A Targeting Self-breakable Agent for Increased Efficacy of Chemotherapeutic Drugs against Caco2 Cells

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Abstract: Many types of nano-sized anti-cancer agents that could increase efficacy of chemotherapeutic drugs have been created and developed in colon cancer treatment over years. Moreover, with the intention of achieving the ideal chemotherapeutic efficacy, nano-sized anti-cancer agents were further designed to have specific functions, efficiently killing colon cancer cells. Our research team focused on two important functions in designing nano-sized agents, controlled drug release and targeting functions. Thus, targeting functional micelles which entrapped chemotherapeutic drug, 7-ethyl-10-hydroxy-camptothecin (SN38) were designed in nano-size and possessed disulfide bonds in this study. In particular, Self-Breakable SN38-loaded micelles (SN/38 micelles), Non-Breakable micelles SN38-loaded (NB/38 micelles) and Folate-targeting Self-Breakable SN38-loaded micelles (FSB/38 micelles) were prepared and tested to the designed agents. The results showed that the folate-decorated functional micelles with disulfide bonds could be an effective chemotherapeutic agent for colon cancer treatment.

1 INTRODUCTION

Chemotherapy is the most common therapy for colorectal cancer, which is an intractable issue for human beings due to its increased chance of death so cancer studies are still ongoing for developing high-efficacy chemotherapeutic drugs(Bala et al., 2013). A good candidate for drug agents is micelle composed of amphiphilic polymers with nanoscale size allowing accumulation of the micelles in the tumor through enhanced permeability and retention (EPR) effect, thereby resulting in high tumor uptake(Joralemon et al., 2010). However, the efficacy of chemotherapeutic drug-loaded micelles without specific functions is unsatisfactory. Recently, the designed micelles with specific functions, for instance, targeting function(Xu et al., 2013), photodynamic function(Peng et al., 2008), and controlled release function(Peng et al., 2010, Peng et al., 2011b) have been created. Such functional micelles were shown to have greater effectiveness in cancer treatments(Sinn Aw et al., 2014).

Folate which has exhibited outstanding ability in increasing cellular uptake of the loaded drug was chosen as the targeting ligands in this study (Khatik et al., 2015, Cuong et al., 2012). In addition, unlike many other targeting ligands used in chemotherapeutic drugs, folate, is safe for human consumption, and has approved by the US Food and Drug Administration for the use in dietary supplements. Based on these reasons, folate is a good choice for a targeting ligand which can be used to modify micelles, which can efficiently increase the cellular uptake and efficacy of the chemotherapeutic drug.

However, even if the folate-decorated functional micelles increase cellular or tumor uptake of functional micelles, the entrapped drug might fail to release owing to its rigid structure, which results in its lower efficacy(Xing et al., 2015). The folate-decorated functional micelles in this study were further designed for successful drug release which plays an important role in achieving optimal efficacy of chemotherapeutic drugs. This is attributed to the fact that drug must reach drug action site in tumor cell for drug action to take place(Kawato et al., 1991). Great efforts have been made to create controlled release micelles which are self-breakable, particularly redox-responsive functional micelles,

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for the enhancement of the drug efficacy. Disulfide bonds created in such functional micelles quickly react with glutathione (GSH) which renders the micelles unstable, thereby enabling them to release the drug spontaneously (Huo et al., 2014, Lai et al., 2014). Therefore, the folate-decorated functional micelles created in this study were designed to be redox-responsive.

We attempted to enhance efficacy of the chemotherapeutic drug, 7-Ethyl-10-hydroxy-camptothecin (SN38), an active metabolite of the clinical drug, irinotecan (CPT-11) which is used in the treatment of colorectal cancer. For this purpose, Folate-targeting Self-Breakable micelles (FSB micelles) consisting of self-degradable copolymers, and targeting copolymer, were created to setup an active drug delivery system using a colorectal cancer cell line, Caco2. FSB micelles could facilitate Caco2 in acidic microenvironment to take up the loaded SN38, resulting in enhanced drug efficacy. In addition, to confirm that FSB micelle can be the best anti-cancer agent, Self-Breakable micelles (SB micelles) without targeting function and Non-Breakable micelles (NB micelles) which have no specific function were also created to evaluate the designed functions of FSB micelles.

