Synthesis of NAD Polystyrene Microspheres and Application as a Carrier of Glucoamyrase Immobilization

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SYNOPSIS

Polystyrene microspheres with a small amount of acrylic acid (200 : 1) incorporated were synthesized by nonaqueous phase dispersion (NAD) polymerization to investigate the feasibility of use as a carrier for immobilized enzyme. Seeded emulsion polymerization was then carried out to obtain larger cross-linked polystyrene spheres, the structure of which was expected to yield a favorable environment to keep the original activity of the immobilized enzyme. Glucoamylase, a particular enzyme catalyzing the hydrolysis of oligosaccharides to glucose, was immobilized through peptide bonding using a carbodiimide activating agent. Thirty percent of the enzyme activity at maximum was retained relative to free glucoamylase. The immobilized enzyme remained active after 4 months, although its poor resistance to heat was not improved. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

Beyond conventional usage, latex particles have acquired a new dimension as microcarriers for various biochemical and biomedical proteins such as enzymes, antigens, and antibodies. According to Singer's review,[1] physical adsorption of physiological proteins onto the surface of various microspheres had been utilized for medical therapy and analysis in the 1940s. Applications to immunoassay and other medical assays have been commercially feasible, taking full advantage of large monosized spheres obtained by the two-step swelling method.[2]

Immobilization of various enzymes on the surface of polymeric microspheres has been reported, and a brief survey of selected articles is summarized in Table I. Generally speaking, immobilization on submicron spheres, ranging from 80 to several hundred nanometers, demonstrates high relative activity compared to free (native) enzyme. Durability and resistance to heat or unfavorable pH are more or less improved after immobilization. Physical adsorption is preferred to chemical bonding as a method of immobilization, though the former is quite susceptible to environmental factors such as pH, ionic strength, and local shear.

As the size of polymeric microspheres increases, even in the range of micrometers, it is difficult to maintain reasonable activity.[11] Recently, huge spheres, 10 μm or larger and composed of natural polymers,[13,14,15] were proved to be far better carriers than are their synthesized counterparts.[12] Being highly porous and rich in functional groups, some of them acting as built-in spacers, those spheres may offer a more physiologically favored environment for enzymes to retain their original conformations.

The only disadvantage seems to arise from the difficulty of obtaining monosized spheres. In general, solvent-evaporation techniques, with abundant variations, have been employed, yielding polydispersity due to the agitation during the processing. Sieving was required to limit the size distribution to a reasonable range.

On the other hand, nonaqueous phase dispersion (NAD) polymerization is capable of providing monodisperse polymeric spheres of several micrometers in a single-step operation. Subsequent seed polymerization can add further dimensions such as incorporation of cross-linking, modification of functionality, and further increase in size. If the activity after immobilization is maintained at a reasonable percentage of free enzyme, monodispersity

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Table I Literature Survey of Enzyme Immobilization on Polymeric Microspheres

Authors	Enzyme Immobilized and Substrate	Microsphere Size and Preparation	Method of Immobilization	Relative Activity
Kitano et al.[3]	Alkaline phosphatase, PNPP[a]	PS-AA-DVB, 0.51 μm, emulsion	Carbodiimide (spacer)	< 10% V_{max} and higher K_m
Ohkubo et al.[4]	Trypsin, BApNA[b]	PS, PMMA, PMA, PS–HEMA, 0.32–0.92 μm, soapless	Adsorption	Max. ca 50%
Ohkubo et al.[5]	Trypsin, BApNA, casein	PMMA, hydrazided, 0.5 μm, soapless	Acyl azidation, $NaNO_2$	50% for BApNA
Ohkubo et al.[6]	Trypsin, BApNA, BAEE[c]	PMMA–NaSS, 0.13–0.42 μm, PS–NaSS?, soapless	Adsorption	50% (PS–NaSS)
Ohtsuka et al.[7]	α-Amylase, starch, etc.	PS–NHMAAm, 1.0 μm, hydrolyzed, emulsion	Woodward-K, carbodiimide	80%
Bahadur et al.[8]	α-Chymotrypsin, GPNA[d]	PMMA–AA/ PMMA, 80 nm, core/shell	Carbodiimide	No direct comparison
Ohkubo et al.[9]	Trypsin, BAEE	PS–GMA, 0.37 μm, soapless, carboxylated	Adsorption	Max. 75%
Beddows et al.[10]	Trypsin (main), BApMA, hemoglobin	PE-*g*-HEMA, ?, hydrolyzed	Carbodiimide	Max. 66%
		PE-*g*-HEMA, emulsion	*p*-Benzoquinon	Max. 25%
Kawaguchi et al.[11]	Glucose oxidase, glucose peroxidase glucose	PS–AAm, 0.38 μm, aminated, soapless	$NaIO_4$ —$NaBH_4$	Max. 30% (GO) Max. 40% (PO)
Skovby and Kops[12]	Lipase, palmitic acid + glycerintrioleate	PMMA–DVB, large and porous, suspension	Adsorption	Max. 17%
Hayashi and Ikada[13]	Papain, ficin, bromelain, BAEE, casein	Chitosan beads with spacer (ACW Chitopal)	$NaBH_4$	More than 50%
Hayashi et al.[14]	Papain	BMLG[e] beads, hydrazidized with spacer	Acyl azidation, $NaNO_2$	Max. 84%
Yamaguchi et al.[15]	α-Amylase	Tannin sphere 16–42 mesh	Adsorption	63%

