Cholesterol modification of an anticancer drug for efficient incorporation into a supramolecular hydrogel system

Communication

Cholesterol-modification of an anti-cancer drug for efficient incorporation into a supramolecular hydrogel system

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Treatment of cancer in the peritoneal cavity may be improved with macroscale drug delivery systems that offer control over intraperitoneal concentration of chemotherapeutic agents. Currently, suitable drug carriers to facilitate a sustained release of small hydrophilic drugs such as mitomycin C are lacking. For this purpose, a pH-responsive supramolecular hydrogel based on ureido-pyrimidinone (UPy) chemistry is utilized here. In order to provide a sustained release profile a lipophilicity-increasing cholesterol conjugation strategy is proposed that enhances affinity between the modified drug (MPC) and the hydrophobic compartments in the UPy gel. Additional advantages of cholesterol conjugation include improved chemical stability and potency of mitomycin C. *In vitro* the tunability of the system to obtain optimal effective concentrations over time is demonstrated with a combinatorial treatment of mitomycin C and MPC in one UPy hydrogel delivery system.

1. Introduction

Colorectal cancer (CRC) is one of the most frequent causes of cancer-related death in Western countries. About 10 % of CRC-patients are diagnosed with metastases to the peritoneal cavity, referred to as 'peritoneal carcinomatosis' (PC), which used to be invariably fatal as conventional treatments such as systemic chemotherapy and palliative surgery appear to be ineffective.[1,2] Locoregional intraperitoneal (IP) chemotherapy is a promising PC treatment, as this exposes the peritoneal surface and microscopic tumor nodules without established vasculature to high drug concentrations, with limited systemic toxicity and adverse side-effects.[3,4] In recent years, controlled macroscale drug delivery systems (DDSs) have emerged to facilitate safe, effective, and sustained IP chemotherapy.[5,6]

Bioresponsive injectable *in situ* gelling hydrogels in particular display desirable features for this purpose. These hydrogels offer; i) therapeutics to be easily mixed in beforehand, ii) applicability via minimally invasive surgical procedures, and iii) a proposedly uniform distribution of the hydrogel drug depot and *in situ* gelation after injection.[6,7]

Currently, much research is being directed towards injectable hydrogels for a variety of chemotherapeutic agents and types of IP cancer.[8–13] Allen and coworkers utilized an injectable chitosan-phospholipid blend (PoLi-gel) for sustained delivery of docetaxel (DTX) following IP administration with favorable drug distribution and antitumor effect in mice.[14,15] In the groups of Ding and Kwon a thermosensitive hydrogel (ReGel) comprising a PLGA-PEG-PLGA triblock copolymer for sustained chemotherapeutics release has been developed.[16,17] ReGel solubilizes hydrophobic compounds and provides sustained release through entrapment in the hydrogel's hydrophobic compartments. An important consideration for this approach is that it requires suitable hydrophobic drugs, since the mechanism for hydrophilic drug release is mainly via diffusion kinetics and cannot be controlled.[18] The hydrophilic drug mitomycin C (MMC), which is increasingly used for IP chemotherapy, is such a drug. MMC is a 335 Da antitumor antibiotic which possesses a tumor penetration

depth of 2 mm and a favorable peritoneal to plasma concentration ratio.[19] In order to increase the efficacy of intraperitoneal MMC, the chemo is often heated to 41 – 42 °C for 90 minutes during surgery; hence the name Hyperthermic Intraperitoneal Chemotherapy (HIPEC).[19] MMC possesses a logP of -0.4 and is highly water-soluble, so it has no intrinsic affinity for hydrophobic compartments of drug depot hydrogels. Furthermore, MMC is prone to degradation, which is accelerated at body temperature and/or slightly acidic conditions.[20]

