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Preparation of multifunctional glyconanoparticles as a platform for potential carbohydrate-based anticancer vaccines

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Dedicated to the memory of Professor Nikolay K. Kochetkov

Abstract—A novel platform for anticancer vaccines has been prepared using glyconanotechnology recently developed in our laboratory. Ten different multifunctional gold glyconanoparticles incorporating sialylTn and Lewis^y antigens, T-cell helper peptides (TT) and glucose in well defined average proportions and with differing density have been synthesised in one step and characterised using NMR and TEM. Size and nature of the linker were crucial to control kinetics of S–Au bond formation and to achieve the desired ligand ratio on the gold clusters. The technology presented here opens the way for tailoring polyvalent anticancer vaccines candidates and drug delivery carriers with defined average chemical composition.

Keywords: Gold glyconanoparticles; Carbohydrate antigens; Synthesis; Multifunctional nanoparticles; Synthetic vaccines

1. Introduction

Metallic nanoclusters functionalised with biomolecules have been a subject of sustained interest for several years.[1] Integrated nanoparticle–biomolecule multifunctional systems constitute useful tools to mimic the behaviour of biomolecules in cells thus helping to explore the mechanisms of biological processes with a variety of potential applications.[2] The preparation of gold nanoparticles protected with self-assembled monolayers of carbohydrate antigens (glyconanoparticles, GNPs) was first reported by us in our continuous search for multivalent systems to prove and evaluate carbohydrate–carbohydrate interactions.[3] These GNPs, which are easily constructed by reducing a gold salt[4] in the presence of thiol functionalised synthetic neoglycoconjugates, are extremely small, water soluble, stable to glycolytic enzymes and can be manipulated as biological macromolecules.[3] Interestingly enough, these GNPs show a permanent magnetism at room temperature, the origin of which is presently being investigated.[5] A review on the preparation, characterization and applications of GNPs has recently appeared.[6]

GNPs provide a multivalent glycocalix-like carbohydrate display with a well defined average chemical composition. They have been used to prove[3a,7] and to quantify[8] adhesion forces between carbohydrate antigens and to interfere with carbohydrate–carbohydrate interaction mediated biological processes.[9] The simple and versatile method of preparation of these biofunctional gold nanoclusters[3,4] allowed to prepare constructs in which the metallic core is protected with mixed monolayers of different carbohydrate and noncarbohydrate ligands including fluorescence probes.[3b] Other authors have used the same synthetic approach to prepare nanoparticles also comprising of a mixed monolayer with functional groups for specific binding to macromolecules.[10]

We now have explored the scope of this preparative strategy by constructing multifunctional gold GNPs

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protected with mixed monolayers of different tumour associated carbohydrate antigens and immunogenic peptides, in well defined average proportions and with differing density, as a new platform for potential anticancer vaccines. To our knowledge, these multifunctional GNPs are the most complex biofunctional nanoclusters that have been prepared so far. In this communication, we report on the preparation and characterization of these multifunctional nanostructures and present preliminary data on the properties of these constructs as potential anticancer vaccine candidates.

Carbohydrates are T cell independent antigens and most carbohydrate based vaccines are conjugate systems in which oligosaccharide or polysaccharide antigens are covalently linked to immunogenic structures.[11] Polysaccharide–protein conjugates are being effectively used in vaccination strategies against bacterial infections.[12] On the other hand, intensive work has been underway for several years to exploit the over expression of tumour associated oligosaccharides to develop anticancer vaccines.[13] In this case, synthetic tumour associated oligosaccharide epitopes are conjugated to a carrier protein, which provides T cell help required for antibody production.[11] A variety of strategies to present these oligosaccharide epitopes for inducing sufficiently strong helper T cell responses have been developed and impressive advances have been achieved in the synthesis of tumour associated glycopeptide and glycolipid structures to construct potential vaccine candidates.[13e,14] While for glycolipids or glycopeptides with large tumour associated oligosaccharide epitopes, single molecule presentation seems to be sufficient for antibody recognition, short haptenic molecules, such as the disaccharide antigen sialyl-Tn (sTn), appear to require being presented as clusters.[15] It has also been observed that even for larger molecules, such as oligosaccharides containing the tetrasaccharide Lewis^y epitope, clustering of the glycodomain is important for antibody production.[16] In this connection, it has been anticipated that multivalent structures may provide more antibody density than monovalent immunizing agents, and that increasing the number of tumour epitopes should result in a broader degree of protection against multiple cancers.[17] Also, incorporating different antigens in a single clustered format has been proposed for the construction of unimolecular vaccines of defined chemical structures.[18]

It has been shown that antigens covalently conjugated to solid core carboxylated polystyrene microspheres of narrowly defined size (0.04–0.05 μm) induce high antibody titres in mice.[19] We envisioned that GNP technology[3] could provide a platform for potential anticancer vaccines if conditions were found to include, in a controlled manner, tumour associated oligosaccharide epitopes and T cell helper peptidic components in the self-assembly process. Therefore, we have investigated the self-assembly of gold nanoclusters comprising mixed monolayers of sTn and Lewis^y antigens in various proportions, a peptide from tetanus toxoid (TT) and glucose as an inert component to control the density of the antigens in the final construct. The sTn epitope is a mucin associated antigen expressed on a variety of epithelial cancer cells[20] and Lewis^y has been identified as an antigen for eliciting antibodies against colon, liver, prostate and ovarian carcinomas.[21]

2. Results and discussion

Neoglycoconjugates **1**–**3** (Chart 1) were synthesised and equipped with an appropriate thiol ended spacer group as previously reported.[3] A C_2 aliphatic spacer for the glucose neoglycoconjugate **1** and a C_5 aliphatic linker for the tumour associated carbohydrate epitopes (**2** and **3**[22]) were chosen. The peptide ligands **4** and **5**, comprising of the sequence FKLQTMVKLFNRIKNNVA, linked through the amino terminal group to a thiol ended C_{11} aliphatic spacer (**4**) or to a mixed hexaethylene glycol-C_{11} aliphatic spacer (**5**), were synthesised on solid phase. The first two amino acids of this polypeptide were included for their ability to be hydrolysed by lysosomal proteases[23] and the remaining 16 amino acids represent αα89–105 from TT.

Neoglycoconjugate **2** was synthesised containing an α oriented thiopentyl spacer group at the reducing end of the disaccharide compound. To this purpose the synthesis was carried out from easily available 2-acetamido-2-deoxy-D-glucose instead of from more expensive *galacto*-derivative. Inversion of configuration at C-4 position of the *gluco*- compound[24] led to pentenyl galactopyranoside **9** in good yield (Scheme 1). Finally, acceptor **10** was prepared by addition of thiolacetic acid to the olefin **9**, catalysed by azobisisobutyronitrile (AIBN), also in good yield.

For α-sialylation[25] of compound **10**, chloride donor **11**[26] was used as depicted in Scheme 2. The yield for the α-anomer was poor and attempts to improve yield and stereoselectivity using more elaborated sialic acid donors,[27] also failed. However, the obtained pure material was enough to continue with the preparation of **2** and we concentrated on the preparation and characterization of the GNPs rather than improving this synthetic procedure. Deprotection of fully protected compound **12** was accomplished in aqueous basic media to obtain neoglycoconjugate **2** in good yield (Scheme 2).

