Lectin recognition of a new SOD mimic bioconjugate studied with surface plasmon resonance imaging†

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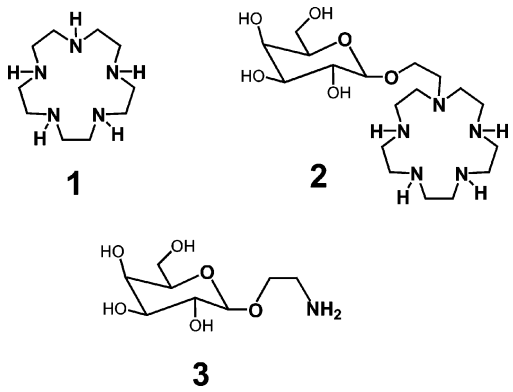
Surface plasmon resonance imaging is used to demonstrate the recognition by the *Ricinus communis* agglutinin of a new SOD mimic, a bioconjugate of the manganese(II) complex of 1,4,7,10,13-pentaazacyclopentadecane with galactose.

Superoxide dismutases (SODs) are a family of enzymes which catalyze the dismutation of superoxide radical anions to hydrogen peroxide and molecular oxygen, and thus protect living cells from toxic oxygen metabolites.[1] An excess of O_2^-, which overwhelms the amount of SOD locally available,[2] is produced by the immune system in such illnesses as inflammation or strokes. This excess damages the surrounding body tissues. Free radical damage has been associated with a growing number of diseases such as rheumatoid arthritis, cancer, neurodegenerative disorders, diabetic complications, strokes, inflammation and reperfusion injury.[3]

SOD native enzymes have shown promising anti-inflammatory properties in both preclinical and clinical studies for the treatment of various diseases, however there were drawbacks and several issues associated with their use as therapeutic agents.[4] For this reason the design of new synthetic, low molecular weight mimics of SOD enzymes (*synzymes*) has been pursued.[5,6] Among the different families of SOD mimics, the manganese(II) complexes of 1,4,7,10,13-pentaazacyclopentadecane[5] (**1**) and its analogous compounds represent a promising new class.[6] Mn(**1**)Cl_2 forms a seven-coordinated Mn(II) complex with *trans*-dichloro ligands and has been shown to be an excellent catalyst for the dismutation of the superoxide anion ($K_{cat} = 4 \times 10^7$ M^{-1} s^{-1}) at the physiological pH. The complex possesses a good thermodynamic stability at physiological pH (log$K = 10.7$) and an excellent kinetic stability. For these reasons, the complex was assessed to test its SOD activity in a variety of *in vitro* and *in vivo* assays. A number of Mn(II) complexes of substituted **1** compounds have been tested in a wide range of animal models of diseases related to the superoxide anion and a potential drug, M40403, has been reported in recent years.[6]

Lectins are a class of proteins which specifically recognize mono, oligo or polysaccharide structures.[7] In recent years it has been shown that the animal lectins, galectins, are important mediators in inflammatory diseases.[8] They are present in epithelium, endothelium and activated macrophages which are central with regard to inflammatory processes. The important role lectins play in recognition processes has prompted efforts to synthesize glycoconjugates of small molecules, proteins or lipids to be specifically bound to selected lectins.[9] In this perspective, we report here on the synthesis, the characterization and the study of the affinity to the *Ricinus communis* agglutinin (RCA_{120}) lectin, of a new galactose-conjugate 1-(2-ethoxy-β-D-galactopyranosyl)-1,4,7,10,13-pentaazacyclopentadecane (**2**) and its Mn^{II} complex [Mn(**2**)Cl_2] as a SOD mimic system (Scheme 1).



Scheme 1

The results reported here provide evidence that the new galactose-conjugate SOD mimic binds the RCA_{120}, that is a model for the animal asialoglycoprotein receptor (ASGP-R) present on the hepatocyte and macrophage surfaces,[8,9d,10] with a constant that is in the range of known binding constants for galactoside–RCA_{120} interactions.[11] The presence of the 1,4,7,10,13-pentaazacyclopentadecane moiety does not alter the ability of lectin to bind the galactose unit even in the presence of the Mn(II) ions. The SOD-like activity of [Mn(**2**)Cl_2] can be thus potentially explicated in the hepatocytes or in other specific inflammatory sites where the galectins and macrophages are present.

