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Peptide functionalized gold nanoparticles: the influence of pH on binding efficiency

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Abstract
We report herein on the synthesis of mixed monolayer gold nanoparticles (AuNPs) capped with both polyethylene glycol (PEG) and one of three peptides. Either a receptor-mediated endocytosis peptide, an endosomal escape pathway (H5WYG) peptide or the Nrp-1 targeting RGD peptide (CRGDK) labeled with FITC. All three peptides have a thiol containing cysteine residue which can be used to bind the peptides to the AuNPs. In order to investigate the influence of pH on peptide attachment, PEGylated AuNPs were centrifuged, the supernatant removed, and the nanoparticles were then re-suspended in a range of pH buffer solutions above, below and at the respective isoelectric points of the peptides before co-functionalization. Peptide attachment was investigated using dynamic light scattering, Ultra-violet visible spectroscopy (UV/Vis), FTIR and photo luminescence spectroscopy. UV/Vis analysis coupled with protein assay results and photoluminescence of the FITC tagged RGD peptide concluded that a pH of ∼8 optimized the cysteine binding and stability, irrespective of the peptide used.

Keywords: gold nanoparticles, PEG, pH, isoelectric point, peptide, attachment, functionalization

(List some figures may appear in colour only in the online journal)

List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AuNPs</td>
<td>gold nanoparticles</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<td>RME</td>
<td>receptor mediated endocytosis</td>
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<tr>
<td>H5WYG</td>
<td>endosomal escape pathway</td>
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<td>RGD</td>
<td>Nrp-1 targeting peptide</td>
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<td>TEM</td>
<td>transmission electron spectroscopy</td>
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<td>DLS</td>
<td>dynamic light scattering</td>
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<td>UV/Vis</td>
<td>Ultra-violet visible spectroscopy</td>
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<td>FTIR</td>
<td>Fourier transform infra-red spectroscopy</td>
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<tr>
<td>pI</td>
<td>isoelectric point</td>
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<tr>
<td>pKa</td>
<td>acid dissociation constant</td>
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<tr>
<td>QDs</td>
<td>quantum dots</td>
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<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
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<tr>
<td>KBr</td>
<td>potassium bromide</td>
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<td>SH</td>
<td>thiol</td>
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</table>

Introduction

Over recent decades AuNPs have emerged as an ideal platform for nanomedicine based cancer therapies. AuNPs can be used for detection of cancers; to treat tumors via targeted drug delivery, photo ablation therapy or by acting as radio-sensitizers [1]. AuNPs are highly attractive agents for nanomedicine due to their biocompatibility, ease of functionalization and the ability to fine-tune their physiochemical properties (e.g. plasmon absorption behavior) [2–5]. AuNPs are essentially non-toxic,
are readily imaged, can be heated by exploiting the plasmon resonance effect and can be functionalized with various groups using thiol chemistry. AuNP based treatments have the potential to improve current cancer therapeutics through the attachment of various ligands to elicit functions including immune system shielding, targeting, cell internalization and/or the delivery of therapeutic payloads [3, 10]. These qualities make AuNPs an optimum choice for optical detection agents, diagnostics, nano-therapeutics, drug delivery systems and imaging agents [6–9].

