Activity of lactoperoxidase when adsorbed on protein layers

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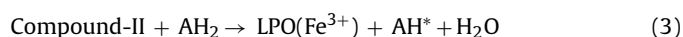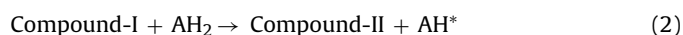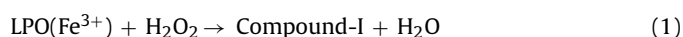
ABSTRACT

Lactoperoxidase (LPO) is an enzyme, which is used as an antimicrobial agent in a number of applications, e.g., food technology. In the majority of applications LPO is added to a homogeneous product phase or immobilised on product surface. In the latter case, however, the measurements of LPO activity are seldom reported. In this paper we have assessed LPO enzymatic activity on bare and protein modified gold surfaces by means of electrochemistry. It was found that LPO rapidly adsorbs to bare gold surfaces resulting in an amount of LPO adsorbed of 2.9 mg/m^2. A lower amount of adsorbed LPO is obtained if the gold surface is exposed to bovine serum albumin, bovine or human mucin prior to LPO adsorption. The enzymatic activity of the adsorbed enzyme is in general preserved at the experimental conditions and varies only moderately when comparing bare gold and gold surface pretreated with the selected proteins. The measurement of LPO specific activity, however, indicate that it is about 1.5 times higher if LPO is adsorbed on gold surfaces containing a small amount of preadsorbed mucin in comparison to the LPO directly adsorbed on bare gold.

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1. Introduction

Lactoperoxidase (LPO) is an enzyme, which has been recognised as one of the innate antimicrobial proteins in addition to lectins, lysozyme, lactoferrin and secretory peroxidases. LPO from bovine milk is a medium size heme-containing protein comprising a single polypeptide chain of 612 amino acids. The molecular mass of the enzyme ranges from 74.5 to 79.2 kDa depending on the degree of glycosylation, which might span from 6.4 to 11.5% of the total mass [1]. At neutral pH LPO is positively charged due to a p*I* ranging from 8.5 to 9.3. The catalytic reaction of LPO can be described by conventional peroxidase reaction Eqs. (1–3), where Compound-I and Compound-II are two- and one-electron oxidised forms of LPO, respectively. AH* represents a one-electron oxidised reducing agent. The catalytic mechanism of the enzyme is, however, much more complicated. LPO shows, e.g., catalase activity [2]. One of the not well-understood phenomena is the relatively low stability of

LPO already at low (μM) concentrations of peroxide, whereas the enzyme has been found to be structurally stable at room temperatures [3].

$$LPO(Fe^{3+}) + H_2O_2 \rightarrow \text{Compound-I} + H_2O \qquad (1)$$

$$\text{Compound-I} + AH_2 \rightarrow \text{Compound-II} + AH^* \qquad (2)$$

$$\text{Compound-II} + AH_2 \rightarrow LPO(Fe^{3+}) + AH^* + H_2O \qquad (3)$$

Despite the relatively low stability of LPO, the enzyme is of high interest for its involvement in the mammalian defence system. The basic antimicrobial principle of action is due to LPO assisted generation of halogens (e.g., I$_2$) and pseudohalogens (e.g., (SCN)$_2$), which react with and inactivate the proteins of bacterial cells. The bacteriostatic or bactericidal effects of LPO-H$_2$O$_2$-SCN$^-$ and LPO-H$_2$O$_2$-I$^-$, respectively, have been demonstrated [4]. Following the current understanding of how LPO acts as antimicrobial agent a number of LPO applications have been proposed in biotechnology, food technology and biomedical technology (e.g., oral hygiene products and salivary substitutes) [5,6]. The majority of the applications rely on the addition of LPO to a homogeneous product phase or immobilisation of LPO on the surface, e.g., of a packaging material [7] or dental (titanium) implants [8]. The development of new methods on how LPO could be introduced into different products is continuously evaluated; one of the recently demonstrated examples describes a possibility of a multilayer assembly of LPO

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in combination with other proteins using layer-by-layer deposition [9]. In this and other applications, where LPO is adsorbed on a surface, the measurements of the enzymatic activity would be of the high interest. Within the present article we describe electrochemical LPO-activity measurements on gold surfaces. The ability of the enzyme to oxidise phenolic compounds, specifically catechol, is exploited for the enzyme activity measurements. The activity of LPO adsorbed on gold is compared to LPO co-adsorbed with other proteins.

