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Matrix metalloprotease selective peptide substrates cleavage within hydrogel matrices for cancer chemotherapy activation

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ABSTRACT

To utilize biologic mechanisms to elicit controlled release in response to disease, protease-sensitive devices have been created. Hydrogels were created with pendant peptide–drug complexes. For the matrix metalloproteases (MMPs) examined, a length of six amino acids greatly improved the specificity of the peptide ($k_{cat}/K_m \sim 2.4 \pm 0.1 \times 10^4\ M^{-1}\ s^{-1}$) over shorter sequences ($k_{cat}/K_m \sim 4.4 \pm 0.2 \times 10^2\ M^{-1}\ s^{-1}$). The peptides did not exhibit anti-proliferative effects upon cancer cells, and peptide–platinum complexes showed similar anti-proliferative effects upon the cancer cells compared to the free platinum drugs. Once the peptide–drug complex was incorporated into the hydrogels, the release was dependent upon the presence of MMP in the solution with approximately 35% of platinum released from hydrogels in the presence of MMP and only 10% without MMP in the week examined. The released drug exhibited the expected anti-proliferative activity over several days of incubation. The MMP selective drug delivery holds much potential for treatment of cancer and other diseases.

1. Introduction

There are a myriad of problems which make successful treatment of cancer difficult. Most cancers are highly invasive and there are problems of recurrence even after surgery, chemotherapy and radiation treatment [6]. Invasion, angiogenesis, and metastasis require extensive degradation and remodeling of the extracellular matrix (ECM). Major contributors implicated in ECM homeostasis are a family of proteolytic enzymes, the matrix metalloproteases (MMPs) or matrixins. Matrix metalloproteases have been particularly implicated in tumor invasion and angiogenesis [12] although these signals both promote and inhibit invasion and angiogenesis [24]. Several MMPs are found to be expressed and active in advanced stage cancer while being minimally expressed in the normal surrounding tissue [6]. The expression and activity of these proteases have been found to increase with tumor progression and grade [22,25]. Comprehension of the exact mechanisms involved in MMP activity has been complicated by the differing expression patterns and roles of these proteases within the tumor [26]. Further complicating the situation, these enzymes have overlapping substrate specificities [18] creating difficulty in designing appropriate inhibitors for only one protease [7]. In addition, the MMPs are present at globally low concentration, but concentrated on the surface of cells at highly elevated and activated concentrations [4]. Due to the complex, but validated target MMPs present [19], alternative methods of targeting MMPs have been developed particularly ones that depend upon liposomal and polymeric vehicles [35].

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In particular, polymeric vehicles are gaining acceptance for use in the biomedical field for controlled and targeted delivery [10]. Following the introduction by Ringsdorf of polymer–prodrugs [23], various polymeric anticancer drug conjugates, for example doxorubicin, paclitaxel, camptothecin, and cisplatin have shown great potential *in vivo* and have reached clinical trials [10]. Although soluble polymer–drug conjugates have been widely investigated for cancer chemotherapy, hydrogels have not been as widely investigated for chemotherapy despite being widely explored for implantation.

