Selective intracellular delivery of proteasome inhibitors through pH-sensitive polymeric micelles directed to efficient antitumor therapy

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ABSTRACT

The ubiquitin-proteasome system is central in the regulation of cellular proteins controlling cell cycle progression and apoptosis, drawing much interest for developing effective targeted cancer therapies. Herein, we developed a novel pH-responsive polymeric-micelle-based carrier system to effectively deliver the proteasome inhibitor MG132 into cancer cells. MG132 is covalently bound to the block copolymer composed of polyethylene glycol (PEG) and polyaspartate through an acid-labile hydrazone bond. This bond is stable at physiological condition, but hydrolytically degradable in acidic compartments in the cell, such as late-endosomes and lysosomes, and thus, it was used for controlled release of MG132 after EPR-mediated preferential accumulation of the micelles into the tumor. MG132-loaded micelles have monodispersed size distribution with average diameter of 45 nm, and critical micelle concentration is well below 10^{-7} M. *In vitro* studies against several cancer cell lines, confirmed that MG132-loaded micelles retained the cytotoxic effect, and this activity was indeed due to the inhibition of proteasome by released MG132 from the micelles. Real-time *in vitro* confocal-microscopy experiments clearly indicated that MG132-conjugated micelles disintegrated only inside the target cells. By intravital confocal micro-videography, we also confirmed the prolonged circulation of MG132 loaded micelles in the bloodstream, which lead to tumor specific accumulation of micelles, as confirmed by *in vivo* imaging 24 h after injection. These micelles showed significantly lower *in vivo* toxicity than free MG132, while achieving remarkable antitumor effect against a subcutaneous HeLa-luc tumor model. Our findings create a paradigm for future development of polymeric-micelle-based carrier system for other peptide aldehyde type proteasome inhibitors to make them effective cohort of the existing cancer therapeutic regiments.

Keywords: Proteasome inhibitor, MG132, polymeric micelle, pH-sensitive, antitumor efficacy.

INTRODUCTION

Proteasomes, *i.e.* large, multicatalytic proteinase complexes present in both the nucleus and the cytoplasm of eukaryotes [1], play an important role in the regulated degradation of intracellular proteins. The ubiquitin-proteasome system, in which damaged proteins are tagged with ubiquitin and delivered to the proteasome for shredding, modulates several fundamental cellular processes, such as inflammation, viral shedding, cell cycle, growth, and differentiation [2]. Inhibition of proteasome in rapidly proliferating malignant cells is crucial, as these cells have an increased requirement for protein synthesis, which would make them more vulnerable to proteasome inhibition. Consequently, proteasome inhibitors have received much interest as potent anticancer agents inducing apoptosis and cell cycle arrest in tumor cells [3], and bortezomib, a dipeptidyl boronic acid derivative, has been recently approved by the US Food and Drug Administration for the treatment of multiple myeloma and mantle cell lymphoma [4]. Bortezomib is based on former peptide aldehydes analogs designed on the preferred substrates of the proteasome's chymotrypsin-like active site [5]. Peptides aldehydes, such as Cbz-leu-leu-leucinal (MG132), Cbz-leu-leu-norvalinal (MG115), Z-Ile-Glu (O-t-Bu)-Ala-Leucinal (PSI), and acetyl-leu-leu-norleucinal (ALLN), which are among the first inhibitors to be developed and are still the most widely used [6-8], are highly potent and rapidly block proteasomes by reacting with the 26S proteasome through its aldehyde functional group, forming a reversible hemiacetal [9]. Nevertheless, they are rapidly oxidized *in vivo* losing their activity and their bioavailability is poor, limiting the administration in the *in vivo* setting [10]. To circumvent these limitations, one approach could be to develop a self-assembled amphiphilic block copolymer micelle-based nanocarrier system to effectively transport these peptide aldehydes in the site of action.

Nanocarriers have shown great potential for delivering bioactive molecules to solid tumors, while protecting them during circulation, to maximize the therapeutic efficacy and minimize side effects [11]. Nanocarriers take advantage of the "enhanced permeability and retention" (EPR) effect, [12] which is based on the pathophysiological characteristics of solid tumor tissues including incomplete vascular architecture, impaired lymphatic drainage system, and increased secretion of

various vascular permeability factors, to preferentially penetrate and accumulate in tumors. Among promising nanocarriers, polymeric micelles, self-assembled from amphiphilic block copolymers, [13, 14] have shown several attractive features for selective tumor delivery of bioactive agents, such as effective protection of their core-loaded cargo, high loading capacity, controlled drug release, prolonged circulation in the bloodstream and selective accumulation in solid tumors. Polymeric micelles, built from poly(ethylene glycol)-*b*-poly(amino acids) (PEG-PAA) copolymers, [15] present unique ability for versatile modifications of the PAA block [16] and engineered drug conjugation, while PEG effectively shields the core due to the high degree of hydration, chain mobility and non-immunogenicity. Several PEG-PAA-based polymeric micelles incorporating the anticancer agents paclitaxel, SN-38, cisplatin, (1,2-diaminocyclohexane)platinum(II) (DACHPt) and epirubicin are being evaluated clinically [17, 18, 19], suggesting the clinical translation capability of PEG-PAA-based polymeric micelles.

