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Reactive bienzyme systems fabricated through immobilization of biotinylated glucose oxidase and peroxidase molecules onto neutralized avidin-conjugated liposomes

Takeshi Ohtsu[a], Saki Shigenari[b], Makoto Yoshimoto[a,b,*], Hiroshi Umakoshi[c]

[a] Department of Applied Molecular Bioscience, Yamaguchi University, Tokiwadai 2-16-1, Ube, 755-8611, Japan
[b] Department of Applied Chemistry, Yamaguchi University, Tokiwadai 2-16-1, Ube, 755-8611, Japan
[c] Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University, Machikaneyama 1-3, Toyonaka 560-8531, Japan

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ABSTRACT

Immobilization of multiple enzymes onto solid surface is of significance for efficient consecutive reactions. In this work, the neutralized avidin (NA) molecules were covalently conjugated at pH 7.5 to the liposomes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 20 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) and 15 mol% *N*-(succinimidyloxy-glutaryl)-L-α-phosphatidylethanolamine, 1-palmitoyl-2-oleoyl (POPE-NHS). The apparent number of NA molecules conjugated per liposome, n_{NA} was estimated on the basis of fluorescence quenching of biotin-4-fluorescein (B4F) in the presence of neutralized avidin-conjugated liposomes (NALs). The NALs possessed the n_{NA} value of $(4.2 \pm 1.8) \times 10^2$ and the mean hydrodynamic diameter of about 150 nm. The biotinylated glucose oxidase and horse radish peroxidase molecules (b-GO and b-HRP) were mixed with NALs at pH 7.5 under the condition that the total concentration of b-GO and b-HRP ([b-GO] = [b-HRP]) is equal to the apparent concentration of NA (500 nM) in the NAL suspension. When the immobilization was performed at [Lipid] = 0.35 mM, a NAL possessed the enzyme activities corresponding to 1.4 free GO and 50 free HRP molecules. The b-GO and b-HRP-immobilized NALs could continuously catalyze the oxidation of glucose with molecular oxygen producing H_2O_2 which was consumed for the catalytic oxidation of 2,2′-Azino-bis(3-ethylbenzthiazoline 6-sulfonic acid) ($ABTS^{2-}$). The results obtained demonstrate that the reactive bienzyme system can be fabricated under mild conditions by using the NALs as colloidal scaffolds.

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1. Introduction

Immobilized enzymes have been extensively studied so far practically because of their improved recoverability from the reaction systems mainly for catalyst reuse as compared to the homogeneously solubilized enzyme molecules [1]. The immobilized enzymes undergo structural alteration and functional modification through the interaction with solid surface or the restriction derived from spatial boundary [2,3]. The enzyme molecules are localized at solid-water interface region [4] or in compartmentalized fine droplets [5] through immobilization. Such features allow fixing the proximity or local concentrations of different enzymes functioning together for catalyzing consecutive reactions [6–11] and can potentially alter the efficiency of catalytic cascade reactions [12]. Therefore, the methodology and functionalized supports, which can precisely control the molecular density and localization of different enzymes, have been received much attention [13].

Among the supports for enzyme immobilization, the porous or fine materials such as mesoporous silica [7] and graphene oxide-based particles [14] are advantageous because of their large specific surface area. Phospholipid vesicles (liposomes) can form a stable colloidal dispersion in an aqueous solution. The surface of liposomes can be modified with the chemically reactive groups [15,16] for conjugating to the specific amino acid residues within enzymes. The liposomes are also modifiable in the density of surface charge by incorporating anionic or cationic lipids [17,18], which is advantageous for controlling the dispersion state of liposomes modified with charged or hydrophobic molecules. The enzyme molecules are, however, more or less deactivated because of the covalent conjugation reactions or in the presence of activating reagents [19]. This is disadvantageous for controlling the molecular density of the biologically active enzyme molecules on liposomes.

* Corresponding author at: Department of Applied Molecular Bioscience, Yamaguchi University, Tokiwadai 2-16-1, Ube, 755-8611, Japan.
E-mail address: yosimoto@yamaguchi-u.ac.jp (M. Yoshimoto).

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Table 1
Conditions for the conjugation of NA to the NHS-bearing liposomes.

no.	Mole fraction of lipids of liposomes, f/–			Total concentration of lipids/mM	Concentration of NA/μM
	POPC (f_{POPC})	POPG (f_{POPG})	POPE-NHS (f_{NHS})		
1	0.75	0.20	0.05	5.0	20
2	0.65	0.20	0.15	5.0	20
3	0.50	0.20	0.30	5.0	20
4	0.85	0	0.15	5.0	100

In this work, two different enzymes were immobilized onto liposomes on the basis of avidin-biotin biospecific affinity [16,20]. If one can densely conjugate the avidin molecules on the surface of liposomes, multiple biotinylated enzyme molecules are potentially immobilized onto the liposomes under mild conditions at various ratios and densities of enzymes. Such liposomal systems may be useful for fabricating the localized multienzyme systems with different catalytic efficiency from the enzymes solubilized in the bulk liquid. The model enzymes employed were glucose oxidase and peroxidase which catalyze consecutive oxidation reactions [4,21].

