Supplementary materials

Sustained Nitric Oxide Release Using Hybrid Magnetic Nanoparticles for Targeted Therapy: An Investigation via Electron Paramagnetic Resonance

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Investigation into NOC-18 stability under the original hMNP synthesis conditions

In our previously published study [7], the preparation of hybrid magnetic nanoparticles (excluding the NO donor) involved the following steps: dissolution, desolvation, heating (1 hour at 55 °C), purification using a Vivaspin centrifugal filter (100 kDa, 20 minutes × 3 cycles at 4000 rpm), and an optional freeze-drying step with sucrose as a lyoprotectant. Notably, certain steps in this protocol might accelerate nitric oxide (NO) release or contribute to the degradation of NOC-18. Heating, a critical step for cross-linking with divinyl sulfone (DVS), is particularly noteworthy.

Two alternative attempts were made to replace the standard 1-hour heating step during the nanoparticle preparation. In the first, DVS cross-linking was performed at room temperature. However, dynamic light scattering (DLS) revealed a distribution peak at ~6 nm by number, which is similar to the peak observed for the free HSA protein (Figure S1) with no indication of cross-linking. In the second attempt, the heating duration was reduced to 30 minutes, resulting in larger nanoparticles (233 ± 30 nm) with a high

polydispersity index (PDI) of 0.36, indicating incomplete cross-linking and partial particle disassembly. Both attempts showed us that 1-hour heating was critical in achieving stable and uniform nanoparticles.

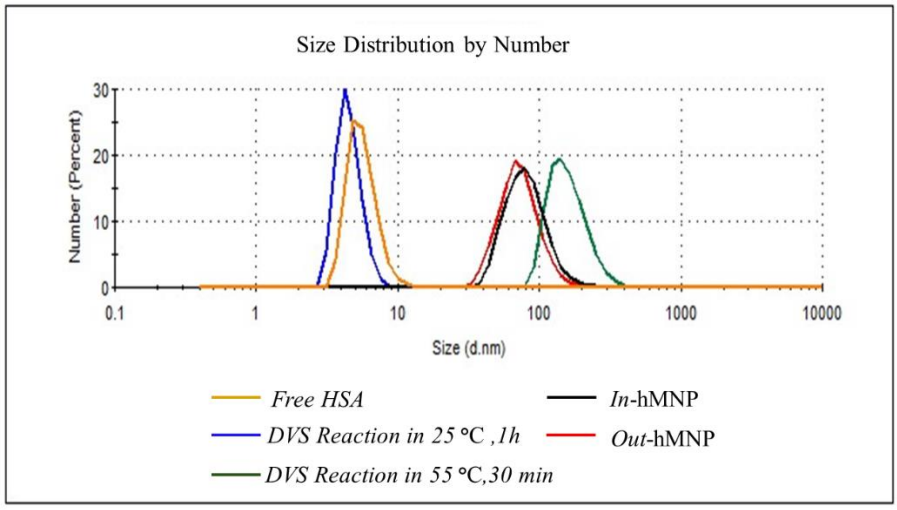


Figure S1. Size distribution (DLS) by number of *Out*-hMNPs, *In*-hMNPs, DVS reaction at 25 °C over 1h; DVS reaction at 55 °C over 30 min, and free HSA (red, black, blue, green, and orange curves, respectively).

It was also necessary to verify the stability of the NO donor following purification with the Vivaspin centrifugal cellulose filter device (100 kDa filter, 20 min × 3, 4000 rpm), as utilized in the previous study. An essential experiment was conducted to evaluate the stability of NOC-18 (at the same concentration as that applied in the reaction). The results, illustrated in Figure S2, demonstrate that NOC-18 passing the filter achieved 50% release immediately at t=0 h, after only 6 minutes of centrifugation, which is substantially shorter than the 60 minutes typically required to wash hMNPs. In contrast, the NOC-18 solution required approximately 30 minutes to achieve the same level of release. Furthermore, it is noteworthy that the NOC-18 within the filter did not reach 100% release, which is probably due to the loss of the NO within the cellulose filter, as shown in Figure S2.

