Targeted Delivery of Boron-10-Loaded Peptide Polymers to Pancreatic Adenocarcinoma Cells Via Bioconjugated Gold Nanoparticles for Neutron Capture Therapy

Sara Althari
Advisor: Andrew C. Webb, PhD

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<td>BPA</td>
<td>boronophenylalanine</td>
</tr>
<tr>
<td>BNCT</td>
<td>boron neutron capture therapy</td>
</tr>
<tr>
<td>BSH</td>
<td>sodium mercaptododecaborate (Na$<em>2$H$</em>{11}^{10}$B$_{12}$SH)</td>
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<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
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<tr>
<td>FT-IR</td>
<td>fourier transform infrared spectroscopy</td>
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<tr>
<td>GNP(s)</td>
<td>gold nanoparticles(s)</td>
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<tr>
<td>GPA33</td>
<td>human glycoprotein A33</td>
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<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma mass spectrometry</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MPI</td>
<td>moxi population index</td>
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<tr>
<td>pAb</td>
<td>polyclonal</td>
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<tr>
<td>PDI</td>
<td>poly dispersion index</td>
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<tr>
<td>PolyGL</td>
<td>D-glutamate:D-lysine peptide polymers</td>
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<tr>
<td>SIA</td>
<td>heterobifunctional cross linker $N$-succinimidyl iodoacetate</td>
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<td>Sulfo-MBS (SMBS)</td>
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ABSTRACT

Of all malignancies, pancreatic cancer is one of the most fatal and aggressive forms. Its rapid metastatic activity, high mutation rate and resistance to radiation-coupled chemotherapy are among the various factors that contribute to its lethal nature. The lack of effective early detection methods and directed therapeutic strategies are highlighted by poor prognoses and increasing death rates. Boron neutron capture therapy (BNCT) is a form of cancer treatment that can be used against such refractory malignancies. BNCT is a binary therapy that relies on the fission of $^{10}$B atoms upon neutron irradiation to alpha particles and lithium-7 nuclei. Due to their high mass and short path length (~10 µm), the cytotoxic effect of these products is limited to a single cell diameter, granting BNCT the selective capacity needed to leave normal tissue unharmed. However, the therapeutic capacity of this form of treatment is fundamentally dependent on the targeted delivery of a sufficient concentration of $^{10}$B to tumor cells, avoiding adjacent normal tissue. Gold nanoparticles (GNPs) have garnered wide interest in the expanding field of oncology as platforms for drug delivery largely because of their low biological toxicity, biocompatibility, high surface-to-volume ratio, and biodistribution properties. Here, we present a method to prepare multifunctional nanovehicles that allow for specific targeting and $^{10}$B delivery by coating the surfaces of gold nanoparticles with targeting and delivering moieties to which boron-loaded peptide sequences are added. Further optimization is required to be able to ultimately utilize these constructs in in vitro and in vivo BNCT studies.
INTRODUCTION

Pancreatic cancer

Despite recent advancements in cancer treatment and diagnosis, the aggressive biology of pancreatic cancer renders it one of the most lethal malignancies. Largely due its asymptomatic nature in its early stages, rapid metastatic growth, high resistance to conventional therapy, pancreatic cancer ranks fourth among cancer-causing mortalities in the United States (Hidalgo 2010). In addition, the molecular biology of pancreatic cancer is not as well understood relative to other malignancies hence the lack of effective biomarkers (Logsdon et al. 2003; Hidalgo 2010). Approximately 45,000 new cases and 38,000 deaths from pancreatic cancer are estimated in the United States in 2013 (NIH 2013). High death rates and poor prognoses underscore the lack of effective early detection methods and directed therapeutic strategies.

Approximately 95% pancreatic tumors are adenocarcinomas, those that arise in the ductal glands of the pancreas (Hidalgo 2010). Patients diagnosed with pancreatic adenocarcinoma have an overall 6% 5-year survival rate (American Cancer Society 2012). The chemotherapeutic agent gemcitabine has shown to modestly lengthen survival for patients who undergo surgery particularly when administered in tandem with erlotinib, an inhibitor of the epidermal growth factor receptor (EGFR) (Burris et al. 1997; Hidalgo 2010). At present, pancreatic cancer treatment is limited to radiation-coupled chemotherapy and surgery, and less than 20% of diagnosed patients are considered candidates for surgical intervention because the cancer is typically detected at advanced metastatic stages (American Cancer Society 2012).

Therefore, improved clinical treatment and prognosis of pancreatic adenocarcinoma is largely dependent upon developing a greater understanding of the underlying molecular
mechanisms involved in regulating this malignancy. This will allow us to exploit the expression patterns of tumor-specific proteins for early detection and targeted drug delivery.

**Boron Neutron Capture Therapy (BNCT)**

Especially for malignancies that are highly refractory to radiation treatment, chemotherapy and surgery, such as those of the pancreas, boron neutron capture therapy (BNCT) seems to be a promising therapeutic approach to the selective eradication of tumor cells. BNCT is a binary therapy based on the nuclear fission reaction that occurs when the nonradioactive isotope of boron-10 ($^{10}$B) is irradiated with epithermal neutrons to produce cytotoxic alpha particles and lithium-7 nuclei (Barth et al. 2005; Pisarev et al. 2007) (Figure 1). These products are heavy, have high linear energy transfer (LET) and a characteristic path length of a single cell diameter (~10 µm), limiting radiation damage to neoplastic tissue in which boron-10 atoms are accumulated, making it an ideal selective therapeutic strategy (Barth et al., 1990). Another feature that renders alpha particles especially efficient in destroying tumor cells is that, unlike X-rays for instance, their biological effectiveness is not compromised in hypoxic cellular environments (Barth et al. 1990). Additionally, alpha particles and lithium nuclei are indiscriminately damaging to both dividing and non dividing cancer cells, in contrast to other forms of radiotherapy, as tumors harbor many viable non dividing cells despite their rapid proliferation (Barth et al. 1990). However, the therapeutic capacity of this procedure relies primarily on the targeted delivery of a sufficient concentration of $^{10}$B to tumor cells, avoiding adjacent normal tissue (Ferro et al. 1995; Barth et al. 2005).
Historically, BNCT was used almost exclusively for the treatment of aggressive brain tumor glioblastoma multiforme (Hawthorne, 1993; Pisarev et al., 2007). In 1951, Sweet and Farr initiated the first BNCT clinical trial at the Brookhaven National Lab to treat patients with high-grade glioblastoma multiforme using thermal neutrons and borax (Na₂B₄O₇•10H₂O) as the boron-containing capture agent (Barth et al. 1990; Sweet 1951). A decade later, Sweet and Brownell conducted further trials at Massachusetts General Hospital using the Massachusetts Institute of Technology Reactor (MITR) (Barth et al. 2005; Asbury et al. 1972). The results of these trials were disappointing, as therapeutic evidence was minimal and mostly insignificant (Barth et al. 1990). Subsequent analyses have shown that the failure of these early trials is largely attributable to 1) high blood boron concentrations at the time of irradiation that damaged the scalp and normal brain tissue, 2) the boron-containing capture agents used did not achieve sufficient tumor-specific localization, and 3) the limited tissue penetration depth of thermal neutrons which only resulted in the destruction superficial tumors (Barth et al. 2005).
To overcome the obstacles that diminished the promising curative capacity of BNCT, different methodological adjustments had to be made. These began with the evaluation of the biodistribution properties of various boron-containing capture agents, particularly with respect to effective tumor localization and minimal vasculature contamination (Hawthorne 1993). The sulfhydryl-containing polyhedral borane (Na$_2$B$_{12}$H$_{11}$SH), given the abbreviation BSH, and boronophenylalanine (BPA) fulfill these requirements to a considerable degree and have been clinically used in the United States, Japan, Europe and Argentina (Barth et al. 2005) (Figure 2). Presently, boronophenylalanine (BPA), sodium mercaptododecaborate (BSH) and disodium-decahydro-closo-decaborate are the only approved $^{10}$B-containing capture agents for human trials (Kueffer et al., 2013). Still, these boron drugs are not considered ideal, only effective relative to other boron-containing capturing agents (Barth et al. 2005). To fully meet the biochemical requirements needed to achieve ideal boron delivery, carrier species such as monoclonal antibodies, receptor-targeting boron-containing bioconjugates and liposomes were tested.
Although highly specific mAbs have the potential to significantly concentrate the amount of boron delivered to the target cancerous tissue, their efficacy is hampered by a limitation in boron loading due to an associated loss of immunoreactivity as the function of most biological macromolecules is conformationally dependent (Ferro et al. 1995; Barth et al. 2005).

