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Sequential enzymatic derivatization coupled with online microdialysis sampling for simultaneous profiling of mouse tumor extracellular hydrogen peroxide, lactate, and glucose

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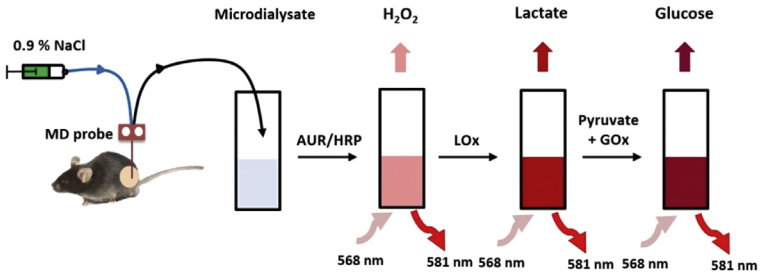
HIGHLIGHTS

- An enzymatic scheme was proposed to sequentially derivatize H_2O_2/lactate/glucose.
- A method for monitoring of tumor extracellular H_2O_2/lactate/glucose was developed.
- The detection limits were 2 μM (H_2O_2), 58 μM (lactate), and 55 μM (glucose).
- The variations of these metabolites for metabolic disturbance were profiled *in vivo*.

GRAPHICAL ABSTRACT



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ABSTRACT

Probing tumor extracellular metabolites is a vitally important issue in current cancer biology. In this study an analytical system was constructed for the *in vivo* monitoring of mouse tumor extracellular hydrogen peroxide (H_2O_2), lactate, and glucose by means of microdialysis (MD) sampling and fluorescence determination in conjunction with a smart sequential enzymatic derivatization scheme—involving a loading sequence of fluorogenic reagent/horseradish peroxidase, microdialysate, lactate oxidase, pyruvate, and glucose oxidase—for step-by-step determination of sampled H_2O_2, lactate, and glucose in mouse tumor microdialysate. After optimization of the overall experimental parameters, the system's detection limit reached as low as 0.002 mM for H_2O_2, 0.058 mM for lactate, and 0.055 mM for glucose, based on 3 μL of microdialysate, suggesting great potential for determining tumor extracellular concentrations of lactate and glucose. Spike analyses of offline-collected mouse tumor microdialysate and monitoring of the basal concentrations of mouse tumor extracellular H_2O_2, lactate, and glucose, as well as those after imparting metabolic disturbance through intra-tumor administration of a glucose solution through a prior-implanted cannula, were conducted to demonstrate the system's applicability. Our results evidently indicate that hyphenation of an MD sampling device with an optimized sequential

Abbreviations: ABTS, 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid); DCFH, 2′,7′-dichlorodihydrofluorescein; TMB, 3,3′,5,5′-tetramethylbenzidine; AUR, Amplex® UltraRed; ECF, extracellular fluid; FIA, flow injection analysis; GOx, glucose oxidase; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; LOx, lactate oxidase; MD, microdialysis; MWCO, molecular weight cut-off; PAES, polyarylethersulfone; PTFE, polytetrafluoroethylene; TRAMP, transgenic adenocarcinoma of the mouse prostate.

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enzymatic derivatization scheme and a fluorescence spectrometer can be used successfully for multi-analyte monitoring of tumor extracellular metabolites in living animals.

1. Introduction

Hydrogen peroxide (H_2O_2), lactate, and glucose are all used as indicative energy metabolites in current cancer biology. Experimental observations have revealed higher-than-common levels of H_2O_2 in cancer cells; it is essential for their development, making them more susceptible to H_2O_2-induced cell death than normal cells [1–3]. In addition, cancer cells generate energy predominantly through converting glucose into lactate, regardless of whether oxygen is available [4]; this synergistic effect of intracellular/extracellular glucose starvation and accumulation of lactate transferred to extracellular space has been correlated with a high incidence of distant metastasis in an early stage [4–6]. Because the levels of these energy metabolites in the tumor extracellular fluid (ECF) can be used to imply tumor malignancy and invasiveness [3,5,6], the ability to identify tumor microenvironments through monitoring of these three representative species can become indispensable for cancer diagnostics and therapeutic applications [5–8].

