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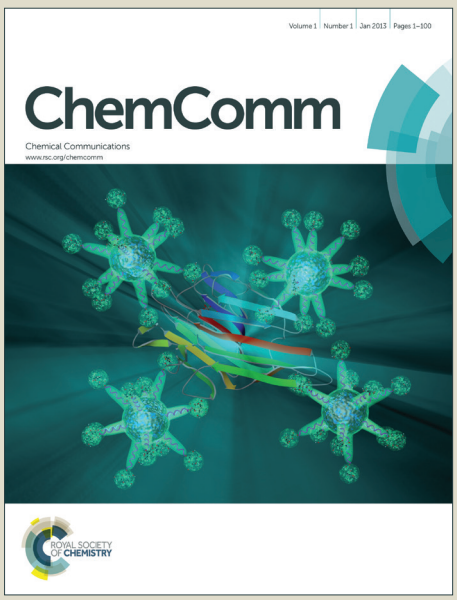

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Electrochromic Polyoxometalate Material as sensor of Bacterial Activity

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L. fermentum, a bacterium of human microbiota, acts as an electron donor to the electrochromic $[P_2Mo^{VI}_{18}O_{62}]^6$. Since, the reductive capacity of *L. fermentum* correlates with its metabolic activity, the reaction with $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ affords a means of evaluating its activity. Following this logic, we have concluded that vancomycin severely affects the activity of *L. fermentum* whereas omeprazole does not.

Polyoxometalates (POMs) have attracted a lot of attention for their applications in diverse fields such as catalysis, biomedicine and material science.[1-10] Their structures can be described as molecular fragments of close-packed metal oxides with the general formula $[X_xM_mO_y]^{n-}$ (where M represents Mo, W, V, etc. and X represents P, Si, As, etc.).[11]

One of the most important properties of POMs, which gives rise to their main applications, is their ability to accept electrons yielding mixed-valence species. This ability means POMs are very good electrochromic materials. A living organism can be the electron-donor to a POM, and this is of paramount interest because in such a case switching of the POM's chromic properties can be directly related to a certain biological activity, i.e., to life itself.

Some microorganisms are known for their ability to reduce metal cations, which has been exploited for the eco-friendly synthesis of zero-valent metal nanoparticles.[12-17] However, this property has not been exploited in other fields beyond the preparation of nanoparticles.

We want to open a new avenue: since the properties of metallic materials depend on the oxidation states of metal cations present in the structure, then the possibility of using living organisms to reduce the metal cations in certain materials offers promising routes to develop new protocols for measuring its biological activity and to be used as a tool to establish an *ex vivo* experimental framework for testing the effect of certain drugs on the metabolism of the microorganism. Detection and determination of bacterial activity are often performed in reference to toxic bacteria. However, the determination of healthy bacteria in some biological fluids and, in particular, the study of how some drugs can affect this healthy function is also of interest.

An approach based on switching the electrochromic properties of a material in order to monitor cell metabolism has been developed for the first time. We have studied how *L. fermentum* is capable of acting as an electron donor towards the electrochromic POM $[P_2Mo^{VI}_{18}O_{62}]^{6-}$. It is well known that this probiotic bacterium has a positive effect on the maintenance of human health since it constitutes part of our natural microbiota, thus playing an important role in promoting immune system activity, defences against infections and anti-inflammatory properties. This protocol can be extended to the observation of other reducing bacteria.

As the reductive ability of *L. fermentum* correlates directly to its bacterial activity (synthesis and secretion of extracellular reducing molecules) the protocol is useful for *ex vivo* screening to measure how specific drugs can affect the bacterium's activity. Thus, the influence of the antibiotic vancomycin and the proton pump inhibitor omeprazole on the activity of this bacterium have been evaluated by simply monitoring the reductive power of *L. fermentum* with respect to $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ while in the presence of these drugs.

The electrochromic POM $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ has been used to assess the activity of the bacteria *L. fermentum*. This POM is reduced by the addition of various specific numbers of electrons, leading to a family of mixed-valence $Mo^{VI}Mo^{V}$ species with a characteristic deep blue colour ("heteropoly blues"), which are easily detected by UV-vis spectroscopy.[18,19]

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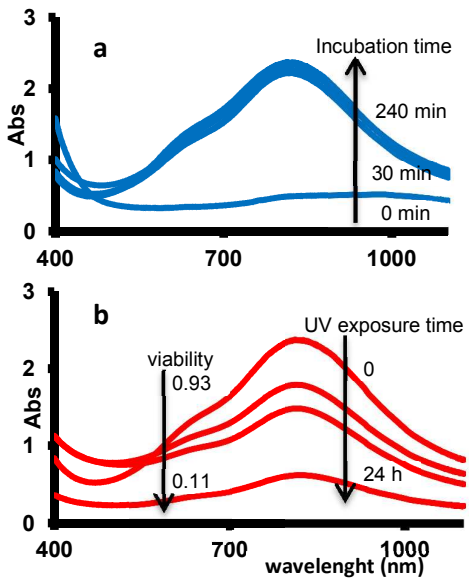