Thus, the two primary limitations of BNCT as a proposed therapeutic strategy remain loading enough $^{10}$B onto directed boron delivering agents to achieve maximum tumor-specific cell kill, as well as accurate ways to determine the biodistribution of these agents upon which irradiation time can then be based. Our study addresses one of those limitations by exploiting the large surface to volume ratio of gold nanoparticles to ultimately achieve optimal $^{10}$B loading and maximize the delivery of boron-containing capture agents.

**Targeted nanoparticles for cancer therapy**

Gold nanoparticles (GNPs) have received great interest in the expanding field of oncology as platforms for drug delivery largely because of their low biological toxicity, high surface-to-volume ratio, biocompatibility, small size, tunable surface functionality and biodistribution properties (Conde et al., 2011; Jain et al., 2012). Thus, the use of monoclonal antibody targeted (mAb) GNP constructs, directed towards a tumor-specific antigen in pancreatic adenocarcinoma cells, can be a particularly effective means of selective $^{10}$B delivery for neutron capture therapy. To this end, a multi-functional nanovehicle capable of delivering high levels of $^{10}$B specifically to pancreatic adenocarcinoma cells was developed (Webb et al., 2012) (Fig 3).

To selectively localize a sufficient concentration of $^{10}$B in the tumor cells at the therapeutic dose ($\geq 20 \, \mu g/g$ tumor tissue or $10^9$ $^{10}$B atoms per cell) for the purpose of our research, we use random D-glutamate:D-lysine peptide polymers (poly-GL) cross-linked with $^{10}$B-enriched BSH (Ferro et al., 1995) (Kueffer et al., 2013) (Fig 2, 4). There are various
advantages to using these synthetic polymers for boron delivery and these include 1) their resistance to proteolytic degradation, a feature that is characteristic of polypeptides made up of the D-isomer form of amino acids and 2) their function as water soluble coupling agents (Ferro et al., 1995). $^{10}\text{B}$ enriched BSH molecules are covalently conjugated to the poly-GL peptide polymers via heterobifunctional crosslinker Sulfo-MBS, which reacts with the primary amino groups (-NH$_2$) in the side chain of lysine residues and forms stable thioether bonds with the sulfhydryl group on the BSH molecules (Fig 4) (Ferro et al., 1995). Another conjugation strategy involves the use of heterobifunctional crosslinker SIA, which, similarly, reacts with primary amino groups present on the side chain of lysine residues and creates thioether linkages by virtue of a nucleophilic substitution of the iodine in its iodoacetyl group upon contact with free sulfhydryls (Rector et al., 1978; Thorpe et al., 1984) (Fig 5). The boronated poly-GL is

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**Figure 3.** Gold nanoparticle (GNP) platform coated with polyethylene glycol (PEG) and decorated with a mixture of targeting (mAb31.1) and delivering (antibiotin) monoclonal antibodies as well as highly boronated D-glutamate:D-lysine polymers. The construct is estimated to have a diameter in the range of 30-40nm and a -10mV zeta potential.
biotinylated and conjugated to the GNPs by way of anti-biotin monoclonal antibodies also linked to the gold surface via a dithiol-PEG-hydrazine linker (Kumar et al., 2008) (Fig 3).

![Image](Figure 4.- The chemical reaction involved in cross-linking $^{10}$B enriched BSH and D-glutamate:D-lysine peptide polymers (poly-GL) via the heterobifunctional linker Sulfo-MBS (m-Maleimidobenzoyl-N-hydroxysulfosuccinimide ester). This linker contains reactive primary amine (S-NHS) and maleimide groups at opposite ends (Ferro et al. 1995).)

Finally, by coating the remaining exposed surfaces of the GNPs with polyethylene glycol (PEG) we are able to increase the biological half life of the construct, reduce nonspecific interaction and increase its biocompatibility considering the hydrophilic nature of the PEG linker (Kumar et al. 2008) (Fig 3). Additionally, through this particular technique, mAb linkage occurs via their Fc carbohydrate, or nonbinding portion of the antibody, and hence orientates the mAbs with their antigen binding portions facing out from the GNPs (Fig 3). Overall, implementing this conjugation strategy allows us to conveniently combine both targeting and delivery antibodies on a single surface.

![Image](Figure 5.- The chemical reaction involved in cross-linking $^{10}$B enriched BSH and D-glutamate:D-lysine peptide polymers (poly-GL) via the heterobifunctional linker SIA (N-succinimidyl iodoacetate). This linker contains a reactive primary amine N-hydroxysuccinimide ester (NHS) and a sulfhydryl-reactive iodoacetyl group at opposite ends.)
Tumor-associated epitopes for directed drug delivery

Human glycoprotein A33 (gpA33) is a gastrointestinal tissue-specific cell surface antigen that is homogenously overexpressed in 95% of colorectal cancers, 63% of gastric tumors and over 50% of pancreatic cancers (Sakamoto et al. 2000). It is not expressed in any other type of epithelial cancer and demonstrates similar restricted expression in normal tissue (Ackerman et al. 2008). GpA33 is a 319 amino acid 43-kDa trans-membrane protein and a member of the immunoglobulin superfamily (Heath et al. 1997). The involvement of gpA33 in cell-cell signaling and/or adhesion has been implied using bioinformatic tools and structural analysis (Heat et al. 1997). However, to date, the precise biological function of this uniquely expressed trans-membrane protein remains unknown despite some Murine monoclonal antibody 31.1, an immunoglobulin (Ig)G2a antibody was developed to specifically target gpA33 (Arlen et al. 1998). Interestingly, upon binding to mAb 31.1, gpA33 molecules become rapidly internalized and sequestered in perinuclear vesicles (Mihaylova 2006, Webb et al. 2012). Extensive biochemical and immunological characterization of gpA33 along with its tumor-specific expression pattern render it a promising detection and drug-targeting candidate.

For our research, two human-derived cell lines from primary pancreatic adenocarcinoma tumors were chosen as in vitro models: CAPAN-2 and BxPC3. These cell lines have markedly different expression patterns of human glycoprotein A33; specifically BxPC3 cells are A33 negative whereas gpA33 is consistently overexpressed in CAPAN-2 cells (Figure 5) (Webb et al. 2012). The overexpression of gpA33 on the surface of CAPAN-2 cells and its ability to become internalized upon antibody binding present this tumor-specific antigen as a remarkably attractive candidate for targeted drug delivery. In addition, previous in vitro analyses have proven the specific uptake of mAb31.1-conjugated GNPs in CAPAN-2 cells (Choi 2011; Webb et al. 2012).
Based on these findings, we rely on the specific and endocytosis inducing mAb31.1-A33 interaction for the immuno-targeted delivery of our GNP construct (Figure 3).

Ultimately, this study aims to selectively deliver and concentrate a sufficient amount of $^{10}$B enriched BSH molecules in CAPAN-2 pancreatic adenocarcinoma cells using a multifunctional nanovehicle delivery system for neutron capture therapy. To this end, GNPs are conjugated with two monoclonal antibodies; one that target the tumor-specific antigen gpA33 on the surface of CAPAN-2 cells (e.g. mAb 31.1) and another (anti-biotin) that facilitate optimal boron loading in the form of biotinylated pGL-BSH peptide. Upon ensuring optimal loading of BSH onto the protein carriers and maximum conjugation of boronated peptides onto the surface of the GNPs, CAPAN-2 cells are exposed to the GNP constructs and irradiated with epithermal neutrons to trigger the emission of destructive alpha particles from the fission of $^{10}$B nuclei. Finally, cell viability is tested using the AlamarBlue assay and a Moxi™ Z automated cell counter to determine the efficacy of the proposed boron delivering strategy.

Figure 6.- Western blot of pancreatic adenocarcinoma cell line (Capan-2 and BxPc3) lysates probed for presence of tissue-associated antigen expression. (Courtesy of Bridget Begg ’13).
**Materials & Methods**

*Cell culture*

Human pancreatic adenocarcinoma cell lines (CAPAN-2, BxPc-3) were purchased from American Type Culture Collection (ATCC). Cell lines were maintained at 37°C and 5% CO₂ using RPMI-1640 Medium (Sigma #R6504) supplemented with 0.2% sodium bicarbonate (Sigma #S8761), antibiotic/antimycotic solution (100 units/ml penicillin, 0.1mg/ml streptomycin sulfate, 0.25 µg/ml amphotericin B; Sigma #A5955), and 10% fetal bovine serum (FBS; Hyclone #SH30070.03). Cells were passaged at 70-80% confluency following trypsinization and assessed for >90% viability by Trypan Blue exclusion assay.

