Bombesin-functionalized water-soluble gold nanoparticles for targeting prostate cancer

Emily J. Simpson,1 Pierangelo Gobbo,1 Fernanda C. Bononi,1 Emily Murrell,1 Mark S. Workentin1 & Leonard G. Luyt1,2*

1 Western University Chemistry, 1151 Richmond Street, London, Ontario N6A 5B7, Canada
2 London Regional Cancer Program, Western University Oncology, 790 Commissioners Rd. E, London, Ontario N6A 4L6, Canada

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Abstract
Cancer targeting can be used for both tumor diagnosis and therapy. Recently, gold nanoparticles (AuNPs) have found utility in this field as they are very small in size, and thus display an enhanced permeability and retention effect, allowing them to be taken up by tumor cells through “passive targeting.” However, this accumulation is non-specific. Conversely, AuNPs functionalized with targeting entities such as peptides, antibodies, or small molecules can specifically target tumors through interaction with cancer-specific protein receptors. In this study, targeted AuNPs were developed using an azide-modified peptide that was able to react with alkyne-functionalized AuNPs through an interfacial strain-promoted azide-alkyne cycloaddition. Small (3 nm) AuNPs were made water-soluble through PEGylation and functionalized with dibenzocyclooctyne to add the alkyne functionality. For the targeting entity, a pan-bombesin peptide ([D-Phe6,β-Ala11,Phe13,Nle14]bombesin(6–14)) was chosen as it binds to all four receptor subtypes of the gastrin releasing peptide receptor, which is highly expressed in prostate cancer. Prostate cancer (PC-3) cells were incubated with the targeted AuNPs and studied via transmission electron microscopy. AuNPs conjugated with bombesin showed higher accumulation in PC-3 cells than either the blocking or control studies. These results suggest that these small, water-soluble, bombesin-functionalized AuNPs have potential applications in targeting prostate cancer as diagnostic or therapeutic entities.

Introduction
Prostate cancer is the most common cancer among American men. It is estimated that one in seven men will develop prostate cancer during their lifetime, and one in 36 will die from the disease (Siegel et al., 2014). If diagnosed early, the survival rate for prostate cancer is high, with the 5-year relative survival rate in the USA being 98.9% (Howlader et al., 2015). However, diagnosis of
prostate cancer is difficult as symptoms are not always present in the early stages. Two common methods of diagnosis are the prostate specific antigen test and the digital rectal exam; however, the accuracy of these clinically used methods has been a subject of debate (Nam et al., 2007; Chodak & Schoenberg, 1989). Detection of cancer via molecular imaging offers a more accurate and less invasive method of diagnosis by targeting receptors that are overexpressed on cancer cells. For example, prostate cancer imaging probes are able to target receptors such as the gastrin-releasing peptide receptor (GRP-R), a G protein-coupled receptor, as well as prostate-specific membrane antigen, a transmembrane protein, which are overexpressed on prostate cancer cells (Sun et al., 2000; Patel et al., 2006; Maurer et al., 2016; Rauscher et al., 2016). Targeting overexpressed receptors can also be used for therapeutic purposes, as was shown by Plonowski et al., where a cytotoxic bombesin analog was developed that targeted GRP-R and inhibited the growth of prostate cancer (Plonowski et al., 2000).

Gold nanoparticles (AuNPs) are widely used for applications in cancer targeting and therapy as they are the most stable metal nanoparticle, have low toxicity, are biocompatible, their surfaces are easily functionalized, and they have tunable photothermal and thermoablative properties (El-Sayed et al., 2005; Rosi & Mirkin, 2005; Rosi et al., 2006; Glazer et al., 2010; Giljohann et al., 2010; Papasani et al., 2012; Naahidi et al., 2013; Abadeer & Murphy, 2016). However, the undecorated AuNP itself can only accumulate in the desired target through a passive type of targeting based on the enhanced permeability and retention effect resulting from tumor angiogenesis (Iyer et al., 2006). Therefore, the visualization and/or treatment of a desired target may be difficult using AuNPs as they can accumulate in various locations. For this reason, development of targeted AuNPs would be advantageous as they could accumulate in higher concentration in the tissue of interest, which could prove useful for both diagnostic and therapeutic purposes.

Nanoparticles show great potential for cancer targeting as they are multifunctional. As a result, they can be used to attach not only a targeting entity to provide probe specificity, but also have the ability to simultaneously attack: an imaging moiety, allowing for external visualization (Manchester & Singh, 2006; Montet et al., 2006; Cheon & Lee, 2008; Brunel et al., 2010); a target for drug delivery (Emerich & Thanos, 2007; Libutti et al., 2010); or a radiotherapeutic isotope (Zhang et al., 2010). Therefore, nanoparticles can simultaneously be targeted while still allowing for imaging and/or therapy. For example, in a study by Morales-Avila et al., 99mTc labeled HYNIC-GGC was attached to AuNPs along with c[RGDFK(C)] to image tumor angiogenesis (Morales-Avila et al., 2011). Through in vivo mouse studies, they found that these nanoparticles showed increased tumor uptake compared to 99mTc labeled HYNIC-c[RGDyK] that was not attached to AuNPs. Therefore, attaching a known targeting peptide to AuNPs can increase the in vivo tumor uptake of the peptide.