Using these components (**1**–**4**) in different ratios, 10 GNPs (**GNP1**–**GNP10**) protected with self-assembled mixed monolayers comprising the different ligands in various proportions (Chart 2 and Table 1) were prepared. Glucose was a major component of all GNPs and all of them contained a low density of TT peptide ligand (3% of the total monolayer). The proportions of the two carbohydrate epitopes in the mixed monolayer

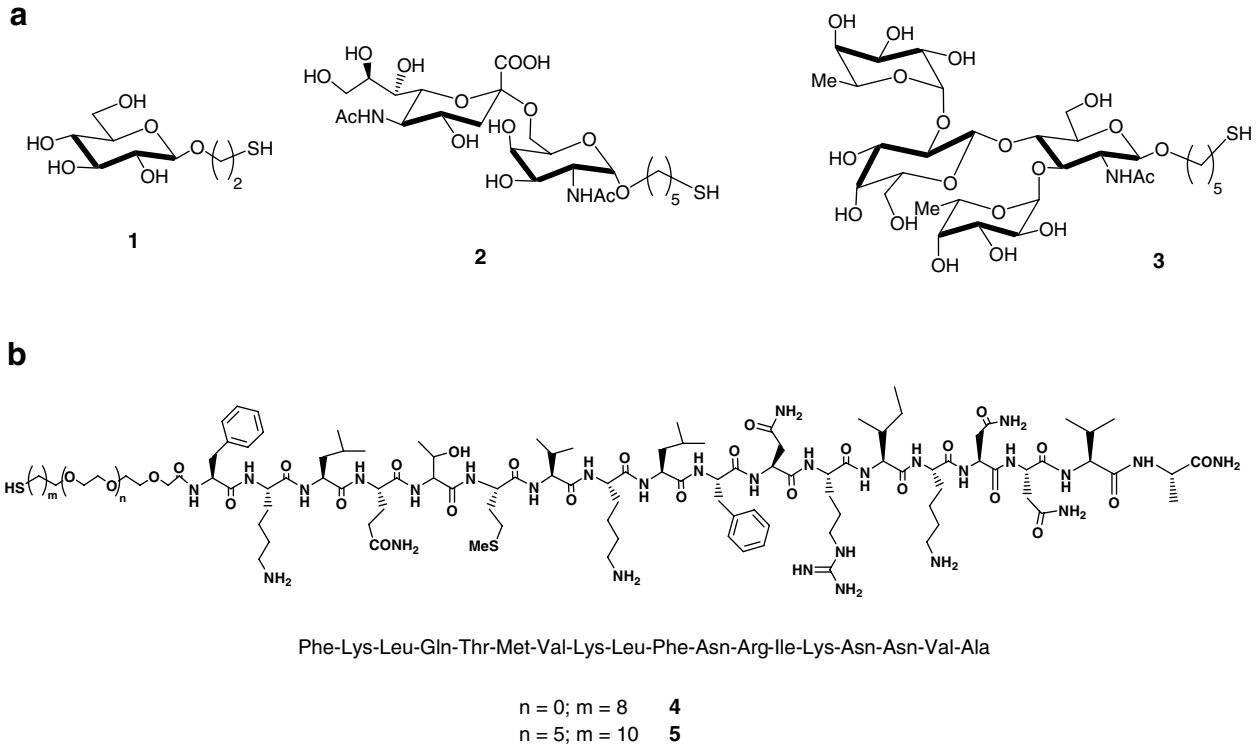
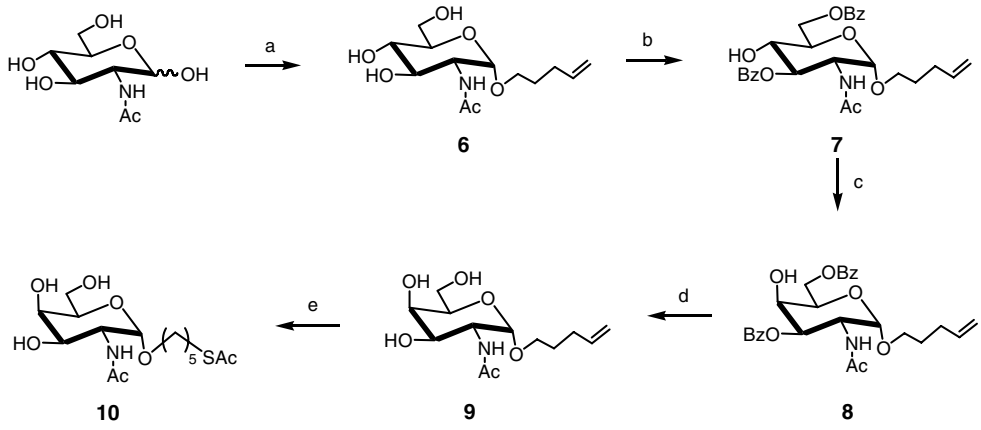


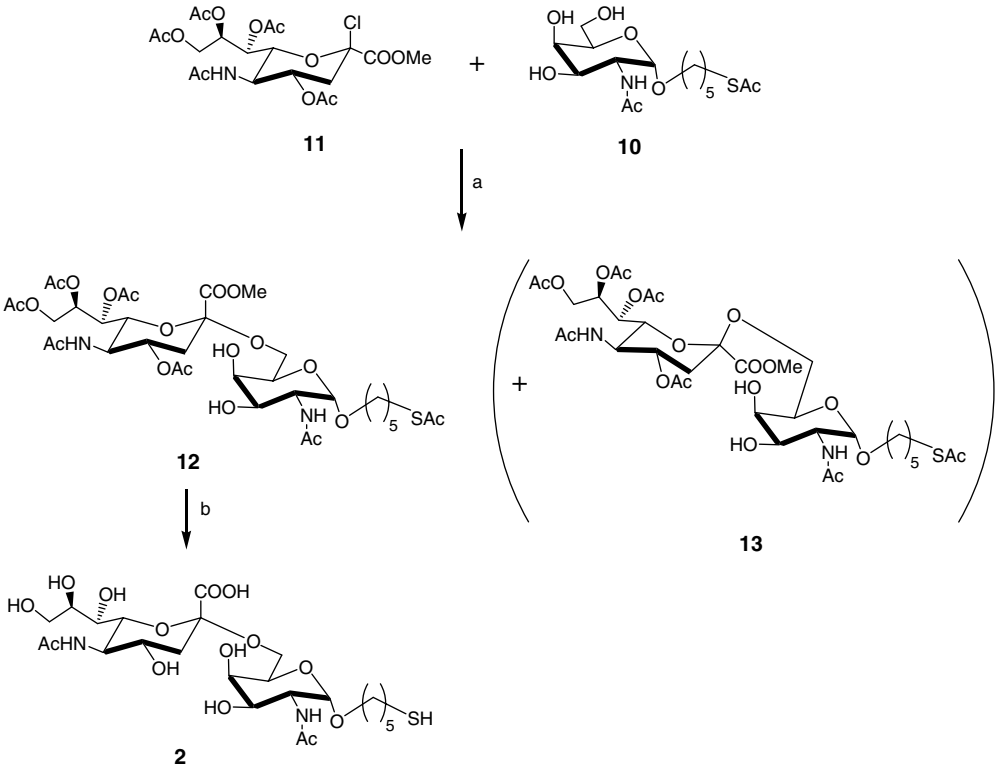
Chart 1. (a) Neoglycoconjugates **1**–**3**; (b) TT peptide ligands.



Scheme 1. Reagents and conditions: (a) IR-120H$^+$, 4-pentenol; (b) BzCl, Py, CH_2Cl_2, −50 °C; (c) i. Tf_2O, Py, CH_2Cl_2, −15 °C, ii. $NaNO_2$, DMF; (d) MeONa/MeOH, CH_2Cl_2; (e) AcSH, AIBN, THF, 65 °C.

ranged from 3% to 30% (Table 1). The composition of the mixed monolayers comprising the various ligands could be controlled by adjusting the initial concentration of the components in the self-assembling process.

