General comments

The manuscript egusphere-2026-3807, entitled “Interference from carbonaceous particles in reactive oxygen species measurements: A case study with Printex 90 and BPEAnit,” investigates the unexpected suppression of fluorescence observed when carbonaceous particles are analyzed using the BPEAnit-based Particle Into Nitroxide Quencher instrument. The authors compare Printex 90 suspensions containing the reactive probe BPEAnit, its fluorescent derivative BPEAnit-Me, and DMSO controls, and they attribute the decreased fluorescence primarily to particle–probe interactions rather than optical scattering or absorption. They further propose using BPEAnit-Me measurements and an empirical correction equation to recover the ROS-related signal. The topic is relevant to online aerosol ROS measurements because particle-induced analytical artefacts could produce substantial underestimation or even apparently negative ROS values. The concentration- and time-dependent experiments provide useful preliminary observations. However, the main mechanistic conclusion and the proposed correction approach rely on assumptions that are not experimentally validated. Equivalent adsorption behavior of BPEAnit and BPEAnit-Me has not been demonstrated, the calculated ROS signal is not independently verified, and the optical-interference controls are insufficient. I therefore recommend major revision. But I would leave that to the editor to decide.

Dear Reviewer,

We would like to thank you for your valuable comments. Based on your suggestions and comments we have conducted series of additional measurements, which have led to revised results and modified conclusions. We present first a summary for quick overview, while below we have addressed all of your comments one by one.

Summary:

- We have performed new series of experiments investigating the probe-particle interactions using the same molar concentration of BPEAnit and BPEAnitMe. The trends and observed mechanisms remained the same, however the differences between BPEAnit-Me and BPEAnit (ROS attributed fluorescence intensity) have different values
- Additional filtration measurements (with and without particles) in BPEAnit-Me confirmed that absorption of light by particles present in solutions is not the main mechanism for the decrease in fluorescence intensity
- Due to particle-probe suppression, ROS generated by Printex90 must be assessed after allowing the time for the reaction (1-3h), which is not captured by online methods.
- We suggest, in parallel to online measurements, a collection of the particles on filters for subsequent offline ROS/OP assessment with correction for observed artefacts.
- A method (first approach) to correct for the carbonaceous particle-induced fluorescent decrease for offline ROS measurements with BPEAnit, namely by using a stable derivative of BPEAnit (i.e. BPEAnit-Me) is suggested
- Our results indicate that using BPEAnit as a probe for offline ROS and OP determinations is possible and should be explored

Once again thank you for your comments and suggestions. We have responded to all of your comments point by point below.

Authors of the manuscript

Major Concerns

1. The proposed correction assumes that BPEAnit and BPEAnit-Me experience identical particle–probe interactions because of their structural similarity. I cannot follow this assumption without direct experimental evidence. The two compounds are used at substantially different concentrations—4 µM BPEAnit versus 130 nM BPEAnit-Me—and they differ in nitroxide functionality, electronic structure, radical character, and potentially surface affinity. Matching their initial fluorescence intensities based on quantum yields does not establish equivalent adsorption, quenching, or surface-site competition. This issue is especially important because adsorption onto carbonaceous particles is commonly concentration dependent and may become nonlinear when surface sites approach saturation. The authors themselves acknowledge that the different probe concentrations may cause differential occupation of adsorption sites and overestimation of the calculated ROS response. Therefore, the current correction equation cannot be considered quantitatively reliable.

Thank you for your comments. We would like to start by clarifying structural similarity of BPEAnit and BPEAnit-Me; below we include schematics of the chemical structure of BPEA and BPEAnit and the fluorescence mechanism leading to BPEAnit-Me. It is included as Fig.1 in the revised manuscript.

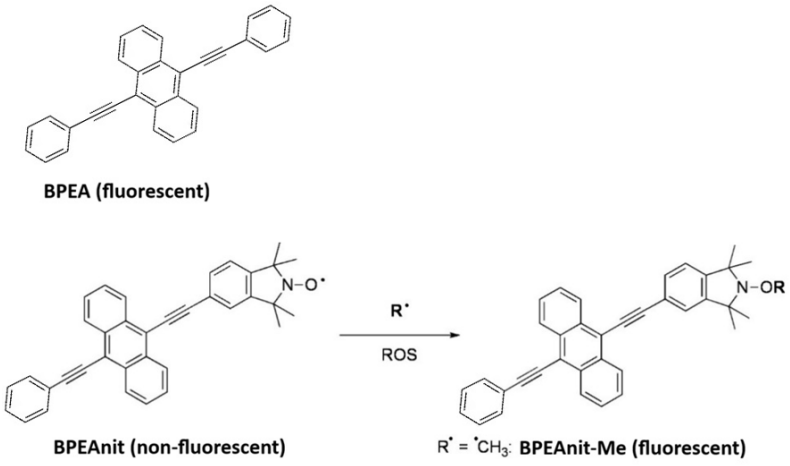



**Figure 1.** Adapted from Elkaee et al., 2025: Chemical structure and fluorescence description of BPEA, BPEAnit, and BPEAnit-R, including the reaction with ROS that elicits BPEAnit-R (primarily BPEAnit-Me).