AuNP synthesis via the Turkevich method produces 10–80 nm diameter, citrate capped particles with a zeta potential of approximately –20 to –40 mV, and the size can be tailored by altering the Au/citrate ratio [11]. The surface can then be functionalized with other ligands such as PEG, peptides or antibodies typically by means of a thiol group. Some researchers suggest that the higher binding energy of the covalent Au–S bond surface relative to the loosely bound citrate results in functionalization via ligand exchange [11–16]. However, in a detailed study using FTIR and XPS, Park and Schumaker-Parry found that a proportion of the citrate oxidizes into acetoacetate on the AuNP surface, and that it is largely this acetoacetate that is replaced by the thiol bound ligands. They also reported that much of the citrate capping remains after ligand attachment, with the citrate and thiol bound ligands forming a regular structure on the AuNP surface. This co-absorption, results in a lower ligand packing density on citrate capped AuNPs compared to that observed with self-assembled monolayers on planar gold. Whilst citrate capped AuNPs form stable colloidal suspensions, they aggregate under physiological salt concentrations and a protein corona is quickly formed in the body which leads to their removal. Aggregation is usually prevented by the repulsive force between particles due to electrostatic repulsion, steric exclusion or the creation of a hydration layer on the nanoparticle surface [7, 17–19]. The amphiphilic polymer, PEG, is widely employed to inhibit aggregation and to increase the circulation time in vivo by preventing non-specific protein attachment. However, while PEG functionalization creates stable AuNPs, it blocks cellular uptake and renders the AuNPs largely biologically inactive. Co-functionalization of partially PEG capped AuNPs with additional biologically active groups such as RME peptides or targeting antibodies allows for the creation of a stable, yet biologically active nanoparticle based therapeutic agent [15].

To create a successful cancer therapeutic, the AuNP must be co-functionalized with a range of moieties to evade detection and removal by the immune system, target cancer cells, initiate uptake, allow for endosomal escape, target the nucleus and/or facilitate the delivery of a payload [20]. Co-functionalization is commonly achieved via electrostatic charge attachment, specific affinity immobilization and/or covalent bonding typically by means of thiol, or less frequently, amine groups [6, 20]. Peptides such as RME peptide can be readily attached to the AuNP surface via the thiol containing cysteine residues [15, 21]. Other methods employ free amine groups to bind directly to the AuNP or by means of a second active functional group such as an NHS ester group on the end of a thiol bound PEG linker [7, 22]. The adsorption of thiol bound ligands onto planar flat gold (111) surfaces has demonstrated that sulfur adsorption is influenced by the rate of oxidation of the gold and that many lattice structures are feasible and can be manipulated via the control of external factors such as pH. L-cysteine forms (√3x√3) R30° adlayers consistent with hexagonal packing, under neutral (H2O) and slightly acidic (0.1 M KClO4 + 1 mM HClO4) conditions [23–27]. While it has been observed that pH plays an important role in controlling ligand attachment, it is not currently possible to predict an optimum pH for attaching a particular peptide. Optimizing the ligand attachment by altering the pH may have the benefit of being able to maximize the binding efficiency with the nanoparticle [7, 15, 16, 20]. The relationship of pH on protein gold binding is not very well understood, it has been previously reported that functionalization can be achieved by, ‘non-specific’ adsorption through altering the pH close to that of the pI of the protein or the nanoparticle so that the electrostatic repulsion is reduced [28]. It has also been suggested that AuNP—moiety surface binding can be initiated by introducing an opposite charge on the desired ligand which is to be attached by van der Waals forces, hydrogen bridges, gold–thiol bonds (from cysteine residues) or by hydrophobic interaction [7]. ‘As synthesized’ citrate capped AuNPs have a zeta potential of approximately –30 mV. Goldman et al 2002 created electrostatic interactions between negatively charged dihydro-lipoic acid (DHLA)-capped CdSe–ZnS core–shell QDs and a positively charged leucine zipper affixed onto the C-terminus of engineered recombinant proteins. It was reported that proteins absorbed onto the nanoparticles by electrostatic attraction if both partners are oppositely charged [29–32]. Adjustment of the pH to below the pI value (pI 10–10.5) has previously been used to bind avidin to AuNPs [29, 33].