Our delivery depot of choice for IP chemotherapy is a supramolecular pH-responsive injectable ureido-pyrimidinone (UPy) based hydrogel, which is equipped with hydrophobic compartments where drugs can be entrapped.[21] UPy-PEG hydrogel systems have been characterized extensively in previous studies.[21–23] Due to the reversible interactions of the supramolecular material a sol – gel transition occurs as a response to either temperature or pH. This switchable behavior is exploited to obtain an *in situ* gelling drug depot that is injectable at pH 9.0 and forms a hydrogel when in contact with tissue at physiological pH.[22] Several types of molecules have been incorporated in the hydrogel that are—in absence of an affinity for the UPy-PEG material—typically released within days via simple diffusion.[22,24,25] Here, it is proposed that conjugation of cholesterol to intrinsically hydrophilic MMC can provide a sustained release from the UPy-PEG hydrogel. Cholesterol conjugation increases the affinity for the hydrophobic compartments; ultimately serving as an anchor to keep the drug retained within the hydrogel. Modification of therapeutics into prodrug-conjugates is a proven strategy to enhance treatment efficacies.[26,27] In literature it has been reported that conjugation of hydrophobic moieties to chemotherapeutic agents can provide an improved entrapment efficiency in lipid nanoparticle vesicles or even supramolecular filaments.[28–32]. However, to our knowledge neither MMC nor other chemotherapeutics have been modified with the goal of acquiring sustained release from macroscale drug delivery depots.

Firstly and mainly, our solution should provide a controlled release of otherwise hydrophilic chemotherapeutics from the UPy-PEG hydrogel; this class of chemotherapeutics has been

underutilized in sustained DDSs until now. Secondly, the modification of MMC into a higher molecular weight drug increases lipophilicity and reduces water solubility.[31,33] It was reported that these properties result in a longer drug residence time within the peritoneum.[34] Thirdly, modification on the aziridine ring is proposed to increase stability. It has been reported that non-reversible substitution on this amine position diminishes biological activity, thus activity of the new molecule mitomycin-PEG_{24}-cholesterol (MPC) should be evaluated.[32]

Design, synthesis, characterization, chemical stability and tumor cell inhibitory effect of MPC are described. Then the release profiles for both MMC and MPC from UPy-PEG hydrogel are determined, to validate if a desirable sustained release can be achieved. Finally, a study was performed to evaluate release of the drugs with efficacy over time, where also an optimal combinatorial treatment of MMC and MPC is proposed.

2. Results and Discussion

Cholesterol was coupled to mitomycin C via a monodisperse poly(ethylene glycol) (mPEG) amine linker (**1**). The synthetic route towards mitomycin-PEG_{24}-cholesterol (MPC) started with the coupling between cholesteryl chloroformate and **1** in $CHCl_3$ via amide bond formation (**Figure 1A**). Purification of the crude product by silica column chromatography afforded a white solid **2** (133 mg, 89 % yield). Hydrolysis of the tert-butyl ester in basic conditions afforded **3** (81 mg, 95 % yield). This intermediate was activated with HATU and coupled to MMC. Purification of the crude product by reversed-phase C18 silica column chromatography afforded MPC (**4**) as a dark red-purple solid (38.5 mg, 53 % yield). 2D NMR spectroscopy confirmed the chemoselectivity of the secondary amine of MMC for the synthesis of MPC. A ^{1}H-^{1}H correlation between this secondary amine and its two neighboring protons was observed on the 2D COSY NMR spectrum of MMC (**Figure 1B**). The ^{1}H NMR spectrum of the product MPC revealed a downfield chemical shift of these two protons next to

the newly formed amide bond. In addition, the ^{1}H-^{1}H correlation from the secondary amine with its two neighboring protons was no longer observed on the 2D COSY NMR spectrum of MPC, endorsing the chemoselectivity of the reaction on the secondary amine (**Figure 1B**).