Monomers: AA = acrylic acid, AAm = acrylamide, DVB = divinyl benzene, E = ethylene, GMA = glycidyl methacrylate, HEMA = 2-hydroxyethyl methacrylate, MA = methyl acrylate, MMA = methyl methacrylate, NaSS = styrene sodium sulphonate, NHMAAm = *N*-hydroxymethyl acrylamide, S = styrene. Substrates: [a] PNPP = *p*-nitrophenyl bihydride sodium salt; [b] BApNA = (*N*-benzoyl-DL-alginine-*p*-nitroanilid)hydrochloride; [c] BAEE = (*N*-benzoyl-L-alginine ethyl ester) hydrochloride; [d] GPNA = *N*-glutaryl-L-phenylalanine-*p*-nitroanilide; [e] BMLG = P(γ-methyl-L-glutamate).

and flexibility of particle designing will be distinct advantages compared to the spheres of natural origin.

It was the purpose of this study to synthesize monosized polymeric microspheres that provide a favorable environment for immobilization of enzymes or other bioactive substances. It may be too early to predict demand for them for disposable columns of high-performance liquid chromatography (HPLC) that provide high resolution with a reasonably low price for quick assays and on-line monitoring. Since the adjustment between high resolution and a tolerable pressure drop becomes the most important issue for designing such a short column, sophistication of polymeric microspheres will be decisive for competition in this market.

In this study, glucoamylase (GluA) was selected as a model enzyme for immobilization. GluA, together with α-amylase (Am), has been commercially used for the mass-conversion of natural starches into glucose or saccharides. Although free (immobilized) Am has been used because of its low production costs, the more expensive GluA must be disposed of after each batch because of its poor stability under heating during hydrolysis. Immobilization of GluA has a certain advantage in cost performance provided that the activity after the treatment remains reasonable, and, hopefully, with increased stability.

Some promising recipes for NAD polymerization with subsequent seed polymerization will be discussed in this article to obtain polystyrene (PS)-based microspheres that have proved effective in maintaining reasonable activity of immobilized GluA in the hydrolysis of oligosaccharides.

EXPERIMENTAL

Materials

Monomers

Styrene (ST) and divinyl benzene (DVB) (Kishida Chemical Co.) were commercial grade and distilled under reduced pressure and stored in a refrigerator prior to use. DVB is a mixture of 55 wt % DVB, 40 wt % ethyl vinyl benzene, and 5 wt % of saturated hydrocarbons. Acrylic acid (AA) was reagent grade and stored in a refrigerator, and polymer was removed by filtration prior to use.

Reagents for NAD and Subsequent Seed Polymerizations

Polymeric stabilizer (ST : AA = 3 : 7 to 6 : 4) was synthesized in this laboratory by thermally initiated bulk copolymerization at 413–453 K. Sodium lauryl sulfate (SLS, Merck) was the grade for biochemical use and analysis. Anhydrous sodium sulfate (Wako Pure Chemicals Co.) was reagent grade. α,α'-azoisobutyronitrile (AIBN) and benzoyl peroxide (BPO, 25 wt % moisture, Kishida) were reagent grade and used as an initiator. Ethanol and n-hexane (Kishida) and toluene (Kokusan Chemicals Co.) were commercial grade and distilled prior to use. n-Heptane, 2-methoxyethanol and acetonitrile (Kishida) were reagent grade and used without distillation. Water was distilled and deionized.

Reagent for Enzyme Immobilization

Glucoamylase (GluA, Gluczeim AF6, Rhizopus delemar, Amano Pharmaceutical Co.) was food-processing grade; a trace amount (unknown) of Am and water-insoluble extenders were in it. Unidentified extenders were removed by filtration from the aqueous suspension prior to immobilization. 1-Ethyl-3-(dimethylaminopropyl)carbodiimide (carbodiimide activator, Sigma Chemical Co.) was toxic and for laboratory use only. It was used as received. Starch, soluble for biochemical use (Wako), was used as the starch source to prepare oligosaccharides for GluA.

Am (*Bacillus* sp., Sigma) was used to hydrolyze soluble starch. Disodium hydrogen phosphate, anhydrous, and potassium dihydrogen phosphate were reagent grade (Kishida) and used for preparation of buffer solutions (pH 5.5, 7.0, and 8.0).

Reagent for Enzyme Assay

Dyestuff Brilliant Blue G (Coomassie Brilliant Blue G, approx. 60 wt % purity, Sigma) was used to determine the percent of immobilization of the enzyme. Phosphoric acid (85%, Kishida) was reagent grade.

Other Materials

Potassium hydroxide of reagent grade was used for conductometric titration.