Another advantage of using AuNPs for targeting relies on their ability to act as phase-transfer agents, increasing the water solubility of certain biomolecules and molecular probes (vide infra) (Yang et al., 2011). Finally, targeted AuNPs can also be used to induce thermal ablation of tumors through irradiation with radio frequency radiation due to their inherent thermoablative properties (Glazer et al., 2010).

Despite the great advantages that derive from the synthesis of targeted AuNPs, the conjugation of molecular systems onto AuNPs still remains a challenge. In fact, attachment of each biomolecule or molecular probe needs to be individually designed, optimized, and characterized. Any changes to the reaction can have a deleterious effect on the stability of the colloidal system, leading to irreversible aggregation or decomposition.

Recently, clickable and bioorthogonal nanomaterial templates have been developed. These are resilient, stable, and biocompatible nanomaterials that display interfacial click or bioorthogonal moieties that are able to react with any molecular system of interest carrying the complementary functional group or chemical reporter. Most importantly, the interfacial chemistry between the two reaction moieties is orthogonal to the surface chemistry of the nanomaterial and leads to the desired (bio)conjugate with no effects on the nanomaterial stability. This approach to conjugation has demonstrated that nanomaterials can potentially be functionalized with any molecular system of interest through easy and reliable chemical reactions (Gobbo et al., 2013; Gobbo et al., 2013; Gobbo & Workentin, 2016; Gobbo et al., 2015).

In this study, this bioorthogonal approach was applied to the modification of small (~3 nm) AuNPs in order to develop targeted AuNPs. Through PEGylation, these nanoparticles were made water-soluble, rendering them suitable for biological studies. As well, they are soluble not only in water, but also in many other organic solvents, facilitating chemical modification. In this study, these AuNPs were functionalized with dibenzocyclooctyne (DBCO) at the interface for use in an interfacial strain-promoted azide-alkyne
cycloaddition (I-SPAAC) reaction with an azide-functionalized peptide in order to form targeted AuNPs. This approach to bioconjugation gives the advantage of using a bioorthogonal “click” reaction that can be performed under copper-free conditions, thus making it more suitable for biological studies than the copper-catalysed azide-alkyne cycloaddition.

Targeting of AuNPs for the detection of prostate cancer can be accomplished using a targeting entity such as bombesin, a tetradecapeptide isolated from the skin of the European fire-bellied toad, Bombina bombina (Anastasi et al., 1971). It specifically targets cell surface GRP-Rs, which are overexpressed in prostate cancer cells, (Reubi, 2003; La Bella et al., 2002; Ferro-Flores et al., 2006; Visser et al., 2007) and has been shown to target prostate cancer when conjugated to nanoparticles (Martin et al., 2010; Steinmetz et al., 2011). There are four known GRP-R subtypes; GRP-R, NMB-R, BRS-3, and BB4-R. It was found that a truncated pan-bombesin, [D-Phe⁶,β-Ala¹¹, Phe¹³, Nle¹⁴]bombesin(6–14) (Fig. 1), successfully targets all four GRP-R subtypes with high affinity (Mantey et al., 1997; Reubi et al., 2002; Pradhan et al., 1998).

Bombesin peptides have been shown to retain high affinity after extensive modifications to the N-terminus (Baidoo et al., 1998; Van de Wiele et al., 2000; La Bella et al., 2002; Smith et al., 2003); therefore, a PEG-azide was added to the N-terminus of the pan-bombesin peptide not only to add the azide functionality, but also to provide distance between the targeting peptide and the nanoparticle. This allowed for attachment of the peptide to the DBCO-functionalized AuNP through an I-SPAAC reaction. After characterization through IR spectroscopy and ζ-potential analysis, these nanoparticles were used for in vitro cell studies using a human prostate cancer cell line (PC-3) and the uptake was visualized through transmission electron microscopy (TEM).

**Results and Discussion**

**Peptide synthesis and model I-SPAAC reaction**

A pan-bombesin peptide, [D-Phe⁶,β-Ala¹¹, Phe¹³, Nle¹⁴] bombesin(6–14), was synthesized with a PEG azide on the N-terminus in order to provide a point of attachment for the peptide to the DBCO-functionalized AuNPs.
through I-SPAAC (Fig. 1). Removal from the resin afforded the desired azide-modified pan-bombesin peptide (1). To verify the ability to perform the I-SPAAC reaction (Scheme 1), bombesin-azide peptide 1 was reacted with a PEGylated DBCO and was found to be complete after 1 h at room temperature. This demonstrated the suitability of the synthesized peptide for an I-SPAAC reaction with DBCO-functionalized AuNPs.