In this study, we developed polymeric micelles incorporating the peptide aldehyde proteasome inhibitor, MG132, where the aldehyde group of MG132 is protected and only becomes activated once it reaches the target site, thus, increasing the bioavailability, selectivity, efficiency and safety of the drug. Therefore, a pH-labile hydrazone linkage between the MG132 and the PAA backbone (**Scheme 1**) was selected as this bond is stable at pH 7.4, *i.e.* during blood circulation, but degrades hydrolytically under pH 4-5, which is the endosomal pH of the targeted cells [20]. We utilized this concept successfully in our former studies where doxorubicin, a widely used anticancer drug, was conjugated to the PAA block through an acid-sensitive hydrazone bond in the core of the polymer micelles. These intracellular pH-sensitive doxorubicin-loaded micelles showed significantly lower *in vivo* toxicity but superior anti-tumor efficacy, compared to that of the free drug [21, 22]. This lower *in vivo* toxicity and superior anti-tumor efficacy can be attributed to the reduction of premature drug leakage during the blood circulation and efficiency in tumor specific drug delivery. NC-6300, an intra-cellular acid-sensitive polymeric-micelle-based nanocarrier, loaded with epirubicin (4'-epimer of doxorubicin), has recently entered phase-1 clinical trial for solid tumors in Japan [19]. We report here on the preparation and physicochemical characterization of pH-responsive polymeric micelles loaded with proteasome inhibitor MG132. The activity of MG132-conjugated micelles (MG132/m) was also evaluated *in vitro* and *in vivo*.

Scheme 1. Preparation of MG132-loaded polymeric micelles. The micelles were self-assembled in water after conjugating MG132 to the hydrazine moieties of poly(ethylene glycol)-*b*-poly(hydrazinyl-aspartamide) copolymer.

MATERIALS AND METHODS

Materials

β -Benzyl-L-aspartate *N*-carboxy anhydride (BLA-NCA) was purchased from Chuo Kaseihin Co., Inc. (Tokyo, Japan). α -Methoxy- ω -amino poly(ethylene glycol) (MeO-PEG-NH₂; M_w = 12000) was obtained from NOF Co, Inc. (Tokyo, Japan). Dimethyl sulfoxide (DMSO), *N,N*-dimethylacetamide (DMAc), Dichloromethane (DCM), acetic anhydride and hydrazine

monohydrate were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Diethyl ether was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). MG132 (Z-Leu-Leu-Leu-H) was purchased from Peptide institute, Inc. (Osaka, Japan). Spectra/Por dialysis tube (MWCO = 6-8,000 Da) was purchased from Spectrum Laboratories (Rancho Dominguez, CA). Amicon stirred cells and filter membrane (MWCO = 100,000 Da) was purchased from Millipore Corporation. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Parma Co. Ltd. (Osaka, Japan). Cell Counting Kit-8 and Hoechst 33342 were purchased from Dojindo Laboratories (Kumamoto, Japan). 20S-Proteasome Activity assay kit was purchased from Merk Millipore. Proteasome-Glo Chymotrypsin like cell based assay kit was purchased from Promega corporation.

Instruments

Proton nuclear magnetic resonance (^1H -NMR) spectra were recorded on a JEOL ECS-400 (400 MHz) spectrometer (JOEL Ltd., Tokyo Japan) and chemical shift was calculated as parts per million (ppm). Molecular weight distribution (MWD) of the obtained polymers were determined by gel permeation chromatography (GPC) using poly(ethylene oxide) standards for the calibration (Polymer Laboratories, Ltd.,UK). Organic phase GPC was performed using TOSOH HLC-8220 system equipped with a RI detector and TSK gel columns (G4000HHR and G3000HHR) at 45 °C eluted with DMF containing lithium chloride (10 mM) at a flow rate of 0.8 mL/min. Aqueous phase GPC was performed using a JASCO HPLC system equipped with a UV detector (at 220 nm) and a Superdex 200 10/300 GL column (GE Healthcare Ltd., UK) at room temperature eluted with PBS buffer (pH 7.4, 10 mM phosphate buffer and 150 mM NaCl) at a flow rate of 0.75 mL/min, room temperature. Cell viability was measured by using a Microplate Reader Bio-Rad Model 680 (BIO-RAD, Hercules, CA).

Ring opening polymerization- Synthesis of MeO-PEG-PBLA polymer

Block-copolymer MeO-PEG-PBLA was prepared following a previously reported procedure [23] with slight modifications. Accordingly, β -Benzyl L-aspartate *N*-carboxy anhydride (3.5 g, 14.0 mmol) was first dissolved in DMF (5 mL), which was then diluted with DCM (30 mL). This mixture was then added to a DCM (35 mL) solution of previously freeze dried MeO-PEG-NH₂ (3.5 mg, 0.29 mmol) under argon atmosphere. After stirring for 2 days at 35°C, the product Methoxy-poly(ethylene glycol)-*b*-poly(β -benzyl-aspartamide) (MeO-PEG-PBLA) was isolated (5.7 g) by diethyl ether precipitation. The composition of MeO-PEG-PBLA copolymer was determined by ^1H -NMR spectra using integral values from the $-\text{CH}_2\text{CH}_2\text{O}-$ proton peaks of PEG and aryl (C_6H_5 -) peak of PBLA block, and from the molecular weight of PEG supplied by the manufacturer. Accordingly, the synthesized MeO-PEG-PBLA has a PEG of 12,000 Da and 42 PBLA units.

