Use of Polyelectrolyte Complex for Immobilization of Microorganisms

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Synopsis

Cells from *Escherichia coli* (IAM 12119) were immobilized with the polyion complex of trimethylammonium glycol chitosan iodide (TGCI) and potassium poly(vinyl alcohol) sulfate (KPVS). The immobilization was carried out at pH 8 by mixing TGCI with the cell suspension, followed by addition of KPVS. The immobilized cells were characterized by investigating the glucose oxidizing activity. The results obtained indicated that the glucose consumption with immobilized cells is due not only to the cells released from the complex support but also to the entrapped cells which are grown in the complex; therefore, the cells entrapped in the complex have the glucose oxidizing activity. The physicochemical studies on the immobilization mechanism showed that cells are immobilized via two stages: the aggregation of cells with TGCI and the entrapment of the aggregates in the TGCI–KPVS complex. In the aggregation process, a part of TGCI which is added to cell suspension adsorbs on the cells and the other remains in the suspension. In the entrapment process, the remainder forms the polyion complex with KPVS added and the aggregated cells are coprecipitated with the complex.

INTRODUCTION

At present, there are numerous reports on the immobilization of enzymes[1,2] and microorganisms.[3–5] The successful methods employed in cell immobilization are based on the adsorption onto suitable adsorbents and on the entrapment in gel of polymeric substances, while enzymes are also immobilized by covalently bonding to solid matrix.

It is well known that the mixing of oppositely charged polyions yields polyelectrolyte complexes which are usually insoluble in aqueous media.[6] The complexation takes place rapidly at room temperature and is little affected by pH if the system is composed of strongly acidic and basic polyions. Moreover, the effects of organic and inorganic compounds which are used as nutrients do not need to be considered so long as a polyion complex is used as support for the immobilization. These facts prompted us to investigate the preparation of immobilized cells by using polyion complex, although little attention has been paid to this immobilization method.

We recently demonstrated that cells from *Nitrosomonas europaea* are successfully immobilized by entrapment in the polyion complex of trimethylammonium glycol chitosan iodide (TGCI) and potassium poly(vinyl alcohol) sulfate.[7] Furthermore, the resulting complex has ammonia oxidizing activity even after total 2000 h of incubation in a batch reactor. It is also interesting to study cell immobilization with polyion complex for other microorganisms in order to

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establish this immobilization method and to clarify the immobilization mechanism.

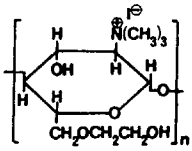
In this study, cells from *Escherichia coli* were immobilized with TGCI–KPVS complex. The characterization of immobilized cells was made by examining the glucose oxidizing activity. The immobilization mechanism was further investigated by means of different physicochemical measurements.

EXPERIMENTAL

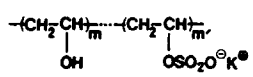
The concentration of polyelectrolyte is reported as the molar concentration of ionizable groups attached to the polymer chain, that is, 1 m*M* KPVS and 1 m*M* TGCI solutions correspond to 166 and 444 mg/L, respectively.

Materials

Chemicals. TGCI and KPVS were the same as used previously.[7–11] The structural formulas for these polyelectrolytes are:



TGCI



KPVS

The amount of trimethylammonium groups in TGCI was 2.25 mmol/g, as determined by colloid titration. The degree of esterification for KPVS, expressed as $m'/(m + m')$, was estimated to be 0.974 from the sulfur content. The number-average molecular weight was 2.54×10^6 for KPVS and 1.03×10^4 for TGCI.

Microorganisms. *Escherichia coli* (IAM 12119) was maintained on agar slants of the following composition (in g/L): meat extract, 10; peptone, 10; NaCl, 5; agar, 15. The culture was restreaked every four weeks. The cells used for experiments were cultivated in the following medium (in g/L): D-glucose, 1.5; K_2HPO_4, 1.0; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.01; $MnCl_2 \cdot 4H_2O$, 0.002; $NaMoO_4 \cdot 2H_2O$, 0.001; $(NH_4)_2SO_4$, 1.0; pH 7. The incubation was carried out in 400-mL Erlemeyer flasks (each containing 100 mL of the medium) at 30°C for 24 h on a rotary shaker. The cells were harvested by centrifugation (10 min, 12,000 rpm) and washed with appropriate buffer. In certain cases of the immobilization experiment, the culture broth was directly used without collection of the cells.

