Direct chemoselective synthesis of glyconanoparticles from unprotected reducing glycans and glycopeptide aldehydes†

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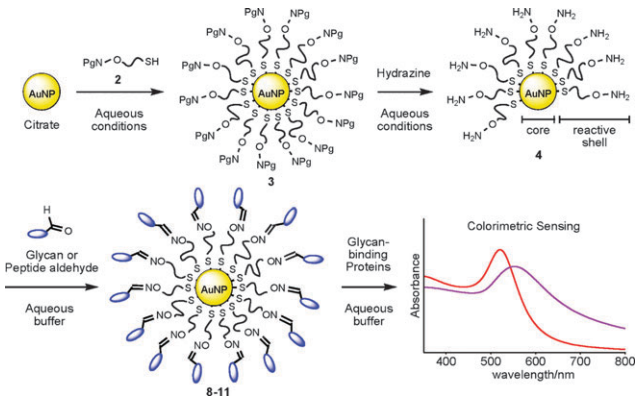
Chemoselective oxime coupling was used for facile conjugation of unprotected, reducing glycans and glycopeptide aldehydes with core–shell gold nanoparticles carrying reactive aminooxy groups on the organic shell.

Covalently bioconjugated nanoparticles, *e.g.* gold nanoparticles (AuNPs), are emerging as tools for detection and modulation of specific interactions between biomolecules. Such systems have been utilized in applications involving *e.g.* oligonucleotide–oligonucleotide,[1] peptide–protein,[2,3] glycan–protein,[4,5] glycan–glycan,[6] and antibody–antigen[7] interactions. Glyconanoparticles are a particularly attractive platform due to the inherent multivalent presentation required for most glycan binding events coupled with facile detection.[8] Here, a protective *shell*, typically consisting of a monolayer of hydrophilic oligomers such as oligo(ethylene glycol) (OEG), is important for optimally presenting the glycan ligands as well as encapsulating the nanoparticle *core* to reduce non-specific interactions and associated aggregation of nanoparticles. Gold glyconanoparticles (glyco-AuNPs) have so far mainly been synthesized either (i) by *in situ* formation of AuNP cores in the presence of *thiol-modified* glycans *via* modifications of the Brust–Schiffrin protocol,[9] or (ii) by introduction of *thiol-modified* glycans onto preformed AuNP cores *via* self-assembly[10] on citrate-stabilized AuNPs[11] (citrate-AuNPs).

Here we present the realization of a novel concept for synthesis of glyco-AuNPs where *unmodified* glycans are anchored *via* a single chemoselective reaction onto the outer nanoparticle shell of reactive core–shell AuNPs. Only a few examples of coupling of glycans to functionalized nanoparticles have thus far been reported, and these cases require multi-step synthetic modifications of the glycans prior to conjugation.[12] We report now that oxime coupling on terminal aminooxy groups allows anchoring of aldehyde-containing biomolecules, specifically unprotected, reducing glycans, peptide aldehydes, and glycopeptide aldehydes, under mild conditions and in aqueous solution thus allowing broad access to glyco-AuNPs (Scheme 1).

The present methodology is based on water-soluble citrate-AuNPs to allow installation of the reactive OEG shell under aqueous conditions. The prerequisite is a novel protecting group approach able to (i) prevent the intermediate, functionalized AuNPs from aggregating in aqueous solution and (ii) allow quantification of ligand density (Scheme 2A). For this purpose we applied a 4-carboxy-phthaloyl protecting group (here abbreviated "Trim" for trimellitoyl), which allows deprotection of aminooxy groups under extremely mild, aqueous conditions without compromising nanoparticle integrity and which enables quantification by HPLC. Self-assembly in the absence of the Trim group (*i.e.* with fully unprotected bifunctional linker) resulted in extensive aggregation. Thus, OEG linker **1**,[5] based on tetra(ethylene glycol) and carrying a tritylated thiol and a free aminooxy group, was protected using trimellitic anhydride with microwave heating[13] in 91% yield. The trityl group was then removed followed by self-assembly of OEG linker **2** onto the surface of citrate-AuNPs (diameter ~12 nm). The functionalized nanoparticles, **3**, were then thoroughly washed, and a comparison of the visible spectrum with that of citrate-AuNPs (see ESI†) showed a shift in surface plasmon band maximum, λ_{SP}, of 4 nm, indicative of thiol self-assembly.[5] Release of the Trim group as the phthalazinedione **5** by treatment of AuNPs **3** with aqueous hydrazine (1 mM) was quantified by HPLC. After 24 h of reaction there was no further increase in the concentration of released **5**, yielding aminooxy-terminated core–shell AuNPs **4** with a *ligand density*, *i.e.* average number of reactive linkers/nanoparticle, of ~435, corresponding to a *surface coverage*, *i.e.* average number of reactive linkers/nm^2 gold surface, of 0.88 (see ESI†). The obtained density is comparable to that obtained in previous studies involving similar nanoparticle cores.[2,5,14]