Preparation of Microspheres

NAD Polymerization

Glass ampules, differing in volume depending on the purpose, were used for the preparation of polymeric microspheres. These spheres were used directly for immobilization or served for further seed polymerization after replacing the continuous phase. Stabilizing polymer was dissolved in ethanol and poured into an ampule. Monomer or its mixture with a good

solvent for polystyrene (PS) was added, and, finally, initiator dissolved in ethanol was added to avoid unnecessarily idle time before heating. After repeated procedures of freeze and thaw cycles, deaerated ampules were sealed and set in a thermostat, allowing the contents to polymerize for 24 h. Instead of a horizontal shaker, a head-over-head rotating disc (Suzuki Industry Co., 25 rev/min) was used with the ampules attached on it.

NAD PS–AA spheres with low cross-linking density were used as seed polymer particles after the replacement of nonaqueous serum with water. Repeated cycles of washing with ultracentrifuge and redispersion under ultrasonification were employed for replacement. Polymer particles of several micrometers were dispersed in the ethanol–water solution of SLS under ultrasonification, and the dispersion was added to a 500 mL glass separable flask. A mixture of monomers, diluent, and initiator was added, and swelling of the polymer particles was done for 18 h while the flask was immersed in an ice bath. The temperature was raised to the reaction temperature, and the seed polymerization was carried out for 24 h at 343 K. The reaction mixture was quite stable during the swelling and polymerization. No nucleation of secondary particles was observed.

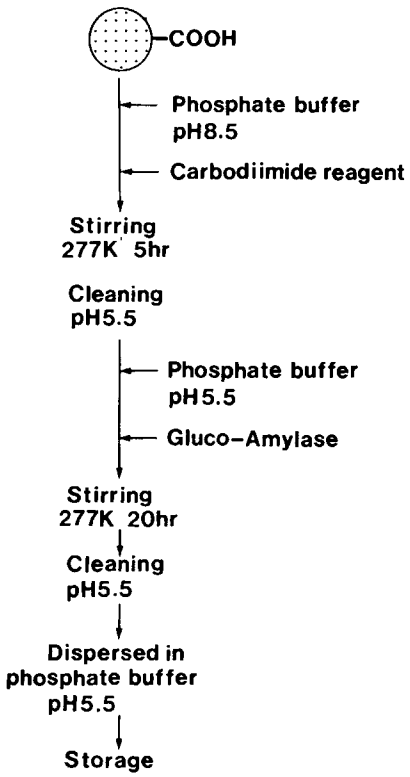

Figure 1 Procedure of enzyme immobilization with carbodiimide reagent.

Immobilization of Glucoamylase

An outline of the immobilization procedure is shown in Figure 1. All processes were carried out in buffer solutions. One gram of the latex particles was dispersed in 20 mL of the phosphate buffer solution of pH 8.5. Normally, 20 mg of a carbodiimide reagent, an excess amount to the equivalent carboxyl group incorporated in the particles, was allowed to activate carboxyls under mild agitation for 5 h at 277 K. The excess reagent was removed by repeated centrifugation, decantation of serum, and redispersion in the phosphate buffer solution of pH 5.5. Ten milliliters of the buffer solution containing the desired amount of GluA was mixed with 10 mL of the latex, and the immobilization was carried out for 20 h at 277 K. The same procedure employed for the removal of the carbodiimide reagent was applied to remove free GluA. Finally, the latex was dispersed in 20 mL of the buffer solution and stored in a refrigerator.

A $NaIO_4$—$NaBH_4$ system,[11] which activates the hydroxyl group in glucose units in enzymes, and eventually bonds with aminated latex particles, was also tried after the incorporation of amino groups on the particles by the Hoffman reaction. This method, if successful, may be preferred to the carbodiimide method because the active enzyme site remains intact by immobilization. The immobilization yield of GluA was always quite reasonable. No noticeable activity, however, remained after the immobilization, and this method was abandoned after several attempts.

Evaluation of GluA Activity Immobilized

The mixture of oligosaccharides was used as the substrate to measure the activity of immobilized GluA. pH of the reaction mixture was adjusted at 5.0 when the latex particles, depending upon the required amount of immobilized enzyme (0.316 mg/100 mL), were dispersed in the substrate solution. Hydrolysis of oligosaccharides was carried out in a stoppered 500 mL glass flask at 313 K under mild agitation until a considerable amount of glucose was formed.

Analyses

Latex Particles and Polymers

Monomer conversion was determined gravimetrically; polymer was precipitated from the latex sam-

ple using methanol, separated with a centrifuge, washed with methanol, and dried in a vacuum oven. Surface features and size of polymer particles were measured with an SEM (JSM-35CFII, JEOL). Molecular weights of polymers were measured with a GPC (HLC-801, Toso Co.) using THF as an eluent. Carboxylic group content incorporated in the polymer particles was measured with a conductivity meter (DS.8F, Horiba) by titration with 0.01*N* KOH solution.