Measurement of Cell Concentration

Cell concentration, expressed in cells/mL, was determined from an empirical relationship between cell number and optical density at 660 nm (see Fig. 1). The cell number was measured by counting cells in a Thoma chamber. Measurement of the optical density was made in a 5-mm-path cell using a Hitachi spectrophotometer model 101.

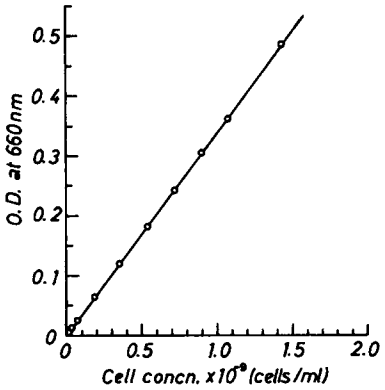


Fig. 1. Linear relationship between optical density and cell concentration.

Cell Immobilization

The immobilization was carried out for two cell samples: one was direct use of the culture broth, and the other was suspension prepared by dispersing the harvested cells into 0.1 *M* phosphate buffer solutions at pH 6–8. The cell concentration for both samples was 5×10^7 cells/mL. The standard immobilization procedure is as follows: The cell suspension (100 mL) was mixed with 8 mL 5 m*M* TGCI solution and then treated by the addition of KPVS (5 m*M*, 8 mL) with stirring to form TGCI–KPVS complex. The resulting complex was separated by decantation, washed with a large amount of phosphate buffer (pH 8), and stored at 3°C before use.

Electron-Microscopic Observation

Immobilized cell sample was fixed with 2% glutaraldehyde for 1.2 h and postfixed with 2% OsO_4 for 1 h. Sodium phosphate buffer (0.1 *M*, pH 8) was used as the solvent and for washing. The specimen was dehydrated through a graded series of ethanol, twice for 15 min each in 30, 50, 70, and 95% and three times in 100%, and then dipped in isoamyl acetate for 1 h to replace ethanol. The dehydrated sample was dried with a Hitachi critical point desiccator model HCP-2. The sample was finally coated with gold and observed by a Hitachi scanning electron microscope model S-450.

Metabolic Activity

The glucose oxidizing activity for immobilized cells was investigated and compared with that found for free cells. The incubation was carried out at 25–35°C for a maximum of 10 h on a rotary shaker. The medium was the same composition as used in the cultivation of the seed culture. To examine the pH effect on the activity, the medium was prepared by using various buffer solutions as the solvent. The buffer solution systems were: CH_3COOH–CH_3COONa, pH 4–6; $NaHPO_4$–KH_2PO_4; pH 6–8; and $NaHCO_3$–Na_2CO_3, pH 9–10. The glucose concentration was measured by the enzymic decomposition method.[12]

Aggregation and Adsorption Experiments

The harvested cells were washed with 0.85% NaCl solution (physiologic saline solution), suspended in the same solution (cell concentration 10^6–10^9 cells/mL), and then adjusted to pH 2–10 with 0.05 *N* HCl or NaOH. The cells in the suspension (40 mL) were aggregated by slow addition of 0.5–5 mL 1 m*M* TGCI or KPVS. After the removal of the aggregates by centrifugation (10 min, 2000 rpm), the amount of the polymer remaining in 20 ml of the supernatant was measured by the colloid titration method. The titration technique has been reported before.[10,13,14] The amount of the polyion required to aggregate cells (or the adsorptive amount of the polyion) was determined from the titration data.

To obtain information about the adsorption mechanism, the reflection infrared spectrum was measured for free and aggregated cells. The measurement was performed for a mixture of the dry cell sample and KBr powder using a Hitachi IR spectrophotometer model 260-50 equipped with a Hitachi powder-diffused reflection apparatus model IRR-8.

