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A novel technique for *in situ* aggregation of *Gluconobacter oxydans* using bio-adhesive magnetic nanoparticles

Huimin Lu^{1,a}, Kefeng Ni^{1,a}, Cunxun Wang¹, Kvar C.L. Black^{2,3}, Dongzhi Wei¹, Yuhong Ren^{1,*}, and Phillip B. Messersmith^{2,3,*}

¹State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China

²Biomedical Engineering Department, Northwestern University, Evanston 60208, USA

³ Chemistry of Life Processes Institute, Northwestern University, Evanston 60208, USA

Abstract

Here, we present a novel technique to immobilize magnetic particles onto whole *G. oxydans in situ* via a synthetic adhesive biomimetic material inspired by the protein glues of marine mussels. Our approach involves simple coating of a cell adherent polydopamine film onto magnetic nanoparticles, followed by conjugation of the polydopamine-coated nanoparticles to *G. oxydans* which resulted in cell aggregation. After optimization, 21.3 mg (wet cell weight) *G. oxydans* per milligram of nanoparticle was aggregated and separated with a magnet. Importantly, the *G. oxydan* aggregates showed high specific activity and good reusability. The facile approach offers the potential advantages of low cost, easy cell separation, low diffusion resistance and high efficiency. Furthermore, the approach is a convenient platform technique for magnetization of cells *in situ* by direct mixing of nanoparticles with a cell suspension.

Keywords

cell aggregation; iron oxide nanoparticles; *Gluconobacter oxydans*; polydopamine

Introduction

Whole cells can be considered as mini-reactors containing all the necessary cofactors and enzymes necessary for use in biocatalysis (Ni and Chen 2004; Park et al., 2006), wastewater treatment (Munoz and Guieysse 2006), pharmaceutical and food industries (Gao et al., 2009; Merfort et al., 2006; Ng and Jaenicke 2009). Easy separation, stability, and repeated reuse play important roles in the large-scale utilization of whole cells as biocatalysts (Verbelen et al., 2006). Although several microbial cell separation techniques including settling tanks (Schilling et al., 2002), hydrocyclones (Pinto et al., 2008), self-aggregation by flocculation (Xu et al., 2005) and centrifuges (Chen et al., 2007) have been evaluated, iron oxide nanoparticles (IONPs) with appropriate surface modification provides an economical, quick, and convenient method for separation of biomacromolecules such as proteins, enzymes, antibodies, as well as cells (Chien and Lee 2008; Kuhara et al., 2004; Li et al., 2009; Xu et al., 2004). Typically, surface modification of IONPs is performed for the purpose of preventing aggregation and improving the stability and mobility of IONPs which is very

*Correspondence to: Y. Ren (Tel.: +86 21 6425 2163, Fax: +86 21 6425 0068) and P. B. Messersmith (Tel.: +1 847 467 5273, Fax: +1 847 491 4928). yhren@ecust.edu.cn (Y. Ren), philm@northwestern.edu (P. B. Messersmith)..

^aThese authors contributed equally to this work.

important for biomedical applications (Amstad et al., 2009; Kuhn et al., 2006). However, in the case of cell separation and catalysis where ease of separation is critical, clusters of nanometer-sized carriers are superior in their binding capacity, magnetic contrast, and diffusion of substrate and product (Lee et al., 2009).

