1 *Original article*

2 *Running head: SMA-cisplatin as a pH-sensitive tumor-selective nanoparticle*

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4 **pH-sensitive polymeric cisplatin-ion complex with styrene-maleic acid**

5 **copolymer exhibits tumor-selective drug delivery and antitumor**

6 **activity as a result of the enhanced permeability and retention effect**

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ABSTRACT

Cisplatin (CDDP) is widely used to treat various cancers. However, its distribution to normal tissues causes serious adverse effects. For this study, we synthesized a complex of styrene-maleic acid copolymer (SMA) and CDDP (SMA-CDDP), which formed polymeric micelles, to achieve tumor-selective drug delivery based on the enhanced permeability and retention (EPR) effect. SMA-CDDP is obtained by regulating the pH of the reaction solution of SMA and CDDP. The mean SMA-CDDP particle size was 102.5 nm in PBS according to electrophoretic light scattering, and the CDDP content was 20.1% (w/w). The release rate of free CDDP derivatives from the SMA-CDDP complex at physiological pH was quite slow (0.75%/day), whereas it was much faster at pH 5.5 (4.4%/day). SMA-CDDP thus had weaker *in vitro* toxicity at pH 7.4 but higher cytotoxicity at pH 5.5. *In vivo* pharmacokinetic studies showed a 5-fold higher tumor concentration of SMA-CDDP than of free CDDP. SMA-CDDP had more effective antitumor potential but lower toxicity than did free CDDP in mice after i.v. administration. Administration of parental free CDDP at 4 mg/kg $\times$ 3 caused a weight loss of more than 5%; SMA-CDDP at 60 mg/kg (CDDP equivalent) $\times$ 3 caused no significant weight change but markedly suppressed S-180 tumor growth. These findings together suggested using micelles of the SMA-CDDP complex as a cancer chemotherapeutic agent because of beneficial properties-tumor-selective accumulation and relatively rapid drug release at the acidic pH of the tumor-which resulted in superior antitumor effects and fewer side effects compared with free CDDP.

Keywords: Cisplatin, Styrene-maleic acid copolymer, Tumor environment-sensitive drug delivery, Tumor-selective drug delivery, EPR effect

1 **1. Introduction**

2 The anticancer drug cisplatin (*cis*-diamminedichloroplatinum[II], CDDP) was
3 discovered by Rosenberg and colleagues in 1965 [1]. CDDP was later shown to exhibit
4 potent anticancer effects. CDDP and its derivatives are now key drugs for treating many
5 malignant diseases, including stomach, esophageal, lung, ovarian, testicular, and head
6 and neck cancers. The mechanism of its anticancer activity is concentration-dependent
7 inhibition of DNA synthesis and tumor cell growth by cross-linking to the DNA
8 guanine base as well as free radical generation [2,3]. However, a major drawback of
9 CDDP is its indiscriminate distribution in normal tissues, which thereby causes adverse
10 side effects such as nephrotoxicity, myelotoxicity, and neurotoxicity; subsequent
11 discontinuation of CDDP therapy results in a poorer therapeutic outcome [4]. In
12 addition, the emergence of drug resistance to CDDP precludes its repeated use [5].
13 Also, the solubility of CDDP in aqueous solution is quite poor (~1 mg/mL), which
14 means that excessive volumes of fluid are required to keep it soluble for infusion. In
15 addition, a large-volume infusion is required to reduce the renal toxicity of CDDP. To
16 overcome these drawbacks of free CDDP, many investigations were undertaken to
17 develop a new drug delivery system that aimed to achieve better selective accumulation
18 of CDDP in tumor tissue while reducing its distribution in normal tissue [6-10].

19 Tumor-targeting strategies are categorized as active targeting and passive
20 targeting. Active targeting attempts to target tumor cells selectively on the basis of
21 antigen-antibody reactions or binding to cancer-specific receptors and ligands.
22 However, recent researches on cancer genomics proved that most human solid tumors
23 undergo extensive multiple genomic diversifications to more than 100 tumor clones. In
24 fact, clinical studies of molecular targeting drugs produced results that were not as

1 successful as had been hoped [11-14]. In contrast, passive targeting to achieve tumor-
2 selective drug accumulation is more widespread: it is attributed to pathophysiological
3 abnormalities such as architectural impairment, hyperpermeability, and hyperplasia of
4 the tumor vasculature. In addition to this hyperpermeability, the extravasated drugs
5 must be retained in tumor tissues for a long time to facilitate more complete tumor
6 eradication. In such circumstances, the concept of the enhanced permeability and
7 retention (EPR) effect of macromolecular drugs or nanomedicines has become so
8 important that is now becoming a basic principle for development of tumor-targeted
9 drug delivery [15-21]. The EPR effect, coupled with a dysfunctional lymphatic system,
10 allows nanomedicines such as liposomes [9-22] and polymeric micelles [23-25] to
11 manifest high intratumor retention for long periods.