*Cell Viability*

To mimic transport to MIT NRL and subsequent post-irradiation conditions, CAPAN-2 cells were incubated at 37°C without CO₂ for a 48-hour period with and without sealing cultures with parafilm. These cells were scored at several time points each day using the Moxi™ Z automated cell counter. Cells were compared to a viable cell population represented by trypsinized cells in an RPMI media suspension after an overnight incubation period at 37°C with 5% CO₂ and to cells induced to undergo apoptotic cell death by treatment with 200µM Etoposide also called VP-16 (Sigma E1383).

*Antibodies*

Several antibodies, both monoclonal (mAb) and polyclonal (pAb) directed against the membrane glycoprotein A33 (gpA33) were used in this study. These include mAb 31.1 a chimeric murine-human IgG1 derived from the clone PCA 31.1 (ATCC HB-12314); mAb As33 a mouse IgG2a
hybridoma isolated from ATCC HB-8779; pAbA33 purchased from Sino Biologicals. The HRP-conjugated secondary antibodies were all polyclonal directed against both IgG heavy and light chains: goat anti-mouse IgG (Thermo Scientific); goat anti-human IgG (Millipore); goat anti-rabbit IgG (Epitomics) and were used at dilutions recommended by the manufacturers.

_Biotinylation and boronation of D-glutamate:D-lysine peptide polymers (poly-GL)._

1-10 mg of poly-GL with a glu:lys ratio of 6:4 and a molecular weight range of 20-50kD (Sigma #P7658) was dissolved in 1 ml phosphate buffer (PB) (0.2 M Na₂HPO₄•H₂O pH 9, 0.2 M NaH₂PO₄•H₂O pH 5) (pH, 6.5). A 5-fold molar excess of Sulfo-NHS-Biotin (Pierce #21217) dissolved in HPLC grade H₂O was added to peptide in solution for preferential biotinylation of N-terminal α-amino groups (Thermo Sci. Tech Tip #46). The reaction was incubated for 24 hrs at 4°C. The solution of biotinylated peptide was incubated with 0.35 mg/ml heterobifunctional crosslinker Sulfo-MBS (Thermo scientific #22312) dissolved in PB (pH, 6.5) for 30 min at RT. 

¹⁰B-enriched BSH (Katchem Ltd., Czech Republic) (0.2mg/ml) was dissolved in HPLC H₂O and added drop-wise in a 3-fold molar excess to the biotinylated peptide solution. The reaction was incubated for 30 min at RT. Zeba™ spin desalting columns (7k MWCO, 5ml) (Pierce #89892) equilibrated with PB (pH, 6.5) were used to remove unreacted biotin, excess cross-linker and excess BSH after each reaction. For larger reaction volumes, Slide-A-Lyzer® Dialysis Cassette (10k MWCO, 3-15ml) (Pierce #66410) were used to remove non-reacted and excess reagents with dialysis stirring in the cold against 2 L of PB. Reactions using SIA (Thermo scientific #22349) crosslinker were conducted similarly; except biotinylated polyGL solutions in PB were recollected in borate buffer (50 mM sodium borate, 5 mM EDTA) (pH, 8.3) using Zeba™ spin desalting columns, to which 10 µl of SIA dissolved in DMSO were added.
**Dot Immunoblot Assay to validate poly-GL biotinylation**

10 µl of biotinylated boron-loaded poly-GL were spotted on a strip of nitrocellulose membrane. The membrane was blocked in 5% Blotting-Grade Blocker (Bio-Rad #170-6404) in TBS (Tris-buffered-saline) for 1 hr shaking at RT, washed several times in TBS containing 0.05% Tween 20 (TBST) and incubated with horseradish peroxidase (HRP) conjugated anti-biotin antibody (Cell Signaling Tech.) at 1:1000 dilution in blocking buffer for 1 hr shaking at RT. After a series of washes with TBST, the bound antibodies were visualized after spraying with chemiluminescent substrate (Luminol Denville Scientific Inc E2400) using the chemiluminescence protocol running on a BioRad GelDoc XR imager.

**Biotin Quantitation Assay**

The FluoReporter® Biotin Quantitation Assay Kit (Invitrogen #F30751) was used to determine the degree of biotin labeling on the poly-GL sequences following manufacturer’s instructions. A standard curve was constructed using supplied biocytin standard (Fig 7). The standard curve was divided into linear fragments. Each linear equation covered a biocytin relative fluorescence range and was used to solve for the amount of biotin (pmol) on the polyGL samples using the relative fluorescence (RF) values from the polyGL reactions that fell within the biocytin RF range. Results are expressed as mean (± standard deviation) number of biotin molecules per peptide sequence(s).

**Spectrophotometric evaluation of ¹⁰B-enriched BSH incorporation**

Fourier Transform Infrared (FTIR) spectroscopy was used to evaluate the success of the BSH-polyGL linkage via incorporated characteristic BSH peaks in the BSH-polyGL reaction spectrum. Spectra of bare poly-GL (Sigma #P7658), ¹⁰B-enriched BSH (Katchem Ltd., Czech
Republic) and boronated biotinylated poly-GL in HPLC H$_2$O were collected at room temperature using an ALPHA platinum ATR FT-IR spectrometer (Bruker). Zeba™ spin desalting columns (Thermo #89892) equilibrated with HPLC H$_2$O were used to prepare boronated poly-GL reactions in water as the solvent for FT-IR analysis.

**Figure 7.** Standard curve for biocytin (biotinylated lysine) used to determine the degree of labeling (DOL) of biotinylated polyGL sequences. Each reaction consisted of a total of 100 µl of 1X PBS, 1X Biotective Green reagent, and biocytin. Following a 5 min incubation period at RT in the dark, fluorescence was measured using SoftMax® Pro on a Molecular Devices M3 microplate reader with excitation/emission maxima of 495/519 nm.

**ICP-MS analysis**

All samples and standards were analyzed for both B isotopes ($^{10}$B and $^{11}$B) on a VG Plasma Quad ExCell ICP-MS in the Department of Earth and Environment at Boston University. The samples were introduced in solution form in 0.1 M NH$_4$OH, through a Meinhard-C concentric nebulizer at a flow rate of ~1 mL/min. Instrumental drift was monitored and corrected for by analyzing a ~5 ng/g natural B standard at various times throughout the run (typically every 5-6 items). A calibration curve was generated by measuring the $^{10}$B enriched BSH standard diluted
to varying concentrations (from 0.5 ng/g to 50 ng/g) interspersed throughout the analytical run, and this curve had a $r^2$ value of 0.9997. BSH concentrations in the samples were determined from the slope and intercept of the calibration curve for $^{10}$B. An analytical blank for B was run concurrently with the samples and was below our detection limit (~0.2 ng/g) and does not contribute to the B concentrations reported.

**Linker attachment to antibodies**

100 ug of antibody were diluted in 100 µl 0.1M sodium phosphate buffer (pH 7.5). While protecting the solution from light, 10 µl 0.1 M sodium periodate was added, mixed and incubated in the dark for 30 min at RT. The reaction was quenched with 500 µl 1x PBS (pH 7.4). Oxidation of the Fc carbohydrate was verified by adding 20 µl antibody solution to 60 µl of freshly prepared 10 mg/ml Purpald (Sigma) dissolved in 1N NaOH. Within 5 minutes, the color of the solution changes to light purple to indicate the presence of aldehydes. 2 µl of a 50mM solution of dithiol-PEG6-hydrazide linker (SensoPath Tech, SPT-0014B) dissolved in 100% ethanol was added to the antibody solution and incubated at RT for 1 hr. 1 ml 40 mM HEPES solution (pH ~8.5) was added to the solution and the reaction concentrated in a Amicon filter (Millipore Biomax 5k NMWL) by centrifugation in a swinging bucket rotor at 4k x g for ~30 min until the volume of retentate was less than 300 µl. The retentate was removed, the concentration of antibody-linker adjusted to 100 µg/ml with 40mM HEPES and then stored at 4°C.