In this study, we propose a new facile approach to aggregate *G. oxydans* using magnetic nanoparticle clusters *in situ*, inspired by the adhesive catechol and amine rich proteins secreted by marine mussels (*Mytilus edulis*) (Lee et al., 2007). Catechol (ortho-dihydroxyphenyl) is the functional group of 3,4-dihydroxy-L-phenyl-alanine (dopa) which is abundant in mussel adhesive proteins, and forms strong bonds with various inorganic and organic surfaces in aqueous media (Lee et al., 2006). The magnetic nanoparticles have a large surface area and are adhesive to cells by virtue of the dopamine coating, which induces cell aggregation (Yang et al., 2011), and confers magnetic activity to cells. Cells containing bound magnetic nanoparticles are easily separated by magnet, facilitating cell reuse and eliminating the costly processes of cell recovery by conventional means. Moreover, cells immobilized in this way are expected to be more stable than free cells and provide less interference with the diffusion of substrate and product. *Gluconobacter oxydans* was selected as a model cell because it acts as a mini-reactor, having been widely used to oxidize sugar, alcohol and aldehyde to produce aldehyde, ketone and acid by its dehydrogenases connected to the respiratory chain (Hölscher et al., 2009; Li et al., 2010; Wu et al., 2010). From a practical perspective, this novel cell purification process using polydopamine-coated IONPs overcomes the common difficulties in the reuse or recycling of *G. oxydans* cells for industrial applications.

Materials and Methods

Materials

Ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), dopamine hydrochloride, Tris buffer and sodium hydroxide were purchased from Sigma-Aldrich (USA). All other chemicals were of the highest grade available. Ultrapure water (resistivity = 18.2 M Ω , pH 6.82) was used in all experiments and obtained from a NANOpure Infinity[®] system from Barnstead/Thermolyne Corporation (Dubuque, IA).

Synthesis of polydopamine coating iron oxide nanoparticles (PD-IONPs) PD-IONPs were prepared by simple coating of adherent polydopamine (PD) film onto magnetic nanoparticles as described in our previous work (Ren et al., 2011) with some modifications. Briefly, 0.5 mmol FeCl_2 and 1.0 mmol FeCl_3 were dissolved in 50 ml ultrapure water under nitrogen at 40°C, and then the pH of the solution was adjusted to 10.0 using 6.0 M NaOH under vigorous stirring. After stirring for 2 hours, the magnetite precipitates were separated and washed several times with ultrapure water by magnetic decantation. The precipitate was dispersed in 50 ml Tris buffer (10 mM, pH 8.5) under ultrasonication for 15 min, after which large precipitates were removed. Dopamine hydrochloride (25mg, 0.5 mg/ml) was added to the remaining IONPs suspension with stirring, and the pH of the solution was kept at 8.5 by addition of 10 mM NaOH. After vigorous stirring for 30 min, the PD-IONPs were collected by magnetic decantation, washed 3 times with ultrapure water, and finally re-dispersed by ultrasonication for 15 min in ultrapure water to final concentration of 1 mg PD-IONPs per milliliter solution.

Preparation of Cells

The strain *G. oxydans* DSM 2003 was used in this study. The cells were cultured in 50 ml fermentation medium containing 80 g/l sorbitol, 20 g/l yeast extract, 1 g/l KH_2PO_4 , 0.5g/l MgSO_4 , 0.1g/l glutamine, and distributed in 500 mL shake flasks. The flasks were sterilized

by autoclaving at 115 °C for 20 min. As *G. oxydans* possesses a natural resistance towards cefoxitin, cultivation was carried out using 50 µg/ml cefoxitin sodium. The cells were incubated at 30 °C with shaking at 200 rpm for 24 h.

Aggregation of *G. oxydans*

G. oxydans aggregation was carried out by adding the suspension of PD-IONPs in ultrapure water to the cell fermentation solution as described above. A fresh solution of the previously described PD-IONPs (2 ml, 1 mg/ml) was added to the cell fermentation solution (25 ml, 2 mg wet cells per ml) at 4 °C. Precipitation was observed immediately as the two solutions mixed. After shaking at 180 rpm for 30 min at 4 °C, the cell-PD-IONPs aggregates were collected using a magnet, washed 3 times with ultrapure water, and stored at 4 °C prior to use. The density of free cells in the solution was determined by measuring the optical density of the cell suspension at a wavelength of 600 nm (OD₆₀₀) with a spectrophotometer (U-2001; Hitachi, Tokyo), both before PD-IONPs were added and after separation. The cell suspension was diluted with ultrapure water to maintain OD₆₀₀ readings in the linear range below 1.0. The difference in cell density was used to calculate the cell-PD-IONPs aggregates.