Figure 1. a) UV-vis spectra of supernatant solution obtained after incubation of *L. fermentum* with $[P_2Mo^{VI}_{18}O_{62}]^{6-}$. The band at 800 nm increases with incubation time. b) UV-vis spectra of supernatant solution obtained after incubation of *L. fermentum* previously exposed to UV light with $[P_2Mo^{VI}_{18}O_{62}]^{6-}$. The band at 800 nm decreases with UV exposure time. Likewise, values of viability, measured as the ration between live and dead bacteria, also decrease. The weaker the metabolic strength of bacteria, the smaller is the UV-vis band at 800 nm.

As shown in Figure 1, when incubating $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ in a culture of *L. fermentum*, a large band centred at 800 nm gradually develops, which confirms the POM is reduced by the bacterium. It means that redox capacity of bacteria is at least higher than 0.5 V vs SCE, which is the potential at which $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ is reduced.[20]

Interestingly, the reductive power of *L. fermentum* is at its greatest under optimal bacteria proliferation conditions. A correlation between the bacterium's biological strength and reductive power is evident: the stronger the metabolic strength of the bacteria, the greater is its reducing capacity. Furthermore, when a culture of *L. fermentum* is exposed to UV light its capacity to reduce $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ decreases with time until it practically disappears (Figure 1 b). The damage and death caused by radiation has implications for the reducing capacity of the bacterial colony, measured in terms of viability. Therefore, measurements of *L. fermentum's* capacity to reduce $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ is a direct evaluation of its metabolism strength.

On the other hand, when the reduction of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ was tested in the absence of the bacteria but in the presence of the extracellular supernatant solution generated by the cultivated bacteria, $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ was still reduced. From these observations it can be concluded that $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ is reduced with the aid of extracellular bacterial reducing agents, while the observations also suggest the existence of a chemical site for binding $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ to the bacterium's external surface, as occurs with other metalates. In this sense, Yamase et al,[21] who were the first to observe the reduction of POM by living organisms, concluded that reduction by Staphylococcus aureus of some POMs proceeds within cells, at the cytoplasmic membrane. However, in contrast to this, in *L. fermentum*, the reduction takes place at extracellular level as demonstrated by the fact that the supernatant solution, containing secreted reducing-molecules, is also capable of reducing $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ and corroborated by Transmission Electron Microscopy (TEM) images, which show reduced $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ material both associated and non-associated to the bacteria (Figure 2).

TEM studies of *L. fermentum* after incubation with $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ demonstrated the rod morphology typical of the Lactobacillus genus with large accumulations of nanoparticles on the bacterium's external surface and outside (Figure 2).

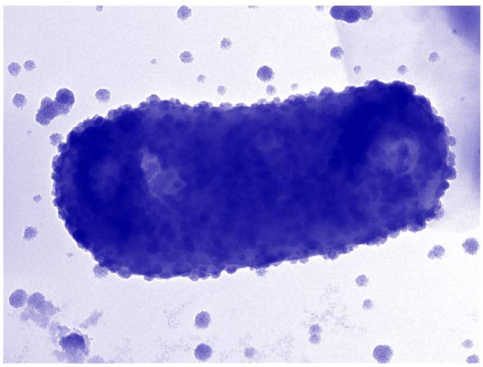

Figure 2. TEM micrograph showing the presence of POM particles on the bacterium's external surface and outside.

The existence of large particles surrounding the bacteria is especially well visualized by High-Angle Annular Dark Field-Scanning Transmission Electron Microscopy (HAADF-STEM). As shown in Figure 3, the bacterium contains bright spherical particles of heterogeneous size distributions, between 20-100 nm. To confirm the presence of reduced POM around the bacteria, energy dispersive X-ray spectroscopy (EDX) experiments were performed. These revealed the juxtaposition of molybdenum (in blue), phosphorous (in green) and the bacterial platform (Figure 3). The particles do not seem confined at a specific baceterial site but asociated to the broad biofilm, which is typic of *L. fermentum*.[22,23]

As the biological strength of *L. fermentum* correlates with its reducing capacity, the methodology we have developed based on monitoring the reduction of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ serves to analyse how specific drugs can affect the activity of *L. fermentum*: the more damage the drug causes to the bacteria, then the bacteria will have less reducing ability. This methodology is therefore useful for screening how some molecules impair *L. fermentum* activity. This is of interest in the context of an improved understanding of the side effects

some drugs have on gut flora, of which *L. fermentum* is one of the main components.

