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A convenient method for synthesis of glyconanoparticles for colorimetric measuring carbohydrate–protein interactions

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ABSTRACT

Carbohydrate functionalized nanoparticles, i.e., the *glyconanoparticles*, have wide application ranging from studies of carbohydrate–protein interactions, in vivo cell imaging, biolabeling, etc. Currently reported methods for preparation of glyconanoparticles require multi-step modifications of carbohydrates moieties to conjugate to nanoparticle surface. However, the required synthetic manipulations are difficult and time consuming. We report herewith a simple and versatile method for preparing glyconanoparticles. This method is based on the utilization of clean and convenient microwave irradiation energy for one-step, site-specific conjugation of unmodified carbohydrates onto hydrazide-functionalized Au nanoparticles. A colorimetric assay that utilizes the ensemble of gold glyconanoparticles and Concanavalin A (ConA) was also presented. This feasible assay system was developed to analyze multivalent interactions and to determine the dissociation constant (K_d) for five kind of Au glyconanoparticles with lectin. Surface plasmon changes of the Au glyconanoparticles as a function of lectin–carbohydrate interactions were measured and the dissociation constants were determined based on non-linear curve fitting. The strength of the interaction of carbohydrates with ConA was found to be as follows: maltose > mannose > glucose > lactose > MAN5.

Introduction

Many metals can be synthesized into nanoscale particles of varying shapes with characteristic optical properties. When the dimensions of the metal are reduced to the nanoscale, the optical properties are dominated by a collective oscillation of conduction electrons in resonance with incident electromagnetic radiation, and this phenomenon is known as surface plasmon resonance [1]. Gold nanoparticles (GNPs), with an inter-particle distance greater than the average particle diameter, appear red as a consequence of the surface plasmon absorption band centered at around 520 nm. As the inter-particle distance decreases to less than the diameter of the particles, coupling interactions result in a broadening and a shift to longer wavelengths of the surface plasmon absorption band, and the solution of aggregated gold nanoparticles changes from red to blue. The color changes associated with Au nanoparticle aggregation have been exploited in the development of colorimetric assays for biomolecular interactions by utilizing the gold nanoparticles functionalized with biomolecules such as protein [2], peptide [3] aptamers [4], and DNA [5].

Carbohydrate functionalized nanoparticles are very useful tools and reagents for proteomic and glycomics research. Glyconanoparticles have potential wide application ranging from studies of carbohydrate–protein interactions [6], glycan biosensors [7,8], in vivo cell imaging [9,10], vaccine development, and drug delivery [11]. In particular, carbohydrate–protein interaction is generally identified with very low affinity between each other. In natural biological system, the low affinity can be compensated by presentation of multiple ligands to individual receptors [12]. The polyvalent interaction between multi-ligands and their receptors can be collectively much stronger than corresponding monovalent interaction [13]. The advantage of glyconanoparticles is that a single nanoparticle with large surface/volume ratio can be coupled with multiple carbohydrate moieties, which provides an increased potential for the enhancement of biomolecular interaction. Thus, glyconanoparticles constitute a good bio-mimetic model of carbohydrate presentation at the cell surface, and are excellent tools for glycobiology, biomedicine, and material science investigations. However, unlike DNA and protein functionalized nanoparticles which have been extensively explored during the last 20 years, the development of carbohydrate functionalized nanoparticles and their applications have just been emerged. Currently very few examples of glyconanoparticles were prepared by conjugating thiol modified carbohydrates onto gold nanoparticle surfaces. To prepare a thiolated carbohydrate, multi-step modifications of carbohydrates moieties were required [7,14–17]. However, the required synthetic manipulations are difficult and time consuming. By considering the fact that carbohydrates

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are the most complex and diverse class of biomolecules, and there are hundreds of biologically interesting carbohydrates, it is impractical to use this approach to prepare a wide range of glyconanoparticles. Here we report a simple and versatile method for preparing Au glyconanoparticles by one-step, site-specific conjugation of unmodified carbohydrates onto hydrazide-functionalized Au nanoparticle under the assistance of convenient microwave irradiation energy, as well as the utilization of the prepared Au glyconanoparticles for colorimetric assays of carbohydrate–lectin interactions.

