COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY and BIOENGINEERING

In Situ Growth of Gold Nanoparticles by Enzymatic Glucose Oxidation Within Alginate Gel Matrix

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Received 16 June 2009; revision received 14 August 2009; accepted 24 August 2009

Published online 28 August 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.22519

ABSTRACT: In situ growth of gold nanoparticles (Au NPs) was performed in an alginate gel matrix co-encapsulating Au NP seeds and glucose oxidase (GOx) for the development of a glucose-sensing platform. We observed a simultaneous growth of Au NPs in the alginate matrix through the reduction of $AuCl_4^-$ by H_2O_2 on Au NP seeds. The detection of the glucose level was carried out successfully via the coupling of Au NP enlargement with the oxidation of glucose catalyzed by the immobilized GOx. The enlargement of Au NPs in the alginate matrix exhibited only a red-shift in absorbance maxima, while the generation of small Au NPs in a free solution caused a blue-shift in higher glucose concentrations. This study shows that the co-encapsulation of metal NPs and bioreceptor in a gel matrix may provide a simple route for the fabrication of an optical biosensor.

Biotechnol. Bioeng. 2010;105: 210–214.

KEYWORDS: nanoparticles; glucose oxidase; encapsulation; biosensors; alginate matrix

Recent advances in nanotechnology enable metal nanoparticles (NPs) to participate in the detection of biorecognition events as a key label (Niemeyer, 2001). The growth of metal NPs coupled with a biocatalytic reaction is considered as a potential sensing technology for the development of future biosensors and bioelectronics (Willner et al., 2006; Zayats et al., 2005). For example, the enlargement of Au NPs was mediated in a free solution by the use of reducing agents, such as neurotransmitters, for triggering the growth of Au NPs (Baron et al., 2005). The colorimetric analysis of the NAD^+-dependent enzymatic reaction was achieved by a NADH-triggered enlargement of Au NPs (Xiao et al., 2004). Other examples include the detection of DNA hybridization using metal NPs-nucleotide conjugate with various methods such as surface plasmon resonance, surface-enhanced Raman scattering, or quartz-crystal microbalance (Cao et al., 2002; He et al., 2000). Moreover, the inhibition of acetylcholine esterase by organophosphate nerve agent was optically measured by the decrease in the growth of Au NPs (Xiao et al., 2005).

Here we report the co-encapsulation of Au NPs and glucose oxidase (GOx) within an alginate gel matrix for the fabrication of a glucose-sensing platform through in situ growth of Au NPs. For the development of enzyme-based sensors, the co-encapsulation of Au NPs and enzymes within a gel matrix may provide an advanced sensory format, as well as a protective carrier for the bioreceptor. We used alginate gel as a model matrix since it is the most commonly used material for the encapsulation of biologicals for various applications such as drug delivery, grafting, or biosensor development (Chen et al., 2004; Coppi et al., 2002; Fernandes et al., 2008; Leonard et al., 2004; Lim and Sun, 1980). The versatility of alginate beads as a carrier for biomolecules comes from the biocompatibility of the polymer and the mild condition for gel formation. According to previous reports (Blandino et al., 2001; Khani et al., 2006), the stability of enzymes, such as GOx, can be enhanced when encapsulated within alginate matrix. Alginate gel can be formed through the crosslink between the guluronate residues in the alginate network and divalent cations such as calcium ions. In this work, we observed a simultaneous growth of Au NPs incorporated in the alginate beads with an increasing concentration of H_2O_2 through the transmission electron microscopy (TEM) analysis and plasmon absorbance measurement. By encapsulating GOx together with Au NPs within alginate beads, the detection of

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Contract grant sponsor: Korea Science and Engineering Foundation (National Research Laboratory Program)
Contract grant number: R0A-2008-000-20041
Contract grant sponsor: The Ministry of Environment (Eco-Technopia 21 project)
Contract grant number: 010-081-036
Contract grant sponsor: Ministry of Knowledge Economy (Fundamental R&D Program for Core Technology of Materials)
Contract grant sponsor: KAIST, Republic of Korea

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the glucose level was performed successfully through the coupling of Au NP enlargement with the oxidation of glucose catalyzed by the immobilized GOx.