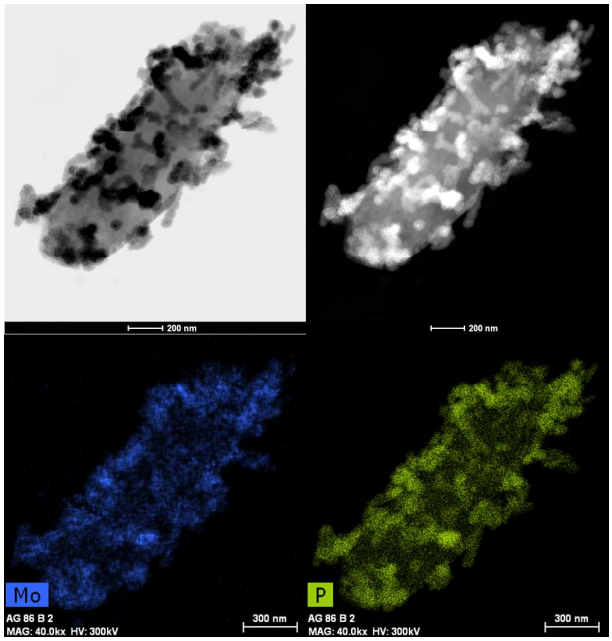

Figure 3. (a) TEM micrograph showing the presence of particles on the bacterium's external surface. (b) HAADF-STEM micrograph of a single bacterium. (c-d) EDX compositional maps of Mo (blue) and P (green) collected over the whole HAADF-STEM image in (b).

Accordingly, we have evaluated the effect of vancomycin and omeprazole on the reducing capacity of *L. fermentum*. Both drugs are on the World Health Organization's List of Essential Medicines, the most important medications required by a basic health system. Vancomycin is an antibiotic used in the treatment of numerous bacterial infections. Orally administered vancomycin is indicated for intestinal infections. Omeprazole is a proton pump inhibitor used in the treatment of gastro-intestinal pathologies. There is some controversy for both vancomycin and omeprazole regarding the existence of side effects on the gut flora.

Experiments employing *L. fermentum* to reduce $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ were carried out in the presence of vancomycin or omeprazole. The effect on the bacterium's reductive activity was monitored by observing increases or decreases in the UV-vis absorbance band centred at 800 nm corresponding to the reduced forms of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$. As shown in Figure 4, the presence of vancomycin drastically affects the reducing power of *L. fermentum*. Thus, even in the presence of just millimolar concentrations of vancomycin, the reductive ability of the bacterium is almost negligible.

However, the presence of omeprazole does not seem to affect *L. fermentum's* ability to reduce the POM $[P_2Mo^{VI}_{18}O_{62}]^{6-}$, since the large band of the UV-vis spectrum that represents the reduced forms barely changes, even at high concentrations of omeprazole (Figure 4).

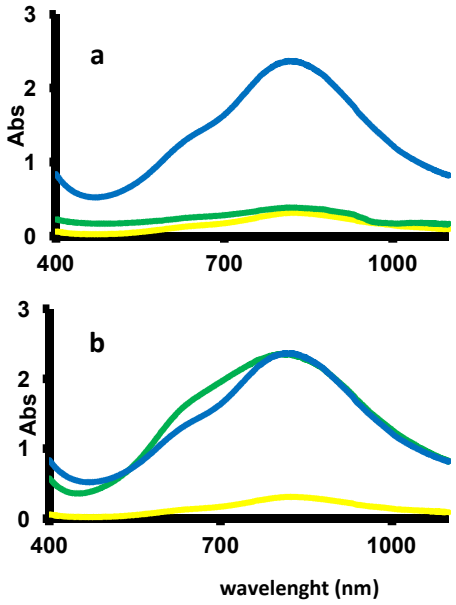

Figure 4. a) UV-vis spectrum for the reduction of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ by *L. fermentum* in the presence of vancomycin (green spectrum). Controls for the no reduction of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ by vancomycin (yellow spectrum) as well as reduction of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ by *L. fermentum* in the absence of vancomycin (blue line) are shown for comparison. Note that in the presence of vancomycin, *L. fermentum* loses its capacity of reducing $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ (green spectrum). b) UV-vis spectra for the reduction of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ by *L. fermentum* in the presence of omeprazole (green spectrum). Controls for the no reduction of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ by omeprazole (yellow spectrum) as well as reduction of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ by *L. fermentum* in the absence of omeprazole (blue line) are shown for comparison. Note that in the presence of omeprazole, *L. fermentum* does not lose its capacity of reducing $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ (green spectrum).