Materials and methods

Materials. $HAuCl_4{\cdot}xH_2O$ was purchased from Alfa Aesar. Di(*N*-succinimidyl)3,3′-dithiodipropionate (DTSP) was obtained from TCI. *N*-(β-maleimidopropionic acid) hydrazide (BMPH) was obtained from PIERCE. Trisodium citrate and 2-(*N*-morpholine) ethanesulfonic acid (MES) were purchased from J.T. Baker. Concanvalin A (ConA), D-(+)-maltose (Mal) and streptavidin were purchased from MP Biomedicals. D-Mannose (Man) was purchased from CALBIOCHEM. 2-Mercaptoethylamine hydrochloride (MEA) and D-(+)-glucose were purchase from Sigma. D-mannopentaose (MAN5) was obtained from V-LABs, Inc. DMF was purchased from EMD. All reagents were used as received without further purification. The water used was purified through a de-ionized water system.

Synthesis and characterization of gold nanoparticles. All glasswares used for preparation of colloids were thoroughly washed with aqua regia (3 parts HCl, 1 part HNO_3), rinsed extensively with distilled water, and then oven dried prior to use. Gold colloids were prepared by sodium citrate reduction of gold salt as reported earlier [18] Briefly, a volume of 200-mL sample of 1 mM $HAuCl_4$ was brought to a vigorous boil with stirring in a round-bottomed flask fitted with a reflux condenser, and 20 mL of 38.8 mM sodium citrate solution was rapidly added to the solution. The solution was boiled for another 15 min, during which the color of the solution was changed from pale yellow to deep red. The solution was allowed to cool to room temperature with continued stirring and then filtered through 0.45 μm nylon filter (Micron Separations, Inc.). The product was stored at 4 °C until further use.

Synthesis of hydrazide-functionalized Au nanoparticles. Fig. 1A outlined the procedures for synthesis of hydrazide-functionalized Au nanoparticles. The detailed procedure was described in the Supplementary material. In brief, the freshly prepared gold nanoparticles were conjugated with bifunctional reagent, DTSP through self-assembling process. The DTSP functionalized Au nanoparticles then were reacted with MEA to generate surface sulfhydryl-functionalized Au nanoparticles. Finally, the sulfhydryl group functionalized Au nanoparticles were then reacted with BMPH via its maleimides group coupling to sulfhydryl group on the Au surface to produce hydrazide-functionalized Au nanoparticles.

Conjugation of unmodified sugar onto hydrazide-functionalized Au nanoparticles under assistance of microwave energy. One microliter of carbohydrate solution, dissolved in 100 mM MES buffer at pH 5.4 was added into 1 mL of hydrazide-functionalized Au nanoparticles solution in a 2 mL of vial. Using microwave heating power of 600W (EMS-820 Precision Pulsed Laboratory Microwave Oven, Electron Microscopy Science), various microwave radiation time