Furthermore, on the basis of the coordination properties of the pentaaza macrocycles the bio-targeting of the ligand **2** could also permit a site specific delivery of other metal ions with different properties.[12]

Compound **2** was synthesized by galactosidation of **1**[13] with 2-bromoethyl-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside.[14] The acetylated derivative was purified by chromatography and then deacetylated by basic hydrolysis. (Details of synthetic procedures and intermediate and final products characterization are reported as electronic supplementary information†).

The 2-amino-ethyl-β-D-galactopyranoside (**3**) was also synthesized to compare the ability of the RCA_{120} to interact with a galactose derivative simpler than **2**.

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The SOD-like activity of [Mn(**2**)Cl_2] was tested by the Fridovich assay as reported elsewhere[15] (see ESI†). The complex shows an $I_{50} = 2.5 \times 10^{-6}$ M and a $K_{cat} = 5.2 \times 10^6$ M^{-1} s^{-1}. Such values are in the range of values reported for other SOD *synzymes*[4] and demonstrates the SOD-like activity of the system.

The interaction between RCA_{120} and **2** or [Mn(**2**)Cl_2] was studied with surface plasmon resonance (SPR) imaging[16] by conducting multiplexed kinetics experiments with which the interactions of RCA_{120} with **1** and **3** were also studied in a single experiment. The comparison of the SPR imaging data allowed us to ascertain the possible influence of the 1,4,7,10,13-pentaazacyclopentadecane unit on the ability of RCA_{120} to interact with the galactose moiety present in **2**. Advantages offered by multiplexed kinetics experiments carried out with SPR imaging on biomolecule arrays have been recently demonstrated.[17] Arrays of **1**, **2** and **3** were obtained by spotting their PBS solutions (1 μl, 10^{-2} M) onto gold surfaces previously functionalized with dithiobis(succinimidyl propionate) (DTSP) (see ESI†). Kinetics data were obtained by plotting the normalized difference in percent reflectivity ($\Delta\%R$) from a selected region of interest (ROI) of the array as a function of time. ROI data were normalized to the average of the $\Delta\%R$ measured for mPEG (methoxypoly(ethylene glycol)) background ROIs adjacent to each array element. This procedure corrected data for nonspecific lectin adsorption and changes in bulk refractive index.

A typical experiment consisted of the injection of a continuous flow of RCA_{120} solution over the array: adsorption of lectin on the glycoconjugate surface occurred. The RCA_{120} solution was then replaced with buffer and the bound lectin was desorbed from the surface. The rate constants k_a and k_d and the equilibrium constants K_A were calculated by fitting adsorption/desorption kinetics data through numerical integration analysis.[18] Separate experiments with RCA_{120} solutions at different concentrations (2.5×10^{-7} M, 5.0×10^{-7} M and 7.5×10^{-7} M) were conducted.

Adsorption/desorption curves observed for the binding of RCA_{120} to **1**, **2**, and **3** are shown in Fig. 1. While the interaction between RCA_{120} and the two compounds carrying a galactose unit (**2** and **3**) produces respectively similar curves, a quite different change in variation of $\Delta\%R$ over time is obtained after the RCA_{120}–**1** interaction. The latter testifies the absence of any specific interactions. Similar qualitative conclusions can be easily drawn from SPR difference images taken after the introduction of RCA_{120} solution to the surface (Fig. 2). The clearly different grey level associated to the region of the array where compounds **2** and **3** were immobilized testifies to their stronger affinity for the RCA_{120}. The more pronounced brightness associated with **3** compared to **2** is a consequence of the more efficient anchoring of **3** to the DTSP functionalized surface due to the presence of the primary amino group.