**Functionalization of gold nanoparticles**

The DBCO-functionalized AuNPs were prepared according to previously published procedures (Gobbo & Workentin, 2012; Gobbo et al., 2014). The DBCO-AuNPs were then reacted with bombesin-azide peptide 1 in an I-SPAAC reaction (Scheme 2), affording a final conjugation yield of 65%, based on recovered unreacted bombesin-azide after centrifuge filtration using Millipore centrifuge filters 10 kDa molecular weight cut-off (MWCO). This yield was comparable to those of other conjugation reactions carried out between clickable and bioorthogonal nanomaterial templates and bulky molecular systems (Gobbo et al., 2014; Milne et al., 2013). This relatively low yield is most likely because of the steric hindrance from the large size of the peptide and the small gold core diameter.

The bombesin-AuNPs were then characterized through IR spectroscopy and ζ-potential measurements. The IR spectrum of the bombesin-AuNPs (5) compared with that of bombesin-azide (1) and DBCO-AuNPs (4) (Fig. 2) clearly showed the absence of the azide stretching mode at 2100 cm⁻¹, a marked increase in the N-H stretching at 3300 cm⁻¹, and the appearance of a signal at 1624 cm⁻¹ corresponding to the amide bond stretch of the peptide, which differs from the amide bond stretch of the DBCO-AuNPs that instead falls at 1660 cm⁻¹. This indicates that the washing procedure used is effective in removing any unreacted peptide and that the peptide has been successfully conjugated to the AuNP surface.

Finally, the success of the I-SPAAC bioconjugation was confirmed by the change in AuNP surface charge. After bioconjugation of the bombesin, the ζ-potential of the AuNPs shifted from −36 ± 5 mV to −59 ± 5 mV, confirming the presence of the peptide on the AuNP surface and demonstrating excellent stability of the AuNP-bioconjugate in aqueous solution.
Cellular toxicity of AuNPs is size-dependent (for reviews see (Boisselier & Astruc, 2009; Khlebtsov & Dykman, 2011; Dreaden et al., 2012; Li et al., 2014; Xia et al., 2016)), as it has been found that AuNPs between 1 and 2 nm are toxic, with nanoparticles that are either smaller or larger being less toxic (Pan et al., 2007). This is in part because of the fact that 1.4 nm AuNPs can fit into the major groove of DNA and coordinate irreversibly (Tsoli et al., 2005). The dimensions of the gold nanoparticles also has significant impact on the eventual biodistribution and organ accumulation of different sized AuNPs, with smaller nanoparticles showing more widespread distribution (Sonavane et al., 2008; Semmler-Behnke et al., 2008). They also display reticuloendothelial system uptake, which causes accumulation in the liver, spleen, bone marrow, and other nontargeted areas. In vivo studies on mice injected with naked AuNPs have revealed that accumulation of differently sized nanoparticles in these organs can be toxic (Chen et al., 2009) as a result of cellular response to nanoparticle invasion (Sabella et al., 2014). However, it was found that coating the nanoparticles, such as through PEGylation or citration of the nanoparticles can reduce toxicity (Fraga et al., 2014) and increase circulation times as the nanoparticles become more hydrophilic and the surface energy is decreased, resulting in less aggregation of the nanoparticles (Jokerst et al., 2011).

This reduction in toxicity through PEGylation also applies to small AuNPs. In a study by Gu et al., it was shown through cell viability tests that PEGylated 3.7 nm AuNPs showed higher than 85% cell viability after incubation with 10 μM nanoparticles for 24 h and 70% cell viability after 72 h (Gu et al., 2009). A previous study was performed with the same 3 nm AuNPs used in the current study, in which the AuNPs were conjugated with a gadolinium(III) chelate for in vivo mouse studies for magnetic resonance imaging (Milne et al., 2013). In this study, 200 μL of 10 mM Gd³⁺-AuNPs was injected into the tail vein of a mouse. It was found that, 24 h post-scan, the mouse showed no ill effects. Therefore, these small, PEGylated AuNPs do not appear to be toxic and targeted versions are expected to be well tolerated for in vivo administration. This was also investigated through a cell viability study, as follow.

**Cell viability**

For this study we chose PC-3 cells, a human prostate cancer cell line that is androgen independent and highly tumorigenic. This cell line expresses high levels of GRP-R (Rogers et al., 2003), which is the desired receptor target for the bombesin-functionalized AuNPs. It has also been shown that there are an average of 44,000 bombesin receptor sites per cell for this cell line (Reile et al., 1994). Cell viability of PC-3 cells when incubated with the AuNPs with bombesin was determined by a Trypan blue exclusion assay. After 24 h, cells incubated with the bombesin-AuNPs were found to be 92.1 ± 4.3% viable, compared with the control PC-3 cell viability of 95.6 ± 1.3% ($P = 0.15$).