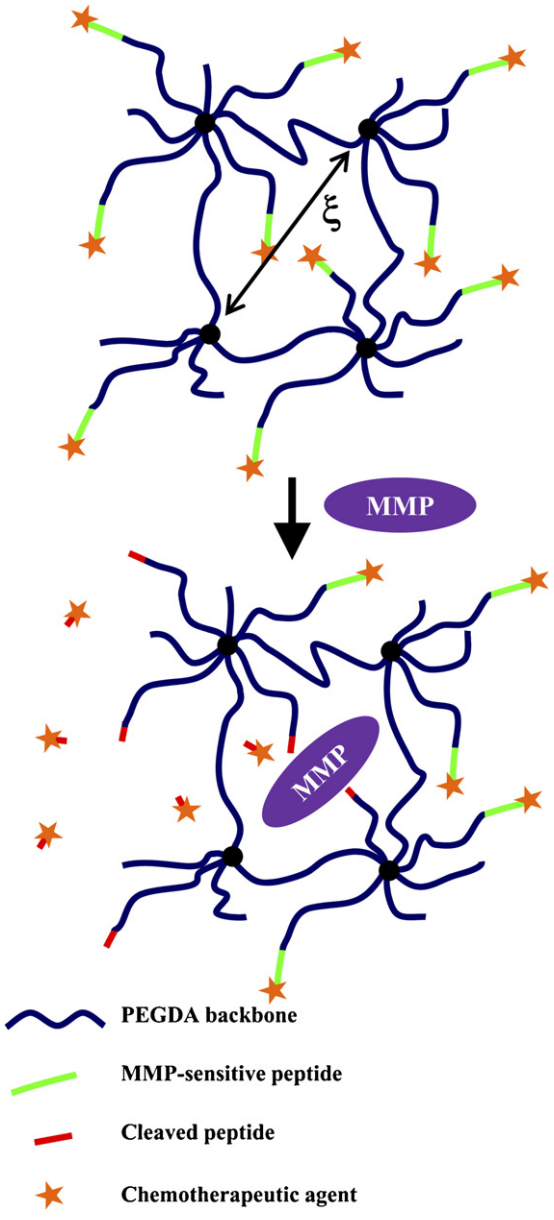

Fig. 1 – Schematic of hydrogel therapeutic paradigm where ξ represents the mesh size. A crosslinked (black circle) poly(ethylene glycol) diacrylate (PEGDA) hydrogel (dark blue lines) was modified with matrix metalloprotease (MMP)-sensitive peptides (light lines) with attached drug (star). The crosslinked system (circles) included pendant PEGDA–peptide–drug and PEGDA backbone. Upon diffusion of matrix metalloprotease into the hydrogel, the peptide could be cleaved releasing the drug with a small peptide fragment (red line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Hydrogels are hydrophilic polymeric systems which are highly biocompatible due to their tissue-like consistency and ability to allow body fluids to move throughout the matrix [20]. The hydrated state of hydrogels allows the diffusion of proteins through the hydrogel at a rate that is dependent upon the properties of the hydrogel matrix. Specifically, the rate of diffusion can be controlled by varying the macromer chain length [8]. Recently, a hydrogel–drug conjugate has been successfully formed [30,31] whereby drug release was controlled by incorporating pendant chains of peptide substrates for MMPs complexed to the chemotherapeutic agent in the hydrogel network (Fig. 1). Similar systems have previously been designed, but for alternate applications including antibacterial [28] and imaging applications [33]. Cleavage of pendant peptides with attached drug by MMPs caused the activation and release of drug from the hydrogel.

The ability of a delivery system to release drug only when activated by MMPs was suggested as a strategy for cancer treatment. Based upon previous studies that confirmed MMP-based chemotherapy release but had a high non-specific release [30,31], this further investigation was primarily designed to improve the specific MMP-based release of bound platinate drugs by utilizing more stable amine–platinum drug attachment and characterizing the protease specificity of MMP-sensitive linkers [32]. Platinum drugs are used as a model chemotherapeutic in this system due to the knowledge of the chemistry and clinical utility. These studies provide further evidence that MMP-2 and MMP-9 expressed in advanced cancer can be used to activate platinates from hydrogels containing MMP substrates. This MMP-sensitive system shows great potential for manipulating release and activation of drug based on biological need.

2. Experimental

2.1. Materials

Peptides were designed based upon the known MMP substrate sequences [34] and were synthesized by the University of Illinois at Chicago Research Resources Center (RRC), identified and purified (approximately 90% pure) by reversed phase high-performance liquid chromatography (HPLC). All peptides were synthesized with a fluorescein containing lysine (K*: where the fluorescein is present on the ε-amino group of lysine) for quantitation. MMP-2 and MMP-9 proenzymes were purchased from Calbiochem (San Diego, CA) and activated as suggested by the manufacturer using 4-(hydroxymercury)benzoic acid. Poly(ethylene glycol) (PEG, MW approximately 8000 g/mol) was purchased from Fluka Biochemika (Buchs, Switzerland) and o-phenylenediamine (oPDA) was purchased from Acros Organics (Morris Plains, NJ). All remaining chemicals were purchased from Fisher Scientific (Fairlawn, NJ) and used without further purification unless specified.

2.2. Peptide cleavage

Prospective MMP substrate peptides, K*PAGLLGC–$CONH_2$, K*AGLLC–$CONH_2$, and K*GLC–$CONH_2$, were dissolved in a protease buffer containing Tris–HCl (50 mM), NaCl (0.2 M), $CaCl_2$ (10 mM), Brij-35 (0.05%) and $ZnSO_4$ (50 μM) (pH 7.4). Activated MMP-2 or MMP-9 was added to the peptide solution at a final concentration of 10 nM. At predetermined time intervals, the reaction was stopped by adding EDTA solution (25 mM). Samples were analyzed using HPLC composed of a gradient pump connected to a scanning fluorescence detector and a photodiode array detector. Peptides were separated on a Zorbax Extend-C18 column (80 Å, 3.6 μm, 4.6 mm × 150 mm; Agilent Technologies, Palo Alto, CA) using a gradient of 30–70% of 0.1% trifluoroacteic acid in acetonitrile against 0.1% trifluoroacteic acid in water over a period of 15 min at a flow rate of 1 mL/min. The expected cleaved fragments, K*PAG, K*AG, and K*G, were synthesized to confirm HPLC retention times. Identity was subsequently confirmed by matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy (MS).

To synthesize platinum (Pt)-complexes, peptides were dissolved in distilled deionized water with an equimolar amount of potassium tetrachloroplatinate (K_2PtCl_4; Acros Organics, Morris Plains, NJ) added to the peptide solution. The solutions were stirred for 20 h and lyophilized. Platinum concentrations were determined spectrophotometrically using the oPDA method [13]. From this, the IC_{50} was calculated using GraphPad Prism (3.02) using the nonlinear curve fitting function.

