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ORIGINAL ARTICLE

Biotinylation of aminopyridine-based macrocycles and metallomacrocycles and inclusion of biotinylated iron(II) complex in avidin

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Abstract A simple methodology for single-site conjugation of macrocycles and metallomacrocycles with biotin is presented. This method has been used to conjugate a redox-active macrocyclic complex and embed it into avidin. The resulting biotin-metal complex-avidin adduct exhibits peroxidase activity as shown by the oxidation of ABTS and pyrogallol with H_2O_2.

Keywords Biotinylation · Macrocycles · Metallomacrocycles · Catalysis

Introduction

Azamacrocycles are known to form stable, well-defined coordination complexes with transition metal and lanthanide ions, giving rise to compounds with potential applications in chemistry, biology and medicine [1, 2]. Of particular interest are pyridine-containing macrocycles whose iron (II/III) metal complexes have been shown to catalyze the decomposition of hydrogen peroxide [3–5], and oxidize olefins [6] and aromatic substrates [4] under mild conditions. Moreover, derivatives of these macrocycles are being considered in radiopharmaceuticals as bifunctional chelators for diagnostic imaging and therapy [7–9].

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Functionalization of macrocyclic ligands is useful in acquiring the desired properties of the metal complex such as redox potential, thermodynamic stability, kinetic inertness, and hydrophobic/hydrophilic character [10–14]. Functional groups in the macrocycle can also be used for conjugation with biomolecules or solid support. Thus, considerable attention is given to the preparation and characterization of functionalized azamacrocyclic ligands. Monofunctionalized macrocycles are particularly attractive, but their synthesis is often challenging.

Our interest in pyridine-containing macrocycles as biomimetic catalysts has led us to develop macrocycles containing a pendant arm [15, 16]. This functionalized macrocycle allows us to encapsulate a redox-active species and embed it into a protein. In order to ensure insertion and localization of redox-active species in the protein cavity, we use the biotin-avidin technology. This supramolecular system takes advantage of the strong affinity ($K_a \sim 10^{15}\ M^{-1}$) of avidin towards biotin [17]. It has been applied in diagnostic, imaging, sensing and therapeutic purposes [18]; however, its use in synthetic chemistry is still being explored. The pioneering work of Whitesides [19] has inspired subsequent applications of biotin-avidin technology in the design of artificial metalloenzymes for enantioselective hydrogenation reactions [20–22]. More recently, Ward et al. have demonstrated its versatility in alcohol oxidation [23], transfer hydrogenation [24] and allylic alkylation reactions [25]. Surprisingly, application of the biotin-avidin technology for biomimetic catalytic oxidation of organic compounds is rare [23, 26, 27].

Here, we propose a very simple methodology for single-site conjugation of macrocycles and metallomacrocycles with biotin, and report a redox-active macrocyclic complex (a known catalyst for H_2O_2 reactions) embedded in avidin.

Experimental

General

ImmunoPure Avidin (salt-free) was purchased from Pierce Biotechnology. All other reagents were obtained from commercially available sources and used without further purification. The pentadentate ligand **L1**, [Ni(**L1**)]$(ClO_4)_2$ and [Fe(**L1**)]$(CF_3SO_3)_2$ complexes were prepared as described elsewhere [6, 15]. UV-vis spectra were acquired on a Jasco V-570 spectrophotometer. IR spectra were recorded on a Mattson RS-1 FTIR spectrometer. Mass spectra were recorded at the University of Minnesota Mass Spectrometry Facility (Minneapolis, MN). High-resolution ESI mass spectra were obtained on a Bruker BioTOF II. ^1H and ^{13}C NMR spectra were recorded on Bruker DPX-300 MHz spectrometer. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory (Woodside, NY).

Syntheses

Caution! Perchlorate salts of metal complexes with organic ligands are potentially explosive. Only small amounts of material should be prepared, and these should be handled with great caution.

