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# Chelating Complex Micelles for Delivering Cytoprotectant Amifostine and its Application in Radiation Protection

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## Abstract

A new nano-size drug carrier was proposed to overcome the shortcomings of conventional drug delivery systems. Amifsotine, a hydrophilic and extremely short half-life cytoprotective agent, was loaded based on chelating complex micelles (CCM) in order to provide the protection from radiation exposure. The preparation of amifostine-loaded CCM (CCM-Ami) was simply mixing ferrous chloride, poly(ethylene glycol)-*block*-poly (glutamic acid) (PEG-*b*-PGA) and amifostine in an aqueous solution without using any organic solvent. The resulting CCM-Ami monodispersed with a mean particle size 25 nm and showed a slow release behavior as compared to amifostine. Furthermore, CCM-Ami pretreatment improved survival rates and median survival in C57BL/6 mice than did treatment with a corresponding dose of amifostine. These results point to a potential use of CCM as a novel drug carrier. Drug molecules that may act as ligands can be considered delivering by this platform technology.

**Keywords:** Chelating complex micelles; Polymeric micelles; Drug delivery system; Amifostine; Radiation protection

**Abbreviations:** CCM: Chelating Complex Micelles; DDS: Drug Delivery System; EE: Encapsulation Efficiency; HPLC: High Performance Liquid Chromatography.

## Introduction

In order to prolong the effective period of drugs, higher dosage or multiple dosing beyond the effective blood concentration are necessary. However, administration of high dosage is potentially toxic to the body and may cause significant adverse effects. To overcome the shortcomings of drug overdose, drug delivery system (DDS) has been developed to enhance drug efficacy and reduce side effects. Polymeric micelles, which are commonly formed by amphiphilic block copolymers, demonstrate excellent potential in the field of DDS. Nano-scaled micelles were designed to extend the half-lives of drugs and reduce the uptake by reticuloendothelial system (RES) [1]. Drugs can be loaded into polymeric micelles through physical encapsulation, covalent bonding, or electrostatic interactions. Physical encapsulation, which requires large quantities of organic solvents, is used to encapsulate hydrophobic drugs due to the intrinsic properties of block copolymer [2-4]. Preparation of polymer-drug conjugate requires complicated synthetic steps and exhibits low conjugating efficiency [5,6]. Electrostatic interaction has also been attempted for the preparation of polymeric micelles. In the appropriate medium, the ionizable regions of the micelles interacted with oppositely charged molecules, thus forming polyion complex micelles (PIC micelles) with a core-shell structure [7,8]. However, it would be more suitable for PIC micelles to incorporate polyelectrolytes or macromolecules like nucleic acids and proteins [9]. As a result, developing a new drug delivery carrier represents a major target for an unmet need within the drug industry [10].

In the study described herein, the chelating complex micelles (CCM) composed of ferrous ion, poly(ethylene glycol)-*block*-poly (glutamic acid) (PEG-*b*-PGA), and cytoprotectant amifostine (WR-2721) are designed for the prevention of radiation damage. Amifostine is a thiophosphate cytoprotective agent developed as a radio protector [11]. First developed by the U.S. Army to protect troops from radiation in the event of nuclear warfare, it is now used to reduce the side effects associated with platinum-containing agents [12]. However, approximately 90% of the amifostine was rapidly cleared from plasma after 6 minutes after intravenous administration [13]. The efficacy of its use for alleviating the side effects of chemotherapy and space travel is also limited by the extremely short half-life. Re-formulations including microcapsules and polymer complex were investigated to assess the efficacy for radioprotection, but no better result was found [14-16]. This article describes the preparation and characterization of amifostine-loaded CCM (CCM-Ami). The safety and effectiveness studies for the prevention of radiation damage were also assessed.

## Materials and Methods

### Reagents

γ-Benzyl L-glutamate was purchased from Carbosynth (Berkshire, UK). Triphosgene was purchased from TCI (Tokyo, Japan). Ethyl acetate and n-hexane was purchased from Macron (St. Louis, USA). Tetrahydrofuran was obtained from Tedia (Fairfield, USA). Methoxy poly (ethylene glycol) amine (mPEG-$NH_2$; Mw 2000) was purchased from SunBio (Gunpo, Korea). Dehydrated dimethyl sulfoxide was obtained from Echo (Miaoli, Taiwan). Methanol and isopropyl ether were purchased from Duksan (Ansan, Korea) and J. T. Baker (Phillipsburg, USA). Ferrous chloride tetrahydrate was obtained from J. T. Baker (Phillipsburg, USA). Amifostine trihydrate was purchased from Runzhong Pharmaceutical (Lianyungang, China). Purified water was obtained by Milli-Q purification system (Millipore Co., Bedford, USA). All other chemicals and reagents were analytical grade and used without further purification.