**Conjugation of targeting and delivery antibodies to gold nanoparticles (GNPs)**

Twenty nanometer GNPs were purchased from BBI International (Ted Pella, Redding, CA). A total of 100 µl of antibody-linker (single or combination) were added to 1 ml of GNP stock (1.0 OD at 525nm = 7x10$^{11}$ particles/ml) and incubated for 20 min at RT on a rotator. The binding of
antibody to the surface of GNPs surface was validated by scanning the GNP solution before and after antibody addition at 450-650 nm. Conjugation of antibody will result in a 3-4 nm red-shift (~524-528 nm) in the plasmon peak due to changes in the local refractive index (Fig 8). The remaining exposed GNP surface between antibody-linker was covered by the addition of 100 µl 10⁻⁵ M methoxy poly (ethylene-glycol)-thiol mPEG-SH 5000 (Laysan Bio) and incubating for 20 min at RT on a rotator. Biotinylated peptide was added to the GNPs via conjugated murine antibi-otin monoclonal antibodies (Jackson ImmunoResearch). The peptides were diluted to a concentration of 0.5 µM in 2% (w/v) PEG 6000 in HPLC water, added to the GNP solution and incubated for a further 20 min at RT on the rotator. Conjugated GNPs were recovered by centrifugation at 4k x g for 20-30 min at 4°C. The light pink supernatant was carefully removed with a fine-tip to not disturb the fluid GNP pellet. The GNP pellet was resuspended in 1 ml of 2% (w/v) PEG in 1x PBS and stored at 4°C in a dark box. GNP concentration was measured by UV-VIS scan in NanoDrop® ND-1000 spectrophotometer to verify absence of a peak shift to >600 nm that might indicate GNP aggregation.

Protein determination by BCA assay

A Thermo Scientific Micro BCA Kit (Thermo #23235) was used to determine the concentration of protein samples both in solution (primary antibodies, polyGl) and also conjugated to GNP. Standard curves were prepared using BSA supplied with the kit at 2 mg/ml and the concentrations of IgG were adjusted for extinction coefficient by a factor of 1.09, 1.18 and 1.12 over BSA values obtained for human, mouse, and rabbit IgG samples, respectively, based upon published data by Thermo Scientific.
Figure 8.- UV-Vis spectra of 20 nm gold nanoparticles (GNPs) before and after the addition of human immunoglobulin G (hIgG) to the particle solution. Immediately upon the binding of antibodies to the surface of the gold, the GNPs experience a 4 nm spectral red-shift in their plasmon absorbance that is associated with GNP functionalization.

**Determination of GNP conjugated antibodies by ELISA**

Each well of 96-well, flat-bottom Immunolon 2HB (Nunc) plates were coated with 100 µl of either 400 ng/ml biotinylated BSA (Thermo) or 1µg/ml protein A/G fusion protein (ProSpec) dissolved in filter sterilized (0.2 µm) PBS overnight at 4°C. Plates were then washed 3X with TBST (0.05% Tween-20 in TBS), prior to the addition of 320 µl/well blocking buffer (TBST containing 2% BSA/dried milk) and incubation at RT for 1 hr. The wells were again washed 3X with TBST before the addition of 100 µl/well in triplicate of antibody or GNP dilutions prepared in TBST containing 0.1% BSA (sample diluent) for 1 hr at RT. Plates were again washed 3X with TBST and in most instances blocked with 250-500µg/ml goat IgG (Vector Labs) in sample
diluent and washed 3X before addition of 100 µl/well of the appropriate secondary antibodies at 1:5000 dilution for 1hr at RT. Further washing (3X) preceded addition of 75 µl/well of TMB ELISA substrate (Thermo Turbo TMB #34022). Plates were covered with adhesive film and shaken gently until the color develops. The reactions were stopped with 100 µl/well 2M sulfuric acid and read at 450 nm in a Molecular Devices SpectraMax 3M plate reader.

Dynamic light scattering (DLS) and Zeta Potential measurements

GNP samples in 2% PEG PBS were pelleted at 4k x g for 20 min at 4°C and resuspended in 1.5ml HPLC H₂O for DLS and zeta potential measurements. Each GNP suspension was diluted 2-fold in HPLC H₂O, filtered through a 0.4-µm cellulose acetate membrane and sonicated at 10-15 pulses prior to the collection of each measurement. The radius of each conjugate was measured using a Malvern Zetasizer Nano-ZS with the gold.sop program and the zeta charge with the gold-zeta program saved on the Zetasizer 6.0.1 software.

BNCT Dosimetry calibrations

Neutron fluxes (2200 m/s) were characterized at the Massachusetts Institute of Technology Nuclear Reactor Lab (MIT NRL) using 24, 48 and 96 well plates to determine thermal neutron and boron dose rates received by each plate. Pieces of bare gold foil weighed to within 1% accuracy (2.8-4.0 mg) were placed into designated wells with 400, 200 and 100ul of RPMI-1640 in each of the 24, 48 and 96 well plates, respectively (Fig 9). Each plate with gold foils was irradiated for 5 minutes at a reactor power of 5.8 MW. The gold reaction is Au-197(n,γ)Au-198 and the activity of the foils is determined with high-purity germanium detectors (HPGE) (Rigues et al., 1994). The detectors are energy and efficiency (at 411 keV as Au-198 emits 411 keV
photons) calibrated and used to determine the decay gamma energy of Au-198 (Rogus et al., 1994). The 2200 m/s neutron flux was then calculated using the following equation:

$$\phi_{2200} = \frac{\text{MW}}{A_v \sigma_{2200}} \left[ \left( \frac{A_{\text{sat}}}{m} \right)_{\text{bare}} - F_{\text{Cd}} \left( \frac{A_{\text{sat}}}{m} \right)_{\text{Cd}} \right]$$

where $\phi_{2200}$ = the 2200 m/s neutron flux averaged over the foil surface (n/cm$^2$s), MW= molecular weight (g/mol), $A_v$= Avogadros number (atoms/mol), $\sigma_{2200}$ = 2200 cm/s absorption cross section, $m$ = mass of foil (g), and where:

$$A_{\text{sat}} = \frac{\lambda C}{\varepsilon(1 - e^{-\lambda t_0})(e^{-\lambda t_1} - e^{-\lambda t_2})}$$

where $\lambda$= decay constant (s$^{-1}$), $t_0$= start of irradiation time (s), $C$= net number of counts (with subtracted background) established with a radiation detector between times $t_1$ and $t_2$, and where $\varepsilon$= overall counting efficiency (Rogus et al., 1994). The 2200 m/s neutron flux is calculated using the cadmium (Cd) difference technique, hence the $A_{\text{sat}}/m$ ratio for Cd-covered foils, which in this experiment was set to equal zero as only bare gold foils were used to for this calibration (Rogus et al., 1994). The 2200 neutron flux values obtained from each well were then used to calculate absorbed thermal neutron and boron dose rates received by the gold foils in each of the wells (cGy/min). The two most crucial ways in which thermal neutrons interact with tissue are via $^{14}\text{N}(n,p)^{14}\text{C}$ and $^1\text{H}(n,\gamma)^2\text{H}$ reactions (Rogus et al., 1994). For the purposes of this study, the $^{14}\text{N}$ dose from thermal neutrons was determined using the kerma factor for $^{14}\text{N}$, which was calculated using the equation:

$$F_n = 1.602E-8 \sigma N_i m^{-1} E_{tr}$$

where $\sigma$= interaction cross section (cm$^2$), $N_i$= number of target atoms in the sample, $m$= mass of the sample (g), and $E_{tr}$= total kinetic energy (MeV) given to charged particles per interaction.
(Rogus et al., 1994). The kerma factor was then multiplied by the 2200 m/s neutron flux determined using gold foils to yield N-14 dose. Similarly, the B-10 dose was calculated using the B-10 kerma factor (Rogus et al., 1994).

Figure 9.- Dosimetry calibrations using weighed gold foils and cell culture medium in 24, 48 and 96 well plates. Each plate was exposed to a 5 minute long 5.8 MW irradiation at the Massachusetts Institute of Technology Nuclear Reactor Laboratory (MIT NRL).