**Transmission electron microscopy**

In vitro cell studies were performed using PC-3 cells. The cells were incubated with the AuNPs in the following manner. The first experiment was a targeting study involving cells incubated with a 1 mg/mL solution of bombesin-conjugated AuNPs. A blocking study was also performed with cells that were incubated with a
50 μg/mL solution of bombesin peptide for 2 h, followed by incubation with a 1 mg/mL solution of bombesin-conjugated AuNPs. Finally, a control study using cells incubated with a 1 mg/mL solution of nontargeted AuNPs (DBCO-AuNP 4) was performed.

The cells were then embedded in epon-araldite plastic and cut into 70 nm slices using an Ultracut ultramicrotome. The slices were collected on copper grids and visualized by TEM at 60 kV. This was performed with the cells incubated with targeted bombesin-conjugated AuNPs, the cells blocked with peptide, and the control cells.

The uptake of AuNPs in cells is predominantly due to the surface chemistry of the particles (Nativo, 2008). In general, nanoparticles are taken up from the cell surface through endocytosis and confined to the endosome. This can occur through either receptor-mediated endocytosis, which was expected for the targeted AuNPs, or through pinocytosis, which was expected for the nonfunctionalized AuNPs. Conversely, the particles can avoid the endosomal pathway by being taken up directly into the cytosol using cell-penetrating peptides (Nativo, 2008). However, the bombesin peptide used is not a cell-penetrating peptide. Therefore, it was expected that the method of cell internalization would be through endocytosis.

The targeted AuNPs showed considerable cell uptake, as would be expected given that they were designed to target the overexpressed GRP-Rs of the PC-3 cells. The pan-bombesin peptide used is a bombesin receptor agonist (Reubi & Maecke, 2008); and once bound to the GRP-Rs found on the surface of the PC-3 cells, the receptor-ligand complex can undergo internalization (Benya et al., 1994). As opposed to compounds that remain on the cell surface, this internalization is ideal as it increases retention in the target tissue (García Garayoa et al., 2007). Evidence for this kind of AuNP internalization is shown in the TEM images of the targeted nanoparticles 5 (Fig. 3a and b) compared to those of the blocking study, where the cells were incubated with bombesin for 2 h before adding the targeted bombesin-AuNPs, resulting in less AuNP retention than with only the targeted nanoparticles (Fig. 3c and d). While there was a reduction in cellular uptake, the multi-valent targeting capability of the bombesin-AuNPs may render it difficult to adequately block the target receptor with a mono-valent peptide ligand. For the control experiment, cells were

**Figure 3.** Transmission electron microscopy images at 60 kV of AuNPs (examples shown by black arrows) in PC-3 cells. (a) Cell incubated with targeted AuNPs conjugated with bombesin at a magnification of 19000 and (b) 64000; (c) blocking study cell at a magnification of 19000 and (d) 64000; (e) control study with nontargeted AuNPs at a magnification of 19000 and (f) 64000.
incubated with nontargeted (untreated) AuNPs and thus are not expected to bind to the GRP-R. As expected, these cells showed minimal retention of the nanoparticles following washing, as there were significantly fewer observed AuNPs in these cells compared with the targeted AuNPs, as observed by TEM (Fig. 3e and f, representative images of samples with additional images of cells provided in Fig. S1). Figure 3 shows TEM images of each study, representative across all cells, where the AuNPs can be seen as small black dots, with black arrows indicating representative AuNPs (also Figs. S1 and S2).

The amount of AuNPs found in the three studies is also evidence for the mode of internalization. As the targeted study involves bombesin-functionalized AuNPs targeting the GRP-Rs overexpressed on the PC-3 cell surfaces, there is potential for them to be internalized (instead of being surface retained) through both receptor-mediated and nonreceptor-mediated pathways, leading to a large amount of internalized AuNPs. For the blocking study, the receptors on the surface are blocked by free bombesin. As a result, it is logical that the amount of AuNPs in the blocked cells decreased as the receptor-mediated pathway is inhibited. However, some nonreceptor mediated retention is still observed, which is also the case with the control experiment using nontargeted AuNPs, likely due to non-specific cell-surface AuNP adhesion.

Grid-based manual counting of the TEM images at a magnification of 64000 (Fig. 3b, d, and f; also Figure S3) was performed in order to semi-quantify the nanoparticle uptake. As the number of large and small clusters appears to be relatively evenly distributed across the cells, clustering of the nanoparticles should not significantly impact the semi-quantitative results. It was determined that the cells showed a ratio of approximately 7:4:2 nanoparticles for targeted:blocking:control, indicating that the targeted AuNPs displayed higher retention than in either the blocking or control studies.