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## Methods

**Synthesis of poly(ethylene glycol)-*block*-poly (glutamic acid) (PEG-*b*-PGA):** γ-Benzyl L-glutamate *N*-carboxyanhydride (Glu(OBzl)-NCA) was synthesized by reacting 16 g of γ-benzyl L-glutamate with 8 g of triphosgene in 140 mL of tetrahydrofuran at 55°C for 3 hours. The mixed solution became clear and was then concentrated to approximately 20 mL followed by recrystallization in 60 mL of 1:1 ethyl acetate/n-hexane (v/v) for 3 times. The crystalline product was washed with n-hexane and then vacuum dried at 40°C for 4 hours. After that, PEG-*b*-PGA was synthesized by ring-opening polymerization of 12 g of Glu (OBzl)-NCA with 2.8 g of mPEG-$NH_2$ as the initiator in 70 mL of dimethyl sulfoxide at 40°C for 16 hours. After concentrating to approximately 30 mL, the product was precipitated in 300 mL of 1:1 methanol/isopropyl ether (v/v). The resulting PEG-*b*-poly (Glu (OBzl)) was collected by filtration followed by hydrolysis with 72 mL of 1*N* NaOH solution and 144 mL of 95% ethanol at 40°C for 4 hours. The mixed solution became clear and was then adjusted to pH 7.0 with 37% HCl. To remove benzyl alcohol and other impurities, the product was dialyzed against 14 L of pure water using Spectra/Por® 6 dialysis membrane (MWCO 3,500 Da). The water was exchanged every hour. After 14 hours of dialysis, the resulting solution was concentrated to density 1.15 g/mL by rotary evaporator and then lyophilized to obtain a white amorphous mass.

**Preparation of amifostine-loaded CCM (CCM-Ami):** CCM-Ami was prepared by mixing amifostine trihydrate, PEG-*b*-PGA, and ferrous chloride tetrahydrate in aqueous solution. PEG-*b*-PGA (330 mg) was dissolved in 10 mL of water. Amifostine trihydrate (145 mg) was dissolved in 2.7 mL of water and then mixed with the PEG-*b*-PGA solution. Ferrous chloride tetrahydrate (84 mg) was dissolved in 3.3 mL of 0.01*N* HCl and then added drop wise to the mixed solution of PEG-*b*-PGA and amifostine. The reaction was stirred at room temperature for 24 hours. To remove the unreacted amifostine and ferrous chloride, the resulting CCM-Ami were then purified by ultrafiltration in Amicon® stirred cell (UFSC05001; MWCO 5,000 Da) using water as diluent. The solution was concentrated to 12 mL followed adding 2 mL of water for 3 times. The volume of CCM-Ami was adjusted to 20 mL, and the theoretical concentration of amifostine was 5.8 mg/mL.

**Characterizations:** $^1$H-NMR spectrum of PEG-*b*-PGA was recorded on a Bruker Avance 600 MHz spectrometer. The molecular weight of PEG-*b*-PGA was analyzed using a gel permeation chromatography (GPC) system consisting of a Jasco PU-2080 Plus pump, a Phenomenex PolySep-GFC-P 3000 column, and a Jasco RI-2031 Plus differential refractive index detector with PEG as a calibration standard (American Polymer Standaeds Corp.). Sodium acetate (25 g/L) adjusted with acetic acid to pH 4.3 was used as an eluent at a flow rate of 1.0 mL/min. The high performance liquid chromatography (HPLC) analyses were conducted for monitoring the unreacted amifostine in the ultrafiltration process (UltiMate 3000 series, diode array detector, Thermo Fisher Scientific Inc.). Inert Sustain C8 column (5 μm, 4.6 × 250 mm, GL Sciences Inc.) eluted with 1.0 mL/min of pH 3.0 1-hexanesulfonic acid sodium salt/$H_3PO_4$/acetonitrile at detection wavelength 210 nm was used for amifostine analysis. The particle size of the CCM-Ami was determined by dynamic light scattering (DLS) instrument (Zetasizer Nano ZSP, Malvern).