The peptide aldehyde[15] Fmoc-Glu-Glu-Gly-Gly-H was used for a detailed study on the efficiency of oxime coupling reactions on the OEG shell of gold nanoparticles. The design



Scheme 1 Strategy for nanoparticle functionalization and detection of protein–glycan interactions. Pg = trimellitoyl (Trim).

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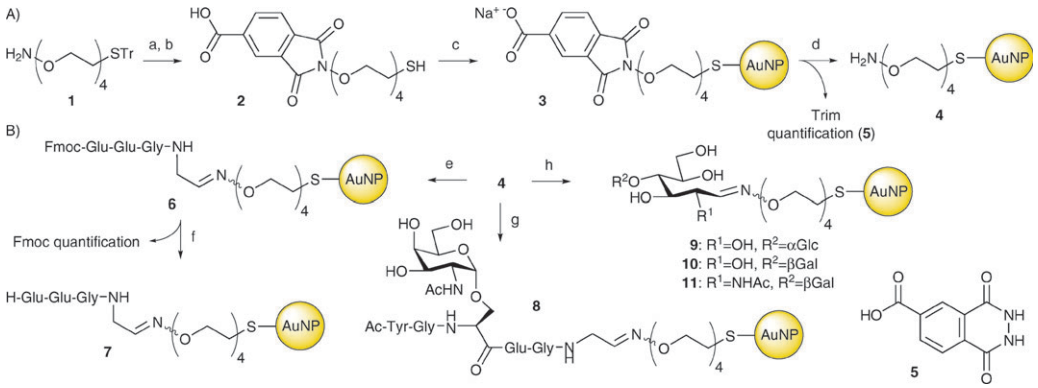

Scheme 2 Synthesis of aminooxy-terminated core–shell AuNPs **4***via* transient Trim protection (A), and chemoselective peptide, glycopeptide, and glycan functionalization (B). Conditions: (a) trimellitic anhydride, pyridine, 180 °C, 30 min, 91%; (b) TFA, triethylsilane, CH_2Cl_2, quant.; (c) citrate-AuNPs, H_2O; (d) 1 mM hydrazine (aq), 24 h; (e) Fmoc-Glu-Glu-Gly-Gly-H (1.25 mM), various temperatures and reaction times, see Table 1; (f) piperidine–H_2O 1 : 1, 20 min; (g) Ac-Tyr-Gly-Ser(αGalNAc)-Glu-Gly-Gly-H (1.25 mM), 40 °C, 16 h; (h) conditions I: glycan (0.5 M), 40 °C, 16 h; or conditions II: glycan (5 mM), 4-anisidine (5 mM), 40 °C, 16 h.