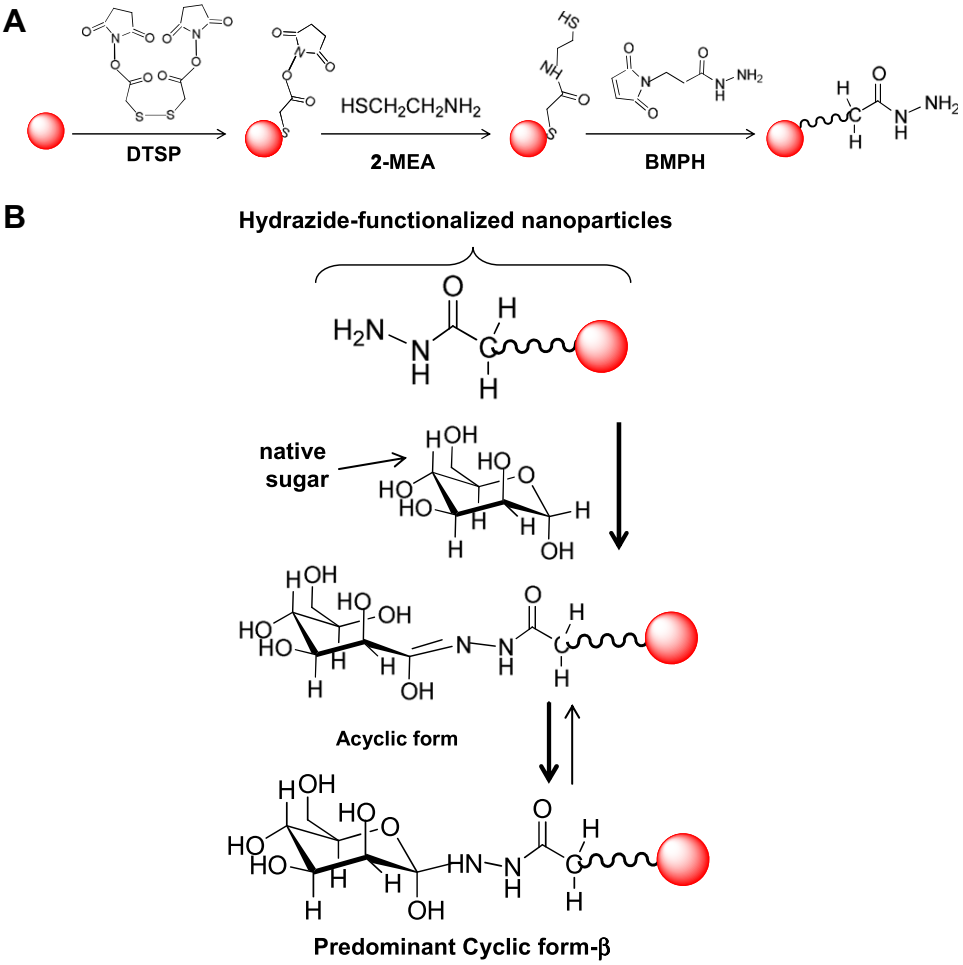

Fig. 1. (A) Synthesis of hydrazide-functionalized nanoparticles; (B) the immobilization mechanism of oligosaccharides onto hydrazide-functionalized nanoparticles.

and temperature were examined for conjugating carbohydrate onto the hydrazide-functionalized Au nanoparticles solution. After microwave conjugation, the solution was centrifugation at the rcf of 15,000 for 20 min, and suspension of prepared glyconanoparticles were purified and re-dispensed into 100 mM MES buffer for measuring UV–vis absorption spectra. The detailed procedure was shown in the Supplementary material.

UV–vis absorption measurements. A Shimadzu UV2450 UV–vis spectrophotometer was used to record both the absorption spectra at room temperature.

Transmission electron microscopy (TEM) measurements. TEM examination of the samples was carried out at 200 KeV with Philips Tecnai 20 electron microscope. For the TEM characterization, a 10 μL drop of the aqueous solutions of the gold nanoparticles or nanoparticle conjugates 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, and excess liquid was removed by contacting the side of the grid with absorbent paper tissue. The real sizes of gold nanoparticles were estimated from TEM images by SigmaScan Pro software.

Colorimetric assays of lectin–carbohydrate interactions with Au glyconanoparticles. A range of concentrations of ConA were prepared (0–100 nM) in TNC buffer (50 mM Tris, 150 mM NaCl, 40 mM $CaCl_2$). Each ConA solution was added to the gold glyconanoparticles under stirring, and the reaction was monitored by UV–vis spectrophotometry. Same concentrations of streptavidin solution were added to the gold glyconanoparticles as negative control in this assay system. Shifts at 620 nm in UV–vis absorption wavelength as a function of ConA–carbohydrate derivatives molar ratios were plotted and the dissociation constants were determined based on non-linear curve fitting with SigmaPlot 10 software.

Results and discussions

Synthesis of hydrazide-functionalized Au nanoparticles

The reduction of gold salt with sodium citrate produced a gold nanoparticle solution of bright red color with an average diameter of 12.33 nm as determined by TEM (Fig. S1a). The gold nanoparticles exhibited an intense surface plasmon absorption band at ca. 520 nm.