*Biotinylated ligand (**L2**)*

(Procedure A): To the DMF solution of ligand (**L1**) (250 mg, 0.782 mmol) and 2,6-lutidine (1.0 mL) was added a solution of (+)-biotin *N*-hydroxysuccinimide ester (530 mg, 1.56 mmol) in DMF (25 mL). The solution was stirred for 24 h at rt under Ar, and then concentrated under vacuo to 3 mL. The remaining solution was treated with diethyl ether (30 mL) to obtain a white precipitate. Yield (385 mg, 90.2%). (Procedure B): NaCN (76 mg, 1.6 mmol) was added to an aqueous solution (25 mL) of [Ni(**L2**)]$(ClO_4)_2$ (208 mg, 0.259 mmol) and the reaction mixture stirred for 1 h at room temperature. The ligand was extracted with CH_2Cl_2 (3 × 10 mL) and the consolidated organic layer was washed with brine, dried over Na_2SO_4 then rotavaped to yield an oily product. The oil was treated with diethyl ether to form a white solid. The product was recrystallized from CH_2Cl_2-diethyl ether. Yield (100 mg, 71%). ^1H NMR (300 MHz, $CDCl_3$, 300 K): $\delta = 7.69$ (t + s, 2H), 7.09 (d, 2H), 6.38 (bs, 1H), 5.45 (bs, 1H), 4.56 (dd, 1H), 4.39 (dd, 1H), 3.91 (m, 2H), 3.43–3.57 (m, 2H), 3.22–3.15 (q, 2H), 2.98–2.72 (m, 3H), 2.53–2.38 (m, 8H), 2.27 (t, 4H), 1.82–1.63 (m, 10H), 1.53–1.46 (m, 8H); ^{13}C NMR (300 MHz, $CDCl_3$, 300 K): $\delta = 174.04$, 164.48, 137.72, 121.34, 62.26, 60.65, 59.56, 56.17, 51.90, 51.77, 45.82, 45.75, 41.04, 37.67, 36.25, 28.66, 28.62, 26.42, 26.26, 23.71; IR (KBr): ν (cm^{-1}) = 3259, 2929, 1646, 1442. HRMS (ESI) *m/z* calcd for $C_{28}H_{48}N_7O_2S$ [$(M + H)^+$] 546.3585, found 546.3611. Anal. calcd. for $C_{28}H_{47}N_7O_2S \cdot 1.5H_2O$: C 58.71%, H 8.80%, N 17.12%; found C 58.76%, H 8.99%, N 16.96%.

*[Ni(**L2**)]$(ClO_4)_2$*

(Procedure A): To the ethanolic solution of **L2** (100 g, 0.183 mmol) was added a solution of $Ni(ClO_4)_2 \cdot 6H_2O$ (67.0 mg, 0.183 mmol) dissolved in 1 mL MeCN. The mixture was stirred for 4 h at rt (any precipitate formed was dissolved by adding MeCN). The yellow-orange solution was evaporated to dryness. The orange solid was recrystallized from MeOH. Yield (0.129 g, 87.7%). (Procedure B): To the solution containing [Ni(**L1**)]$(ClO_4)_2$ (300 mg, 0.523 mmol) and 2,6-lutidine (1 mL) in MeCN was added a solution of (+)-biotin *N*-hydroxysuccinimide ester (200 mg, 0.585 mmol) dissolved in 5 mL DMF. The solution was stirred at rt for 24 h, and then the solvent was removed under vacuo. The oily residue was redissolved in MeOH–H_2O (1:5) and washed with CH_2Cl_2. The solvent was evaporated, and the orange solid residue was washed with EtAc, then hexane. Orange crystals were recrystallized from MeOH. Yield (208 mg, 49.9%). IR (KBr) ν (cm^{-1}) = 3332, 2937, 1648, 1465; HRMS (ESI) *m/z* calcd for $C_{28}H_{47}ClN_7NiO_6S$ [$(M\text{-}ClO_4)^+$] 702.2345, found 702.2341; calcd for $C_{28}H_{47}N_7NiO_2S$ [$(M\text{-}2ClO_4)^{2+}$] 301.6427, found 301.6440. Anal. calcd. for $C_{28}H_{47}Cl_2N_7NiO_{10}S \cdot 2H_2O$: C 40.06%, H 6.12%, N 11.68%, Ni 6.99%; found C 40.04%, H 6.24%, N 11.38%, Ni 6.91%.