IONP Material Property Characterization

To characterize aggregate morphology, scanning electron microscopy (SEM) images of the cell aggregates were acquired on a Hitachi S4800 scanning electron microscopy (Hitachi, Japan). SEM specimens were prepared by casting drops of dilute dispersion of the aqueous cell aggregate solution onto a silicon wafer and drying under 4°C. To characterize the composition of the PD-IONP aggregates, thermo gravimetric analysis (TGA) was carried out on a SDT 2960 model from TA Instruments (USA). All samples were dried under vacuum overnight to remove moisture before analysis and were heated from 50 °C to 750 °C at 10 °C /min under a nitrogen flow. Magnetic measurements were recorded on a vibrating sample magnetometer (VSM, Lake Shore, USA) at room temperature.

Dynamic Light Scattering (DLS)

DLS experiments were carried out on a Nicomp 380 ZLS (PPS, USA). The PD-IONPs and IONPs were dispersed at a concentration of 100 µg/ml in ultrapure water under ultrasonication for 15 minutes.

Activity Assay of Free Cells and the Cell-PD-IONPs Aggregates

The activity of cells was measured at 30 °C in phosphate buffer (pH 6.5, 10 mM) through the catalytic conversion of glycerol to dihydroxy acetone (DHA). A typical reaction volume was 1 ml, and the concentration of glycerol in the mixture was 10 g/l. After shaking for 60 minutes at 30 °C in a thermomixer (HLC, German), the free cells were separated by centrifugation and the cell-PD-IONPs aggregates were separated by a magnet. The reaction products were analyzed by HPLC using a COREGEL 87H3 column (Transgenomic, USA). Each injected sample (10 µl) was eluted at 35 °C with 4 mM H₂SO₄ at a flow rate of 0.4 ml/min, and the products were detected at 210 nm. The retention time of DHA was 20.2 minutes under these conditions.

Effect of Ionic Strength, pH, and Temperature

The effect of ionic strength on the cell aggregation by IONPs and PD-IONPs was determined by mixing the cell and the particles in the NaCl solution ranging from 0 to 1.0 M at 4 °C for 30 min, followed by measuring the optical density of the cell suspension at a wavelength of 600 nm (OD₆₀₀) after separation as described in the cell aggregation assay section. The effect of pH on the cell aggregation by IONPs and PD-IONPs was determined

by mixing the cells and the particles at 4 °C in the solution at pH ranging from 6 to 10 by addition 10mM NaOH or 10 mM HCl for 30 min, followed by measurement of the suspension OD₆₀₀ as described above. The effect of temperature on the cell aggregates mixed with either IONPs or PD-IONPs was determined by mixing the cells and particles in solution (pH 7.0) at temperatures ranging from 4 °C to 50 °C for 30 min, followed by measurement of the suspension OD₆₀₀ as described above.

Recovery and Reuse of Cell Aggregates

The stability of free cells and the cell aggregates under conditions of repeated magnetic isolation and reuse was studied under the same conditions as described in activity assay section. After each cell run, the cell aggregates were magnetically isolated and washed twice with ultrapure water to remove any remaining substrate and product species before the next experiment. The residual activity of the cell aggregates after each cycle was normalized to the initial value (the initial activity was defined as 100%).

Results and Discussion

Synthesis and Characterization of PD-IONPs

IONPs were prepared by alkaline co-precipitation of Fe (II) and Fe (III) and then incubated in an alkaline dopamine solution for a given time to create an adherent PD film on IONP clusters. The size and morphology of the IONPs and PD-IONPs were observed by secondary electron microscopy and TEM in our previous work (Ren et al., 2011). The average size of the PD-IONPs clusters is about 320 nm, larger than as-synthesized IONPs clusters (average diameter ~ 150 nm) due to PD induced partial aggregation of the IONP clusters.