As mentioned above, the reactivity of the three neoglycoconjugates (**1**–**3**) and the peptide conjugates (**4** and **5**) equipped with this set of linkers permitted the initial ratio of the different components in the reaction mixture to be conserved in the final construct. The ratio of the different ligands on the nanocluster surface could be assessed by comparison of the ^{1}H NMR spectra of the initial mixtures, the formed GNPs and the recovered mother liquors after the self-assembly process (see Supplementary data). In some cases, some proportion of the peptide ligands (**4** or **5**) in excess precipitated after the nanoclusters had been formed and the amount of the peptide component remaining in the mother liquors after nanoparticle formation could not be assessed from the NMR spectra. However, the signals corresponding to other components present in the mother liquors indi-



Scheme 2. Reagents and conditions: (a) DTBP, AgOTf, THF; (b) NaOH aq, MeOH.

cated a ligand ratio almost identical to that of the initial mixture (Fig. 1).

The obtained GNPs were water soluble and stable for months in solution. They were characterised by transmission electron microscopy (TEM) (Fig. 2). The mean gold core diameters ranged from 2.25 to 1.45 nm (Table 1 and Supplementary data), which corresponds to an average of 309 and 116 gold atoms, respectively.[28] In theory, gold nanoclusters of this size can bind between 92 and 53 thiol chains to their surface (Table 1). As an example, **GNP1** with a 28/1/1 glucose/sTn/peptide ratio and a mean core diameter of 2.25 nm would have an estimated number of 92 thiol chains, which would correspond to approximately 86 glucose, 3 sTn and 3 peptide ligands.

These multifunctional GNPs are being tested in vivo using 8-week-old female Balb/c mice and various experimental protocols. Preliminary data indicate that reactive antisera are generated that detect sTn/Le^y epitopes on the surface of the nanoparticles. The results will be reported in due course.

In conclusion, these results provide experimental evidence of the potential of the GNP technology. Multifunctional hybrid nanoparticles comprising up to more than four different ligands with well defined average chemical composition can be prepared in a one-step procedure. Under carefully controlled experimental conditions, the type, the ratio and the density of carbohydrate antigens and T-cell helper peptides on the multivalent construct can be varied in a straightforward way during the self-assembly process. This methodology allows the tailoring of complex bio-functional nanoclusters incorporating a set of different ligands in a controlled way and it complements the dynamic place-exchange reaction. In the place-exchange reaction, the number of exchanged ligands is determined by the nature of the gold cluster previously formed and not by an under-controlled design. Potential polyvalent vaccine candidates and polyvalent drug carriers with chemically defined average composition can be created using this technology.

3. Experimental

3.1. General[†]

TLC was performed on Silica Gel 60 F_{254} precoated on aluminium plates (E. Merck) and the compounds were detected by staining with 1:9 H_2SO_4–EtOH or with anisaldehyde soln (anisaldehyde (25 mL) with H_2SO_4 (25 mL), EtOH (450 mL) and CH_3COOH (1 mL)) followed by heating at over 200 °C. Column chromatography was carried out on Silica Gel 60 (E. Merck)

[†] For compounds missing the required elemental microanalysis, i.e. **6**–**10**, pertinent NMR spectra run at high sensitivity have been provided in the Supplementary data section.

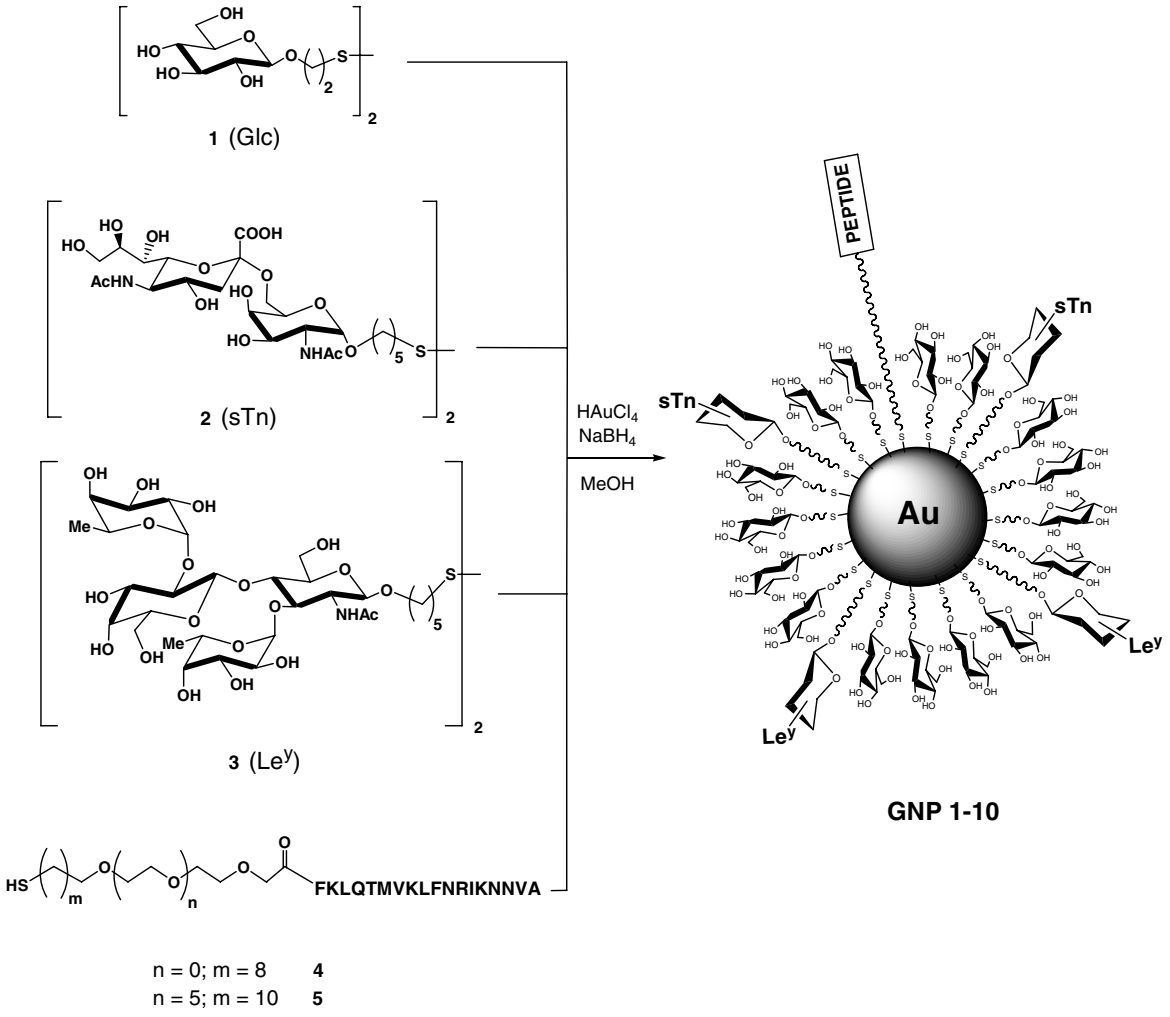


Chart 2. One-step synthesis of glyconanoparticles incorporating neoglycoconjugates and TT peptide ligands in a controlled way.