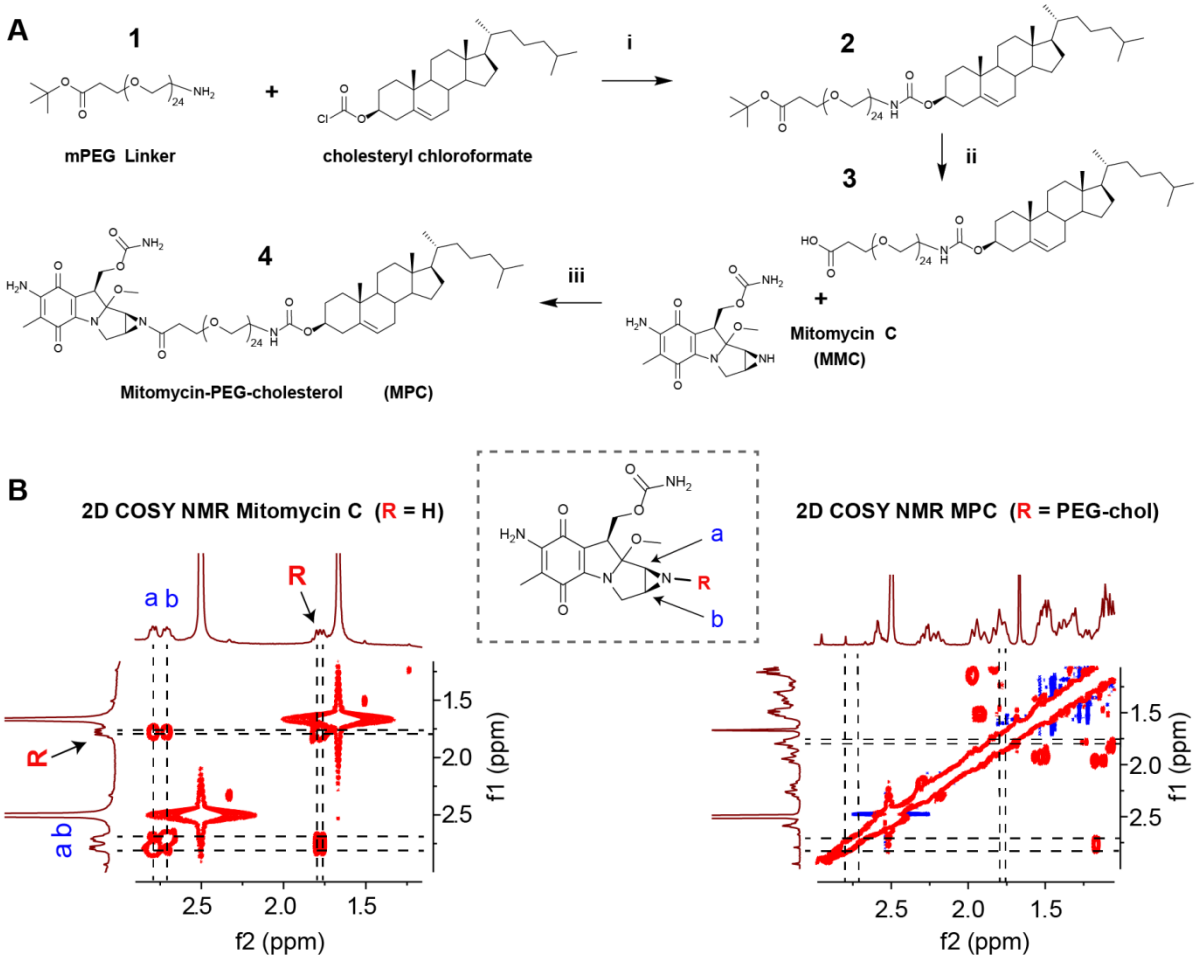

Figure 1. Synthesis and 2D COSY NMR characterization of MPC. **A**. Reaction scheme for the synthesis of mitomycin-PEG$_{24}$-cholesterol (**4**) from mitomycin C (MMC), cholesteryl chloroformate and an mPEG amine linker (**1**). **i** DIPEA, $CHCl_3$, room temperature, overnight, yield 89 %. **ii** NaOH, MeOH/H_2O, 50 °C, overnight, yield 95 %. **iii** HATU/DIPEA, DMF, 50 °C, overnight, yield 53 %. **B.** Highlighted the loss of ^{1}H-^{1}H correlation between the secondary amine and its two neighboring protons.

Chemical stability of MPC was evaluated from the UV absorption spectrum, and compared to MMC. As expected, MMC and MPC were found to be relatively stable at 4 °C and 37 °C when kept at neutral pH (**Supplementary Figure 1**). In PBS pH 6.5 however, the degradation

of MMC can easily be identified; two new maxima at 315 nm and 250 nm appeared, the minimum at 290 nm shifted to 280 nm, and the distinctive peak at 363 nm disappeared (**Figure 2A**). In contrast, MPC barely showed any changes in UV absorption for up to two weeks (**Figure 2B**). Based on the decrease of the characteristic 363 nm peak the rate of mitosane degradation can be determined, as was performed previously.[35] It was calculated that only 19.6 % of MPC had degraded after 13 days, compared to 73.9 % degradation for MMC (**Figure 2C**). With MALDI-TOF it was confirmed that no profound changes take place after storing MPC in PBS pH 6.5 at 37 °C, validating the UV absorption measurements (**Supplementary Figure 2**). The tertiary amide in MPC bestows it a reduced rate of degradation.

