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# New approach for natural products screening by real-time monitoring of hemoglobin hydrolysis using quartz crystal microbalance

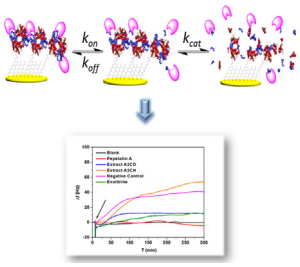
Vivian E. Cornelio [a,1], Mariele M. Pedroso [a,1], André S. Afonso [a], João B. Fernandes [a], M.Fátima G.F. da Silva [a], Ronaldo C. Faria [a,*], Paulo C. Vieira [a,*]

[a] Department of Chemistry, Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil

## HIGHLIGHTS

- The methodology allows to monitor the Hb hydrolysis by cathepsin D in real-time.
- The label-free approach greatly simplifies the procedure.
- The methodology allows QCM screening of NPs products inhibitors in real-time.
- $K_d$ and $k_{cat}$ are followed through the mass changes (frequency variation) on QCM.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The hemoglobin (Hb) released from erythrocytes is a primary nutritive component for many blood-feeding parasites. The aspartic protease cathepsin D is a hemoglobinase that is involved in the Hb degradation process and is considered an interesting target for chemotherapy intervention. However, traditional enzymatic assays for studying Hb degradation utilize spectrophotometric techniques, which do not allow real-time monitoring and can present serious interference problems. Herein, we describe a biosensor using simple approach for the real-time monitoring of Hb hydrolysis as well as an efficient screening method for natural products as enzymatic inhibitors using a quartz crystal microbalance (QCM) technique. Hemoglobin was anchored on the quartz crystal surface using mixed self-assembled monolayers. The addition of the enzyme caused a mass change (frequency shift) due to Hb hydrolysis, which was monitored in real time. From the frequency change patterns of the Hb-functionalized QCM, we evaluated the enzymatic reaction by determining the kinetic parameters of product formation ($k_{cat}$). The QCM enzymatic assay using immobilized human Hb was shown to be an excellent approach for screening possible inhibitors in complex mixtures, opening up a new avenue for the discovery of novel inhibitors.



## 1. Introduction

Hemoglobin (Hb) digestion is an essential process for the survival of blood-feeding parasites, such as schistosomes, blood-feeding ticks, and plasmodium species, among others [1–3]. In most cases, Hb hydrolysis is used by parasites primarily to provide the amino acids needed for the synthesis of their own proteins, which is fundamental for parasite development [4]. In the

* Corresponding author at: Rodovia Washington Luís, km 235, SP-310, CEP: 13565-905, São Carlos, São Paulo, Brasil. Tel.: +55 16 3351 8061.
*E-mail addresses:* rcfaria@ufscar.br (R.C. Faria), dpcv@ufscar.br (P.C. Vieira).
[1] These authors contributed equally.

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digestion process, proteases, such as cysteine, aspartic, and metalloproteases, play important roles [5–10].

In schistosomes, the proteins ingested in blood by the parasite, including Hb, are sources of nutrients, and the process of digestion involves a range of gut-associated proteases. These peptidases are thought to be related to the proteolysis of ingested host proteins and include orthologs of mammalian endopeptidases, such as cathepsins B, D and L, and exopeptidases, such as cathepsin C [11,12].

Several studies of cathepsin D-like (CD), belonging to a Clan AA aspartic protease that is present in both *Schistosoma japonicum* (SjCD, U90750) and *Schistosoma mansoni* (SmCD, U60995), have shown differences in human Hb preferences hydrolysis sites between parasites and human proteases [11,13,14]. In addition, the existence and proper functioning of these proteases are crucial for the maintenance and survival of parasites, making hemoglobinases important alternative targets for schistosomiasis chemotherapy [15,16].

Natural products (NPs) and their synthetic derivatives have been widely used in drug discovery because of their structural diversity and their highly selective and specific biological activities [17–19]. Numerous NPs, semi-synthetic NPs and NP-derived compounds are undergoing clinical testing [20–22]. Worldwide, between 2005 and 2010 alone, nineteen new NP-based drugs were approved for marketing [23]. Cragg and Newman continuously present updates on NP drugs based on traditional medicine both as pharmaceutical agents and/or as leads for bioactive molecules against serious diseases [18,24]. It is anticipated that nature will continue to be a major source of new structural leads, and effective drug development will depend on multidisciplinary collaborations. A wide number of studies, including *in vitro*,*in vivo* and enzymatic experiments, have reported effective activity in NP extracts and have isolated compounds from them [25–28].