**Determination of BSH toxicity by the alamarBlue assay**

100 µl of the cell suspension (~5,000 cells/ml in RPMI-1640) was added to each well of a 96-well microplate. A serial dilution of 1 mM BSH was added in 10 µl aliquots in duplicate. As a positive control, 10 µl of a serial dilution of the pro-apoptotic agent Etoposide (VP-16 Sigma #P4405) were added to cells in duplicate. The assay scheme also included untreated controls, a negative control of media without cells and a positive control of alamarBlue™ Reagent (Thermo #88951). After incubation at 37°C in a 5% CO2 atmosphere for 1hr, 10 µl Alamar Blue were added to each well with the exception of the positive control wells to which 10 µl of HPLC H2O were added. After an overnight incubation period, the change of color of the solution was measured at A570nm and A600nm with the Molecular Devices M3 plate reader running SoftMax Pro. The percentage reduction of AlamarBlue™ Reagent was calculated according to the formula provided by the manufacturer (Thermo Scientific #88951).
RESULTS

The functionalization of gold nanoparticles for the selective delivery of a sufficient concentration of $^{10}$B enriched BSH molecules to pancreatic tumor cells for successful BNCT is a multistep process that requires exhaustive validation and quantitation following each step of the protocol. Firstly, polyGL peptide sequences - the boron carriers in this study - were biotinylated and boronated. The biotinylation of these sequences was confirmed and the amount of biotin molecules per polyGL was quantified. The success of boron linkage was assessed both spectrophotometrically by FT-IR and quantitatively by ICP-MS. Successfully biotinylated and confirmed boron-carrying polyGL sequences were loaded onto the surface of functionalized GNPs by interaction with antibiotin antibodies. The effectiveness of the biotin-antibiotin reaction based loading strategy was assessed via the observation of a change in size and surface charge of the GNP complexes before and after functionalization and loading. Additionally, preliminary gold surface saturation analyses were conducted to determine the concentrations of glycoprotein A33 targeting antibody (mAb31.1) and thiol methoxy-PEG needed to sufficiently coat the surface and yield stable GNPs that would not aggregate in physiological media. Thusly, we were able to synthesize multifunctional boron-delivering GNP constructs for BNCT experiments.

Before using the GNP constructs for the delivery and localization of boron to CAPAN-2 pancreatic adenocarcinoma cells and commencing irradiation experiments at the MIT NRL, it was necessary to perform dosimetry calibrations, since the reactor facility has not been operated for BNCT since the year of 2000. These dosimetry measurements were used to calibrate the beam intensity and calculate the neutron and boron doses received by various multi-well plate configurations that we anticipated might be used for the proof-of-principle experiments in this study. In addition, we conducted cell viability assays that allowed us to test cell survival under sub-optimal conditions (namely without CO$_2$ and temperature fluctuations ranging between 37°C
and RT) resembling the environment they were exposed to in the process of transporting cells to MIT from Wellesley as well as during irradiation and over the course of post-irradiation analyses. Altogether, the results presented below establish a foundational framework for pursuing GNP platform-based BNCT experiments to achieve maximum cytotoxicity.

**Biotin validation and quantitation**

Biotin molecules were added to polyGL peptide sequences under defined experimental conditions (pH 6.5, 24 hr reaction at 4°C, and a biotin:peptide molar ratio of 5:1) for selective labeling of the N-terminal alpha-amino group of each sequence to leave the maximum number of primary amino groups on the side chains of lysine residues accessible for BSH cross-linkage (Sélo et al., 1996). A nitrocellulose membrane dot immunoblot assay was used to confirm the presence of biotin molecules on the polymers. The sample was probed for biotin using horse-radish peroxidase (HRP) conjugated antibiotin antibodies. The intense signal detected from the biotinylated polyGL sample on the dot blot indicates successful biotinylation of the polymers (Fig 10).

However, a quantitative analysis was necessary to determine whether polyGL sequences were in fact preferentially labeled with a single biotin molecule at the N-terminal alpha-amino group. To this end, the FluoReporter® Biotin Quantitation Assay Kit, a highly sensitive fluorometric assay that provides an accurate quantitation of biotin molecules on protein, was used. It does so, briefly, based upon the displacement of ligands tagged with quencher dye from biotin binding sites of Biotective™ Green reagent, which also consists of avidin labeled with fluorescent dye, the signal from which is quenched by the ligand through fluorescence resonance energy transfer (FRET). This assay suggested that the labeling of polyGL polymers occurred at a ratio of approximately 0.28 ± 0.07 biotin molecules (pmol) per polyGL sequence (pmol).
Figure 10. Dot immunoblot of conjugated 20 nm GNP samples probed with HRP-conjugated antibiotin antibody. 5 or 10µl of each sample was spotted onto a nitrocellulose membrane. The dots contain the following complexes: (1) mPEG coated GNPs (2) biotinylated boronated polyGL (3) & (4) 5 & 10 µl of GNPs conjugated with monoclonal antibiotin antibodies (5)&(6) 5&10 µl of antibiotin antibody conjugated GNPs with biotinylated boron-loaded polyGL (7) 5 µl biotinylated protein standard.

Spectrophotometric assessment of boron-polyGL linkage and quantitation of boron molecules using Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Upon confirming biotinylation, the polyGL sequences were loaded with $^{10}$B enriched BSH molecules using one of two different heterobifunctional crosslinkers, Sulfo-MBS and SIA (see Materials and Methods) (There were no analyses conducted to confirm that the biotin labeling did in fact occur at the N-terminal alpha-amino groups on the peptide sequences). These linkers react with primary amines from one end to form amide bonds with the peptides and with sulphydryl groups from the opposite end to form stable thioether bonds with the BSH molecules (Fig 4 & 5). IR spectroscopy was used to confirm the attachment of BSH to the polyGL sequences. The spectra of solutions of BSH (0.2 mg/ml), unreacted polyGL sequences, biotinylated polyGL with each of the linker molecules and
Figure 11.- FT-IR spectra of polyGL, $^{10}$B enriched sodium mercaptododecaborate (BSH) (1 mM), biotinylated polyGL with each of the heterobifunctional crosslinkers used to link BSH to the polymers, and biotinylated polyGL with BSH linked via the crosslinkers. (A) using Sulfo-MBS and (B) using SIA as the crosslinker between the polyGL sequences and BSH. Arrows labeled A indicate thiol (S-H) group peaks (2500 cm$^{-1}$). Arrows labeled B indicate overlapping spectral regions between BSH and the biotinylated boron-carrying peptides. Samples were all in HPLC H$_2$O.
biotinylated boron-carrying polyGL were collected to show BSH incorporation. As illustrated in Figure 11, the peak observed in BSH spectra (highlighted in red) that is characteristic of the thiol (-SH) group (~2500 cm⁻¹) is not present in the spectra of boronated polyGL, indicating the successful coupling of BSH to the polyGL via crosslinker reactions. Overlapping spectral regions within the frequency range of 700-1700 cm⁻¹ when comparing BSH and boronated polyGL spectra also suggest BSH incorporation. To distinguish peaks potentially indicative of BSH linkage to polyGL from vibrations specific to linker-polyGL complexes, we superimposed the spectra from biotinylated polyGL sequences with crosslinkers attached before the addition of BSH (highlighted in green) with biotinylated boronated peptides (Fig 11). As shown in Figure 11, the polyGL-linker spectra differ from bare polyGL spectra by small vibrations in the 2000-2500 cm⁻¹ frequency range, which suggests that observed peaks attributed to boron linkage in the boronated complexes are in fact unique to BSH coupling.

The concentration of ¹⁰B enriched BSH attached to polyGL sequences was quantified in parts per million (ppm) and parts per billion (ppb) using inductively coupled plasma mass spectroscopy (ICP-MS) at the Department of Earth and Environment at Boston University (Fig. 12). A calibration curve was contructed using BSH standard (0.2 mg/ml) with a >99.95% enrichment factor for the ¹⁰B isotope. A boron-11 (¹¹B) count was also generated from a natural boron standard at the BU facility and used to quantitify ¹¹B concentrations in the BSH standard. Biotinylated peptides linked to BSH with either of the two crosslinkers, SIA or Sulfo-MBS, in HPLC H₂O and biotinylated polyGL sequences linked to BSH via Sulfo-MBS in phosphate buffer were subjected to ICP-MS analysis. Based on the BSH concentrations obtained using ICP-MS, Sulfo-MBS appears to be a more effective crosslinker than SIA as a greater concentration of BSH is detected on polyGL sequences to which BSH was linked via Sulfo-MBS. Additionally,
BSH molecules are present at higher concentrations on peptides in phosphate buffer than they are on those in HPLC H$_2$O suggesting decreased stability of polyGL-BSH linkages in water.

\[
y = 0.000127x + 0.195119 \\
R^2 = 0.999686
\]

**Figure 12.-** Determination of the concentrations of $^{10}$B enriched BSH linked to polyGL sequences using inductively coupled plasma mass spectrometry (ICP-MS). A calibration curve was created using 0.2 mg/ml BSH standard (>99.95% $^{10}$B enrichment). The table shows various dilutions and concentrations of BSH attached to biotinylated polyGL sequences using one of two cross linker, SIA or Sulfo-MBS (SMBS) (Courtesy of Thomas Ireland, BU Dept. of Earth and Environment).
**GNP functionalization/polyGL loading**

Successful GNP functionalization was assayed and quantitated in two ways: by total protein determination using the BCA assay, and specific antibody loading by ELISA.

