# In Vitro Evaluation of HPMA-Copolymers Targeted to HER2 Expressing Pancreatic Tumor Cells for Image Guided Drug Delivery[a]

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Personalized medicine for the treatment of pancreatic cancer is one potential avenue which can prevent the dire outcome of this difficult to treat disease. Image guided drug delivery (IGDD) is a method allowing real-time imaging of drug therapy in order to predict the potential efficacy and safety of a given treatment. Water soluble macromolecular drug carriers such as N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers provide multifunctional platforms for the construction of such IGDD systems. HPMA copolymer conjugates containing gemcitabine, a targeting ligand for HER2 receptors overexpressed in some pancreatic cancers, and an $^{111}In^{3+}$ chelating agent are synthesized, characterized, and evaluated in vitro for their potential use as an IGDD system for pancreatic tumors. The conjugates are capable of binding to pancreatic tumor cell lines which express HER2. In vitro drug release is achieved under physiological and acidic pH environments. The chelated radioisotopes are stable in the presence of mouse serum. The conjugates are effective in killing pancreatic tumor cell lines in vitro. These copolymers have potential for further preclinical evaluation in pancreatic tumor models.

Image Guided Drug Delivery Polymer
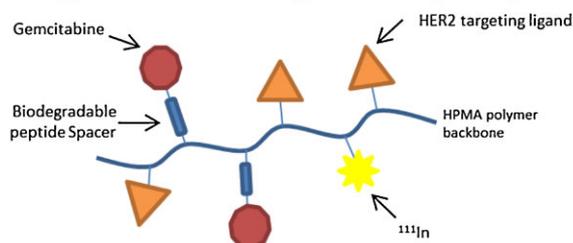
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1. Introduction

Successful treatment of pancreatic cancer is in dire need of improvement. Patients have an abysmal prospect when diagnosed based on the current 5 year survival rate of only 6%.[1] Not only is there a low probability of survival but incidence rates have been increasing by 1.5% since 2004.[1] Many of the current therapies for the treatment of pancreatic cancer are designed as one size fits all approach. However, heterogeneity of the cancer patient population, tumor type, origin and microenvironment contribute to differences in response to any particular therapy. There remains a need to predict and assess therapies administered to patients in real-time and provide information of the potential efficacy and safety of drug conjugates. Advanced knowledge of a potentially successful treatment will provide better efficiency, efficacy, and safety for

anti-cancer drug therapies thus individualizing treatment for each patient.

One approach for accomplishing individualized medicine is to use image guided drug delivery (IGDD). Imaging is being incorporated into cancer treatment at an ever growing rate.[2] It has the capability of providing clinicians information for diagnosis and potentially selecting a particular therapy based on the imaging results. This approach has been used in the clinic to improve cancer therapy. One example is the use of radio-immunotherapy for B-cell lymphomas such as Bexxar and Zevalin.[3] This radio-immunotherapy includes a two part system. First, a patient is given an imaging version of the labeled CD-20 targeted antibody and imaged by gamma camera. Depending on the biodistribution data acquired, the patient is then qualified for the therapeutic radiolabeled antibody based on safety parameters defined in the imaging version. A similar approach can be used for drug conjugates. A multifunctional carrier that can carry both an imaging agent and a drug can be combined to provide both diagnostics and therapeutics in one carrier. This allows real-time assessment of drug safety and potential efficacy.[4–7] Systems based on this technology can be of particular use for clinicians in selecting patients for anti-cancer therapies.

Macromolecular water soluble polymers such as *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers are known to increase the solubility of many hydrophobic drugs and exhibit passive targeting to tumor tissues due to the enhanced permeability and retention (EPR) effect.[8,9] HPMA copolymers, prepared via controlled polymerization techniques such as RAFT, are suitable for IGDD due to their biocompatibility, their controlled molecular weight and molecular weight distribution, and incorporation of multifunctional components such as drugs, imaging agents and active targeting ligands. The use of cancer specific targeting ligands conjugated to the backbone of HPMA copolymers has shown to increase its localization in the tumor tissue.[10]

One potential target for pancreatic cancer that has recently been explored is HER2.[11–13] HER2 is a member of the human epidermal growth factor receptor family of tyrosine kinases. HER2 receptors are overexpressed in many cancer types including pancreatic cancer. However, there exists a reported range (16–61%) of pancreatic tumors having high expression of HER2.1.[14–18] Recently a small peptide ligand KCCYSL was reported to have selective specificity to HER2 receptors overexpressed in breast cancer tissues confirmed by in vivo imaging techniques.[19] KCCYSL can easily be conjugated to HPMA copolymers in a similar manner as described for other peptide ligands.