RESULTS AND DISCUSSION

Preparation of Immobilized Cells

Cells suspended in a phosphate buffer solution at pH 6–8 were aggregated by the addition of TGCI. TGCI was also effective for the aggregation of cells from the culture broth obtained by cultivation of the seed culture. However, KPVS did not bring about the aggregation at the neutral pH region. Thus, TGCI was chosen as a reagent for the cell aggregation.

When KPVS was added to the suspension (pH 6–8) containing the cells aggregated with an excess of TGCI, an amorphous complex resulted from complexing KPVS with the excess of TGCI. After the removal of the resulting complex by decantation, no free cells remained in the supernatant; therefore, the aggregated cells are mostly or entirely entrapped in the polyion complex of TGCI and KPVS. Figure 2 shows scanning electron micrographs for immobilized cells. It is seen that the cells are situated in the pores and on the surface of the complex support.

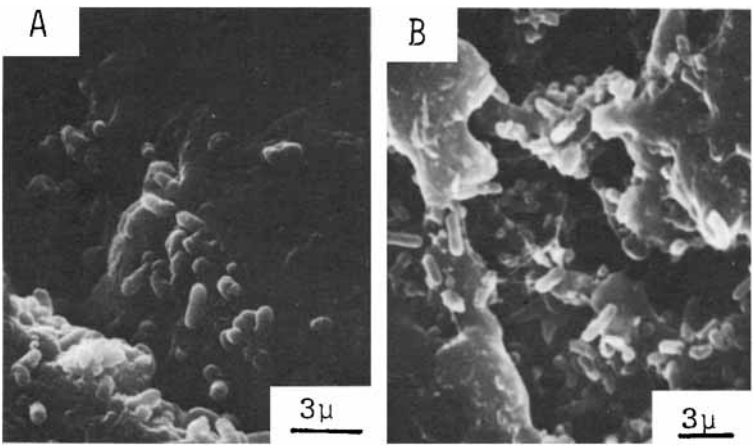


Fig. 2. Scanning electron micrographs of immobilized *E. coli* cells. A and B show the surface and inside of TGCI–KPVS complex support, respectively.

Aggregation and entrapment at pH 7–8 can be made by 2–5 mmol per 1 g dry cells (corresponding to 2.47×10^{12} cells). However, the results of the preliminary experiments showed that about 20 mmol of both polymers is required for the preparation of a stable support; thus, the standard procedure described in the experimental section was determined. Unless otherwise noted, the immobilized cells used here were prepared from a culture broth adjusted to pH 8 according to the standard procedure. The direct preparation of immobilized cells from the culture broth seems to be one of the principal advantages of the present method, because other immobilization methods require the collection of cells before entrapment.

Characterization of Immobilized Cells

Immobilized cells were characterized by investigating the glucose oxidizing activity. Figure 3 shows the time courses of glucose consumption with free and immobilized cells, together with growth curves of free cells and cells released from the complex support. No remarkable difference is observed in the glucose concentration profiles between free and immobilized cells, whereas the amount of released and grown cells is smaller than that of free cells. These results indicate that the glucose consumption with immobilized cells is due not only to

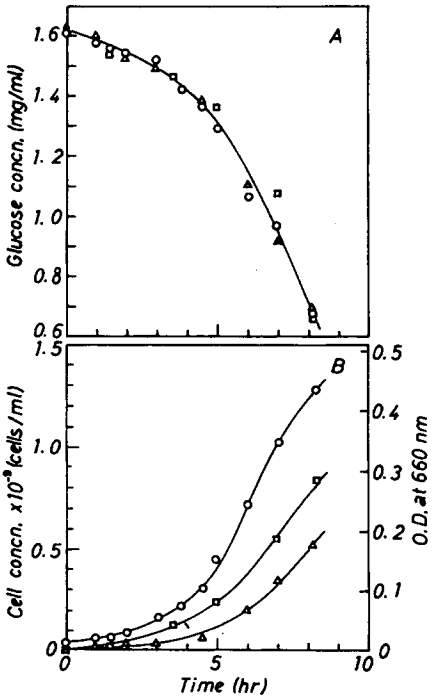


Fig. 3. Time courses of glucose consumption (A) and cell growth (B): (○) free cells; (□) freshly prepared immobilized cells; (△) immobilized cells after completion of the original incubation. The growth curve of immobilized cells was expressed in the concentration of the cells which were released from the support and grown in the medium. Incubation was made at 30°C on a rotary shaker. The culture medium (100 mL) used contained 163 mg D-glucose and the other nutrients mentioned in the experimental section. Initial cell number was 5×10^9 for the incubations of free cells and for the freshly prepared immobilized cells; the latter contained about 20 mg TGCI–KPVS complex.