Thermogravimetric analysis (TGA) (Figure 1) performed on the nanoparticles shows that the mass loss of both the IONPs and the PD-IONPs begins at 105 °C due to the evaporation of bound water. The mass of the IONPs becomes stable when the temperature reaches 410 °C with a final 12.1% weight loss. However, a large mass loss of the PD-IONPs is observed from 620 °C to 660 °C which is presumed to result from decomposition of PD. The final mass loss of the PD-IONPs is 48.8%, indicating that PD comprises more than one third of the mass of the PD-IONPs.

A typical magnetization curve of IONPs, PD-IONPs, cell-IONPs aggregates and cell-PD-IONPs aggregates is shown on figure 1B. The hysteresis loop revealed the superparamagnetic behavior (no hysteretic behavior) of IONPs at room temperature before and after coating with PD. The saturated magnetization (M_s) of IONPs and PD-IONPs were 69.8 emu/g and 64.7 emu/g (at 15000G), respectively. The results suggested that PD did not significantly influence the magnetic properties of IONPs.

Aggregation of *G. oxydans* Using PD-IONPs

The strain *G. oxydans* was cultured using normal methods. Cells and PD-IONPs were combined in suspension and then the pH of the solution was adjusted to 7.0 using 100 mM NaOH. The binding of PD-IONPs and cells was rapid, resulting in precipitation of iron-bound cell aggregates soon after mixing. The percentage of aggregated cells increased over the first 30 minutes of the reaction, reaching a final value of 85.2% (Figure 2). In contrast, cells forming aggregates through binding with unmodified IONPs was significantly lower (19.2%) and was complete within 1 min, presumably by physical adsorption due to electrostatic forces between cell membranes and the IONPs. The difference in the aggregation process suggests that the mechanism of PD-IONPs binding to cells was different than with unmodified IONPs. We speculate binding may be due to the reactivity of PD toward nucleophiles such as primary amines and thiol groups on the cell surface, rather

than solely through electrostatic adsorption. The residual quinones in the PD coating, which are reactive toward nucleophilic amino or thiol groups, can give rise to covalent linking to nucleophilic biomolecules through Michael addition and/or Schiff base formation (Lee et al., 2006; Yang et al., 2011). Thus it is likely that covalent as well as noncovalent interactions between the surface of the PD-IONPs and the cells result in binding of PD-IONPs to the cell surface, leading to the formation of cell aggregates (Figure 3A).

The morphologies of the cell-IONPs aggregates and the cell-PD-IONPs aggregates were observed by SEM (Figure 3C and 3D, 3E and 3F). Clusters of IONPs covering almost the entire cell surface were observed, whereas clusters of PD-IONPs with sizes ranging from 150 nm to 400 nm surrounded and adhered to part of the surface of the cells and induced cell-to-cell aggregation. This indicates that PD-IONPs adhere to the cells' surface differently than unmodified IONPs, potentially through both covalent and noncovalent mechanisms, providing superior cell aggregation efficiency. Magnetic isolation of the cell-PD-IONPs aggregates was accomplished by placing a magnet adjacent to a vial containing cells suspended in the culture medium (Figure 3B). Within 15 seconds the solution became clear as a result of movement of the aggregated cells towards the magnet, demonstrating simple and rapid isolation of the cell aggregates using a magnetic field. Removal of the magnet followed by agitation led to re-suspension of the cell-PD-IONPs aggregates.

The superparamagnetic behavior of IONPs remained after binding to cells (Figure 1B). The M_s of cell-IONPs aggregates and cell-PD-IONPs aggregates were 25.1 emu/g and 14.0 emu/g (at 15000G), respectively, which was significantly lower than that of particles. The decrease of M_s was due to the increased amount of cell in the cell-particle aggregates because the weights of all samples used for the measurement of magnetic properties were constant in this experiment. Importantly, the presence of the magnetic moments in the cell-particle suspensions indicate that the IONP clusters retain their superparamagnetic properties even upon coating with PD and adhering to cell surfaces, which can be used to separate cells in suspension using a magnet.