We employed commercial available heterobifunctional crosslinkers to generate hydrazide-functionalized Au nanoparticles. Fig. 1A illustrated the procedures to generate the functionalized Au nanoparticles. The freshly prepared Au nanoparticles were self-assembled with bifunctional reagent, DTSP, onto the Au surface through the strong sulfur–Au interaction. DTSP is a water insoluble, homobifunctional NHS ester, known as Lomant's reagent. DTSP can form a self-assembly monolayer on gold surfaces, through the disulfide group [19], so that the terminal succinimidyl groups allow further covalent bonding with amino-containing MEA molecules through acylation of free primary amino groups which produced free sulfhydryl groups on the Au nanoparticle surface. The sulfhydryl-functionalized Au nanoparticle was then reacted with BMPH, a heterobifunctional crosslinker containing sulfhydryl-reactive maleimide and carbonyl-reactive hydrazide moieties to generate the hydrazide-functionalized Au nanoparticle. TEM images of BMPH-functionalized gold nanoparticles also showed the nanoparticles did not change the spherical shape (Fig. S1b).

Conjugation of unmodified carbohydrates onto hydrazide-functionalized Au nanoparticles under assistance of microwave energy

The immobilization of carbohydrates onto hydrazide surface is based on the hydrazone formation between a highly reactive amine group of the nucleophilic hydrazide and the carbonyl group at the reducing end of suitable carbohydrates via irreversible condensation, as deciphered in Fig. 1B. Reactions of hydrazide groups with free oligosaccharides are known to be chemoselective and have been used for the synthesis of various glycoconjugates [20,21]. It is known that cyclic adducts with β-configurations are produced predominantly in reactions of carbohydrates with hydrazide-containing compounds (Fig. 1B) [22,23]. However, the reaction of reducing sugar with the hydrazide group required long heating process.

Microwaves (~0.3–300 GHz) lie between the infrared and radio frequency (RF) electromagnetic spectrum. In the past two decades, the use of microwave radiation has greatly increased in its application to accelerating reactions in synthetic organic chemistry [24], nanomaterial synthesis [25], and biochemistry [26]. The "Technology Vision 2020" of the US chemical industry believes that microwave heating will soon replace traditional heating techniques in chemical and biochemical synthesis. It is widely thought that microwaves accelerate chemical reactions by increased rate of mutarotation (the process of equilibration between unreactive closed ring structures that proceeds *via* the reactive open chain intermediate [27]) above that achieved by equivalent thermal heating, and by accelerated solvent heating which increases the rate of mutarotation.

The use of microwave radiation energy to accelerate the conjugation of reducing sugars onto hydrazide-functionalized nanoparticles has yet been reported. To investigate the utilization of microwave radiation energy to conjugate carbohydrates onto hydrazide-functionalized Au nanoparticles, we used UV–vis spectrometer to record spectra of the conjugation of mannose to the hydrazide-functionalized Au nanoparticles at different reaction time. As shown in Fig. 2A, microwave heating time of 15 min gave