2.3. Peptide and peptide–platinate activity

As a model for cancer, a malignant glioma cell line (U-87 MG, ATCC #HTB-14) was grown and maintained in Eagles minimum essential media with 10% fetal bovine serum, 1% penicillin/streptomycin, sodium pyruvate (110 mg/L), L-glutamine (292 mg/L), and nonessential amino acids at 37 °C in 5% CO_2. One day prior to the test, cells were plated at 50,000 cells/mL in supplemented media on 96-well plates. Peptides at different concentrations were incubated with cells for 24 h. Cell viability, with respect to the untreated control, was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cell Titer 96® Aqueous One solution Cell Proliferation Assay; Promega; Madison, WI) [2].

2.4. Hydrogel synthesis and characterization

Poly(ethylene glycol) diacrylate (PEGDA) was synthesized using PEG and acryloyl chloride [16]. Briefly, PEG (2.5 mmol) was dried in benzene by azeotropic distillation. Dried PEG was dissolved in 50 mL methylene chloride was placed in a three neck flask. The solution was flushed with nitrogen for 30 min. Triethylamine (TEA; 15 mmol) and acryloyl chloride (15 mmol) were sequentially added drop wise. The reaction was stirred overnight at room temperature. The TEA-chloride was filtered and PEGDA was precipitated using diethyl ether. The precipitate was filtered and purified by dissolving in methylene chloride and reprecipitated in diethyl ether. The purified product was filtered and dried *in vacuo*. The product was analyzed by Fourier-transform infrared and Raman spectroscopy (data not shown, Nexus 870 FT-IR with Raman accessory, Thermo Electron Corporation, Madison, WI).

Peptide (2 mM) was added to PEG(8000)DA (8 mM) in phosphate-buffered saline (pH 7.4) and stirred for 4 h. The solution was then polymerized using ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine at 37 °C which resulted in peptide being incorporated as pendant groups within the hydrogel matrix [30,31]. Hydrogels were prepared between two glass slides and cut into 6 mm discs using a biopsy punch. To control the thickness, silicon spacers were used. To load Pt, the hydrogels were washed repeatedly in double deionized water and dried *in vacuo*. The dried hydrogels were immersed in a solution of K_2PtCl_4 (0.2 mg/mL) and allowed to swell for 20 h. The hydrogels were then repeatedly washed in phosphate-buffered saline to remove excess Pt. Platinum and peptide incorporated into the hydrogels was determined by mass balance. Hydrogels with and without the peptide were characterized with respect to swelling ratio which was then used to estimate the mesh size [5]. Although this method does not take into consideration the pendant chains or the ionic groups, the estimate can be use to explain the protein entry into the hydrogel.

2.5. Platinum release

Hydrogels (containing peptide and/or Pt) were placed in 0.5 mL of buffer (pH 7.4) containing Tris–HCl (50 mM), NaCl (0.2 M), $CaCl_2$ (10 mM), Brij-35 (0.05%), and $ZnSO_4$ (50 μM). Activated MMP-2 or MMP-9 was added individually at a final concentration of 10 nM. Aliquots of the supernatant were taken at predetermined time intervals and peptide amount, platinum amount, and identity in the release media was determined by fluorescence intensity, oPDA method, and HPLC, respectively, as previously described. Based upon preliminary studies, samples were spiked with additional MMP every 32 h to maintain enzymatic activity. Release was compared to Pt release from hydrogels with entrapped Pt; no peptide was present in the hydrogel for entrapped Pt studies which act as a diffusional release control.

2.6. Hydrogel activity

Hydrogels (PEG), hydrogels with peptide (PEG–P), and hydrogels with peptide complexed Pt (PEG–P–Pt) were incubated with U-87 MG cells in supplemented serum free media for 48 h. Matrix metalloprotease (10 nM) or media (no MMP) was added to media for specific wells to supplement the MMPs expressed by U-87MG cells. Cells treated with matrix metalloprotease (10 nM) without hydrogels was examined as a control. Additional MMPs were added at 32 h to maintain MMP activity. Cell viability with respect to the controls was calculated using a modified MTT assay [2].

To determine activity of Pt released longer than 48 h, hydrogels were placed in serum free media in the presence and absence of MMP-2 or MMP-9. Activated MMPs were added every 32 h as in the previous studies. All conditions were maintained similar to the release studies. At 48 and 96 h, the supernatant media was added to adherent U-87 MG cells in a

96-well plate. After 24 h of treatment, cell viability with respect to the controls was calculated using a modified MTT assay [2].

2.7. Statistical analysis

All experiments were performed in triplicate at a minimum ($n = 3$). Analysis of variance (ANOVA) was used to determine significant difference between groups. Tukey's test was used for post hoc analysis with a p-value less than 0.05 being statistically significant.