Amount of Immobilized Enzyme

After the immobilization procedure, the amount of free enzymes remaining in the serum was determined by the Coomassie Brilliant Blue (CBB) method as proteins.[16] Brilliant Blue G, 100 mg, was dissolved in 50 mL of 95 wt % ethanol. Added was 100 mL of 85 wt % phosphoric acid, which was then diluted to 1 L with distilled water. This solution was stored in a refrigerator.

The sample solution, 0.1 mL, containing 10–100 μg of proteins was diluted to 1 mL with the phosphate buffer solution of pH 7.0 and thoroughly mixed with 5 mL of CBB solution. Absorbance at 595 nm was measured after 5 min. The amount of immobilized enzyme was determined by subtracting the amount of free enzyme from that of the initial feed.

Oligosaccharides

HPLC (HPLC-8010, Toso Co.) packed with polyamide gel (TOSOH Amido-80) was used to separate oligosaccharides. Oligosaccharides composed of up to 10 glucoside units were detected quantitatively by the differential refractive index (TOSOH RI-8010). Mixed solvent (acetonitrile/water = 60 : 40 v/v) was used as an eluent.

Evaluation of GluA-immobilized Microspheres

For quantitative correlations between the performance of immobilized GluA (Im-GluA) and the immobilization recipe including the shape and size of polymer particles, the following values were defined for detailed discussions:

Relative activity (R_A):

$$R_A = \frac{\text{(Initial rate of Glu formed by 3.16 mg/L of Im-GluA)}}{\text{(Initial rate by the same wt of free GluA)}} \quad (1)$$

Number of feed GluA molecules assigned for a single carboxyl group (β_0):

$$\beta_0 = \frac{[\text{GluA}]_0}{[-\text{COOH}]} = \frac{\text{(No. feed GluA molecules)}}{\text{(No. carboxyls on the surface)}} = \frac{\text{(Wt of feed GluA)} \times \text{(Avogadro no.)}/M_W}{[\text{C.D.}] \times (10^{18}) \times (6/\bar{d}_p)} \quad (2)$$

Surface coverage of immobilized GluA (ϕ):

$$\phi = \frac{\text{(Surface area occupied by immobilized GluA)}}{\text{(Total surface area of 1 g solid Lx)}} = \frac{\text{(No. Im-GluA molecules)} \times a_G \times (10^{-18})}{6/\bar{d}_p} \quad (3)$$

where Glu and solid-Lx are abbreviations of glucose and solid in latex, respectively; [C.D.], charge density, $\bar{d}_p$, average diameter of microspheres (μm); M_w, molecular weight of GluA; and a_G, coverage area of GluA (nm^2).

There are limited data available for GluA: 58,000 Daltons of M_w was given by Nakamura et al.[17] and the tridimensional size was assumed to be 5.1 × 5.0 × 5.0 nm^3 from the specific volume[18]; hence, a_G = 5.1 × 5.0 = 25.5 nm^2.

RESULTS AND DISCUSSION

General Features of PS–AA–DVB Microspheres

Selected recipes of NAD polymerization without subsequent seed polymerization are shown in Table II. The AA to ST ratio was fixed at 1/200, and 1 wt % of DVB was added as a cross-linker. Polymerizations, carried out with a stirred tank reactor (STR), were able to produce monodisperse spheres (#6200 and 6300), although technical difficulty in preventing the evaporation of ethyl alcohol during polymerization discouraged further employment of the STR. Particles of regular-shaped coagulum, composed of one larger globule to which several numbers of smaller particles were adhered, were obtained from sealed-ampules shaken horizontally in the thermostat (#5701 and 0041). Addition of only 1 wt % DVB yielded the spheres (#0041) as shown in Figure 2.

Immobilized GluA on these particles definitely retained activity to the hydrolysis of oligosaccha-

Table II Selected Recipes of NAD Polymerization

	Run No.				
	6201	6301	5321	5701	0041
Polymerization	STR	STR	Shake	Shake	Shake
Monomer					
ST (g)	29.55	44.30	3.92	3.94	3.94
AA (g)	0.15	0.20	0.04	0.02	0.02
DVB (g)	0.30	0.50	0.04	0.04	0.04
Solvent					
Ethanol (g)	256	232	15.2	16.0	15.2
Toluene (g)	—	9	0.8	—	0.8
Stabilizer					
ST/AA = 3 : 7 (g)	6.0	6.0	1.0	1.0	1.0
Initiator					
AIBN (g)	1.50	1.50	0.1	0.1	0.1
Polymer feature	Sphere mono	Sphere mono	Reg. sp.[a] poly	Reg. sp. mono	Reg. sp. mono
$\bar{d}_p$ (μm)	3.1	3.3	—	2.5	2.3
Mol. Wt.					
$M_w \times 10^{-4}$	4.20	4.62	8.00	11.7	4.01
$M_n \times 10^{-4}$	0.75	0.83	1.62	2.12	1.16
M_w/M_n	5.63	5.57	4.95	5.50	3.46
Max. R_A (%)	4.62	4.78	0.50	1.41	14.3

[a] Regulated coagulum of microspheres; several smaller spheres are firmly attached to the largest sphere.
[b] After the synthesis, all latices were processed with steam stripping to remove unreacted monomers and inert solvent.

rides, in particular, #0041 recorded 12.9% of relative activity. However, the limitation of low DVB content, combined with the uncertainty of obtaining reproducibility, discouraged the synthesis of microspheres with the STR or shaking glass ampules.