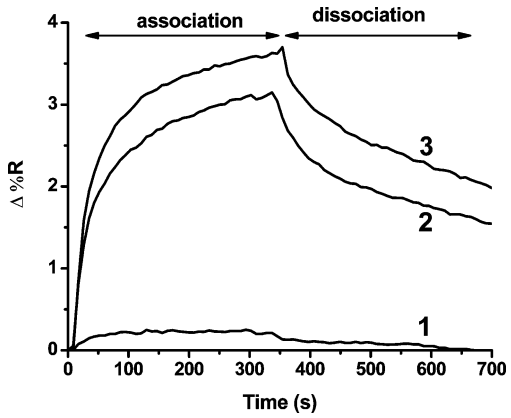

Fig. 1 Kinetics data for the adsorption and desorption of RCA_{120} (750 nM) to **1**, **2**, and **3**.

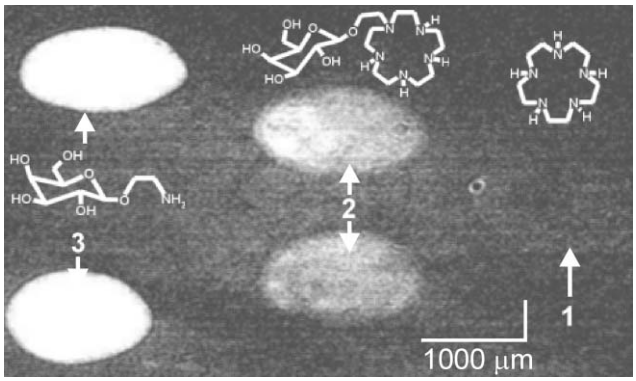

Fig. 2 SPR difference images taken after the flowing of a 750 nM RCA_{120} solution on to the arrayed chip surface. Spots were carried out by using a micro-tip equipped pipette. The different scale bars for the *x* and *y* directions shown in the image are a consequence of the distortion in the SPR image caused by the SPRI optical setup.

Similar SPR difference images and kinetics data were obtained when the interactions of [Mn(**1**)Cl_2] and [Mn(**2**)Cl_2] with the RCA_{120} were investigated.

The best-fit of the kinetics data for the interaction of RCA_{120} with **2**, [Mn(**2**)Cl_2] and **3** were respectively obtained when a bivalent interaction model was taken into account (Fig. 3). Carbohydrate–lectin interactions often deviate from pseudo first-order kinetics.[19] Such an effect is mostly based on the multivalency of the interaction and, more generally, on the existence of interactions which differ in stoichiometry from the 1 : 1 ratio

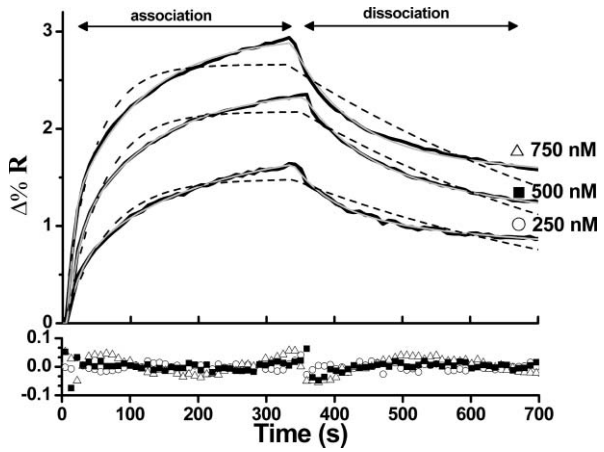

Fig. 3 Kinetics data for the adsorption and desorption of different concentrations of RCA_{120} onto **2**. Data were fitted (grey line) by assuming the interaction of RCA_{120} with one or two galactose units on the chip surface. Residuals are also shown. Dashed lines show the best-fit obtained by assuming pseudo first-order kinetics.