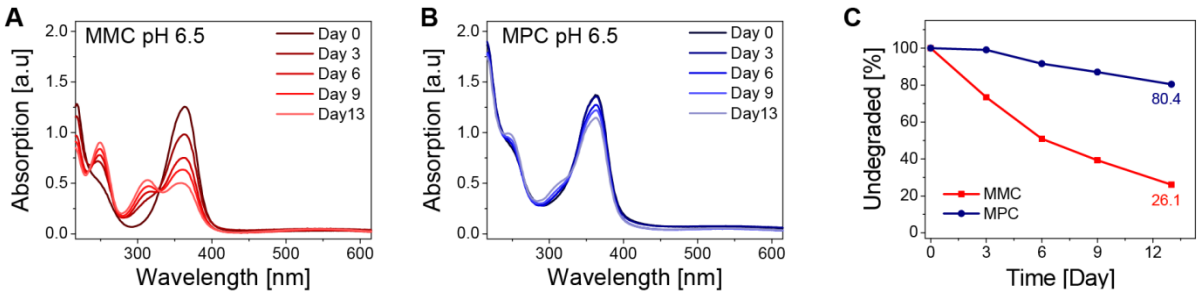

Figure 2. UV/VIS absorption spectra of MMC and MPC stored in PBS pH 6.5 at 37 °C, displaying the progression of acid catalyzed degradation over time. **A.** MMC. **B.** MPC. **C.** Percentage undegraded MMC and MPC over time. [35]

Next, the cytotoxic effect of MPC was evaluated and compared to the activity of MMC using the colorimetric MTT assay on CC531 colon carcinoma cells (**Figure 3A**). A concentration of 30 µM MPC results in almost complete cell-death, whereas the effect of MMC at that concentration is only 60 %. At the highest concentration of 300 µM 97 % cell death was observed for MPC. In order to quantify the increase in effect, dose response curves were fitted. From these curves an EC_{50} of 19.6 ± 3.2 µM and 3.8 ± 1.2 µM were determined for MMC and MPC, respectively. It demonstrates that MPC has an enhanced effect on the tumor

cells compared to MMC. Thus while both drugs display approximately 100 % efficacy, MPC performs much better with a 5.2 times increase in potency. An identical procedure was performed on 3T3 fibroblasts (**Figure 3B**) and HK-2 epithelial cells (**Figure 3C**). The EC_{50}-MMC to EC_{50}-MPC ratios are 4.2 and 1.1 for 3T3 cells and HK-2 cells, respectively. Thus, the potency of MPC towards these two cell lines was also amplified, but to a smaller extent than potency amplification occurred towards the carcinoma cell line. This finding might be explained by an enhanced cellular uptake of the cholesterol-conjugated compound by cancer cells. Cancer cells overexpress low-density lipoprotein (LDL) receptors and consume higher amounts of cholesterol; a concept that has previously been exploited for drug delivery.[36,37] It is currently unknown when and/or if MPC is degraded intracellularly to form parent MMC.