A new thermostat that allows vertical rotation of glass ampules during polymerization was tried next and provided good reproducibility and stable operation, though still ineffective in producing monodisperse and highly cross-linked microspheres.

Two-step polymerization, synthesis of low cross-linked seeds by NAD polymerization, and subsequent growth by aqueous phase emulsion polymerization, controlled the degree of cross-linking in microspheres and probably in the microporous structure. Highly cross-linked structure was incorporated in the second-stage polymerization after the seeds were swollen with DVB and the diluent, *n*-hexane (HX) or *n*-heptane (HP). Recipes to obtain highly cross-linked spheres are listed in Table III.

Note that AA was not added in the seed emulsion recipe. Addition of ethanol in the continuous phase promoted swelling of the mixture of monomer and diluent, which lasted for 18 h. No secondary particles were formed during the seed polymerization even though the concentration of SLS was quite high. Average diameters of the seeds increased from 1.2 to 1.3 times after the seed polymerization, indicating that the swollen monomer was all spent in the growth of seed particles.

SEM photographs of the final microspheres with different degrees of cross-linking are shown in Figure 3. All the spheres retained good monodispersity. As

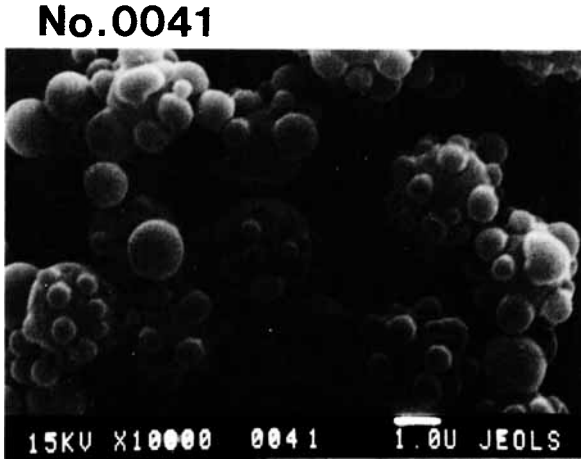

Figure 2 SEM photograph of nonspherical yet orderly NAD microspheres. Magnification = 10,000×.

Table III Recipes of NAD Polymerization and Subsequent Seed Emulsion Polymerization

(a) NAD Recipe

	Run No.			
	0540	0550	0620	0690
ST (g)	4.96	4.96	4.96	4.96
AA (g)	0.024	0.024	0.024	0.024
DVB (g)	0.015	0.015	0.015	0.015
Ethanol (g)	20.0	20.0	20.0	20.0
Toluene (g)	1.0	1.0	1.0	1.0
ST/AA = 3 : 7 (g)	1.25	1.25	1.25	1.25
AIBN (g)	0.125	0.125	0.125	0.125
$\bar{d}_p$ (μm)	2.40	3.00	2.67	2.19
$M_w \times 10^{-4}$	4.27	4.55	5.15	5.38
$M_n \times 10^{-4}$	1.38	1.02	1.49	1.42
M_w/M_n	3.10	4.44	3.46	3.78

(b) Seed Emulsion Recipe

	Run No.			
	0541	0551	0621	0691
Seed[a] (g)	18.0	18.1	18.0	19.5
ST (g)	18.0	16.9	14.4	9.75
DVB (g)	1.2	1.1	3.6	9.75
BPO (g)	0.75	0.72	0.72	0.78
HX or HP (g)	HX 4.0	HP 3.6	HP 9.0	HP 19.5
Water (g)	250	250	250	250
Ethanol (g)	25	25	25	25
SLS (g)	0.5	1.0	1.0	2.0
Na_2SO_4 (g)	0.1	0.1	0.1	0.2
$\bar{d}_p$ (μm)	3.04	3.70	3.44	2.88
$M_w \times 10^{-4}$ [b]	5.19	4.81	5.87	5.13
$M_n \times 10^{-4}$	1.27	1.29	1.34	1.28
M_w/M_n	4.07	3.72	4.38	4.01

[a] Solid content in emulsion.
[b] Molecular weight of THF soluble fraction.

the cross-linking density increases, the diameters of the final spheres decrease and the spheres (#0551) become skewed, gradually shifting to ellipsoids (#0691). Though not shown here, further increase of DVB and HP enhanced the phase separation and rough surface feature and eventually yielded a capsulelike structure.[19] In principle, these microcapsules consist of a cross-linked polymer shell and a liquid (HP) core, a similar structure to the PS–isooctane microcapsules reported by Hatate et al.[20] However, genuine microcapsules were not observed in this work, probably due to the unbalanced development of the strain during the phase separation process, leaving ripped-off shells or hollow spheres. More precise formulation of ST, DVB, and the diluent is required to obtain appropriate microspheres as enzyme carriers. This investigation is now under progress.