We have improved the description of BPEAnit in the introduction. It now reads:

*"PINQ uses the chemical probe BPEAnit dissolved in dimethyl sulfoxide (DMSO) (Brown et al., 2019b). BPEAnit is a pro-fluorescent nitroxide that is sensitive to a broad range of ROS and is stable to auto-oxidation (Stevanovic et al., 2012). BPEAnit contains a nitroxide group (Fig. 1), which quenches the fluorescence of the molecule. When BPEAnit reacts with ROS, the nitroxide group undergoes either reduction or oxidation, which eliminates the quenching effect, thereby restoring fluorescence. The assay's sensitivity is enhanced by using dimethyl*

*sulfoxide (DMSO) as a solvent, which acts as a mediator in reactions between the BPEAnit probe and various ROS. The reaction is rapid due to a diffusion-limited mechanism, making it suitable for online analysis. The BPEAnit-reacted molecule becomes fluorescent as a stable derivative (primarily the methylated derivative BPEAnit-Me, see Fig. 1), allowing for ROS quantification. BPEAnit-Me is typically synthesized to generate a calibration curve for PINQ, which is then used to calculate the equivalent ROS concentration from the PINQ measurements"*

Addressing the comment: We do think that the similarities between the two probes molecules make it highly probable that the interactions between probe and soot are equivalent. We have performed additional experiments (Fig A3 in the revised manuscript) that show clear similarities between BPEAnit-Me and BPEA, indicating that it is the fluorophore part of the molecule that is responsible for the interactions with the particle surface, presumably through π-π-interactions. To our knowledge there isn't any experimental procedure that would prove the role of the nitroxide group and the radical in surface interactions, therefore, we mention this as a caveat in the discussion.

*"Note that this is a first attempt at correcting for the observed artefact, and further investigations are required to validate the correction method and the equations "*

*"No experimental validation was performed to confirm that the newly formed fluorescent product interacts with the particles to the same extent as the probes, however, this is considered likely due to their chemical similarities under normal operating conditions."*

*"The decrease in fluorescence for BPEA was very similar to that of BPEAnit-Me (Fig. A3), indicating that the reduction in fluorescence is due to interaction with the fluorophore, and not the nitroxide."*

We are aware that matching the quantum yield does not guarantee identical particle-probe interaction (whether it is adsorption or other mechanisms).

After your highly relevant feedback, we repeated all experiments with equal probe concentration (4 µM), which have led to revised resultsand changed correction method. The relative fluorescence for BPEAnit-Me became higher compared to before, when we used 130 nM as probe concentration. The difference between BPEAnit-Me and BPEAnit became much lower, and a clear difference in fluorescence (that we attribute to ROS formation) was only seen after extended residence time. The correction and the discussion regarding its interpretation have now been changed accordingly.

2. I am confused by the relationship between Equation 1, Figure 4, and Figure 5. Equation 1 uses two normalized fluorescence ratios and therefore produces a dimensionless quantity. For the example presented in Figure 4, values of approximately 0.84 and 0.63 would yield a corrected value of roughly 0.33. However, Figure 5 reports corrected fluorescence values in arbitrary units ranging from approximately (10^4) to above (10^6). The manuscript does not explain how the dimensionless output from Equation 1 was transformed into these absolute

fluorescence values. This inconsistency prevents reproduction of the analysis and raises concerns about whether the correction was applied to normalized or raw fluorescence data. The problem becomes particularly severe at 25 µg mL-1, where the BPEAnit-Me denominator approaches zero and the corrected value becomes mathematically unstable. The authors acknowledge this singularity but nevertheless present the resulting values as meaningful ROS responses.

The connection between the equation and these two figures, and the change of units, was improved. Thank you for making us aware of this issue. In the revised manuscript, we have now introduced a set of new equations, which shows how the normalization was done (Eq. 2 and 3), and the connection between counts and relative fluorescence (Eq. 6). All equations have been moved to the Method section. Figure 6 has been updated to visualize the equations. With the higher concentration of BPEAnit-Me used in our new experiments, the probe depletion (denominator close to zero) was not as apparent as before. Furthermore, in our suggested correction method (described in the Discussion), we say that you should not use the equations if the remaining fraction of BPEAnit-Me is below 80 % of fluorescence intensity of particle free BPEAnit-Me, since this could give unreliable results. As mentioned above, this should be seen as a first attempt to introduce a method for correcting and not as concluded results of the ROS formation potential of Printex 90, our main objective for this study was describing the observed effect.