***In vitro* release:** Twenty milliliter of CCM-Ami and amifostine (5.8 mg/mL) solution were placed in separate dialysis tubes (MWCO 3,500 Da), respectively. The dialysis tubes were immersed in 200 mL of purified water, and the unloaded free drug passed through the semi-permeable membrane was quantified by HPLC. The released ratio of iron was monitored by ICP-OES (iCAP 6000 series, Thermo Fisher Scientific Inc.).

**Acute toxicity studies and irradiation study:** The acute toxicity studies were conducted across several testing sites with different testing animals, including Level Biotechnology Inc. (Taiwan), Joinn Laboratories (Suzhou, China), and I-Shou University (Taiwan). The design of pharmacokinetics and effectiveness studies were described elsewhere [17]. Dose range of acute toxicity studies, 15 mg/kg to 77 mg/kg, were selected in rats (Sprague Dawley, Level Biotechnology Inc.), mice (C57BL/6, Joinn Laboratories), dogs (Beagle, Joinn Laboratories) and rabbits (New Zealand White, I-Shou University). All described experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at each site. Animals were administrated with various doses of CCM-Ami by a single intravenous infusion. The irradiation study was conducted at the $^{60}$Co gamma-radiation facility of National Tsing Hua University (Nuclear Reactor & Radiation Isotope Lab, Hsinchu, Taiwan), and the potency of the radiation source was validated annually with certification (Atomic Energy Council, Taiwan). The radiation dosage was measured by electronic dosimeter (Thermo Scientific EPD-G; Thermo Fisher Scientific, UK), which was validated regularly by the Institute of Nuclear Energy Research, Atomic Energy Council, Taiwan.

## Results and Discussion

### Characterization of PEG-*b*-PGA

To obtain PEG-*b*-PGA, Glu (OBzl)-NCA was polymerized according to the previous reports with some modifications [18,19]. The resulting PEG-*b*-poly (Glu (OBzl)) was deprotected by alkaline hydrolysis. As shown in Figure 1, the PGA methylene ($CH_2$) and methine (CH) groups together with the PEG ($OCH_2CH_2$) groups are resolved, which mean the diblock copolymer was completely dissolved in $D_2O$ without forming self-assembled micelles. The degree of polymerization is also estimated by $^1$H-NMR spectrum according to the peak intensity of methoxy proton and methine proton from PEG and PGA segments, respectively. The resulting PGA with 32 repeating units is close to the initial mole ratio of Glu (OBzl)-NCA to mPEG-$NH_2$. Figure 2 shows the GPC chromatograms overlay of mPEG-$NH_2$ and PEG-*b*-PGA. PEG-*b*-PGA shows a monomodal trace with no trace of the mPEG-$NH_2$. The weight average molecular weight ($\overline{M}_w$) and the polydispersity index (PdI) determined by GPC are 8050 and 1.15, respectively.

### Preparation and characterizations of CCM-Ami

As shown in Figure 3, the block copolymer of PEG-*b*-PGA is designed as a functional excipient to deliver amifostine. The preparation of CCM is much easier than physical encapsulation and chemical modification of drugs. Amifostine and PGA segments in PEG-*b*-PGA chelate the ferrous ions while mixing in the water, thus resulting in the formation of CCM-Ami. This crosslinking structure also prevents the burst effect, which is usually found in conventional polymer drugs [20]. The PEG segments, by contrast, extend outside of the chelating region to enhance the micelle stability. As shown in Figure 4, the effect of reaction time on the particle size of CCM-Ami was investigated by DLS technique. On considering with reaction in the early stage, the particle size was difficult to be measured because of still being in the molecular state. The micelle formation met instrument quality criteria after 14 hours, and reached about 25 nm in the end of reaction. The size of CCM-Ami was increased approximately in a linear fashion over time. The unloaded amifostine was also monitored by HPLC to ensure