12 To achieve EPR effect-based tumor delivery, macromolecular drugs must have
13 adequate stability during circulation but must be able to release the active drug
14 component in the tumor tissue. The interstitial pH of tumors is known to be lower than
15 the pH in circulating blood [26,27]. In addition, endosomes and lysosomes, which are
16 involved in post-intracellular uptake by endocytosis, occur in acidic conditions (pH 5.0–
17 6.0). In these conditions, having lower pH-sensitive linkers in polymeric micelles is
18 desirable [28,29].

19 Accordingly, we designed a polymeric CDDP micellar complex by using styrene-
20 maleic acid copolymer (SMA), named SMA-CDDP, which has excellent properties
21 including (i) a much wider safety margin than free CDDP; (ii) a tumor-selective
22 accumulation based on the EPR effect [15-21, 30-34]; (iii) a simple preparation
23 protocol, so the cost of manufacturing is quite economical; (iv) much greater liberation
24 of free CDDP at acidic pH than at neutral pH; (v) immunostimulating activity such as

1 activation of macrophages, T-cells, and NK cells by SMA copolymers [35,36]; (vi)
2 much higher intracellular uptake rate via endocytosis than that for polymeric micelles
3 using poly(ethylene glycol) [37-39]; and (vii) less multidrug resistance because as a
4 macromolecule [40-44] it does not fit the efflux transporter system (*e.g.* P-glycoprotein)
5 [45-47].

6 Here, we report the synthesis of this new SMA-CDDP micelle, as well as its
7 physicochemical, pharmacokinetic, tumor-selective, and other advantageous properties
8 compared with parental low-molecular-weight (low-Mw) CDDP.

9 10 **2. Materials and methods**

11 *2.1. Materials*

12 Anhydride SMA with a mean molecular size of 1898 Da (Mw/Mn: 1.26) was
13 supplied by Kuraray Ltd., Kurashiki, Japan. SMA used in this experiment was
14 synthesized by conventional radical condensation as described earlier [48], with
15 continuous alternating styrene/maleic anhydride. It was purified by using large-scale
16 column chromatography with stepwise increases of solvent. The product thereby
17 obtained was as good as a RAFT-synthesized polymer, as described in previous
18 literature [32].

19 CDDP, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and
20 trypsin were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan; 3-(4,5-
21 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from
22 Dojindo Chemical Laboratory, Kumamoto, Japan. All other chemicals were of reagent
23 grade from commercial sources and were used without further purification.

2.2. *Synthesis of the SMA-CDDP complex*

SMA (anhydride) copolymer 0.5 g (2.47 mmol, assuming a mean repeating units of 9 SMA monomer per SMA chain), and CDDP, 0.2 g (0.67 mmol), were dissolved in 0.1 M Na₂CO₃, and the mixture was then sonicated for 5 min. The mixture was stirred with a magnetic bar at 45 °C for 3 days at 200 rpm in the dark. During this process, the mixture gradually became a clear solution. Then, the solution was lyophilized. The lyophilized product was dissolved in distilled water, and the solution was stirred at 200 rpm in the dark at room temperature for 1 day, followed by ultrafiltration using the Millipore Labscale TFF system (Millipore, Billerica, MA, USA) with a membrane having an Mw cutoff of 5 kDa to remove free CDDP, free SMA, and their breakdown products from the SMA-CDDP micelles, after which lyophilization was performed to obtain SMA-CDDP powder.

2.3. *Characterization of the SMA-CDDP complex*

2.3.1. *Size exclusion chromatography of SMA-CDDP*

Sephadex G-25 Fine chromatography (column size: 41 cm × 1.5 cm; Pharmacia LKB, Uppsala, Sweden) was performed to confirm the uniform size distribution of the SMA-CDDP complex. The column was eluted with 10 mM sodium phosphate buffer (pH 7.4). The absorbance at 300 nm (λ_{max} of CDDP) and at 258 nm (λ_{max} of SMA) was measured for each 6-mL fraction collected.

2.3.2. *UV-visible spectrophotometry and infrared (IR) spectroscopy*

UV/visible absorption spectra were obtained by means of a spectrophotometer (U-2000A; Hitachi Ltd., Tokyo, Japan). IR spectra were recorded with an FTIR-4200

1 spectrometer (JASCO Corp., Tokyo, Japan) by using KBr discs of samples.

