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Copper binding properties of metformin – QCM-D, XPS and nanobead agglomeration

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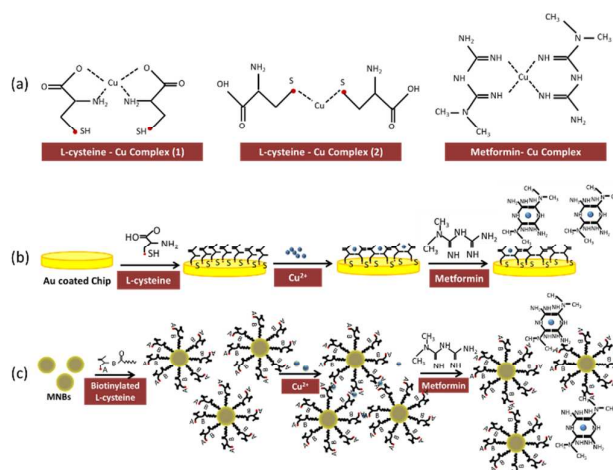
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Study on copper binding properties of metformin is important for revealing its mechanism of action as first-line type-2 diabetes drug. A quantitative investigation of interactions between metformin and L-cysteine-copper complexes was performed. The results suggest that metformin could interact with biological copper, which plays a key role in mitochondrial function.

According to the estimation of the International Diabetes Federation (IDF), in 2014 there were 387 million people suffering from diabetes worldwide, and more than 90 % of the patients had type-2 diabetes (T2D).¹ The biguanide derivative metformin has been used as the first-line drug for treatment of T2D worldwide for several decades.² Moreover, metformin has attracted attention due to its ability to decrease tumorigenesis and cardiovascular-related mortality.²⁻⁵ However, the molecular mechanism of action still remains unclear, hindering the development of novel, targeted therapies and new drugs.

A recent report has shown that metformin acts on mitochondrial copper ions, which may suppress mitochondrial function.⁶ Investigation of the copper-binding properties of metformin might clarify the inhibition mechanisms of this drug. Furthermore, free copper ions hardly exist at the cellular level in living organisms due to binding of the ions to copper proteins.⁷⁻¹⁰ In this scenario, metformin will be obliged to interact with protein/amino acid-bound copper ions, particularly Cu(I) ions, which are the vast majority of intracellular copper ions.¹¹ To shed more light on the behaviour of metformin metal complexes,¹²⁻¹⁹ a variety of analytical techniques have been used, such as nuclear magnetic resonance, electron paramagnetic resonance, and infrared spectrometry, as well as protease assay, voltammetry, and mass spectrometry.



Scheme 1 (a) Schematic representation of the molecular structure of copper complexes: L-cysteine-copper complex under carboxylic/amino group (1) and sulphur (2) coordination, as well as metformin-copper complex. Proposed functional mechanism of metformin interaction with (b) L-cysteine-copper complex (1) on an L-cysteine functionalized gold coated surface, and (c) L-cysteine-copper complexes on biotinylated L-cysteine functionalized magnetic nanobeads (MNBs) (A=NH₂, B=S for complex 1; A=SH, B=NH for complex 2).

However, the conclusions are not directly valid for the biological systems, because the above mentioned studies apply free copper ions, while in vivo copper is always coordinated by proteins and amino acids. Therefore, it is important to evaluate and compare the copper binding affinities between metformin and proteins or amino acids to understand the biological activity of metformin.

In biological systems, cysteine functions as an important ligand in copper proteins;^{7, 20, 21} as a result 35 % of the copper ligands are cysteine residues.²² Previous investigations on the interaction of biguanide with glycine and glycyglycine was unable to differentiate between binding to Cu(I) and Cu(II).^{15, 23} Hence, a study on the interaction between metformin and cysteine-copper complex, which binds Cu(I) and Cu(II) ions would provide new understanding of the functional mechanism of metformin. Since L-cysteine is readily available and its copper chelating function is well known,¹⁰ it was chosen as the model compound in our study. As shown in Scheme 1(a), there are two predominant coordination types to

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form L-cysteine-copper complex. The 2:1 stoichiometry of L-cysteine-copper(II) complex (1) coordinated by the carboxylic and amino groups has been elucidated using cysteine modified gold electrodes.²⁴ The stability constant (pK) for formation of the complex was estimated to be 5-6.²⁵ Although the corresponding pK value for the complex formation in solution is not available, it can be estimated to be of the same order of magnitude as the pK for alanine (14-15).^{25, 26} In the other structure of the L-cysteine-copper complex (2), two cysteine molecules bind copper in a nearly linear geometry (S-Cu(I)-S),²⁷ providing a strong stability constant (pK ~17-19).²⁸⁻³¹ This is one of the common copper binding domains in copper containing proteins.¹⁰ Metformin-copper complex has been shown to form electron delocalised planar ring structures^{11, 12} (illustrated in Scheme 1(a)) and the determined stability constant (pK) varies between 14.3-18.5.³²