Based on the published number of particles/ml ($7 \times 10^{11} / \text{ml}$) for these GNPs (Ted Pella, BBI data), the number of IgG molecules with MW of 150kD per 20nm particle was calculated. These values varied between 20-67 IgG/GNP depending on the preparation and antibody preparation that was conjugated to the GNP. These values are within the range of the 48 IgG molecules conjugated to BBI 20nm GNP as published by Ted Pella (http://www.tedpella.com/gold_html/gold-tec.htm).

GNPs conjugated with both anti-biotin (mouse) and anti-A33 (human, mouse, or rabbit) antibodies were evaluated by ELISA assay after either biotinylated BSA or protein A/G capture to 96-well plates validated the attachment of both antibodies (Fig 13 & 14).

**Figure 13.-** ELISA following biotinylated BSA capture of GNP conjugated with mouse antibiotin and mouse mAb As33 with and without quenching with goat IgG.
PolyGL sequences that were validated for both biotinylation and $^{10}$B enriched BSH linkage were loaded onto the surface of antibiotin conjugated GNPs via interaction with the antibiotin antibodies. Changes in the hydrodynamic diameter and the overall surface charge of the GNP constructs were measured as evidence for the success of the antibiotin-biotin interaction based loading strategy. Table 1 shows the mean diameters of each construct as determined by dynamic light scattering (DLS) and the mean zeta potential values of the same constructs. An increase in the expected size of the non-functionalized GNPs (~20 nm) was observed with each conjugation. Of all the measured GNP conjugates, the final construct, to which boronated peptides were attached, had the largest mean hydrodynamic diameter (39.35 nm). Likewise, the zeta potential of the GNPs increases or approaches neutrality with functionalization, yielding an overall charge of -9.4 mV after the addition of polyGL-BSH complexes to their surfaces. Both
increases in diameter size and zeta potential measurements suggest surface functionalization of the GNPs by antibiotin antibodies coupled with biotinylated boron-carrying peptides.

Table 1. Hydrodynamic diameter and zeta potential measurements of GNP conjugates.

<table>
<thead>
<tr>
<th></th>
<th>Diameter (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNP</td>
<td>21.16 ± 6</td>
<td>-45.4 ± 13</td>
</tr>
<tr>
<td>GNP-mPEG</td>
<td>33.72 ± 17</td>
<td>-24.6 ± 17</td>
</tr>
<tr>
<td>GNP-antibiotin</td>
<td>32.39 ± 19</td>
<td>-15.2 ± 10</td>
</tr>
<tr>
<td>GNP-antibiotin-PolyGL-BSH</td>
<td>39.35 ± 21</td>
<td>-9.4 ± 5</td>
</tr>
</tbody>
</table>

PDI values: GNP = 0.090, GNP-mPEG = 0.166, GNP-antibiotin = 0.209, GNP-antibiotin-PolyGL-BSH = 0.216

In addition to size and charge characterization, another assessment of successful polyGL-BSH loading onto GNP surfaces involved spotting the conjugated GNP solutions onto a nitrocellulose membrane and probing for biotin molecules using horse-radish peroxidase (HRP) conjugated antibiotin antibodies (Fig. 7). Presumably, a signal from biotin molecules attached to the polyGL sequences and recognized by the antibiotin antibodies conjugated to the surface of GNPs should be detected using this assay. However, as seen in Figure 7, a signal was not detected from these samples (dots 5 & 6). The lack of a biotin detection signal can be attributed to either an insufficient concentration of antibiotin antibodies on the surface of the particles or that the biotin molecules are shielded from the HRP-antibiotin antibodies by the conformation of the antibody binding of the GNP-conjugated antibiotin molecules.

Preliminary analyses for BNCT experiments at MIT NRL

In the process of transporting cells from Wellesley College to the MIT NRL and over the course of irradiation and post-irradiation analyses at MIT, cells are housed at temperatures ranging from 22°C to 37°C without CO₂. To determine the effect of these sub-optimal conditions on the cells, we performed a cell viability assay wherein CAPAN-2 cells were incubated at room
temperature without CO₂ for a 48-hour period (Fig 15). These cells were scored at several time points using a Moxi™ Z automated cell counter, which reports cell concentration, average diameter of the cell population in µm and moxi population index values (MPI). The MPI is a quantitation of culture health that is determined by the ratio of counted cells to the entire particle population (i.e. including small particles, debris and contaminants which increase in number as a result of cell shrinkage and/or apoptotic cell death) (ORFLO Technologies). CAPAN-2 cells

![Figure 15](image)

**Figure 15.- Cell viability assay using a Moxi™ Z automated cell counter.**

CAPAN-2 cells were cultured on multi-well plates at room temperature (RT) with no CO₂ for the duration of 48 hrs with and without sealing the culture plates with parafilm. (A) Cells scored at time point zero (B) cells scored after 24 hrs (C) Cells scored after 48 hrs (D) Cells treated with 200 µM etoposide (VP-16). The table shows average Moxi generated values from cell populations scored at three different time points over 48 hrs as well as controls cells scored at time point zero and 3 hrs after VP-16 treatment. T: time point; MPI: Moxi Population Index.

<table>
<thead>
<tr>
<th>Time</th>
<th>Cell concentration (cell/ml)</th>
<th>Average size (µm)</th>
<th>MPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>2.24x10⁶</td>
<td>14.27</td>
<td>0.94</td>
</tr>
<tr>
<td>T1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parafilm</td>
<td>1.39x10⁵</td>
<td>14.79 ± 0.06</td>
<td>0.79 ± 0.03</td>
</tr>
<tr>
<td>w/o parafilm</td>
<td>3.25x10⁵</td>
<td>14.70 ± 0.2</td>
<td>0.64 ± 0.1</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parafilm</td>
<td>1.86x10⁵</td>
<td>15.70 ± 0.4</td>
<td>0.81 ± 0.1</td>
</tr>
<tr>
<td>w/o parafilm</td>
<td>1.67x10⁵</td>
<td>14.70 ± 0.3</td>
<td>0.63 ± 0.1</td>
</tr>
<tr>
<td>T3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parafilm</td>
<td>1.28x10⁶</td>
<td>15.59 ± 0.2</td>
<td>0.69 ± 0.1</td>
</tr>
<tr>
<td>w/o parafilm</td>
<td>7.92x10⁵</td>
<td>15.00 ± 0.9</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>VP-16 treated</td>
<td>4.91x10⁶</td>
<td>17.50</td>
<td>0.49</td>
</tr>
</tbody>
</table>
scored at time point zero, immediately after trypsinization and suspension in cell culture media after an overnight incubation at 37°C and 5% CO₂, are presented as the healthiest population. Moxi readings from the same cells treated with 200 µM of etoposide (VP-16) and incubated at RT for 3 hours are presented as the most unviable cell population. It appears that culturing CAPAN-2 cells under non-favorable conditions with respect to CO₂ and temperature does not significantly impact cell viability when culture plates are sealed with parafilm (Fig 15). Even with unsealed cultures, only after an entire 48 hours do they exhibit signs of cell death as indicated by the low mean MPI value (see T3).

To ensure that cell kill from irradiation experiments results primarily from the fission of ¹⁰B atoms that are selectively delivered and localized in the cells via our multifunctional nanovehicles, CAPAN-2 cell viability was assessed after incubation with ¹⁰BSH-treated tissue culture media using the alamarBlue™ cell viability assay (Fig 16). It is a sensitive and reliable test that is based on a change in fluorescence/color of the alamarBlue reagent, a blue colored non-fluorescent oxidized indicator that experiences a color change to red and becomes fluorescent when incubated in the reducing environment of viable cells (Thermo scientific #88951). As a positive control, CAPAN-2 cells were incubated with cytotoxic apoptosis-promoting agent, etoposide (VP-16). AlamarBlue reagent was added to the cells after they were incubated with the chemical compounds for 1 hour at 37°C and 5% CO₂. Changes in the color/fluorescence of the reagent were detected with absorbance measurements at 570 nm and 600 nm after 72 hours. Absorbance readings were used to calculate the percent reduction of alamarBlue reagent. As shown in Figure 16 (B), ¹⁰BSH molecules do not seem to have a toxic effect on cells when present in the media unlike the positive control samples to which VP-16 was added that yielded as low as a 10% reduction of the reagent. These data suggest that the majority
of cell death that would be detected after thermal neutron irradiation can largely be attributed to the targeted delivery and internalization of $^{10}$BSH via GNP carriers.