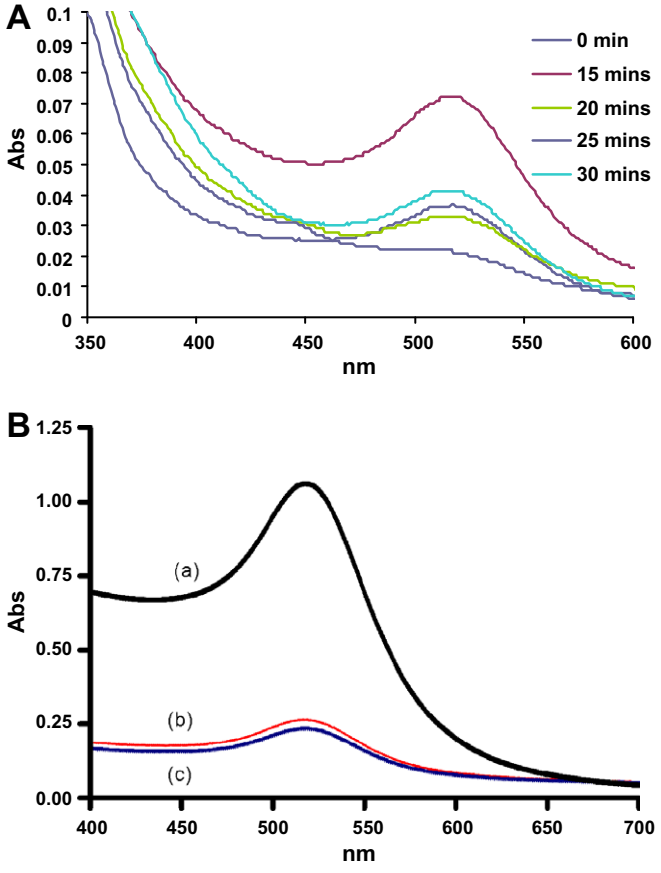

Fig. 2. (A) UV–vis spectrum of the suspension of mannose binding to BMPH-Au at different microwave heating time at 55 °C: (a) 0; (b) 15; (c) 20; (d) 25; (e) 30 min; (B) UV–vis absorption spectra of (a) gold nanoparticles (5.9 nM), (b) maltose-conjugated gold nanoparticles, and (c) mannose-conjugated gold nanoparticles.

best conjugation as evidenced by the highest Plasmon peak at 520 nm. Longer microwave heating time and higher reaction temperature did not increase the conjugation. In contrast, longer microwave heating time and higher reaction temperature caused the surface coating of gold nanoparticles to peel off from the surface of gold nanoparticles. After testing different heating time and temperature, it showed that the conjugation of carbohydrates onto hydrazide-functionalized Au nanoparticles is optimal by microwave radiation of 15 min at 55 °C. Following the conjugation of the mannose or maltose onto the hydrazide-functionalized Au nanoparticles, the surface plasmon absorption band of gold nanoparticles at ~520 nm did not have significant shift (Fig. 2B), which indicated that the prepared gold glyconanoparticles remain the optical properties gold nanoparticles. TEM images of mannose and maltose-conjugated gold nanoparticles also showed the glyconanoparticles were monodisperse as were the hydrazide-functionalized Au nanoparticles (Fig. S1c).

Gold glyconanoparticle for colorimetric assays of carbohydrate–protein interactions

We used a representative ConA lectin as a model protein for evaluating the biological activities of prepared Au glyconanoparticles. At pH < 5, ConA is a active dimmer lectin, with each of the two subunits containing an α-D-mannose binding site [28]. These sites enable binding to the mannose ligands on a nanoparticle surface leading to aggregation of the nanoparticles in solution caused by the formation of polymeric ConA–glyconanoparticle hybrids. TEM images of the aggregation of mannose and maltose-conjugated gold nanoparticles upon addition of the ConA also confirmed the formation of the nanoparticle hybrids (Fig. S1d). Upon formation of the ConA–glyconanoparticle hybrids, a red-shift in wavelength and a broadening of the surface plasmon absorption band occurs. Fig. 3A and B showed the typical UV–vis spectra of the mannose-conjugated Au nanoparticles (mannose-GNP) and maltose-conjugated Au nanoparticles (maltose-GNP) on addition of ConA. It showed that there are significant changes in absorbance intensity at 620 nm for the interactions between the ConA and mannose-GNPs and maltose-GNPs. These changes provide method for the quantitative measurement of the ConA lectin. As a negative control, the addition of streptavidin into the mannose-GNP and maltose-GNP solutions did not cause changes in absorbance intensity at 620 nm (spectra not shown in Fig. 3). To investigate the lectin–glyconanoparticle interactions, the plasma shifts at 620 nm of varying concentrations of ConA to both mannose-GNP and maltose-GNP were assessed. Fig. 4 shows the quantitative relationship between shift absorbance wavelength at 620 nm and the different concentration of ConA for maltose-conjugated gold nanoparticles. Therefore, K_d can be derived from non-linear regression fitting using SigmaPlot 10 software. The typical non-linear curves displayed in Fig. 4A and B are based on the fitted parameters. The dissociation constant for mannose-conjugated Au nanoparticles and ConA interaction was thus determined to 12.88 nM using this approach. Encouraged by the above results, we prepared five kinds of glucoside containing carbohydrates-conjugated gold nanoparticles (i.e., mannose-GNP, maltose-GNP, glucose-GNP, lactose-GNP, and