In vitro and in vivo imaging of HPMA copolymers has been performed for many studies.[20] Imaging agents such as fluorophores, MRI contrast agents and radionuclides have proven valuable in evaluating the uptake of HPMA copolymer conjugates in tumor cells and localization in animal models.[20–23] Nuclear medicine techniques such as single photon emission computerized tomography (SPECT) and positron emission tomography (PET) are advantageous based on their ability to provide high resolution and quantifiable images of radionuclide labeled HPMA copolymer distribution in vivo.[5] For example, we have used gamma scintigraphy to monitor the tumor localization of targeted HPMA copolymers in various tumor animal models.[5,24,25] However, these studies were limited to planar gamma scintigraphy techniques which only give information on the localization but cannot be used quantitatively.^{111}In is an optimal radionuclide to attach to the HPMA copolymers due to its clinical utility and accessibility, and its 2.8 day half-life which is sufficient for monitoring the bio-distribution for a few days in vivo by SPECT/CT which can give more accurate and quantitative results.

Gemcitabine is the first line therapy for pancreatic cancer but suffers from poor stability in the blood stream.[26] It is a cytidine analogue and during cell division can inhibit DNA chain elongation thus triggering apoptosis.[27] This requires the drug to traverse the blood stream to the tumor site and migrate into the tumor cell for it to be effective. However, it is known that gemcitabine is rapidly metabolized in the blood stream to an inactive product. Another factor limiting gemcitabine efficacy is its dose-limiting toxicity associated with myelosuppression.[26] Gemcitabine delivery to the tumor cell may be improved when incorporated into the side chains of HPMA copolymers via a lysosomal degradable linker. Including this drug in the side chains of the copolymer can serve three purposes: First, it can prevent rapid metabolism in the blood stream because the HPMA copolymer can reduce the interactions of the drug with the enzymes responsible for its inactivation. Second, covalently bound gemcitabine can potentially prevent free drug from diffusing into the normal vasculature. This can limit the dose-limiting toxicity and allow a higher amount of drug to be administered to the patient thus improving efficacy. Third, site specific release can be achieved by attachment of targeting moieties and degradable spacers. This will ultimately improve the safety and efficacy profile of gemcitabine therapy in the treatment of pancreatic cancer. The goal of this study was to synthesize and characterize HPMA copolymers containing gemcitabine, chelator of ^{111}In^{3+}, and KCCYSL in the side chains. To our knowledge, this is the first example of an HPMA copolymer simultaneously containing anti-cancer drug, tumor targeting peptide and imaging agent. A series of experiments were conducted to evaluate the radionuclide stability, drug release and targetability of these systems in vitro.

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2. Experimental Section

2.1. Materials

2.1.1. Chemicals

Gemcitabine HCl was obtained from LC Laboratories (Woburn, MA). Amino acids for peptide synthesis were obtained from AAPPTec (Louisville, KY). N-[(R)-2-Amino-3-(p-isothiocyanatophenyl)propyl]-trans-(S,S)-cyclohexane-1,2-diamine-N,N-N',N',N''N''-pentaacetic acid (p-SCN-CHX-A''-DTPA) was obtained from Macrocyclics (Dallas, TX). N-(3-Aminopropyl)methacrylamide hydrochloride (APMA) was acquired from Polysciences (Warrington, PA). 2,2'-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) was obtained from Wako Chemicals (Richmond, VA). $^{111}InCl_3$ was obtained from the Intermountain Radiopharmacy (Salt Lake City, UT). All other reagents were of reagent grade and obtained from Sigma-Aldrich (St. Louis, MO).

2.1.2. Synthesis of Comonomers

N-(2-hydroxypropyl)methacrylamide comonomer (HPMA) and N-methacryloylaminopropyl-2-amino-3-(isothiourea-phenyl)propyl-cyclohexane-1,2-diamine-N,N-N',N',N'',N''-pentaacetic acid (APMA-CHX-A''-DTPA) were synthesized according to published methods[28,29] (Figure 1). The intermediate N-methacryloylglycylphenyl-alanylleucylglycine (MA-GFLG-OH) was synthesized via solid phase synthesis. Briefly, amino acids were sequentially added from N-terminus to C-terminus using a typical solid phase synthesis strategy.[30] The final addition involves the coupling of methacrylic acid to the peptide resin employing O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) as a coupling reagent. The MA-GFLG-OH was removed from the resin by treatment with trifluoroacetic acid in the presence of a polymerization inhibitor, t-octyl pyrocatechine. MA-GFLG-OH was converted

to N-methacryloylglycylphenylalanylleucylglycyl-p-nitrophenyl ester (MA-GFLG-ONp) according to published methods.[31] The final steps for synthesis of the drug-comonomer N-methacryloylglycyl-phenylalanylleucylglycyl-gemcitabine (MA-GFLG-Gem) were performed by aminolysis of the MA-GFLG-ONp with excess gemcitabine (1:1.5 ratio respectively). The product was purified by column chromatography (silica gel, eluent: ethylacetate/methanol). The final product was confirmed by electrospray ionization mass spectrometry (ESI-MS) m/z calculated for $C_{32}H_{41}F_2N_7O_9$ 705.7062, found 705 $[M]^+$, 727 $[M+Na]^+$.