3. Results

3.1. Peptide cleavage

As expected, comparison of the specificity (k_{cat}/K_m) of the peptides suggested that the hexapeptide was the best substrate of the peptides tested (Table 1). The longest peptide showed higher reaction rate (k_{cat}) for both MMP-2 and MMP-9. Although the affinity or binding strength (K_m) for the hexapeptide was not significantly higher than the other peptides, the turnover number (k_{cat}) was significantly higher than the other peptides. This resulted in high substrate specificity (k_{cat}/K_m) for K*PAGLLGC–$CONH_2$. This peptide had better affinity and specificity for MMP-9 than MMP-2 but this peptide would be considered a good substrate for both MMPs. These results supported the fact that the length of the amino acid sequence around the cleavage site was important for recognition of the substrate by the enzymes. Therefore, K*PAGLLGC was chosen for incorporation into PEGDA hydrogels for further studies.

The parent peptide, GPAGLLG, from which the three peptides were designed was known to be cleaved between glycine and leucine by MMP-2 [34]. Comparison of the HPLC chromatogram of the synthesized potential cleaved fragment K*PAG with chromatograms of the peptide cleaved by MMP-2 and MMP-9 confirmed that K*PAGLLGC was cleaved between glycine and leucine (Fig. 2). The cleavage site (PAG∼LLG) was not altered by fluorescein modification or addition of the amino acids on the terminal ends of the peptide. The HPLC of the parent peptide and smaller fragments confirm that further purification is necessary for further analysis; however, the rank-order and magnitude of the differences in the cleavage rates are expected to be maintained even with purer starting materials. Another concern is that dimerization may influence the cleavage. The conditions were maintained to minimize dimerization, but HPLC and MALDI did detect some products that could be attributed to the dimerized peptides.

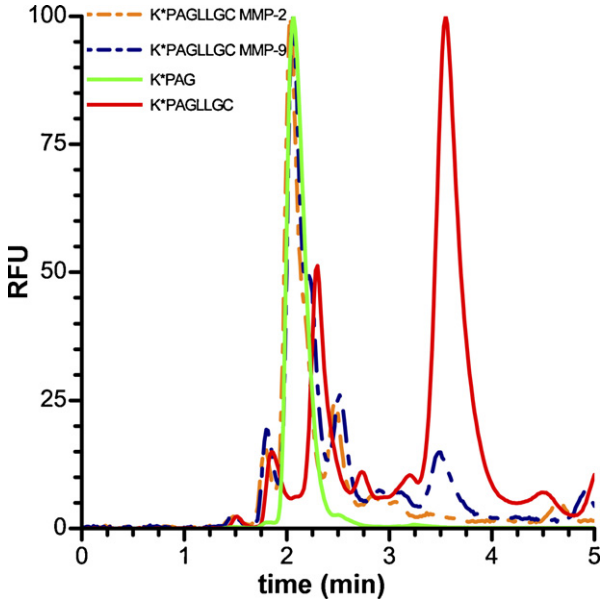

Fig. 2 – HPLC chromatograms of the full length peptide (——), K*PAGLLGC, the expected cleaved fragment (——), K*PAG, and the full length peptide cleaved with (– –) MMP-9 and (– –) MMP-2 for 2 h. Only the relevant section of the chromatogram is shown as no other peaks are present. A single HPLC chromatogram (at 260 nm) is shown, but the experiment was replicated at least three times.

3.2. Peptide and peptide–platinate activity

The intact peptides did not show any toxicity on U-87 MG cells after incubation for 24 h (Fig. 3A). At concentrations as high as 200 μM, cell viability was equivalent to the untreated control for all peptides. Similarly, all of the cleaved fragments did not show any toxic effects on cells (Fig. 3B). These results suggest

Table 1 – Kinetic parameters for K*PAGLLGC–$CONH_2$, K*AGLLC–$CONH_2$, and K*GLC–$CONH_2$ cleavage to K*PAG, K*AG, and K*G with MMP-2 and MMP-9 ($n = 3$)

	$V_{max} \times 10^{-9}$ (M/s)	$K_m \times 10^{-3}$ (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
K*PAGLLGC				
MMP-2	270 ± 120	1 ± 0.5	10.7 ± 4.8	10700 ± 1000
MMP-9	240 ± 50	0.4 ± 0.1	9.6 ± 2.1	24000 ± 1000
K*AGLLC				
MMP-2	6.02 ± 2.1	1.4 ± 0.4	0.24 ± 0.08	170.7 ± 15.2
MMP-9	0.99±0.2	0.16 ± 0.1	0.04 ± 0.01	251.6 ± 18.6
K*GLC				
MMP-2	0.88 ± 0.1	0.081 ± 0.02	0.035 ± 0.006	438.6 ± 20
MMP-9	5.3 ± 1	0.6 ± 0.1	0.21 ± 0.04	358.2 ± 10