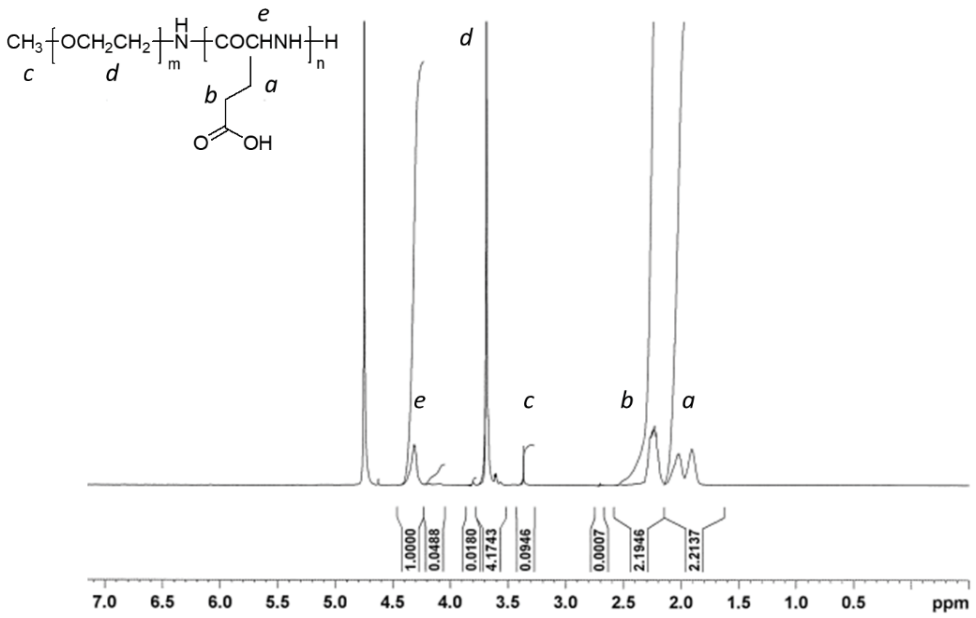


**Figure 1:** $^1$H-NMR (600 MHz) spectrum of the PEG-b-PGA copolymer in $D_2O$.

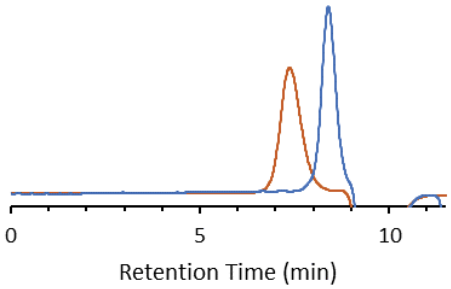


**Figure 2:** GPC chromatograms overlay of mPEG-$NH_2$ (blue line) and PEG-*b*-PGA (orange line). The $\overline{M}_w$ and PdI were 8050 and 1.15, respectively.

the reaction end point. Amifostine with a retention time of 5.3 minutes was quantified and about 3% of the amifostine was remained in the solution (Figure 5). No further significant decrease was observed over time. Encapsulation efficiency (EE %) of 97% is therefore calculated from the HPLC chromatogram using the following formula:

$$\text{Encapsulation efficiency (EE \%)} = (W_i\text{-}W_f)/W_i \times 100\% \quad (1)$$

$W_i$ is the total quantity of drug added initially during preparation.

$W_f$ is the amount of free drug determined by HPLC.

After an ultrafiltration purifying process, the amount of free amifostine was reduced to 0.5 wt%. The particle size of CCM-Ami was measured with a DLS technique, and the result indicated that the CCM-Ami possessed a uniform small size around 25 nm with a PdI of 0.162 (Figure 6).

## *In vitro* release of CCM-Ami

An *in vitro* release experiment was also performed to determine the release behavior for CCM-Ami and free amifostine. As shown in Figure 7a, 56% free drugs diffused from the dialysis tube to the outside water in 2 hours, while CCM-Ami released only 5% amifostine in the same time period. This result indicated that amifostine was successfully encapsulated in the CCM, and no burst effect was observed in contrast to conventional polymer micelles. Furthermore, only 0.3% of the iron was found outside the dialysis tube after 2 hours of dialysis (Figure 7b). The iron was incorporated into CCM-Ami and retained inside the dialysis tube. Iron that acts as a Lewis acid is the linkers between amifostine and PEG-*b*-PGA. The release rate of the iron also reveals the structural integrity of the CCM-Ami.

## Acute toxicity and total body irradiation

Acute toxicity studies were conducted to further evaluate the safety of CCM-Ami. Table 1 shows the survival and conversion dose for 14 days of acute toxicity [21]. The single-dose toxicity studies in rats (Sprague Dawley), mice (C57BL/6), and dogs (Beagle) were conducted in compliance with good laboratory practice (GLP) in contract research organizations, whereas rabbits (New Zealand White) were not required a full compliance with GLP and conducted for pilot tests. Survival rates of 100% with NOAEL (no observed adverse effect level) were found in each toxicity study in different species.