The experimental scheme for the growth of Au NPs within GOx-encapsulated alginate beads is illustrated in Figure 1. The GOx encapsulated in the Ca–alginate gel beads produces H_2O_2 by the oxidation of glucose, which then reduces $AuCl_4^-$ into Au (0) on Au NP seeds that result in the enlargement of Au NPs within the alginate matrix. We treated Au NPs-incorporated alginate matrix beads with growth solutions including $HAuCl_4$, with different H_2O_2 concentrations (0, 5, 20, 60, 120, 240, and 400 μM). As shown in Figure 2, alginate gel beads turned into a reddish color as the H_2O_2 increased in the growth solution, which indicates an increase of reduced Au species in the alginate matrix as the concentration of H_2O_2 increased. We observed a gradual increase and a red-shift of the maximum absorbance at around 520–540 nm, implying the simultaneous enlargement of Au NPs in the alginate matrix (Link and El-Sayed, 1999). The absorbance value of the resulting alginate beads at 535 nm was generally proportional to the concentration of H_2O_2 from 0 to 150 μM and tended toward a saturation at higher concentrations of H_2O_2 (Fig. 2, inset).

In order to investigate Au NPs in alginate beads after the growth reaction, we examined alginate matrices containing Au NPs by TEM. The specimen was prepared by deforming an alginate matrix after the growth reaction and drying the deformed matrix on a TEM grid. Figure 3 shows images of representative Au NPs within an alginate gel matrix, reacted with a H_2O_2 concentration of 0 μM (A), 60 μM (B), 120 μM (C), and 240 μM (D) in the growth solution, respectively. The overall size of Au NPs in the alginate matrix was gradually enlarged with the increasing amount of H_2O_2. The average size of Au NPs for each case was 14 nm (A), 16 nm (B), 25 nm (C), and 33 nm (D), respectively, indicating the enlargement of Au NPs in alginate matrix. According to Willner et al., Au NPs were grown in a free solution by the generation of Au clusters near Au NPs seeds in the presence of a reducing agent (Zayats et al., 2005). In the alginate matrix, however, the approach of $AuCl_4^-$ anions to activated Au NPs for the generation of Au clusters might be hindered by the charge-to-charge repulsion between COO^- in the alginate and $AuCl_4^-$. We suppose that the encapsulation of Au NPs in the alginate bead matrix can be facilitated by the electrostatic attraction of Ca^{2+} to both COO^- on alginate and anionic Au NPs, as illustrated in Figure 1. The lowered electrostatic repulsion between $AuCl_4^-$ and COO^- by the presence of Ca^{2+} may enable an easier approach of $AuCl_4^-$ into an alginate gel matrix to induce the reduction of $AuCl_4^-$ on the Au NPs. According to Pal et al. (2005), Au NPs synthesized by a photochemical reaction stayed stable in an alginate solution for months; they attributed this result to the stabilization of Au NPs by the presence of alginate. Thus, in our study, the alginate gel should work for the stabilization as well as the encapsulation of Au NPs within the matrix.

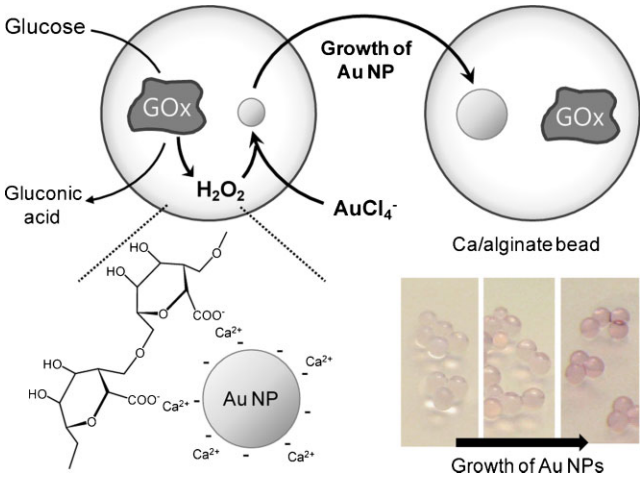

Figure 1. Schematic of the growth of Au NPs in an alginate matrix with co-encapsulated glucose oxidase (GOx) and Au NP seeds. The GOx-mediated oxidation of glucose produces H_2O_2, a reducing agent that induces the growth of Au NPs in the presence of $AuCl_4^-$. The encapsulation of Au NPs was facilitated by the electrostatic interaction of Ca^{2+} with anionic Au NPs and COO^- groups in the gel network. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

