Electrochimica Acta 252 (2017) 362–367

Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

Electrochemical assay of lipid kinase activity facilitated by liposomes

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ARTICLE INFO

Article history:
Received 3 July 2017
Received in revised form 30 August 2017
Accepted 31 August 2017
Available online 1 September 2017

Keywords:
lipid kinase
electrochemical biosensor
liposome
biomimetic membrane
signal amplification

ABSTRACT

Lipid phosphorylation plays central regulatory roles in diverse fundamental cellular processes. However, there are still many challenges remained to investigate these effector enzymes, lipid kinase. So we report a simple, sensitive and effective electrochemical method assisted by liposome that can provide biomimetic membrane environment. In this work, the liposome is designed to not only provide favorable catalytic environment for the assay of lipid kinase, but also act as a carrier of abundant signal molecules to enhance the electrochemical signal. So, the problems involved in lipid kinase assay can be addressed, and very high sensitivity of the assay is ensured owing to the enrichment of signal molecules (methylene blue, MB). Compared with the currently-used methods, this new method avoids complex treatments of lipid substrats/products, thus fewer steps of the assay procedure are required. In this work, a vital lipid kinase, sphingosine kinase 1 (SphK1), has been selected as the assay target. Facilitated by liposome-based electrochemical signal amplification, a relative wide detection range from 10 pmol min^{-1} mg^{-1} to 12 nmol min^{-1} mg^{-1} and a low limit of detection (LOD) down to 2.33 pmol min^{-1} mg^{-1} have been achieved. This method can be effectively utilized for SphK1 detection, and further employments may also hold great promise for the analysis of other lipid kinases and even lipid metabolites in the future.

1. Introduction

Lipid kinase, such as sphingosine kinase 1 (SphK1) [1], phospholipase D (PLD) [2] and phosphoinositide 3-kinases (PI3Ks) [3], is a significant class of metabolizing enzymes to produce signaling molecules in diverse biological processes [4,5]. Dysfunction of these lipid kinases and corresponding phosphorylation reactions may lead to abnormal cell proliferation, cell death and even tumorigenesis [6]. So, monitoring the activity of lipid kinases is essential in biomedical researches and clinical diagnosis. However, detection of lipid kinase activity remains a challenge, since (1) many lipid kinases need specific catalytic environment, and (2) lipid substrates/products are difficult to be quantified with simple techniques. So, for the assay of lipid-metabolizing enzymes, current methods are still rely on the determination of phospholipids by phosphate-specific labeling. For example, ^{125}I-labelled immunoassay is performed for plasma 1,25-dihydroxyvitamin D detection [7], and ^{3}H-phosphatidylethano is utilized to evaluate the activity of phospholipase D [8]. Therefore, time-consuming preparation of these labels and complicated substrates/products separation steps are required in these methods, which usually lead to relatively low detection efficiency.

Many challenges are involved in the design of a method to assay the activity of a lipid kinase, including the catalytic environment of lipid kinase and signal readout of the assay method. At the first place, the catalytic reaction of lipid kinases usually takes place at the surface of plasma membrane, so it is necessary to provide these kinases with membrane mimetic environment. Currently, available methods for the fabrication of membrane environment are bilayer lipid membrane (BLM) [9–11], Langmuir-Blodgett layers [12,13] and artificial lipid membranes [14]. To date, liposome [15,16] has been used not only as carriers of molecules in vessels for drug or gene delivery [17], but also as simplified model of cell membrane [18]. So, we propose that the two properties of liposome, if integrated, could be perfect to provide favorable catalytic environment and enable signal readout. Therefore, in this work, the lipid bilayer of the liposome is designed to embed the lipid substrate. Consequently, the substrate of lipid kinase can be preserved intact in lipids through hydrophobic interaction, while the lipid catalytic environment can be simulated to facilitate the

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http://dx.doi.org/10.1016/j.electacta.2017.08.190
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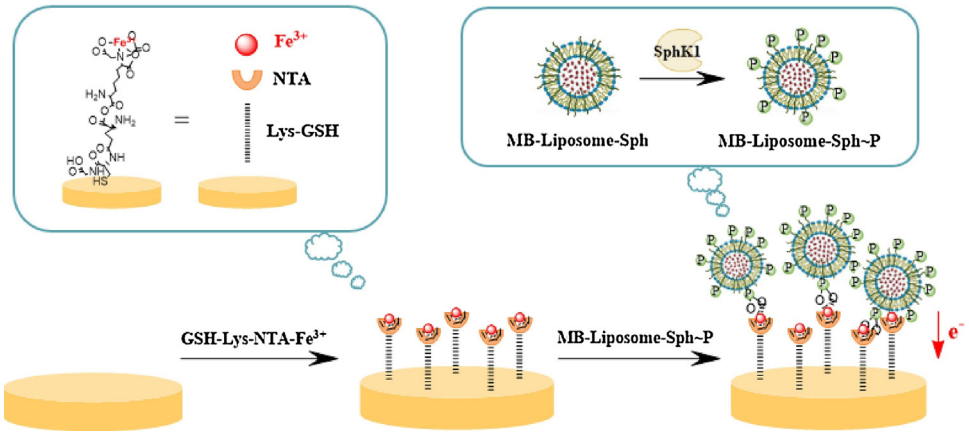

Scheme 1. Schematic illustration shows the principle and fabrication procedures for lipid kinase activity based on liposome-assisted electrochemical assay.

catalysis reaction. Besides, solubility problem of the lipid substrates [19] can be solved in this way.