2. Experimental

2.1. Chemicals

Na_2HPO_4, NaH_2PO_4, $CaCl_2$, $NaCl$, H_2O_2, H_2SO_4, $K_4[Fe(CN)_6]$, 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), catechol, hydroquinone, phenol, *p*-cresol, and hexaamineruthenium(II) chloride of analytical or higher grade were obtained from Sigma–Aldrich Sweden AB (Stockholm, Sweden). Deionized water (18 MΩ) used for preparation of all solutions was purified with a PURELAB™ UHQ system (ELGA LabWater, UK).

2.2. Proteins

Bovine lactoperoxidase (LPO) (L8257, from bovine milk), bovine mucin (BSM) (M3895, from bovine submaxillary glands, Type I-S), and bovine albumin (BSA) (A8531, from bovine serum) were purchased from Sigma–Aldrich. Human salivary high molecular weight mucin (MUC5B) was purified from collected saliva according to Wickstrom et al. [10] and dialyzed using a Spectra/Por® Dialysis Membrane, MWCO: 6–8000 from Spectrum Europe (Breda, The Netherlands) according to Lindh et al. [11]. All protein solutions were prepared freshly before each experiment.

The specific activity of lactoperoxidase in homogeneous solutions towards different substrates was determined spectrophotometrically in 10 mM phosphate buffer, pH 7.0 containing 100 mM NaCl using a Ultrospec II Biochrom "LKB" (Bromma, Sweden) spectrophotometer. The absorbance change at different wavelengths was measured at 25 °C for 60 s, *viz.* ABTS ($\lambda = 420$ nm, $\varepsilon = 36000$ M^{-1} cm^{-1}) and $K_4[Fe(CN)_6]$ ($\lambda = 420$ nm, $\varepsilon = 1040$ M^{-1} cm^{-1}). One unit of activity is defined as the amount of lactoperoxidase oxidising 1 μmol of substrate per min at room temperature (approximately 21 °C). Specific activities are expressed as units of activity per mg of protein.

2.3. Gold surfaces

The gold substrates were manufactured in a Balzers UMS 500 P system by electron-beam deposition of 2000 Å of gold onto silicon (1 0 0) wafers, precoated with a 25-Å-thick titanium adhesion layer (Laboratory of Applied Physics, Linköping University, Sweden). Prior to each experiment the gold surface was cleaned electrochemically in 0.5 M H_2SO_4 by means of cyclic voltammetry.

2.4. Ellipsometry measurements

The adsorption of proteins on to gold surfaces was studied with *in situ* ellipsometry using a Rudolph thin film automated ellipsometer (type 43063-200E, Rudolph Research, Fairfield, NJ, USA) equipped with a xenon lamp. With a fixed angle of incidence (67.8°) the light was detected at 442.9 nm employing an interference filter with ultraviolet and infrared blocking (Melles Griot, Netherlands). The gold surface was vertically mounted into a glass trapezoid cuvette (Hellma, Germany) containing 5 mL of solution, which was

thermostated at 25 °C and stirred using magnetic stirrer with rotation speed of 325 rotations per minute. In order to determine the refractive index of the surface, prior to each measurement, a two-zone surface characterization in buffer solution was carried out. After a stable baseline was acquired, protein stock solutions were added to the cuvette containing 10 mM phosphate buffer with 100 mM NaCl and 1 mM $CaCl_2$, pH 7.0 and formation of the adsorbed protein film was monitored. In the case of LPO the adsorption was carried out for 60 min from a 10 μg/mL enzyme solution, which corresponds to the concentration presents in human saliva [4]. Combined protein layers with lactoperoxidase were realised by 60 min preadsorption from solutions containing 50 μg/mL of BSM, BSA or MUC5B, respectively. In addition, a lower concentration of MUC5B (0.5 μg/mL) was also investigated. Each protein adsorption was followed by 5 min cuvette rinsing with phosphate buffer at a continuous flow of 18 mL/min and monitoring for 15 min before the LPO addition. After LPO adsorption the cuvette was rinsed for 5 min and monitored for 30 min. From ellipsometric data the thickness and the adsorbed amount was calculated using the value of 0.18 mL/g [12] as a refractive index increment (dn/dc) with protein concentration. All ellipsometry experiments were performed at least in duplicate.