The efficiency of cell binding and aggregate formation, as well as the activity of the cell-PD-IONPs aggregates at pH 7.0, is shown in Table 1. In the absence of PD treatment, only 19.2% of the cells were purified with the magnet due to the weak physical adsorption between cells and the magnetic IONP clusters. Through the use of PD, the amount of cells able to be purified with the magnet increased by as much as 4-fold due to strong interactions between the PD-IONPs and the cells. The data of activity recovery showed that the *G. oxydans*-PD-IONPs aggregates retained 97.1% of the activity for the conversion of glycerol to dihydroxy acetone (DHA) compared to the same mass of free cells. These results indicate that *G. oxydans* formed aggregates via PD-IONPs with high efficiency and low diffusion resistance.

Effect of Dopamine Concentration and Incubation Time During the Preparation of PD-IONPs on *G. oxydans* Aggregation Efficiency

The effect of dopamine concentration used during the preparation of PD-IONPs on the amount of cell aggregation was investigated and the results are shown in Figure 4A. Increasing the concentration of dopamine used in preparing the PD-IONPs in the range 0 – 0.5 mg/ml resulted in an increase in the percentage of aggregated cells from 19.2% to 85.2%, indicating that the coating of IONPs with PD increased dramatically their binding capability to cells. However, the binding capability of the PD-IONPs began to decrease above dopamine concentration of 1mg/ml, which we believe to be due to the increase in mean diameter of the PD-IONPs clusters at high dopamine concentration (Figure 4B and 4C). Cell aggregation efficiency was also found to be dependent on the PD coating reaction time onto IONPs, as illustrated by preparation of PD-IONPs using a 0.5 mg/ml dopamine

solution (Figure 5). Increasing the PD coating time in the range 0–30 min resulted in an increase in the cell aggregation capacity, although cell aggregation efficiency began to decrease above 30 min reaction (Figure 5A). The results imply that the cell aggregation efficiency increased with thicker PD coatings on IONP clusters until the mean diameter of the PD-IONPs clusters reached a critical size around 30 minutes, which caused cell aggregation efficiency to decrease (Figure 5B and 5C).

Effect of Ionic Strength, pH and Temperature on Efficiency of *G. oxydans* Aggregation

As shown in Figure 6A, 6B, and 6C, the cell aggregation efficiency of both IONPs and PD-IONPs was found to depend on ionic strength, pH, and temperature of the solution during the aggregation process. For IONPs, the cell aggregation efficiency rapidly decreased upon increasing the concentration of NaCl from 0 to 100 mM, and increased with increasing pH. However, for PD-IONPs, little change was observed with increasing the concentration of NaCl from 0 to 250 mM, and the best performance was observed near neutral pH. Similar results were found in $(\text{NH}_4)_2\text{SO}_4$ solution (data not shown). Correlating with the results already described, these results suggest that the mechanism of the cell aggregation by PD-IONPs is different compared to unmodified IONPs. As mentioned previously, it is hypothesized the difference is caused by covalent as well as noncovalent interactions between the surface of the PD-IONPs and the cells, compared to solely electrostatic adsorption between the bare IONPs and the cells. The effect of temperature on the cell aggregation efficiency was similar for both IONPs and PD-IONPs, with neither adversely affected at temperatures below 20 °C, dropping significantly above 37 °C to a low of 50.1% at 50 °C.

Magnetic Isolation and Reuse

We investigated reuse of the cell-PD-IONPs aggregates in view of their intended use for multiple cycles of magnetic isolation and reuse for conversion of glycerol to DHA. The activities of free cells, and cell aggregates using IONPs or PD-IONPs clusters, are shown in Figure 7. The activities of cells toward conversion of glycerol to DHA began to decrease after 6, 8 and 12 cycles, for free, IONPs and PD-IONPs, respectively. Compared to the free cells which were separated by centrifugation, the cell-PD-IONPs aggregates purified with a magnet displayed excellent stability over many cycles, and the conversion of glycerol was more than 70%, even after 15 cycles. The drop in activity of recycled cells may be caused by cell lysis, as increased protein levels were detected in the supernatant after many cycles (data not shown). The increased cycles of activity of the cell aggregates compared to the free cells may be due to decreased damage of cell's membrane by DHA after aggregates are formed.