Secondly, signal generation, or even signal amplification of the catalytic reaction is the key point of an assay method; however it is not easy to obtain a signal for the assay of lipid kinase. Herein, liposome is once again utilized as the signal molecule carrier, in which large quantities of signal molecules (MB) is encapsulated. In this way, after catalytic reaction of the lipid kinase, the phosphorylated lipids on liposomes can be recognized and captured *via* the specific interaction between coordinated metal ions and phosphorylated products [20]. Thus, the reacted liposomes are dragged to the electrode sensing surface, and the electrochemical signal of MB can thereupon be transmitted from the liposomes to the electrode surface directly. Thus, the enriched MB in liposomes can greatly enhance electrochemical signal, ensuring high sensitivity of the assay.

As is shown in Scheme 1, all the steps in this new method are based on practical principles: (1) The catalytic environment is stimulated by the liposomes, (2) the phosphorylated sites are recognized with high affinity through NTA-Fe (III) coordinated interaction, and (3) the quantitative signal is significantly enhanced by MB encapsulated liposomes. As the results indicated in the later part of this paper, the lipid kinase detection can be realized more efficiently with fewer steps, and a high detection sensitivity is ensured.

2. Experimental Section

2.1. Materials and reagents

The sphingosine kinase 1 (SphK1) was purchased from Sino Biological Inc. and D-erythro-sphingosine was from J&K Scientific Ltd. Protein kinase A (PKA), methylene blue hydrate (MB), glutathione (GSH), Nα,Nα-Bis-(carboxymethyl)-L-lysine hydrate (LysNTA) and N-Ethyl-N-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich. Soy Lecithin and cholesterol were purchased from AVT Pharmaceutical Technology Co., Ltd. All reagents were of analytical grade. Ultrapure water was purified by a Milli-Q system (>18.2 MΩ) and used throughout the experiments.

2.2. Preparation and characterizations of the MB-encapsulated liposomes

MB-encapsulated liposomes were prepared by adding 6 mg soy lecithin, 2 mg cholesterol and 0.3 mg sphingosine into 2 mL $CHCl_3$ solution containing 5 mM methylene blue. After well vortexing, this mixture was mixed and evaporated under 37 °C to dryness by a rotary evaporator. Then, the dry residue was resuspended with 1 mL 20 mM phosphate buffer (150 mM NaCl, pH 7.4) and sonicated for 30 min to obtain the desired compositions [21]. Purification of the modified liposomes was performed by a 10 K centrifugal filter device (Millpore Amicon Ultra) through filtering and recovery steps.

The liposomes were dispersed in deionized water for characterization. Fluorescent images were measured on a IX73 fluorescent microscope with DP80 digital camera (Olympus, Japan). Transmission electron microscopy (TEM) observations were carried out on a JEM-2100 microscope. The dynamic light scattering (DLS) measurements were performed on a particle size analyzer (Brookhaven 90Plus, USA) at 25 °C. The fabricated liposomes were stored at 4 °C for following experiments.

2.3. Preparation of NTA-Fe (III)-immobilized gold electrode

Gold electrode was treated according to the previous method with slightly modification [22]. Specifically, the gold electrode (3 mm in diameter) was first cleaned with piranha solution (98% H_2SO_4: 30% H_2O_2 = 3:1, *v/v*) for 5 min followed by rinsing with deionized water. Then, it was polished to a mirror sheen on sand paper and alumina powder of 1.0, 0.3 and 0.05 μm in sequence. After successive sonication in ethanol and deionized water for 5 min, the gold electrode was immersed in 50% (*v/v*) nitric acid for 30 min. After that, it was electrochemically cleaned with 0.5 M H_2SO_4 followed by drying with high purity nitrogen.

The above cleaned gold electrode was immediately immersed into 5 mM GSH solution at −4 °C for 12 h to obtain a well-aligned monolayer, followed by rinsing the electrode with water to remove the non-covalent binding GSH. After activated by 5 mM EDC and 10 mM NHS in pH 6.0, the electrode was immersed into 5 mM LysNTA solution (pH 7.4) for 1 h and into 5 mM $FeCl_3$ for 1 h for the following experiments.

2.4. SphK1 activity assay

The assay procedure is illustrated in Scheme 1. The SphK1 reaction mixture was first prepared with SphK1 of a desired concentration in 20 μL reaction buffer (0.5 mM ATP and 50 mM $MgCl_2$ in 20 mM Tris-HCl buffer, pH 7.4). Then, 5.0 μL of the prepared liposomes were incubated with the reaction mixture at 37 °C for 1 h. After that, the reaction mixture was diluted to 100 μL. The modified electrode was dipped in this mixture for 1.0 h,