Acetylation of the terminal amine function of MeO-PEG-PBLA

MeO-PEG-PBLA (Mw of PEG: 12,000; PBLA units: 42; 1 g) was dissolved in DMSO (10 mL) with gentle heating. Catalytic amount of DMAP was added to a DMSO (2 mL) solution of acetic anhydride (120 μL , excess) and the resulting mixture was added to the DMSO solution of MeO-PEG-PBLA. This was then left stirring for 3 h at room temperature. Cold (Et)₂O (120 mL) was slowly added to the reaction mixture with stirring, the resulting white precipitate was washed with 3 batches of fresh cold (Et)₂O (3 $\times$ 100 mL) and collected by centrifugation. The acetylated product (MeO-PEG-PBLA-Ac) was then dried under high vacuum.

Aminolysis reaction with hydrazine

Aminolysis reaction of MeO-PEG-PBLA-Ac was done using a previously described procedure [24] with moderate modifications. MeO-PEG-PBLA-Ac (400 mg) was dissolved in DMSO (5 mL) with gentle heating and then excess of hydrazine monohydrate (200 μL) was added. The reaction mixture was left stirring for 4 h at 40°C. Cold diethyl ether (50 mL) was slowly added

to the reaction mixture with stirring, the resulting white precipitate was washed with 3 batches of cold (Et)₂O (3×50 mL) and collected by centrifugation.

Quantification of hydrazide function by acetylation

An aliquot of the resultant polymer (15 mg) was dissolved in DMSO (1 mL) and acetic anhydride (200 μ L) was added, the reaction mixture was left stirring for 3 h at room temperature. The reaction mixture was subjected to precipitation formation with cold ether and the product was collected by centrifugation. The number of acetyl unit was calculated by ¹H-NMR spectra comparing peak intensity of PEG protons with the acetyl protons.

Drug-conjugation reaction

MeO-PEG-PAsp-hydrazide (400 mg) was dissolved in DMAc (40 mL) and MG132 (200 mg) was added. The mixture was left stirring for 48 h at 40°C, and was used directly for micelle preparation. For ¹H-NMR analysis, an aliquot of the DMAc solution of MG132-polymer conjugate was subjected to ether precipitation, and dried rigorously under high vacuum.

Micelle preparation

MG132-polymer conjugate in DMAc solution (40 mL) was drop-wise added to water (360 mL) with constant stirring. This solution was then dialyzed against water for 48 h in a 7,500 D molecular weight cut off (MWCO) dialysis bag. The dialyzing media was changed 5 times during the dialysis procedure. The solution inside the dialysis bag was filtered (0.22 μ m) before concentrating by ultrafiltration using 100K MWCO filter membrane.

Size and PDI measurement by dynamic light scattering technique

Size and polydispersity of the micelles was determined by dynamic light scattering (DLS) technique at 25 °C (unless otherwise mentioned) using Zetasizer nano ZS (Malvern instruments, UK) at a detection angle of 173° with a He-Ne laser (633 nm) as the incident beam.

Transmission electron microscopy (TEM)

The morphology of micelle was observed on a Transmission Electron Microscope (JEM-1400, JEOL, Japan) operated with 100 kv acceleration voltages and 40 uA beam current. Diluted micelle was stained by mixing with uranyl acetate solution (2%, w/v) and placed on 400-mesh copper grids.

Total drug loading and loading efficiency

A known amount of micelle solution in water was freeze-dried and weighed. This sample was then subjected to ¹H-NMR analysis. The average number of conjugated drug units in each polymer chains can be calculated, by comparing the peak intensity of 18 methyl protons (at around 0.7-0.95 ppm) arising from three of the isopropyl group of MG132 with PEG protons (3.5 ppm) of polymer; the weight ratio of polymer and drug can now be calculated by using the molecular weight of polymer and MG132.

$$\% \text{ Drug loading} = \frac{\text{MW of MG132} \times \text{No of drug units}}{\text{MW of PEG-Pasp-hydrazide polymer} + (\text{MW of MG132} \times \text{No of drug units})} \times 100$$

$$\% \text{ Drug loading efficiency} = \frac{\text{MW of MG132} \times \text{No of drug units conjugated to polymer}}{\text{Amount of MG132 added to the drug conjugation reaction}} \times 100$$

In vitro cytotoxicity

In vitro cytotoxicity of proteasome inhibitor MG132 and MG132-conjugated micelles (MG132/m) were evaluated against mesothelioma cells (MSTO-211H), breast cancer cells (MDA-MB-231), prostate cancer cells (PC-3) and cervical cancer cells (HeLa-luc), using cell-counting kit-8. MSTO cells (2500 cells/well), PC-3 cells (5000 cells/well) and HeLa-luc cells (2500 cells/well) were cultured in DMEM medium and MDA-MB-231 cells (5000 cells/well) were cultured in RPMI medium, with 10% FBS, in 96-well-plates. The cells were then exposed to free MG132 and MG132 conjugated micelles. Cell viability was measured after 24, 48 and 72 h of incubation by measuring the formazan absorbance at 450 nm.

***In vitro* proteasome activity assay using 20S-proteasome activity kit**

HeLa-luc cells (2.5×10^5 cells/well) were plated in 6 well plates and incubated overnight. Cells were then treated with MG132/m solution (16.4 μ M). Cells were incubated for 2, 4, 8 and 24 h (n=3) and collected using lysis buffer. Cells were washed twice with PBS, lysed ((pH 7.5, 50mM HEPES, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100) for 30 minutes on ice, centrifuged at 15,000 rpm for 15 minutes at 4°C. The supernatant was used, for the proteasome activity assay, according to manufacturer protocol. Cells treated with only cell culture medium was used as control for every time points.