Results of Immobilization

Characteristics of microspheres, initial feed, immobilized weight of GluA, and other variables are listed in Table IV. Immobilization was always carried out using 1 g (dried weight) of microspheres. Due to the small scale, the weight of immobilized GluA was not consistent with that of feed, as shown in Table IV. A number of plots were attempted to grasp the general tendency of immobilization, and the correlation shown in Figure 4 emerged as most comprehensive.

Yield of immobilized GluA, based on the unit dry weight of microspheres, was plotted against the molecular ratio of feed GluA to the carboxyl group on the surface. As shown in Table IV, since the charge density changed only slightly with the microspheres and the number of carboxyl groups assigned for single GluA molecule, β_0 and β after immobilization, were high enough, the GluA yield may be considered to be dependent on the amount of feed GluA. The data points in Figure 4 are scattered and hardly imply a straightforward relationship between log(Im-GluA yield) and log β_0. The correlation analysis, however, revealed a positive correlation coefficient of 0.615, and the Im-GluA yield was proportional to $0.41th$ power of β_0 rather than the direct proportionality.

Performance of Im-GluA

Performance and activity of Im-GluA were investigated from the hydrolysis of 10 g/L oligosaccharide solution measured at pH 5.0 and 313 K. Typical profiles of oligosaccharides, G_j (j = 1–8), are plotted in Figure 5 against the elapsed time. Only the data points of G_1 are shown as open circles, whereas those of the other G_j are shown as smoothed curves to avoid confusion due to so many data points. Generally, separation of the individual peaks by HLPC analysis was quite good, each G_j being easily identified. Im-GluA 0541-4, the amount of which was