This study will therefore focus on tailoring the pH of the AuNP solution in relation to the pI values of a range of peptides to investigate if there is an optimum pH for peptide attachment, and if this varies depending upon the peptide characteristics. As zwitterionic molecules, peptides can display both negative and positive charges on different functional groups simultaneously depending on pH. However, the ratio of functional groups bearing a charge changes with pH, resulting in peptides being predominantly positively charged at pH values below the peptides pI and negatively charged above. At the pI the overall net charge is zero. This offers the possibility to attract peptides electrostatically to negatively charged nanoparticles [7]. The attachment of the peptides (RME, HS5WG and RGD) to partially PEG capped AuNPs were studied in a range of pH buffers above, below and at the pIs of the peptides. The peptides used were the RME sequence CKKKKKKSEDEYPYVPN which has an overall net charge of +2.9 and a pI of 9.6. The buffers used for investigation of RME peptide were pH 8, 9, 9.2, 9.4, 9.6, 9.8,
An endosomal escape pathway H5WYG peptide sequence GCGLFHAIAHFIHGGWHGLIHGWYG with a net charge of +0.4 and a pl of 7.75 was also studied. The buffers used for investigation of H5WYG peptide were pH 6, 7, 7.25, 7.5, 7.75, 8, 9, 10 and 11. Finally, the Nrp-1 targeting peptide used for investigation of H5WYG peptide were pH 6, 7, 7.25, 7.5, 7.75, 8, 9, 10 and 11. An endosomal escape pathway H5WYG peptide targeting peptide and (c) the RME peptide all constructed from carbon (green), hydrogen (gray), nitrogen (blue), oxygen (red) and sulphur (yellow).

Methods/experimental

Chloroauric acid (HAuCl4·3H2O), SH-PEG (5000 Mw), citric acid/sodium hydroxide buffer solution pH 6, borax/sodium hydroxide buffer solution pH 8 and 10, di-sodium hydrogen phosphate/sodium hydroxide buffer solution pH 12, and sodium citrate were obtained from Sigma Aldrich UK. The 0.01 wt% chloroauric acid and 1 wt% sodium citrate solutions were prepared using distilled water. RME peptide (sequence CKKKKKSSEDYPPVPN), H5WYG peptide (sequence GCGLFHAIAHFIHGGWHGLIHGWYG) and the FITC functionalized RGD peptides (sequence FITC-Ahx-CRGDK) were custom synthesized by BIOMATIK (USA), the pl and net charge of each of these peptides was calculated using PepDraw© 2011 created by Tulane University.

Synthesis and washing of AuNPs

Prior to synthesis, all the glassware was washed with aqua regia, rinsed with distilled water and then dried overnight at 80 °C–100 °C. 11 of 0.01 wt% chloroauric acid solution was heated to a rolling boil under reflux in a 1 l round bottom flask using a temperature controlled round bottom heating mantle (MESE-Ltd) with continuous stirring by magnetic flea. 22.5 ml of 1 wt% sodium citrate solution was then added to the boiling chloroauric acid solution and heating continued under reflux for 2 h to enable reaction completion. The solution was left to cool overnight with stirring in order to yield citrate capped AuNPs.

To create PEGylated AuNPs, 10.68 μg ml⁻¹ (μg per ml of as synthesized colloidal gold) of free SH-PEG was dissolved in 1 ml of distilled water and added to the citrate capped AuNPs. As described in our previous publication, this level of PEG addition yields a partial PEG coating (~50% saturation) [12]. In this previous paper, UV/Vis analysis of the sample supernatant coupled with thermal gravimetric analysis of dried AuNPs was used to investigate saturation of the nanoparticle surface with PEG. The PEGylated solution was then left to stir for 8 h at room temperature after PEG addition, to allow for exchange of the citrate molecules with PEG.

The AuNP-PEG solutions were then collected and aliquoted evenly into 50 ml centrifuge tubes and centrifuged for 90 min at 13 500 RCF. 90% of the supernatant was then decanted and discarded leaving the AuNPs as a pellet in the bottom of the centrifuge tube. Each individual centrifuge tube was then refilled to the initial volume using either distilled water or a pH buffer as required and then vortexed to re-disperse the AuNPs.