Table 1. Glyconanoparticles prepared by varying the percentages of different components

GNPs	Mean core diameter (nm)	Average number of gold atoms[a]	Average total number of chains[a]	Initial molar ratio in GNP formation Glc:sTn:Le^y:peptide[b]
GNP1	2.25	309	92	28:1:0:1
GNP2	1.45	116	53	20:9:0:1
GNP3	2.05	225	71	18:10:1:1
GNP4	1.81	201	71	18:1:10:1
GNP5	1.80	201	71	28:1:0:1
GNP6	1.55	140	53	20:9:0:1
GNP7	2.19	309	92	18:10:1:1
GNP8	1.77	201	71	18:1:10:1
GNP9	1.64	140	53	28:0:1:1
GNP10	1.79	201	71	20:0:9:1

[a] Calculated according to Ref. 28.
[b] **GNP1**–**GNP4**: peptide **4** (endowed with a C11 aliphatic spacer); **GNP5**–**GNP10**: peptide **5** (endowed with a mixed EG_6-C11 aliphatic spacer).

(0.2–0.5 mm, 0.2–0.063 mm or 0.040–0.015 mm; Merck). Optical rotations were determined with a Perkin–Elmer 341 polarimeter. ^1H and ^{13}C NMR spectra were acquired on Bruker DPX-300, DRX-400 and DRX-500 spectrometers and chemical shifts are given in parts per million (δ) relative to tetramethylsilane as an internal reference or relative to D_2O. Mass spectra (fast atom bombardment, HRFABMS) were carried out by the Mass Spectrometry Service, Facultad Química, Seville, with a Kratos MS-80 RFA spectrometer. For all experi-

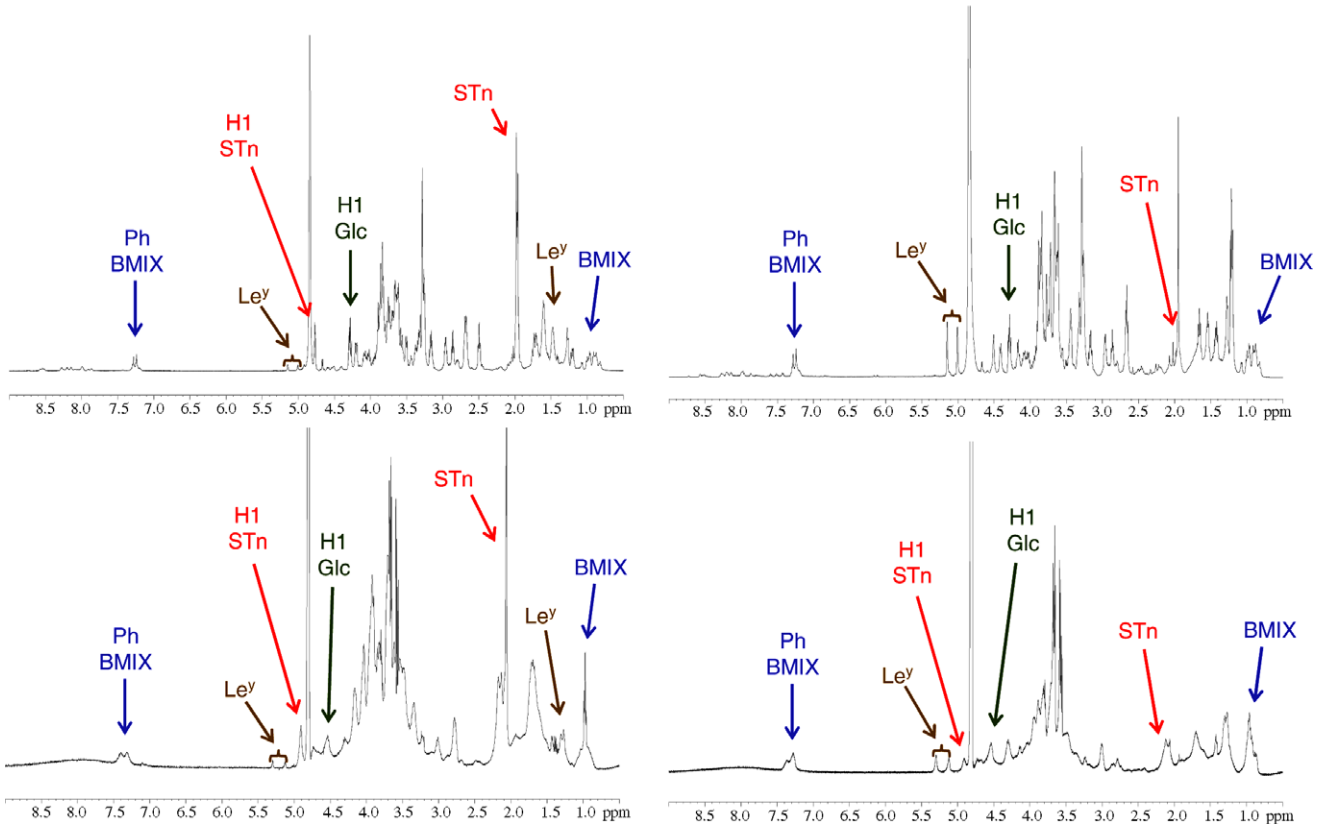


Figure 1. Example of ^{1}H NMR analysis. Up: ^{1}H NMR spectrum of the initial mixture, left **GNP7**, right **GNP8**. Down: ^{1}H NMR spectrum of glyconanoparticle, left **GNP7**, right **GNP8**.

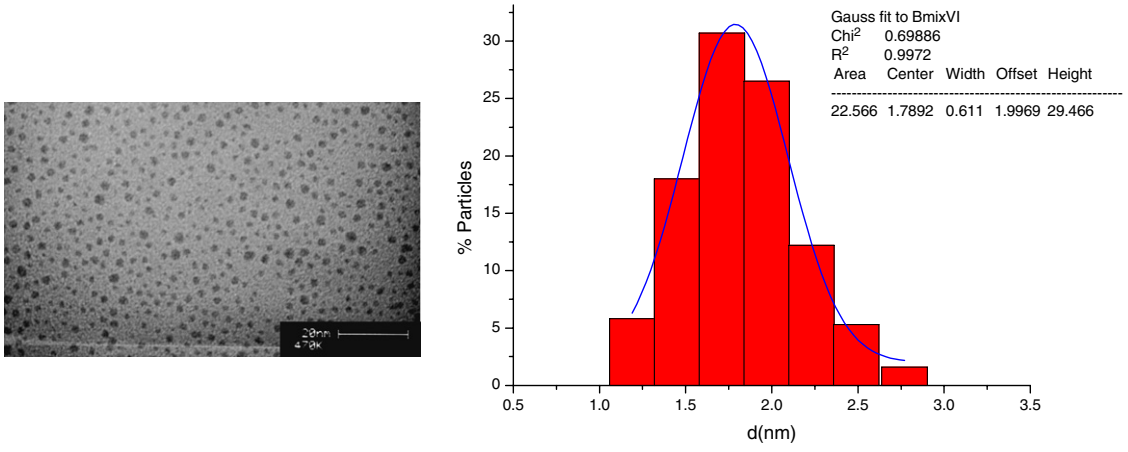


Figure 2. Example of TEM and size distribution histogram of **GNP10**.

ments and solns, Nanopure water (18.1 mΩ) was used. Peptides **4** and **5** were prepared in the Unitat de Síntesis de Péptidos of the University of Barcelona. For TEM examinations, a single drop (20 μL) of the aqueous soln (ca. 0.1 mg/mL) of the gold glyconanoparticles was placed onto a copper grid coated with a carbon film. The grid was left to dry in air for several hours at room temperature. TEM analysis was carried out in a Philips CM200 microscope working at 200 kV. The particle size distribution of the gold nanoparticles was evaluated from several micrographs by means of an automatic image analyser.

