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A high-affinity recombinant antibody permits rapid and sensitive direct detection of myeloperoxidase

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ABSTRACT

Over the past 10 years, a growing field of research supporting the value of myeloperoxidase (MPO) as a prognostic indicator in acute cardiac pathophysiologies has emerged. The availability of a rapid and disposable MPO detection platform would enable research clinicians to more readily assess MPO indications for guiding therapy and also facilitate clinicians at the patient interface to readily adopt MPO testing and potentially drive more informed prognoses. Here we describe the isolation of a high-affinity avian MPO-specific recombinant antibody panel using phage display. Rapid isolation of a suitable single-chain variable fragment (scFv) antibody was facilitated using a surface plasmon resonance (SPR)-based "off-rate ranking" screening process. The selected scFv was then successfully incorporated into a rapid, simple, and sensitive one-step lateral flow immunoassay (LFIA) for the detection of MPO. This "one-step" feature of the developed assay was made possible by the scFv's strong affinity for MPO, obviating the need for sandwich signal enhancement steps. The assay's rapid performance was also further enhanced by exploiting the intrinsic enzymatic properties of MPO in its final detection. Use of the optimized LFIA facilitated the sensitive detection of MPO in MPO-depleted serum within clinically relevant reference ranges.

Cardiovascular disease (CVD)[1] is the cause of nearly half of all deaths in the Western world (49%) [1] and is estimated to cost the European Union approximately 192 billion euro every year [1]. Despite the success achieved in employing the cardiac troponins in the diagnosis of myocardial infarction (MI), serious myocardial damage may have already occurred before troponin levels become noticeably elevated. Consequently, intense research has focused on the identification of a serum marker with the ability to identify patients at risk for plaque rupture. The discovery of such a marker would enable the implementation of a more accurate and efficient triage of patients with symptoms of acute coronary syndrome (ACS) in the ambulance or emergency department. Myeloperoxidase (MPO) has been shown to be a strong predictor of adverse events in patients with ACS [2,3], chronic heart failure [4], acute myocardial infarction [5], and of future coronary artery disease (CAD) in a healthy population [6]. It is a well-characterized enzyme, released mainly by activated neutrophils, characterized by powerful pro-oxidative and pro-inflammatory properties. Native MPO (150 kDa) is a dimer consisting of two 15-kDa light chains and two variable-weight glycosylated heavy chains bound to a prosthetic heme group. Following on from seminal studies at the beginning of this decade [2,3], evidence is now growing in support of MPO measurements to inform risk stratification in acute cardiac pathophysiologies [7–9]. To develop a rapid direct binding assay platform for MPO detection, it was necessary to first isolate a suitably high-affinity anti-MPO antibody. We used chickens as the host species to immunize because they can often produce a more vigorous antibody response to highly conserved mammalian antigens due to their large phylogenetic distance from humans. Several additional features of the avian genus render them particularly suitable for production of antibodies for use in diagnostic platforms [10,11]. Lateral-flow assays, which have been used as diagnostic tools for a wide range of applications [12–20], provide a robust and user-friendly immunoanalytical

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[1] *Abbreviations used:* CVD, cardiovascular disease; MI, myocardial infarction; ACS, acute coronary syndrome; MPO, myeloperoxidase; CAD, coronary artery disease; ELISA, enzyme-linked immunosorbent assay; scFv, single-chain variable fragment; LFIA, lateral flow immunoassay; cDNA, complementary DNA; RT-PCR, reverse transcriptase polymerase chain reaction; SOE, splice-overlap-extension; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HA, hemagglutinin; mAb, monoclonal antibody; PNPP, *p*-nitrophenyl phosphate; EDTA, ethylenediaminetetraacetic acid; CM, carboxymethylated; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*′-(dimethyl-aminopropyl) carbodiimide hydrochloride; FC, flow cell; TB, Terrific Broth; NTA, nitrilotriacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IgG, immunoglobulin G; HRP, horseradish peroxidase; TMB, tetramethylbenzidine; SPR, surface plasmon resonance; RU, response units; HTS, high-throughput screening; CLT, central laboratory testing; TAT, turnaround time; POC, point-of-care.

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method suitable for specific semiquantitative detection of analytes. This technique needs only low-cost instrumentation and enables significant reduction of the assay duration when compared with enzyme-linked immunosorbent assays (ELISAs) on microtitration plates. In this study, our aim was to isolate a high-affinity single-chain variable fragment (scFv) and incorporate it into an optimized one-step lateral flow immunoassay (LFIA) for the detection of MPO from human serum.

Materials and methods

Construction and selection of anti-MPO scFv library

Antibody production and selection were performed as described by Andris-Widhopf and coworkers [21]. RNA was isolated from the spleens and bone marrow of two MPO-immunized chickens and converted to complementary DNA (cDNA) by reverse transcriptase polymerase chain reaction (RT-PCR) using a SuperScript III kit (Invitrogen). Antibody variable heavy- and light-chain genes were amplified from the cDNA template using the primer sets described by Andris-Widhopf and coworkers [21]. The scFv gene product was amplified using splice-overlap-extension (SOE) PCR, cloned into the pComb3X vector, and electroporated into *Escherichia coli* XL1-Blue cells (Stratagene), producing a library size of approximately 2.5×10^7 clones. Enrichment of the scFv library was achieved by performing four rounds of VCMS13-facilitated phage display biopanning with increasing stringency against solid-phase immobilized MPO. The final enriched phage pool was infected into the nonsuppressor *E. coli* strain Top10F′ (Invitrogen) for soluble scFv expression.