*[Mn(**L2**)]Cl_2*

$MnCl_2 \cdot 4H_2O$ (9.6 mg, 0.0485 mmol) and **L2** (26.4 mg, 0.0485 mmol) were dissolved in MeOH (10 mL) and refluxed under N_2 for 1 h. The solution was then added dropwise to diethyl ether (50 mL) to precipitate the complex. Yield (28.7 mg, 88.6%). IR (KBr) ν (cm^{-1}) = 3227, 2928, 1636, 1442; HRMS (ESI) *m/z* calcd for $C_{28}H_{46}MnN_7O_2S$ [(**L2**-H + Mn)$^+$] 599.2814, found 599.2809; calcd for $C_{28}H_{47}ClMnN_7O_2S$ [$(M\text{-}Cl)^+$] 635.2581, found 635.2586. Anal. calcd. for $C_{28}H_{47}Cl_2MnN_7O_2S$: C 50.07%, H 7.05%, Mn 8.18%, N 14.60%; found C 46.69%, H 6.67%, Mn 8.60%, N 12.30%.

*[Fe(**L2**)]$(CF_3SO_3)_2$*

In a glovebox, a 0.5 mL DMF solution of (+)-biotin *N*-hydroxysuccinimide ester (33 mg, 0.0967 mmol) and 2,6-lutidine (0.125 mL) was added to the 2.0 mL MeCN solution of [Fe(**L1**)]$(OTf)_2 \cdot 2MeCN$ (65 mg, 0.0967 mmol), and stirred for 24 h at rt. The yellow solution formed was

then added dropwise to 50 mL diethyl ether to precipitate the biotinylated complex, which was filtered and dried. Yield (80 mg, 92%). IR (KBr) $\nu = 3253, 2933, 1642, 1454$; HRMS (ESI) *m/z* calcd for $C_{28}H_{46}FeN_7O_2S$ [(**L2**-H + Fe)$^+$] 600.2783, found 600.2791. Anal. calcd. for $C_{30}H_{47}F_6FeN_7O_8S_3 \cdot 2CH_3CN$: C 41.59%, H 5.44%, Fe 5.69%, N 12.84%; found C 41.63%, H 5.84%, Fe 5.90%, N 12.56%.

HABA assay

To a mixture of HABA (300.0 μM) and avidin (7.4 μM) in potassium phosphate buffer (0.01 M, pH 7.4, 2 mL) were added 1–2 μL aliquots of the [Fe(**L2**)]$(CF_3SO_3)_2$ complex (0.01 M) in 1-min intervals. The formation of biotin-Fe(II)-avidin adduct was indicated by the decrease in absorbance at 500 nm. Binding stoichiometry of [Fe(**L2**)]$(CF_3SO_3)_2$ to avidin was determined by plotting $-\Delta A_{500\ nm}$ versus [[Fe(**L2**)]$(CF_3SO_3)_2$]:[avidin].

Oxidation of ABTS

To a fresh solution of ABTS (0.70 mM) and H_2O_2 (3.5 mM) in 1.5 mL MeCN was added [Fe(**L2**)]$(CF_3SO_3)_2$-avidin complex (0.22 mM, 3 equiv biotin per avidin) in 0.50 mL phosphate buffer (0.01 M, pH 6.8). The absorbance was measured at 414 nm.

Oxidation of pyrogallol

To a fresh aqueous solution of pyrogallol (4.5 mM) and H_2O_2 (18 mM) was added [Fe(**L2**)]$(CF_3SO_3)_2$-avidin (0.22 mM, 3 equiv biotin per avidin) in 1.0 mL phosphate buffer (0.01 M, pH 6.8). The absorbance was measured at 420 nm.

Results and discussion

While several methods of derivatization of azamacrocycles have been reported [28–38], regioselective monofunctionalization of azamacrocycles continues to be a challenging task. For example, cyclen and cyclam have many potential reactive sites (usually, NH-groups) which can lead to multiple products with varying degrees of conjugation. Recently, Todd, Watkinson et al. [39] reported synthetic methods for biotinylation of azamacrocycles involving multiple steps: protection of several amines in the cyclic framework, selective alkylation or acylation to introduce the linker, then finally attaching the biotin moiety. The number of synthetic steps can be reduced if the linker containing a free amino group is already present in the macrocycle. One such macrocycle is the aminopyridine ligand **L1** [15] (Fig. 1). This macrocycle features a metal-binding site composed of the aminopyridine framework, and a bioconjugating site in the form of an alkyl amine arm. With this macrocycle, it is possible to bioconjugate the ligand selectively due to the difference in reactivity between the primary amine in the pendant arm and the secondary amines of the macrocyclic framework.