Co-functionalization with peptide was achieved by returning the re-dispersed AuNP-PEG samples to a round bottom flask. While continually stirring, 4.45 μg ml⁻¹ of free RME peptide, 5.86 μg ml⁻¹ of free H5WYG peptide or 2.31 μg ml⁻¹ of free FITC labeled RGD peptide (dissolved in 1 ml of the relevant water/buffer) were added to the PEGylated AuNP. Using the molecular weight of the various peptides, this is the amount estimated to yield 50% surface saturation (based on a previous publication from our lab which coupled thermal gravimetric analysis with UV/Vis of the supernatant to determine the point at which the nanoparticle was saturated with 5000 Mw PEG) [14]. The samples were left for 8 h at room temperature to allow for complete reaction. The process of centrifugal washing was then repeated to remove any unattached reactants. The supernatant of the FITC labeled RGD peptide was retained for pl measurements. The samples are
named as follows: citrate capped AuNP, AuNP SH-PEG (PEGylated AuNPs), AuNP SH-PEG and RME, AuNP SH-PEG and H5WYG, AuNP SH-PEG and FITC-RGD.

Characterization of AuNPs

The AuNPs were characterized using TEM, FTIR, DLS, UV/Vis, pl, and BCA protein quantification assay.

Citrate, SH-PEG, SH-PEG and RME conjugated samples were dried on carbon coated copper grids and imaged using a JEOL 2100F transmission electron microscope at 200 kV and analyzed using ImageJ software. FTIR was obtained using a Varian 640-IR FTIR spectrometer analyzed using ImageJ software. FTIR was obtained using a Varian 640-IR FTIR spectrometer analyzed using ImageJ software.

Results

For size and zeta potential measurements, a Malvern Zetasizer Nano-ZS series (Worcestershire, UK) was used with disposable cuvettes of 2 ml and 700 µl respectively. DLS was conducted at 25°C using a refractive index of 0.310 and an adsorption of 1.00 at a measurement angle of 173°. The average sizes are reported as the DLS number diameter with an ‘n’ value of 9. pl was recorded using an in-house system. The software used was Solis 4.19.30 with acquisition mode DDG on, accumulation number 20, slit 500 µm, gain 50, transistor-transistor logic width 400, and a system temperature of ~10°C.

Peptide concentrations were calculated using a Micro BCA kit (Thermo Scientific Micro BCA Protein Assay Kit, Pierce Biotechnology). Standard protocols in the kits were used in this study and standard concentrations of BSA were used as the calibration standards across all peptide types. The experiments were performed in 96-well microplates in triplicate. After incubating the assays for 2 h, the absorbance was measured at 562 nm. The concentrations quoted were calculated using the BSA calibration curve.

TEM images for citrate capped, AuNP SH-PEG, AuNP SH PEG and RME capped AuNPs are shown in figure 2. The citrate capped AuNPs show an average particle size of 13.21 nm ± 1.31 nm by TEM. The PEG functionalized and RME co-functionalized samples do not show a significant increase in size (d) and (e) which illustrate the average diameters to be 14.3 nm and 16.5 nm respectively. This is expected as the acceleration voltage used is not sensitive to the organic coating.

FTIR spectra of the washed and dried AuNPs, AuNP SH-PEG, AuNP SH-PEG RME, AuNP SH-PEG H5WYG and AuNP SH-PEG RGD are shown in figure 3. Prominent differences are visible in the spectra for the PEG capped AuNP spectrum compared to that for the citrate capped AuNP. The presence of the mono-functional PEG (SH-PEG) on the AuNP SH-PEG is confirmed by the peaks associated with C=O stretching (1600 cm⁻¹) and bending (1380 cm⁻¹) and the presence of additional peaks relating to N–H bending (1600 cm⁻¹) and N–H wagging (600–900 cm⁻¹) [15]. The characteristic vibrations found in ethylene glycol moieties are observed at 1106 cm⁻¹ (C–O–C stretching) and a narrowing of the CH₂ stretching mode (2850 cm⁻¹) [13, 14, 34–36].