2 3 *2.3.3. Measurement of platinum content and CDDP loading in SMA-CDDP*

4 The SMA-CDDP complex was dissolved in 0.15 M saline at 0.5 mg/mL and was
5 applied to the Model AA-6700 flameless atomic absorption spectrophotometer (FAAS)
6 (Shimadzu, Kyoto, Japan). The amount of CDDP in this sample was estimated from the
7 measured platinum content as CDDP-equivalent weight by using the chemical formula
8 of CDDP ($\text{Pt}(\text{NH}_3)_2\text{Cl}_2$), after which CDDP loading in the SMA-CDDP complex was
9 calculated as compared with the amount of tested SMA-CDDP.

10 11 *2.3.4. Size distribution and zeta potential*

12 The size distribution and *zeta* potential of SMA-CDDP were measured by means
13 of an electrophoretic light scattering analyzer (Model ELSZ; Photol Otsuka Electronics,
14 Osaka, Japan), which is equipped with He/Ne laser optics, by using cumulant and
15 histogram methods. The analysis was performed with 10 mg/mL SMA-CDDP prepared
16 in 10 mM phosphate-buffered 0.15 M saline (PBS), pH 7.4, measured at 25 °C. The
17 sample solution was filtered through a 0.22- μm filter immediately before measurement.

18 19 *2.3.5. Dynamic light scattering (DLS) and static light scattering (SLS)*

20 The DLS and SLS measurements were performed on Photol DLS-7000 HLs
21 instrument (Otsuka Electronics, Osaka); equipped with a He-Ne laser (10 mW) at a
22 wavelength of 632.8 nm. The scattering angle was fixed at 90° for DLS and multi-angle
23 (20° ~ 150°) was used for SLS measurement at 25°C. DLS was carried out using 4
24 mg/ml sample prepared in PBS (pH 7.4), and SLS was carried out using samples of 1, 2,

1 3, 4 mg/ml in PBS (pH 7.4). The sample solution was filtered through a 0.22- μ m filter
2 immediately before measurement. For SLS analysis, the differential index of refraction
3 (dn/dc) of SMA-CDDP solution in PBS (pH 7.4) was determined by using a differential
4 refractometer (DRM-3000, Otsuka Electronics, Osaka)

6 2.3.6. *Transmission electron microscopy*

7 A drop of SMA-CDDP (0.05 mg/mL) was applied to a copper grid coated with
8 carbon film and was air-dried. The image and size of the SMA-CDDP complex were
9 analyzed by means of a transmission electron microscope (Tecnai F20; FEI, Hillsboro,
10 OR, USA).

12 2.3.7. *Titration of hydrolyzed SMA and SMA-CDDP*

13 A 10-mL solution of hydrolyzed SMA (8 mg/mL) that was prepared according to
14 previously published methods [31] and SMA-CDDP (10 mg/mL, containing 8 mg/mL
15 SMA) were prepared with deionized water. Titration was carried out by using 0.01 N
16 HCl or NaOH (standard titration solution) under a nitrogen barrier with stirring, and the
17 pH values of sample solutions were monitored.

19 2.3.8. *Release rate of CDDP from SMA-CDDP*

20 To determine the release rate of hydrated CDDP derivatives from the SMA-CDDP
21 complex *in vitro*, 26 mg of the SMA-CDDP complex, which contained 20% (w/w)
22 CDDP, was dissolved in 1 mL of PBS without magnesium and calcium and placed in a
23 sealed dialysis tube (Mw cutoff of 500 Da; Spectra/Por; Spectrum Laboratories Inc.,
24 Rancho Dominguez, CA, USA). The dialysis tubes were submerged in a 50-mL glass

1 test tube containing 25 mL of PBS, and the pH was adjusted to either pH 5.5 or pH 7.4.
2 The dialysis tubes were then incubated for several hours at 37 °C in the dark with
3 reciprocal shaking at 1 Hz. Hydrated CDDP derivatives released from the dialysis tubes
4 were collected at predetermined time intervals and were quantified by using an FAAS.

6 *2.4. In vitro cytotoxicity*

7 *In vitro* cytotoxicity of free CDDP and SMA-CDDP was analyzed by using the
8 MTT method [49] with HeLa cells. Three thousand cells/well were plated in Falcon 96-
9 well culture plates (BD Labware, Franklin Lakes, NJ, USA) and were cultured
10 overnight in DMEM with 10% fetal calf serum at 37 °C in an atmosphere of 5% CO₂-
11 95% air. The cells were then incubated for 24 h in PBS at pH 5.5 or pH 7.4 with various
12 concentrations of free CDDP or SMA-CDDP. After the overnight incubation, the
13 medium was replaced with fresh medium for another 24 h. Then, the MTT assay was
14 performed and the toxicity was quantified as the fraction of surviving cells relative to
15 no-drug controls.