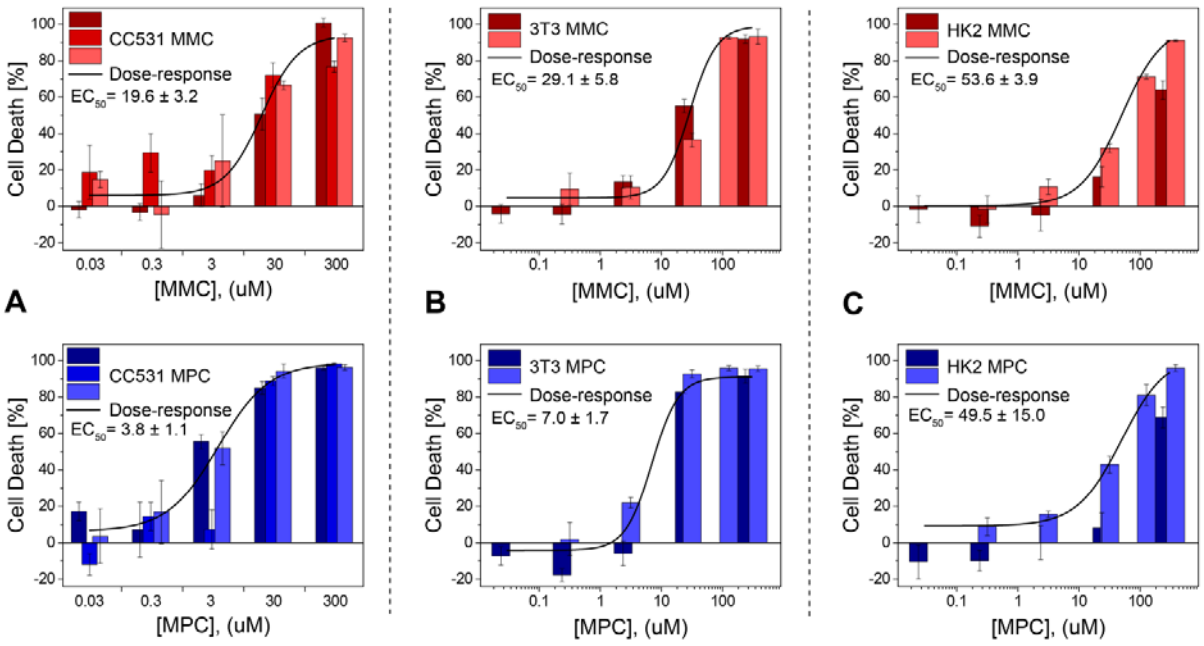

Figure 3. MTT assays of MPC and MMC on three cell lines, represented with mean ± sd, n = 6. Added are corresponding dose response curves and EC_{50}. **A.** CC531 cells. **B.** 3T3 cells. **C.** HK2 cells.

Release rates for both MMC and MPC from a UPy-PEG hydrogel were determined to disclose whether molecules can freely diffuse or have an affinity for the hydrogel network. It demonstrates that unmodified is released in 24 h via a typical diffusion profile and thus

experiences no affinity (**Figure 4A**). MPC yields a completely different release profile, with a burst release of only 7 % in the first day. After that, a steady controlled release of MPC was observed of just over 1 % per day (**Figure 4A**). After two weeks, the remaining MPC could be retrieved almost completely by dissolving the hydrogel—indicating that the drug endures encapsulation in the hydrogel without severe degradation. Since MPC is an amphiphile, it is probable that the drug forms micelles or liposomes in aqueous solution. Hypothetically, if large particles were present, physical interactions with the hydrogel network could significantly slow down the release by diffusion. In that case, release rate over time would have been affected by changes in the hydrogel's physical properties from swelling and degradation. However, the percentage based release of MPC is at an equal rate according to the erosion of the hydrogel itself.[24] Judging by this extremely slow but steady release rate, it is projected that the hydrophobic interactions between cholesterol and the core of the UPy fibers results in a strong affinity. Moreover, a control conjugate molecule of mitomycin C and PEG without cholesterol (MMC-PEG_{24}) was released within two days (**Supplementary Figure 3**); further strengthening this conclusion.

Since release of the drug is dictated by erosion, it is imperative for future work to obtain information about *in vivo* characteristics like the long-term erosion rate of the hydrogel. In order to monitor erosion rate we have recently introduced a method for supramolecular labeling of the UPy-based hydrogel with reporter molecules such as MRI contrast agents, which we plan to utilize for this purpose.[25] Next, a sustained release experiment was initiated with simultaneous activity measurements. An experiment was set up with hydrogels containing MMC or MPC, where drug that is released can subsequently be taken up by cells. Based on the release rates of MPC the drug-loaded hydrogels were transferred to fresh cells every three days, with an extra time point after the first day to distinguish the effect of initial burst release. MMC loaded hydrogels induced almost complete cell death at day 1, 3 and 6 (**Figure 4B**). Thereafter, besides a small effect on day 12, the MMC hydrogels no longer

influenced cell viability**.** For MPC loaded hydrogels the result of the much smaller initial burst release was clearly noticeable, with only 25 % cell death at the first time point. Notwithstanding, the consecutive data points demonstrate the effect of a slow and controlled release over time. After two more days incubation, the MPC loaded hydrogels induced 63 % cytotoxicity, whereafter every three-day interval resulted in complete cell death up to a measured 18 days (**Figure 4B**).