3.2. 4-Pentenyl 2-acetamido-2-deoxy-α-D-glucopyranoside (6)

A suspension of 2-acetamido-2-deoxy-D-glucose (10 g, 45 mmol) and Amberlite IR-120H$^+$ (2 g) in 4-pentenol (42 mL, 0.58 mol) was stirred at 75 °C overnight. Then, the mixture was filtered and the solvent removed under diminished pressure. The resulting syrup was purified by flash chromatography (9:1 → 5:1 CH_2Cl_2–MeOH) to yield **6** (6 g, 46%). $[\alpha]_D$ +76.0 (*c* 0.3, MeOH). ^{1}H NMR (300 MHz, MeOD): δ 5.84 (m, 1H, –*CH*=CH_2); 5.08–4.96 (m, 2H, –CH=*CH*$_2$); 4.80 (d, 1H, $J_{1,2}$ 3.5 Hz, H-1); 3.84–3.57 (m, 7H, H-2, H-3, H-4, H-5, H-6a, H-6b, –CH_2–O–); 3.58 (m, 1H, –CH_2–O–); 2.17 (m, 4H, *J* 7.5 Hz, –$(CH_2)_2$–); 2.00 (s, 3H, –$COCH_3$); 1.73 (m, 2H, *J* 7.0 Hz, –(CH_2)–). HRFABMS: Found: *m*/*z* 290.1606 [M+H]$^+$. Calcd for $C_{13}H_{24}NO_6$: 290.1604.

3.3. 4-Pentenyl 2-acetamido-3,6-di-*O*-benzoyl-2-deoxy-α-D-glucopyranoside (7)

To a soln of **6** (3.2 g, 11 mmol) in a mixture 1:1 of dry CH_2Cl_2 and dry pyridine (72 mL) at −50 °C was added dropwise benzoyl chloride (2.81 mL, 24.2 mmol). The resultant suspension was stirred at that temperature for 2 h and then was warmed gradually to room temperature. Upon addition of MeOH (3 mL), the mixture was concentrated to dryness. Flash chromatography of the residue (2:1 hexane–EtOAc) yielded **7** (4.1 g, 74%). $[\alpha]_D$ +88.0 (*c* 0.6, CH_3Cl). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.10–7.26 (m, 5H, Ph); 5.82–5.77 (m, 2H, NH, –*CH*=CH_2); 5.36 (m, 1H, dd, $J_{3,4}$ 9.2, $J_{2,3}$ 10.5 Hz, H-3); 5.08–4.99 (m, 2H, –CH=*CH*$_2$); 4.88 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1); 4.80 (dd, 1H, $J_{5,6a}$ 4.0, $J_{6a,6b}$ 12.1 Hz, H-6a); 4.56–4.44 (m, 2H, H-2, H-6b); 4.02 (m, 1H, H-5); 3.86–3.74 (m, 1H, H-4, –CH_2–O); 3.48 (m, 1H, –CH_2–O–); 2.17 (m, 4H, *J* 6.3 Hz, –$(CH_2)_2$–); 1.89 (s, 3H, –$COCH_3$); 1.75 (m, 2H, *J* 7.0 Hz, –(CH_2)–). HRFABMS: Found: *m*/*z* 498.2129 [M+H]$^+$. Calcd for $C_{27}H_{32}NO_8$: 498.2128.

3.4. 4-Pentenyl 2-acetamido-3,6-di-*O*-benzoyl-2-deoxy-α-D-galactopyranoside (8)

To a soln of triflic anhydride (2.2 mL, 13 mmol) in CH_2Cl_2 (50 mL) at −15 °C was added dropwise pyridine (2.1 mL, 25.9 mmol). Then a soln of **7** (4.1 g, 8.2 mmol) in CH_2Cl_2 (20 mL) was added, and the resulting mixture stirred at −15 °C for 1 h. The reaction mixture was diluted with CH_2Cl_2 (100 mL), washed successively with 5% hydrochloric acid, aq $NaHCO_3$, water, dried (Na_2SO_4) and filtered. The filtrate was evaporated to obtain 4-pentenyl 2-acetamido-3,6-di-*O*-benzoyl-2-deoxy-4-*O*-trifluoromethanesulfonyl-α-D-galactopyranoside as a yellow-brown syrup, which was used immediately in the next step without purification.

The syrupy filtrate thus obtained was dissolved in DMF (18 mL). Sodium nitrite (6 g, 86 mmol) was added and the mixture was stirred at room temperature for 16 h. After dilution with $CHCl_3$ (200 mL), the reaction mixture was washed thoroughly and successively with brine and water, filtered and evaporated. The resulting mixture was purified by flash chromatography (1:1 → 1:2 hexane–EtOAc) to yield **8** (2.7 g, 66%). $[\alpha]_D$ +83.6 (*c* 0.6, CH_3Cl). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.09–7.23 (m, 5H, Ph); 5.84–5.70 (m, 2H, NH, –*CH*=CH_2); 5.34 (m, 1H, dd, $J_{3,4}$ 2.8, $J_{2,3}$ 10.2 Hz, H-3); 5.04–4.86 (m, 4H, –CH=*CH*$_2$, H-1, H-2); 4.63 (m, 1H, dd, $J_{5,6a}$ 5.5, $J_{6a,6b}$ 11.3 Hz, H-6a); 4.50 (m, 1H, dd, $J_{5,6b}$ 6.9, $J_{6a,6b}$ 11.4 Hz, H-6b); 4.26–4.24 (m, 2H, H-4, H-5); 3.76 (m, 1H, –CH_2–O–); 3.48 (m, 1H, –CH_2–O–); 2.17 (m, 4H, *J* 7.5 Hz, –$(CH_2)_2$–); 1.87 (s, 3H, –$COCH_3$); 1.72 (m, 2H, *J* 6.9 Hz, –(CH_2)–). HRFABMS: Found: *m*/*z* 498.2136 [M+H]$^+$. Calcd for $C_{27}H_{32}NO_8$: 498.2128.

3.5. 4-Pentenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (9)

A soln of **8** (2.5 g, 5 mmol) in 1:9 CH_2Cl_2–MeOH (20 mL) was treated with MeONa (2 mL of a 1 M soln of MeONa in MeOH). After stirring for 1 h, the reaction was neutralised with Amberlite IR-120H$^+$, filtered and concentrated to dryness to yield **9** (1.45 g, 100%). $[\alpha]_D$ +5.6 (*c* 0.2, MeOH). ^{1}H NMR (300 MHz, MeOD): δ 5.85 (m, 1H, –*CH*=CH_2); 5.07–5.00 (m, 2H, –CH=*CH*$_2$); 4.83 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1); 4.26 (m, 1H, dd, $J_{1,2}$ 3.9, $J_{2,3}$ 8.9 Hz, H-2); 3.92–3.70 (m, 6H, H-3, H-4, H-5, H-6a, H-6b, –CH_2–O–); 3.41 (m, 1H, –CH_2–O–); 2.18 (m, 4H, *J* 7.4 Hz, –$(CH_2)_2$–); 2.01 (s, 3H, –$COCH_3$); 1.71 (m, 2H, *J* 7.5 Hz, –(CH_2)–). HRFABMS: Found: *m*/*z* 290.1608 [M+H]$^+$. Calcd for $C_{13}H_{24}NO_6$: 290.1604.