2 EXPERIMENTAL SECTION

2.1 Synthesis of the Self-degradable, Non-self-degradable Copolymers and Targeting Copolymers

The non-self-degradable (ND) copolymers, methoxyPolyEthylene Glycol-PolyCaproLactone, (mPEG-PCL) was synthesized as described in our earlier research(Chen et al., 2015). The self-degradable (SD) copolymers, methoxyPolyEthylene Glycol-S-S-PolyCaproLactone (mPEG-S-S-PCL) was primarily obtained via two chemical reactions. MPEG-SH was reacted with excess 2-mercaptoethanol in deionized water to obtain mPEG-S-S-C_2H_4OH. Then, MPEG-S-S-PCL was obtained via the ring-opening polymerization.

To prepare the targeting copolymer, Folate-Poly(Ethylene Glycol)-Poly(CaproLactone) (F-PEG-PCL), FMOC-NH-PEG-PCL was used to synthesize as described (Peng et al., 2011a). F-PEG-PCL was obtained by conjugating the de-protected polymer, NH_2-PEG-PCL, with folate via an amide bond. The designed copolymers were characterized by ^{1}HNMR, FT-IR, and Gel Permeation Chromatography (GPC) was used to determine the molecular weight (MW) of copolymers.

2.2 Characteristics of Self-breakable, Non-self-breakable, and Targeting Self-breakable Micelles

The NB micelles prepared from ND copolymers and SB micelles prepared from SD copolymers in this study were prepared to evaluate the function of SB micelles in triggering the release of loaded drug in the presence of GSH in cancer cells. The FSB micelles prepared using a mixture containing 80% (w/w) SD and 20% (w/w) targeting copolymers were designed to have targeting and self-breakable function for achieving the best chemotherapeutic efficacy in cancer treatment.

SB micelles, NB/38 micelles, SB/38 micelles, and FSB/38 micelles were prepared using a lyophilization-hydration method. The SN38-loaded micelle formulations containing 10mg/mL of polymer and 1mg/mL of SN38 in PBS were filtered using 0.22μm filter to remove non-loaded SN38. Then, the size of micelles was determined by Transmission Electron Microscopy (TEM), and Dynamic Light Scattering (DLS). Loading Efficiency (LE) and Drug Content (DC) were determined using the calibration curve based on maximum absorption values of SN38 in DMSO. Critical Micelle Concentration (CMC) of the micelles was determined using pyrene as described elsewhere.

2.3 Physical and Chemical Stability of the Micelles

To access whether SB/38 micelles, and NB/38 micelles were self-breakable agents or not, the micelles were incubated with or without 10mM DTT in phosphate buffered saline (PBS) which was used to simulate glutathione (GSH) in cells. Incubation with or without DTT was conducted at 37°C over specific time periods. Then, the micelle stability was determined by their size and polydispersity (PdI). At select time points, the size and PdI of the micelles were determined by DLS.

To prove that disulfide bonds designed in the SD copolymers could be broken up by GSH in the cells, the MW of copolymers self-assembling into SB micelles were analysed via GPC after the incubation with 10mM DTT. In brief, SB/38 micelles were lyophilized after incubation at 37°C for 24 h. The lyophilized SB/38 micelles were dissolved in THF,

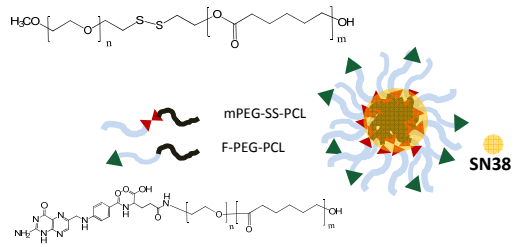
and then the MW of the polymers of SB/38 micelles was determined via GPC.

