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Letters to *Analytical Chemistry*

Finding Out Egyptian Gods' Secret Using Analytical Chemistry: Biomedical Properties of Egyptian Black Makeup Revealed by Amperometry at Single Cells

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Lead-based compounds were used during antiquity as both pigments and medicines in the formulation of makeup materials. Chemical analysis of cosmetics samples found in Egyptians tombs and the reconstitution of ancient recipes as reported by Greco-Roman authors have shown that two non-natural lead chlorides (laurionite Pb(OH)Cl and phosgenite $Pb_2Cl_2CO_3$) were purposely synthesized and were used as fine powders in makeup and eye lotions. According to ancient Egyptian manuscripts, these were essential remedies for treating eye illness and skin ailments. This conclusion seems amazing because today we focus only on the well-recognized toxicity of lead salts. Here, using ultramicroelectrodes, we obtain new insights into the biochemical interactions between lead(II) ions and cells, which support the ancient medical use of sparingly soluble lead compounds. Submicromolar concentrations of Pb^{2+} ions are shown to be sufficient for eliciting specific oxidative stress responses of keratinocytes. These consist essentially of an overproduction of nitrogen monoxide (NO°). Owing to the biological role of NO° in stimulating nonspecific immunological defenses, one may argue that these lead compounds were deliberately manufactured and used in ancient Egyptian formulations to prevent and treat eye illnesses by promoting the action of immune cells.

Figure 1. Water-trough carrying girl dressed with the black makeup (Polychromatic wood, ~2000 B.C. Reprinted with permission from the Louvre Museum B.C. collections, C2RMF, D. Bagault). Among many others, this representation testifies that the use of the black makeup was not restricted to the highest Egyptian classes.

In ancient Egypt, many written texts, paintings, statues, and toilet accessories support the fact that green and black makeup were extensively used for their aesthetic, religious, and therapeutic properties.[1] These cosmetics played an important role in everyday practices (Figure 1) as well as during ritual and burial ceremonies. Indeed, besides the purely cosmetic functions that we may fully appreciate today, it is enough to think about Queen Nefertiti's stupendous gaze, ancient Egyptians also associated a magic role with these cosmetics according to which their bearers would be directly protected by Horus and Ra against several illnesses.

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Analysis by scanning electron microscopy and quantitative X-ray diffraction of 52 samples taken from makeup containers preserved in the Louvre museum showed that their formulation involved a restricted range of lead-based chemicals.[2] They were mostly prepared by mixing four lead(II) species: galena (PbS) for dark tones and gloss and three white materials: cerussite ($PbCO_3$), phosgenite ($Pb_2Cl_2CO_3$), and laurionite (Pb(OH)Cl).

The presence of two lead chlorides in many Egyptian makeup formulations was unexpected because of their non-natural occurrence in and around Egypt. Ancient texts from Roman authors of the first century A.D., including Pliny the Elder[3a] and Dioscorides,[3b] indicate that that these compounds were intentionally synthesized for their medical properties. For example, Dioscorides (5, 102) mentioned that they "appear to be good medicine to be put in eyes, and for foul scars, and for faces wrinkled and full of spots". Dioscorides also provided detailed descriptions of the large-scale synthesis of these compounds as was required to supply the needs of a large fraction of the population. This was rooted in simple but extremely delicate wet chemistry: lead oxide powders (PbO, litharge) were stirred energetically with rock salt (NaCl), sometimes together with natron (Na_2CO_3 and $NaHCO_3$), in warm water (eqs 1 and 2):

$$\mathrm{PbO + H_2O + NaCl \rightarrow Pb(OH)Cl + NaOH} \qquad (1)$$

$$\mathrm{PbO + H_2O + NaCl + 1/2\ Na_2CO_3 \rightarrow 1/2\ Pb_2Cl_2CO_3 + 2\ NaOH} \qquad (2)$$

This solid−liquid process was delicate because the pH had to be maintained within the neutral range to avoid the formation of undesired lead hydroxides (Figure 2). However, from the stoichiometry of the reactions, it is clear that the pH should spontaneously increase as the reaction proceeds (see blue arrow in Figure 2). Hence, the supernatant had to be frequently removed as soon as its causticity increased and fresh water and rock salt added. After a few weeks of reaction, litharge was entirely consumed and replaced by a white precipitate. During the exact reconstitution of the ancient protocol at the laboratory scale, the precipitation of the two chlorinated products found in Egyptian cosmetics was indeed observed.[2]