According to the pharmacokinetic results, CCM-Ami group showed a higher plasma WR-1065 concentration than that of amifostine group [17]. C57BL/6 mice treated with total body irradiation (TBI) were then used to assess the survival benefit of CCM-Ami compared to amifostine. Previously published studies utilized TBI from 5-12 Gy mostly for 10 minutes (0.6-2.2 Gy/min) [14,22,23]. However, longer radiation exposure times with lower dose rates are more representative of a nuclear accident. Accordingly, the test subjects were irradiated with total 8.48 Gy for 4 hours except for a short exposure of the control group (10 minutes). There were no survival for both of the short (10 minutes) and long (4 hours) exposure control groups. The median suvival time of short and long exposure control groups was 9 days and 13 days, respectively. A statistically significant survival advantage was observed for the long exposure time compared to the short exposure time ($P < 0.05$, Log-rank (Mantel-Cox) test). On the other hand, CCM-Ami given intravenously at a dose of 40 mg/kg and 45 mg/kg

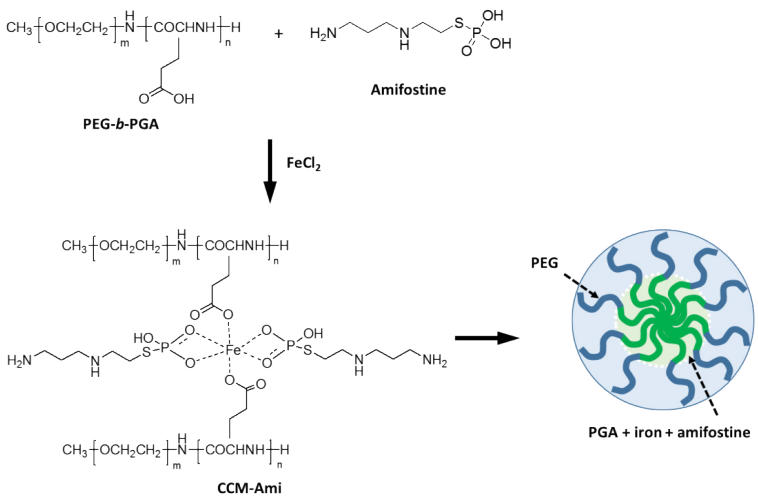


**Figure 3:** The synthetic route of CCM-Ami and a diagrammatic representation of their core-shell structure.

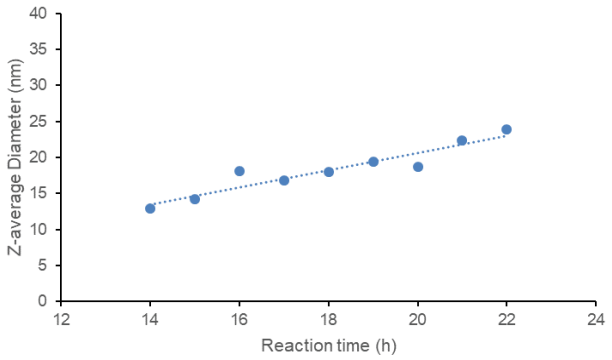


**Figure 4:** The relationship between particle size and reaction time for the preparation of CCM-Ami. The particle size was increased approximately in a linear fashion over time.

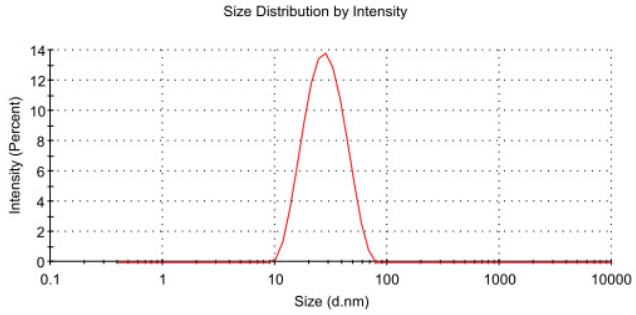


**Figure 6:** The size and size distribution of CCM-Ami were determined by DLS technique. The particle and PdI were 25 nm and 0.162, respectively.