***In vitro* proteasome activity assay using Proteasome-Glo**

MDA-MB-231 cells (5000 cells/well) were plated in 96 well plates. After incubation of 18 h, cells were treated with free MG132 solution (0.43 μ M) and MG132/m solution (4.6 μ M). Treated cells were then incubated for additional periods and proteasome activity was measured using Proteasome-Glo according to the manufacturers protocol in different predetermined intervals (n=4) for a total time period of 48 h. Cells treated with only cell culture medium was used as control for every time points.

***In vitro* Confocal microscopy experiment**

The dissociation of BODIPY TR-MG132/micelle in HeLa-luc cells were observed using CLSM (LSM780, Carl Zeiss, Oberkochen, Germany). HeLa-luc cells (3×10^4 cells/well) were plated on a 35 mm glass-based dish and immersed in DMEM containing 10% FBS. For cellular internalization study, we incubated the cell nuclei with CellLight™ Nucleus-GFP (10 μ L/mL medium) for 48h. Subsequently, the medium was washed and refilled with Opti-MEM. BODIPY TR tagged MG132/m were then added to the sample, at a concentration of 10 μ M. Confocal Microscopy study was then conducted (for the whole experimental time the cells were incubated in the BODIPY TR tagged, MG132/m). Cells were observed using CLSM equipped with a C-Apochromate 40X/1.2 W objective, Ar-laser (458 nm/488 nm/514 nm) and DPSS laser (561 nm). ZEN 2010 software was used to process the imaging data. To confirm the presence of disintegrated micelle only in the intracellular compartment, the HeLa-luc cells were seeded as described above, but the cellular membranes were stained with CellLight™ Plasma membrane-GFP (10 μ L/mL medium) for 48h. Fresh medium with Hoechst 33342 (10 μ L/mL medium) nuclear stain was added and incubated for 3 minutes. This medium was then replaced with Opti-MEM. BODIPY TR tagged MG132/m were then added (10 μ M) and cells were observed using CLSM with a Plan-Apochromate 63X/1.4 Oil objective, diode laser (405 nm) and the above mentioned lasers. To observe the subcellular distribution of the micelle, the late endosomes in HeLa-luc cells were stained with CellLight™ Late Endosomes-GFP (10 μ L/mL medium). After 48h incubation, the culture medium was replaced with a fresh one, and BODIPY TR tagged MG132/m were then added (10 μ M). The cells were examined using CLSM as described above.

***In vivo* laser scanning microscopy**

In vivo laser scanning microscopy was conducted using Nikon A1R confocal laser scanning microscope system attached to an upright ECLIPSE FN1 (Nikon Corp., Tokyo, Japan) and

equipped with Plan Apo VC 20x DIC N2 Nikon lens (numerical aperture- 0.75). All pictures/movies were acquired as spectral images using 10 μ m of confocal slices. Laser with 640 nm was used to excite Alexa 647. Alexa 647-labeled MG132/m was administered *via* a tail vein catheter. Acquired data were processed using Nikon NIS Elements (ver. 4.00.06).

Biodistribution of Alexa 647-labeled MG132 micelles

Alexa 647-labeled MG132/m was prepared to investigate the accumulation in a HeLa-Luc subcutaneous tumor. Alexa 647-labeled MG132/m (12 mg/kg on MG132 basis) were intravenously injected to a tumor bearing mouse and the mouse were imaged after 24 h to track the accumulation of micelles in the whole body by using IVIS imaging system (excitation: 640 nm; emission: 720 nm; exposure time: 1 s; field of view: 18.1 cm). Then, the mice were sacrificed and the organs (liver, kidney, heart, lung and spleen) and the tumor were imaged *ex vivo* to evaluate the accumulation of micelles.

***In vivo* antitumor activity of Free MG132 and MG132-conjugated micelle (MG132/m) against s.c. HeLa-luc tumor model.**

BALB/c-nu/nu mice (female) were inoculated subcutaneously with HeLa-luc cells (5×10^6 cells/per mouse, $V = 100 \mu$ l, in cell culture medium DMEM). Tumors were allowed to grow for 5 days (average tumor size was 55 mm^3). Subsequently, mice were treated with 6 times at 1-day intervals (day 0, 2, 4, 6, 8 and 10), at doses of 16mg/kg and 32mg/kg ($n = 7$) for free MG132 (intraperitoneal injection in 50μ l DMSO) and MG132/m at 16 mg/kg, 32 mg/kg and 64 mg/kg doses ($n = 7$, tail vein injection in 200μ l water), calculated as the amount of conjugated drug. The same treatment regime was conducted using PBS solution (intravenous, 200μ l) to a control group ($n = 6$). The antitumor activity was evaluated in terms of tumor size (V), as estimated by the following equation:

$$V = a * b^2/2$$

where, a and b are the major and minor axes of the tumor measured by a caliper, respectively. The body weight was measured simultaneously and was taken as a parameter of systemic toxicity.

RESULTS

Synthesis and characterization of block copolymers

Methoxy-poly(ethylene glycol)-*b*-poly(β -benzyl L-aspartate) (PEG-PBLA; M_w of PEG = 12 kDa; polymerization degree of PBLA = 42) was synthesized as the base for preparation of the micelle, by ring-opening polymerization of β -benzyl aspartic acid *N*-carboxyanhydride, initiated by the terminal primary amino function of α -methoxy- ω -amino poly(ethylene glycol) (**Scheme 2**). The resulting block copolymer showed narrow and unimodal distribution of molecular weight, evident by GPC analysis (molecular weight distribution (MWD), M_w/M_n : 1.10). The degree of polymerization of the PBLA block was determined by comparing the peak intensity of the β -benzyl residue (7-7.5 ppm) and PEG protons (-O-CH₂-CH₂-; 3.5 ppm) in the ¹H-NMR spectrum.