As can be seen in Figure 4a-d, the AuNPs accumulate to some extent in the endosomes, with the control cells in Figure 4b showing densely packed, aggregated nanoparticles in a significant amount in endosomes. The

![Figure 4. Transmission electron microscopy images at 60 kV showing AuNP accumulation in endosomes and cytosol of PC-3 cells. (a) Cell incubated with targeted AuNPs conjugated with bombesin at a magnification of 10500 and (c) 19000; (b) control study with nontargeted AuNPs at a magnification of 10500 and (d) 19000.](image-url)
targeted AuNPs also show some accumulation in endosomes, but to a lesser extent. In both the control and targeted studies, as well as the blocking study in Figure 3, the AuNPs are also found distributed throughout the cell in the cytosol. As well, many of the AuNPs present in the endosomes are surrounded by an additional membrane, indicating they have been taken up into the endosome from the cytosol (Nativo, 2008). However, many AuNPs are present in endosomes in the absence of a second membrane, indicating they are taken up through endocytosis. While we expected exclusive endosomal localization, a relatively uniform distribution of AuNPs throughout the cells was also found. As many AuNPs were also observed in the cytosol, it is likely that the endosomal membranes are being disrupted by the nanoparticles or bombesin, leading to endosomal escape and the presence of AuNPs in the cytosol (Nativo, 2008; Martens et al., 2014). To the best of our knowledge, this is the first example of in vitro visualization of cell uptake of bombesin-conjugated AuNPs via TEM.

Bombesin has been conjugated to AuNPs for prostate cancer imaging in the past (Chanda et al., 2010; Hosta-Rigau et al., 2010; Ocampo-Garcia et al., 2011; Jiménez-Mancilla et al., 2013). However, these previous examples were not PEGylated. PEGylation not only lowers the toxicity but also increases the solubility of the AuNPs. As such, our bombesin-functionalized AuNPs have solubility >10 mg/mL in water, whereas the best solubility previously reported for bombesin-functionalized AuNPs was <3 mg/mL in phosphate-buffered saline (PBS) (Chanda et al., 2010). Solubility in water is a very important consideration when using nanoparticles for animal studies; therefore, these novel, water-soluble, bombesin-functionalized AuNPs are much more suitable for in vivo studies due to their increased solubility in water.

Peptides are most commonly conjugated to AuNP surfaces through a thiol, typically a cysteine (Hosta-Rigau et al., 2010) or disulfide (Chanda et al., 2010), or an N-terminal primary amine such as the ε-amine of a lysine (Ocampo-Garcia et al., 2011; Jiménez-Mancilla et al., 2013). A much more convenient method of functionalizing AuNPs is through the use of click chemistry, which is inherently quick, easy, and reproducible. Our AuNPs are functionalized with DBCO, allowing for the use of I-SPAAC for conjugation of an azide-modified peptide to the AuNP. Azide modification of peptides is easily accomplished through the addition of a commercially available PEG azide. The use of strain-promoted click chemistry has the added advantage of not requiring a copper catalyst to drive the reaction, further enhancing its compatibility for in vivo studies.

**Conclusions**

An azide-modified pan-bombesin peptide was successfully synthesized and conjugated to DBCO-functionalized AuNPs, creating targeted, water-soluble AuNPs. These were formed through I-SPAAC, providing a quick, easy, and bioorthogonal method of conjugation of a peptide to AuNPs. The bioconjugation was confirmed through IR spectroscopy and ζ-potential measurements, showing that a stable bioconjugate was formed. Through in vitro TEM studies, it was shown that targeted AuNPs are more strongly retained by PC-3 cells, resulting in higher observed concentration than either the blocking or control studies, which we interpret as internalization of the targeted AuNPs. Bombesin has been shown to target GRP-Rs that are over-expressed on PC-3 cells; through this study, it has been shown that this is still the case when bombesin is conjugated to small, water-soluble AuNPs. This provides a new platform for the conjugation of bombesin (and other targeting systems) to AuNPs while also allowing for addition of a radioisotope, dye, or drug through I-SPAAC with free DBCO on the nanoparticles, allowing them to be utilized for targeting of PC-3 cells. This work establishes a methodology that has potential to be applied to other cell-targeting moieties, with the eventual application for targeting prostate and other cancer cells for both diagnosis and therapy.