3.6. 5-Thioacetylpentyl 2-acetamido-2-deoxy-α-D-galactopyranoside (10)

To a soln of **9** (1.45 g, 5 mmol) in dry THF (65 mL), AcSH (1.5 mL, 20 mmol) and catalytic recrystallised AIBN were added. The reaction was stirred at 65 °C overnight, then concentrated to dryness. Flash chromatography of the residue (9:1 → 3:1 CH_2Cl_2–MeOH) yielded **10** (1.62 g, 89%). $[\alpha]_D$ +94.0 (*c* 0.7, MeOH). ^{1}H NMR (300 MHz, MeOD): δ 4.82 (d, 1H, $J_{1,2}$ 3.9 Hz, H-1); 4.25 (m, 1H, dd, $J_{1,2}$ 3.9, $J_{2,3}$ 11.1 Hz, H-2); 3.91–3.70 (m, 6H, H-3, H-4, H-5, H-6a, H-6b, –CH_2–O–); 3.41 (m, 1H, –CH_2–O–); 2.90 (t, 2H, *J* 7.1 Hz, –CH_2–S–); 2.32 (s, 3H, –$SCOCH_3$); 2.01 (s, 3H, –NHC-OCH_3); 1.66–1.45 (m, 6H, –$(CH_2)_3$–). HRFABMS: Found: *m*/*z* 365.1586 [M+H]$^+$. Calcd for $C_{15}H_{28}NO_7S$: 365.1586.

3.7. 5-Thioacetylpentyl *O*-(methyl(5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)uronate)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (12) and 5-thioacetylpentyl *O*-(methyl(5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosyl)uronate)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (13)

To a stirred mixture of **10** (270 mg, 0.74 mmol), powdered molecular sieves (1 g), AgOTf (270 mg, 1.05 mmol) and DTBP (280 μL, 1.24 mmol) in dry THF (2mL) was added dropwise a soln of donor **11** (444 mg, 0.87 mmol) in dry THF (0.8 mL) during 30 min. After stirring for 16 h at room temperature in the darkness, more AgOTf (270 mg, 1.05 mmol), DTBP (270 μL, 1.20 mmol) and **11** (370 mg, 0.72 mmol) were added. The reaction mixture was stirred for 24 h more and then filtered through Celite and evaporated. Flash chromatography of the residue (15:1 CH_2Cl_2–MeOH) yielded firstly **13** (85 mg, 13%) and then **12** (135 mg, 22%). Unreacted acceptor was lastly recovered (150 mg, 56%).

Data for the α-anomer **12**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.93 (d, 1H, $J_{1,2}$ 8.6 Hz, –NH–); 5.57 (d, 1H, J 9.7 Hz, –NH′–); 5.34–5.26 (m, 2H, H-7′, H-8′); 4.82 (m, 1H, H-4′); 4.75 (d, 1H, $J_{1,2}$ 3.4 Hz, H-1); 4.31 (dd, 1H, $J_{1,2}$ 2.1, $J_{1,2}$ 12.4 Hz, H-9′a); 4.26 (m, 1H, H-2); 4.10–3.99 (m, 3H, H-5′, H-6′, H-9′b); 3.89–3.58 (m, 6H, H-3, H-4, H-5, H-6a, H-6b, –CH_2–O–); 3.77 (s, 3H, $COOCH_3$); 3.34 (m, 1H, –CH_2–O–); 3.22 (br s, 1H, OH-4); 2.83 (m, 2H, –CH_2–S–); 2.54 (dd, 1H, $J_{3,4}$ 4.5, $J_{3eq,3ax}$ 12.8 Hz, H-3′eq); 2.28 (s, 3H, –$SCOCH_3$); 1.92 (m, 1H, H-3′ax); 2.09, 2.08, 2.01, 1.99, 1.98, 1.83 (6s, 18H, 6 –$COCH_3$); 1.58–1.38 (m, 6H, –$(CH_2)_3$–). ^{13}C NMR (125 MHz, $CDCl_3$): δ 196.0 (SC=O); 172.1, 170.9, 170.9, 170.4, 170.2, 170.1, 168.1 (C=O); 98.8 (C-2′); 97.3 (C-1); 72.8; 70.7; 69.2; 69.1; 68.5; 68.3; 67.6; 67.5; 63.6 (C-6); 62.5 (C-9′); 53.0 (OCH_3); 50.5 (C-2); 49.3 (C-5′); 37.5 (C-3′); 30.6; 29.3; 28.8; 28.7; 25.3; 23.4; 23.1; 21.1; 20.9; 20.8; 20.7. HRFABMS: Found: *m*/*z* 861.2944 $[M+Na]^+$. Calcd for $C_{35}H_{54}N_2O_{19}S$: 861.2939.

Data for the β-anomer **13**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.8 (d, 1H, J 8.1 Hz, –NH′–); 6.06 (d, 1H, J 9.3 Hz, –NH′–); 5.44–5.18 (m, 3H, H-4′, H-7′, H-8′); 4.85 (m, 1H, H-9′a); 4.72 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1); 4.46–3.60 (m, 10H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-5′, H-6′, H-9′b, –CH_2–O–); 3.34 (m, 1H, –CH_2–O–); 2.87 (m, 2H, –CH_2–S–); 2.44 (dd, 1H, $J_{3,4}$ 4.9, $J_{3eq,3ax}$ 12.9 Hz, H-3′eq); 2.32 (s, 3H, –$SCOCH_3$); 1.86 (m, 1H, H-3′ax); 2.11, 2.08, 2.05, 2.01, 1.98, 1.79 (6s, 18H, 6 –$COCH_3$); 1.62–1.40 (m, 6H, –$(CH_2)_3$–). ^{13}C NMR (75 MHz, $CDCl_3$): δ 196.6 (SC=O); 173.0, 172.0, 171.2, 171.1, 170.8, 170.7, 168.7 (C=O); 98.5 (C-2′); 97.9 (C-1); 73.1; 71.7; 70.7; 69.6; 69.1; 68.7; 68.4; 68.1; 6.1; 53.2; 50.6; 49.1; 37.7 (C-3′); 31.0; 30.1; 29.7; 29.2; 29.1; 25.6; 24.0; 23.3; 21.4; 21.3; 21.2. HRFABMS: Found: *m*/*z* 861.2941 $[M+Na]^+$. Calcd for $C_{35}H_{54}N_2O_{19}S$: 861.2939.

3.8. 5-Thiopentyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)uronic acid-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (2)

A soln of **12** (29 mg, 34 μmol) and aq NaOH 1 M (1.2 mL) in MeOH (4 mL) was stirred for 1 h. Then, the reaction was neutralised with Amberlite IR-120H$^+$, filtered and concentrated to dryness to yield **2** (20 mg, 95%). ^{1}H NMR (300 MHz, MeOD): δ 4.81 (d, 1H, $J_{1,2}$ 2.4 Hz, H-1); 4.24 (m, 1H, dd, $J_{1,2}$ 3.5, $J_{2,3}$ 10.5 Hz, H-2); 3.95–3.37 (m, 14H, H-3, H-4, H-5, H-6a, H-6b, H-4′, H-5′, H-6′, H-7′, H-8′, H-9a′, H-9b′, –CH_2–O–); 2.77–2.50 (m, 3H, –CH_2–S–, H-3′eq); 2.03–2.00 (m, 7H, 2 –$NHCOCH_3$, H-3′ax); 1.71–1.45 (m, 6H, –$(CH_2)_3$–). ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.1, 172.9, 172.4 (C=O); 100.3 (C-2′); 97.4 (C-1); 71.5; 69.3; 68.9; 68.5; 68.0; 67.4; 63.1; 62.7; 52.8; 50.3; 41.1; 33.5; 28.6; 28.5; 24.6; 23.6; 21.3; 21.3; 19.4. HRFABMS: Found: *m*/*z* 637.4499 $[M+Na]^+$. Calcd for $C_{24}H_{42}N_2O_{14}S$: 637.4519.