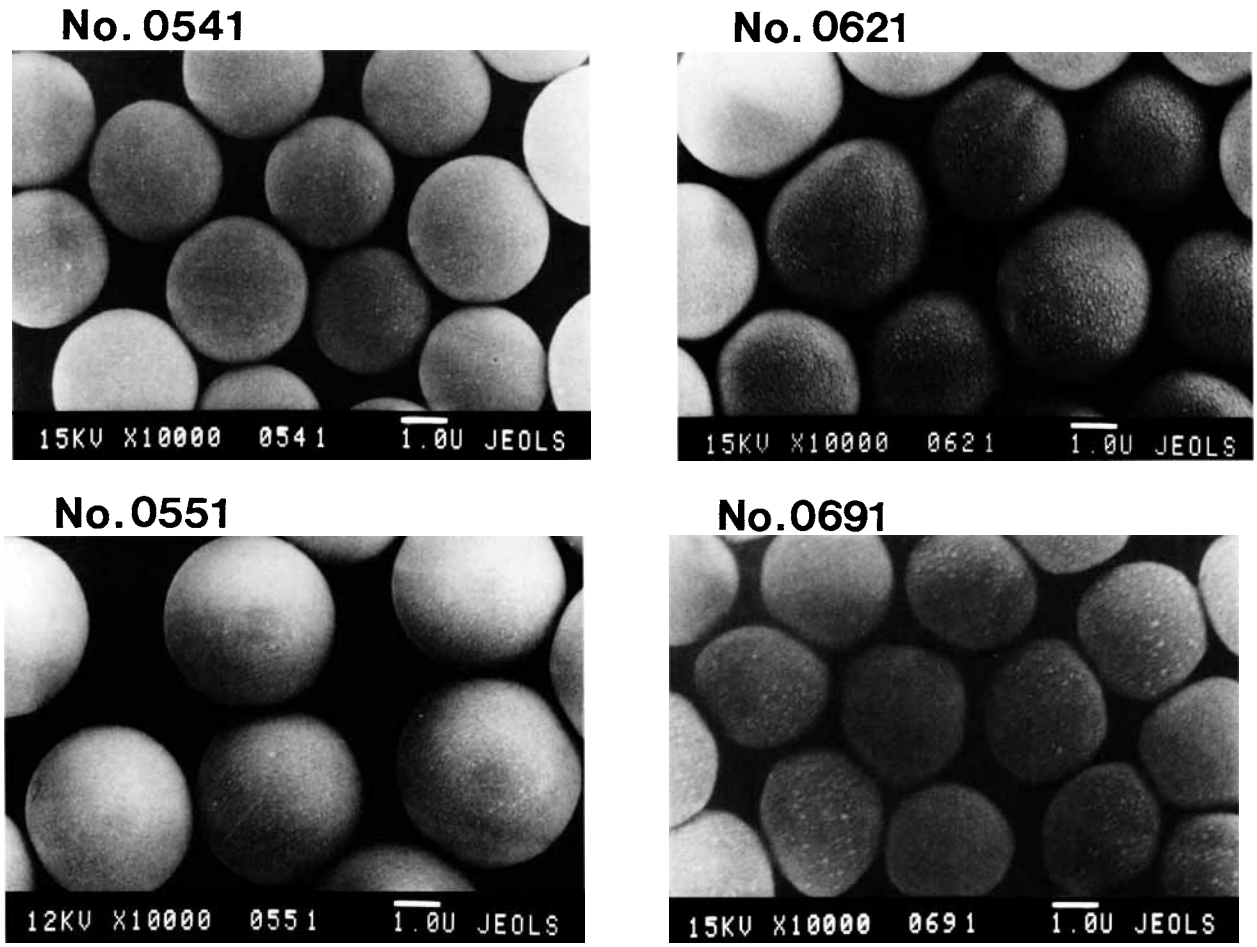

Figure 3 SEM photographs of cross-linked microspheres prepared by seed emulsion polymerization. Magnification = 10,000×. Wt % of DVB in the seed polymerization recipe (based on total monomer): (a) #0541 6.25; (b) #0551 6.11; (c) #0621 20.0; (d) #0691 50.0.

adjusted so that exactly 0.316 mg of GluA is present in 100 mL of the mixture, was employed. G_3 and G_6 were two dominant components in the initial oligosaccharides with considerable amounts of G_2, G_4, and G_5. The initial formation of glucose (G_1) progressed almost linearly, and the profiles of G_2 to G_4 stayed almost unchanged, indicating the balanced rate between the formation by the degradation of higher G_j and the disappearance by their own hydrolysis. Initially, G_5 increased up to 2 h and then decreased steadily while G_6 and G_7 decreased from the beginning. G_8 was detected only in the initial 4 h. Although free GluA was capable of converting all G_j ($j > 1$) to G_1 after 24 h, an appreciable amount of G_2 (1.05 g/L) still remained after 120 h. Glucose yield increased steadily: 7.04 g/L after 24 h, 8.43 g/L after 72 h, and 8.95 g/L after 120 h.

It is well known that GluA is able to cleave α-1,4 and α-1,6 glucoside linkages; therefore, the rate of hydrolysis should be based upon the overall numbers of glucoside linkage cleaved. However, as shown in Figure 5, numerical calculations based upon an adequate kinetic model are necessary to estimate the overall rate of hydrolysis. As a compromise solution at the present stage, the relative activity of Im-GluA was evaluated as defined in eq. (1) and is listed in Table IV. Mathematical simulation concerning the overall kinetics of the hydrolysis will be presented later.[21]

The first thing to be recognized is that a high yield of immobilization does not necessarily assure high R_A. Although the trend of the data in Table IV is by no means clear, it can be said that low surface coverage by Im-GluA (Runs #0541-2, -4, -5, and #0621-2) is preferred for R_A rather than the higher coverage (Runs #0541-8, and #0621-6). Whether a minimum R_A really exists at intermediate values of the surface coverage remains uncertain because the

Table IV Result of Enzyme Immobilization

Microsphere No.	Average Diameter $\bar{d}_p$ (μm)	Charge Density[a] (U/nm^2)	Feed Wt of GluA[a] (mg)	Immobilized Wt of GluA[a] (mg)	Relative Activity[b] R_A (%)	$\frac{[GluA]_0}{[-COOH]^c}$ $\beta_0 \times 10^3$	Surface Coverage of GluA ϕ (%)
0541-1	3.04	9.90	1.0	0.769	7.6	0.532	10.3
-2			5.0	1.125	15.1	2.66	15.1
-3			7.0	2.485	4.7	3.72	33.3
-4			10.0	1.310	20.8	5.32	17.6
-5			12.0	1.068	14.0	6.37	14.3
-6			15.0	2.745	6.8	8.00	36.8
-7			20.0	3.080	10.7	10.6	41.3
-8			40.0	4.120	7.9	21.3	55.3
0551-1	3.70	13.35	1.0	0.417	7.4	0.480	6.8
-2			5.0	1.900	5.2	2.40	31.0
-3			10.0	2.190	6.3	4.78	35.7
-4			15.0	2.535	7.2	7.19	41.4
-5			20.0	2.360	4.9	9.62	38.5
0621-1	3.44	13.38	3.0	2.334	5.6	1.34	35.0
-2			5.0	0.530	20.2	2.22	8.0
-3			7.0	2.128	3.5	3.12	31.9
-4			10.0	1.940	3.0	4.44	29.1
-5			12.0	1.584	3.9	5.35	23.7
-6			15.0	3.180	2.2	6.67	47.7
0691-1	2.88	11.39	3.0	1.998	2.5	1.44	25.6
-2			5.0	1.440	2.8	2.40	18.4
-3			7.0	0.805	8.3	3.36	10.3
-4			10.0	0.825	4.4	4.81	10.9
-5			12.0	2.112	1.6	5.75	27.0
-6			15.0	1.020	3.5	7.19	13.0

[a] Based on 1 g of dried polymer particles.
[b] Rate of glucose formation divided by that of free GluA of identical weight.
[c] No. feed GluA molecules assigned to each carboxyl group on the surface.