Scheme 2. Preparation of poly(ethylene glycol)-*b*-poly(hidrazinyl-aspartamide-MG132).

After terminating the ω -amine functionality by acetylation, the benzyl esters of PBLA side chain was substituted to hydrazinyl by the quantitative ester-amide exchange reaction according to our previous work with slight modification [24], which resulted in almost quantitative conversion without any sign of intermolecular crosslinking, evident by $^1\text{H-NMR}$ spectrum and GPC analysis (M_w/M_n : 1.03, **Supplementary Figure S1a**). The number of hydrazide functions was quantified separately by acetylating the hydrazide groups of the PEG-*b*-poly(hydrazinyl-aspartamide). By calculating the ratio of the peak intensity of the acetyl protons (1.7 ppm;) and the PEG protons in the $^1\text{H-NMR}$ spectrum (**Supplementary Figure S1b**), we found that 39 hydrazide units were introduced.

Preparation and physicochemical characterization of MG132-loaded micelles

Conjugation of MG132 with the hydrazide polymer was done in dimethylacetamide (DMAc) at 40°C for 48 h. The amount of conjugated drug in the polymer backbone was calculated by comparing the peak intensities of the three-isopropyl group protons of MG132 with the PEG protons in $^1\text{H-NMR}$ spectra (**Supplementary Figure S2**). Accordingly, by controlling the amount of MG132 in the reaction mixture, the drug loading was precisely modulated (**Table 1**). For reaction mixtures with excess hydrazide units, all MG132 was conjugated to the block copolymer, as confirmed by the complete disappearance of the aldehyde peak of MG132 (9.35ppm; multiplet) in the $^1\text{H-NMR}$ spectra, allowing us to proceed to next step without further purification. Conversely, despite of the excess amount of MG132 in the reaction mixture, the MG132 excess reaction did not proceed to completion, probably because of the steric hindrance from the already attached drug molecules, which required further purification process. Thus, for the ease of preparation of MG132-polymer conjugate, 2:1 mixing ratio, which provides 33% total drug loading (% weight of MG132 compared to the total micelle weight), was chosen to be optimal and used this composition for the subsequent analysis. This composition also yields micelles with smallest diameter, compare to the other compositions attempted (**Table 1**).

Table 1. MG132/hydrazide molar ratio, and % weight of MG132 compare to that of the total polymer weight added to the reaction mixture corresponds to conjugation efficiency

Mixing	molar	Conjugated	Conjugation	%Weight	Z-average
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ratio MG132 to Hydrazide	[MG132]/[Hydrazide] molar ratio	efficiency	of MG132 ^a	diameter (nm) ^b
1:7	0.15	100 %	14	64
1:4	0.25	100%	21	62
1:2	0.50	100%	33	45
1:1	0.73 ^c	73%	42	80
1:0.6	0.75 ^c	75%	44	80

^a % weight of MG132 compared to the total micelle weight, after micelle preparation

^b Determined by dynamic light scattering

^c After purification.

Polymeric micelles incorporating MG132 (MG132/m) were prepared by drop-wise addition of the MG132-conjugated block copolymer in DMAc to water under constant stirring to a final 1:10 volume ratio. Then, the organic solvent was completely removed by extensive dialysis, and the micelle solution was purified and concentrated to the desired amount by pressure-induced ultrafiltration with a membrane having 100,000 Da molecular weight cut off. The micelles remained stable during the dialysis and ultrafiltration procedures. Dynamic light scattering (DLS) measurements revealed narrowly distributed assemblies (PDI: 0.13) with Z-average diameter of approximately 45 nm (**Figure 1**). Moreover, Transmission Electron Microscopy (TEM) analysis revealed the diameter was 15-25 nm, corresponding to the core of the micelles, as the PEG shell cannot be observed by TEM (**Supplementary Figure S3**).

Figure 1. Size distribution by intensity of MG132 conjugated polymeric micelles, determined by DLS.

By using static light scattering technique, we studied the changes in the apparent molecular weight, $M_{w,app}$, of the micelles with their concentration (C) from the detection-angular dependence of the excess Rayleigh ratio ($\Delta R(\theta)$). By extrapolating the detection angle (θ) to zero on the linear plots of $KC/\Delta R(0)$ vs. $\sin^2(\theta/2)$, the following Debye equation can be derived:

$$\frac{KC}{\Delta R(0)} = \frac{1}{M_{w,app}} + 2A_2C \quad (1)$$

The change in $KC/\Delta R(0)$ was evaluated by varying the concentration of the micelles from 0.04 to 0.001 mg/ml (**Figure 2**). According to Eq. (1), the $M_{w,app}$ of the micelles was determined to be 8.53×10^5 g/mol by extrapolating the concentration of the solution to zero. Therefore, the number of associated drug-conjugated block copolymers in each micelle is 30, considering an average loading of 24 MG132 molecules per block copolymer, as determined by $^1\text{H-NMR}$. Moreover, MG132/m did not show any abrupt change in $M_{w,app}$ (or $KC/\Delta R(0)$) within this concentration range of 0.04 to 0.001 mg/ml [25], indicating the maintenance of their micellar structure even at 0.001 mg/ml (3.5

10^{-8} M). That means, the critical micelle concentration (CMC) of MG132/m is much lower than the typical range, 10^{-6} to 10^{-7} M, generally suggested for polymeric micelles in the literature [16].