Based on the stability constants mentioned above, in solution the stability of metformin-copper complex can be assumed to be of same order of magnitude as L-cysteine-copper complex (1) coordinated by the carboxylic and amino groups, whereas the sulphur coordinated complex (2) has a somewhat higher stability. However, considering that in the biological systems copper is primarily coordinated by cysteine molecules in copper containing proteins, the comparison between the stability constants in solution is not relevant. Hence, to evaluate the ability of metformin to abstract copper from amino acid complexes in proteins, the closest analogy can be achieved by studying surface immobilised complexes (1) and (2). To obtain a qualitative understanding of metformin copper binding, we use here two independent approaches to study in real-time the ability of metformin to abstract copper from surface bound L-cysteine-copper complexes showing the extent and kinetics of the process.

Quartz crystal microbalance with dissipation (QCM-D) provides information on both the frequency and dissipation change in real-time during the occurring processes, simultaneously responding to changes in adsorbed/desorbed mass and conformation of surface bound molecules. Scheme 1(b) illustrates the strategy of the approach to study the ability of metformin to abstract copper from L-cysteine-copper complex (1). First, the surface of an Au coated QCM chip was functionalized with an L-cysteine self-assembled monolayer (SAM). Then, the binding sites of the amino acids were saturated with copper ions to form the L-cysteine-copper complex followed by exposure to metformin solution. Monitoring of the surface change of the functionalized QCM chip allows an effective investigation of the extent to which metformin can abstract copper from surface bound L-cysteine-copper complexes. Subsequently, X-ray photoelectron spectroscopy (XPS) was applied for elemental analysis to evaluate the quantitative change in copper and sulphur species during the process. In addition, for verifying the quality of the formed L-cysteine SAM on the gold chips, the functionalization process was investigated by QCM-D (Fig. S2-1) and XPS (Fig. S2-2) to confirm the high level of homogeneity and reproducibility of the resulting L-cysteine SAMs on the Au surfaces.

To our best knowledge, there are no reports on the stability constant of surface bound L-cysteine-copper complex (2) in comparison with metformin-copper complex. Due to the analogy to proteins where the cysteine ligands are coupled to a large molecule, possibly influencing the stability constant, it is highly relevant to evaluate the ability of metformin to abstract copper from surface bound complexes coordinated by sulphur atoms. Because of the

favoured formation of strongly bound thiolates on gold, we used streptavidin functionalized magnetic nanobeads (MNBs) to form the L-cysteine-copper complex (2). It is worthy to note that, for keeping thiol side chain of L-cysteine free, the biotinylated L-cysteine molecules were formed by using N-hydroxysuccinimide (NHS) esters of biotin, with which L-cysteine forms stable amide bonds. For comparing the ability of metformin in abstracting copper from two different L-cysteine-copper complexes, N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio) propionamide (EZ-link HPDP) biotin was applied to link the MNBs and L-cysteine forming the L-cysteine-copper complexes (1).

For monitoring the abstraction of copper, we applied an agglutination type assay scheme^{33, 34} with a novel readout.³⁵ As illustrated in Scheme 1(c), the MNBs were modified with biotinylated L-cysteine followed by incubation in copper solution to form the complex, which results in clustering of the MNBs. Since the copper bound MNB clusters cannot follow the external magnetic field variation, the modulated optical signal is reduced due to the decrease in the quantity of freely rotating MNBs. During incubation with metformin, the abstraction of copper ions breaks the clusters, resulting in an increase in the signal amplitude due to

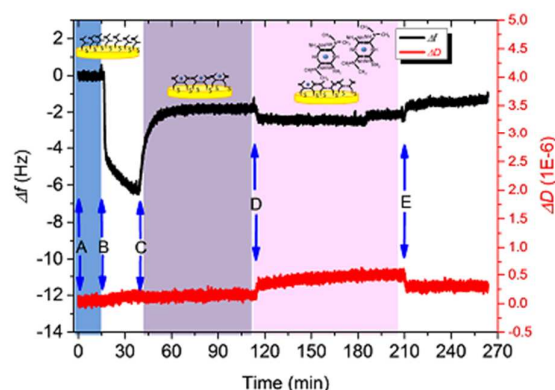


Fig. 1 QCM-D frequency and dissipation data (overtone 7): (Step A) perfusion with PBS; (Step B) addition of 100 μM buffered $\text{Cu}(\text{NO}_3)_2$ solution; (Step C) rinsing with PBS; (Step D) injection of 10 mM buffered metformin solution; (Step E) rinsing with PBS.

an increased quantity of free MNBs.