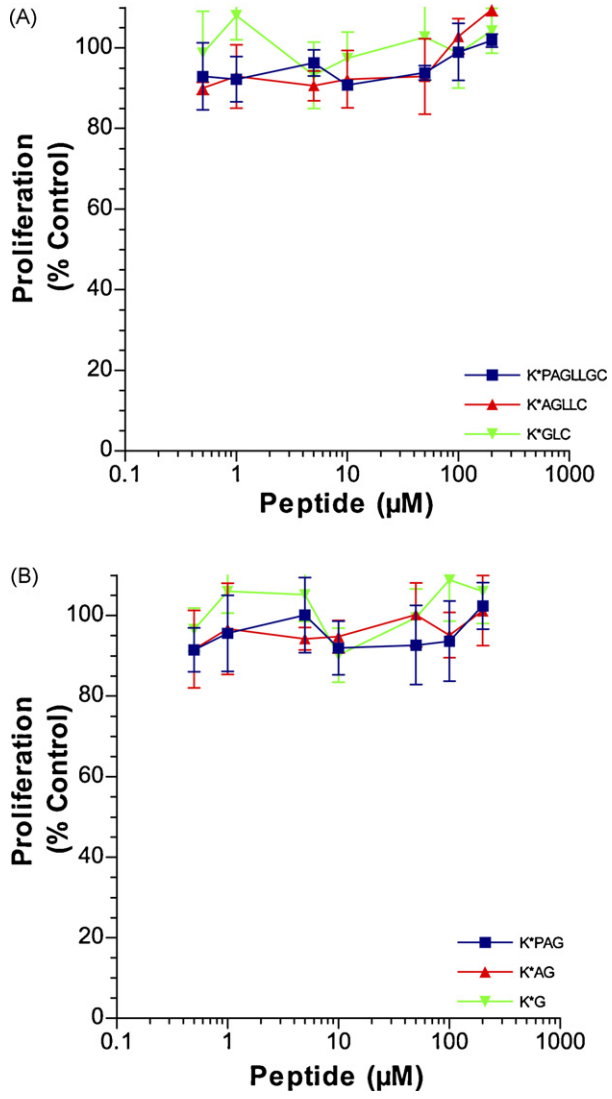

Fig. 3 – (A) Cytotoxicity of intact peptides on U-87 MG cells: K*PAGLLCC (■), K*AGLLC (▲), and K*GLC (▼). (B) Cytotoxicity of cleaved fragments on U-87 MG cells: K*PAG (■), K*AG (▲), and K*G (▼) ($n = 3$; average ± S.D.). No platinum agents were used in these studies.

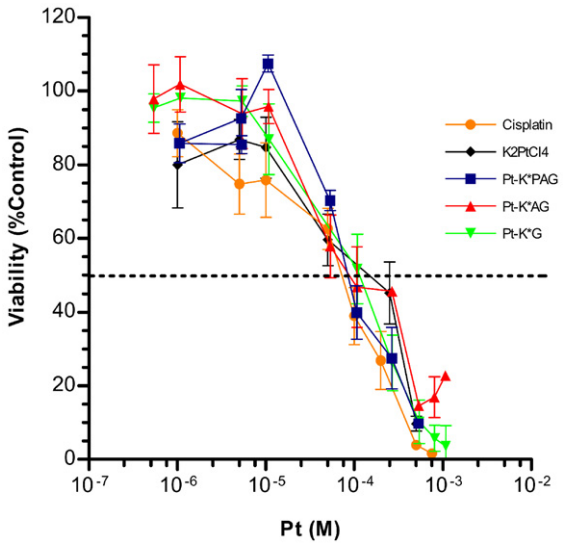

Fig. 4 – Activity Pt–peptide complexes, (▼) Pt–K*PAG, (▲) Pt–K*AG, (■) Pt–K*G, and platinum agents, (◆) tetrachloroplatinum, and (●) cisplatin ($n = 3$; average ± S.D.).

that there should be no toxicity arising from the peptides themselves. Any activity should be due to the complexed platinates once the drugs are attached or included.

The parent drug, cisplatin ($IC_{50} = 46.9 \pm 15$ μM), was more active than K_2PtCl_4 ($IC_{50} = 70.7 \pm 15$ μM) used to create the complexes although the difference was not significant. Platinum-complexes showed slightly reduced activity ($p > 0.05$ for all groups compared to K_2PtCl_4), less than an order of magnitude decrease compared to cisplatin and K_2PtCl_4 (Fig. 4) with IC_{50} values of 101.8 ± 13 μM, 116.4 ± 33 μM, and 80.2 ± 18 μM for Pt–K*PAG, Pt–K*AG, and Pt–K*G, respectively. The lines drawn o the graph do not exactly match the values presented as the values presented were calculated using nonlinear curve fitting of all data points in the curve. Reduction in activity was expected as the complexed peptide may form the *trans*-form of platinum which is less active at crosslinking DNA than the *cis*-form [3,15] or alteration of transport and activity due to the presence of the peptide sequence. However, only subtle and non-statistically significant ($p > 0.05$) reduction in activity was observed. Some reduction in activity may be tolerated in order to reduce toxicity to normal cells and have reduced systemic toxicity. Since this system is intended for local delivery, fewer total drug molecules would be required to be administered compared to the intravenous dose due to the greatly restricted volume being treated. Hence, this reduction in activity may not adversely impact therapeutic efficacy if a local concentration can be achieved.