3.9. Synthesis of GNP1

Peptide **4** (3.1 mg, 1.31 μmol) was dissolved in CF_3COOD (100 μL) and the soln was concentrated under an argon stream until formation of an oil was observed. Compounds **1** (8.8 mg, 36.7 μmol) and **2** (0.8 mg, 1.31 μmol) were then added and the mixture was dissolved in CD_3OD (500 μL). The ^{1}H NMR spectrum showed a ratio 28:1:1 between the signals of **1**, **2** and **4** (see Supplementary data).

The soln was diluted with MeOH (2.8 mL) and the pH value was adjusted to 1 by addition of trifluoroacetic acid. An aqueous soln of $HAuCl_4$ (286 μL, 0.025 M) was added. Then, 1 M aqueous soln of $NaBH_4$ (157 μL) was added in several portions with rapid shaking. The black suspension formed was shaken for an additional 2 h and the methanolic layer was separated by decantation. The black solid was dissolved in water (700 μL) and purified by centrifugal filtering (AMICON MW 10,000, 30 min, 14,000 rpm). The process was repeated twice, until the nanoparticles were free of salts and starting materials. The residue in the AMICON filter was dissolved in 500 μL of water and lyophilised to afford 1.2 mg of **GNP1**.

TEM: average diameter 2.25 nm, 309 gold atoms, 92 chains, MW = 90,586 (see Supplementary data).

^{1}H NMR (500 MHz, D_2O): see Supplementary data.

3.10. Synthesis of GNP2

Peptide **4** (4.0 mg, 1.7 μmol) was dissolved in CF_3COOD (100 μL) and the soln was concentrated under an argon stream until the formation of an oil was observed. Compounds **1** (8.1 mg, 33.9 μmol) and **2** (9.3 mg, 15.2 μmol) were then added and the mixture was dissolved in CD_3OD (500 μL). The ^{1}H NMR

spectrum showed a ratio of 20:9:1 between the signals of **1**, **2** and **4** (see Supplementary data).

The soln was diluted with MeOH (3.7 mL) and the pH value was adjusted to 1 by addition of trifluoroacetic acid. An aqueous soln of $HAuCl_4$ (368 μL, 0.025 M) was added. Then, 1 M aqueous soln of $NaBH_4$ (202 μL) was added in several portions with rapid shaking. The black suspension formed was shaken for an additional 2 h and the methanolic layer was separated by decantation. The black solid was dissolved in water (500 μL) and purified by centrifugal filtering (AMICON MW 10,000, 30 min, 14,000 rpm). The process was repeated twice, until the nanoparticles were free of salts and starting materials. The residue in the AMICON filter was dissolved in 500 μL of water and lyophilised to afford 1.8 mg of **GNP2**.

TEM: average diameter 1.45 nm, 116 gold atoms, 53 chains, MW = 45,358 (see Supplementary data).

^{1}H NMR (500 MHz, D_2O): see Supplementary data.

3.11. Synthesis of GNP3

Peptide **4** (2.8 mg, 1.2 μmol) was dissolved in CF_3COOD (100 μL) and the soln was concentrated under an argon stream until the formation of an oil was observed. Compounds **1** (5.1 mg, 21.3 μmol), **3** (0.9 mg, 1.2 μmol) and **2** (7.3 mg, 11.8 μmol) were then added and the mixture was dissolved in CD_3OD (500 μL). The ^{1}H NMR spectrum showed a ratio 18:10:1:1 between the signals of **1**, **2**, **3** and **4** (see Supplementary data).

The soln was diluted with MeOH (2.4 mL) and the pH value was adjusted to 1 by addition of trifluoroacetic acid. An aqueous soln of $HAuCl_4$ (256 μL, 0.025 M) was added. Then, 1 M aqueous soln of $NaBH_4$ (142 μL) was added in several portions with rapid shaking. The black suspension formed was shaken for an additional 2 h and the methanolic layer was separated by decantation. The black solid was dissolved in water (500 μL) and purified by centrifugal filtering (AMICON MW 10,000, 30 min, 14,000 rpm). The process was repeated twice, until the nanoparticles were free of salts and starting materials. The residue in the AMICON filter was dissolved in 500 μL of water and lyophilised to afford 0.5 mg of **GNP3**.

TEM: average diameter 2.05 nm, 225 gold atoms, 71 chains, MW = 76,661 (see Supplementary data).

^{1}H NMR (500 MHz, D_2O): see Supplementary data.

3.12. Synthesis of GNP4

Peptide **4** (2.7 mg, 1.1 μmol) was dissolved in CF_3COOD (100 μL) and the soln was concentrated under an argon stream until the formation of an oil was observed. Compounds **1** (4.9 mg, 20.6 μmol), **3** (8.8 mg, 11.4 μmol) and **2** (0.7 mg, 1.1 μmol) were then added and the mixture was dissolved in CD_3OD (500 μL). The ^{1}H NMR spectrum showed a ratio 18:1:10:1 between the signals of **1**, **2**, **3** and **4** (see Supplementary data).

The soln was diluted with MeOH (2.3 mL) and the pH value was adjusted to 1 by addition of trifluoroacetic acid. An aqueous soln of $HAuCl_4$ (248 μL, 0.025 M) was added. Then, 1 M aqueous soln of $NaBH_4$ (137 μL) was added in several portions with rapid shaking. The black suspension formed was shaken for an additional 2 h and the methanolic layer was separated by decantation. The black solid was dissolved in water (500 μL) and purified by centrifugal filtering (AMICON MW 10,000, 30 min, 14,000 rpm). The process was repeated twice, until the nanoparticles were free of salts and starting materials. The residue in the AMICON filter was dissolved in 500 μL of water and lyophilised to afford 1.2 mg of **GNP4**.

TEM: average diameter 1.81 nm, 201 gold atoms, 71 chains, MW = 75,409 (see Supplementary data).

^{1}H NMR (500 MHz, D_2O): see Supplementary data.

3.13. Synthesis of GNP5

Peptide **5** (3.5 mg, 1.31 μmol) was dissolved in CF_3COOD (100 μL) and the soln was concentrated under an argon stream until formation of an oil was observed. Compounds **1** (8.8 mg, 36.6 μmol) and **2** (0.8 mg, 1.31 μmol) were then added and the mixture was dissolved in CD_3OD (500 μL). The ^{1}H NMR spectrum showed a ratio 28:1:1 between the signals of **1**, **2** and **5** (see Supplementary data).

The soln was diluted with MeOH (2.7 mL, total volume: 3.2 mL) and the pH value was adjusted to 1 by addition of trifluoroacetic acid. An aqueous soln of $HAuCl_4$ (314 μL, 0.025 M) was added. Then, 1 M aqueous soln of $NaBH_4$ (157 μL) was added in several portions with rapid shaking. The black suspension formed was shaken for an additional 2 h and the methanolic layer was separated by decantation. The black solid was dissolved in water (700 μL) and purified by centrifugal filtering (AMICON MW 10,000, 30 min, 14,000 rpm). The process was repeated twice, until the nanoparticles were free of salts and starting materials. The residue in the AMICON filter was dissolved in 500 μL of water and lyophilised to afford 1.0 mg of **GNP5**.