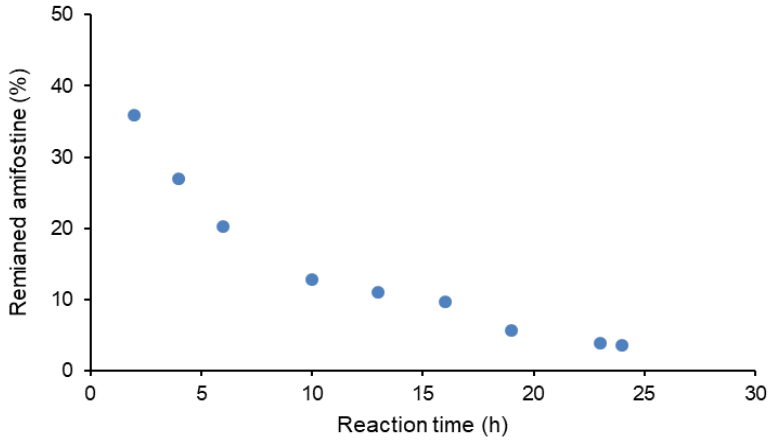


**Figure 5:** The ratio of unloaded amifostine in the reaction solution over time. Three percent of amifostine was determined after 24 hours of reaction.

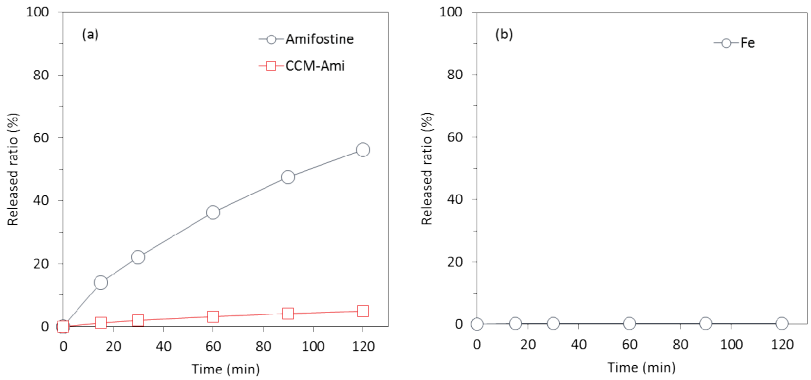


**Figure 7:** (A) The release profiles of amifostine and CCM-Ami determined by loading each into dialysis tubes (MWCO 3,500 Da). The released amifostine was monitored by HPLC. (B) The amount of iron outside the dialysis tube was analyzed by ICP-OES. The iron was incorporated into CCM-Ami, and almost all of the iron was retained inside the dialysis tube.

| Species | Strain | Dosage (mg/kg) | Survival rate | Conversion dose (mg/kg) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Human | Rat | Mice | Dog | Rabbit |
| Rat | Sprague Dawley | 77.8 | 100% [a] | 12.6 | - | 155.6 | 23.3 | 38.9 |
| | | 64.7 | 100% [a] | 10.5 | - | 129.4 | 19.4 | 32.4 |
| | | 53.9 | 100% [a] | 8.7 | - | 107.8 | 16.2 | 27 |
| Mice | C57BL/6 | 40 | 100% [a] | 3.2 | 20 | - | 6 | 10 |
| | | 50 | 100% [a] | 4.1 | 25 | - | 7.5 | 12.5 |
| | | 55 | 100% [a] | 4.5 | 27.5 | - | 8.3 | 13.8 |
| Dog | Beagle | 20 | 100% [a] | 10.8 | 66.7 | 133.3 | - | 33.3 |
| | | 25 | 100% [a] | 13.5 | 83.3 | 166.7 | - | 41.7 |
| | | 30 | 100% [b] | 16.2 | 100 | 200 | - | 50 |
| Rabbit | New Zealand White | 15.7 | 100% [b] | 5.1 | 31.4 | 62.8 | 9.4 | - |

**Table 1:** The survival and conversion dose of 14 days of acute toxicity. [A] GLP acute toxicity studies. [B] Pilot test for acute toxicity.