The continuity of practices for synthesizing and using lead chlorides for ophthalmologic and skin care traces back to the most ancient Egyptian medical reports, as attested by the Ebers papyrus, dated to the 16th century B.C.[3c] The important documentation left by Greco-Roman authors testifies to the probable handing down to the Roman period of this expertise in chemistry and ophthalmology originally developed in Egypt,[4] a country renowned in Mediterranean antiquity for its treatment of the eyes.[5] In particular, during the flooding of the Nile, inhabitants of Egypt suffered from numerous eye diseases and inflammations such as bacterial conjunctivitis. Recipes to prevent or cure such bacterial inflammations reported in the medical papyri fully document this. For example, the Ebers papyrus details recipes for eye drops, plaster dressings, and cosmetics for the eyes and eyelids that were to be prescribed for the treatment of a number of ailments.[3c] The use of lead chlorides and their manufacture have thus been of unambiguous interest to physicians for over 36 centuries!

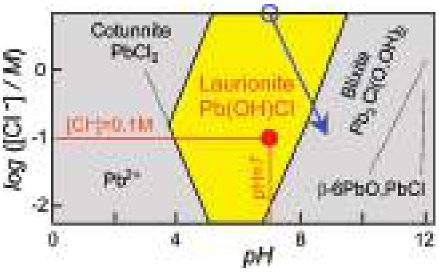

Figure 2. Pourbaix diagram of lead(II) chlorides. The stability range of laurionite is shown in yellow. This diagram evidences that for accessing laurionite as a stable phase, the pH had to be maintained below 7.5−8. The red circle in the diagram corresponds to the conditions prevailing in lachrymal fluid ([Cl$^-$] ≈ 0.1 M, pH ≈ 7). See the Supporting Information for the construction of this Pourbaix diagram.

Today the well established toxicity of lead compounds (e.g., saturnism) has completely overshadowed their potential benefits for health. Lead poisoning symptoms from lead based pigments (mainly lead white) have been described since the modern period.[6] This became a severe issue during the 20th century

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(8) For the artificial synapse method applied to oxidative stress, see. (a) Arbault, S.; Pantano, P.; Jankowski, J. A.; Vuillaume, M.; Amatore, C. *Anal. Chem.* **1995**, *67*, 3382–3390. (b) Amatore, C.; Arbault, S.; Bruce, D.; De Oliveira, P.; Erard, M.; Sojic, N.; Vuillaume, M. *Analusis Eur. J. Anal. Chem.* **2000**, *28*, 506–517. (c) Amatore, C.; Arbault, S.; Bruce, D.; De Oliveira, P.; Erard, M.; Vuillaume, M. *Faraday Discuss.* **2000**, *116*, 319–303. (d) Amatore, C.; Arbault, S.; Bruce, D.; De Oliveira, P.; Erard, M.; Vuillaume, M. *Chem.−Eur. J.* **2001**, *7*, 4171–4179. (e) Arbault, S.; Sojic, N.; Bruce, D.; Amatore, C.; Sarasin, A.; Vuillaume, M. *Carcinogenesis* **2004**, *25*, 509–515. (f) Amatore, C.; Arbault, S.; Bouret, Y.; Cauli, B.; Guille, M.; Rancillac, A.; Rossier, J. *ChemPhysChem* **2006**, *7*, 181–187. (g) Amatore, C.; Arbault, S.; Bouton, C.; Coffi, K.; Drapier, J.-C.; Ghandour, H.; Tong, Y. *ChemBioChem.* **2006**, *7*, 653–661. (h) Rancillac, A.; Guille, M.; Tong, X.-K.; Geoffroy, H.; Hamel, E.; Amatore, C.; Arbault, S.; Rossier, J.; Cauli, B. *J. Neurosci.* **2006**, *26*, 6997–7006. (i) Amatore, C.; Arbault, S.; Chen, Y.; Crozatier, C.; Tapsoba, I. *Lab Chip* **2007**, *7*, 233–238. (j) Verchier, Y.; Lardy, B.; Nguyen, M. V. C.; Morel, F.; Arbault, S.; Amatore, C. *Biochem. Biophys. Res. Commun.* **2007**, *361*, 493–498. (k) Amatore, C.; Arbault, S.; Bouton, C.; Drapier, J. C.; Ghandour, H.; Koh, A. C. W. *ChemBioChem* **2008**, *9*, 1472–1480. (l) Amatore, C.; Arbault, S.; Erard, M. *Anal. Chem.* **2008**, *80*, 9635–9641. (m) Ferreira, D. C. M.; Tapsoba, I.; Arbault, S.; Bouret, Y.; Alexandre Moreira, M. S.; Ventura Pinto, A.; Goulart, M. O. F.; Amatore, C. *ChemBioChem* **2009**, *10*, 528–538.