Compound screening using natural products represents an important source of new enzymatic inhibitors. Most enzymatic assays are performed using spectrophotometric or radioactive techniques. Among assays using human Hb as the substrate and proteases as the target, absorbance or fluorescence techniques are most commonly used [2,15,29]. However, compounds can be discarded due to the presence of absorbance or fluorescence at the same wavelength as the product obtained from hydrolysis. This problem has led to false results due to the complexity of crude plant extracts, which include secondary metabolites. Furthermore, compounds that have a structure similar to that of the cleavage product can also be discarded [30]. This limitation prompted us to study new techniques for the development of assays free of interferences using Hb as a substrate, with the possibility of studying enzymatic kinetic parameters in real time. Here, we propose a simple, focused and effective screening approach for enzymatic assays of NPs using an electrogravimetric technique.

The quartz crystal microbalance (QCM) provides a means to measure Hb hydrolysis in real time. The technique is based on variations in the quartz crystal frequency related to the amount of mass deposited on the quartz crystal surface. QCM approaches for investigating biomolecular interactions offer high sensitivity and real-time analysis [31], and also offers rapid, cost-effective and reliable results. Furthermore, QCM is able to measure tiny mass changes in real time through the label-free detection of molecules [32]. Recently, QCM has been used to determine the kinetic constants of numerous biomolecules [33–36], including enzymes such as endo- and exo-type proteases [37–39] and β-amylase [40], with the intention of developing reliable protocols and methods to measure enzymatic activity [32].

Usually, enzymatic reactions performed in bulk solution are kinetically studied using the Michaelis–Menten equation (1) assuming a steady-state approach, in which the concentration of the enzyme–substrate complex (ES) is approximated to be constant during the reaction because of the relative difficulty of detecting this complex.

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} ES \overset{k_{cat}}{\rightarrow} P \quad (1)$$

By monitoring the enzymatic reaction, it is possible to obtain the initial rate ($v_0$) and values of $k_{cat}$ and $K_m$, where $K_m$ is a complex value composed of $k_1$ ($k_{on}$), $k_2$ ($k_{off}$) and $k_3$ ($k_{cat}$), as demonstrated in Eq. (1). However, these latter constants are usually not followed independently, limiting the analysis of the reaction progress. In general, enzymes interact in specific and particular ways with their respective substrates. The dissociation constant $K_d = k_{off}/k_{on}$, a parameter not obtained in real-time solution reactions, represents the rates of binding and release of the enzyme and provides a greater understanding of enzyme-substrate interactions.

To ensure the above condition for the kinetic model, it is possible to design a biosensor on QCM functionalized surface strategically to satisfy the model's boundary condition. By using a protein-immobilized QCM, these kinetic parameters ($K_d$ and $k_{cat}$) can be followed in real-time through the mass changes (frequency variation) on the quartz crystal as a function of time. Thus, if the formation and decomposition of the ES complex can be followed directly during the reaction, then more accurate kinetic constants might be obtained.

In this study, we developed a new approach for inhibitory assays based on the use of a QCM to monitor human Hb hydrolysis in real-time. For this, human Hb was covalently immobilized on the quartz surface, and the hydrolysis process was evaluated by measuring the frequency as a function of time after the enzyme bovine cathepsin D (CatD), either inhibited or not inhibited by extracts of plants or pure compounds, was injected. We discuss the necessary conditions for assays using an immobilized substrate based on previous reports of Hb in solution assays and provide an analysis of the kinetic parameters ($K_d$ and $k_{cat}$) obtained by QCM. The method proved to be useful for the screening of compounds from NPs without interference. The QCM assays showed the ability to identify potential inhibitors in complex matrices, opening up a new avenue for the screening of plant extracts that has not been proposed hitherto.

## 2. Materials and methods

### 2.1. General methods

The CatD and human Hb used in this work were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The solvents used in the chromatographic process were HPLC grade from J.T. BAKER, TEDIA and PANREAC. 1D and 2D NMR experiments were performed on a Bruker DRX 400 (9.4 T) instrument using deuterated solvents from Aldrich Chemical Company, Acros Organics, and Cambridge Isotope Laboratories, Inc. The reagents used in the immobilization process were from Sigma and/or Fluka. The quartz crystal used in this study was 16 mm in diameter, AT-cut, and had a fundamental frequency of 5 MHz (the polished quartz crystals were provided by the QCM Stanford company with gold electrodes). The measurements were performed using a QCM 200 Quartz Crystal Microbalance (Stanford). The degradation of Hb by CatD in solution was recorded using a Hewlett Packard model HP8454 UV–visible