16 In some experiments, the cytotoxicity of the mixture of SMA and CDDP was
17 investigated to compare it with that of the SMA-CDDP complex. After HeLa cells were
18 incubated overnight, the SMA-CDDP complex or a mixture of SMA with CDDP (the
19 same amounts of SMA and CDDP as the amounts in the SMA-CDDP complex) was
20 added to the cells, and after 48 h of treatment, the MTT assay was performed.

22 *2.5. In vivo antitumor effect of the SMA-CDDP complex*

23 Mouse sarcoma S-180 cells (2×10^6 cells), which were maintained as an ascitic
24 tumor that was passaged every 7-10 days in ddY mice, were implanted subcutaneously

1 in the dorsal skin of 6-week-old male mice. When tumors were 5-6 mm in diameter,
2 mice were subjected to drug treatment, administered via the tail vein, at a dose of 8 or
3 60 mg/kg (CDDP equivalent) in three equal doses every 3 days. The day of initial
4 administration was set as day 0, followed by administration on days 3 and 6. Tumor
5 growth was determined as the tumor volume (V) measured with a digital caliper; the
6 volume was estimated by using the longitudinal cross section (L) and transverse section
7 (W) according to the formula $V (mm^3) = W^2 \times L / 2$. Tumor volumes at each time point
8 were normalized by using the initial tumor volume and were presented as means $\pm$
9 standard error (SE). The weight of each mouse was measured as an index of acute
10 toxicity, and it was normalized to the initial weight, as with the tumor volume.

11

12 *2.6. Tissue distribution of the SMA-CDDP complex after i.v. injection*

13 The mouse sarcoma S-180 solid tumor model was also used in this part of the
14 study. To obtain enough tumor tissue for the distribution assay, when tumors were 8-10
15 mm in diameter, free CDDP or SMA-CDDP was administered to mice via the tail vein
16 at dose of 5 mg/kg (CDDP equivalent). After 24 h, mice were killed, blood samples
17 were drawn from the inferior vena cava, and mice were subjected to reperfusion with 10
18 mL of saline containing heparin (5 units/mL) to remove blood components in the blood
19 vessels of each tissue. Then, tumor tissues as well as normal tissues, including the liver,
20 spleen, kidney, intestine, colon, heart, lung, brain, and muscle, were collected and
21 weighed. Whole blood was centrifuged at 5000 rpm for 20 min, and plasma was frozen
22 at -80°C until analysis. To each 100-mg sample of tissue, 1 mL of 6.7% nitric acid was
23 added, followed by incubation for 2 h at 80°C , after which samples were kept at room

1 temperature for 1 week before analysis. Then, samples were homogenized and
2 centrifuged ($12,000 \times g$, 25 °C, 10 min) to precipitate insoluble tissue debris. The
3 supernatant as well as the plasma (1.5 mL) was diluted 10 times with 6.7% nitric acid,
4 and the platinum content in each tissue or plasma sample was quantified by using an
5 atomic absorption spectrophotometer (AA-890; Thermo Fisher Scientific K.K.,
6 Yokohama, Japan).

8 2.7. Statistical analyses

9 Data were analyzed by using ANOVA followed by the Bonferroni *t*-test. A
10 difference was considered to be statistically significant when $p < 0.05$.

12 3. Results and Discussion

13 3.1. Synthesis of SMA-CDDP

14 Figure 1 shows the synthetic scheme for SMA-CDDP. An important point in this
15 scheme is that the maleyl dicarboxyl group of SMA formed a dicarboxylate ion
16 complex via hydrolysis of SMA (anhydride form) in basic conditions, at about pH 10.0.
17 The dicarboxylate form of SMA can bind to the bivalent cation of CDDP in aqueous
18 solution to form ion complexes (via chelation), which are stable in normal aqueous
19 systems including dialysis. We expected that the SMA-CDDP complex was formed
20 through ionic interaction between Pt^{2+} and SMA with ionized maleyl residues, with
21 CDDP being chelated either with the same SMA molecule or with a different SMA
22 molecule, to form a polymeric complex as illustrated in Fig. 1. The transmission
23 electron microscopic findings of SMA-CDDP that more electron dense structure (due to
24 Pt^{2+}) were seen in the outer surface of the particle (Fig. 1), supported our expectation of

1 the possible structure of SMA-CDDP, in which styrene residues of SMA formed a
2 hydrophobic core and CDDP distributed throughout the complex but tended to be more
3 oriented toward the outer surface, as it interacted with negatively charged maleyl groups
4 of the SMA-CDDP complex (Fig. 1).