**Experimental**

**Materials and Equipment**

All chemicals were purchased from Sigma-Aldrich, Caledon, Cambridge Isotope Laboratories, Commercial Alcohols, BDH, Spectra/Por, Novabiochem, Aapptec, Peptides International, Chem-Impex, Wisent Inc., and Life-technologies and were used without further purification unless indicated. The HPLC-MS system (Waters, Canada) system consisted of a Waters 2998 Photodiode Array Detector and a Waters 2767 Sample Manager, a Sunfire RP-C18 4.6 × 250 mm, 5 μm column was used for analytical work, and a Sunfire RP-C18 19 × 150 mm, 5 μm column was used for preparative separations. A gradient solvent system consisting of acetonitrile (ACN) + 0.1% trifluoroacetic acid (TFA) (solvent A) and H₂O + 0.1% TFA (solvent B) was used. Analytical UHPLC-MS (Xevo-QToF, Waters, Canada) used an Acquity UPLC BEH C18 2.1 × 50 mm, 1.7 μm column.
with a gradient solvent system consisting of ACN + 0.1% formic acid (solvent C) and H2O + 0.1% formic acid (solvent D). An INOVA 400 NMR spectrometer (Varian, Canada) was used for ¹H NMR studies. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS, 0.00 ppm). For electro-spray ionization (ESI) mass spectra, a Micromass Quattro Micro API (Waters, Canada) mass spectrometer was used. For TEM, an Ultrarect ultramicrotome (Leica, Germany) was used for sample preparation and a CM10 TEM (Philips Electronics, The Netherlands) was used for imaging. Infrared spectra were recorded using a Bruker Vector 33 spectrometer (Bruker, Canada) by placing a thin film of sample on a KBr disk. ζ-potential measurements were performed at 25°C using a Zetasizer Nano-ZS (Malvern Instrument, UK), and measurements were repeated at least three times. A solution of AuNPs in PBS (pH 7.0) was prepared with a concentration of 0.5 mg mL⁻¹. This solution (1 mL) was inserted in a latex folded capillary cell equipped with electrodes and the ζ-potential was calculated by employing the Hückel approximation.

**Bombesin-azide peptide (1)**

The peptide was synthesized on-resin using standard Fmoc solid-phase peptide chemistry by manual synthesis using Fmoc protected Rink amide MBHA resin (0.5 mmol, loading 0.52 mmol/g). The Fmoc protecting group was removed by treatment with 20% piperidine in N,N-dimethylformamide (DMF) for 5 and 15 min, followed by rinses with dichloromethane (DCM) and DMF. Fmoc protected amino acids were then coupled from C-terminus to N-terminus for 1 h, followed by a second coupling for 45 min, using 3 equiv. of Fmoc amino acid, 3 equiv. of 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylsilane (TMS, 0.00 ppm). For TEM, an Ultrarect ultramicrotome (Leica, Germany) was used for sample preparation and a CM10 TEM (Philips Electronics, The Netherlands) was used for imaging. Infrared spectra were recorded using a Bruker Vector 33 spectrometer (Bruker, Canada) by placing a thin film of sample on a KBr disk. ζ-potential measurements were performed at 25°C using a Zetasizer Nano-ZS (Malvern Instrument, UK), and measurements were repeated at least three times. A solution of AuNPs in PBS (pH 7.0) was prepared with a concentration of 0.5 mg mL⁻¹. This solution (1 mL) was inserted in a latex folded capillary cell equipped with electrodes and the ζ-potential was calculated by employing the Hückel approximation.

**Synthesis of PEG-DBCO (2)**

This oxidation was adapted from a procedure developed by M. Hunsen (Chen et al., 2009). Periodic acid (5.00 g, 21.8 mmol) was dissolved in 30 mL of dry acetonitrile, followed by addition of triethylene glycolmonomethyl ether (1.6 g, 10 mmol). The reaction was then cooled in an ice bath and a solution of pyridinium chlorochromate (PCC, 55 mg, 2.5 mol%) in dry acetonitrile (10 mL) was added dropwise over 15 min. After 3 h, the reaction was diluted with ethyl acetate (60 mL) and washed with saturated sodium bisulphite solution (75 mL). The organic layer was collected and the aqueous layer was extracted twice with ethyl acetate (30 mL). The organic fractions were combined, washed with brine, and dried with magnesium sulphate, before solvent removal under vacuum. This resulted in Me-EG₃-COOH as a yellow oil in 78% yield. ¹H NMR (CDCl₃): δ 4.17 (s, 2H), 3.67 (m, 8H), 3.38 (s, 3H). ¹³C NMR (CDCl₃): δ 173.1, 71.3, 69.98, 69.86, 68.1, 58.5. MS (Cl): m/z calculated for C₁₁H₂₄O₅, 178.0841 [M + H]⁺; observed, 178.0921 [M + H]⁺.

In dry acetonitrile (3 mL), Me-EG₃-COOH (12.5 mg, 0.07 mmol) was dissolved. DIPEA (49 μL, 0.28 mmol) was added, and the mixture cooled to 0°C. O-Benzotriazolo-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU, 53 mg, 0.14 mmol) dissolved in dry acetonitrile (2 mL) was added and allowed to stir for 15 min at 0°C. Dibenzocyclooctyne-amine (DBCO-amine, 20 mg, 0.07 mmol) dissolved in dry acetonitrile (3 mL) was added to the reaction flask and the ice bath removed. The reaction proceeded overnight at room temperature. The solvent was then removed under vacuum, giving crude 2, which was purified by column chromatography using 3:1 acetone to CH₂Cl₂ as the eluent (Rₛ = 0.5). This afforded 2 as a pale yellow oil. ¹H NMR [(CD₃)₂O]: δ 7.58 (m), 5.13 (d), 3.48 (m), 2.74 (s), 2.56 (m), 1.97 (m), 1.47 (d). ¹³C NMR [(CD₃)₂O]: δ 171.5, 169.9, 152.7, 149.5, 133.4, 130.4, 129.75, 129.05, 129.8, 127.8, 126.1, 124.91, 124.07, 123.1, 119.3, 115.3, 116.7, 108.9, 72.6, 71.66, 71.08, 70.9, 58.8, 55.92, 55.25, 38.8, 35.53. MS (Cl): m/z calculated for C₂₅H₂₈N₅O₅, 437.2091 [M + H]⁺; observed, 437.2076 [M + H]⁺.