Conclusions

We described a facile method for adhesion of magnetic nanoparticles onto cells and the formation of cell aggregates through an adhesive PD film. Our aggregation experiments show that PD-IONPs exhibit high *G. oxydans* loading capacity due to high surface area and strong adhesive interactions between the cells and PD. Aggregation does not affect the activity of *G. oxydans* for the catalytic oxidation of glycerol, and the *G. oxydans* aggregates show high specific activity and good reusability due to the convenience of the magnetic recovery method facilitated by binding of PD-IONPs onto cells. Cells aggregated by the optimized protocol show better stability than free cells, leading to maintenance of high cell biocatalytic activity over more cycles. These results demonstrate that aggregation of cells via PD coated magnetic iron oxide nanoparticles is economical, facile and efficient. This strategy could be used in the future as a convenient technique for immobilization of

enzymes (Lee et al. 2009; Ren et al. 2011), DNA (Ham et al. 2011), antibodies (Black et al. 2012), and other biomolecules onto magnetic nanoparticles.

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Figure 1.

A: TGA curves of the IONPs (red dots) and PD-IONPs (black squares). B: Hysteresis loop of magnetic nanoparticles before and after binding to cells.

Figure 2.
Effect of incubation time of the PD-IONPs with cells on the amount of cell aggregation.

Figure 3.

A: Illustration of the aggregation of *G. oxydans* using PD-IONPs. B: Photograph of a suspension of free cells in culture medium (left) and PD-IONP aggregated cells after magnetic isolation (right). C and D: SEM images of the cell-IONPs aggregates. E and F: SEM images of the cell-PD-IONPs aggregates.

Figure 4.

A: Effect of dopamine concentration used during preparation of PD-IONPs on cell aggregation efficiency. B: Effect of dopamine concentration used during preparation of PD-IONPs on the mean diameter of the PD-IONPs. C: Particle size distributions of IONPs (black), and PD-IONPs formed at 0.5 mg/ml (red) and 5.0 mg/ml (green) initial dopamine concentrations after stirring for 30 min at room temperature.

Figure 5.

A: Effect of the PD coating time on the cell aggregation efficiency of PD-IONPs. B: Effect of the PD coating time on the mean diameter of the PD-IONPs. C: DLS of IONPs (black), and PD-IONPs formed by coating for 45 min (red) or 120 min (green) at an initial dopamine concentration of 0.5 mg/ml.

Figure 6.

Effect of ionic strength, pH, and temperature on cell aggregation efficiency of IONPs and PD-IONPs, respectively. A: Effect of ionic strength on cells aggregation efficiency. B: Effect of pH on cell aggregation efficiency. C: Effect of temperature on cell aggregation efficiency.

Figure 7. Activity of cells as a function of the number of cycles of isolation and reuse. The free cells were isolated by centrifugation, whereas the PD-IONP aggregated cells were recovered by magnetic isolation. The total elapsed time of the experiment was less than 30 hours.

Table 1
The efficiency of the cell aggregation and the activity of the cell-PD-IONPs aggregates.

Materials	Amount of Nanoparticles (mg)	Added Cells (mg)	Aggregated Cells (mg)	Cell Recovery (%)	Activity Recovery (%)
IONPs	2	50	9.6±0.9	19.2±1.8	18.3±0.5
PD-IONP ^[a]	2	50	42.6±1.2	85.2±2.4	82.7±1.0
Free cells		50			100

^[a]The PD-IONPs were prepared using 0.5 mg/ml initial dopamine and cell aggregation performed using 50 mg wet cells and 2 mg materials at pH 7.0 at 4 °C for 30 min.