Identification of MPO-specific clones via direct ELISA

Crude bacterial lysates from expressed clones were tested for binding in a direct ELISA on Nunc MaxiSorp™ plates previously coated with 200 ng/well of MPO (Biodesign International) and blocked with phosphate-buffered saline (PBS) containing 2.5% (w/v) milk (Marvel) and 2.5% (w/v) bovine serum albumin (BSA). A 100-μl sample of each of the bacterial lysates was added to the corresponding coated/blocked wells of the plate and incubated for 1 h at 37 °C. Antibody fragments were expressed with a hemagglutinin (HA) fusion tag and detected with an alkaline phosphatase-labeled mouse anti-HA monoclonal antibody (mAb). Absorbance values were read at 405 nm on a Safire 2 plate reader (Tecan) following a 20-min incubation with *p*-nitrophenyl phosphate (PNPP) substrate (Sigma).

Biacore studies

Analysis was carried out on a Biacore 3000 instrument using a research-grade CM5 sensor chip (GE Healthcare). The running buffer for all Biacore experiments (unless otherwise specified) was HBS buffer (pH 7.4) containing 10 mM Hepes, 150 mM NaCl, 3.4 mM ethylenediaminetetraacetic acid (EDTA), and 0.05% (v/v) Tween 20. The running buffer was freshly prepared, filtered (0.22 μM cutoff), and degassed using vacuum filtration apparatus (Millipore). Preconcentration experiments were performed prior to immobilization according to the manufacturer's instructions.

Immobilization of capture mAb for analysis of MPO-specific clones

All of the scFv constructs used throughout contained a C-terminal tag comprising the amino acid sequence YPYDVPDYA, which is an epitope sequence derived from the HA protein. To facilitate oriented capture of the HA-tagged scFv antibodies, a generic capture format was prepared by immobilizing an anti-HA antibody on a CM5 sensor chip surface.

The carboxymethylated (CM) dextran matrix on the sensor chip was activated by mixing equal volumes of 100 mM *N*-hydroxysuccinimide (NHS) and 400 mM *N*-ethyl-*N*′-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) and injecting the mixture over the sensor chip surface for 10 min at a flow rate of 5 μl/min. The HA-specific mAb (Affinity Bioreagents) used for scFv capture was diluted in 10 mM sodium acetate (pH 4.0) to a concentration of 60 μg/ml and injected over the activated chip surface for 20 min at a flow rate of 5 μl/min. The surface was capped using an 8-min injection of 1 M ethylenediamine (pH 8.5). The reference flow cell to be used for the analysis was also activated with EDC and NHS chemistry and then capped with ethylenediamine to reduce any potential nonspecific binding of the MPO to the CM dextran surface.

Off-rate analysis of anti-MPO scFv clones

Crude scFv-containing bacterial lysates containing 12 mg/ml BSA and 12 mg/ml CM dextran were subsequently injected (10 μl at a flow rate of 10 μl/min) over flow cell 1 (FC1) and FC2 using reference subtraction of FC2–FC1. Next, MPO at a concentration of 3 nM was passed over the captured scFv at a flow rate of 30 μl/min. The association and dissociation times used were 3 and 10 min, respectively. Regeneration of the chip surface was accomplished with 2×30-s injections of 20 mM NaOH.

Large-scale protein expression and extraction from bacterial cultures

An overnight culture of the candidate clone was inoculated into 500 ml of sterile Terrific Broth (TB) medium containing 100 μg/ml carbenicillin, 1 mM $MgSO_4$, 0.5% (v/v) glycerol, and 0.05% (w/v) glucose. The culture was incubated at 37 °C while shaking at 250 rpm until the cells reached approximately 0.6 (OD_{600} nm), after which expression of the scFv was induced. Bacterial cell pellets were harvested by centrifuging at 15,000*g* in a GSM rotor for 20 min. The cell pellet was thoroughly resuspended in 30 ml of sonication buffer ($1\times$ PBS [pH 7.4], 0.5 M NaCl, and 20 mM imidazole) and sonicated using a microtip Vibra-Cell™ sonicator for periplasmic extraction of the expressed scFv. Clear scFv-rich lysate was harvested after centrifugation of the lysed cells, followed by filtration of the supernatant through a 0.2-μM filter.

Purification of scFv from bacterial lysate via immobilized metal affinity chromatography

A 3-ml aliquot of Ni^+–NTA (nitrilotriacetic acid) agarose resin (Qiagen) was added to a 20-ml column and equilibrated with 20 ml of running buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 7.5). The filtered scFv-rich lysate was then applied to the equilibrated column, and the flow-through was collected. The column was then washed with 30 ml of wash buffer A (50 mM NaH_2PO_4, 1 M NaCl, 10% [v/v] glycerol, 20 mM imidazole, and 1% [v/v] Triton X-100, pH 7.5). A second wash step was then performed with 30 ml of wash buffer B (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 7.5). Elution of the scFv was achieved with 15 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 7.5). The eluted product was then thoroughly buffer exchanged against filtered $1\times$ PBS using a 5-kDa cutoff Vivaspin 6 column and quantified using a Nanodrop ND-1000 system (Thermo Fisher Scientific).

SDS–PAGE

Proteins were separated using a 12.5% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the methods described by Laemmli [22].