2.5. Electrochemical studies

2.5.1. Cyclic voltammetry

Electrochemical cleaning of the gold surfaces was carried out in 0.5 M H_2SO_4 by means of cyclic voltammetry, i.e., by sweeping the applied potential between 0 and 1.9 V *vs.* NHE with scan rate of 100 mV/s for 30 min. A three-electrode potentiostat (AMEL Instruments, Model 2059, Milano, Italy) with a SCE as a reference and platinum as a auxiliary electrode was used. An electroactive area of gold electrodes was determined electrochemically *via* analysis of the current peak that was due to the electrochemical reduction of surface bound gold oxide. The method is based on the assumption that a monolayer of oxygen is chemisorbed onto the gold film in a relation of 1:1 with the surface gold atoms. Thus integration of the area of the reduction peak from a current–potential plot provided the charge passed during the gold oxide reduction. The value was related to the theoretical value (390 ± 10 μC) required for the coverage of 1 cm^2 of polycrystalline gold with oxide film [13].

2.5.2. Amperometry

In all experiments, a one-compartment electrochemical cell (volume of 10 mL) was used. The equilibrium current values were registered under aerobic conditions at room temperature. A two-electrode system with the enzyme-modified electrode as a working electrode and a silver wire as a combined reference and counter electrode was connected to a potentiostat (ZPta Elektronik, Höör, Sweden). The current responses were recorded on a strip chart recorder (Kipp & Zonen, Delft, The Netherlands). The applied potential was −50 mV in the supporting electrolyte: 10 mM phosphate buffer, pH 7.0 with 100 mM NaCl and 1 mM $CaCl_2$. The error bars presented on figures correspond to the highest and the lowest measured values, whereas each data point represents the average of three independent measurements.

3. Results and discussion

### 3.1. Adsorption of lactoperoxidase on bare gold surfaces

Previous investigations on lactoperoxidase adsorption on hydrophilic and hydrophobic silica have shown very high LPO surface affinity and formation of a monolayer at low (0.5 μg/mL) enzyme concentrations [14]. Higher adsorbed masses, up to
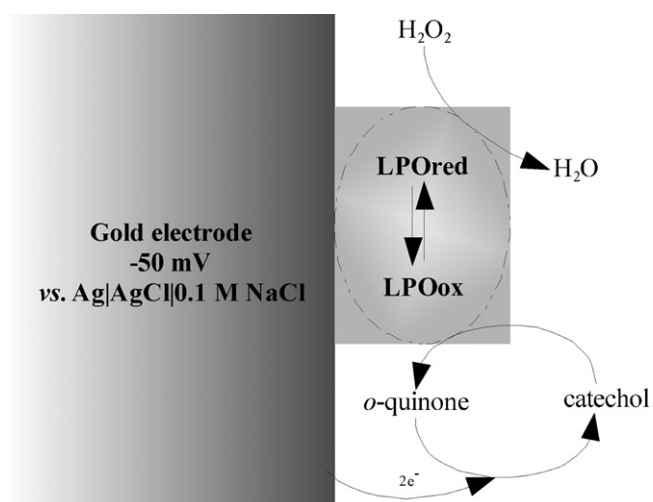
Fig. 1. Schematic representation of electroreduction of LPO-produced *o*-quinone at a gold electrode.

3.2 mg/m^2 of LPO depending on enzyme concentration in solution, were obtained on hydrophilic surfaces [15]. One problem with silica surfaces is that it is difficult to measure the activity of the adsorbed enzyme, which is of obvious importance for the development of antimicrobial surfaces. In this work LPO adsorption was studied on gold substrates which due to the electrical conductivity provide possibilities to measure the activity of surface bound LPO by electrochemical means, as illustrated by Fig. 1 (described in Section 3.3), based on our earlier electrochemical studies of peroxidases [16,17].