Peptide comonomers N-methacryloylglycylglycyllysylcy-stylcystyltyrylserylleucine (MA-GG-KCCYSL) and N-methacryloyl-glycylglycyllysyltyrylleucylcystylserylcysteine (MA-GG-KYLCSC) were also synthesized via solid phase synthesis on a PS3 Peptide Synthesizer (Protein Technologies, Inc., Tucson, AZ) with addition of methacrylic acid as the final peptide residue as described above in the MA-GFLG-OH synthesis. Both products were confirmed by ESI-MS m/z calculated for $C_{38}H_{59}N_9O_{12}S_2$, 898.0582, found 898.3 $[M]^+$.

Polymerization of HPMA conjugates: Copolymerization was performed using the reversible addition-fragmentation chain transfer (RAFT) method in order to control the size and polydispersity of the copolymers.[32] Briefly, HPMA monomer, MA-GFLG-Gem, MA-GG-KCCYSL (or MA-GG-KYLCSC for control) and APMA-CHX-A''-DTPA comonomers were combined with the initiator VA-044 and the chain transfer agent 2-cyano-2-propyl dodecyl trithiocarbonate (CPDT) in 40/60 DMF/methanol in a nitrogen purged sealed glass ampule (Figure 1). The feed ratio of comonomers were kept constant at 83:10:5:2 (mol%), respectively. Pilot batches were synthesized in order to determine the optimal polymerization ratios of monomer, CPDT and VA-044. Ultimately, a ratio of [300:1:1] monomers:CPDT:VA-044 with monomer concentration held constant at 1 M provided the desired results. The ampule was placed in a 40 °C oil bath for 24 h, after which the resulting polymer was collected by precipitation and washed with
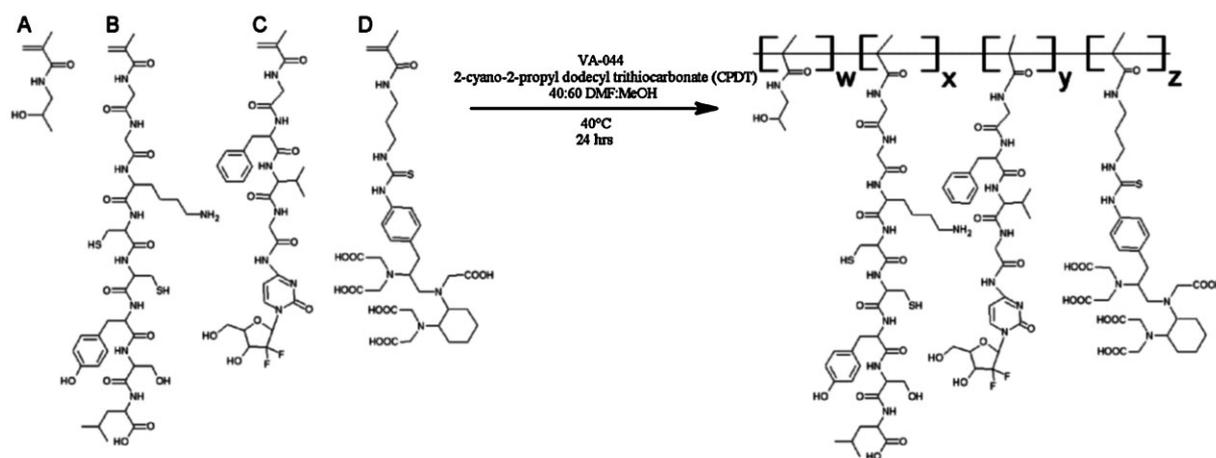

Figure 1. Comonomers used for the synthesis of the polymers and RAFT synthesis scheme. A) HPMA comonomer which affords water solubility constituting the main part of the backbone. B) MA-GG-KCCYSL comonomer used for active targeting of HER2 receptors. C) MA-GFLG-Gem comonomer capable of lysosomal release of gemcitabine within the tumor cell. D) APMA-CHX-A''-DTPA comonomer for chelation of $^{111}In^{3+}$ for imaging by SPECT/CT. For RAFT polymerization monomers were kept constant at 1 M concentration with a ratio of [300:1:1] monomer:CPDT:VA-044.

diethyl ether. The resulting copolymers' average molecular weight ($\overline{M}_{w}$) and polydispersity ($\overline{M}_{w}/\overline{M}_{n}$) were estimated by size exclusion chromatography (SEC) using a Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare, Piscataway, NJ) equipped with a Superose 12 column calibrated with fractions of known molecular weight HPMA homopolymers. Control conjugates with either the scrambled peptide sequence (KYLCSC), a lack of targeting peptide or lack of drug were also synthesized in a similar manner.