**Model 1-SPAAC reaction (3)**

In acetonitrile (250 μL), PEG-DBCO (2) (3.2 mg, 0.007 mmol) was dissolved. Bombesin peptide 1...
Acid group in the carboxy-terminated ligands. These tons belonging to the alpha carbon of the carboxylic broad peak at 4.11 ppm corresponding to the two pro-

2014). Briefly, a solution of HOOC-EG₄-AuNPs (65 mg, 30 μmol of -COOH) in dry DMF (9 mL) was added DIPEA (16 μL, 90 μmol) and the reaction was cooled to 0°C in an ice bath. HBTU (23 mg, 60 μmol) in dry DMF (5 mL) was then added to the AuNP solution and allowed to stir for 15 min at 0°C. DBCO-amine (17 mg, 60 μmol) in dry DMF (3 mL) was added and the ice bath removed. The reaction was allowed to proceed overnight under an inert atmosphere, and was then dialysed (MWCO 6000-8000 Da) against DMF. The DMF was changed twice at 2 h intervals. Finally, the sample was dialysed against water overnight. As reported previously, the DBCO-AuNPs prepared following this procedure were found to contain DBCO in a concentra-

linear gradient of 20-80% solvent C in D). The reaction was complete after 1 h. MS (ESI+): m/z calculated for C₁₀₄H₁₄₅N₂O₂₆, 2090.06 [M + H]+, 1045.54 [M + 2H]²⁺; observed 2090.08 [M + H]+, 1045.53 [M + 2H]²⁺.

Triethylene glycol monomethyl ether AuNPs (Me-EG₃-AuNPs)

According to our previously established procedure, Me-EG₃-AuNPs were synthesized (Gobbo & Workentin, 2012). Briefly, HAuCl₄·3H₂O (19.3 mg, 49 μmol) was dis-
solved in dry MeOH (7.5 mL) and glacial acetic acid (1.25 mL). To this mixture, triethylene glycol monomethyl ether thiol (Me-EG₃-SH) (26.3 mg, 146 μmol) was added. The solution was stirred vigorously for 1 h. NaBH₄ (18.5 mg, 490 μmol) dissolved in nanopure water (1.25 mL) was added to the reaction mixture dropwise. The reaction was stirred overnight at room temperature and was then concentrated under vacuum. Brine (20 mL) was then added and the nano-
particles were exhaustively extracted with toluene. The solvent was removed under vacuum, leaving a film of AuNPs that was then washed with hexanes to remove excess Me-EG₃-SH. The nanoparticles were then redissolved in nanopure water and purified by dialysis overnight (MWCO 6000-8000 Da).

The ¹H NMR spectrum showed the presence of three broad peaks: one at 3.34 ppm that corresponded to the methyl group at the nanoparticle interface and two at 3.58 ppm and 3.66 ppm related to the protons of the ethylene glycol units.

HOOC-EG₄-AuNPs

According to our previously established procedure, HOOC-EG₄-AuNPs were synthesized (Gobbo et al., 2014). Briefly, to a solution of Me-EG₃-AuNPs (73.9 mg) dissolved in DCM (5 mL), HOOC-EG₄-SH (10.9 mg) was added, the reaction was allowed to proceed for 30 min, and the solvent was removed under vacuum. The AuNP film was washed with hexanes and isopropyl alcohol and the HOOC-EG₄-AuNPs were then redissolved in MilliQ water. The ¹H NMR spectrum of the clean HOOC-EG₄-AuNPs showed the appearance of a small, broad peak at 4.11 ppm corresponding to the two protons belonging to the alpha carbon of the carboxylic acid group in the carboxy-terminated ligands. These AuNPs contain the carboxyl groups in a concentration of 0.46 μmol mg⁻¹.

DBCO-AuNPs (4)

According to our previously established procedure, DBCO-AuNPs were synthesized (Gobbo et al., 2014). Briefly, to a solution of HOOC-EG₄-AuNPs (65 mg, 30 μmol of -COOH) in dry DMF (9 mL) was added DIPEA (16 μL, 90 μmol) and the reaction was cooled to 0°C in an ice bath. HBTU (23 mg, 60 μmol) in dry DMF (5 mL) was then added to the AuNP solution and allowed to stir for 15 min at 0°C. DBCO-amine (17 mg, 60 μmol) in dry DMF (3 mL) was added and the ice bath removed. The reaction was allowed to proceed over-

night under an inert atmosphere, and was then dialysed (MWCO 6000-8000 Da) against DMF. The DMF was changed twice at 2 h intervals. Finally, the sample was dialysed against water overnight. As reported previously, the DBCO-AuNPs prepared following this procedure were found to contain DBCO in a concentra-