LPO is an asymmetric, oval molecule of dimensions 55 Å × 81 Å × 78 Å and a partial specific volume of 0.721 mL/g [14]. Keeping in mind these geometric characteristics of the enzyme Mårtensson et al. [14] have proposed two models for molecular organization of LPO on hydrophilic and hydrophobic silicon dioxide surfaces, i.e., "side-on" and "end-on" orientation. An average thickness of 33 Å of a lactoperoxidase layer on gold was estimated from our ellipsometric measurements, Fig. 2. The final value of the adsorbed amount of 2.9 mg/m^2 corresponds to 4466 Å^2 per LPO molecule, which is practically the same as the area occupied by the "end-on" adsorbed enzyme molecule, 55 Å × 81 Å = 4455 Å^2. Data in Fig. 2 show that adsorption of LPO on gold is a rapid process and is finished within 10–15 min. The apparent lack of adsorption reversibility and very small desorption of the protein from the surface can also be observed, Fig. 2.
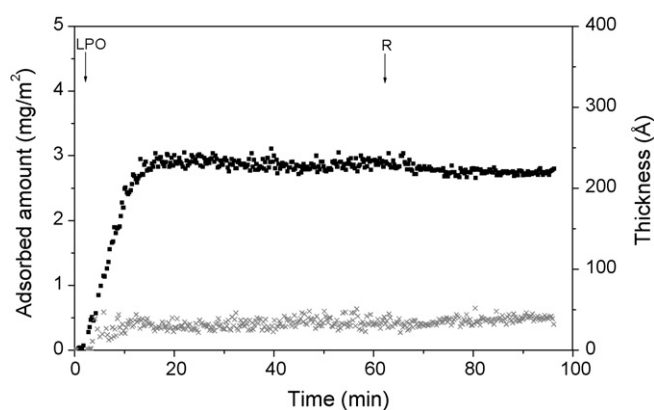
Fig. 2. Adsorbed amount (■) and thickness (×) versus time of adsorption of LPO on a gold electrode surface. Arrows indicate protein addition or rinsing with phosphate buffer (R).
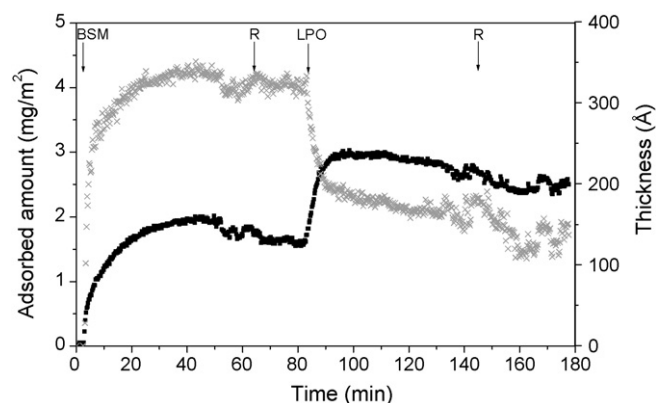
Fig. 3. Adsorbed amount (■) and thickness (×) versus time of adsorption for sequential adsorption of BSM and LPO on a gold electrode surface. Arrows indicate protein addition or rinsing with phosphate buffer (R).

### 3.2. LPO adsorption on gold surface with other pre-adsorbed proteins

The gold surface was modified with other proteins (BSM, BSA or MUC5B) and the subsequent adsorption of lactoperoxidase was studied. The most important question was to understand if the BSM-LPO, BSA-LPO, or MUC5B-LPO layers will show substantial differences in the specific activity or stability of the peroxidase as compared to LPO adsorbed directly on gold.

3.2.1. LPO adsorption on Au-BSM surface

Bovine mucin from the submaxillary glands exhibits structural properties similar to some salivary mucins, e.g., MUC7 (MG2). It is known that from synergistic interactions BSM can form complexes with other proteins [18,19]. Castillo et al. [20] have proposed the three-layer model for the *in vitro* bovine mucin adsorption on the surface of a contact lens with the top layer consisting of BSM gel layers capable of desorption. The proposed model confirmed previous *in situ* studies on the adsorption of BSM at solid/liquid interfaces showing the presence of a loosely bound mucin layer in addition to irreversibly bound BSM [21]. In general, a surface bound BSM layer is considered to be much softer than a rigid layer of surface bound BSA [19].