the cells released from the complex support, but also to the cells entrapped. That is to say, the entrapped cells have glucose oxidizing activity. Another important feature of Figure 3 is that the number of released and grown cells is diminished by the repeated incubation of freshly prepared immobilized cells, although there is no change in consumed glucose, indicating apparent growth of cells in the complex support.

In order to confirm the glucose oxidizing activity of immobilized cells, the rate of substrate consumption (dS/dt) was estimated by graphic differentiation of the curves in Figure 3(a) and was plotted against the concentration (X) of cells in the medium. Here, for the immobilized cell system, X represents the concentration of released and grown cells in the liquid phase. The results obtained are shown in Figure 4. When microorganisms are grown with unlimited supply of nutrient, the relationship between dS/dt and X in the simplest case of the exponential growth is represented by[15]

$$\frac{-dS}{dt} = \left(\frac{\mu}{Y}\right) X \tag{1}$$

where μ and Y denote specific growth rate and yield factor, respectively. The plots of $\Delta S/\Delta t$ vs. X for free and immobilized cells are straight lines passing through the origin, indicating that the glucose consumption in both cell systems can be characterized by eq. (1). Comparison of the results in Figure 4 shows that the slope of the straight line for immobilized cells is larger than that for free cells. Furthermore, the slope for immobilized cells increases as incubation is repeated. This could signify that the entrapped cells have glucose oxidizing activity and also that they grow in the complex support, because it seems reasonable to consider that the term μ/Y is essentially unaltered by the entrapment of the cells.

To further characterize immobilized cells, the effects of temperature and pH on glucose consumption were investigated and compared with the results for free cells (Figs. 5 and 6). With immobilized cells, part of the cells were released from the support and grown in the medium during the incubation (see above). The comparison was thus made under condition where the medium contains a constant amount (1 g dry weight) of free cells or released and grown cells. According

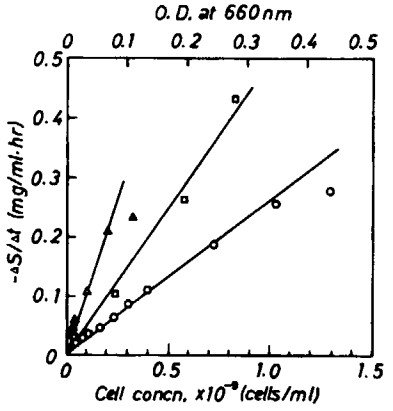


Fig. 4. Plots of $\Delta S/\Delta t$ vs. X for free and immobilized cells estimated from the results in Fig. 3. Symbols correspond to those in Fig. 3.

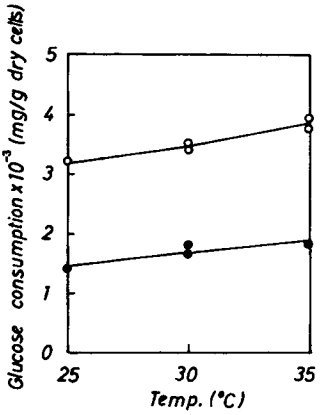


Fig. 5. Effect of temperature on glucose consumption with free (●) and immobilized (○) cells. The glucose consumed with immobilized cells was expressed on the basis of released and grown cells in the medium but not on the basis of entrapped cells in the support. "Incubation was made in the same manner as described in Fig. 3, except for the temperature.

to this analytic method, the difference in the glucose consumption with free and immobilized cells can be related to the amount of glucose consumed with the entrapped cells. From Figures 5 and 6, the glucose consumed with immobilized cells is found to be larger than that with free cells. This finding again indicates retention of the glucose oxidizing activity for the entrapped cells.