of the peptide incorporates in a simple form (i) a C-terminal aldehyde functionality, (ii) an Fmoc protecting group for spectrophotometric quantification of oxime coupling yields, and (iii) two carboxylate groups for aqueous solubility and net negative charge.[2,16] The feasibility of Fmoc cleavage from AuNPs was initially confirmed in experiments with piperidine–H_2O 1 : 1 on peptide conjugated AuNPs **6**, that had been prepared from a pre-assembled peptide linker conjugate of Fmoc-Glu-Glu-Gly-Gly-H and OEG linker **1** in solution (data not shown). We then applied novel oxime coupling with Fmoc-Glu-Glu-Gly-Gly-H on aminooxy-terminated core–shell AuNPs in phosphate buffer (Scheme 2B, pathway e) under various conditions. The efficiency of the oxime coupling reaction was monitored by Fmoc quantification (Scheme 2B, pathway f, and Table 1). The coupling was essentially quantitative when the reaction was performed at 40 °C for 16 h; however, at room temperature (16 h) and under microwave heating at 60 °C (1 h) the reaction did not proceed to completion. Coupling with an analogous octa(ethylene glycol) linker provided similar results (data not shown). In a control experiment, thiolytic release[5] of the organic shell after oxime coupling, and analysis by HPLC-MS confirmed that peptide oxime bonds were indeed formed on the OEG shell (data not shown). Additionally, oxime coupling with the peptide aldehyde was corroborated by dynamic light scattering measurements, which showed an increase in hydrodynamic radius, as well as by a spectrum subtraction of AuNPs before (**6**) and after (**7**) Fmoc deprotection, which clearly revealed a characteristic Fmoc signal in the UV-Vis spectrum of AuNPs **6** (see ESI†). Transmission electron microscopy (TEM) analyses confirmed that gold cores were unchanged during installation and functionalization of the reactive shell (see ESI†).

Table 1 Oxime coupling with Fmoc-Glu-Glu-Gly-Gly-H on aminooxy-terminated core–shell AuNPs

Entry	Conditions	Ligand density (peptides/AuNP)	Surface coverage (peptides/nm^2)	Yield (%)[a]
1	25 °C, 16 h	157	0.32	36
2	60 °C[b], 1 h	162	0.33	37
3	40 °C, 16 h	427	0.86	98

[a] Calculated from Fmoc quantification relative to Trim quantification. [b] By microwave heating.

Using the established conditions for peptide oxime coupling, we investigated glyco-AuNP generation with the glycopeptide aldehyde Ac-Tyr-Gly-Ser(αGalNAc)-Glu-Gly-Gly-H containing the Tn antigen (Scheme 2B, pathway g).[17] After extensive washing of resulting AuNPs **8** with phosphate buffer to remove excess glycopeptide, a colorimetric assay with the αGalNAc-binding protein *Helix aspersa* agglutinin (HAA) was performed by monitoring the changes in λ_{SP} relative to control protein (BSA). The surface plasmon band shift values, $\Delta\lambda_{SP}$, of lectin-induced aggregation followed apparent first order kinetic profiles in $\Delta\lambda_{SP}$.[18] Glycopeptide-functionalized AuNPs **8** displayed a dramatic shift, $\Delta\lambda_{SP}$, of 16 nm in the presence of 5 μM HAA relative to BSA (<1 nm) (Fig. 1A), confirming the correct assembly of glycopeptide AuNPs **8**.

Encouraged by these results, we applied oxime coupling for anchoring of glycans with subsequent colorimetric detection of lectin interactions. We have previously demonstrated that carbohydrate oximes, when presented on AuNPs, may be recognized by lectins with a very high degree of specificity.[5] Thus, aminooxy-terminated core–shell AuNPs **4** were reacted with unprotected, reducing glycans (maltose, lactose, and *N*-acetyllactosamine) under two different sets of conditions to promote glycan oxime formation: (i) a vast excess (0.5 M) of glycan (conditions I),[19] or (ii) nucleophilic catalysis by 4-anisidine with 5 mM glycan (conditions II),[20] both at 40 °C for 16 h (Scheme 2B, pathway h). After extensive washing of AuNPs **9–11** with phosphate buffer to remove excess glycans, colorimetric assays were performed directly by addition of various lectins and monitoring of $\Delta\lambda_{SP}$. The terminal glucose-binding lectin Concanavalin A (Con A) from *Canavalia ensiformis* and the terminal galactose-binding lectin *Erythrina cristagalli* agglutinin (ECA) were used to assess glycan functionalization. The apparent first order rate constant, k_{obs}, and the maximal surface plasmon band shift, $\Delta\lambda_{SP,max}$, were used to describe the aggregation process (see ESI†).[18] Maltose-functionalized **9**(conditions I) and