2.4 Drug Release Profile

In vitro SN38 release profiles of SB/38 and NB/38 micelles, dispersed in PBS with or without DTT, were analysed using a modified dialysis-bag diffusion technique at 37°C. The dialysis tube containing 0.4 mL of the micelle formulation was suspended in 100 ml PBS in a closed bottle. A magnetic mixer was introduced into the bottle and incubated at 37°C. Every 1ml of aliquot was withdrawn from the external media and refilled with 1ml of fresh PBS at select time intervals. The SN38 concentration was determined by fluorescence intensity at 427nm (excitation at 390nm). All experiments were conducted in triplicate.

2.5 In Vitro Cytotoxicity

The human colon cancer cell line, Caco2, was cultured in a humidified 5% CO_2 incubator at 37 °C in Minimum Essential Media, MEM (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 20% heat-activated fetal bovine serum (FBS), 1% non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, and 1% (v/v) Penicillin-Streptomycin Amphotericin B Solution (GIBCOBRL). Initially, the Caco2 cells were seeded onto 96-well plates at a density of 10,000 cells per well and cultured. After 24 h, cells were incubated in media containing different concentrations of SN38 for 6h. Then, the cells were washed three times with PBS to remove the suspended SN38 and cultured with fresh medium for another 48 h. Cell viability was assessed using MTT assay with a scanning multi-well ELISA reader (Microplate Autoreader EL311, Bio-Tek Instruments Inc., Winooski, VT, USA). The cytotoxicities of SB micelles, NB micelles, FSB/38 micelles, SB/38 micelles and NB/38 micelles were also evaluated by the same method.



Scheme 1: Structure of a targeting self-breakable drug-loaded micelle.

3 RESULTS AND DISCUSSION

3.1 Synthesis of the Self-degradable, Non-self-degradable Copolymers and Targeting Copolymer

The FSB micelles composed of SD copolymers and targeting copolymers were used as a targeting self-breakable agent for the enhancement of drug efficacy and were loaded with the chemotherapeutic drug, SN38, used to treat colon cancer in this study (scheme 1). The ^{1}H-NMR results revealed that SD copolymer was successfully synthesized and had a MW of 8,530 g/mol (data not shown). GPC analysis indicated SD copolymer had a molecular weight, 11,253 g/mol, and a narrow PolyDispersity (PD) of 1.15 (Table 1). The FT-IR spectra showed the linkage of NH_2PEG-PCL with folate via an amide bond which indicated the successfully synthesis of F-PEG-PCL (Figure 1).

3.2 Characteristics of Self-breakable, Non-self-breakable, and Targeting Self-breakable Micelles

The characteristics of the SB/38, NB/38 and FSB/38 are shown in Table 2. In terms of the size, the size of NB/38 micelles, NB/38 micelles, and FSB/38 micelles were determined to be all about 130nm at 10:1 ratio of polymer/drug in PBS. The TEM images further supported this finding as the results show that the actual sizes FSB/38 micelles used as a targeting self-breakable agent were the same as that determined via DLS (Figure 3). Comparison of the in vitro and in vivo test results involving the use of each of these three SN38-loaded micelles ruled out the possible issues associated with the difference in size, perhaps due to their similarity in size. In addition, owing to their uniform nano-size, these SN38-loaded functional micelles could successfully accumulate in the tumor via the EPR effect and thus the enhance efficacy of drug, SN38.

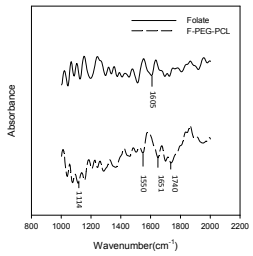


Figure 1: FT-IR spectra of folate-PEG-PCL and folate.

Table 1: Molecular characteristics of mPEG-S-S-PCL, mPEG-5,000, and SB/38 micelles incubated with DTT for 24 h.