5 Table 1 summarizes the physicochemical characteristics of SMA-CDDP as
6 compared with SMA. The content of CDDP in SMA-CDDP was relatively high, about
7 $20.1 \pm 0.84\%$ (w/w) of CDDP in the complex, as determined via FAAS. The yield of
8 SMA-CDDP was approximately 75% based on CDDP by weight. In addition, SMA-
9 CDDP had excellent solubility in aqueous solution compared with free CDDP (SMA-
10 CDDP > 5.2 mg/mL; free CDDP < 1.0 mg/mL).

12 *3.2. Characterization of SMA-CDDP*

13 *3.2.1. Size exclusion chromatography*

14 As Fig. 2A shows, the SMA-CDDP complex clearly manifested an increase in
15 molecular size compared with free CDDP, according to Sephadex G-25 Fine
16 chromatography. SMA-CDDP appeared relatively stable and had a monodisperse
17 macromolecular size distribution.

19 *3.2.2. Particle size and surface charge of SMA-CDDP*

20 As Fig. 2B illustrates, SMA-CDDP had a monodisperse peak with a mean
21 diameter of 102.5 ± 43.4 nm, as measured by electrophoretic light scattering in PBS at
22 pH 7.4. Having the size of a macromolecule is a critical parameter for the operation of
23 the EPR-based drug delivery to tumors, so as to avoid renal excretion and maintain a
24 high plasma concentration of the drug. The EPR effect consequently facilitates effective

1 accumulation in the tumor tissue [50], as described below. In addition, by DLS the
2 average hydrodynamic radius (R_h) was found to be 61.4 nm which is similar to the size
3 determined by electrophoretic light scattering (Table 1). The dn/dc of SMA-CDDP were
4 0.1414, and radius of gyration (R_g) of SMA-CDDP was then determined to be 67.3 nm
5 by SLS (Table 1). Regarding the R_g/R_h value, although generally $R_g/R_h = 1$ means
6 hollow spheres, i.e., vesicles [51,52], this may not be applicable to our SMA-CDDP
7 complex because it is consisting of two components (SMA and Pt) and Pt has much
8 larger scattering length than other organic component. When the outer region of a
9 micelle has a large scattering length than the inner region, R_g becomes close to R_h , i.e.,
10 $R_g/R_h = 1$. Therefore, our result of R_g/R_h value (1.09) suggested that SMA-CDDP in
11 aqueous solution (i.e., PBS) behaves as micelles with Pt mostly distributing in the outer
12 surface, which is consistent to the findings of transmission electron microscopy (Fig. 1)
13 and supported our estimation of the structure of SMA-CDDP as shown in Fig. 1.

14 The zeta potential, which reflects the surface charge of particles, of SMA-CDDP
15 was -20.86 mV in PBS; SMA-CDDP thus had a less negative charge compared with
16 free SMA alone (-37.81 mV in PBS). The negative charge of SMA is due to the
17 carboxylate of maleic acid, so the zeta potential finding may indicate formation of an
18 ionic bond between SMA and CDDP, which resulted in partial neutralization of
19 carboxylate. Also, titration of free SMA and SMA-CDDP showed that SMA-CDDP had
20 consumed less alkaline for complete deprotonation as revealed by titration curve
21 (Supplemental data Fig. S1). Moreover, isoionic pH of SMA and SMA-CDDP was
22 found to be ~ 7.4 and ~ 8.8 respectively (Table 1). These findings further support the
23 complex formation of SMA-CDDP through ionic bond.

24 In general, positively charged particles are eliminated rapidly from the blood

1 circulation and excessively negatively charged particles are easily taken up by the liver
2 [53]. The present finding of an almost 50% reduction in the negative charge of the
3 carboxylate ion contributes to a longer circulation time and an improved EPR effect for
4 drug targeting to tumors.

6 3.2.3. *UV-visible and IR spectroscopy*

7 As seen in Fig. 3A, hydrolyzed SMA alone had an absorption λ_{max} at 258 nm in
8 water at pH 8.5 but no absorption above 300 nm. However, the UV spectrum of SMA-
9 CDDP had a peak at 258 nm (corresponding to SMA) and a broad shoulder at about 300
10 nm (corresponding to CDDP). Although no clear peak or hidden peak was observed in
11 the shoulder, these findings may indicate the presence of both components (SMA and
12 CDDP) as a complex.