| Groups | Survival (n=5/group) | Median survival | Statistics | | | |
|---|---|---|---|---|---|---|
| | | | Long exposure | Amifostine | CCM-Ami (mg/kg) | |
| | | | | | 40 | 45 |
| Short exposure | 0% (0/5) | Day 9 | ** (p=0.0021) | No significance | ** (p=0.0021) | ** (p=0.005) |
| Long exposure | 0% (0/5) | Day 13 | - | No significance | ** (p=0.0018) | * (p=0.035) |
| Amifostine (45 mg/kg) | 20% (1/5) | Day 9 | No significance | - | * (p=0.0133) | No significance (p=0.056) |
| CCM-Ami (40 mg/kg) | 100% (5/5) | undefined | ** (p=0.0018) | * (p=0.0133) | - | No significance |
| CCM-Ami (45 mg/kg) | 80% (4/5) | undefined | * (p=0.035) | No significance (p=0.056) | No significance | - |

**Table 2:** The survival statistics of TBI (8.48 Gy) effectiveness study.

90 minutes before irradiation elicited a net survival of 100% and 80% with no statistical significance. Survival in the group given amifostine at a dose of 45 mg/kg was 20%, and the median survival time was 9 days. CCM-Ami pretreatment improved survival rates and median survival than did treatment with a corresponding dose of amifostine (Figure 8). These results demonstrated that a single injection of CCM-Ami successfully prevented the radiation damage from TBI. Significant improvements of survival rate and median survival of C57BL/6 mice were found as compared to the amifostine and control groups (Table 2). An investigation that takes into consideration treatment of radiation exposure might aid the design of future CCM-Ami.

## Conclusion

In conclusion, the hydrophilic drug amifostine was successfully incorporated into a polymeric drug carrier CCM. The results of acute toxicity and effectiveness studies indicated that this platform technology has made markedly progress in nano-medicine. Drug molecules with functional groups that can donate lone pair of electrons, such as carboxylic acids, hydroxy ketones, and phosphonic acids can be considered as the chelating ligands. For instance, the preferential sites for binding iron with doxorubicin, ciprofloxacin and amifostine are shown in Figure 9. Either hydrophilic or hydrophobic drug molecules that may act as ligands are capable of being loaded in CCM. The drugs will be released due to the removal of iron by transferrin, an iron-binding blood plasma glycoprotein in the body [24]. The result of TBI effectiveness study also demonstrated that CCM-Ami provided a more sustained potency than that of free amifostine. These promising results open a variety of applications in the field of drug delivery systems. Replacing iron with gadolinium or technetium-99m will also lead to interesting future applications for real-time monitoring and therapy.

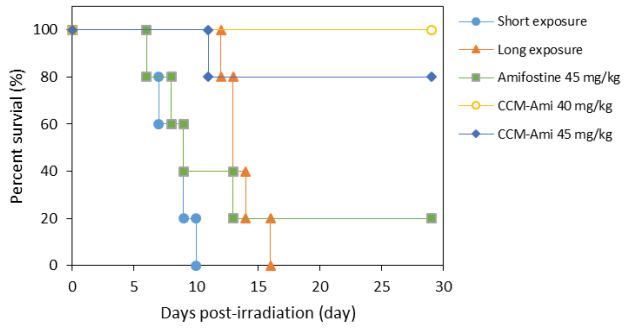


**Figure 8:** Overall survival of mice subjected to 8.48 Gy TBI. The graph shows percent survival as a function of days after irradiation. Five groups of C57BL/6 mice were exposed to TBI for 10 minutes with no treatment (blue circles), TBI for 4 hours with no other treatment (orange triangles), intravenous amifostine at a dose of 45 mg/kg 90 min prior to TBI for 4 hours (green squares), intravenous CCM-Ami at a dose of 40 mg/kg (open yellow circles) and 45 mg/kg (blue diamonds) 90 min prior to TBI for 4 hours.

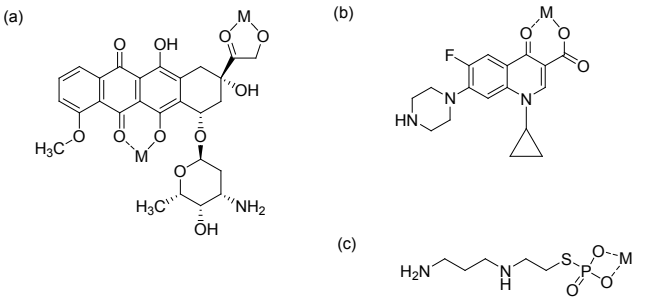


**Figure 9:** Structural formulas of (A) doxorubicin, (B) ciprofloxacin, and (C) amifostine showing the sites of preferential binding of iron ions. M represents ions of transition metals.

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