TEM: average diameter 1.80 nm, 201 gold atoms, 71 chains, MW = 63,300 (see Supplementary data).

^{1}H NMR (500 MHz, D_2O): see Supplementary data.

3.14. Synthesis of GNP6

Peptide **5** (3.5 mg, 1.31 μmol) was dissolved in CF_3COOD (100 μL) and the soln was concentrated under an argon stream until the formation of an oil was observed. Compounds **1** (6.3 mg, 26.2 μmol) and **2** (7.2 mg, 11.7 μmol) were then added and the mixture

was dissolved in CD_3OD (500 μL). The ^1H NMR spectrum showed a ratio 20:9:1 between the signals of **1**, **2** and **5** (see Supplementary data).

The soln was diluted with MeOH (2.7 mL, total volume: 3.2 mL) and the pH value was adjusted to 1 by addition of trifluoroacetic acid. An aqueous soln of $HAuCl_4$ (314 μL, 0.025 M) was added. Then, 1 M aqueous soln of $NaBH_4$ (157 μL) was added in several portions with rapid shaking. The black suspension formed was shaken for an additional 2 h. In this case it was impossible to separate the methanolic layer by decantation. Then, the volume was reduced to 1 mL, water (700 μL) was added and purified by centrifugal filtering (AMICON MW 10,000, 30 min, 14,000 rpm). The process was repeated until the nanoparticles were free of salts and starting materials. The residue in the AMICON filter was dissolved in 500 μL of water and lyophilised to afford 2.5 mg of **GNP6**.

TEM: average diameter 1.55 nm, 140 gold atoms, 53 chains, MW = 50,567 (see Supplementary data).

^1H NMR (500 MHz, D_2O): see Supplementary data.

3.15. Synthesis of GNP7

Peptide **5** (3.9 mg, 1.46 μmol) was dissolved in CF_3COOD (100 μL) and the soln was concentrated under an argon stream until the formation of an oil was observed. Compounds **1** (6.3 mg, 26.2 μmol), **3** (1.1 mg, 1.46 μmol) and **2** (9.0 mg, 14.6 μmol) were then added and the mixture was dissolved in CD_3OD (500 μL). The ^1H NMR spectrum showed a ratio 18:10:1:1 between the signals of **1**, **2**, **3** and **5** (see Supplementary data).

The soln was diluted with MeOH (3.2 mL, total volume: 3.7 mL). An aqueous soln of $HAuCl_4$ (350 μL, 0.025 M) was added. Then 1 M $NaBH_4$ (193 μL) was added in several portions with rapid shaking. The black suspension formed was shaken for an additional 2 h and the methanolic layer was separated by decantation. The black solid was dissolved in water (500 μL) and purified by centrifugal filtering (AMICON MW 10,000, 30 min, 14,000 rpm). The process was repeated twice, until the nanoparticles were free of salts and starting materials. The residue in the AMICON filter was dissolved in 500 μL of water and lyophilised to afford 2.8 mg of **GNP7**.

TEM: average diameter 2.19 nm, 309 gold atoms, 92 chains, MW = 103,569 (see Supplementary data).

^1H NMR (500 MHz, D_2O): see Supplementary data.

3.16. Synthesis of GNP8

Peptide **5** (3.7 mg, 1.38 μmol) was dissolved in CF_3COOD (100 μL) and the soln was concentrated under an argon stream until the formation of an oil was observed. Compounds **1** (6.0 mg, 24.8 μmol), **3** (10.7 mg, 13.8 μmol) and **2** (0.85 mg, 1.38 μmol) were then added and the mixture was dissolved in CD_3OD (500 μL). The ^1H NMR spectrum showed a ratio 18:1:10:1 between the signals of **1**, **2**, **3** and **5** (see Supplementary data).

The soln was diluted with MeOH (3.0 mL, total volume: 3.5 mL) and the pH value was adjusted to 1 by addition of trifluoroacetic acid. An aqueous soln of $HAuCl_4$ (330 μL, 0.025 M) was added. Then 1 M $NaBH_4$ (182 μL) was added in several portions with rapid shaking. The black suspension formed was shaken for an additional 2 h and the methanolic layer was separated by decantation. The black solid was dissolved in water (500 μL) and purified by centrifugal filtering (AMICON MW 10,000, 30 min, 14,000 rpm). The process was repeated twice, until the nanoparticles were free of salts and starting materials. The residue in the AMICON filter was dissolved in 500 μL of water and lyophilised to afford 1.8 mg of **GNP8**.

TEM: average diameter 1.77 nm, 201 gold atoms, 71 chains, MW = 75,934 (see Supplementary data).

^1H NMR (500 MHz, D_2O): see Supplementary data.

3.17. Synthesis of GNP9

Peptide **5** (3.7 mg, 1.4 μmol) was dissolved in CF_3COOD (100 μL) and the soln was concentrated under an argon stream until formation of an oil was observed. Compounds **1** (9.4 mg, 39.1 μmol) and **3** (1.1 mg, 1.4 μmol) were then added and the mixture was dissolved in CD_3OD (500 μL). The ^1H NMR spectrum showed a ratio 28:1:1 between the signals of **1**, **3** and **5** (see Supplementary data).

The soln was diluted with MeOH (3.0 mL, total volume: 3.5 mL) and the pH value was adjusted to 1 by addition of trifluoroacetic acid. An aqueous soln of $HAuCl_4$ (331 μL, 0.025 M) was added. Then, 1 M aqueous soln of $NaBH_4$ (182 μL) was added in several portions with rapid shaking. The black suspension formed was shaken for an additional 2 h and the methanolic layer was separated by decantation. The black solid was dissolved in water (700 μL) and purified by centrifugal filtering (AMICON MW 10,000, 30 min, 14,000 rpm). The process was repeated twice, until the nanoparticles were free of salts and starting materials. The residue in the AMICON filter was dissolved in 500 μL of water and lyophilised to afford 0.7 mg of **GNP9**.

TEM: average diameter 1.64 nm, 140 gold atoms, 53 chains, MW = 45,568 (see Supplementary data).

^1H NMR (500 MHz, D_2O): see Supplementary data.

3.18. Synthesis of GNP10

Peptide **5** (3.5 mg, 1.31 μmol) was dissolved in CF_3COOD (100 μL) and the soln was concentrated under an argon stream until the formation of an oil

was observed. Compounds **1** (6.3 mg, 26.2 μmol) and **3** (9.2 mg, 11.8 μmol) were then added and the mixture was dissolved in CD_3OD (500 μL). The ^1H NMR spectrum showed a ratio 20:9:1 between the signals of **1**, **3** and **5** (see Supplementary data).

The soln was diluted with MeOH (2.7 mL, total volume: 3.2 mL) and the pH value was adjusted to 1 by addition of trifluoroacetic acid. An aqueous soln of $HAuCl_4$ (314 μL, 0.025 M) was added. Then 1 M aq $NaBH_4$ (157 μL) was added in several portions with rapid shaking. The black suspension formed was shaken for an additional 2 h. In this, it was impossible to separate the methanolic layer by decantation. Then, the volume was reduced to 1 mL, water (700 μL) was added and purified by centrifugal filtering (AMICON MW 10,000, 30 min, 14,000 rpm). The process was repeated until the nanoparticles were free of salts and starting materials. The residue in the AMICON filter was dissolved in 500 μL of water and lyophilised to afford 1.8 mg of **GNP10**.

TEM: average diameter 1.79 nm, 201 gold atoms, 71 chains, MW = 73,864 (see Supplementary data).

^1H NMR (500 MHz, D_2O): see Supplementary data.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.11.018.

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