From the comparison of the curves in Figure 5, no remarkable difference is observed in the temperature effects on glucose consumption with free and immobilized cells. On the other hand, the pH activity profile shows that the glucose oxidizing activity for free cells is inhibited completely at pH 10. As described below (see Fig. 9), this could be due to alkaline denaturation of *E. coli* cells. In contrast to free cells, the immobilized cells have an activity even in the basic range which is comparable to that found for free cells at optimum pH. This seems an advantage of the cell entrapment, which arises from the protection of cells with complex support.

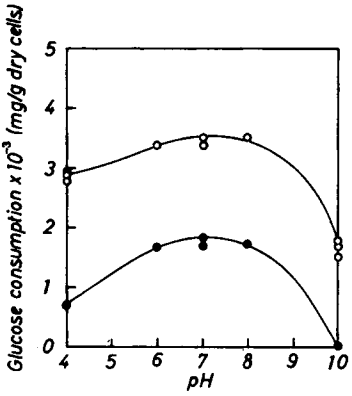


Fig. 6. Effect of pH on glucose consumption with free (●) and immobilized (○) cells. The glucose consumed with immobilized cells is expressed in the same way as in Fig. 5. Incubation was made under the same conditions as described in Fig. 3, except for the use of various buffer solutions to control the pH of the medium.

Mechanism of Cell Immobilization

The process of cell immobilization with TGCI–KPVS complex seem to be divided into two stages: the aggregation of cells with TGCI and the entrapment of the aggregated cells in the complex. Cell aggregation, which is characteristic of the present immobilization method, can be expected to facilitate the entrapment of cells with polyion complex, because the concentration of cells takes place in this stage. Thus, it should be preferable to investigate in detail the aggregation of cells with polyion in order to clarify the immobilization mechanism.

As described above, KPVS is not effective for cell aggregation in the neutral pH range. In the acidic range, however, the cells are aggregated with KPVS. Thus, KPVS was used for the study of cell aggregation, together with TGCI. The relationship between the polyelectrolyte amount (A_p in mol) required to aggregate cells and the weight of dry cells is shown in Figure 7. Plots of A_p vs. cell weight are straight lines passing through the origin, which could mean that the polyion adsorbs quantitatively onto the cell surface and the adsorption is due to a strong chemical force.

To obtain information about the adsorption mechanism, the infrared spectrum for the aggregated cells with TGCI was compared with that for free cells. The spectral measurements were made via the reflection method, because spectra obtained via transmission method are affected by chemical species inside the cells. Figure 8 depicts the spectra. A discernible shoulder at 1720 cm^{-1}, which can be assigned to carboxyl groups on the cell surface, is observed in the spectrum of free cells washed with HCl solution (pH 3). The existence of carboxyl groups is also confirmed by the following fact: When free cells were washesd with NaOH solution (pH 8), the spectrum revealed an absorption band at 1390 cm^{-1}, assigned to the carboxylate ions, instead of the disappearance of the shoulder at 1720 cm^{-1}. On the other hand, the spectrum for the cells aggregated with TGCI at pH 8 and washed with HCl solution (pH 3) shows absorption at 1390 cm^{-1} but not at 1720 cm^{-1}. The spectrum of the aggregated cells further shows a shoulder at 1100 cm^{-1}, which could be assigned to the ether groups of TGCI. On the basis of these spectral changes, the adsorption of TGCI can be interpreted in terms of salt-linkage formation of $^{+}N(CH_3)_3$— groups in TGCI with acidic groups (at least

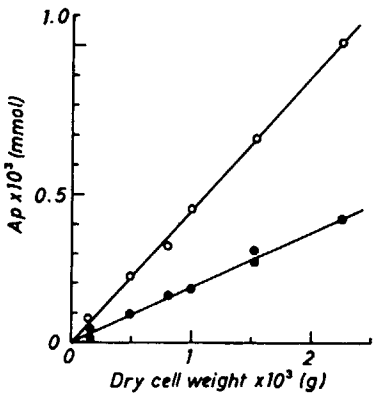


Fig. 7. Dependence of A_p on the weight of dry cells. A_p represents the amount of TGCI (○) or KPVS (●) required to aggregate the cells and is expressed in moles of ionizable groups attached to the polymer. The aggregation with 1 m*M* TGCI and KPVS was made at pH 8 and 2, respectively.