Copolymer/micelle	Mn	Mw	Mp	P.D.
mPEG-S-S-PCL	9,742	11,253	13,699	1.15
mPEG-5000	6,679	7,015	7,606	1.05
SB/38 micelles (mPEG-S-S-PCL) +10mM DTT	7,115	7,792	7,569	1.08

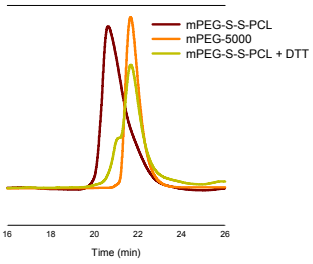


Figure 2: GPC elugram of mPEG-S-S-PCL, mPEG-5,000, and SB/38 micelles incubated with DTT for 24 h.

3.3 Physical and Chemical Stability of the Micelles

To prove that SB micelles can be a self-breakable agent, the SB/38 micelles, and NB/38 micelles were incubated with DTT which was used to simulate GSH with thiol groups in cells. SB/38 micelles with DTT became larger than those without DTT over time (Figure 4A). The PdI data shown in Figure 4B indicated that SB/38 micelles with DTT had a wide range of particle distribution (PdI : over 0.3) 3h after the start of the test. As expected, these results were due to the thiol groups in DTT reacting with disulfide bonds in SB/38 micelles, causing SB/38 micelles to be relatively unstable and aggregate. In contrast, the presence of DTT did not affect the stabilities of NB/38 micelles during the course of the whole experiment. Compared with SB/38 micelles, NB/38 micelles remained stable.

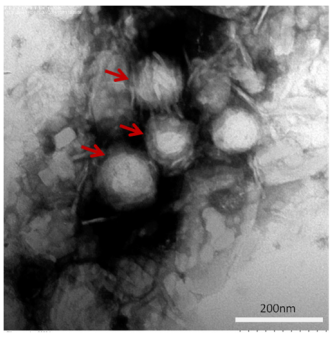


Figure 3: TEM image of FSB/38 micelle.

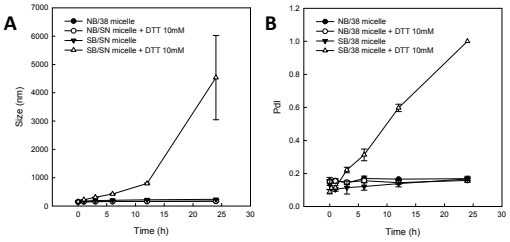


Figure 4: Stability of SB/38 micelles or NB/38 micelles incubated with or without DTT for 24h was determined by DLS in terms of size (A) and PdI (B).

Moreover, the GPC analysis (Table 1 and Figure 2) was performed to confirm that SB micelles composed of mPEG-S-S-PCL could be a self-breakable agent indicated that the disulfide bonds designed in SB copolymers were broken up by DTT after incubation of the SB/38 micelles with DTT, resulting in a significant decrease in molecular weight.

3.4 Drug Release Profile

Drug release profiles of SB/38 and NB/38 micelles with or without DTT were conducted to determine the micelle's ability to release drug. As shown in Figure 5, only SB/38 micelles successfully released SN38 with DTT over time. DTT reacted with the disulfide bonds in SB/38 micelles, which resulted in a significant drug release. In contrast, the other micelles released little amount of SN38 with or without DTT (i.e., < 5% of SN38 released) over 96h. This implies NB/38 micelles were relatively stable regardless of the presence or absence of DTT. These results are in accord with those obtained via DLS and GPC informed us that unstable SB/38 micelles will release drug. This proved again the efficacy of SB/38 micelles to be used a potent drug for colon cancer treatment.

Table 2: Characteristics of NB/38 micelle, SB/38 micelle, and FSB/38 micelle.