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S–Au bonds are expected to appear at 1777 or 1545 cm⁻¹. All conjugates present noise at approximately 1777 cm⁻¹. While the noise may indicate the presence of Au–S bonds it cannot be confirmed. The successful conjugation of all three peptides to the nanoparticle surface was confirmed by the appearance of new amide bands around 1670 cm⁻¹ and 1550 cm⁻¹ for amide I and II respectively. Amide II bands are mainly associated with the C=O stretching vibration relating to the backbone conformation, while Amide II bands result from N–H bending and C–N stretching. Co-functionalization was further confirmed through the observation of N–H wagging peaks (600–900 cm⁻¹) on the peptide functionalized samples, with a C–H bend at approximately 1376–1387 cm⁻¹ and the C–H alkene bend (992 cm⁻¹). Amide III bands found on the co-functionalized samples (approximately 1400–1300 cm⁻¹ region) are complex bands resulting from a mixture of several coordinate displacements. Successful attachment of the PEG was further confirmed by an increase in hydrodynamic area, by DLS after co-functionalization, from a size of 17.5 nm to approximately 35.4 nm.

UV/Vis is commonly used to investigate the aggregation states of colloidal gold. The unique optical properties of metal nanoparticles, which result from the SPR effect, offers a simple means to study size, functionalization and aggregation states with high sensitivity [37, 38]. AuNP aggregation is accompanied by a red-shift in the plasmon peak, as well as broadening of the adsorption peak, and a decrease in absorbance which can be easily monitored by UV/Vis spectroscopy [39]. An increase in size resulting from aggregation is also evident visually with a change in color of the solution from red to blue/purple [40]. Complete aggregation and settling out of the AuNP will result in a loss of color and diminution of the characteristic peak in UV/Vis. Each sample was measured after synthesis; following PEGylation, after redispersion in the various pH buffer solutions, and after peptide co-functionalization. We used the intensity and peak location (Amax) of the plasmon peak in the UV–vis spectra as indicators of AuNP aggregation state.

Figure 4 displays the UV/Vis spectra for AuNP SH-PEG which has been re-suspended in the various buffer solutions. In agreement with a number of publications the characteristic peak at 520 nm is clearly visible for all AuNP samples illustrating a degree of stability across the pH range tested [11, 37, 41, 42]. The results show that the PEGylated colloidal AuNP solutions have maintained a high degree of stability following centrifugal washing and re-dispersion in the buffer solutions. Extremely acidic pH were not measured,
as Das et al (2012) observed that the SPR band remained stable except at extreme acidic pH conditions. It was reported that at a pH of 1, the characteristic SPR peak was shifted to 537 from 527 nm and an extra peak at 649.80 nm was observed [43].