because of the massive use of lead in industry (paints, plumbing, etc.). In fact, lead ions target many biomolecules that are apt to interact with the most common divalent cations (calcium, zinc, iron, copper, magnesium),[6] thus challenging the specific functionalities of ion channels, enzymes, and proteins. Because lead ions were seldom encountered during natural selection due to the fact that most lead compounds are fully insoluble, finely tuned biological mechanisms may not have evolved to protect cells against lead ions in the same way, for example, as for the selectivity of K^+ vs Na^+ or between the common divalent cations. In the absence of specific selection mechanisms, (bio)chemical confusion is expected between Pb^{2+} and Ca^{2+}; they have identical charges, similar atomic radii, and identical coordination numbers with water molecules (6, 8, or 10).

Since micromolar variations of Ca^{2+} elicit several biological functions including the activation of NADPH-oxidases and NO-synthases, two major machineries of the nonspecific immunological system in aerobic organisms, one may suppose that similar levels of Pb^{2+} concentrations may play an important role in the onset of natural immunologic defenses, thus explaining the use of partially soluble lead compounds by ancient physicians for the prevention of bacterial infections. In fact, naturally occurring lead salts are very insoluble under physiological conditions (solubility constants: $K_s = 7.40 \times 10^{-14}$ for $PbCO_3$; $K_s = 3 \times 10^{-28}$ for PbS). Conversely, laurionite exhibits some solubility, although it is low and dependent on both pH and chloride concentration. The Pourbaix diagram of Pb(OH)Cl (Figure 2) predicts a maximum concentration of Pb^{2+} ions of about 10^{-4} M at equilibrium in the lachrymal fluid (pH $\approx$ 7, $[Cl^-] \approx 0.1$ M). This has to be considered as an uppermost value certainly not achieved in the lachrymal fluid of a makeup bearer. Indeed, on the one hand the makeup was lined up on the eyelid junction with the eye surface and thus offered a minimal exchange surface with a constantly renewed liquid film, and on the other hand this value does not account for the possible interference with dissolved CO_2. Hence, it is expected that the maximal concentration of Pb^{2+} in the lachrymal fluid of a makeup bearer was at most in the micromolar range. Thus, if Pb^{2+} had any protective influence on the eyes of ancient Egyptians it must have done so at concentrations in this range or below. Yet, considering the remark above about calcium ability to elicit important changes in cellular activity following micromolar changes of its concentration, this does not appear unreasonable at all. We thus undertook an investigation of the effect of submicromolar Pb^{2+} on the oxidative stress response of human keratinocytes (HaCaT cell line). We specifically looked for oxidative stress responses since, to the best of our knowledge, this is the most direct function that may be related to nonspecific protection of the makeup bearer's eye and unprotected soft skin tissues through its direct connection with the nonspecific immunological system involving phagocytes.

To detect possible cellular responses elicited by exposure to submicromolar Pb^{2+} concentrations, we relied on the use of platinized carbon fiber ultramicroelectrodes. These were positioned 5 μm above single cells (Figure 3a) so as to create an artificial synaptic cleft in which any electroactive species released by the cell could be detected with subfemtomole resolution.[7,8] Figure 3b shows that as soon as a submicromolar solution of lead acetate was puffed around a cell, the latter released an oxidative stress burst that lasted for ~30 min. The particular kinetic features of the release varied from cell to cell reflecting individual cellular variability, although on average (15−30 cells per lead concentration tested) the response increased when the Pb^{2+} concentration was increased (compare two representative typical responses for $[Pb^{2+}] = 0.2$ and 0.4 μM in Figure 3b). Importantly, such brief Pb^{2+} stimulations did not introduce any changes beyond these responses in the cellular behavior. This was checked by comparison to controls when the stimulated cells were followed for 3 days after placing them back in the culture medium.