Figure 2. Change in $KC/\Delta R(0)$ value with the concentration of MG132 incorporated micelle.

MG132/m can be stored in aqueous solution at 4°C for at least two months, without changes in the diameter and PDI as determined from DLS measurements (**Figure 3a**). The size and PDI of the micelles were also monitored at defined time periods for confirming the stability of micelles in quasi-extracellular physiological conditions, *i.e.* 10 mM phosphate buffer plus 150 mM NaCl at 37°C. The size of the micelles remained unchanged throughout 48 h, with a PDI of approximately 0.14. Moreover, the light scattering intensity also remained in a narrow range (**Figure 3b**) for the whole trial period, confirming the stability of the micelles in physiological conditions.

Figure 3. Time-dependent change in size and relative scattering intensity of MG132 conjugated polymeric micelles under (a) storage condition (b) under physiological condition.

Furthermore, MG132/m was stable enough in the laboratory-mimicked physiological conditions that almost no drug leakage was observed (**Supplementary Figure S4a**) when dialyzed against PBS buffer at 37°C. While, drug release was impeded even at acidic pH as in the micelle form, the release of the drug from the polymer-drug conjugate in an organic solvent was triggered in acidic condition, and the recovery of the therapy significant aldehyde group of MG132 was confirmed by ¹H-NMR analysis (**Supplementary Figure S4b**).

Time dependent release of MG132 from the polymer-drug conjugate in an acidic condition

Freeze-dried MG132 loaded micelles (containing 19 units of MG132) were dissolved in DMSO and then dialyzed against acidified and hydrated DMSO solution (1% TFA and 1% water, V/V) in a 6000-8000 MWCO dialysis bag. Four individual samples were prepared for time-dependent analysis in four time points (24, 48, 72 and 96 h). At each time point, the dialyzing medium was changed to deionized water and then the DMSO free sample inside the dialysis bag was freeze-dried and the drug content was measured by ¹H-NMR analysis. The results of time dependent release of MG132 from the polymer-drug conjugate are summarized in **Table 2**.

Table 2. Remaining drug units and % remaining drug unit in the polymer-drug conjugate, during the drug release experiment.

Time Point (h)	Remaining Drug unit ^a	% remaining drug unit
0	19	100
24	15	78
48	11	58
72	9	47
96	6	32

^aDetermined by ¹H-NMR

In vitro cytotoxicity and proteasome inhibition of MG132/m

MG132/m were found to be active against various cancer cells. The 50% inhibitory concentration (IC₅₀) of the micelles against mesothelioma cells (MSTO-211H), breast cancer cells (MDA-MB-231), prostate cancer cells (PC-3) and cervical cancer cells (HeLa-luc) decreased with the exposure time (**Table 3**) to approximately 4 μM for all cells at 72 h. The cytotoxicity of the MG132/m was 10-fold lower than that of the free MG132 (**Table 3**), probably because the micelles are gradually internalized by endocytosis and slowly release the drug intracellularly, while MG132 rapidly penetrate the cells [26, 27].

Table 3. Fifty-percent inhibitory concentration (IC₅₀) of free MG132 and MG132/m (n = 8 wells).

Cell line	IC ₅₀ (μM) ^a			
	MG 132		MG132/m	
	48 h	72 h	48 h	72 h
MSTO	0.46	0.46	4	4
MDA-MB-231	0.43	0.43	17	4.7
PC-3	0.44	0.44	16	4.6

^aDetermined by cell-counting kit.

To confirm the cytotoxic effect of MG132/m was due to the inhibition of proteasome, the proteasome activity in HeLa-luc and MDA-MB-231 cells was monitored after incubation with free MG132 and MG132/m using 20S-proteasome activity assay kit, according to manufacturer's protocol. For HeLa-luc cells, by using 16.4 μ M MG132/m on a MG132 base, which is the IC₇₅ at 24 h, the proteasome activity was gradually inhibited to less than 70% of the activity of untreated cells after 8 h incubation, and below 40% after 24 h incubation (**Figure 4a**), confirming the inhibitory effect of the micelles. For MDA-MB-231 cells, we used the IC₅₀ values observed at 72 h, and the proteasome activity was followed for 34 h (**Figure 4b**). Accordingly, while both free MG132 and MG132/m comparably inhibited the proteasome activity for the first 16 h, the micelles inhibited the proteasome more efficiently than the free drug after 16 h incubation.