As shown in Fig. 1, in preparation for the QCM-D investigation, the system was stabilised during perfusion of 50 mM phosphate buffered saline (PBS, pH = 6) for 20 - 30 min (step A). Then, L-cysteine-copper (II) complex was formed by injecting 100 μM $\text{Cu}(\text{NO}_3)_2$ solution (buffered in PBS) for 20 min (Step B). This was followed by perfusion with PBS to remove the unbound copper ions until a stable baseline was generated (step C). Based on Sauerbrey equation,³⁶ the observed ~ 2.0 Hz net decrease in frequency after this step corresponds to a wet mass adsorption of ~ 32 ng/cm^2 (including the coupled solvent). This indicates that the copper ions were adsorbed and chelated by the L-cysteine on the Au surface. Since no significant change in ΔD was observed during the copper binding process, the L-cysteine-copper (II) complex forms a compact film. When 10 mM metformin in PBS was introduced (Step D), an initial levelling off was observed followed by a slight increase in both frequency and dissipation during ~ 90 min. This can be explained by the interaction between metformin and L-cysteine

bound copper ions. The abstraction of copper from the L-cysteine functionalized surface reduces the mass resulting in a less compact film. After the final rinsing with PBS, a frequency increase of ~ 1.2 Hz was obtained corresponding to a wet mass desorption of ~ 20 ng/cm² from the surface due to formation of metformin-copper complex, which was removed by the perfused buffer. The observed mass decrease corresponds to ~ 62.5 % removal of copper from the surface. The small increase in ΔD indicated that the L-cysteine monolayer became slightly less compact compared the L-cysteine-copper complex (1) monolayer.

Since the mass changes determined by QCM-D are influenced by the adsorbed water, the correlation between the mass decrease and copper desorption from the surface bound L-cysteine complex (1) was verified by recording XPS spectra for copper and sulphur before and after incubation with copper and metformin. Fig 2(a) shows Cu 2p XPS spectra acquired after different metformin incubation times. Two distinct copper species are observed in the Cu 2p region. The signal in the 934 eV - 936 eV range is assigned to Cu²⁺, while the peak at the lower binding energy (932.2 eV) is assigned to Cu⁺. An increase in the Cu⁺ peak intensity is observed with increased XPS irradiation time, which may be caused by photo-reduction of Cu²⁺ (Fig S2-1).³⁷ However, a significant decrease in both Cu peak intensities is observed when the metformin incubation time is increased from 0 min to 90 min. This indicates that metformin is able to abstract both Cu(II) and Cu(I), which has significance in the biological systems.³⁸

Peak intensities in XPS spectra are directly correlated to the amount of a specific species. Therefore, the amounts of both Cu species and S adsorbed at the surface are extracted from the area under the Cu 2p and S 2p peaks. Fig. 2(b) shows the integrated peak areas of Cu 2p and S 2p change as function of metformin incubation time. With an increased incubation time, the integrated area of the S peaks remains relatively stable regardless of the incubation time, being indicative of a stable L-cysteine SAM. On the other hand, a dramatic decrease ($\sim 75\%$) in the integrated area of the Cu peaks was observed with increased incubation time from 0 to 90 min, demonstrating the extent to which L-cysteine bound copper was removed from the surface due to the interaction with metformin.

To evaluate the interaction between metformin and L-cysteine-copper complexes, we studied agglomeration of MNBs using a novel optomagnetic readout method. The details of measurements