2.2. Methods

2.2.1. Characterization of Gemcitabine and Peptide Contents of HPMA Copolymer Conjugates

Gemcitabine content of the conjugates was determined by enzymatic release of free drug from the HPMA copolymer backbone as described previously.[33,34] Treated samples were subjected to HPLC analysis using a mobile phase consisting of deionized water with 1% trifluoroacetic acid (TFA) and acetonitrile (ACN) with 1% TFA according to the following gradient: 0 min, 2% ACN to 90% ACN over 20 min. HPLC analyses were performed with an Agilent Series 1100 HPLC (Agilent Technologies, Wilmington, DE, USA) equipped with an Alltima C18 5 μm 150 × 4.6 mm column and a photo diode array detector. A flow rate of 1.0 mL min^{-1} was utilized with an injection volume of 20 μL. UV absorbance of 267 nm was used for quantification of gemcitabine. Peptide content was determined by amino acid analysis performed in the University of Utah Core Research Facilities (Salt Lake City, UT).

2.2.2. Radiolabeling and Stability of the Conjugates

Each copolymer was dissolved in 400 μL of 1.0 M sodium acetate buffer pH 5.0 and added to 5.0 mCi of ^{111}InCl$_3$ stock solution which was also previously treated with 200 μL of 1.0 M sodium acetate buffer pH 5.0. The reaction mixture was heated at 50 °C for 45 min after which 50 μL of 0.05 M EDTA solution was added to scavenge any free ^{111}In ions. The radiolabeled copolymer was then purified using Sephadex G-25 PD-10 columns (GE Life sciences, Piscataway, NJ). Radiostability was evaluated by incubating radiolabeled copolymer at 37 °C in the presence of mouse serum. Samples were collected at 24, 48, and 72 h and a comparison between ^{111}In labeled copolymers and free ^{111}In^{3+} was performed by size exclusion chromatography using PD-10 columns. Each fraction was subsequently measured using a Cobra II Auto-gamma-counter (Canberra Industries, Inc., Meriden, CT).

2.2.3. Cell Lines

CAPAN-1 pancreatic adenocarcinoma cells (ATCC, Manassas, VA) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (ATCC, Manassas, VA) supplemented with 20% (v/v) fetal bovine serum (FBS) and 1:100 penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO$_2$ (v/v). PANC-1 pancreatic duct carcinoma cells (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Medium (DMM) (ATCC, Manassas, VA) supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO$_2$ (v/v). For all experimental procedures, the confluent cultures were harvested by treatment with TrypLE^{TM} Express (Invitrogen, Grand

Island, NY) and subsequent dilution in their respective medium or phosphate buffered saline (PBS).

2.2.4. Relative Expression of HER2 by Flow Cytometry

Anti-HER2 (Neu) and normal mouse IgG$_1$ (negative control) monoclonal antibodies labeled with phycoerythrin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Samples of CAPAN-1 and PANC-1 cells were harvested, washed with fresh media followed by PBS, and then incubated with antibodies for 30 min at 4 °C. Cells were washed with PBS and then fixed in 1% formaldehyde in PBS. Samples were analyzed by flow cytometry using a FACScan System (BD Biosciences, San Jose, CA).

2.2.5. Affinity of KCCYSL-containing Copolymers

Copolymers containing KCCYSL in the side chains were assayed by performing an in vitro blocking experiment. CAPAN-1 cells were harvested and re-suspended in serum free IMDM media. Each sample was combined with ^{111}In labeled KCCYSL copolymers and 100 and 1 000 fold excess concentrations of free KCCYSL peptide or KYLCSC as control. The samples were incubated at 4 °C for 18 h and then cells were centrifuged and the supernatant removed. The cells were subsequently washed three times with saline until no further activity could be removed from the resulting cell pellet. The resulting cell pellet was then counted using a CAPTUS® 3000 Well Counting System (Capintec, Ramsey, NJ). The percent of HPMA copolymer conjugate bound to the cells relative to control was reported. The assay was conducted in triplicate.