Table V Effect of Carbodiimide Reagent Concentration on GluA Immobilization

	Carbodiimide[a]						
Run No.	mg	$\frac{[Carbo]}{[-COOH]^d}$	Feed Wt of GluA[a] (mg)	Immobilized Wt of GluA[a] (mg)	Relative Activity[b] R_A (%)	$\frac{[GluA]_0}{[-COOH]^c}$ $\beta_0 \times 10^3$	Surface Coverage of GluA ϕ (%)
0551-2	20.0	2.90	5.0	1.900	5.2	2.40	31.0
-6	5.0	0.73	5.0	1.815	3.9	2.40	29.6
-7	2.0	0.29	5.0	3.285	2.6	2.40	53.6
-8	1.0	0.15	5.0	1.475	6.5	2.40	24.1
-3	20.0	2.90	10.0	2.190	6.3	4.80	35.7
-9	10.0	1.45	10.0	1.200	13.0	4.80	19.6
-10	5.0	0.73	10.0	0.770	29.1	4.80	12.6
-11	2.0	0.29	10.0	0.220	27.9	4.80	3.6

[a] Based on 1 g of dried polymer particles.
[b] Rate of glucose formation divided by that of free GluA of identical weight.
[c] No. feed GluA molecules assigned to each carboxyl group on the surface.
[d] No. carbodiimide molecules assigned to each carboxyl group.

lowest data of R_A were all obtained from the microspheres of highest cross-linking (#0691). The effect of cross-linking is now under investigation and will be reported later. Ohkubo et al.[6] reported that the activity of immobilized trypsin became higher as the enzymes were closely packed on the surface. Also, the trend of our data at higher surface coverage appears, to some extent, to confirm the claim by Ohkubo et al; however, the maximum (#0541-7) never reached to the level attained at the lower surface coverage.

Effect of Carbodiimide Concentration

As all the immobilization experiments listed in Table IV were conducted with constant and excess concentration of the carbodiimide reagent, the effect of the reagent concentration on GluA immobilization and the resulting R_A was investigated. Table V indicates that the amount of carbodiimide reagent clearly affects the yield of immobilization and R_A, in particular, when the feed weight of GluA was 10 mg. The data also support the preference of lower surface coverage to retain high activity of GluA after immobilization. Table V also suggests that, in order to obtain reliable data, the yield of immobilization should be controlled by the concentration of the activated carboxyl group on the surface of the microspheres rather than on the amount of feed GluA.

CONCLUSION

Procedures to prepare PS–AA microspheres adequately designed as a carrier for immobilizing GluA were defined. Minor adjustments to control the amount of AA and to introduce appropriate microporous structure remained to be investigated.

It seems that less surface coverage of Im-GluA favored retaining high activity of GluA after the immobilization (Tables IV and V). Optimal formulation for the immobilization, feed concentrations of carbodiimide reagent relative to the carboxyl group on the carriers, and native GluA seems to be located in a limited area and is being investigated. The effect of incorporating more advanced cross-linking in the spheres is also to be investigated.

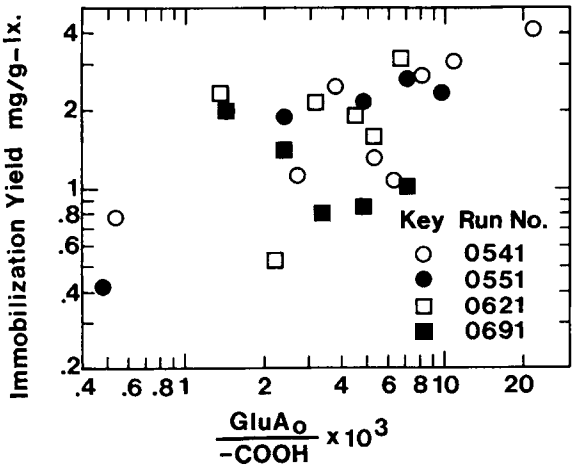

Figure 4 Immobilization yield of GluA vs. feed concentration of GluA. Feed GluA was expressed as an assigned value for each carboxyl group.

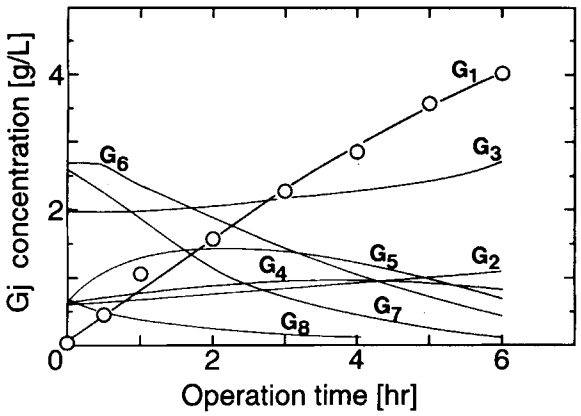

Figure 5 Concentration profiles of oligosaccharides (G_j) with elapsed time. Im-GluA = 0541-3, 3.16 mg/L. Initial concentration of saccharides = 10 g/L, pH 5.0, T = 313 K. Data points other than G_1 are shown as smoothened curves.

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