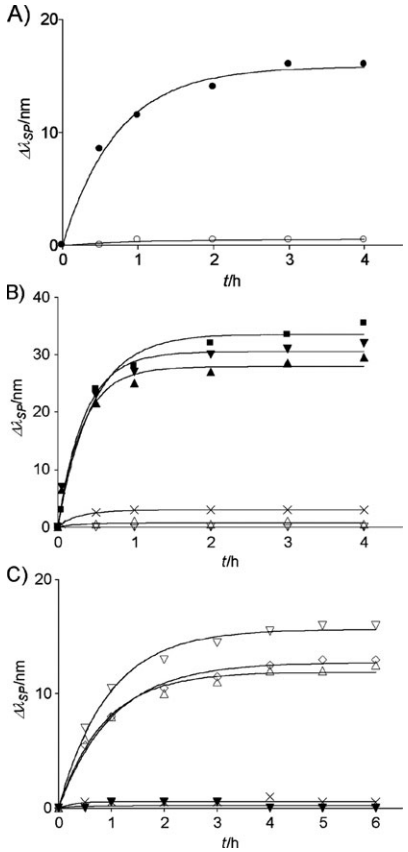

Fig. 1 Colorimetric detection assays for HAA (●) and BSA (○, control) with glycopeptide-functionalized AuNPs **8** (A), and Con A (B) and ECA (C) with glyco-AuNPs **9–11**. Legend: **4** (×, control), **9**[5] (■), **9**(I) (▲), **9**(II) (▼), **10**(I) (△), **10**(II) (▽), **11**(II) (◇); (I): conditions I, (II): conditions II.

9(conditions II) displayed similar $\Delta\lambda_{SP,max}$ of ~30 nm and k_{obs} of ~3 h^{-1} with Con A (5 μM), essentially identical to previously reported maltose oxime glyco-AuNPs (**9**, $\Delta\lambda_{SP,max}$ ~33 nm and k_{obs} ~2.5 h^{-1}, ligand density ~300 glycans/nanoparticle[5]), indicating that glycan oxime formations proceeded to high degrees of conversion under both conditions (Fig. 1B). The detection limit for Con A was ~100 nM (see ESI†). Lactose-functionalized AuNPs, **10**(conditions I and II), were completely devoid of a shift in λ_{SP} with Con A ($\Delta\lambda_{SP,max}$ <1 nm), as expected. In the presence of ECA (5 μM) the selectivity was completely opposite; lactose AuNPs, **10**(conditions I and II), showed $\Delta\lambda_{SP,max}$ of ~14 nm and k_{obs} of ~1 h^{-1}, whereas maltose AuNPs **9**(conditions I) were completely devoid of a shift ($\Delta\lambda_{SP,max}$ <1 nm) (Fig. 1B). Interestingly, oxime coupling with the less reactive *N*-acetyllactosamine and subsequent lectin recognition was comparable to that of lactose, as judged by the similar profiles of **11**(conditions II) and **10**(conditions I and II). Control experiments with aminooxy-terminated AuNPs **4** showed only minute shifts in λ_{SP} of ~3 nm and <1 nm upon treatment with Con A and ECA, respectively (Fig. 1A and B).

In summary, we have developed a novel method for anchoring of unmodified, reducing glycans and glycopeptide aldehydes directly to AuNPs with a reactive core–shell architecture.[21] This approach avoids synthetic modifications of glycans prior to conjugation and allows direct evaluation of *e.g.* glycan–protein interactions with high specificity. Additionally, the methodology allows quantitation of the ligands installed in the shell. We envision that the methodology may be applied for synthesis of glyco-AuNPs of complex glycans and glycopeptide aldehydes, as well as in a parallel format to facilitate rapid screening of biomolecular interactions.

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