2.2.6. Gemcitabine Release in Various Media

The rate of gemcitabine release in vitro from copolymers was examined in buffer solutions or cell culture media. The release of the drug from copolymers was carried out by dissolving the polymers (3 mg mL^{-1}) each in acetate buffer solution (pH 5.0), phosphate buffered saline (PBS) solution (pH 7.4), Dulbecco's Modified Eagle's Medium (DMEM) with 10% of fetal bovine serum (FBS) and Iscove's Modified Dulbecco's Medium (IMDM) with 20% of fetal bovine serum, respectively. The samples were kept at 37 °C. At scheduled time intervals, 20–60 μL solutions were withdrawn from the samples. Gemcitabine contents were analyzed by HPLC under the same conditions as described above.

2.2.7. Cytotoxicity of Conjugates with Gemcitabine in the Side Chains

The in vitro cell growth inhibition of gemcitabine and copolymers was evaluated using a Cell Counting Kit-8 (CCK-8) assay (Donjindo Molecular Technologies, Inc., Rockville, MD). Briefly, PANC-1 and CAPAN-1 cells were seeded into 96-well plates at an initial density of 2 000 cells/well and allowed to adhere for 24 h. Stock solutions of free gemcitabine, conjugates and controls were prepared in dimethylsulfoxide (DMSO) and subsequent dilutions were done to a final concentration of 0.5% (v/v) in media. Seeded cells were incubated for 72 h with increasing concentrations of gemcitabine or copolymers in fresh media containing 0.5% (v/v) DMSO. The drug containing media were removed and incubated in fresh media for another 48 h. The media was removed and 100 μL of medium

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solution containing 10% CCK-8 reagent was added. The cells were incubated at 37 °C for a further 1–4 h. Cell viability was obtained by scanning with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) at 450-630 nm. Each experiment was performed three times in triplicate.

3. Results and Discussion

HPMA copolymer conjugates containing gemcitabine, chelator of $^{111}In^{3+}$, and a HER2 targeting peptide were successfully synthesized by RAFT copolymerization techniques with characteristics shown in Table 1. The yield was low for these polymers perhaps due to the possible degradation of the RAFT agent by primary amines contained in the comonomers. This low yield may also be due to steric effects based on the bulkier comonomers. However, sufficient quantities were obtained in order to evaluate these conjugates in vitro. The design criteria for a potentially translatable and successful IGDD system based on HPMA copolymers are as follows: First, the copolymer must be synthesized in a size-controlled and reproducible manner. Second, the polymer-drug constructs must have a biological half-life that allows enough circulation time in the blood stream in order to allow sufficient time to encounter and accumulate within the diseased tissue. The constructs on the other hand must be eliminated from the kidney in a timely manner in order to reduce background signal in the blood during imaging and reduce the longtime exposure of HPMA copolymer conjugates to non-targeted tissues in the body. Previous studies have shown that HPMA copolymers generally can be filtered through the kidney and excreted in the urine when the $\overline{M}_w$ is less than 45 kDa.[9] Therefore, the copolymer conjugates were synthesized around a size range of 20–30 kDa (Table 1). The final criteria needed in a successful IGDD HPMA copolymer system
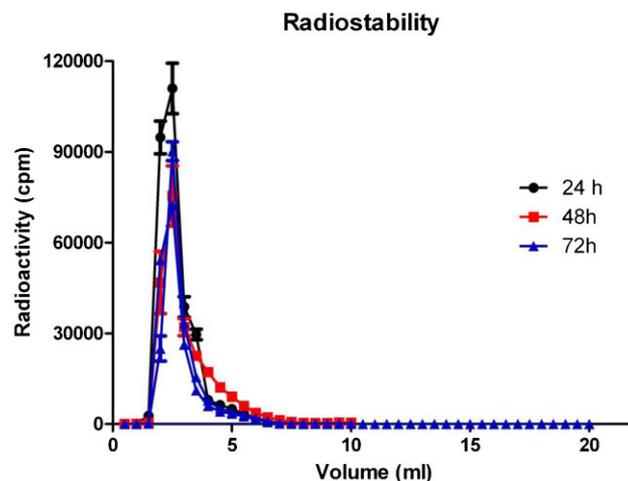

Figure 2. Stability of the [^{111}In] radiolabeled HPMA copolymer conjugate. HPMA copolymers were incubated in the presence of mouse serum for up to 72 h. After SEC purification each fraction was measured. Each time point was performed in triplicate.

is stability of the drug and imaging components in conditions simulated to the blood stream and site-specific drug release within the targeted tissue. Each of these issues was examined as discussed below.