To test our hypothesis, we decided to biotinylate **L1** with biotin *N*-hydroxysuccinimide ester, a reagent typically used in conjugating proteins at the primary amine of lysine residues [40] (Scheme 1, Route A). In a one-pot synthesis, (+)-biotin *N*-hydroxysuccinimide ester dissolved in DMF was reacted with **L1** in the presence of 2,6-lutidine. This led to the smooth formation of biotinylated ligand **L2** in 90% yield. This strategy demonstrates that we can selectively acylate the ligand at the primary amine in the presence of secondary amines. With the new ligand at hand, we then proceeded with the binding of metal ions, Ni(II), Mn(II) and Fe(II). Results show that Ni(II) and Mn(II) ions bind effectively, forming biotinylated metal complexes, [Ni(**L2**)]$(ClO_4)_2$ and [Mn(**L2**)]Cl_2 in 88 and 89% yield, respectively. The binding of Fe(II), however, proved problematic, as we recovered mostly the free biotinylated ligand after reaction of $Fe(CF_3SO_3)_2$ with **L2**. This led us to explore an alternative strategy to prepare biotinylated metal complexes.

Recently, our group discovered that it is possible to selectively monofunctionalize metal complexes of **L1** (R = H, Me) at the pendant arm with excess acylating agents [16]. For example, when [Ni(**L1**)]$(ClO_4)_2$ complex was reacted with acetic anhydride or benzoyl chloride, only the primary amine at the pendant arm formed an amide (Scheme 2). This regiospecific *N*-acylation can be attributed to the reduced nucleophilicity of the secondary amine groups in the macrocyclic framework brought about by strong coordination to the nickel ion [16, 34]. Although the primary amino group of the pendant arm can also coordinate to the metal center, this interaction is weaker compared to that of the other amino groups in the macrocycle [6].

This finding led us to formulate an alternate strategy to synthesize biotinylated metal complexes wherein the sufficient reactivity of the amine pendant arm would allow us to prepare metal complexes first, and attach biotin next