In the present investigation it has been observed, that after BSM adsorption on gold, the amount as well as the thickness of adsorbed bovine mucin decreased slightly with time (Fig. 3.), achieving an approximate surface concentration of 1.6 mg/m^2 after rinsing with phosphate buffer. The decrease of initially adsorbed amount of BSM probably indicates that the thick layer of BSM is composed of both strongly and weakly bound glycoprotein molecules where the latter are removed upon protein rearrangement at the surface. Subsequent addition of lactoperoxidase to the mucin layer resulted in an increase in the total adsorbed amount on the gold surface. However, LPO adsorption simultaneously leads to a reduction of the thickness of the film. A noticeable compaction of the protein bi-layer may be expected by the incorporation of LPO molecules into the mucin layer. Similar compaction behaviour of a surface bound BSM layer was previously observed upon subsequent addition of BSA [19] or chitosan [22]. The adsorption conditions might be of high importance in determining the interactions between biopolymers at surfaces since a swelling of an adsorbed BSM layer upon subsequent adsorption of chitosan has been also reported [23,24]. The structural changes of the BSM layer upon LPO adsorption might be a consequence of electrostatic interactions between the oppositely charged bovine mucin and the bovine LPO.
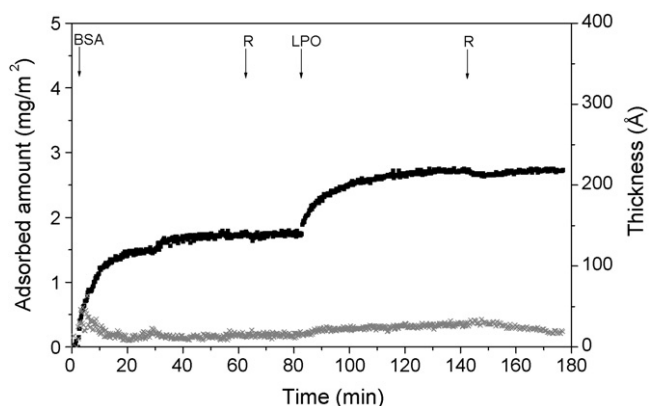
Fig. 4. Adsorbed amount (■) and thickness (×) for sequential adsorption of BSA and LPO on a gold electrode surface. Arrows indicate protein addition or rinsing with phosphate buffer (R).

### 3.2.2. LPO adsorption on Au-BSA surface

Bovine serum albumin was used as a control as it is used in surface preparations or as a "blocking agent" in ELISA methods or biosensor applications. Earlier studies of the adsorption of BSA on gold and stainless steel have shown that albumin adsorbs strongly on metals [25]. The adsorption profile of BSA on the gold surface and sequential LPO adsorption is presented in Fig. 4. A thin albumin layer remained on the surface despite buffer rinsing and an increase of adsorbed amount and of the film thickness was observed after LPO adsorption. A substantial difference between LPO adsorption on Au-BSA (Fig. 4) can be seen compared to LPO adsorption on Au-BSM surfaces (Fig. 3).

3.2.3. LPO adsorption on Au-MUC5B surface

Iontcheva et al. [26] have shown that human salivary mucin MG1 (MUC5B) selectively forms hetero-complexes with amylase, proline-rich proteins, statherin, and histatins. Further molecular mapping of statherin- and histatin-binding domains in human salivary mucin MG1 by the yeast two-hybrid system has been studied [27]. Wickstrom et al. [28] demonstrated macromolecular organization of saliva and identification of "insoluble" MUC5B assemblies and non-mucin proteins in the gel phase. It has earlier been reported that MUC5B and lactoperoxidase can be used to build multilayers on hydrophobic and hydrophilic silica surfaces [9]. In our studies human salivary mucin (MUC5B) adsorption on a gold surface has been investigated (Fig. 5). Two different mucin concentrations in the reaction solution: $50\,\mu g/mL$ and $0.5\,\mu g/mL$ have been tested. For the higher MUC5B concentration, amounts adsorbed of over $3\,mg/m^2$ have been obtained, which can be compared to $2.5\,mg/m^2$ previously observed at hydrophobized silica [11]. However, a small decrease in this value and in layer thickness can be seen after buffer rinsing. Sequential addition of LPO did not significantly change the ellipsometric parameters of the film indicating no or minor LPO adsorption. Therefore, adsorption from a 100 times lower concentration of MUC5B solution was used to modify the gold surface. A much lower MUC5B adsorption was observed, which was followed by the highest adsorbed amount of LPO recorded in the present study, in fact the amount of LPO adsorbed is close to the value recorded for pure gold which might be expected due to the small amounts of MUC5B adsorbed.