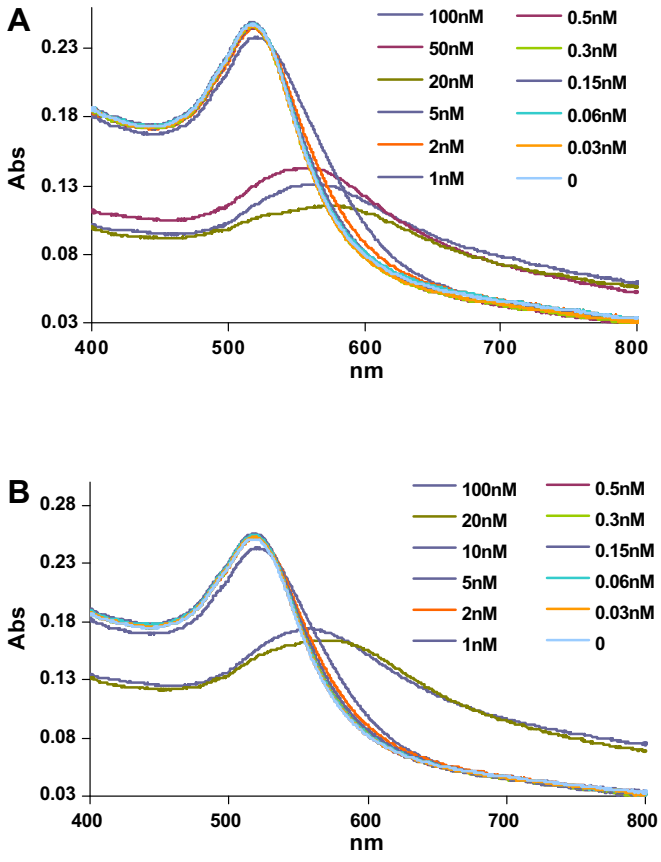

Fig. 3. Changing UV–vis spectra following addition of increasing concentrations of ConA to (A) maltose-conjugated gold nanoparticles, and (B) mannose-conjugated gold nanoparticles. Spectra were obtained 2 h following addition of ConA. Ten concentrations of ConA from 0 to 100 nM was used in both cases.