We applied the growth reaction of Au NPs within an alginate matrix by co-encapsulating GOx for the enzymatic detection of the glucose level. Alginate beads co-encapsulating Au NP seeds and GOx were incubated with glucose solutions at different concentrations, and $HAuCl_4$ and cetyltrimethylammonium chloride (CTAC) were added for the subsequent enlargement of Au NPs. The absorbance changes at 535 nm indicate that Au NPs in the alginate beads were successfully grown as the concentration of glucose increased (Fig. 4B). The absence of either glucose or GOx resulted in no color change at the reaction condition for the enlargement of Au NPs in the alginate matrix, which shows that only enzymatic oxidation of glucose induced the enlargement reaction. When we conducted a control experiment consisting of GOx in alginate without Au species, there was no change of absorbance at different glucose concentration (data not shown), indicating that Au species are essential for the detection of glucose. The absorbance value of alginate beads after the growth reaction including Au species increased proportionally with the glucose concentration from 0 to 800 μM, and the increase in absorbance saturated at higher glucose concentrations. The range of glucose concentration tested in this study is suitable to detect the blood glucose level for the diagnosis of diabetes (general range: 0–20 mM) (Bruulsema et al., 1997).

We observed the color of the resulting beads became purple and the absorbance maxima of the alginate only exhibited a red-shift with the increase of glucose concentration. The absorbance maxima for the growth of Au NPs in the alginate by the addition of H_2O_2 also exhibited a red-shift as the concentration of the reducing agent increased (Fig. 2). For a comparison, we conducted the growth of Au NPs in a free solution including GOx and glucose.

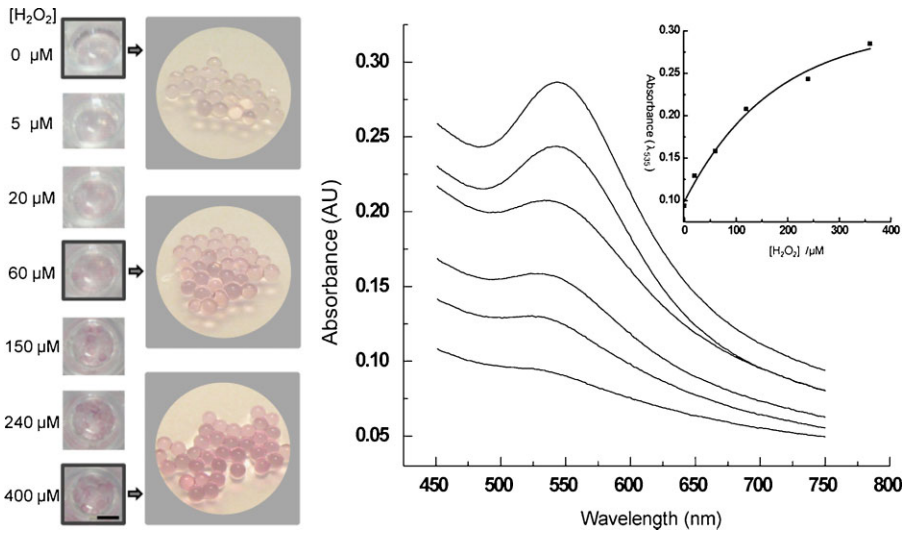

Figure 2. Optical images and UV absorbance spectra of Au NPs-incorporated alginate beads after the growth reaction. Each number indicates the concentration of H_2O_2 in the growth solution. Alginate beads encapsulating Au NPs were immersed in the growth solution containing $HAuCl_4$ and CTAC at 35°C for 1 h. The scale bar is 2 mm. The inset shows the absorbance changes at 535 nm for resulting beads at different H_2O_2 concentrations. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

According to our result, the absorbance maxima in the free solution reaction followed a red-shift at low concentrations of glucose and a blue-shift at higher concentrations (Fig. 4A). TEM images indicate that the red-shift is accompanied by the enlargement of Au NPs (Fig. 4A, a and b) and further blue-shifts at higher glucose concentration is associated with the increasing number of small Au NPs co-existing with large Au NPs in the solution (Fig. 4A and C). We attribute the red-shift to the enlargement of the Au NPs by the reduction of Au Cl_4^- on the Au NPs, while the blue-shift is due to the formation of Au NPs with smaller dimension together with enlarged Au NPs. Thus, the only red-shift observed in the GOx-mediated reaction within alginate matrix implies that the enlargement of Au NPs is dominant in the gel beads rather than the formation of smaller Au NPs, even at high glucose concentrations, as TEM images also support (Fig. 4B,a–c). In addition, according to Figure 4Bc, the red-shift might be caused by the aggregation of newly formed Au NPs within the alginate matrix. In a free solution, Au species should be detached by an interaction with the surfactant, CTAC, resulting in the generation of small free Au NPs. In the case of Au NP growth in an alginate matrix, the encapsulated Au NPs may become less susceptible to the interaction with the surfactant when surrounded by a gel network, thus, the detachment of Au species should be hindered to result in the formation of large aggregates.

