [10]. During the formation of covalent product (CD-GO-EDTA-SN38 and CD-GO-Fe_3O_4-EDTA-SN38), the SN38 characteristic peak at 3550 disappeared which asseverate a broken O–H band and formation of the covalent bond with EDTA. However, this band still existed in the noncovalent product (CD-GO-Fe_3O_4-SN38). This result confirmed the successful conjugation of SN38 to GO through EDTA as linker. The unique peak at 570 cm^{-1} corresponds to F–O bands stretching and confirmed the presence of Fe_3O_4 NPS in both covalent/noncovalent complexes.

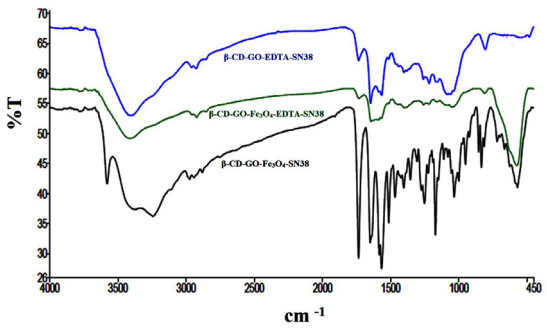

Fig 3. FTIR spectra of β-CD-GO-Fe3O4-SN38, β-CD-GO-EDTA-SN38 and β-CD-GO-EDTA- n Fe3O4-SN38

Both size and morphology of the newly synthesized nanomaterials were investigated using SEM. To prepare samples, one drop of the aqueous samples solution was deposited onto the aluminum foil and dried and sputter coated with gold. Figure 4 represented the SEM images of GO, β-CD-GO and β-CD-GO-EDTA-SN38-Fe_3O_4. SEM image of GO shows some wrinkles on the sheet-like surface (Fig 4A) while the β-CD-GO image indicates that cyclodextrin covered the rough surface of GO (Fig 4B). SEM image of β-CD-GO-EDTA-SN38-Fe_3O_4 confirmed homogeneous spherical shapes on the flat structure of GO, which could be attributed to the attachment of different nanoparticle on the surface of GO (Fig 4C).

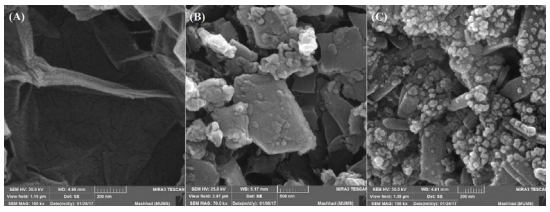

Fig 4. SEM images of GO (a), β-CD-GO (b) β-CD-GO-EDTA-Fe3O4-SN38 (c)

The particle size of new formulations GO with acceptable size distribution was shown in Table 1. The particle size and zeta potential were measured by Malvern Nano ZS instrument and DTS software. The mean particle size of the product and β-CD-GO were approximately 127 and 117 nm, respectively and the zeta potential measurement for two samples indicated negative values.

Table 1. Average particle size and zeta potential of β-CD-GO and β-CD-GO-EDTA-Fe3O4-SN38.

Sample	Average size(nm)	Zeta potential(mV)
β-CD-GO	116.2±13.92	(-14.96)±1.67
β-CD-GO-Fe_3O_4-EDTA-SN38	126.8±27.313	(-13.63)±0.75

Drug content and release study

We evaluated the loading efficiency of the composite which was covalently attachment to SN38. In the covalently attached formulation, SN38 solution in DMSO was conjugated to EDTA solution in deionized water *via* esteric bond formation. Then the complex was covalently attached to β-CD-GO at room temperature. After dialysis, the UV absorbance of the loaded SN38 was evaluated using UV–Vis spectroscopy at 390 nm. The drug content of covalently attached formulation for β-CD-GO-EDTA-Fe_3O_4-SN38 was 11%.

Cancerous tissue is slightly acidic (pH of 6.5-7.2) but the pH of endosomes is 5.5-6.8 [23]. Besides, it is known that after endocytosis of the nanoparticles, they are entered into the endosomes which are finally matured into acidic vesicles and might/might not fuse with lysosomes. The intracellular fate of the endosomal content is significantly important for successful drug delivery. The release of the chemotherapeutic agent in early endosomes provides their efficient cytotoxicity. In the current study, we used model buffers at pH 5.5 and 7.4 to simulate the pH of endosomes of the tumor cells and the blood, respectively and to determine the release behavior of drug from the produced structure [24]. Next, *in vitro* drug release profile of β-CD-GO-EDTA-Fe_3O_4 covalently attachment to SN38 at pH 5.5 and pH 7.4 were measured for 8 days and the results were shown in Fig 5 and 6, respectively.