Finally, preliminary dosimetry calibrations were conducted at the MIT NRL to characterize the neutron flux using the decay gamma energy of pre-weighed gold foils that were distributed in 24, 48 and 96 well plates. The exact distribution of the gold foils was predetermined from previous dose calibrations and BNCT experiments at the MIT NRL from which they were able to trace the way in which the flux varies from the center to the edge of a 96 well plate. Characterizing the intensity of the beam is essential to constructing a map of the dosage received by specific wells on each of the multi-well plates, which is precisely what is illustrated in Figure 17. The rates at which thermal neutron and boron doses were received by the designated wells was calculated as previously described in Material and Methods. These values will dictate the format of the multi-well plate used for irradiation experiments as well as the

Figure 16.- Determination of $^{10}$B enriched BSH toxicity using alamarBlue™ Cell Viability Reagent. CAPAN-2 cells in media were exposed to various concentrations of (A) Etoposide (VP-16) and (B) BSH, for 1 hr at 37°C and 5% CO$_2$. AlamarBlue Reagent was added to treated cells, which were incubated at 37°C 5% CO$_2$ for 72 hrs, and then measured at $A_{570}$ and $A_{660}$ using SoftMax® Pro on a Molecular Devices M3 microplate reader to calculate % reduction of the reagent as a function of cell viability.
locations and distribution of the experimental samples and controls on that particular plate configuration.

**Figure 17.- Absorbed thermal neutron and boron dose rates in 24, 48 and 96 well plates.** The thermal neutron (blue) and boron (red) dose rates were calculated using 2200 m/s neutron flux values that were experimentally characterized using gold foils (2.8–4.0mg) in cell culture medium weighed to 1% accuracy and positioned in designated wells across each plate. Each plate was irradiated with a reactor power of 5.8 MW for 5 minutes at the Massachusetts Institute of Technology Nuclear Reactor Lab (MIT NRL).
**DISCUSSION**

By and large, the promising potential of BNCT based cancer treatments has yet to be fully realized because of the challenges faced with preferentially or selectively concentrating therapeutic doses of $^{10}$B-containting agents in target tumor tissue (Kueffer et al., 2013). In this study, we attempt to overcome this longstanding limitation by establishing a framework for the synthesis and design of immunotargeted GNP based $^{10}$B delivery systems for neutron capture therapy experiments (Fig 3).

To grant GNPs the necessary targeting capacity, their surfaces were coated with targeting monoclonal or polyclonal antibodies directed against GI tract tumor-associated cell surface antigen gpA33 that is uniquely upregulated in our pancreatic adenocarcinoma cell line, CAPAN-2. Therapeutic delivering antibodies, in this instance monoclonal antibiotin antibodies, were also linked to the surface of GNPs. These antibiotin antibodies are considered the delivering moieties in this system because $^{10}$B enriched sodium mercaptododecaborate (BSH) molecules were loaded onto the particles via the interaction between biotinylated synthetic peptide polymers (polyGL) carrying BSH molecules with antibiotin antibodies.

A series of confirmatory analyses were conducted to validate and quantify antibody conjugation, biotinylation of the boron-carrying peptides and covalent coupling of BSH molecules to polyGL sequences. In addition, we performed a dosimetry calibration and cell viability assays to prepare for neutron capture experiments at the MIT NRL. Despite evidence of the successful construction of this delivery system and of the sensitivity of the proposed and tested post-irradiation cell death detection assays, further optimization and confirmation is required to carry out *in vitro* BNCT experiments using these multifunctional nanovehicles for proof-of-principal before pre-clinical testing in an *in vivo* model.
Biotinylation

Biotinylation of the polyGL sequences was initially confirmed by an intense signal detected by HRP-conjugated antibiotin antibodies on a nitrocellulose membrane in a dot immunoblot assay (Fig 10). Further, the precise degree of biotin labeling was also determined and shown to take place at a ratio of $0.28 \pm 0.07 \text{ pmol of biotin molecules per pmol of polyGL}$. Although the biotinylation protocol adopted in this study is based on creating reaction conditions that allow for the selective labeling of N-terminal $\alpha$-amino groups of short peptides, the quantitation suggests that labeling of the polyGL sequences occurred at a lower than expected rate (Sélo et al., 1996). The rational for this approach was to limit and direct biotinylation such that the free amines on lysine residues in the polyGL were available for BSH reactivity. The calculations used to derive the concentration of the polyGL solution prior to biotinylation were based on an assumed molecular weight (35kD) in the middle of the polyGL molecular weight range (20-50kD) supplied by the vendor (Sigma P7658). Moreover, all subsequent calculations pertaining to the biotin quantitation assay were also based on the same molecular weight assumption, including the polyGL picomole values used to determine the degree of biotin labeling. In order to make more accurate quantitations, it would be worthwhile to determine which end of the MW range the majority of the peptide sequences lie by size separation techniques such as gel electrophoresis$^1$. Alternatively, using size exclusion chromatography would allow for the selection of peptides of a known MW, from which peptide concentrations can be more precisely calculated.

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$^1$ However, prior electrophoretic analysis of the same polyGL product in the Webb lab suggested the 25kD form of this material is the most prominent.
Boronation

Initially, boron loading can be verified using spectrophotometric analyses such as UV-visible and FT-IR spectroscopy (Mandal et al., 2011; Ananthanarayanan, 2008). These provide a general indication of the association of $^{10}$B enriched compounds with loading platforms especially and more effectively when compared to the absorption spectra or vibration frequencies of those particular platforms prior to boron loading. As confirmed via FT-IR analysis, we were able to successfully link $^{10}$B enriched BSH to polyGL sequences and further verify this finding by superimposing the vibrational frequencies from BSH stock solutions and polyGL sequences both prior to and following the addition of heterobifunctional linkers (Fig 11). Mandal et al. (2011) also used UV-visible spectroscopy for the same verification using $^{10}$B enriched 4-Borono-L-phenylalanine (BPA) as their $^{10}$boron-containing agent. They linked BPA to polyelectrolytes and added the boronated polyelectrolytes in a layer-by-layer fashion to the surface of GNPs. By observing the characteristic ~268nm peak from $^{10}$BPA solution in the spectra of functionalized GNPs, they were able to verify BPA loading onto the surfaces of the particles (Mandal et al., 2011).

However, in addition to relatively crude and easily obtained spectrophotometric confirmations, more definitive and quantitative analyses are necessary to be able to determine the concentration or amount of loaded $^{10}$B atoms. To this end, our boronated peptide sequences were subjected to ICP-MS analysis at the BU Department of Earth and Sciences, from which we were able to obtain numerical evidence for successful BSH linkage (Fig 12). Moreover, through ICP-MS analysis, we detected the presence of relatively low (ppb) concentrations of $^{10}$B on boronated polyGL sequences in HPLC H$_2$O. Based on FT-IR readings, one interpretation is that the BSH molecules were dissociated from the peptide sequences as a result of being stored in water for an extended period of time. The spectra from these boronated peptides in water appeared identical
to spectra collected from polyGL sequences to which only SIA or SMBS linkers were attached without BSH, hence the conclusion made about the stability of the linkage in HPLC H$_2$O.

According to Kriz and Cadova (2000), BSH is in fact highly stable in water solution as indicated by negligible levels of its oxidation products when stored in HPLC grade water at -22°C for 177 days. Despite the stability of the compounds themselves in water, it is still very likely that the stability of the thioether bonds, created between the crosslinkers used in this study, SIA or SMBS, and the BSH molecules decrease in stability when stored in water and not in their respective conjugation buffers.

Furthermore, ICP-MS analysis revealed that more $^{10}$BSH compounds were loaded onto longer polyGL sequences with a MW range of 75-125kD and a 1:4 ratio of glutamate to lysine supplied by the vendor (Sigma P8619). More specifically, the concentration of $^{10}$B attached to the longer peptides was approximately 2.5X greater (7.13 ppm) than the concentration detected on the shorter sequences (2.99 ppm) (Fig 12). However, a variety of issues were encountered using the longer polyGL sequences as boron-carrying agents including solubility in conjugation buffer and the adherence of presumably exposed polyGL lysine residues to the sides of eppendorf tubes when loading the boronated sequences onto the GNPs.