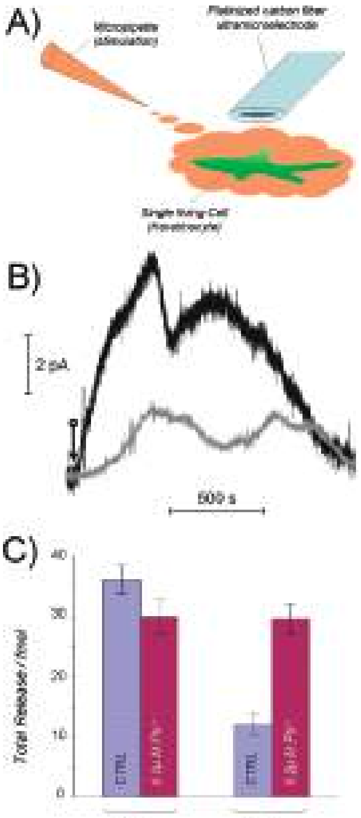

Figure 3. Amperometric detection of the stimulation of oxidative stress responses by Pb^{2+} in single HaCaT cells. (A) Principle of the measurement using the artificial synapse configuration.[7,8] (B) Representative oxidative stress responses following the stimulation (vertical arrow) of a single HaCaT cell by lead acetate 0.2 (gray curve) or 0.4 μM (black curve) as detected by a 10 μm platinized carbon fiber electrode poised at 850 mV vs SSCE; the electrode−cell distance was 5 μm. (C) Average quantities of superoxide ion and nitric oxide released by a single HaCaT cell without (CTRL (blue bars), controls) or following their stimulation by 0.2 μM Pb^{2+} (dark pink bars). Fluxes of superoxide ion and nitric oxide were reconstructed from the responses measured independently for each secondary component (H_2O_2, $ONOO^-$, NO°, and NO_2^-) and application of the stoichiometry of their production.[8,9]

Cell responses such as those shown in Figure 3b characterize the transient behavior that follows their stimulation by Pb^{2+}. These

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(9) For experimental details and relevant data not shown here, see the Supporting Information.

responses were measured at 850 mV vs SSCE, a potential where all species that are components of ordinary oxidative stress responses could be detected, namely, H_2O_2, $ONOO^-$, NO°, and NO_2^-.[8,9] These secondary products arise from the spontaneous evolution of two primary species, superoxide ion and nitric oxide produced by the simultaneous activation of NADPH-oxidases (yielding $O_2^{\circ-}$) and NO-synthases (yielding NO°).[8] To characterize the possible presence and to determine the average individual fluxes of these four secondary species, a statistical averaging of a few tens of cell responses measured at a set of four different potentials (300, 450, 650, and 850 mV vs SSCE)[8k] was undertaken.[9] By application of the stoichiometries of the follow-up reactions yielding each detected subproduct, this allowed the reconstruction of the primary fluxes of superoxide ion and nitric oxide elicited by the Pb^{2+} stimulation (Figure 3c).[8,9] It was observed that Pb^{2+} did not induce any significant change in the normal production of superoxide ion but that it led to an increased (~240% for 0.2 μM Pb^{2+}) and longer lasting production of NO°.[9] Longer incubation times led to a reinforcement of these amperometrically detected responses indicating an increase in the oxidative stress cellular status. This was confirmed by the internal precipitation of formazan when the cells were simultaneously stained with nitroblue tetrazolium.[9,10]

NO° is an important messenger in the nonspecific immune system. It signals infection to immune cells including macrophages, increases the blood flow in capillaries,[8f,h] hence the supply of phagocytes, and promotes their crossing of capillary walls. The present electrochemical data thus establish that the eyes of Egyptians bearing the black makeup were presumably prone to immediately resist a sudden bacterial contamination with extreme efficiency through the spontaneous action of their own immune cells. Indeed, it is well recognized today that in most tropical marshy areas, such as was the Nile area during floods, several bacterial infections are transmitted to humans following any accidental projection of contaminated water drops into one's eye.

These data fully support that Horus' and Ra's protection that ancient Egyptians associated with this makeup and particularly with its laurionite component was real and effective, despite the fact that its "magic" implications seemed a priori totally irreconcilable with our modern scientific views and contrast with our present understanding of the toxicity of lead ions. One cannot evidently go as far as to propose that laurionite was purposely introduced into the composition of the makeup because of any recognized antibacterial properties. Yet, one can presume that ancient Egyptian "chemists" recognized empirically that whenever this "white precipitate" was present in the makeup paste, their bearers were enjoying better health and thus decided to amplify this empirical protective function by specifically manufacturing laurionite. Many examples of such subtle observations and medical conclusions that would have a priori been surprising can be found even in our recent history. It is sufficient, for example, to think about the historical origin of penicillin, aspirin, or quinine... Anyway, whether or not the manufacture of these lead chlorides was deliberately connected to preventive health care by Egyptians, it is clear that such intentional production remains the first known example of a large scale chemical process. It is no wonder that "kemē", the Egyptian word that referred to the Egyptian land and to the black earth of the Nile valley, was handed to us via the Greeks and then the Arabs to eventually coin our present "chemistry".

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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