3.3. *Hydrogel synthesis and characterization*

After repeated washings, the amount of peptide incorporated within the PEG(8000)DA hydrogel was found to be 65.2 ± 1.3% of the original amount added before polymerization. The amount of Pt found to be retained as a complex with the peptide in the PEG(8000)DA hydrogel was found to be 16.4 ± 1.2 μg per disc (0.065 μg/mm^3, 0.3 nmol/mm^3). This amount (84 nmol) is higher than the theoretical amount of peptide (43 nmol) in each disc. This may be due to some adsorption of Pt within the hydrogel or attachment of excess Pt to other groups, i.e. two moles of Pt complex with one peptide at carboxyl, thiol, or alternate amine groups [1,9,38]. Future work in our lab will investigate each of the forms of platinum compounds that were formed; however, the major released form of platinum is the monoplatinum compound that is described as confirmed my MALDI-MS (data not shown).

There was no significant difference in the molecular weight between crosslinks or mesh size of the hydrogels, which was expected as the pendant peptides are at a relatively low concentration compared to the crosslinking macromer, PEGDA. The estimated mesh size for PEG(8000)DA was 12.6 ± 0.7 nm

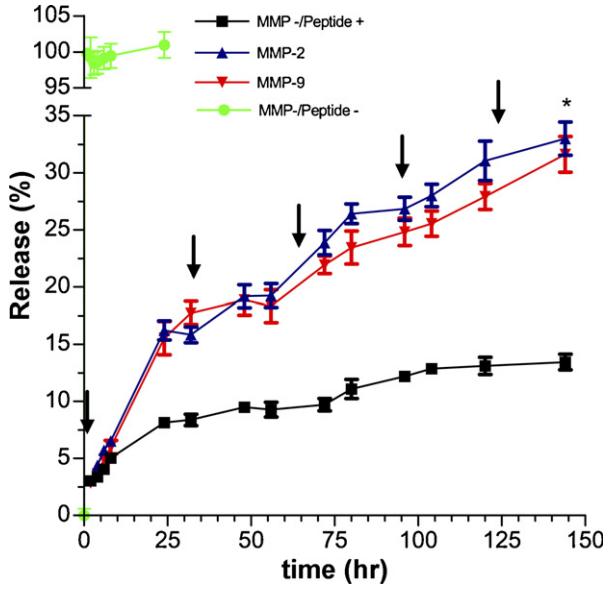

Fig. 5 – Release of the cleaved fragment from PEG(8000)DA hydrogels in the presence of (▲) MMP-2 (10 nM) and (▼) MMP-9 (10 nM) compared to non-specific release (■) in the absence of MMPs. Also shown is (●) Pt release when no peptide is present. Arrows indicate addition of MMP to maintain constant activity, and *indicates significant difference from non-specific release ($p < 0.05$, $n = 3$).

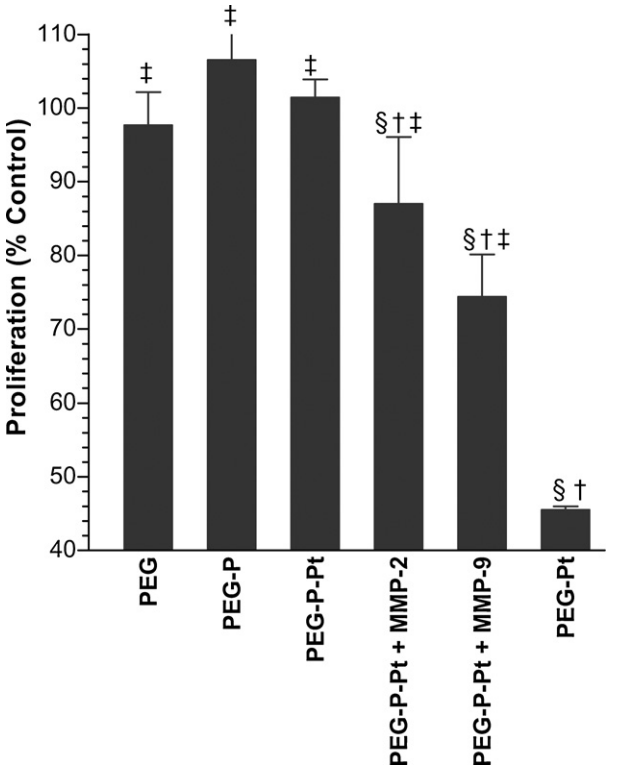

Fig. 6 – Activity of PEG(8000)DA hydrogels toward U-87 MG cells. Hydrogels without Pt or peptide (PEG), hydrogels with peptide incorporated but without Pt (PEG–P), hydrogels with peptide–Pt complex in the absence (PEG–P–Pt) and presence of MMP-2 (PEG–P–Pt + MMP-2) or MMP-9 (PEG–P-Pt + MMP-9), and hydrogels with entrapped Pt without peptide for complexation (PEG–Pt) where § indicates significant difference from hydrogels with peptide incorporated but without Pt (PEG–P), † indicates significant difference from hydrogels with peptide–Pt complex in the absence of MMP (PEG–P–Pt) and ‡ indicates significant difference from positive control, hydrogels with entrapped Pt without peptide for complexation (PEG–Pt) ($p < 0.05$, $n = 3$).

and 13.1 ± 0.4 nm for hydrogels with and without peptide, respectively.