Figure 4. (a) Time profile of the *in vitro* proteasome activity of MG132/m against HeLa-luc cells at a concentration of 16.4 μ M (IC₇₅ at 24 h). (b) Direct comparison of time-dependent *in vitro* proteasome activity of MG132 and MG132/m against MDA-MB-231 cell lines, at a concentration equal to that corresponding to their IC₅₀ values at 72 h. Proteasome activity of untreated cell was taken as 100%. (Student's t-test * $P < 0.05$)

Cellular internalization of MG132/m

The cellular internalization and the dissociation of MG132/m were investigated in real-time with confocal laser scanning microscopy (CLSM). For this study, we prepared dye-labeled MG132/m by conjugating BODIPY TR at the ω -amino end of the block copolymer (**Supplementary Scheme S1**). Thus, as the dyes are maintained in the core of the micelles during micelle form, the self-quenching of BODIPY TR is induced due to their close proximity [28]. Fluorescence quenching of the polymer-conjugated dyes after micelle formation was confirmed by fluorescence spectroscopy (**Supplementary Figure 5**). We have previously used this approach for following the intracellular dissociation of polymeric micelles in real-time [29]. Accordingly, HeLa-GFP cells were treated with BODIPY TR-labeled MG132/m (BODIPY TR-MG132/m) and the real-time fluorescence intensity of BODIPY TR was monitored for a total time period of 24 h by time-lapse CLSM (**Supplementary Video S1**). While the cells remained incubated with BODIPY

TR-labeled MG132/m containing cell culture medium for the whole experimental period, the fluorescence from the micelles in the cell culture medium was not visible throughout the experiment due to the quenching of the core-conjugated BODIPY TR. The fluorescence from BODIPY TR was only visible in the intracellular space and the fluorescence intensity increased gradually with time throughout the experimental time frame (**Figure 5**). This gradual increase of the fluorescence intensity corresponds with the dequenching of BODIPY TR fluorescence driven by the disintegration of BODIPY TR-labeled MG132/m inside the cells.

Figure 5. Real-time *in vitro* CLSM of the intracellular disruption of BODIPY TR-labeled MG132/m. (a, b, c, d and e) snapshots of the cellular uptake and disruption of BODIPY TR-labeled MG132/m (red) in HeLa-GFP cells (green; nuclei) at various time points. (f) Mean intensity increment, with time, of red-fluorescence from the disintegrated BODIPY TR-labeled MG132/m in the intracellular space of HeLa-GFP cells (n = 30) was measured for a 16 h time frame.

To further demonstrate the disintegration of the micellar structure only occurs after cellular entry and not on the cellular surface, we marked the cell membrane of HeLa-luc cells through the expression of GFP in the myristoylation/palmitoylation sequence from Lck tyrosine kinase by using CellLight Plasma Membrane-GFP. After incubating the cells for 16 minutes with BODIPY TR-labeled MG132/m, fluorescence from BODIPY TR was only visible inside the cells, but not on the plasma membrane (**Figure 6**). This observation confirms that the micellar structure was intact in the cell culture medium and during the cellular entry, and that micelles disintegrated only after cellular uptake.

Figure 6. *In vitro* CLSM study of dissociation of BODIPY TR-labeled MG132/m (red) in HeLa-luc cells. Cell membrane was tagged with GFP (green) and nucleus with Hoechst (blue). Absence of

colocalization of micelles and cell membrane confirms the disintegration of the micelles only in the intracellular space.

The dissociation of BODIPY TR-labeled MG132/m, in the late-endosomal compartments of HeLa-luc cells, was confirmed in more detail by marking Rab7a with CellLight Late Endosomes-GFP and using CLSM with high magnification. Thus, red-fluorescence from the BODIPY TR of dissociated drug-conjugated polymer was observed as dots inside the late-endosomes (**Figure 7**) after 30 min incubation.

Figure 7. *In vitro* CLSM study of the dissociation of BODIPY TR-labeled MG132/m (red) in late-endosomal compartments, which were marked with GFP (green), enlarge section is shown in the flap.

***In vivo* biodistribution and blood circulation profile of Alexa 647-labeled MG132/micelles**

For the *in vivo* biodistribution and blood circulation study of MG132/m, we conjugated Alexa 647 instead of BODIPY TR at the ω -amino end of the block copolymer, and fluorescent MG132/m micelle was prepared accordingly. In this case the micelles remained fluorescence (**Supplementary Figure 6**) unlike the BODIPY TR-labeled MG132/m. By fluorescence imaging of the whole body of a HeLa-luc subcutaneous tumor-bearing mouse, we evaluated the *in vivo* distribution of Alexa 647 labeled MG132/m. Selective accumulation of Alexa 647-labeled MG132/m was confirmed by strong fluorescence signal at the tumor site, 24 h post tail vein injection (**Figure 8a**). Tumor specific accumulation was also confirmed by imaging; intense fluorescence signal was observed from the tumor part compared to that from the other tissues, *i.e.* spleen, liver, heart and kidneys (**Figure 8b**).

Prolonged blood circulation profile of MG132/m was examined by intravital confocal micro-videography technique. Immediately after intravenous injection, Alexa 647-MG132/m was observed flowing inside the blood vessels within the earlobe area of the experimental mouse and remained in the blood circulation even after 16 h (**Figure 9** and **Supplementary Video S2**).

Figure 8. Biodistribution of Alexa 647-labeled MG132/m. (a) Whole body fluorescent image of subcutaneous HeLa-luc tumor bearing mouse, 24 h after the injection of Alexa 647- MG132/m. The micelles are detected specifically at the tumor site. (b) *Ex vivo* fluorescent imaging of tumor part and the other vital organs.

Figure 9. *In vivo* CLSM observation of Alexa 647-labeled MG132/m in blood vessels. (a) Three regions of interest (ROI) are selected respectively as an artery (blue), vein (green), and extravascular skin tissue (yellow). (b) Fluorescence intensity in these ROIs plotted against time (Y-axis is in logarithmic scale).

***In vivo* antitumor activity**

The antitumor activity of free MG132 and MG132/m was evaluated against subcutaneous cervical cancer (HeLa-luc) model at various dosages (**Figure 10**). Drugs were intravenously injected 6 times every second day, *i.e.* on days 0, 2, 4, 6, 8 and 10 from the start of the experiment.