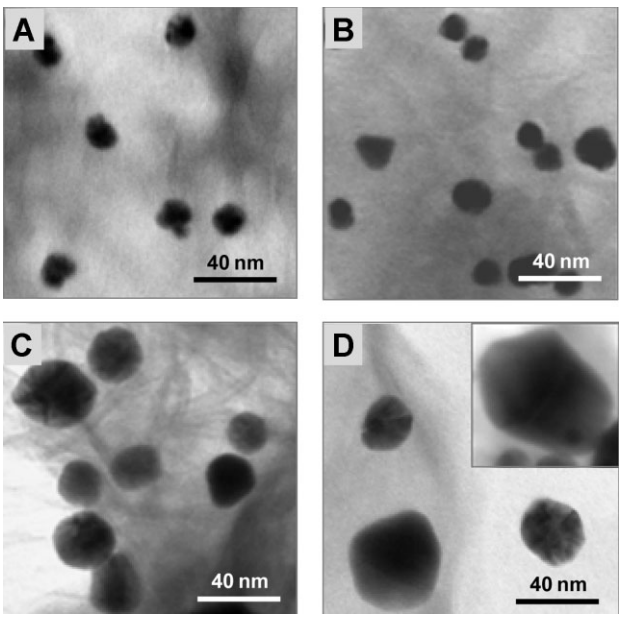

Figure 3. Representative TEM images of Au NPs formed in alginate beads after the growth reaction with H_2O_2 concentrations of (**A**) 0, (**B**) 60, (**C**) 120, and (**D**) 240 μM. The scale bar is 40 nm.

In summary, we have demonstrated a simultaneous growth of Au NPs through the biocatalytic oxidation of glucose by GOx encapsulated within the alginate matrix. A gradual enlargement of Au NPs was observed with the increasing concentration of H_2O_2 through TEM analysis and UV absorbance measurement as well as the color changes of the resulting gel beads. By encapsulating GOx together with Au NPs in the alginate gel matrix, the detection of glucose levels was performed successfully via the coupling of Au NP enlargement with the GOx-mediated oxidation of glucose. The generation of H_2O_2 accompanies many other biocatalytic reactions driven by various oxidases, thus, the

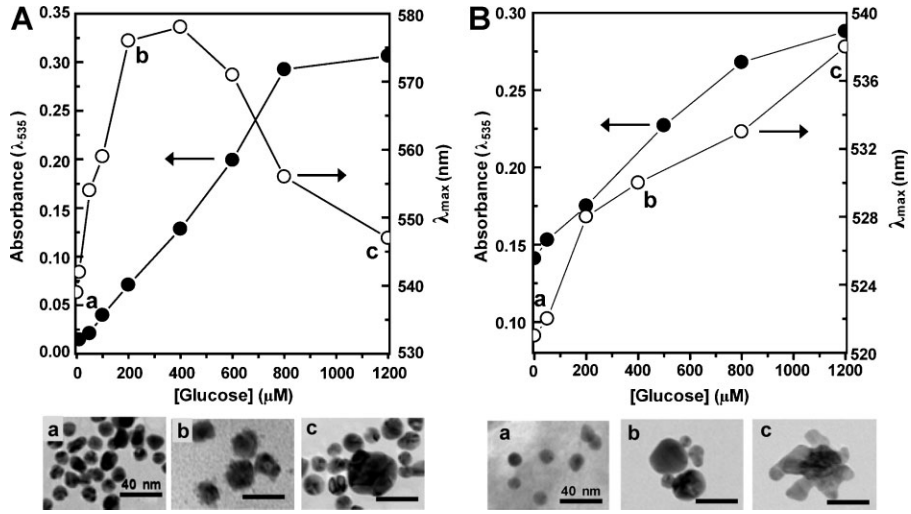

Figure 4. The absorbance changes at 535 nm (closed circles) and the wavelength of the absorbance maxima (open circles) for a free solution (**A**) and for an alginate matrix encapsulating GOx and Au NPs (**B**) after the growth reaction of Au NPs by the biocatalytic oxidation of glucose. TEM images of resulting Au NPs in each reaction are shown at different glucose concentrations.

co-encapsulation of metal NPs and oxidase within gel matrix should provide a simple route for the fabrication of optical biosensor.