Drug release profiles were shown to be pH dependent. The release of SN38 from β-CD-GO-Fe_3O_4-EDTA-SN38 formulation at physiologic pH is more than that in acidic pH which could be attributed to the solubility of SN38 under neutral conditions.

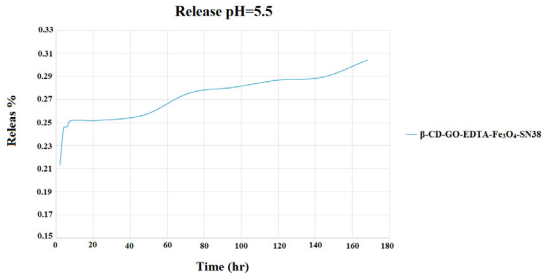

Fig 5. Release profiles of β-CD-GO-EDTA-Fe3O4-SN38 in citrate buffer (pH 5.5)

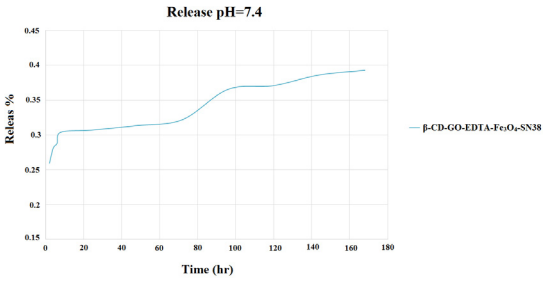

Fig 6. Release profiles of β-CD-GO-EDTA-Fe3O4-SN38 in PBS (pH 7.4)

Cytotoxicity of β-CD-GO-EDTA-Fe_3O_4-SN38

We evaluated the cytotoxicity of covalently attached SN38 to GO-CD composite. The results

of the *in vitro* cytotoxicity of β-CD-GO-Fe_3O_4-EDTA-SN38, and free SN38 on HT-29 cells using MTT assay are depicted in Figure 9. Our previous results indicated that CD-GO and GO were relatively not toxic to HT-29 cells above 62.5 µg/ml [14]. In another word, nanocarrier alone did not show cytotoxic activity against the HT-29 cells. The cytotoxicity results of covalently attached SN38 to nanocarrier (β-CD-GO-Fe_3O_4-EDTA-SN38) at various concentrations of SN38 showed equal cytotoxicity in comparison with free SN38 and it significantly increased when chemo-photothermal therapy was employed. The combined chemo-photothermal effect of each formulation was indicated in Figure 8. The PTT/PDT results confirm that β-CD-GO-Fe_3O_4-EDTA-SN38 showed highest cytotoxic effects on HT-29 cells.

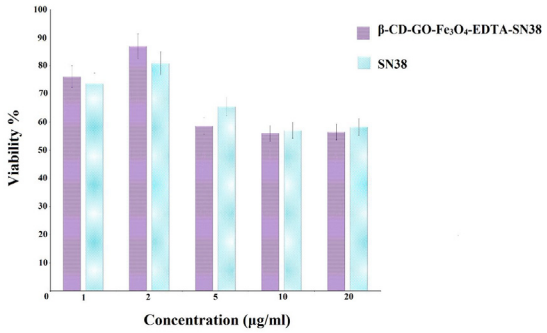

Fig 7. Cytotoxicity of β-CD-GO-EDTA-Fe3O4-SN38 on HT-29 cell line after 24 h of incubation

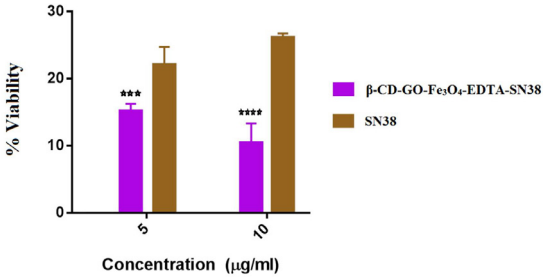

Fig 8. PDT and PTT effect of β-CD-GO-EDTA-Fe3O4-SN38 conjugates on HT-29 cells after 24 h of incubation

CONCLUSION

The aim of the present work was to employ graphene oxide (GO) to improve SN38 drug delivery to the cancer cells. Our strategy was to overcome the challenges in SN38 drug delivery including protecting the drug from degradation and introducing biocompatible formulation. The developed formulation (β-CD-GO-EDTA-Fe_3O_4-SN38) could improve water solubility, provide more biocompatibility and protect the drug by cyclodextrin-functionalized graphene oxide. β-CD-GO-EDTA-Fe_3O_4-SN38 with particle sizes of 127 nm demonstrated strong synergistic activity compared to free SN38 in inhibiting the proliferation of HT-29 cells *in vitro*.

The results of our study showed that the new formulation possessed a great potential as chemo-photothermal-photodynamic therapy agents in synergistic phototherapy.

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