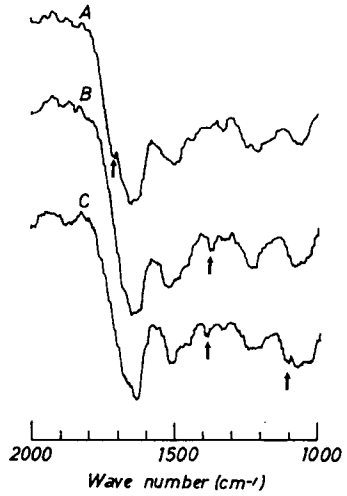


Fig. 8. Reflection infrared spectra of free and aggregated cells. A and B show the spectra of free cells washed with HCl solution (pH 3) and with NaOH solution (pH 8), respectively; C is the spectrum of cells aggregated with TGCI at pH 8 and washed with HCl solution (pH 3).

carboxyl groups) on the cell surface. The adsorption of KPVS may also be understood by assuming the salt-linkage mechanism, although spectral data were not obtained because unambiguous assignment of basic groups on the cell surface was difficult to make from infrared analysis.

The salt-linkage formation between cells and polyions was further studied by measuring the amount of $^{+}N(CH_3)_3$— or $^{-}OSO_3$— groups (M_s) bound to the cells at different pH values. Here, the value of M_s (in mol/g dry cells) corresponds to that of A_p at 1 g dry cells. The results are shown in Figure 9. The curve of M_s vs pH with KPVS depicts a monotonous decrease in M_s with increasing pH, while the curve with TGCI shows a gradual increase in M_s with increase of pH, followed by a rapid increase of the curve at pH above 9. These can be related to the changes in the dissociation of both acidic and basic groups on the cell surface, since it has become apparent that the dissociation of $^{+}N(CH_3)_3$— groups in TGCI and of $^{-}OSO_3$— groups in KPVS is almost independent of pH.[11] A crossing point observed at pH 3.7 could be assigned to an isoelectric point of *E. coli* cells. This value compares favorably with isoelectric

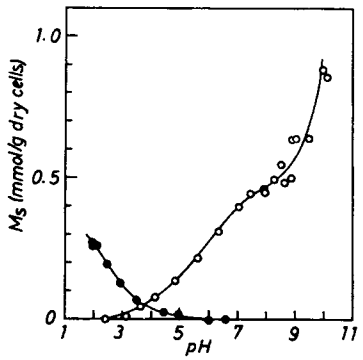


Fig. 9. Curves of M_s vs. pH. M_s represent the amount of ionizable groups in TGCI (○) or KPVS (●) which are bound to 1 g dry cells.

points for Gram-negative bacteria, which has been reported to be in the range of pH 2–4.[16] Another remarkable feature of Figure 9 is that the curve with TGCI shows a rapid increase in M_s at pH above 9. In this pH region, the glucose oxidizing activity of *E. coli* cells was inhibited completely (see Fig. 6). These results can be understood by the explanation that the membrane structure on the surface of the cells is denatured with alkali in the medium.

From the results mentioned above, it is clear that the adsorption of polyelectrolyte onto *E. coli* cells is caused by the salt linkages of polyions with ionizable groups on the cell surface and that this brings about the cell aggregation. Taking into account these facts, the mechanism of cell immobilization can be explained as follows: In the aggregation process, when TGCI is mixed with the cell suspension, part of TGCI is adsorbed on the cells, which results in aggregation, and the other part remains in the suspension. In the entrapment process, the remainder forms the polyion complex with added KPVS and the aggregated cells are coprecipitated with the complex. These chemical changes take place at room temperature and are little afected by pH, small ions, and organic compounds which vary with the cultivation conditions. Therefore, this important feature makes it possible to prepare immobilized cells by the direct use of culture broth under mild conditions.

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