13 IR spectroscopy showed that the SMA anhydride had only an extremely weak
14 peak at about 3400 cm^{-1} (Supplementary Materials, Fig. S2A). However, hydrolyzed
15 SMA and SMA-CDDP had broad peaks that corresponded to O-H stretching because of
16 maleic acid (Supplementary Materials, Fig. S2A). Moreover, hydrolyzed SMA and
17 SMA-CDDP had slight differences at 1400 cm^{-1} (Supplementary Materials, Fig. S2B).
18 These findings may indicate that the carboxyl group in hydrolyzed SMA plays a role in
19 complexing with CDDP. The free CDDP spectrum showed no clear IR peak at about
20 1400 cm^{-1} in contrast to the SMA-CDDP spectrum, which did have such a peak (Fig.
21 3B). These results also partly support the formation of an SMA-CDDP complex.

23 3.2.4. *Release of free CDDP from SMA-CDDP*

24 Figure 4 indicates the release rate of hydrated CDDP derivatives from the SMA-

1 CDDP complex *in vitro* by using dialysis tubes. Hydrated CDDP derivatives were
2 released much more slowly from the SMA-CDDP complex in PBS at pH 7.4 (0.75%
3 per day) than at pH 5.5 (4.4% per day). This finding suggests that the acidic conditions
4 occurring in tumor tissues (pH about 6-7) are favorable for release of CDDP from
5 SMA-CDDP. After internalization of SMA-CDDP into tumor cells via endocytosis, the
6 lower pH (~5) of the phagolysosomal system will facilitate a more rapid release of
7 hydrated CDDP derivatives. The much lower release of CDDP derivatives at neutral or
8 physiological pH (7.4), as in circulating blood, is again a favorable characteristic of this
9 SMA-CDDP complex, with its better stability. When the complex reaches the tumor
10 tissue, CDDP derivatives will be released rapidly in response to the tumor's acidic
11 environment, and therefore the SMA-CDDP complex possesses another beneficial
12 property: greater tumor-selective efficacy.

13 Utilizing the acidic tumor pH-responsive feature is one of the promising strategies
14 for enhancing the efficacy of polymeric antitumor drugs via tumor-targeted release of
15 active drugs; this approach has been investigated and verified in our laboratory as well
16 as by other research groups [54,55]. Other strategies for developing tumor environment-
17 responsive polymer conjugates include utilizing cleavable polypeptide linkers between
18 polymers and drugs, for example, a cathepsin B-cleavable peptide linkage
19 (glycylphenylalanylleucylglycine, GFLG) [56]. All these strategies will ensure rapid
20 and selective release of active drugs in tumor tissues, thereby resulting in a superior
21 antitumor effect.

22

23 3.3. Anticancer activity of SMA-CDDP *in vitro* and *in vivo*

24 *In vitro* study: Figure 5 provides data for the cytotoxic activity of free CDDP and

1 SMA-CDDP against HeLa cells, for treatments at both pH 5.5 and pH 7.4. As expected,
2 free CDDP exhibited anticancer activity regardless of the pH of the medium. However,
3 the cytotoxicity of SMA-CDDP was critically influenced by the different pH values.
4 SMA-CDDP had a more potent effect at the acidic pH but was much less toxic at the
5 neutral or physiological pH. These results are consistent with the rate of drug release
6 from SMA-CDDP at a lower pH value. Therefore, our results indicated that SMA-
7 CDDP could achieve pH-dependent controlled drug release.

8 In addition, when we compared the cytotoxicity of the SMA-CDDP complex with
9 that of the mixture of SMA with CDDP, we found that the mixture showed much
10 stronger cytotoxicity than did the SMA-CDDP complex, results that are similar to the
11 result for free CDDP (Supplementary Materials, Fig. S3). These data may also provide
12 some evidence to support formation of a micellar SMA-CDDP complex, probably via
13 ionic interaction, rather than just a simple mixture. Our future studies will continue the
14 characterization of the ion-complex formation.

15 *In vivo study:* To evaluate antitumor activity *in vivo*, we measured the volume of
16 S-180 solid tumors in ddY mice. As Fig. 6A shows, SMA-CDDP had dose-dependent
17 antitumor activity. Also, SMA-CDDP given at a total of 180 mg/kg of CDDP equivalent
18 (given as 60 mg/kg $\times$ 3, i.v.) resulted in almost complete suppression of tumor growth.

19 Both the profile of release of free CDDP from SMA-CDDP at the acidic tumor pH
20 and the *in vitro* cytotoxicity are consistent with the ability of SMA-CDDP to inhibit
21 tumor growth *in vivo*. Here, tumor-selective accumulation of SMA-CDDP, based on the
22 EPR effect, is a prerequisite for these remarkable results, without visible adverse
23 effects. That is, as seen in Fig. 6B, the group given SMA-CDDP demonstrated no
24 difference in normalized body weight during the study. However, mice treated with free

CDDP had a significant weight loss when the body weight on day 0 was compared with that on days 5-8 (Fig. 6B). This finding indicated that SMA-CDDP was well tolerated, even more so than the 15-fold CDDP equivalent dose, and thus had a much greater safety window than did free CDDP.