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, lot 16096811FL, 99.7%, lot 14096811FL, 99.8%), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG, lot 15066951L, 99.4%) and *N*-(succinimidyloxy-glutaryl)-L-α-phosphatidylethanolamine, 1-palmitoyl-2-oleoyl (POPE-NHS, lot 1509696LN5, > 85%) were obtained from NOF (Tokyo, Japan). Neutralized and deglycosylated avidin from egg white (NA, M_r = 58,800 in the tetrameric form, lot SAR0481, lot LKF6845, lot PDR2114), avidin from egg white (lot CTJ1047) and horse radish peroxidase (HRP, lot SAK1219, lot PDK5778) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Glucose oxidase from *Aspergillus sp.* (GO, lot 94810) was from Toyobo (Osaka, Japan). Biotin glucose oxidase conjugated (b-GO, lot #36370) was obtained from Rockland Immunochemicals (Limerick, PA, USA). Biotinylated peroxidase (b-HRP, lot RE237223, lot QA214870, lot RC234541) was obtained from Invitrogen (Rockford, IL, USA). Biotin-4-fluorescein (B4F, lot 1731053) was obtained from Molecular Probes (Eugene, OR, USA). 2,2′-Azino-bis(3-ethylbenzthiazoline 6-sulfonic acid) diammonium salt ($ABTS^{2-}(NH_4^+)_2$, lot 12774722) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). All chemicals were used as received.

2.2. Covalent conjugation of NA to NHS-bearing liposomes

A mixture of POPC, POPG and POPE-NHS, which was weighed into a 100-mL round bottom flask at the total lipid amount of 50 μmol, was dissolved in 4.0 mL of chloroform and the solvent was evaporated under the reduced pressure. The lipids were solubilized in 4.0 mL of diethylether and the solvent was removed. This procedure was repeated twice to form the thin lipid film on the wall of flask. The residual solvent molecules remained within the film were removed with a freeze-dryer instrument for 2 h. The flask was covered with an aluminum foil for shielding the light. The dry lipid film was hydrated with 2.0 mL of 10 mM phosphate buffer solution (pH 7.5). Then, the lipid suspension was frozen for 7 min in a dry ice/ethanol and incubated for 7 min in a water bath at 37 °C. This freeze-thaw cycle was performed 7 times to induce the removal of lipid film from the wall of flask and to obtain homogeneous suspension of large multilamellar vesicles (MLVs). The MLVs were transformed into smaller vesicles by passing through a polycarbonate membrane with the nominal pore diameter of 200 nm. For the extrusion, the handy type extruder instrument Liposofast from Avestin (Ottawa, Canada) was used. The concentration of POPC was measured enzymatically with a kit Phospholipids C from Wako Pure Chemicals Industries. The liposome suspension and NA in the phosphate buffer solution were mixed in a glass test tube to give the concentrations of total lipids and NA of 5.0 mM and 20 or 100 μM, respectively. The concentration of NA was determined on the basis of absorbance at 280 nm taking 1.66 L (g cm)$^{-1}$ with respect to the comparable protein [22]. The measurement was performed with the spectrophotometer instrument V-630BIO from JASCO (Tokyo, Japan). The total volume of above mixture was 1.0 mL. The mole fraction of each lipid, f and detailed conditions of the liposome/NA mixtures are shown in Table 1. A part of experiments was performed with avidin (100 μM) instead of NA and the liposomes composed of POPC:POPE-NHS (85:15 in molar ratio, [lipid] = 5.0 mM). The mixture was incubated at ambient temperature for 3 h to induce the covalent boding between the NHS-bearing liposomes and the primary amines within the proteins [23]. The free (unbound) protein molecules were removed from the neutralized avidin-conjugated liposomes (NALs) with a sepharose 4 B column collecting each 1.0-mL fraction giving a total elution volume of 20 mL. For the separation, 50 mM Tris-HCl buffer solution (pH 7.5) was used as eluent.

2.3. Measurements of size distribution and ζ-potential of liposomes

The mean hydrodynamic diameter D_h and size distribution of liposomes were measured by the dynamic light scattering (DLS) method with the instrument ELSZ2-plus from Otsuka Electronics (Hirakata, Japan). The ζ-potential of liposomes was measured by the laser-Doppler method with the above instrument [24]. Each measurement was performed in triplicate.

2.4. Quantification of the concentration of NA

The fluorescence of B4F is quenched by binding to avidin, which is applicable to determination of the number of biotin-binding sites of the avidin-containing systems [25]. The 50 mM Tris-HCl buffer solution (pH 7.5) containing B4F was filtrated by passing through 0.22-μm membrane pores using the Milex PVDF from Merk Millipore (Carrigtwohill, Co. Cork, Ireland). The concentration of B4F in the filtrated solution was determined on the basis of absorbance at 495 nm (ε_{495} = 68,000 M^{-1}cm^{-1} [25,26]). The B4F solution was mixed with the free NA solution to give the fixed B4F concentration of 8.0 nM and various concentrations of NA up to 6.0 nM. The mixture (3.0 mL) was incubated for 30 min and the fluorescence intensity I_{B4F} was measured at the wavelengths of excitation and emission of 495 and 525 nm, respectively with the spectrofluorometer FP-8200 from JASCO. The measurements were also performed with respect to the NALs. The degree of quenching Q_{B4F} is calculated as $Q_{B4F} = (I_0 - I_{B4F})/I_0$, where I_0 is the fluorescence intensity of 8.0 nM B4F in the absence of NA. The apparent number of NA molecules conjugated per liposome was determined by correlating the con-

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