Materials and Methods

GOx type II-S, hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot 3H_2O$), alginic acid sodium salt, calcium chloride ($CaCl_2$), sodium citrate tribasic dihydrate, and CTAC were purchased from Sigma–Aldrich (St. Louis, MO), and they were used without further purification. Au NPs (diameter: 13 ± 2 nm), stabilized with citrate, were synthesized according to the previous literature (Grabar et al., 1995). The concentration of the Au NP solution was determined by the number of Au NPs in the solution, which is calculated from total numbers of Au ions incorporated in the synthesis reaction and Au atoms in a single NP of which the dimension was analyzed from the TEM image (13 nm, with the assumption that all Au ions were reduced for the synthesis of Au NPs). The prepared Au NPs solution was kept stable for at least 1 month and used within 2 weeks of its preparation.

To encapsulate Au NPs in an alginate matrix, alginic acid sodium salt was dissolved in deionized water to make the solution at a final concentration of 5% (w/v). Au NP solution (40 μL, 1.2×10^{-8} M) and 260 μL of deionized water were mixed with 200 μL of an alginate solution. The alginate mixture was added into 100 mM $CaCl_2$ solution by using a syringe equipped with a 0.5 mm-diameter needle with a coaxial flow of N_2 at a constant flow rate of 0.8 mL/s. The average diameter of the alginate beads was 0.8 ± 0.1 mm, which was left at 4°C for 15 min and shaken at 60 rpm for gel bead formation. Alginate beads co-encapsulating GOx and Au NPs were prepared by mixing 40 μL Au NPs (1.2×10^{-8} M), 30 μL GOx solution (1 mg/mL), and 230 μL distilled H_2O with 200 μL alginate solution and dropping the mixture into 100 mM $CaCl_2$ solution. The solution for the growth of Au NPs consisted of 0.2 mM $HAuCl_4$ and 2 mM CTAC in a phosphate buffer (0.01 M, pH 7.0). CTAC is one of common surfactants utilized in the enlargement of Au NPs (Zayats et al., 2005). The alginate beads were placed in a 96-well plate with ~50 beads per well after a washing with deionized water. Then, 150 μL of the growth solution with different concentration of H_2O_2 was introduced to wells, and the plate was incubated at 35°C for 1 h. For the growth of Au NPs initiated by the GOx-mediated oxidation of glucose, ~50 alginate beads encapsulating GOx and Au NPs were immersed in the 10 mM phosphate buffer (pH 7.0) with different concentrations of glucose at 30°C for 5 min under an O_2 atmosphere, then $HAuCl_4$ (0.2 mM) and CTAC (2 mM) were added, and kept at 35°C for 1 h. The resulting beads were washed with a 10 mM phosphate buffer (pH 7.0). For the growth of Au NPs mediated by the oxidation of glucose in a free solution, a 10 mM phosphate buffer, containing GOx (0.03 mg/mL), Au NPs (3×10^{-10} M), and glucose (at various concentrations), was kept at 35°C for 30 min under an O_2 atmosphere, followed by the addition of growth solution to the mixture and incubation at 37°C for 1 h.

UV–Vis spectra and absorbance at 535 nm were measured by using a UV–Vis spectrophotometer (Cary 100, Varian, Palo Alto, CA) and a plate reader (1420 multi-label counter, PerkinElmer, Waltham, MA), respectively. For the absorbance measurement, resulting gel beads were filled into a quartz-cuvette with the path length of 1.0 mm. For the TEM

analysis of Au NPs in an alginate matrix, alginate beads after the growth reaction were deformed, 3 μL of which were applied to a carbon-coated copper grid, and dried in a vacuum at room temperature for 3 h. For the TEM analysis, we used a scanning TEM (HD-2300A, Hitachi, Tokyo, Japan) operating at 200 kV.

This work is supported by the Korea Science and Engineering Foundation (KOSEF) National Research Laboratory Program (R0A-2008-000-20041-0), the Eco-Technopia 21 project (010-081-036) from the Ministry of Environment, the Fundamental R&D Program for Core Technology of Materials from the Ministry of Knowledge Economy, and the EEWS Initiative (EEWS0913) from the office of KAIST, Republic of Korea.

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