### 3.3. Activity measurements of immobilised lactoperoxidase

The enzymatic activity of surface bound LPO was evaluated from measurements of the current generated by electrochemical reduction of the enzymatically oxidised electron donor as
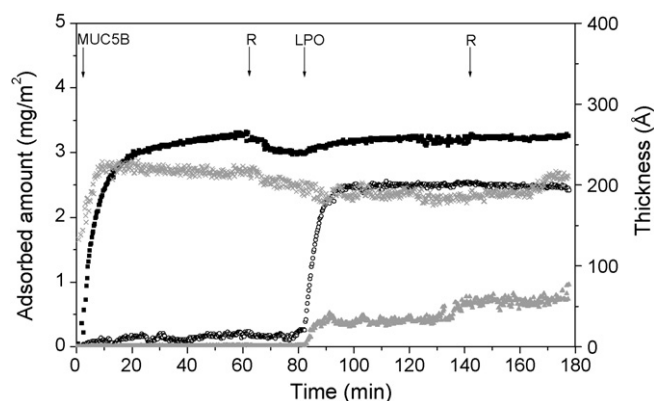
Fig. 5. Comparison of the adsorption behaviour upon sequential adsorption of human mucin (MUC5B) and LPO on gold surfaces at two (■, ×: $50\,\mu g/mL$ and ○, ▲: $0.5\,\mu g/mL$) concentrations of MUC5B (adsorbed amount (■, ○) and thickness (×, ▲) versus time). Arrows indicate protein addition or rinsing with phosphate buffer (R).

shown in Fig. 1. The feasibility of current measurements has been tested with electron donors (called electron transfer mediators) such as ABTS, $K_4[Fe(CN)_6]$, hydroquinone, phenol, p-cresol, hexaamineruthenium(II) chloride, and catechol. The highest current response has been achieved with catechol. Therefore the activity of LPO adsorbed on various surfaces was monitored electrochemically using catechol. From Fig. 6 significant differences in current responses (presented as current densities) can be observed at different electrodes. The current response varied between 0.2 and $5.5\,\mu A/cm^2$, being the highest from LPO adsorbed on gold, modified with a low amount of MUC5B. The current response is proportional to the activity of the enzyme and to the amount of the adsorbed enzyme (Table 1). Since the amount of the enzyme can be estimated from ellipsometric measurements, the specific activity of LPO on different surfaces was calculated.

The fact, that the current densities (Fig. 6) are about one order of magnitude below what could be expected from diffusion-controlled currents, allows us to consider the responses as being kinetically limited. Thus, electrochemical current measurements can be used to estimate the specific activity of adsorbed LPO accord-
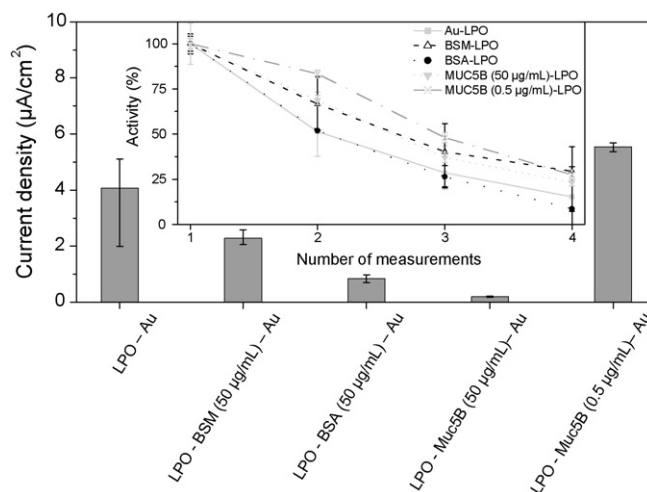
Fig. 6. Current density of LPO adsorbed on bare gold and gold with preadsorbed proteins immediately after ellipsometric measurements; 10 mM phosphate buffer, pH 7.0, 100 mM NaCl, 1 mM $CaCl_2$, 0.1 mM catechol, applied potential: $-50\,mV$ vs. Ag|AgCl|0.1 M NaCl. Insert: operation stability of LPO adsorbed on bare gold and on protein-modified gold electrodes for four subsequent activity measurements.