Micelle	Size (nm)[a]	PdI[a]	LE(%)[b]	DC(%)[c]	CMC [d] (wt%)
NB/38	130.0±2.1	0.14±0.03	94±4.3	8.6±0.21	0.0021
SB/38	132.4±1.2	0.10±0.04	93±4.1	8.5±0.13	0.0025
FSB/38	131.5±2.3	0.13±0.02	92±3.9	8.4±0.24	0.0023

3.5 In Vitro Cytotoxicity

To evaluate the cytotoxicity of the free drug, the designed nano-sized agents, free SN38, SB micelles, NB micelles, SB/38 micelles, NB/38 micelles and FSB/38 micelles were incubated with Caco2 cell

under conditions mimicking in vivo tumor environment at a low pH(Vaupel et al., 1989, Estrella et al., 2013). No toxicity was observed over 24 h in NB micelles and SB micelles (data not shown), which confirmed that SB micelles could be nontoxic owing to their biocompatibility. Free SN38 achieved the highest efficiency in killing cancer cells, which was expected in this study, as it is known to be the most toxic in vitro in cellular experiments (Figure 6B). However, it is not clinically used. Among the designed anti-cancer drugs without a targeting function, the toxicity of SB/38 micelles was significantly higher than that of NB/38 micelles which was due to their successful self-controlled drug release. Regarding to FSB/38 micelles, it was found that they were able to achieve the highest level of effectiveness in killing cancer cells among the anti-cancer drugs studied. This can be attributed to the fact that FSB/38 micelles had decorated-folate on their surface which caused the Caco2 cells to take up more FSB/38 micelles, resulting in the much higher efficacy of SN38. In addition, to evaluate the effect of medium pH on the cytotoxicity of FSB/38 micelles, a comparison of the cytotoxicity of FSB/38 micelles at a medium pH of 7.4 with that of FSB/38 micelles at a medium pH of 6.7 or 6 was conducted. The highest cytotoxicity of FSB/38 micelles was observed at pH 6 (Figure 6A). This could be

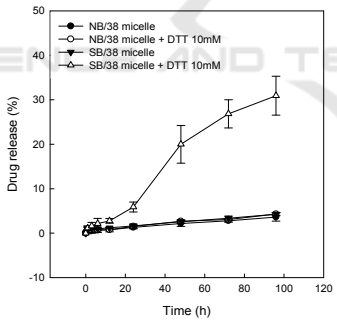


Figure 5: SN38 release profile.

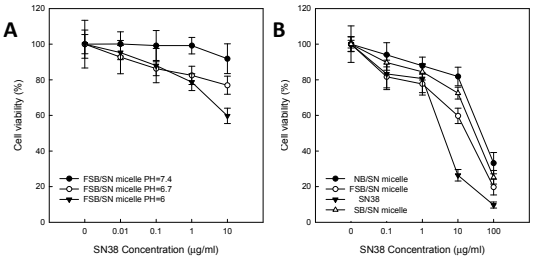


Figure 6: Cell viability of FSB/38 micelles was evaluated in media with different pH values, pH = 6, 6.7, and 7.4 (A). Cell viability of SN38, NB/38 micelles, SB/38 micelles, and FSB/38 micelles (B).

attributed to the fact that Caco2 cells quickly took up folate in the medium at low pH, resulting in more uptake of the folate-decorated FSB/38 micelles. These results show that FSB/38 micelles could be an effective drug for colon cancer treatment.

4 CONCLUSIONS

In this study, the nano-micelles were designed to be self-breakable micelles, called SB/38 micelles. The intention of creating SB/38 micelles was to improve the drug release and enhance drug efficacy. The results of DLS and GPC prove that SB/38 micelles disassembled with DTT which was used to simulate GSH with thiol groups in the cells, resulting in drug release. Furthermore, the release profiles showed that not only SB/38 micelles successfully released SN38, but also a great amount of SN38 was released with DTT. To effectively kill cancer cells and thus ensure better results of cancer treatments, a targeting smart anti-cancer agent, FSB/38 micelles, which consisted of 80% SD and 20% targeting copolymers were successfully designed and produced. It was then confirmed that FSB/38 micelles are an ideal anti-cancer drug through cytotoxicity experiments and cellular uptake experiments. The *in vitro* cytotoxicity results showed that FSB/38 micelles achieved the best effectiveness in killing colon Caco2 cells among the designed anti-cancer drugs. Hence, these findings confirm the efficacy of FSB/38 micelles as an effective chemotherapeutic drug for colon cancer treatment.

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