Radiolabeling of the HPMA copolymers with ^{111}In was found to be quite efficient with a decay corrected radiochemical yield of $83.1 \pm 0.2\%$. Based on the feed ratio of the APMA-CHX-A''-DTPA and the specific activity provided by the supplier, approximately 17% of chelators contained an indium atom. Stability was assessed in mouse serum as displayed in Figure 2. DTPA is known to be a stable chelator of ^{111}In and other radionuclides.[35] No free $^{111}In^{3+}$ was detected from the size exclusion column purifications over the 72 h. The results confirm that chelation with DTPA is stable. The HPMA copolymer-KCCYSL-DTPA conjugates

Table 1. Summary of the HPMA copolymer characteristics.

Polymer	Monomer Feed Ratio[a] [mol%]	$\overline{M}_w$ [b] [kDa]	$\overline{M}_n$ [b] [kDa]	PDI [$\overline{M}_w/\overline{M}_n$]	Expected Peptide Content [wt%]	Measured Peptide Content[c] [wt%]	Expected Gemcitabine Content [wt%]	Measured Gemcitabine Content[d] [wt%]	Polymer Yield
HPMA-KCCYSL-Gem-DTPA	83:5:10:2	26.8	18.7	1.4	14.3	13.6	6.2	4.7	18%
HPMA-KYLCSC[e]-Gem-DTPA	83:5:10:2	29.9	21.4	1.4	14.3	14.8	6.7	5.4	19%
HPMA-Gem-DTPA	88:10:2	23.6	21.0	1.1	–	–	6.5	5.0	11%
HPMA-KCCYSL-DTPA	93:5:2	20.4	16.8	1.2	18.4	19.9	–	–	28%

[a]Total monomer concentration 1 M for RAFT polymerization. Solvent = 40:60 Dimethylformamide:Methanol; [b]Estimated by size exclusion chromatography; [c]Determined by amino acid analysis; [d]Determined by enzymatic release followed by HPLC analysis; [e]KYLCSC is non-targeted scramble peptide sequence.

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contain the HER2 targeting peptide sequence discovered by Karasseva, et al.[19] This peptide was included because of its potential in targeting HER2 overexpressed in some tumor types such as pancreatic cancer. Several tumor cell lines have been reported to express different levels of HER2 as investigated by flow cytometry.[36] The results are shown in Figure 3. PANC-1 and CAPAN-1 were selected for our study based on their tumorogenicity and expression levels of HER2. CAPAN-1 was shown to have a significantly higher expression of HER2 than PANC-1 ($p < 0.001$) and control ($p < 0.0001$), which was consistent with previous reports.[36] No significant difference in HER2 expression was observed between PANC-1 and control antibodies, therefore PANC-1 cell line was used as a negative control for HER2 expression for in vitro assays.

The ability of the KCCYSL containing HPMA copolymers to bind HER2 expressing cell lines was investigated, because the HPMA copolymer may change or prevent the affinity of the KCCYSL peptide contained on its side chains. No natural ligand is known for HER2 receptors that can be used in a competitive binding assay.[37] Therefore a blocking experiment was performed where excess free peptide was incubated in the presence of ^{111}In labeled HPMA copolymer-KCCYSL-DTPA conjugates with CAPAN-1 cells in serum free media. Figure 4 shows the results of the blocking study. In summary, binding of the CAPAN-1 cell lines known to express HER2 were effectively blocked when excess peptide was incubated in the presence of HPMA copolymer-KCCYSL-DPTA conjugates. Scramble peptide KYLCSC was used as a control and therefore suggests that the KCCYSL containing copolymer retains a measurable binding affinity to the HER2 receptor.

Drug release was investigated in complete cell culture media and also in buffered solutions of pH 5.0 and 7.4. Results are shown in Figure 5. The conjugates were most stable in the low pH 5.0 media which is consistent with an amide bond formed between the amide of the pyramidine ring of gemcitabine and the C terminus of the GFLG linker.

Figure 4. Binding affinity of HPMA copolymer-KCCYSL-DTPA conjugate. Control (blue bar) includes 1000-fold excess KYLCSC scramble peptide. Increasing amounts of 100 and 1000-fold excess of free KCCYSL peptide increasingly blocked the binding of HPMA-KCCYSL-DTPA. Data represented as the mean and SEM ($n = 3$). Statistical significance performed using one-way ANOVA with Tukey's post-test ($* = p < 0.01$ and $*** = p < 0.001$).