Table 1
Adsorbed amounts of LPO on bare gold and on gold with preadsorbed protein layers after buffer rinsing

Surface	Concentration of the surface modifier in the stock solution (μg/mL)	Adsorbed amount of LPO (mg/m^2)
Au	0	2.93 ± 0.24
Au-BSM	50.0	1.01 ± 0.17
Au-BSA	50.0	0.74 ± 0.24
Au-MUC5B	50.0	0.22 ± 0.15
Au-MUC5B	0.5	2.41 ± 0.22

Note: LPO concentration in the stock solution used for surface modification was 10.0 μg/mL.

ing to a simple equation (Eq. (4)):

$$\text{LPO activity (U/mg)} = \frac{\text{Current density}\,(\mu A/cm^2) \times 60\,s}{\text{Amount of LPO}\,(mg/cm^2) \times n \times F} \quad (4)$$

where $n = 2$ is a number of electrons by which catechol is enzymatically oxidised, F is the Faraday constant. In Fig. 7 the estimates for the specific activities of LPO are shown for all surfaces studied. It can be seen that the specific activity varied from 2.7 up to 7.9 U/mg. The simple conclusion that can be made is that the specific activity of the enzyme is relatively well preserved on all surfaces, i.e., varies less than a factor of three. The highest estimated specific LPO activity was found on gold surfaces pretreated with BSM or low amounts of MUC5B. It is important to note that at these surfaces the specific activity is higher than that found for LPO adsorbed on bare surfaces. The specific activities estimated on gold electrodes pretreated with BSA and high concentrations of MUC5B were the lowest. It is not straightforward to interpret these results. One should keep in mind that the amounts of adsorbed LPO used in the estimation of the specific activity are subject to some uncertainty. First, we might expect that some of the initially adsorbed protein, i.e., BSA, BSM or MUC5B, could be exchanged by LPO. Not accounting for this would lead to an overestimation of the specific enzymatic activity rather than an underestimation. Second, the amounts were based on assumption of the same dn/dc for all proteins. For example, the dn/dc for mucin has been reported to 0.16 mL/g [9], resulting in a possible underestimation of the amount of mucin of about 10% but provided that no exchange takes place this does not affect the activity data. In the case of MUC5B adsorption at the low concentration (0.5 μg/mL),
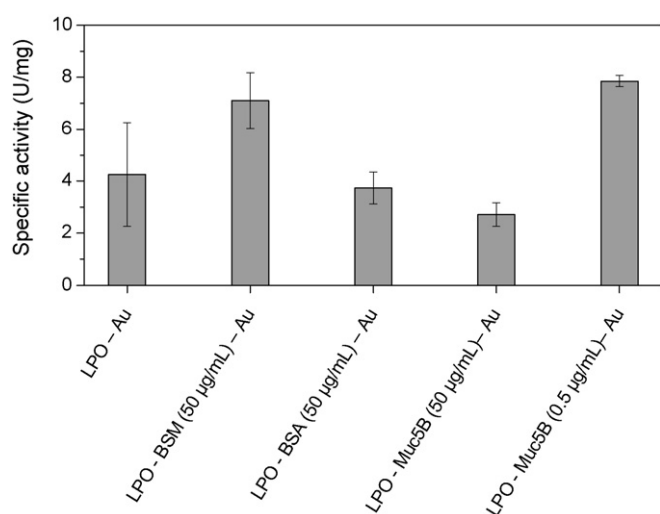
however, the small amount of adsorbed (exchangeable) MUC5B will make the possible error small.

In summary, the specific activities of bovine LPO on bare Au and protein modified Au surfaces are comparable (Fig. 7). The stability of adsorbed LPO was found to be almost independent of surface modification (Fig. 6, insert) and relatively poor as such. On average about 25% of the activity is lost after each electrochemical enzyme activity assay.

4. Conclusions

In this paper we demonstrate that LPO adsorbs on bare gold and gold surfaces pre-treated with other proteins such as BSA, BSM and MUC5B. The amount of adsorbed LPO on gold is close to a monolayer. LPO adsorption on a BSM layer imposes substantial compaction of the protein bi-layer indicating strong (possibly electrostatic) interactions between LPO and bovine BSM or substantial exchange of BSM by LPO.

Specific activities of LPO on different surfaces were determined using hydrogen peroxide as oxidant and catechol as electron donor. On all surfaces studied the specific activity was found to be in the range of 2.7–7.9 U/mg. The LPO activity was about 1.5 times higher on gold surfaces carrying a small amount of preadsorbed human mucin (MUC5B) in comparison to LPO directly adsorbed on bare gold. The observed activity variations are too low to prove any beneficial interaction between LPO and studied salivary proteins in terms of lactoperoxidase activity or stability.

Acknowledgements

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Fig. 7. Specific activity of LPO adsorbed on bare gold and on protein-modified gold electrodes.

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