Thus, free MG132 at 32 mg/kg resulted in acute toxicity as all animals died after the 2nd injection. Moreover, significant toxicity was also observed for free MG132 at 16 mg/kg, as mice presented severe body weight losses and two mice died at day 10. On the other hand, MG132/m at 16, 32 and 64 mg/kg did not show any systemic toxicity. Moreover, MG132/m at 16 mg/kg and 32 mg/kg showed significant antitumor activity, which was found to be comparable. MG132/m at 64 mg/kg presented the strongest tumor growth suppression, and the average tumor volume for this group, at the end of the experiment, was more than 3-fold lower than that of the control group. This non-linear dose response (in the range of 16-64 mg/kg), which is usually displayed by cytotoxic chemotherapeutics, could be a factor of the multiple inhibitory sites of MG132 [2, 30] and its complex apoptotic mechanism in cancer cells [31]. Thus, these findings indicate that MG132/m can significantly increase the treatment efficacy without any toxicity, expanding the therapeutic window of MG132.

Figure 10. Antitumor activity of Free MG132 and MG132 conjugated micelle against s.c. HeLa-luc tumor model. (* $P < 0.01$, ** $P < 0.0001$ students t test). (b) Body weight of the mice (†: 7 of 7, ‡: 1 of 7 and ⊙: 2 of 7 mice dead.)

DISCUSSION

Our findings highlight the effectiveness of MG132/m for enhancing the *in vivo* antitumor activity and expanding the therapeutic window of MG132. Accordingly, MG132/m at 4-fold higher dose than the maximum tolerated dose of free MG132, showed significantly higher antitumor activity than that of the free drug without any body weight loss. Thus, while the conjugation of MG132 to the micelles structure prevented drug leakage and protected the active aldehyde function of the drug from inactivation, the enhanced proteasome inhibition of MG132/m after achieving selective intracellular dissociation can be directly associated with the micelles superior *in vivo* efficacy.

MG132/m present several benefits as a drug delivery formulation. Whilst the prolonged shelf life of MG132/m suggests their applicability as a ready-to-use formulation, their high stability

at extracellular physiological conditions and their relatively small size are substantial advantages for systemic drug delivery and tumor targeting. Accordingly, we have recently observed that polymeric micelles in the sub-50 nm size range may efficiently penetrate solid tumors, even in tumors with reduced vascular permeability [32]. Thus, MG132/m having 45-nm diameter may be able to reach cells that are distant from the vasculature, achieving a homogeneous intratumoral distribution of the drug.

The time-dependent inhibition of the proteasome activity by MG132/m indicates the controlled release of the drug at the intracellular environment, after dissociation of the micelles at the late endosomal compartment. It is worth noticing that the proteasome inhibition induced by the micelles was stronger than that produced by the free drug, at equitoxic concentrations. This activity enhancement may be due to various factors, such as the protection of the inactivation of MG132, sustained levels of the drug inside the cells due to gradual release from the polymer-drug conjugates or selective subcellular targeting of MG132/m to proteasomes. The exact mechanisms that triggered the dissociation of MG132/m at the endosomal compartment and the subcellular fate of the drug is yet to understand with full details, and experiments in this direction are ongoing in our laboratory.

Proteasome inhibitors have been recognized to have a broad therapeutic potential beyond cancer treatment. Thus, proteasome inhibitors have been successfully used in rodent models of autoimmune diseases [33], as well as, for organ transplant in human patients, selective destruction of antibody-producing plasma cells [34]. Moreover, the ability of proteasome inhibitors to reduce inflammation has been used for treating inflammatory diseases such as arthritis [35], asthma [36], colitis [37] and psoriasis [38], as well as reperfusion injury after stroke [39]. This anti-inflammatory effect of proteasome inhibitors is mainly endorsed to the attenuated activation of nuclear factor kappa B (NF- κ B), a central modulator of inflammatory response [40, 41], resulting in down-regulation of anti-apoptotic Bcl-2 and MDR1 (P-glycoprotein) expression [42, 43]. These proteasome-mediated down-regulation of NF- κ B and associated genes, is also a potential strategy for overcoming inherent and acquired resistance to chemotherapy [44, 45]. In this way, proteasome inhibitors have been demonstrated to sensitize resistant cells to several anticancer agents, such as anthracyclines [46], topoisomerase inhibitors [47] and taxanes [48] in addition to sensitizing cancer cells to radiation therapy [49, 50]. Thus, polymeric micelles for safe and specific delivery of proteasomes inhibitors present high potential not only for enhancing their antitumor efficacy, but also for playing an exceptional role in combination therapy against cancer and in non-toxic targeted treatments of non-cancerous diseases.

CONCLUSION

We successfully developed polymeric micelles for intracellular delivery of proteasome inhibitor MG132 to cancer cells, avoiding drug leakage and protecting the active aldehyde function of the drug until reaching the therapeutic target. Moreover, the enhanced *in vivo* efficacy and safety of MG132/m support our strategy. Thus, this system may open the opportunity for other peptide-aldehyde based proteasome inhibitors to be included in the anticancer therapeutic arsenal.

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Graphical Abstract

Proteasome-inhibitor MG132 conjugated-micelles disintegrate only after cellular internalization through endocytosis in the target cells, where they induce cytotoxic effects and, thus, exert superior anti-tumor efficacy, avoiding systemic toxicity.