In conclusion, *L. fermentum* acts as an electron donor towards the electrochromic POM $[P_2Mo^{VI}_{18}O_{62}]^{6-}$. The method developed in this paper can be used to measure *L. fermentum* activity and to characterise its behaviour in the presence of certain drugs, such as the antibiotic vancomycin and the proton-pump inhibitor omeprazole. Thus, using this method we have concluded that the antibiotic vancomycin severely affects the bacterial activity of *L. fermentum* whereas omeprazole does not. This protocol can be extended to the characterisation of other metal-reducing bacteria (with either negative or positive effects on human health) in the field of medical analysis. In this sense, future prospects of this work is to create new nano-devices based on the approach and methodology described in this article for screening *ex vivo* activity of new antibacterial materials.

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Notes and references

Experimental: $Na_6[P_2Mo^{VI}_{18}O_{62}]\cdot(H_2O)_{24}$ was synthesized as reported in the literature.[24] The purity of the compound was confirmed by the IR spectrum, which showed the characteristic bands of the polyanion. IR (KBr pellet, cm^{-1}): 1078 (ν P-O, vs), 941 (ν Mo=O, vs), 906 (ν Mo-O-Mo, w), 778 (ν Mo-O-Mo, vs).

L. fermentum bacteria were grown in anaerobiosis conditions in a synthetic medium at 37ºC with orbital agitation for 24h in an initial concentration of 1mg bacteria in 1mL of medium. The synthetic medium consisted of (g/L) Na_2HPO_4 – 5.0, KH_2PO_4 – 6.0, tris-ammonium citrate – 2.0, sucrose – 50.0, $MgSO_4$ – 1.0 and trace elements solution – 10 ml (consisting of (g/L): $MnSO_4$ – 2.0, $CoCl_2$ – 1.0, $ZnCl_2$ – 1.0 dissolved in 0.1 N HCl solution). The medium with an initial pH 6.7 was sterilized at 121 ºC. The final *L. fermentum* cell concentration was $3.3\cdot10^8$ CFU/mL, which was used for all the experiments.

POM reduction by the bacterium: 100µL of a 10mM stock solution of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ were added to 900µL of *L. fermentum* culture to obtain a final concentration of 1mM of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$. Bacteria with POM were incubated for 24h and then centrifuged. *L. fermentum's* ability to reduce the POM was measured at 30, 60, 240 and 1440 min of incubation by UV-vis spectroscopy through the apparition of a band centred at 800 nm due to reduced POM forms. A control of culture media with no bacteria reductive activity was included.

L. fermentum culture exposed to UV light: 3 bacteria culture aliquots of 900µL were exposed to UV light for 60, 240 and 1440 min before the incubation at 37ºC. After UV exposition, the cultures were incubated for 24h at 37ºC with orbital agitation and then were added 100µL of a 10mM stock solution of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$. After centrifugation, the reductive activity was measured by the UV-vis absorbance band centred at 800 nm of the supernatant solution. A control of no UV light exposed culture was included.

Viability of the bacterial colony was defined as the ratio between live and dead bacteria, measured by flow cytometry. Values of 0.93, 0.70, 0.63 and 0.11 were obtained from the different UV exposed experiments at 0, 60, 240 and 1440 min.

Vancomycin and omeprazole effect on the reducing capacity: vancomycin and omeprazole in a final concentration of 0.2mg/mL were added to two independent cultures. The cultures were incubated for 24h at 37ºC with orbital agitation and then, 100µL of a 10mM stock solution of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ were added. The reductive activity in the presence of each drug was measured at 4h by the UV-vis absorbance band centred at 800 nm at the supernatant solution obtained after centrifugation. Controls for the reduction of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ by *L. fermentum* with no drugs and reduction of $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ by the drugs with no bacteria were included.

Viability values corroborated the effect of each drug of *L. fermentum*. Thus, almost null viability (0.07) was obtained for the experiment done in the presence of vancomycin whereas a value close to the control (in the absence of drug) was obtained for the experiment done in the presence of omeprazole (0.91).

Electronic Microscopy: For TEM grid preparation a drop of *L. fermentum* after incubation with $[P_2Mo^{VI}_{18}O_{62}]^{6-}$ was placed onto a carbon-coated Cu grid (200 mesh) and was blotted with filter paper. Samples were observed with a FEI TITAN G2 microscope and HAADF-STEM and EDX map were done with the same equipment.

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