Because of their small dimensions and rapid signal response, implantable electrochemical biosensor arrays can allow simultaneous multi-analyte monitoring of chemical substances in living biological systems [9–13]. Nevertheless, the coexistence of complicated biological matrix/redox species and fouling of the sensing units is unavoidable, making it difficult to quantify these tumor extracellular metabolites through the signals acquired from implanted biosensors [14–16]. Thus far, these implantable biosensors have rarely been applied to directly explore chemical substances in intact solid tumors. The coupling of microdialysis (MD) sampling techniques with a diverse array of analytical methods and apparatus has evolved into alternative clinical tools for studying the chemistry of extracellular substances related to homeostatic regulation and the biochemical processes of disease development [14,17–19]. With its great ability to exclude almost all extracellular biomolecules from the sampling site, while also significantly minimizing interference and fouling of the sensing components, MD sampling is now widely applied for the identification and reliable calibration of extracellular substances in both anesthetized and freely moving animals [20–22]. Because enzyme-based electrochemical sensing strategies are highly specific, sensitive, and conveniently integrated and miniaturized [23], coupling an MD sampling device with an electrochemical biosensor array might facilitate interference-free, multi-analyte monitoring of tumor extracellular substances.

Multi-analyte monitoring using enzyme-incorporated electrochemical methods, however, remains impractical because of serious cross-talk among the immobilized enzymes, mutually disturbing their sensing abilities, especially for the simultaneous determination of lactate and glucose [9–13,24–27]. In addition to direct sensing of electrochemically active species (e.g., H_2O_2), many chromogenic and fluorogenic reagents that behave as suitable substrates for horseradish peroxidase (HRP) can be converted into colorful and brightly fluorescent emitting species in the presence of H_2O_2 (or the H_2O_2 generated from biochemical oxidation) [28]. Using spectrophotometric methods to detect the derivatives obtained following enzymatic derivatization becomes an alternative approach that has attracted considerable interest because of its methodological specificity, sensitivity, adaptability, convenience, and diversity [23,29,30]. Moreover, using a step-wise series of biochemical reactions to sequentially derivatize multiple analytes within the same analytical cycle would be beneficial in terms of decreasing total analysis times, chemical consumption, and labor intensity.

To demonstrate that reporting biochemically generated H_2O_2 with a commercial H_2O_2-sensing molecular probe [Amplex® UltraRed (AUR), a fluorogenic substrate for HRP] can be a universal strategy for enabling multi-analyte determination of chemical substances through the incorporation of their specific oxidases, in this study we designed a smart derivatization scheme involving sequential loading of AUR/HRP, microdialysate, lactate oxidase (LOx), pyruvate, and glucose oxidase (GOx) to allow simultaneous determination of mouse tumor extracellular H_2O_2, lactate, and glucose. Here we applied, for the first time, pyruvate, the product of LOx-mediated lactate oxidation [31], to diminish cross-talk among HRP, LOx, and GOx. After optimizing the parameters for the MD sampling system, the sequential enzymatic derivatization scheme, and the fluorescence determination system, we evaluated the analytical performance of the proposed H_2O_2/lactate/glucose monitoring system. In addition, we demonstrated the system's applicability through not only spike analyses of offline-collected mouse tumor microdialysate but also *in vivo* monitoring of the basal concentrations and dynamic variations of mouse tumor extracellular H_2O_2/lactate/glucose following intra-tumor administration of a glucose solution through a prior-implanted cannula.

2. Materials and methods

2.1. Chemicals

H_2O_2 (216769), HRP (P8250), D-(+)-glucose (G8270), GOx (G7141, from *Aspergillus niger*), L-(+)-lactic acid (L1750), LOx (L0638, from *Pediococcus* sp.), pyruvate (P2252), sodium chloride (NaCl; S5886), potassium chloride (KCl; 05257), trisodium citrate dihydrate (C8532), sodium dihydrogen phosphate (NaH_2PO_4; S8282), and disodium hydrogen phosphate (Na_2HPO_4; S7907) were purchased from Sigma–Aldrich. AUR Reagent (A36006) was purchased from Thermo Fisher Scientific. All chemical solutions were prepared using water purified through a Milli-Q Integral water purification system (Merck Millipore). The AUR working solution was kept in the dark as much as possible. To avoid bubble formation during fluorescence analysis, all of the system's carrier solutions were purged with high-purity N_2 gas in advance.

2.2. Apparatus and methods

The proposed H_2O_2/lactate/glucose monitoring system comprised an MD sampling device, a sequential enzymatic derivatization system, and a fluorescence spectrometer (Fig. 1). A commercial MD probe, featuring a 4-mm-long, 500-μm-diameter polyarylethersulfone (PAES) membrane having a molecular weight cut-off (MWCO) of 20 kDa (8010435; CMA 20, CMA Microdialysis), was selected for sampling of tumor extracellular substances. To provide a reaction chamber capable of sequential loading of reaction medium [10 mM phosphate-citrate (PC) buffer (pH 7) and AUR/HRP], microdialysate, and others reagents, a quartz cuvette