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Parallel assay of oxygen equilibria of hemoglobin

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ABSTRACT

Methods to systematically analyze in parallel the function of multiple protein or cell samples in vivo or ex vivo (i.e., functional proteomics) in a controlled gaseous environment have so far been limited. Here, we describe an apparatus and procedure that enables, for the first time, parallel assay of oxygen equilibria in multiple samples. Using this apparatus, numerous simultaneous oxygen equilibrium curves (OECs) can be obtained under truly identical conditions from blood cell samples or purified hemoglobins (Hbs). We suggest that the ability to obtain these parallel datasets under identical conditions can be of immense value both to biomedical researchers and clinicians who wish to monitor blood health and to physiologists who are studying nonhuman organisms and the effects of climate change on these organisms. Parallel monitoring techniques are essential in order to better understand the functions of critical cellular proteins. The procedure can be applied to human studies, where an OEC can be analyzed in light of an individual's entire genome. Here, we analyzed intraerythrocytic Hb, a protein that operates at the organism's environmental interface and then comes into close contact with virtually all of the organism's cells. The apparatus is scalable and establishes a functional proteomic screen that can be correlated with genomic information on the same individuals. This new method is expected to accelerate our general understanding of protein function, an increasingly challenging objective as advances in proteomic and genomic throughput outpace the ability to study proteins' functional properties.

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Oxygen (O_2)[1] uptake from the environment and use by the organism are essential to nearly all of the planet's animal species, many of which rely on blood to bind and transport O_2 throughout the body. The hemoglobin (Hb) O_2-binding system is key to organisms' abilities to operate because of the necessity for O_2 in energy metabolism as a final electron receptor in ATP synthesis [1]. Red blood cells (RBCs), which carry Hbs, contact the environment at the lungs or gills and circulate to subserve every tissue in the body [2,3]. An exquisitely tuned O_2, carbon dioxide (CO_2), and nitric oxide (NO) sensor, Hb enables organisms to sense and adapt to their environments [4]. The functional flexibility in the O_2-binding abilities of Hb are clearly modulated in both short- and long-term environmental changes [5].

Reliable methods do currently exist for studying O_2-binding of blood and Hb samples, and a vast body of literature supports fundamental discoveries concerning Hbs of humans and other species. These studies have been carried out largely or exclusively on single samples from individuals. Simultaneous samples from many individuals within a population are infrequent, owing in part to the inability to perform many O_2-binding curves in true parallel. To date, no method can easily or practically handle replicate samples simultaneously, or multiple samples under identical conditions, a prerequisite for high-throughput approaches. These limitations, together with a lack of the instrument portability needed to study samples and the need for a specialized apparatus, have also limited the ability to widely obtain O_2 equilibrium curves (OECs)—an important clinical and biological property—in the clinic and the field.

The development of this parallel monitoring apparatus presents numerous potential applications for O_2-binding studies. In addition to revealing changes in blood O_2-binding properties with changing environmental conditions, an equally important current challenge is to understand how blood O_2-binding behaviors change with blood aging, particularly during blood banking. For example, depletion of intraerythrocytic 2,3-diphosphoglycerate (2,3-DPG)

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[1] *Abbreviations used:* O_2, oxygen; Hb, hemoglobin; RBC, red blood cell; CO_2, carbon dioxide; NO, nitric oxide; OEC, O_2 equilibrium curve; ATP, adenosine triphosphate; PBS, phosphate-buffered saline; DTPA, diethylenetriaminepentaacetic acid; Hct, hematocrit; HbA_0, hemoglobin A_0; PO_4, inorganic phosphate; N_2, nitrogen; sccm, standard cubic centimeters per minute; pO_2, partial pressure of oxygen; metHb, methemoglobin; P_{50}, pO_2 (mm Hg) at 50% HbO_2 saturation.

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and adenosine triphosphate (ATP) increases the O_2 affinity of stored RBCs. A similar "storage deficit" occurs within hours in the intracellular levels of the NO derivative of Hb, *S*-nitrosohemoglobin (SNO-Hb). Studies are currently under way to identify interventions to eliminate this deficit [6–8]. More broadly, hospitals and blood banks must ensure that the stored blood they provide to transfused patients is safe and functional in terms of its O_2-binding and related properties. To date, no technology for OECs is sufficiently widely available or efficient to guide medical decision making or quality assurance in transfusion medicine. Similarly, single-sample throughput has limited the ability of scientists to determine the basis for interindividual changes in OEC behavior during RBC storage.

Growing evidence suggests a relationship between morbidity and mortality in patients receiving blood as a function of storage time [9,10]. There is substantial variation among the biological properties of RBC units stored conventionally [11]. A practical test capable of characterizing the OEC unique to a given unit of RBCs could, in principle, promote superior outcomes with RBC transfusion. Readily available OEC data from the recipient could further personalize this approach while aiding in inventory management of this precious medical resource.