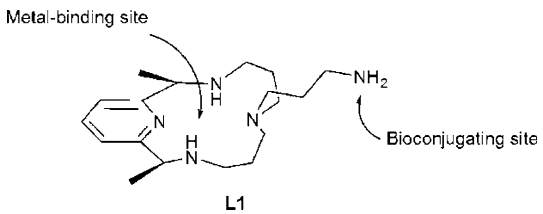
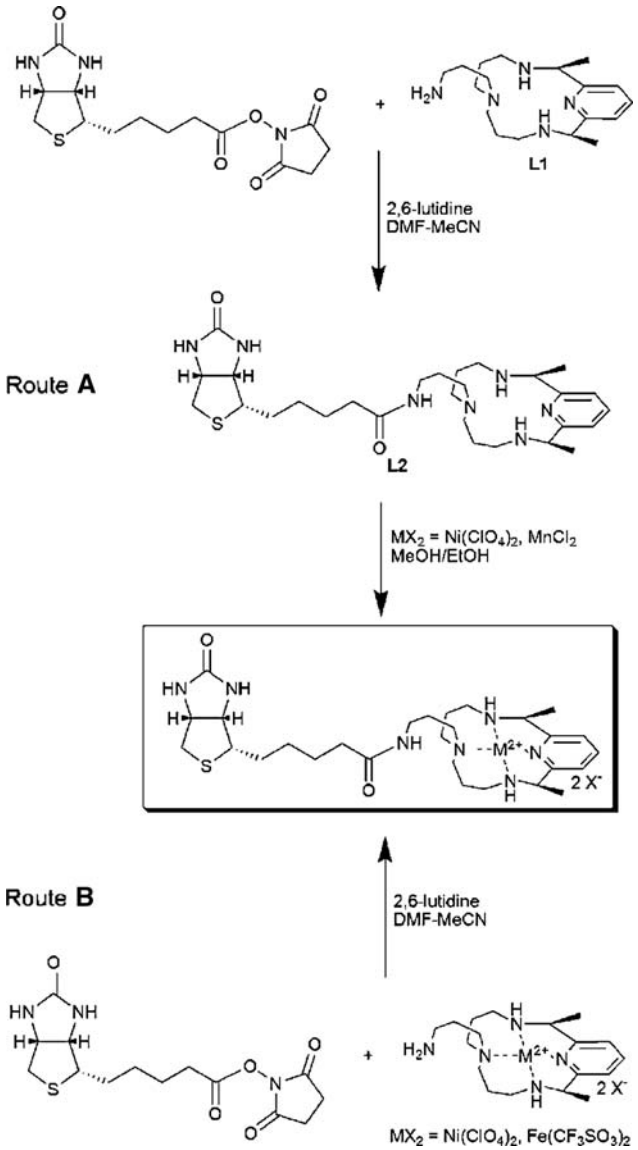
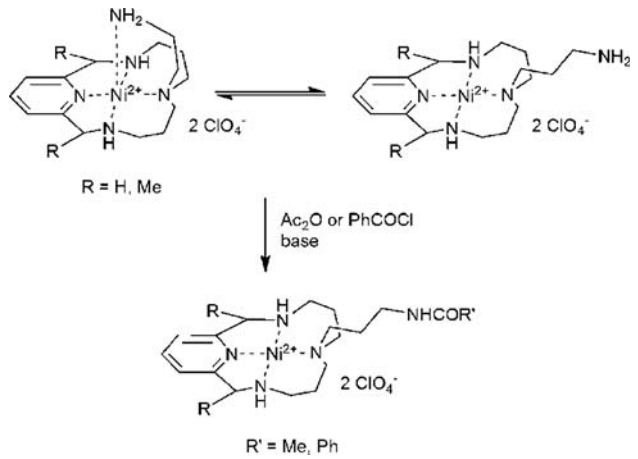


Fig. 1 An aminopyridine-based bifunctional macrocycle



Scheme 1 Synthesis of biotinylated metal complexes



Scheme 2 *N*-acylation of [Ni(**L1**)]$(ClO_4)_2$ complex at the aminopropyl pendant arm

(Scheme 1, Route B). Hence, the biotin reagent was reacted with an equivalent amount of [Ni(**L1**)]$(ClO_4)_2$ or [Fe(**L1**)]$(CF_3SO_3)_2$ in the presence of 2,6-lutidine. The reaction proceeded smoothly with the formation of biotinylated metal complexes, [Ni(**L2**)]$(ClO_4)_2$ and [Fe(**L2**)]$(CF_3SO_3)_2$, in 50 and 92% yield, respectively.

The binding of biotinylated iron (II) complex to avidin was investigated using a standard HABA (4′-hydroxyazobenzene-2-carboxylic acid) assay [40]. Typically, the HABA dye is added to an avidin solution and binds to the protein, giving rise to an absorption feature at 500 nm. Upon addition of biotin to the HABA-avidin reagent, the HABA is displaced by biotin due to a stronger biotin-avidin complexation, resulting in decreased absorbance at 500 nm. In this work, addition of [Fe(**L2**)]$(CF_3SO_3)_2$ to the HABA-avidin solution led to a decrease in absorbance, indicating that the biotinylated iron complex was able to displace HABA from avidin. The plot of $-\Delta A_{500\ nm}$ versus [[Fe(**L2**)]$(CF_3SO_3)_2$]:[avidin] showed an equivalence point at [[Fe(**L2**)]$(CF_3SO_3)_2$]:[avidin] = 4:1 (Fig. 2). Similar binding stoichiometry was also found for [Ni(**L2**)]$(ClO_4)_2$. Since avidin has four biotin-binding sites [41], this result suggests that appending a metal–ligand complex to biotin does not significantly affect its ability to bind with avidin. It also indicates that quantitative encapsulation of biotin into avidin is possible even in the presence of the 2+ charge of the complex. This observation is consistent with several reported cationic biotinylated metal complexes [22, 42, 43].