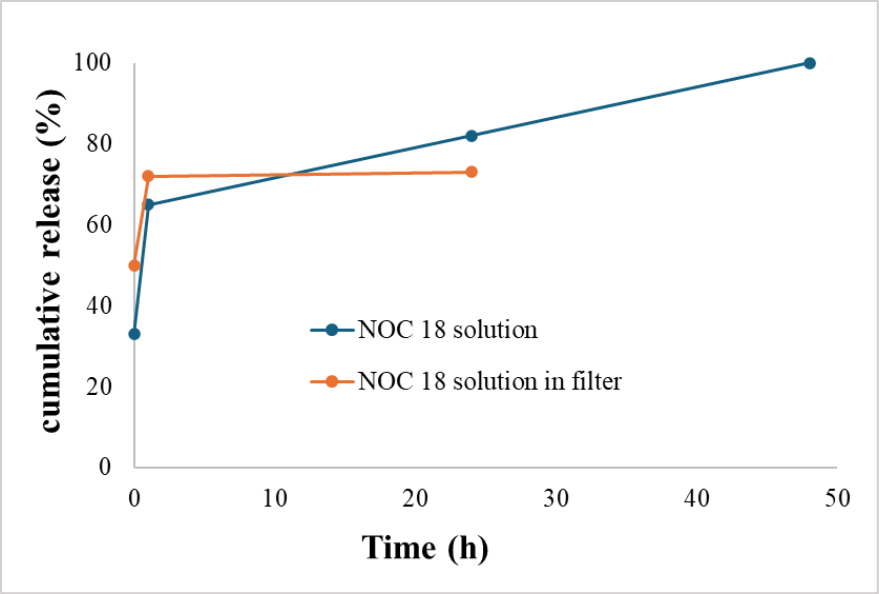


Figure S2. Cumulative release of NO from NOC-18 solution and NOC-18 purified in a filter.

Consequently, we examined the third step to assess sucrose's impact on the stability of NOC. According to the Griess assay, the simple mixing of NOC-18 and sucrose resulted in 85% release at t=0 h, indicating a rapid release (data not shown). As reported by Brodsky et al., sucrose can degrade NO donors. This suggests that either the cellulose filter or sucrose accelerates the release of NO from NOC-18 and interacts with or degrades the NO donor [35]. However, we cannot monitor this release during these steps because the entire NO amount has already been released.

A possible explanation for this phenomenon involves interference with the internal hydrogen bonds within the NOC-18 molecule. NOC-18 is a triamine molecule with pKa values ranging from ~9 to 11 [55]. In physiological conditions, most of the molecules are double-charged, which means that the N-oxide group in NOC-18 can be stabilized by two hydrogen bonds with the ammonium salt hydrogens (Figure S3). Without these hydrogen bonds, the release of NO is faster [56].

Sucrose contains primary hydroxyl groups, which can be radical and can be oxidized to aldehyde groups [57]. Aldehydes are known to react spontaneously and very quickly with primary amine groups. The NOC-18 molecule has two primary amine groups that may produce a Schiff base upon interaction with aldehydes of oxidized sucrose. Therefore, these primary amines are no longer hydrogen bond donors, leading to a higher overall effect in disrupting the internal hydrogen bonds in NOC-18.

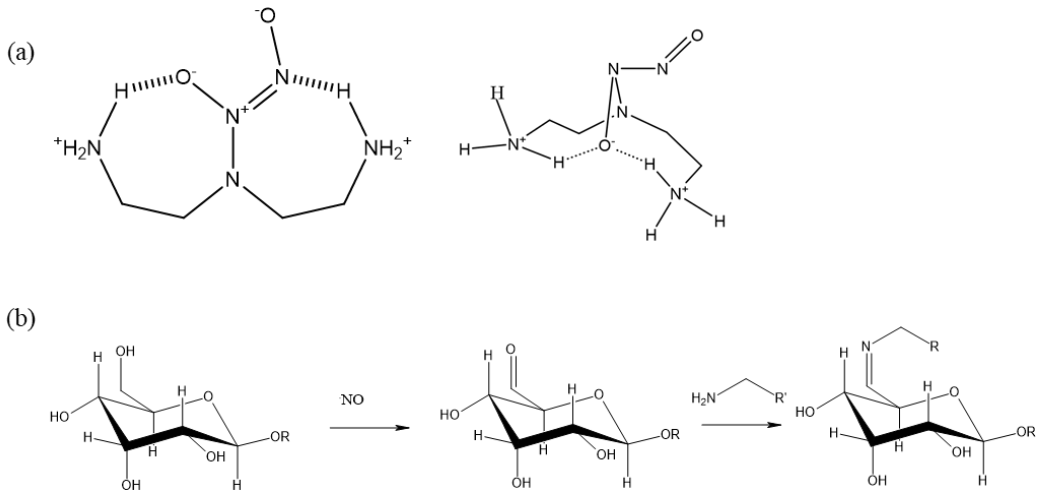


Figure S3. Possible molecular structures of stabilized NOC-18 (a) and mechanism of Schiff base formation (b).