However, some drug instability was found in cell culture media. The amide bond may unexpectedly be less stable due to electron delocalization related to the pyrimidine ring of the drug. On another note, the drug released faster in cell culture media than PBS. This result is consistent with previously reported results with a HPMA copolymer-docetaxel conjugate containing the same linker[34] and may be because there are various enzymes and proteins in the media. However, the HPMA copolymer docetaxel
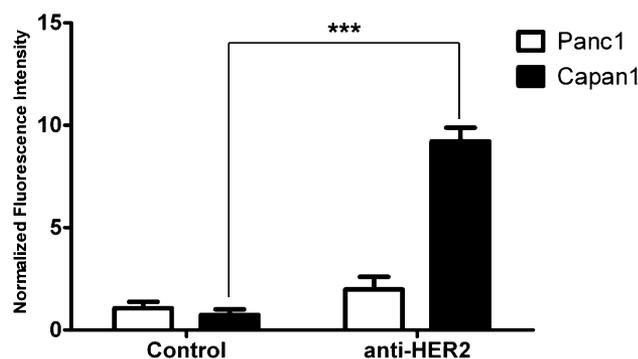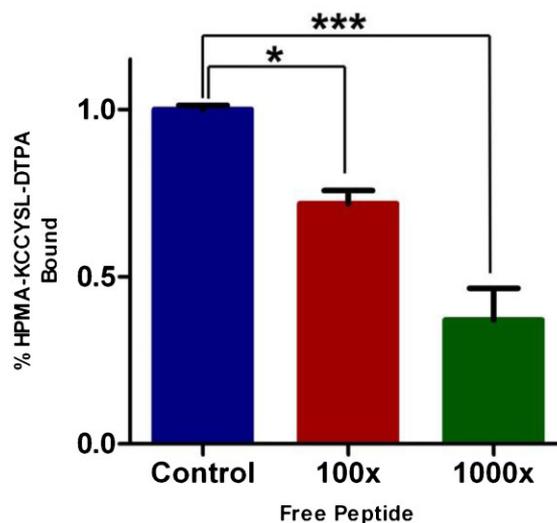

Figure 3. Relative expression of HER2 on Pancreatic Cancer Cell Lines. Only HER2 expression on Capan-1 cell lines showed a significant difference ($*** = p < 0.0001$) than that of control.
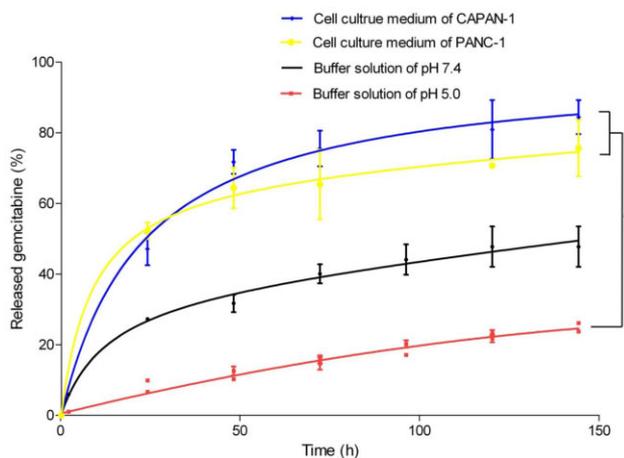

Figure 5. Gemcitabine release from the conjugates. Data expressed as the mean and SEM ($n = 3$). Statistical significance performed using 1 way ANOVA with Tukey's post-test. $* = p < 0.01$.

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conjugates with RGDfK ligands for targeting neovascular tissue demonstrated an enhanced anti-cancer effect, indicating that future in vivo experiments with targeted HPMA copolymers with gemcitabine may demonstrate a similar result. The stability of the gemcitabine conjugate is sufficient for drug delivery using HPMA copolymers, but perhaps could be improved by development of more stable linker chemistries.

Cytotoxicity of HPMA copolymer constructs was evaluated in both cell lines including PANC-1 (negative HER2) and CAPAN-1 (positive HER2). Results are shown in Figure 6 and calculated IC_{50} values are displayed in Table 2. IC_{50} values were consistently in the nM range, similar to results reported previously.[36] However, minor differences were observed between HER2 positive and HER2 negative cell lines, for reasons that are not entirely clear. Data suggests that HER2 positive cell lines are more sensitive to gemcitabine therapy. Some studies have suggested that

Table 2. IC_{50} values of HPMA copolymer conjugates in HER2 negative and HER2 positive cell lines. Statistical analysis was performed using one way ANOVA with Tukey's post-test. Free drug was statistically different than all three conjugates in both cell lines ($*** = p < 0.001$). HPMA conjugates were not statistically significant from each other.