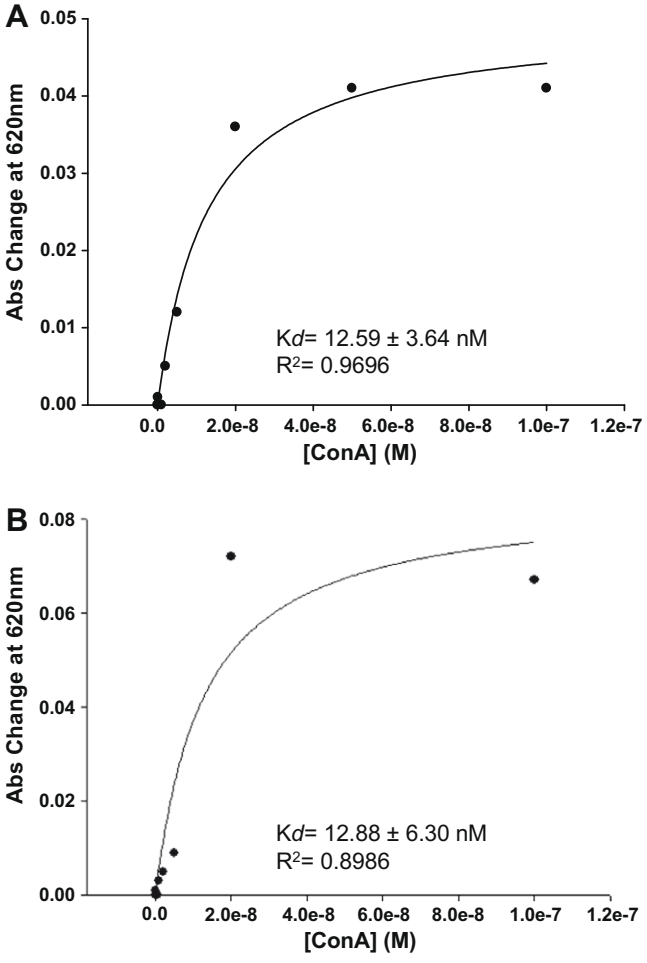

Fig. 4. Non-linear regression fitting for ConA binding to (A) maltose-conjugated gold nanoparticles, and (B) mannose-conjugated gold nanoparticles.

Table 1
Dissociation constants (K_d) of carbohydrates and ConA. The various concentrations of ConA from 0 to 100 nM were used in this affinity assay.

Carbohydrate	Lectin	K_d (R^2)[a]	Assay platform	Reference
Maltose	ConA	12.59 ± 3.64 nM (0.9696)	In this research	
Mannose	ConA	12.88 ± 6.30 nM (0.8986)	In this research	
Glucose	ConA	14.55 ± 2.3 nM (0.9827)	In this research	
Lactose	ConA	20.10 ± 47.61 nM (0.8637)	In this research	
MAN5	ConA	25.84 ± 9.30 nM (0.9655)	In this research	
Mannose	ConA	200 ± 50 μM	Surface plasmon resonance image	[29]
Galactose	Jacalin	16 ± 5 μM	Surface plasmon resonance image	[29]
Mannose	ConA	83 nM	Carbohydrate microarray	[30]

[a] The dissociation equation was shown in supporting information.

MAN5-GNP), and the dissociation constants of these five glyco-GNPs with ConA were also determined by this method. The dissociation constants of the five glyco-GNPs with ConA proteins were shown in Table 1. By comparing the dissociation constants (K_d) of mannose with ConA obtained with the mannose-GNP in this study (12.88 nM) and the reported data determined by two-dimensional carbohydrate microarrays (200 μM in glycan microarray employed surface plasmon image [29] or 83 nM in glycan microarray employed fluorescent detection [30]), one can see that the three-dimensional, water soluble, stable, and biologically active glyconanoparticles can be a better tool for measuring the low-affinity carbohydrate–protein interactions than the two-dimensional glycan microarrays, because the glyconanoparticles provide a solution-like environment and polyvalent interactions. Overall, the strength of the interaction of carbohydrates with ConA was found to be as follows: maltose > mannose > glucose > lactose > MAN5. This result is, respectively, consistent with the literatures [30–34].

Conclusions

Our results have shown that microwave radiation energy is convenient mean to assist the conjugation of free carbohydrates onto hydrazide-functionalized nanoparticles surface, thus a wide range of glyconanoparticles (e.g., Au glyconanoparticle library) can be easily prepared without the chemical modifications of carbohydrates. The prepared Au glyconanoparticles have been demonstrated for colorimetric measuring carbohydrate-specific proteins with a minimum of substance consumption and time spent. The water soluble, stable, and biologically active glyconanoparticles provide customizable tools for in vivo and *in vitro* diagnosis, imaging, and biolabeling. More importantly, the glyconanoparticles present the carbohydrate antigens in a three-dimensional and polyvalent format conferring biological specificity. The combination of hydrazide surface chemistry with the microwave radiation energy can also be used as a versatile method for facile preparation of a wide range glycan conjugated nanoparticles, such as fluorescent glyconanoparticles, and magnetic glyconanoparticles.

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Appendix A. Supplementary data

A detailed description of the synthesis of glyconanoparticles as well as the calculations of dissociation constants between glyconanoparticles and lectins can be found in the online version.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.08.079.

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