EPR was also used to follow up on NO release. Figure 4S-a shows the EPR measurement of the NOC-18 solution with a radical trap (PTIO) initially at t=0, where there is mainly a PTIO signal. Over time, the PTI signal increases, and it stays stable up to 3 h. However, when using a cellulose filter, there is a mixed signal from both PTIO and PTI at t=0 (Figure 4S-b), indicating instant nitric oxide (NO) release. After 1 hour, the PTI signal becomes clearly visible (with seven distinct peaks), while the PTIO signal significantly weakens. After 3 hours, both signals fade. The presence of the PTI signal at t=0 suggests that the filtration process likely accelerates NOC-18 degradation. The decline in the PTI signal at t=3 is likely due to the release of a reductant into the solution, which reduces PTI to its hydroxylamine form, an EPR-silent species, thus causing the PTI signal to diminish (Figure 4S-b). When sucrose is mixed with NOC-18 solution, at t=0, there are mainly PTI signals (Figure 4c); however, after 1 hour, the signal has declined (Figure 4S-c). We suspect that here, too, sucrose enhances NOC-18 degradation, leading to the reduction of PTIO and PTI to their EPR-silent signals by the degraded products. The faster degradation of NOC-18 when mixed with sucrose, compared to its slower release using a cellulose filter, is due to immediate and uniform contact in solution, while the filter limits interaction, resulting in a more gradual release.

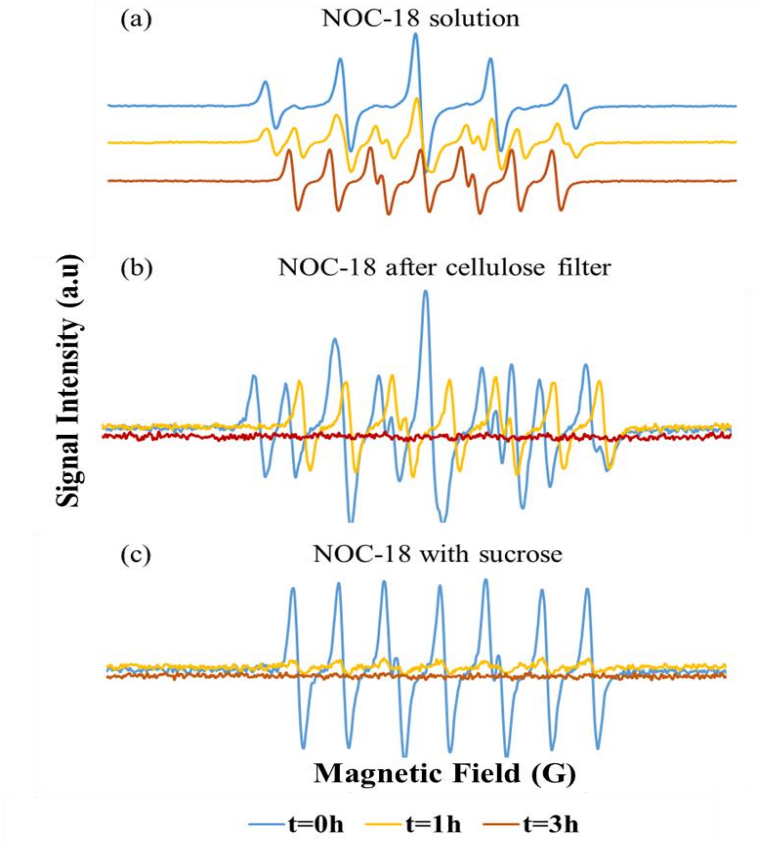


Figure S4. EPR measurement of NOC-18 solution (a); NOC-18 purified in a filter (b); NOC-18 with sucrose (c).

Reference

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