3.4. *Platinum release*

Addition of MMP-2 and MMP-9 showed a significantly higher release of cleaved fragment from the PEG(8000)DA hydrogels compared to the control (Fig. 5). The release did not plateau before 144 h as long as additional MMP was added. There was increased response to addition of MMPs every 32 h. The increased response indicated that a continuing release of Pt could be obtained with availability of active MMPs. Analysis of the supernatant from 6 days with HPLC confirmed that the cleaved fragment Pt–K*PAG was released (data not shown). This data further supported the fact that MMPs were able to show activity on the peptides incorporated in the hydrogel. Non-specific release of Pt from amino groups of the peptide was very low indicating a strong peptide–Pt complex. In contrast to the complexed Pt, all entrapped Pt was instantaneously released within 24 h.

3.5. *Hydrogel activity*

PEG(8000)DA hydrogels without Pt (PEG–P) did not show any activity toward cells. Entrapped Pt (PEG–Pt) showed the greatest activity due to instantaneous release of Pt. Since the amount of Pt loaded within the hydrogel was nearly equivalent to that loaded in the other hydrogels, the activity of the released Pt was only approximately 45% and did not completely inhibit cell growth. When MMP selective hydrogels were incubated with the cells for 48 h, hydrogels with complexed Pt (PEG–P–Pt) showed significantly higher activity only in the presence of MMP-2 and MMP-9 compared to those without peptide and Pt (PEG) (Fig. 6). There was also significantly higher activity with addition of MMPs than in the absence of MMPs with hydrogels containing peptide and Pt (PEG–P–Pt).

When hydrogels were incubated for longer periods with MMPs and the released drug then incubated with cells, a similar trend was observed (Fig. 7); this is similar to the previous figure (Fig. 6) except the release was conducted in a cell free environment and the cells exposed to the released solution. There was significantly higher activity seen with longer incubation times (96 h) in the presence of MMPs. The actual activity (viability of 69 ± 4% with MMP-2 and 59 ± 4% with MMP-9) was nearly equivalent to the activity of the free drug that was calculated to be released in an equivalent amount of time from the release experiment (approximately 66% viability). Cells in the presence of PEG–P–Pt hydrogels with added MMPs exhibited morphology typical of dying cells while

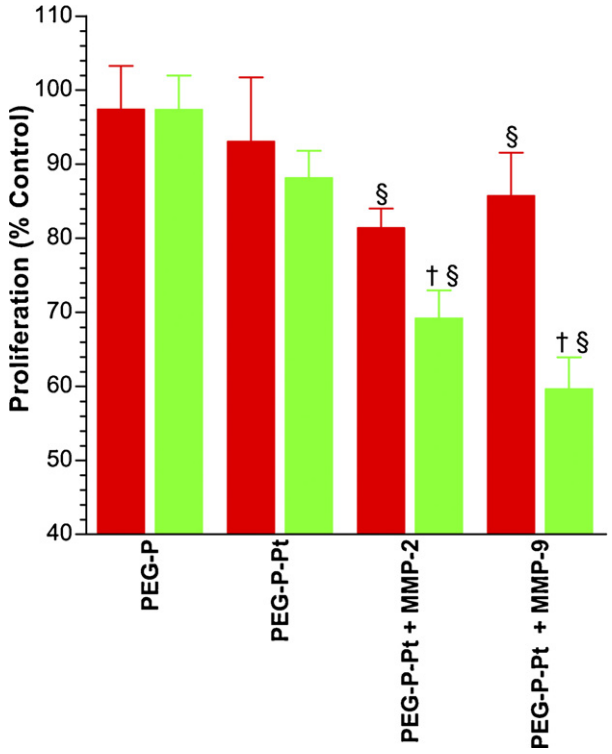

Fig. 7 – Activity of Pt released from PEG(8000)DA hydrogels into media after 48 h (dark/red bars) and 96 h (light/green bars) on U-87 MG cells. Hydrogels with peptide incorporated but without Pt (PEG–P), hydrogels with peptide–Pt complex in the absence (PEG–P–Pt) and presence of MMP-2 (PEG–P–Pt + MMP-2) or MMP-9 (PEG–P–Pt + MMP-9) where § indicates significant difference from hydrogels with peptide incorporated but without Pt (PEG–P) and † indicates significant difference from hydrogels with peptide–Pt complex in the absence of MMP (PEG–P-Pt) ($p < 0.05$, $n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

cells in contact with hydrogels without Pt and PEG–P–Pt hydrogels in the absence of MMPs show normal morphology (data not shown).