Peroxidase activity of the [Fe(**L2**)]$(CF_3SO_3)_2$ complex embedded in avidin was determined using typical peroxidase substrates 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) [44] and pyrogallol [45] (Scheme 3). The oxidation of the substrates was carried out

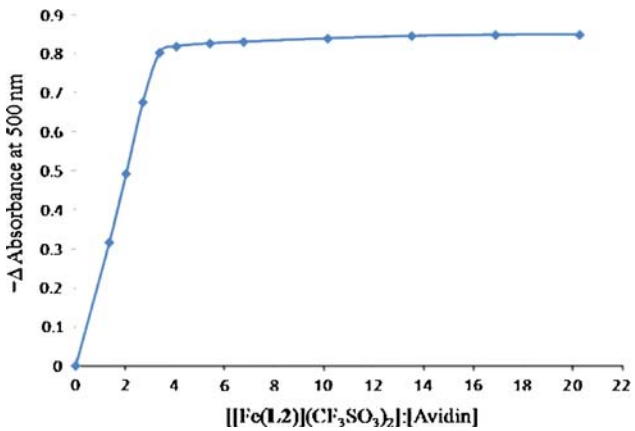
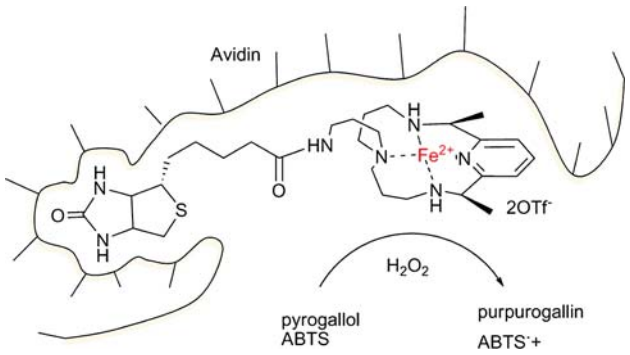


Fig. 2 Absorption titration curve for the titration of HABA-avidin complex with [Fe(**L2**)]$(CF_3SO_3)_2$. Experimental conditions: 1–2 μL aliquots of [[Fe(L2)]$(CF_3SO_3)_2$] (0.01 M) were added to a pre-mixed solution of HABA (300.0 μM) and avidin (7.4 μM) in phosphate buffer (0.01 M, pH 7.4) at 25 °C



Scheme 3 Oxidation of ABTS and pyrogallol with H_2O_2 in biotinylated [Fe(**L2**)]$(CF_3SO_3)_2$-avidin system

using H_2O_2 as oxidant. Figures 3 and 4 illustrate the oxidation of ABTS and pyrogallol, respectively. Results show that the biotinylated complex [Fe(**L2**)]$(CF_3SO_3)_2$ with avidin can catalyze the oxidation of both substrates. The catalytic effect of [Fe(**L2**)]$(CF_3SO_3)_2$ is found to be significantly lower in the presence of avidin, compared to the free biotinylated iron complex. This observation strongly suggests that the catalyst is encapsulated deep in the avidin cavity, and that the oxidation of the substrates takes place in the vicinity of the metal center within the protein. Lower activities of encapsulated catalysts due to restricted diffusion of substrates were previously reported [46, 47]. We also noted that the decrease in catalytic activity due to encapsulation of the catalyst in avidin is smaller for pyrogallol (70% decrease with respect to free catalyst) compared to ABTS (90%). The effect on substrate size may imply a constricted space around the active site of the