Figure 5 displays the UV/Vis spectra for AuNPs co-functionalized with PEG and either RME or H5WYG peptide in various buffers 24 h after functionalization. Tables 1 and 2 show the results in tabular form listing the peak positions and absorbance values of the samples, both before and after co-functionalization with peptide. The conjugation of these samples was investigated by comparing the UV/Vis spectra of each sample to the PEGylated AuNP prior to the peptide.
addition. Upon successful binding of ligands to the AuNP surface, the SPR spectra will red-shift by a few nanometers. This shift is a result of an increase in the local refractive index at the AuNP surface, and is consistent with peptide attachment. A very large upshift in peak position and a reduction in absorbance at the AuNP surface, and is consistent with peptide attachment. This shift is a result of an increase in the local refractive index and/or loss of stability. From figure 5 and table 1, it is apparent from the maintenance of a significant peak, that RME co-functionalized AuNPs demonstrated significant stability at all pH levels tested. Optimum co-functionalization was indicated via a characteristic shift in peak position occurring at a pH of 8, accompanied by a modest reduction in absorbance. In the case of the H5WYG, it can be seen that many of the buffers tested resulted in a very significant or complete loss of the plasmon peak, or a large upshift in peak position indicating aggregation. Only the samples functionalized at pH 6 and 8 displayed a small (~2–3 nm) upshift in peak position; however, pH 6 showed a large change in absorbance while pH 8 maintained significant absorbance, consistent with successful functionalization and stability. The sample prepared at pH 12 showed only a small drop in absorbance after peptide addition, but the upshift in peak position from 521.6–540 suggests a significant degree of aggregation. For both peptides a pH of 8 (underlined in tables 1 and 2) was concluded to be the optimum pH for successful cysteine conjugation of peptides to the nanoparticle surface. The reduced stability observed with the H5WYG peptide may be as a result of the lower net charge of +0.4 compared to 2.9 for the RME peptide. As the AuNPs are slightly negatively charged (~5 mV) after PEGylation the higher positive charge on the RME will result in more electrostatic attraction between the RME and PEGylated AuNPs. The H5WYG will also be less stable in solution as there will be little repulsion between the peptide chains due to their low net charge. In addition, the H5WYG peptide contains a higher proportion of hydrophobic residues (namely A, F, I, L, W and Y) than RME rendering the peptide less water soluble.

Figure 5. UV/Vis absorbance spectra for AuNP SH-PEG co-functionalized with (a) RME and (b) H5WYG for each buffer solution.

To confirm the optimum pH for peptide attachment via the cysteine residues as being approximately pH 8; a third peptide was investigated. The RGD Nrp-1 targeting peptide (CRGDK) was modified with the fluorescent ligand FITC. The AuNP SH-PEG and RGD sample was analyzed via UV/Vis in triplicate and the spectra’s obtained are plotted in figure 7. The UV/Vis spectra for pH 7, 8 and 8.6 display a peak at 495 nm corresponding to the FITC and the characteristic peak at ~520–525 nm, for AuNP. For the remaining buffer solutions, due to poor stability, the FITC peak dominates the spectra [44, 45].

Initially, pH 7, 8 and 8.6 maintains the highest absorption profile with respect to both AuNP and FITC. For pH values of 6 and 9–12 there is no AuNP signal present, overall there is a gradual decrease in the fluorescent intensity due to the absorbed FITC as the pH drops towards 6. However, after 24 h the absorption profile for pH 7 and 8.6 greatly diminishing with only the sample prepared at a pH of 8 retaining the characteristic profile of AuNP at 520 nm. For the remaining samples only the absorption peak at approximately 495 nm is apparent. It is noteworthy that a loss of stability was not observed at the pH of 8.6. This may be due to the high solubility of the peptide. Short peptides are inherently highly water soluble and the sequence contains a high proportion of hydrophilic residues and no hydrophobic residues. The addition of the water soluble FITC tag may also have increased the stability of the peptide at the pH.
Table 1. Changes in peak position and absorbance for each pH for AuNP SH-PEG and RME.

<table>
<thead>
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<th>pH</th>
<th>SH-PEG and Buffer</th>
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<td>0.92</td>
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<tr>
<td>11</td>
<td>521.9</td>
<td>0.40</td>
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Table 2. Changes in peak position and absorbance for each pH for AuNP SH-PEG and H5WYG.

<table>
<thead>
<tr>
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<th>PEG, Buffer and H5WYG</th>
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<td>11</td>
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</table>

Figure 6. AuNP SH-PEG RME and AuNP SH-PEG H5WYG co-functionalized AuNPs in each buffer solution.
Figure 8 shows the fluorescence spectra of the supernatants after centrifugal removal of the FITC labeled RGD and SH-PEG capped AuNPs. After co-functionalization with the RGD peptide in a range of buffer solutions, the samples were centrifuged in 1.5 ml Eppendorf’s at 10 000 RPM for 30 min, to remove the co-functionalized AuNPs [40]. The amount of unbound free FITC labeled RGD peptide following centrifugation was determined from the fluorescent intensity of FITC left in the supernatant. A clear emission peak was noted around 520 nm for all sample types. This is typical for green fluorescein FITC [44, 45].