Both drug loaded hydrogels function on a different time scale, in agreement with the results obtained from the release study. MMC loaded hydrogels induced a fast response but for a relatively short period, whereas MPC loaded hydrogels offer a slow start but a prolonged activity. In an attempt to expand the therapeutic window further a new set of hydrogels was loaded with both MMC and MPC in a 1:1 molar ratio. Excitingly, by combining the burst release from MMC and controlled release of MPC, cell death of at least 75 % was induced at all time points (**Figure 4B**). This means that simply by combining both chemotherapeutics a third effective timeframe of drug release and activity can be achieved within the proposed strategy.

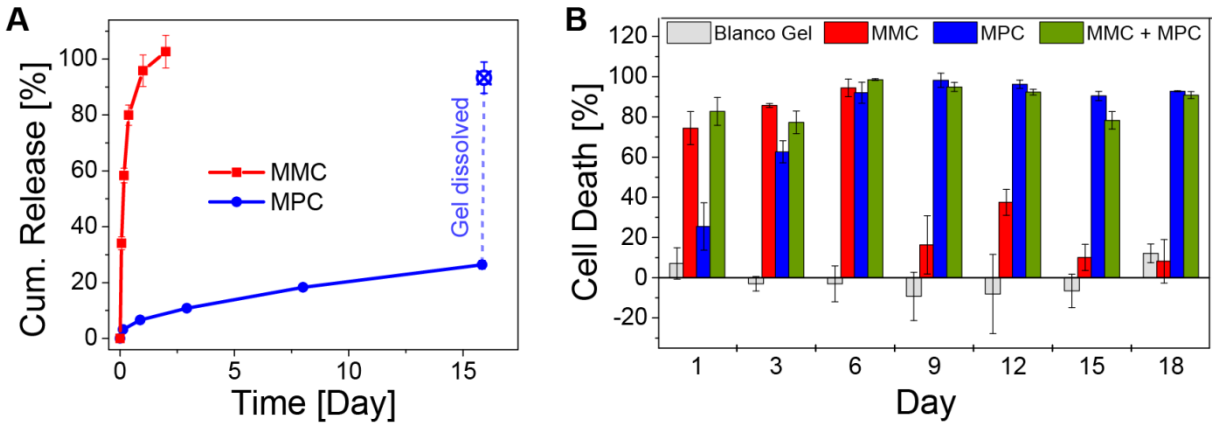

Figure 4. Release and activity over time. **A.** Release of MMC or MPC at 3 mM from UPy-PEG hydrogel. **B.** Drug release from hydrogels and subsequent cytotoxicity on CC531 cells**.** Hydrogels were loaded with 1.8 mM MMC, MPC, or 0.9 mM of both. At each time point the hydrogels were transferred to freshly seeded cells and MTT assays were performed on the cells that had been exposed to the hydrogels, data represents mean ± sd, n = 3.

3. Conclusion

Cholesterol-modification of hydrophilic small molecule drugs is an innovative method to radically enhance the retention in hydrogels that contain hydrophobic compartments. Here, sustained release of a cholesterol-modified MMC was achieved from a pH-responsive supramolecular UPy-PEG hydrogel. The newly designed molecule MPC is mostly insensitive to acid catalyzed degradation and exhibits a five times increased potency towards a colon carcinoma cell line. Acknowledging the limitations of *in vitro* studies, the data provides valuable information on the mechanism behind drug incorporation and release, whereas the UPy-PEG hydrogel system has previously proven its *in vivo* potential for drug release in large animal studies. The formulation with MPC can provide and improve long-term drug presence compared to a single or multiple conventional dose(s) without carrier. In addition, a UPy-PEG hydrogel formulation with MMC can be utilized when short-term release is desired. MMC and MPC can be loaded in one hydrogel formulation to tune total active drug release; combining the burst from MMC with a subsequent controlled release from MPC for optimal

coverage over time. Since standardized IP chemotherapy has not been established up to date, this tunable drug delivery system can be a valuable asset improving the treatment of patients suffering from peritoneal cancer.

Supporting Information

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

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Keywords: mitomycin C, intraperitoneal chemotherapy, controlled release, supramolecular hydrogel, drug delivery, cholesterol

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