3. The authors claim that the higher fluorescence observed for BPEAnit relative to BPEAnit-Me represents ROS generated by Printex 90. However, no independent measurement confirms that the difference is caused by ROS. Differential adsorption, static or dynamic fluorescence quenching, electron transfer, chemical reduction of the nitroxide, or different surface-induced changes in quantum yield could also produce unequal responses.The argument that Printex 90 generates ROS in other assays does not demonstrate that the observed difference in the present DMSO/BPEAnit system is specifically due to ROS. The manuscript currently treats this interpretation as established rather than hypothetical.

To answer your question we would like to go back to our explanation to your first question i.e. about the structural similarity of the probes used. This clarifies why increased fluorescence in BPEAnit and BPEAnit-Me is caused by ROS, in the manuscript we also refer to articles with details about how the probe and its stable derivative was developed and tested. After updating the experiments and using the two probes at identical concentrations, the results show smaller differences between the fluorescence intensity. Based on additional measurements performed we provide evidence that ROS attributed fluorescence is a major part of the difference. Over time, we see that the fluorescence from the BPEAnit-particle-suspension becomes higher than particle-free solution, indicating that the probe has reacted with the particles.

4. The manuscript repeatedly concludes that fluorescence suppression is primarily caused by adsorption or “ad/absorption” of the probes onto Printex 90 surfaces.

However, the experiments only demonstrate a decrease in fluorescence; they do not measure adsorption or probe depletion directly. Fluorescence loss alone cannot distinguish among adsorption, fluorescence quenching without adsorption, electron transfer, chemical transformation, aggregation-induced effects, or photochemical degradation. The supporting literature on adsorption of aromatic compounds or fluorescence quenching by graphene does not establish the mechanism for BPEAnit and BPEAnit-Me under the present conditions. Nevertheless, the conclusion states that probe inactivation by particle adsorption is the primary mechanism.

We agree, we relied mainly on previous experience and knowledge of most likely soot particles surface interaction, however we cannot prove it, hence in the revised manuscript we call it "suppressed fluorescence" or "interaction" as suggested (thank you) and mention adsorption as one of the possible mechanism that can contribute

The photochemical stability of BPEAnit and BPEAnit-Me is well studied (Wang et al., 2025) and is not likely cause of the reduction in fluorescence.

In the Discussion we mention this as a limitation and emphasize the need to investigate this further:

*"Adsorption of the probe to particle surfaces is one hypothesized mechanism of probe suppression, but further studies are needed to conclude the reduction mechanism"*

5. The authors use DMSO with and without Printex 90 to conclude that scattering and absorption are negligible. I cannot follow this logic because DMSO itself has essentially no relevant fluorescence emission comparable to the probe. Comparing two weak DMSO spectra does not directly determine whether Printex 90 attenuates the 450 nm excitation beam or absorbs/scatters BPEAnit emission near 483 and 515 nm. Inner-filter effects must be evaluated in a fluorescent matrix, not solely in the nonfluorescent solvent. Moreover, the optical assessment was not systematically performed across all three particle concentrations. Carbon black is a strong broadband absorber, and even modest attenuation could become significant at 25 µg mL-1 or after deposition inside the flow cell.

Figure A1 was included to show that there is no sudden drop in incoming laser light, although it is not the best representation due to the limited wavelength covered. In the revised manuscript we have removed the graph, and instead added results from additionally performed filtration experiments for three different concentrations of Printex90 in BPEAnit-Me (Table A1 and Fig A1). The result show very little difference in fluorescence between filtered and unfiltered suspension. Deposition on the inside of the flow cell has not been viewed as an issue, since the repeated experiments with particle-free solution would allow us to detect such anomalies. The flow-through cell was also rinsed and checked continuously to control for potential deposition. In the methods, we have now described this in more detail.

There is also a new figure (Fig. A3), that shows fluorescence at different probe concentrations. The probe depletion is apparent and dependent on probe concentration for given Printex 90 concentrations. If absorption was the main mechanism the fluorescence level would remain at the same level for a given particle concentration regardless of probe concentration.

6. The comparison between filtered and unfiltered BPEAnit-Me suspensions is interpreted as evidence that optical effects account for only approximately 3% of the fluorescence reduction. This experiment is not sufficient for that conclusion. First, filtration through a 0.22 µm membrane does not guarantee complete removal of Printex 90 primary particles or smaller aggregates. No post-filtration particle concentration, turbidity, absorbance, or size distribution is reported. Second, if BPEAnit-Me had already adsorbed onto particles before filtration, removal of the particles would also remove the adsorbed fluorophore. Consequently, the fluorescence would not return to its initial value even if optical attenuation had contributed substantially before filtration. The authors later acknowledge that removing particles does not restore fluorescence, but this observation cannot by itself discriminate adsorption from other irreversible processes.