For the samples prepared using pH 9–12 there were high intensities of fluorescing FITC found in the supernatant, consequently indicating large amounts of unbound peptide. Whereas the pH 6–8.6 samples had significantly lower intensities, with pH 7 and 8 having the lowest intensity. These results confirm optimal attachment at pH values of 7 and 8 reaffirming the result observed with the RME and H5WYG peptides, however due to the instabilities identified previously, of the buffers tested, pH 8 has been identified as being the most effective for attaching the peptide.

pI could not be used to confirm binding for the remaining two peptides due to the lack of a fluorescent tag, therefore to confirm optimized binding efficiency at pH 8 for the RME and H5 peptides; protein estimation was carried out using a Micro BCA Protein Assay Kit for the RME and H5WYG peptides at a pH range of 6, 8 and 10. Although biuret based protein assays are theoretically applicable to peptide measurement, there is a high level of inter-peptide variation, determined largely by peptide hydrophobicity. This variation in peptide reactivity can be significantly reduced by heat-denaturation of peptides at ~95 °C for 5 min in the presence of 0.1 M NaOH containing 1% (w/v) SDS, Therefore prior to incubation with BCA, each peptide was denatured at 90 °C [46].

1 ml of each sample was aliquoted into a 1.5 ml Eppendorf tube and centrifuged at 10 000 RPM for 30 min, 900 μl of the supernatant was then removed and labeled as the supernatant counterpart to be measured. The remaining pellet was re-dispersed. 100 μl of each peptide ‘as synthesized’, and their respective supernatants after centrifugation, were then pipetted into a 96 well plate in quadruplicate. After incubating the assays for the time specified in the kit; the absorbance was measured at 562 nm and the OD was converted to concentration (μg ml⁻¹) using the standard curve generated from BSA. The results for both peptides are displayed in table 3.

Approximately 19% and 18% of the H5 peptide added was measured in the supernatant for the samples functionalized at a pH of 6 and 10 respectively; whereas only 10% of the overall peptide added was found to be present in the supernatant for pH 8. Thus, indicating that the remaining 5.24 μg ml⁻¹ had successfully attached to the AuNPs, in comparison to the 4.64 μg ml⁻¹ and 4.59 μg ml⁻¹ for pH 6 and 10 respectively. A similar trend was observed for the RME peptide. Lower attachment percentages were observed in general for RME peptide in comparison to the H5WYG, however it was still apparent that the most efficient attachment occurred at a pH of 8. When combined with the pI data this confirms that for the pH levels tested, a pH of 8 results in the most efficient attachment for all three peptides.

After a period of 28 d under dark ambient conditions (figure 9), the AuNP solutions were imaged to monitor long term stability, with changes in solution color indicative of aggregation. The AuNPs functionalized with either RGD or H5WYG peptide at a pH of 8 remained stable over 28 days. Whereas samples prepared at a pH distant from this optimum...
value displayed either a change in color or a reduction in intensity. The long term stability of RME capped AuNP-PEG was less sensitive to the pH used during capping.