Table 1 Kinetics data for the interaction of the RCA_{120} with **2**, **3** and [Mn(**2**)Cl_2]

	$k_{a1}/M^{-1}\ s^{-1}$	k_{d1}/s^{-1}	K_{A1}/M^{-1}	$k_{a2}/M^{-1}\ s^{-1}$	k_{d2}/s^{-1}	K_{A2}/M^{-1}
2	$2.4\ (\pm0.1) \times 10^4$	$1.8\ (\pm0.5) \times 10^{-3}$	$1.3\ (\pm0.4) \times 10^7$	$3.1\ (\pm0.3) \times 10^5$	$1.2\ (\pm0.1) \times 10^{-2}$	$2.5\ (\pm0.6) \times 10^7$
3	$3.9\ (\pm0.4) \times 10^4$	$5.5\ (\pm1.8) \times 10^{-4}$	$7.1\ (\pm2.4) \times 10^7$	$3.3\ (\pm0.9) \times 10^5$	$3.4\ (\pm1.4) \times 10^{-3}$	$9.7\ (\pm4.8) \times 10^7$
[Mn(**2**)Cl_2]	$1.9\ (\pm1.2) \times 10^4$	$1.7\ (\pm0.8) \times 10^{-3}$	$1.1\ (\pm0.9) \times 10^7$	$1.2\ (\pm0.4) \times 10^5$	$5.7\ (\pm0.9) \times 10^{-3}$	$2.1\ (\pm0.8) \times 10^7$

between the lectin and the anchored receptor.[7a] The interaction model considered in our case takes into account that RCA_{120} possesses two different binding sites.[20] The interaction of RCA_{120} with one or two galactose units on the chip surface is quite possible. The poor results obtained by fitting data assuming pseudo first-order kinetics are evident from Fig. 3 (dotted lines). Rate and equilibrium constants obtained for the interaction of the RCA_{120} with **2**, [Mn(**2**)Cl_2] and **3** are reported in Table 1. No constants for the interaction with **1** and [Mn(**1**)Cl_2] are reported due to the absence of interactions. k_{a1}, k_{d1} and K_{A1} represent respectively the association rate constant, the dissociation rate constant and the equilibrium constant, calculated as k_{a1}/k_{d1}, respectively for the interaction between RCA_{120} and one galactose unit. The constants k_{a2}, k_{d2} and K_{A2} refer to the interaction between RCA_{120} and two galactose units.

The calculated values for the equilibrium constants (K_{A1}, K_{A2}) of the interaction between RCA_{120} and **2** or [Mn(**2**)Cl_2] range between $1.1\ (\pm0.9) \times 10^7\ M^{-1}$ and $2.5\ (\pm0.6) \times 10^7\ M^{-1}$ (Table 1). Such values are in agreement with those reported for the interaction of RCA_{120} with glycoside ligands[9e,f,10] and demonstrate that the pentaazamacrocyclic component of **2** does not significantly modify the ability of RCA_{120} to interact with the galactose unit. Such conclusions are also valid for [Mn(**2**)Cl_2]. The slightly higher K_{A1} and K_{A2} values ($7.1\ (\pm2.4) \times 10^7\ M^{-1}$ and $9.7\ (\pm4.8) \times 10^7\ M^{-1}$, respectively) calculated for the RCA_{120} interaction to **3** are very likely caused by the difference in the structures of **2** and **3** which are expected to have different steric requirements. The absence of an interaction between **1** or [Mn(**1**)Cl_2] and the RCA_{120} further supports the galactose driven interaction between the new SOD mimic presented here and the RCA_{120} lectin.

In conclusion we have shown that the new SOD mimic bioconjugate **2** interacts with the RCA_{120} lectin, that is a model for the animal ASGP-R. The 1,4,7,10,13-pentaazacyclopentadecane moiety does not significantly alter RCA_{120} affinity toward the bioconjugate. Such evidence is expected to improve the therapeutic applicability of the promising SOD mimic based on the Mn(II) complex of **1**. In addition we have shown that SPR imaging of arrayed surfaces may be used to study the interaction between the patterned compounds and selected analytes using a powerful multiplexed approach for simultaneously measuring the binding of different glycoconjugates and RCA_{120} lectin.

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