Overall, these results show that the ionic complex of SMA polymer with CDDP, with an apparent particle diameter of above 100 nm, would achieve a significantly better therapeutic effect and alleviation of the distressing adverse reactions of free CDDP by utilizing EPR effect-based tumor targeting.

3.4. Tissue distribution of SMA-CDDP

Figure 7 shows that SMA-CDDP had a significantly higher accumulation in tumor tissues at 24 h after i.v. administration than did free CDDP (~5-fold higher). Higher accumulation of SMA-CDDP compared with free CDDP also occurred in the liver and spleen, which are rich in reticuloendothelial system (RES) components, and many polymeric drugs including SMA conjugates are known to be captured by the RES [57-59]. SMA-CDDP exhibited a little higher accumulation, but not a significant one, in the kidney compared with free CDDP (Fig. 7). In most other normal tissues, no difference in distribution was noted between SMA-CDDP and free CDDP (Fig. 7). More important, the concentration of SMA-CDDP in tumor tissues was much higher than that in most normal tissues (Fig. 7)

These findings are in agreement with the functioning of the EPR effect, that is, SMA-CDDP behaves as a macromolecule that selectively accumulates in tumor tissues but accumulates less in normal tissues, the results being a marked antitumor effect and less cytotoxicity compared with free CDDP (Fig. 6). Although SMA-CDDP also had a

1 high level of accumulation in the liver and spleen, no apparent side effects were
2 observed, even at the dose of 180 mg/kg (Fig. 6B). These data may support the
3 relationship between pH and the effects of SMA-CDDP, in that much less CDDP is
4 released at the neutral pH of normal tissues, which results in little cytotoxicity, but the
5 acidic pH of tumor tissues triggers the release of CDDP, which leads to tumor-selective
6 cytotoxicity.

7 In addition, the tumors used in the tissue distribution study (8-10 mm) were larger
8 than those used in the *in vivo* treatment study (5-6 mm). However, such differences in
9 tumor size will not influence the EPR effect. Tumor angiogenesis and the EPR effect
10 have been observed in tumors larger than 0.1-1.0 mm in diameter and even in smaller
11 tumors [60]. Also, experimentally induced tumors smaller than 1 cm in diameter are
12 usually suitable for EPR-based drug delivery, but tumors larger than 3 cm in diameter
13 may show extensive heterogeneity of the EPR effect in some necrotic and avascular
14 regions [60].

16 **4. Conclusions**

17 We successfully developed a simple and efficient method to synthesize a high-
18 loading, water-soluble polymer complex of CDDP by preparing an ionic complex with
19 dicarboxylate residues of maleyl groups in the SMA chain. The SMA-CDDP complex
20 had the desired controlled release profile of CDDP under weakly acidic pH values.

21 Our SMA-CDDP complex also had relative stability in the blood circulation at
22 neutral pH, with much less drug release in circulation and thus less toxicity compared
23 with free CDDP. Also, because of its macromolecular nature, it could take advantage of
24 the EPR effect and had better pharmacokinetics than did free CDDP. The marked

1 antitumor activity of SMA-CDDP occurs via liberation of free CDDP from the
2 complex, especially in the acidic tumor milieu, and probably depends on hydrolysis of
3 the maleyl linkage in the ionic complex (Fig. 6B).

4 Also, like other SMA micelles, SMA-CDDP was rapidly incorporated into cancer
5 cells by means of endocytosis [38,39], with quick release of free CDDP occurring via
6 endosomes. In addition, this SMA-CDDP complex had significantly improved water
7 solubility compared with free CDDP, which has poor water solubility, so it is much
8 easier to administer large doses of the SMA-CDDP complex at one time, which is not
9 possible with free CDDP.

10 Together the present data suggest that SMA-CDDP may become an EPR effect-
11 based tumor-targeted drug with high tumor selectivity. Additional investigations with
12 different tumor cell lines and tumor models including human tumor xenografts and
13 carcinogen-induced cancers will be performed, with aims to accomplishing translational
14 research and clinical evaluation.

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No potential conflicts of interest were disclosed.

