

Photodelivery of Nitric Oxide from a Nitrosothiol-Derivatized Surface

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Nitric oxide (NO) is a messenger molecule with multiple physiological roles, such as neurotransmission, regulation of muscle tone, and cell-mediated cytotoxicity against bacteria and tumor cells.^{1,2} The metabolic pathways, physiological action, and biochemistry of NO in living tissues are being actively investigated, particularly the way in which NO is produced and released by the cells.^{3–6}

Although there is an active search for versatile and biocompatible NO generation techniques, almost all of the procedures used to determine the action, transport, occurrence, and kinetics of NO in living tissues have poor resolution. Photochemical release of NO using inorganic complexes⁷ allows some spatial resolution, since a tissue zone can be illuminated while other areas are kept dark. However, the NO dispenser substance must still be present in all of the tissue simultaneously.

Although NO microsensors with cellular resolution have been developed,⁸ to our knowledge no method able to deliver NO in a controlled way into a very small area has been reported.

In this work we demonstrate the possibility of achieving a controlled way of binding the NO function onto a solid substrate previously derivatized to obtain an exposed thiol group. The nitrosothiol function so formed is thermally stable, but NO can be photoreleased by means of visible light. The method is a first step in the development of a molecular dispenser of NO with submicrometer resolution and is also relevant to the subject of surface-initiated reactions.

To detect the mass changes due to the substrate derivatization and further NO attachment and photorelease, the quartz crystal microbalance⁹ (QCM) was used. It consists of a piezoelectric quartz crystal which oscillates at a frequency that changes linearly with the deposited mass,¹⁰ $\Delta f = -k(\Delta m/A)$ where $k = 0.226 \text{ Hz ng}^{-1} \text{ cm}^2$ for the quartz crystals used. We used a 50 μL flow injection QCM cell with 14 mm gold coated quartz crystals ($f = 10 \text{ MHz}$) mounted on the cell by sealing nitrile O-rings, with only one face in contact with the carrier. A 1 L carrier reservoir (0.1 M HCl + 0.1 M KCl in water) was placed at 1 m over the level of the QCM cell and its drain. The hydrostatic pressure so generated produced a very stable flow of 8 $\mu\text{L/s}$. The temperature of the carrier was stabilized by means of an 80 cm tubing coil immersed in a room temperature equilibrated water bath (23 °C),

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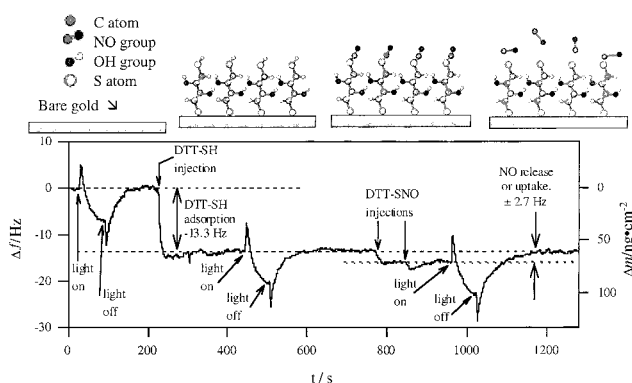


Figure 1. Quartz crystal microbalance frequency and mass changes during dithiothreitol (DTT-SH) adsorption, transnitrosation with *S*-nitroso dithiothreitol and NO photorelease. Three visible light irradiation periods are also shown. See that the last one promotes NO photorelease.

placed immediately before the QCM cell. The reactants were injected into the mainstream using a 100 μL syringe. A cold light source (a 30 W tungsten lamp through fiber optic bundle) was used to perform the photodelivery of NO from the thiol-derivatized gold surface. All chemicals were analytical grade (Aldrich).

To ensure that the light absorbed by the water, the crystal, and the associated elements of the system did not alter the QCM measurements, a control experiment was done over a bare crystal by turning the light on and off, while recording the QCM frequency signal. Figure 1 ($t = 20 \text{ s}$) shows the transients due to irradiation on the gold-covered quartz before thiol adsorption. The transients were mainly due to a tiny temperature change of the cell that produced viscosity changes of the carrier solution. It can be seen that after irradiation the frequency recovered exactly the same value as the one observed before turning the lamp on. Therefore, changes in mass induced by irradiation can be measured by comparison of the corresponding frequencies.

To estimate the mass and photostability of the thiol adsorbed layer, 100 μL of a 2 mM solution of dithiothreitol (DTT-SH) in carrier were injected in the flow system. Figure 1 ($t = 220 \text{ s}$) shows the decrease of the resonant frequency of the QCM ($\Delta f = -13.3 \text{ Hz}$), which corresponds to a mass increment of 58.9 ng/cm^2 of DTT-SH, that is, a coverage of 0.38 nmole/cm^2 . This latter value can vary with the gold surface cleanliness and its oxidation state, but is always lower than the coverage of a monolayer of aliphatic thiol,¹¹ $\sim 0.9 \text{ nmole/cm}^2$. This low coverage is possibly due to the presence of the lateral hydroxyl groups in the DTT-SH molecule, which would tend to form hydrogen bonds with neighboring molecules, producing a more open monolayer structure.

After the new constant frequency value is reached, a second injection of DTT-SH shows no significant mass increase; only a very small transient is observed (Figure 1, $t = 310 \text{ s}$), indicating that a complete monolayer has been formed.

Thiol layers adsorbed onto gold are very stable, as previously reported.^{12,13} However, in a flow system, the continuous presence of a thiol free carrier promotes the desorption of short chain thiols. For mercaptoacetic acid monolayers, 30% of the total adsorbed mass is lost in 47 min at the flow rate used, while aminoethanethiol monolayers lose 30% of its mass in 29 min. For these

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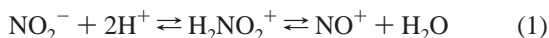
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short chain thiols, the coverage varies between 0.8 and 1.2 nmole/cm², as expected for very compact layers.