Ideally, however, to accumulate even higher concentrations of boron-10 containing capture agents in target cells, it would be best to, instead of using delivering antibodies as intermediates, link boron-loaded polyGL sequences directly onto the surface of GNPs. This can be accomplished via a heterobifunctional linker (NHS-SH) with a reactive primary amine $N$-hydroxysuccinimide ester (NHS) that would covalently bind primary amino groups on the side chain of polyGL lysine residues and a thiol (SH) group that would attach to the gold surface with high affinity near-covalent gold-thiol bonds (Kumar et al., 2008). To avoid cross reactions between the linker thiol groups and the BSH sulfhydryls, employing this loading strategy would
require attaching the linkers to surface of the gold first, after having added a sufficient amount of targeting antibodies to the surface, and performing the polyGL-BSH linkage reactions separately, which would then be added to the linker and antibody coated GNPs. We are presently having this NHS-PEG-SH linker synthesized by SensoPath Technologies, Inc., the supplier of the dithiol-PEG-hydrazine linker used for orientated antibody conjugation to our GNP.

In one very recent in vivo application of BNCT, researchers were able to accumulate $^{10}$B atoms in target mouse mammary adenocarcinoma solid tumor cells (EMT6), inoculated into female BALB/c mice, at concentrations (67 µg/g) much greater than the suggested optimal therapeutic dose ($\geq$ 20 µg/g) (Kueffer et al., 2013). They were able to localize these amounts using unilamellar liposomes as boron delivery constructs, relying on their passive accumulation by the leaky neo-vasculature of tumors for specific uptake, and $^{10}$B-enriched polyhedral boranes $[\text{closo-B}_{10}H_{10}]^{2-}$ and $[\text{closo-B}_{12}H_{12}]^{2-}$ as boron-containing capture agents, which were encapsulated in the aqueous core of the liposomes.

Two of the major drawbacks of using this delivery system are 1) the inability to immunodirect liposomes, a feature that would otherwise significantly enhance their targeting capacity and specific neoplastic cell uptake, and 2) the inherently limited BNCT agent loading capacity of the liposome structures, as only a small portion of compounds become encapsulated. However, they were able to address and overcome the latter issue, and also slightly improve specific targeting, by incorporating a stable lipophilic agent K[$\text{nido-7-CH}_3(\text{CH}_2)_{15-7,8-\text{C}_2\text{B}_9\text{H}_{11}}$] into the liposome vesicle bilayer. Including this amphiphilic nido-carborane in the bilayer, selective buildup of boron concentrations in tumors was achieved either using the liposomes alone or in tandem with boron salts encapsulated in liposomes (Kueffer et al., 2013). In addition, cells are able to internalize liposomes more efficiently as the compounds impart a negative
charge to the liposome structure, which is a feature that has been shown to maximize the rate of clathrin-mediated endocytosis of the boron-carrying liposomes (Kueffer et al., 2013).

**GNP functionalization/polyGL loading**

Quantifying the number of $^{10}\text{B}$ enriched compounds successfully loaded onto the surface of GNPs via polyGL sequences is crucial to being able to ultimately determine the amount of $^{10}\text{B}$ atoms per gram of tumor tissue. Moreover, this quantitation can also be used to further assess and compare the efficiency of boron loading techniques, either using antibodies as intermediate delivering moieties or via direct linkage of boron-carrying polyGL sequences using an NHS-PEG-SH linker. This numerical data can be obtained by ICP-MS analysis of GNP solutions after conjugation with $^{10}\text{BSH}$-linked polyGL sequences. Another significant quantitation to consider for future studies is the number of particles associated with and/or internalized by each cell. We are currently performing pull-down assays in effort to make relatively accurate estimations of the amount of particles per cell.

In terms of validating the attachment of biotinylated polyGL-BSH linkages to antibiotin antibody coated GNPs, a more reliable technique needs to be adopted, as the use of antibiotin antibodies for the detection of biotin molecules on polyGL sequences proved ineffective (Fig 7). As previously mentioned, it is highly likely that the biotin molecules were made inaccessible to the HRP-conjugated detection antibodies used in the dot immunoblot assay by the antigen-binding site of the antibiotin antibodies on the GNP surfaces. For this reason, we are currently having antibodies synthesized that are targeted against the polyGL sequences themselves. These will allow for a more sensitive detection of boronated biotinylated polyGL association with GNPs in assays such as a dot immunoblot and ELISA. Furthermore, these antibodies will assist in determining the ratios needed to functionalize the surface of GNPs in a manner such that their
surfaces are coated with the maximum possible number of delivering moieties to localize as many boron atoms as possible in target cells and the least number of targeting antibodies.

On the other hand, dynamic light scattering (DLS) and zeta-potential analyses gave an indication of successful GNP functionalization by the binding of antibiotin antibodies, methoxyPEG linkers and biotinylated boronated polyGL sequences to GNP surfaces. PDI values listed suggest that GNP constructs did not aggregate during and after synthesis and functionalization, as they are measures of particle monodispersity (Mandal et al., 2011). As anticipated, the hydrodynamic diameter (nm) and zeta-potential (mV) measurements of GNPs before functionalization were 21.16 ± 6 and -45.4 ± 13 respectively. The surface charge decreased with the addition of mPEG and even more so after linking antibiotin antibodies to the surface (Table 1). The antibodies are approximately 10 nm in diameter and the observed diameter increase following their conjugation with GNPs was about 11 nm. The diameter of GNPs coated with mPEG alone increased by about 12 nm not unexpectedly considering the hydrophilic nature of mPEG molecules which, as a result, causes them to project outward in solution rather than orient themselves in a folded confirmation. More importantly, however, for the purpose of this investigation, we observed that, upon polyGL-BSH binding via interaction with antibiotin antibodies, the particle diameter and zeta-potential values increased to 39.35 ± 21 and -9.4 ± 5, respectively.

Taken together, these data suggest nearly optimal cell uptake of GNPs as previous studies have shown that tissue penetration reaches the maximum as the size of the particles approaches a 40-50 nm range (Chithrani et al., 2006; Chen et al., 2009). In terms of surface charge, a slightly negative, close to neutral, charge is thought to be ideal because a degree of repulsion from the negative cell membrane potential is maintained to ensure that specificity with respect to targeting is for the most part due to antigen recognition and binding. Alterations in surface charge after
exposure of GNP conjugates to biological macromolecules in tissue culture media should be explored in future studies, as a significant change in the overall charge may impact penetration/targeting efficiency.

**Future Irradiation experiments**

For the initial round of BNCT experiments, we propose the use of a 96-well plate out of the various multi-well plate configurations. Originally, 24 and 48-well culture plates were considered in order to recover a sizeable cell pellet following trypsinization post-irradiation for Moxi analysis. However, using the alamarBlue cell viability assay, which proved to be a reliable indicator of cell death, would not require the detachment of the cells to cytotoxicity (Fig 16). The other major advantage of alamarBlue vs Moxi viability analysis following irradiation, is that the non-toxic nature of the dye means that the temporal scale of cell death can be monitored on the same plate over a period of days if necessary. Although, to better mimic the conditions at the reactor site, the alamarBlue assay should be repeated in the absence of CO$_2$, which, as indicated by the Moxi data collected over a period of 48 hours (see Fig 15), should not significantly impact cell viability.

In addition, based upon the dosimetry calibrations and absorbed neutron and boron dose rate calculations, we would load samples in an 8x8 block in the center of a 96-well plate avoiding the first and last couple of rows that would likely receive very low dose rates due to the structure of the plate holder apparatus and circular nature of the beam (Fig 17). A set of five experimental samples and controls would be subjected to a neutron flux within a 4-7 x10$^9$ cm$^2$ s$^{-1}$ range and would include CAPAN-2 cells that are 1) untreated to assess the direct effect of exposure to thermal neutron flux on cell viability 2) exposed to untargeted GNP-polyGL-BSH constructs in the media to evaluate the targeting capacity of the construct and the impact of
irradiated BSH in media on the viability of the cells, and 3) treated with targeted boron-delivering GNPs in the media to determine the efficacy of our delivery system. As controls, BxPC-3 cells in media and BxPC-3 cells in media treated with immune-targeted boron loaded GNP constructs should also be included in the experiment. We would expect these BxPc-3 controls to yield similar results in terms of viability to the untreated CAPAN-2 cells and the CAPAN-2 cell in media with non-targeted boron-loaded GNPs since BxPc-3 cells do not express the target antigen.
REFERENCES