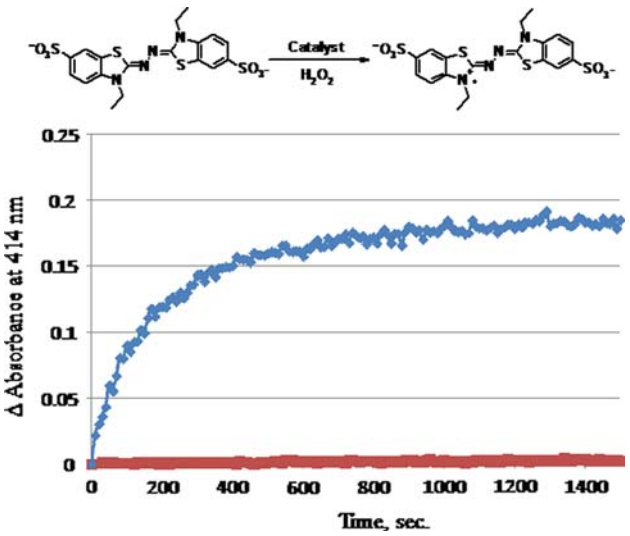


Fig. 3 Absorbance versus time curve for the oxidation of ABTS with H_2O_2 without catalyst (■), and with [Fe(**L2**)]$(CF_3SO_3)_2$ catalyst in avidin (◆). Reaction mixtures contain ABTS (0.70 mM), H_2O_2 (3.5 mM), [Fe(**L2**)]$(CF_3SO_3)_2$-avidin complex (0.22 mM, 3 equiv biotin per avidin), phosphate buffer (0.01 M, pH 6.8)-MeCN (3:1)

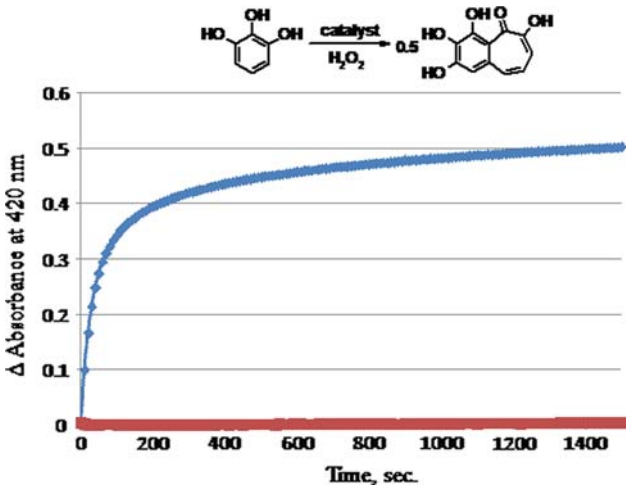


Fig. 4 Absorbance versus time curve for the oxidation of pyrogallol with H_2O_2 without catalyst (■), and with [Fe(**L2**)]$(CF_3SO_3)_2$ catalyst in avidin (◆). Reaction mixtures contain pyrogallol (4.5 mM), H_2O_2 (18 mM) and [Fe(**L2**)]$(CF_3SO_3)_2$-avidin (0.22 mM, 3 equiv biotin per avidin), phosphate buffer (0.01 M, pH 6.8)

catalyst-avidin adduct. Hence, the smaller pyrogallol molecule can penetrate through the cavity walls of avidin and reach the active site easier than the larger ABTS molecule. On the other hand, it is also possible that oxidation of the biotin moiety and amino acid residues in avidin took place, as suggested in the work by Leonard [48]. In this case, when mass transport limits accessibility of the substrate to the active site, H_2O_2 decomposition and/or protein oxidations become more important. Further addition of H_2O_2 to the reaction mixture caused a slight increase in the absorbance of the product (data not shown). This indicates that the catalyst is still active, and suggests that the initial H_2O_2 added was consumed in processes other than oxidation of the substrate.

Summary and outlook

We have synthesized biotinylated metal complexes based on macrocyclic aminopyridine ligands through direct conjugation at the pendant arm of the ligand. The positively charged biotinylated metallomacrocycles have been shown to bind with avidin. Moreover, the biotinylated iron complex exhibits catalytic properties in the oxidation of ABTS and pyrogallol with aqueous hydrogen peroxide. While there is a need to improve the catalytic performance of the biotin-iron complex-avidin adduct, our methodology of site-specific monofunctionalization of (metallo)macrocycles may also be useful for immobilizing catalysts or metal complexes in solid supports, polymers or biomolecules. Consequently, complexes containing radioisotopes such as ^{64}Cu can also be prepared for positron emission

tomography (PET) imaging and targeted radiotherapy [49–53], while lanthanide-bound conjugates can serve as diagnostic agents in magnetic resonance imaging (MRI) [7, 54, 55].

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