In addition to applications for human blood, this parallel and potentially high-throughput method can be used in research stations to analyze all types of nonhuman blood for comparative studies and to discover physiological responses to changing environments. As our planet moves into an era of increasing temperature and rapid environmental change, we must develop successful methods for understanding mechanisms behind the effects of these changes on organismic physiology [12]. Global climate change has become a critical issue in discussions of the future of our planet, for both humans and other species, and may seriously affect numerous organisms and ecosystems, with varying effects within a single species. For example, fish RBCs show relatively frequent polymorphisms in Hb types, demonstrating individual-to-individual variations within a single species [13]. It has not been possible to date, however, to extensively study O_2-binding properties of the RBCs from individual fish or to simultaneously analyze replicate samples from multiple species living under varying environmental conditions. Nor has it been possible to perform OECs "in the field," free of potential transport-associated artifacts, including the passage of time.

Here, we describe and present results from a microplate-based O_2 tonometric approach that allows, for the first time, parallel monitoring of samples. Our findings support the facility and replicability of this approach. The device and approach can promote accelerated functional studies of proteins and cells so as to keep pace with the rapid growth of proteomic and genomic knowledge.

Materials and methods

Sample preparation

Animal and human blood samples were obtained under protocols approved by the institutional animal care and use committee and the institutional review board. Standard antecubital phlebotomy and heparin-coated syringes were used for human blood draws, and samples were typically used within 4 h of acquisition. Blood was centrifuged for 3 min at 2500 RCF at room temperature (25 °C), plasma and white blood cells were removed, and then RBCs were washed with phosphate-buffered saline (PBS, pH 7.4). The chelating agent diethylenetriaminepentaacetic acid (DTPA, 0.1 mM) was typically added to inhibit metal-dependent methemoglobin formation (Hb oxidation). RBCs were suspended at a hematocrit (Hct) of 20 to 25% (v/v), which was ideal for examining the "A" and "B" visible (500–650 nm) spectral bands. No significant difference in the resulting binding curves was seen when varying spectral regions (Soret vs. A/B visible) were used to calculate the degree of Hb O_2 saturation.

RBC hemolysates were prepared by hypotonic lysis in 4 volumes of water containing DTPA (0.1 mM). Chromatographically purified hemoglobin A_0 (HbA_0) was a kind gift from Curacyte/Apex Biosciences (Durham, NC, USA). Chemical modifications were performed in order to modify intraerythrocytic Hbs and create well-characterized changes in O_2-binding characteristics. These modifications allowed us to create our own examples of Hb with known differences in O_2 equilibria.

Chemicals

All chemicals were obtained from Sigma Chemical (St. Louis, MO, USA) unless otherwise noted. In some cases, HbA_0 or hemolysates were dialyzed in various buffers (as described in each accompanying figure legend). Specifically, thin-film dialysis of the Hb versus Hepes or Bis–Tris buffer of varying inorganic phosphate (PO_4) concentrations with DTPA present (0.1 mM) was used to change the buffered PO_4 concentrations in order to study the effect of PO_4 concentration on O_2 affinity of HbA_0.

Analyte preparation

A standard, microplate-based spectrophotometer (Molecular Devices' SpectraMax 190 or BMG Labtech's FLUOstar Omega) was used to obtain the spectra needed to construct OECs from blood and Hb samples. Temperature was kept constant by the instrument's thermostatically controlled sample chamber, and experiments were done at temperatures ranging from 18 to 37 °C.

The multicuvette tonometer cell was designed and built by two of the current authors, J. Bonaventura and L. Perez. Fig. 1 shows a typical 8-well tonometer used for some of the experiments. The tonometer's dimensions are typically approximately $127 \times 85.5 \times 17$ mm, and the drawer of a standard microplate reader readily accommodates the device. The cell's length and width are nearly identical to those of a typical microplate. The height is slightly greater but does not impede these readers. Currently, the novel tonometer can analyze 8 to 24 samples simultaneously, depending on the layout of the inner microcuvette-holding insert plate. The number of simultaneous O_2 equilibria can theoretically be increased further by raising the number of, and shrinking the size of, the inserted microcuvettes. A simple modification of the drawer's "door" intended to seal the loaded microplate reader will allow tubing (for gas flow) to remain connected to the cell throughout an experiment.

Microcuvette and cell assembly

Samples were prepared in microcuvettes constructed for the tonometry cell using a technique developed at the Duke University Marine Laboratory. In summary, a suspension of hemolysate, RBCs, or other O_2-binding protein is sandwiched between two sheets of Teflon, Saran Wrap, or another appropriate O_2-permeable membrane with a maximal thickness of 1 μm. Saran Wrap was used for the experiments reported here. To prepare samples, the O_2-permeable membrane is stretched over a black plastic ring and secured in place with an O-ring. Sample (10 μl) is pipetted as a drop onto this first membrane layer. A second membrane layer is prestretched and secured on a larger plastic ring. This second layer is carefully lowered onto the droplet of Hb sample, flattening it to produce a thin sample layer. The cuvette is secured and completed by the stacked application of a second O-ring. Each assembled microcuvette is then placed into a clear plastic insert secured in the multicuvette tonometer (Fig. 1B and C). The positions of cuvette