Polymer	Panc-1 IC_{50} [nM]	Capan-1 IC_{50} [nM]
Gemcitabine (Free drug)	28.8***	5.3***
HPMA-KCCYSL-Gem-DTPA	142.0	18.2
HPMA-KYLCSC[a)]-Gem-DTPA	108.0	12.4
HPMA-Gem-DTPA	104.0	14.6

[a)]KYLCSC is non-targeted scramble peptide sequence.

overexpression of HER2 in pancreatic cancer patients correlates with poor prognosis.[38,39] Regardless, more in depth analysis is needed to validate this hypothesis. The results also show a difference between the free drug and the HPMA copolymer species. This is typical of HPMA copolymer-drug conjugates since the free drug enters the cell by passive diffusion compared to endocytosis of the conjugates by the macromolecular system. No significant difference was observed between the KCCYSL and KYLCSC (scrambled) peptides due to the fact that the drug is gradually released in the cell culture media over the 72 h. The available drug activity over this time frame is prolonged and therefore local concentration of gemcitabine to a tumor cell may be lower thus explaining the difference between the free drug and the polymer-drug conjugate.

The in vitro results demonstrate that gemcitabine activity is still retained when bound to the HPMA copolymer. However, the drug is gradually released in physiological media. Gemcitabine is rapidly metabolized in the bloodstream thus losing its anti-cancer activity. Therefore high doses must be administered to patients in order to achieve efficacy, but that in turn also increases toxicity such as myelosupression.[26] However, because the drug is released slowly from the conjugates, it should be protected from metabolic enzymes until encountering the tumor in the in vivo situation. Subsequent release once in the tumor environment may increase the local active concentration of gemcitabine, thus providing a potentially more efficacious and safe drug therapy. Active targeting of the drug to tumor cells under study did not show an increased efficacy. Reports in the literature regarding active and passive targeting of drugs are mixed.[40] In the current study a possible explanation of lack of difference between actively or passively targeted copolymers in terms of cytotoxicity is the premature
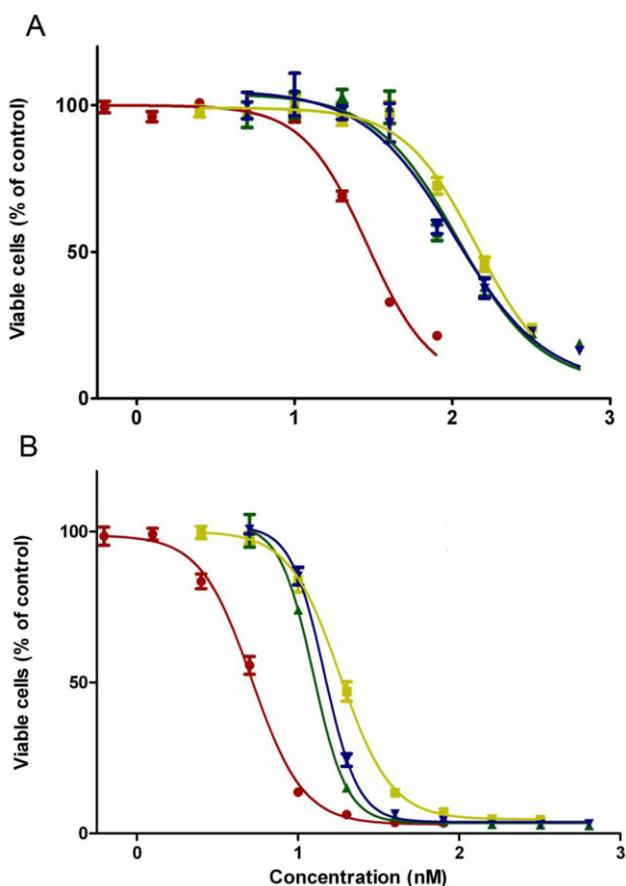

Figure 6. Cytotoxicity of HPMA copolymer conjugates containing gemcitabine. A) PANC-1 and B) CAPAN-1 graphs demonstrating the cytotoxicity of each HPMA copolymer species. Red circle is free Gemcitabine. Yellow square is HPMA copolymer-KCCYSL-Gem-DTPA. Green diamond is HPMA copolymer-KYLCSC-Gem-DTPA (scramble). Blue triangle is HPMA copolymer-Gem-DTPA. Data is represented as the mean and SEM ($n = 9$).

release of the drug. Future in vivo studies will need to be conducted to evaluate the value of this targeting strategy in relevant preclinical animal models and perhaps to consider alternative targeting strategies for targeting pancreatic tumors.

4. Conclusion

HPMA copolymer–gemcitabine conjugates containing chelator of $^{111}In^{3+}$ and HER-2 targeting peptides in the side chain were synthesized in a size and content-controlled manner. The conjugates showed efficacy against pancreatic tumor cell lines. However attachment of the targeting peptides did not improve cytotoxicity. Premature release of the free drug was observed that can in part contribute to cytotoxicity. In vitro radiostability of the ^{111}In complex was sufficient for imaging over a relevant time. These studies have set the stage for further optimization and evaluation of the constructs for image guided delivery in pancreatic tumor models.

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