DTT-SH monolayers are exceptionally stable, and the mass keep within the QCM resolution (0.3 Hz, 5% of the adsorbed mass) for more than 4 h. This stability is probably another consequence of the hydrogen bond stabilization due to the lateral hydroxyl groups of DTT-SH.

Once modified with DTT-SH, the crystal was irradiated as in the control experiment. Figure 1 ($t = 440$ s) shows no change in the mass after irradiation. The experiment was repeated for several on-off cycles, with excellent reproducibility. Thus, we conclude that no desorption of thiol is produced upon illumination.

In a second stage, the aim was to generate an *S*-nitroso functionality on the thiol-derivatized surface. A first attempt was made on the basis of the known properties of nitrite solutions, which behave as efficient nitrosating agents toward thiol species in acid media.¹⁴



The procedure was not successful for obtaining a monolayer of *S*-nitrosodithiothreitol (DTT-SNO) attached to gold. When 100 μL of a 1 mM solution of NaNO₂ in the carrier solution was injected to the flow line, the frequency first decreased, showing a mass gain probably due to a fast NO linkage to the external thiol groups. However, for later times, the frequency increased again up to the same value achieved before thiol adsorption, as expected for a total surface stripping. Therefore, in contrast to the behavior in aqueous solutions, nitrite cannot be used as a nitrosating agent for the gold-attached DTT-SH; probably, desorption of the thiol is obtained through the cleavage of the Au-S bond leading to an *S*-nitroso derivative in the solution. The treatment with nitrite was however quite useful for efficiently cleaning the gold surface, detaching the previously adsorbed thiols; this was systematically used for that purpose before every experiment.

Thus, we searched for an alternative NO donor. It is known that nitrosothiols themselves can be used as effective NO donors provided that transnitrosation reactions are very fast and efficient.¹⁴⁻¹⁶ In these reactions, one molecule of thiol acts as a NO-donor and another one as a NO-acceptor:



R₁ and R₂ may be different or the same residues. We selected aqueous DTT-SNO itself as a nitrosating agent. This solution was obtained as described elsewhere¹⁴ by mixing 2 mL of a 10 mM carrier solution of DTT-SH with an equal amount of a 10 mM NaNO₂ in carrier solution. The 5 mM reddish DTT-SNO solution was immediately injected into the flow line, while registering the resonance frequency of the previously thiolated gold surface. Figure 1 ($t = 770$ s) shows the decrease in frequency upon injection of aqueous DTT-SNO; it can also be seen that a second

injection ($t = 850$ s), although giving a transient, does not change the final mass of the layer. The change in resonant frequency, $\Delta f = -2.7$ Hz, corresponds to a mass increase of about 11.9 ng/cm², or 0.41 nmole/cm² on replacement of H by NO, in very good agreement with the previously measured thiol coverage of 0.38 nmole/cm². Thus, a trans-nitrosation reaction occurred, as in eq 3, between the DTT-SH layer and the DTT-SNO solution reactant, with formation of an adsorbed DTT-SNO layer, which appears as quite stable (for most experiments, the baseline remains unchanged up to 40 min).

DTT-SNO solutions show two characteristic absorption bands at 332 nm ($\epsilon = 544 \text{ M}^{-1} \text{ cm}^{-1}$) and 545 nm ($\epsilon = 17.4 \text{ M}^{-1} \text{ cm}^{-1}$).¹⁴ Considering this low extinction coefficient for visible light, and even if a maximum quantum yield was to be assumed, only 1 every 25 000 visible light photons would be able to photolyze the nitrosothiol. This low efficiency can be counteracted by means of a high power light source or by increasing the irradiation time. For applications in which the spatial and temporal resolution are important, the use of high power sources would be preferred.

It can be seen that after one on-off irradiation cycle (Figure 1, $t = 970$ s), the measured mass coincides with the mass registered after the DTT-SH adsorption, showing that the bound NO was totally released during photolysis. A second irradiation period (not shown) does not lead to a further change in mass, since visible light does not break the thiol layer itself, as previously determined. As found with related nitrosothiols, a homolytic decomposition ensues, thus generating mainly NO and the thiyl radical, with a high quantum yield.^{17,18}

Once NO is photoreleased, a further addition of DTT-SNO does not lead to renitrosation of the thiol monolayer. However, if a previous treatment with DTT-SH or 2 mercaptoethanol is done, the surface can be reconstituted using DTT-SNO. This strongly suggests that the primary photoproduct, the thiyl radical, dimerizes on the surface forming the disulfide group, which does not react rapidly with DTT-SNO. By reducing the disulfide with a thiol, the surface is transformed back to the free thiol stage.

We conclude that stable monolayers of DTT-SH onto gold are easily formed in aqueous solution and that they can be used as a basement for molecular modified surfaces. Nitrosation of the surface can be achieved through addition of DTT-SNO previously prepared in the solution. The new DTT-SNO layer is thermally quite stable, but NO can be released in a controlled way by irradiating with visible light, through a process that resembles the behavior of nitrosothiols in aqueous solutions. The QCM gold-covered crystals are good substrates to investigate the chemistry of these monolayers. However, any other derivatizable material (e.g., glass) could be also used and would be a better choice to develop a molecular dispenser of NO with high spatial resolution. Further investigations on this issue are being carried out, including the use of polymeric matrixes that attach higher amounts of NO, to achieve higher efficiency in the photodelivery process.

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