Bombesin-AuNPs (5)

In a typical bioconjugation procedure, DBCO-AuNPs (4) (20 mg, 2 μmol of DBCO) were mixed with bombesin-

azide 1 (9.9 mg, 6 μmol) in dry methanol (5 mL). The reaction was allowed to proceed for 2 h, after which, the methanol was evaporated and the bombesin-AuNPs and excess bombesin-azide 1 were redissolved in a 1:1 mixture of MeOH:H₂O (4 mL). The excess peptide was then removed by centrifugation (6000 rpm for 7.5 min) using Millipore centrifuge filters (MWCO 10000 Da). Bombesin-AuNPs were never allowed to dry completely on the filter, taking care that 500 μL of sol-

vent was still present inside the filter after centrifugation. Bombesin-AuNPs were washed four times and finally transferred to a vial. The solvent was completely evaporated and the bombesin-AuNPs were redissolved in MilliQ water to obtain a final concentration of 10 mg/mL. This solution was stored at 4°C.

In order to calculate the l-SPAAC reaction yield, the purifica-
tion was carefully evaporated. This resulted in 4.7 μmol of bombesin-azide 1 being recovered, indicating that 65% of the interfacial DBCO reacted with the peptide.

Cell viability

Prostate cancer cells were grown in Ham’s F-12 media supplemented with 10% fetal bovine serum and after 3-4 passages were seeded into 6-well plates at
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300,000 cells/well in 2 mL of media. After 24 h, 10 μL of a 10 mg/mL solution of AuNPs with bombesin in methanol was added to each well (n = 6) for a final concentration of 50 μg/mL. As a control, 10 μL of methanol was added to the remaining wells (n = 6). The cells were incubated at 37°C for 24 h, at which point the cells were counted for viability. Adherent cells in each well were lifted with 500 μL of a 0.25% Trypsin-EDTA solution and added to the supernatant media. The total number of cells and number of nonviable cells for each well were counted using Trypan blue staining and a hemocytometer. Cell viability was determined by an average of the percent of viable cells from the total number of cells for each well. A paired t-test was performed on the two sets of data.

Cell preparation for TEM

Prostate cancer cells were grown in T-75 flasks using Ham’s F-12 media supplemented with 10% fetal bovine serum. Cells (400,000-500,000, cell passage 6-7) were incubated with a 1 mg/mL solution of AuNPs either with or without bombesin to a final concentration of 50 μg/mL at 37°C for 24 h. For blocking studies, bombesin was added to the cells to a final concentration of 50 μg/mL for 2 h prior to the addition of the bombesin-conjugated nanoparticles. After 24 h, the cells were gently washed with PBS, gently lifted using a 0.05% trypsin-EDTA solution, and centrifuged at 1200 rpm for 5 min. The cell pellet was washed twice with PBS and fixed with 2% glutaraldehyde in PBS solution overnight. Cells were then washed three times with PBS to remove the fixing agent, resuspended in PBS, and analysed by TEM.

Transmission electron microscopy

Once the cells were fixed with a 2% glutaraldehyde in PBS solution, they were washed three times with PBS buffer and resuspended in 2% OsO4 for 1 h. They were then washed three times with double distilled water. The cells were set in a 1% agarose solution that was then cut into small cubes and subjected to dehydration with an acetone series; percent acetone in water: 20%, 50%, 70%, 90%, 95%, 100%, 100%, and 100%. The cells were then embedded with a series of epon-araldite plastic in acetone; 50%, 66%, 100%, and 100%. The samples were then set in a form using epon-araldite plastic. The molds were then cut into 70 nm slices using an Ultracut ultramicrotome. The slices were collected on copper grids and visualized using a Phillips CM10 TEM at 60 kV. This process was performed in duplicate, with approximately 100 cells being observed for consistency of ratiometric (grid-based counting) determination.

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Conflict of Interest

The authors report no conflict of interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1: TEM images at 60 kV of AuNPs in PC-3 cells. a) Cell incubated with targeted AuNPs conjugated with bombesin at a magnification of 19000, b) blocking study cell at a magnification of 25000, and c) control study cell with non-targeted AuNPs at a magnification of 25000.
**Figure S2:** TEM images at 60 kV of AuNPs in PC-3 cells. a) Cell incubated with targeted AuNPs conjugated with bombesin at a magnification of 46000, b) blocking study cell at a magnification of 46000, and e) control study cell with non-targeted AuNPs at a magnification of 46000.

**Figure S3:** TEM images at 60 kV of AuNPs in PC-3 cells. a) Cell incubated with targeted AuNPs conjugated with bombesin at a magnification of 64000, b) blocking study cell at a magnification of 64000, and e) control study cell with non-targeted AuNPs at a magnification of 64000.