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24

Figure Legends

Fig. 1. The scheme of synthesis of the SMA-CDDP complex from styrene-maleic acid (SMA) and *cis*-diamminedichloroplatinum(II) (CDDP). Maleic anhydride residues in SMA were hydrolyzed in 0.1 M Na₂CO₃ at pH 10.0, and CDDP was added and stirred at 45 °C for 3 days (see text for details). A hypothetical structure of the SMA-CDDP complex is given at the top right. CDDP or its moieties, either the hydrated form or the ionized form of platinum (Pt in blue circles), will form complexes with carboxyl groups of maleyl residues. The hydrophobic styrene residues and protonated carboxyl groups of the maleyl residues (H in blue circles) tend to be clustered inside of the complex. The hydrophilic ionized carboxylate residue of the maleyl groups are interacted with ionized form of platinum (CDDP derivatives) which distribute throughout the complex but tend to be more oriented toward the outer surface of water phase. A transmission electron micrograph of this SMA-CDDP micellar complex is at the lower right (see text), in which more electron dense structure (due to Pt²⁺) were seen in the outer surface of the particle.

Fig. 2. Sephadex G-25 Fine chromatography (A) and particle size distribution (B) of SMA-CDDP. (A) A Sephadex G-25 column of 1.5 × 41 cm was used, and SMA-CDDP or free CDDP was eluted with 10 mM sodium phosphate buffer at pH 7.4. For (B), each sample (10 mg/mL) was prepared in PBS, pH 7.4, and was subjected to electrophoretic light scattering.

Fig. 3. UV-visible spectroscopy (A) and IR spectra (B) of SMA-CDDP. (A) Samples of SMA, free CDDP, and SMA-CDDP were prepared in 0.1 M Na₂CO₃. (B) IR spectra were recorded by using KBr discs.

Fig. 4. Release of hydrated CDDP derivatives from SMA-CDDP. (A) Release at pH 7.4 and at pH 5.5. Results are expressed as the mean $\pm$ SD ($n = 3$). $*p < 0.05$; $**p < 0.01$. (B) Mechanism for releasing hydrated CDDP derivatives from SMA-CDDP. In acidic conditions (medium containing chloride ions), hydrated CDDP derivatives were rapidly released from SMA-CDDP.

Fig. 5. *In vitro* cytotoxic effects of SMA-CDDP and free CDDP on the human cervical cancer HeLa cell line. Each test drug was incubated for 2 days at 37 °C at pH 5.5 or pH 7.4, after which it was added to the cells, which were treated for 72 h. Results are expressed as the mean $\pm$ SD. Data are expressed as mean $\pm$ SD. ($n = 8$)

Fig. 6. *In vivo* antitumor effect (A) and toxic effect (B) of SMA-CDDP. Different doses of CDDP and SMA-CDDP were administered to 6-week-old male ddY mice bearing sarcoma S-180 tumor, and the tumor sizes and body weights of mice were monitored. (A) Normalized tumor volume was evaluated as a function of time as a measure of antitumor activity. Results are expressed as the mean $\pm$ SE. (B) Normalized body weight compared with pretreatment weight was assessed as a marker of general acute toxicity. Results are expressed as the mean $\pm$ SD ($n = 4-5$).

Fig. 7. Tissue distribution of SMA-CDDP and free CDDP in S-180 tumor-bearing male

1 ddY mice. SMA-CDDP or free CDDP was injected i.v. at 5 mg/kg of CDDP equivalent.
2 At 24 h after administration of drugs, mice were killed, and samples of blood, tumor,
3 liver, and other normal tissues and organs were collected. Platinum in each tissue or
4 organ was then measured by using an atomic absorption spectrophotometer. Results are
5 expressed as the mean $\pm$ SE ($n = 3-4$).
6
7
8

1 **Table 1.** Physicochemical characteristics of SMA-CDDP (compared with SMA)¹.

Parameters	SMA	SMA-CDDP
CDDP content (w/w) ² in the complex	–	20.1 ± 0.84
Particle size ³	~ 2 nm	102.5 ± 43.4 nm
Zeta potential	-37.81 mV	-20.86 mV
Radius of gyration (R _g) ⁴	–	67.3 nm
Hydrodynamic radius (R _h) ⁴	–	61.4 nm
Differential index of refraction (dn/dc)	–	0.1414
Clouding point (pH)	2.2 – 2.3	3.2 – 3.3
Precipitation start at pH	1.9 – 2.0	2.1 – 2.2
Color	Colorless	Pale yellow

2 ¹ Samples are dissolved in PBS (pH 7.4).

3 ² CDDP content was calculated as defined by the formula in Section 2.3.3.

4 ³ Data are from electrophoretic light scattering

5 ⁴ Data are from DLS (R_h) and SLS (R_g).

6

7

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Graphical Abstract