**Discussion**

The binding efficiency of a ligand to a nanoparticle surface depends upon a range of parameters including the nanoparticle size, the length of the ligands, the rotational flexibility of the bond, steric effects as well as the type of bonding used in the coupling chemistry [40, 47]. Whilst coupling chemistry is often considered as the dominant factor affecting ligand binding, it has been reported that in the case of lectin that ligand arrangement can profoundly affect binding and stability. As previously reported there are several strategies for conjugation of peptides and proteins to AuNPs resulting in distinct types of binding. Van der Waal forces and hydrogen bridges result in weak binding forces between the conjugate and nanoparticle (NP), whereas covalent bonding produces stronger conjugation. For any of these binding modes to occur, repulsion between the NP and ligand should be minimized in a manner that maintains repulsion between the individual particles, to minimize aggregation. This can be achieved by comparing the pIs and selecting a pH where materials being joined have opposite surface charges, but are still charged, as uncharged material reduces repulsion and can lead to precipitation of the uncharged component. In the case of proteins being conjugated, consideration of the pI of the peptide in isolation may be erroneous as proteins contain a range of functional groups that can form different bonds at the NP surface. Thus the dissociation constant (pK) of the various functional groups in each amino acid sequence should be considered. The pK values for the reactive groups have been reported as 1.71, 8.37 and 10.78 for carboxyl, ammonium and thiol groups respectively [48]. As such in the pH range investigated carboxylic acids will predominantly be dissociated whereas both amino and sulphur resides will be predominantly protonated and available for surface binding. Compared to the other free reactive groups present thiol has a much higher binding affinity to Au. Thiol molecules are chemisorbed on Au by strong thiolate–Au bonds (40–50 kcal mol$^{-1}$) compared to NH$_2$ forming weak covalent binding with Au–N bond strengths estimated to be 8 kcal mol$^{-1}$ [49, 50]. It is suggested that all three peptides bind to the AuNP surface via the free thiol of the cysteine residues [51, 52]. This binding was observed be optimized by adjusting the pH to 8 which results in the protonation of the thiol group. Since all three peptides bind via the same chemistry, it is the appropriate protonation of the thiol group which determines the optimum pH for binding, and not the overall pI value of the peptide. However, it may not always be possible to create stable peptide modified AuNPs by selecting a pH in the region of 8. If this pH is close to the pI value of the peptide used for surface modification the material may show limited stability under such conditions. The net charge and water solubility of the peptide used in surface modification are also important factors contributing to protein functionalized AuNP stability. It was observed that proteins with a higher positive net charge displayed a higher degree of stability. This could be expected given a higher positive net charge will result in greater attraction between a protein to the negatively charged AuNP leading to increased protein loadings. Likewise surface functionalization with proteins with

<table>
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<th>pH</th>
<th>As synthesized ($\mu$gm l$^{-1}$)</th>
<th>Supernatant ($\mu$gm l$^{-1}$)</th>
<th>% in supernatant</th>
</tr>
</thead>
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<td>6</td>
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<td>1.03</td>
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<tr>
<td>pH</td>
<td>As synthesized ($\mu$gm l$^{-1}$)</td>
<td>Supernatant ($\mu$gm l$^{-1}$)</td>
<td>% in supernatant</td>
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</tbody>
</table>
larger net charges can lead to greater stability in solution due to the larger electrostatic repulsions between peptide chains.

**Conclusions**

pH plays a critical role in controlling peptide binding and subsequent protein modified AuNPs stability; PEG capped AuNPs are stable between pH values of ∼6–12. Peptides containing a cysteine residue can be readily bound to the PEG capped AuNPs by means of the thiol group on the cysteine. Adjusting the pH to ∼8 optimizes this binding, but care should be taken to avoid the pl of the peptide as this may lead to instability. This method of peptide attachment can be used to create AuNPs capped with PEG and one or more peptides, allowing the creation of stable AuNPs with the desired biological activity.

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**Disclosure**

The authors report no conflicts of interest in this work.

**Authors’ contributions**

Emma Harrison: was responsible for the synthesis and characterization (apart from TEM analysis) of all the samples listed previously and the interpretation of the results. With assistance from D Dixon she was also responsible for producing a draft of the manuscript.

Jeremy Hamilton: assisted with interpretation of the results and drawing conclusions drawn from the study.

Manuel Macias-Montero: was responsible for the TEM imaging and analysis.

Dorian Dixon: was responsible for the proposed research plan for this study and oversaw the writing and the interpretation of the results.

All authors were involved in the review of the final draft of this paper.

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