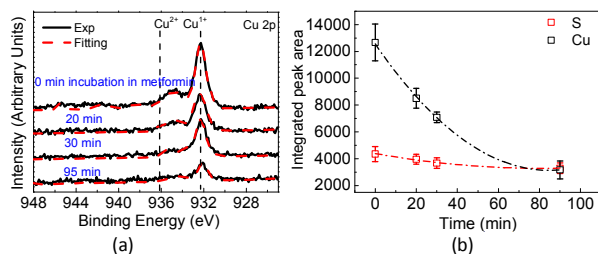
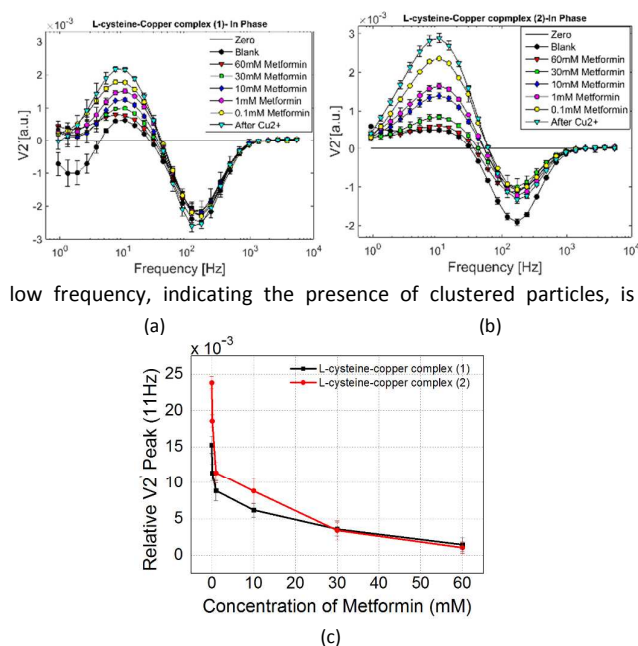


Fig. 2 (a) Cu 2p XPS spectra for different metformin incubation times. (b) Integrated area of C 2p and S 2p peaks as function of metformin incubation time. The error bars represent the standard deviation for triplicate measurements.

are presented in the Supplementary material S1. In brief, the readout is based on the measurement of the modulation of light

beam ($\lambda = 405$ nm) passing through a suspension of MNBs actuated with a uniaxial oscillating magnetic field. The second harmonic in-phase and out-of-phase components (V_2' and V_2'') measured at different frequencies give information on the extent of agglomeration of the MNBs.^{39,40}

Fig 3 shows the in-phase (V_2') spectra for suspensions of 1mg/ml streptavidin modified MNBs with and without incubation in 500 μ M Cu(NO₃)₂ solution buffered in 50 mM 2-(N-morpholino) ethanesulfonic acid (pH = 6) as well as after the subsequent incubation in metformin solutions having the indicated concentrations (pH = 6). MNBs and Cu (Volume ratio: 1:1) were incubated for 2 min and then loaded in the disc followed by 3 minutes incubation under a couple of permanent magnets (total field at the centre 60 mT) used to enhance MNBs agglutination⁴⁰. Metformin has been added to the MNBs and Cu mixture (volume ratio 2:1) and incubated for another 2 minutes followed by loading in the disc and incubation in between permanent magnets of 3 minutes. After incubation in copper solution, a reduction of the high frequency peak amplitude, corresponding to the amount of free MNBs in solution and the rise of a strong positive intensity peak at



low frequency, indicating the presence of clustered particles, is

Fig. 3 V_2' spectra of 200 nm MNB suspensions (1 mg/mL) incubated in Cu formed L-cysteine-copper complex (1) (a) and complex (2) (b), respectively, and subsequently metformin solutions of the indicated concentrations measured after incubation in a strong magnetic field.

observed. This indicates that the MNBs mainly form agglomerates due to the formation of L-cysteine-copper complexes. After metformin incubation, the intensity of the peak corresponding to clustered particles decreases compared to a negative control without incubation in metformin solution. This effect is due to the disruption of the MNB clusters caused by the interaction between metformin and L-cysteine-copper complex (2). However, compared to the low frequency signal measured at 11 Hz from blank samples (i.e. no copper incubation), about 59 % and 63 % of the MNB clusters were disrupted by metformin after the incubation in 10

mM metformin for L-cysteine-copper complex (1) and complex (2), , respectively. It is indicative of the similarity of the efficiency of metformin in abstracting copper from L-cysteine-copper complexes regardless of the significant difference of the L-cysteine-copper complexes in the stability constant. The result for complex (1) is in the line with those obtained from QCM-D technique. Furthermore, the results from dose response curves demonstrate that metformin is not able to abstract copper from the two complexes completely even after incubation in 60 mM metformin.

In conclusion, two independent approaches were used to investigate of efficiency of metformin in abstracting copper from surface bound L-cysteine-copper complexes. The presented results show that despite the higher affinity of metformin to copper in comparison with surface bound L-cysteine, metformin could only partially abstract copper from the L-cysteine-copper complexes. Moreover, the extent of copper abstraction from the sulphur coordinated complex, which is analogous to the protein bound copper in the biological systems, was similar as abstraction from L-cysteine-copper complexes coordinated by carboxyl/amino groups. This provides a fresh insight into the molecular behaviour of metformin interaction with copper with relevance to the biological systems. These novel detection strategies could be used to study the metal binding properties of metformin and its analogues in investigations related to drug discovery.

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