4. Discussion

Over the last several decades, research has shown that the delivery of chemotherapeutics can be improved by targeting the activation or release of the chemotherapeutic at a desired site via active tumor mechanisms [10]. These systems utilize different characteristics of tumor biology to improve the outcome for cancer patients. Since local delivery in the form of a polymeric implant has proven to be effective in the treatment of some tumors [37], novel methods of combing local treatment with drug activation should not be overlooked. Such a system, once implanted will act as a drug reservoir that would release active drug in response to tumor invasion, angiogenesis and recurrence. This may ensure long-term survival of the patient with reduced adverse reactions.

To allow long-term delivery, suitable materials must be utilized as the base for any therapeutic system. PEGDA hydrogels have been widely explored and found to have properties useful for many biomedical applications [21] and among these properties are the mesh size. Hydrogel characteristics, specifically mesh size, can be utilized to control delivery of drugs and proteins [8,36] by controlling their diffusion or by controlling of diffusion of proteases into and within a hydrogel [30,31]. Hydrogels designed and developed in this study showed that adequate mesh size for protease entry into the hydrogel (greater than 10 nm) can be attained for MMP diffusion into the hydrogel as MMP-2 and MMP-9 have a longest dimension if approximately 10 nm [11,17]. The diffusing MMPs can cleave and subsequently release active platinate from the hydrogel matrix.

But to instill MMP sensitivity into the hydrogels, control over more than just the hydrogel parameters was necessary. Peptide substrates were designed considering the data obtained from extensive knowledge about substrate specificities and selectivity [34,35]. The choice of substrate is critical in developing a matrix metalloprotease-sensitive system. Peptide cleavage and kinetics supported the fact that reducing the specific amino acids around the cleavage site significantly reduced the specificity of the substrate for the enzyme. The peptide with the longest amino acid sequence, K*PAGLLGC, was found to be the best MMP-2 and MMP-9 substrate among those studied. K*PAGLLGC showed the highest turnover rate, affinity and specificity for both the proteases and was therefore incorporated into the hydrogels.

Once appropriate hydrogel and peptide conditions were identified, development of a successful matrix metalloprotease-sensitive hydrogel system depended on both the release of cleaved fragment from the hydrogel in response to presence of MMPs and the activity of the Pt complexed to the cleaved fragment. The peptides and the respective cleaved fragments were not toxic to U-87 MG cells. When Pt was complexed to the cleaved fragment, activity of Pt in the cells was found to be lower than but close to the activity of potassium tetrachloroplatinate which was used to synthesize these complexes. Reduction in activity may be due to the presence of the residual-cleaved fragment complexed to Pt or by the formation of complexes with multiple peptides or *trans*-platinum like complexes which may not be as active as cisplatin [3,15]. The cleaved peptide fragment may cause some level of steric hindrance to the interaction of Pt with DNA [27]. Cisplatin alone is known to be very active [14]. Hence, slight reduction in the activity of the complexes may be acceptable to prevent adverse effects to normal tissue.

In addition to the specificity of cleavage, the chemistry for complexing Pt to the peptide was also critical to specifically obtain MMP controlled release of platinate. Results from similar studies using the carboxy–Pt complex showed a significantly higher non-specific release of Pt as compared to the amine–Pt complex [30,31]. By minimizing non-specific release, the complex ensured that the release and activity of platinate from the hydrogel would be biologically controlled by the growth of the tumor. Unfortunately, accompanying the decrease in non-specific release, a related decrease in apparent activity was

observed. This decrease was proportional to the decreased loading of the platinum within the hydrogels. Future work developing this idea will focus upon controlling the loading of peptide, and hence, Pt available for release; however, the current work does accomplish the necessary task of reducing non-specific release and allowing MMP-based release. Interestingly, the earlier platinum chemistry resulted in cisplatin analogs [29] that were significantly less active than the amine-based cisplatin analogs formed here. This finding is greatly encouraging in the development of peptide–drugs for release from such systems.

The main focus of this work was to confirm that MMPs can be used as a triggering mechanism and Pt was only utilized as a model drug. Much optimization of activity and choice of active agent is possible to most effectively utilize this mechanism.

5. Conclusion

An improved strategy to mimic the natural homeostatic nature of extracellular proteases for cancer treatment has been developed. This system has been designed to utilize the biology of cancer by using the overactivity of extracellular protease to activate a prodrug from a hydrogel matrix. A model drug, platinum, can be retained in PEGDA hydrogels by complexation with lysine containing peptides with minimum non-specific release. A level of specificity control was achieved by manipulating the length of the peptide sequence utilized. When MMPs were present, drug was released at an accelerated rate. Thus, PEGDA hydrogels containing peptide prodrugs have shown great promise for locally controlling the release of chemotherapeutics. Although the current system shows vast improvement over the earlier systems with respect to non-specific release of the agents, further control is necessary. This system warrants future research for other proteases and drugs and should be further explored. The potential is great particularly for systems where known proteases are localized at the site of disease.

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