   Particles passing through the 0.22 µm filter are expected to be predominantly smaller than 0.22 µm. At these sizes, their contribution to light absorption or scattering is expected to be limited. Therefore, we claim that the loss of fluorescence seen in the filtered suspensions is due to suppressed fluorescence, and not optical attenuation. In revised manuscript, we have added data from filtration experiments with three particle concentrations, and the results are consistent and donot show Printex 90 concentration dependence. We revised the results section and the description of possible optical effects have changed based on results from the additional measurements, which prove that optical effects are not the major mechanism of fluorescence reduction. As mentioned above, it is correct that we cannot prove that adsorption, and not other irreversible processes, is the reduction mechanism. This is now changed throughout the revised manuscript.

7. The concentration of BPEAnit-Me was selected to reproduce the baseline fluorescence intensity of 4 µM BPEAnit. While this facilitates visual comparison, it does not establish equivalent analytical behavior. The authors appear to assume that matching the baseline signal and approximately compensating for the quantum-yield difference are sufficient to compare particle-induced losses. This approach neglects differences in detector linearity, fluorescence lifetime, molar absorption coefficient, adsorption affinity, concentration quenching, and the response of each compound to the local carbon surface environment. A 30-fold concentration difference may also lead to very different surface coverage. In addition, the manuscript does not report complete calibration curves, limits of detection, linear ranges, or instrument stability for either compound.

   As mentioned above, we have now made the measurements at equal particle concentration to avoid concentration-dependent differences in surface

adsorption (or other particle-probe interactions). In the updated manuscript we emphasize that the correction method is only a first approach to account for this artefact.

Calibration curves from earlier papers (Miljevic et al., 2010) and standard calibration show that BPEAnit and BPEAnit-Me are in the linear range at their current concentrations. Molar absorption coefficient is similar for BPEAnit and BPEAnit-Me(Fairfull Smith, 2008). Fluorescence lifetime is not relevant here as we have continuous excitation and steady-state fluorescence.

8. The same suspensions were measured repeatedly at 5 min, 30 min, 1 h, 2 h, 3 h, and 24 h. However, the PINQ measurement cell continuously withdraws liquid at 1 mL min-1, and the measured liquid is discarded. Each measurement lasts at least two minutes. It is therefore unclear how repeated sampling affected the remaining volume, particle concentration, probe concentration, headspace, and oxygen availability. The suspensions were also repeatedly vortexed, which can alter aggregate size, expose new particle surface area, and increase oxygen transfer. I am also unable to determine whether particle-free BPEAnit and BPEAnit-Me controls were measured at every corresponding time point. Normalization to a single initial blank would be inappropriate if the probes undergo storage-related fluorescence drift or auto-oxidation over 24 h.

   Repeated sampling does not alter the response from the control samples, which were measured as frequently as the particle suspensions. We have never encountered any issues arising from repeated sampling.

   Based on our experience, the vortexing does not affect the suspensions in such a way that unreliable results are attained. The sonication of the stock suspension is the main method of de-aggregation. The vortexing is done to ensure a homogenous suspension (for shorter residence time it is probably unnecessary, but there is a risk for some sedimentation over a time span of hours). However, we have never experienced differences in the results depending on time of vortexing. Despite that, we kept the vortexing time consistent between all experiments. Moreover, all methodology was identical between BPEAnit, BPEAnit-Me and BPEA, minimizing the risk of unknown factors influencing the result.

   In the Method section in the revised manuscript, we have now clarified how the particle-free solutions were measured. No single, initial blank was used, instead they are measured before and after each set of experiments (1,5 and 25µg/mL), and the average are used as normalization value.

9. The authors state that measurements performed within five minutes are comparable to PINQ measurements, although the online instrument reportedly analyzes the aerosol within approximately one minute after collection and mixing. Given that the particle–probe interaction changes rapidly during the initial period, a fivefold difference in reaction time may be analytically important. The manuscript does not provide measurements at the actual PINQ residence time. Manual suspension experiments also do not reproduce aerosol collection, mixing efficiency, particle deposition, tubing losses, cell fouling, or possible memory

effects during continuous operation. Carbonaceous particles deposited on tubing or the optical cell could progressively reduce the signal and might be mistaken for solution-phase probe interactions.

The experiments with suspensions that are presented in the manuscript are different to PINQ analysis in several ways. We were not claiming that the performed experiments are exactly the same as for online PINQ measurements. However, the observed and described effect of Printex 90 particles on BPEAnit is relevant to understand when using PINQ,since that effect is visible during PINQ operation at certain circumstances. The different conditions between our suspension test and PINQ measurements are stated as a limitation of the study in the discussion.

This study is exploratory with the use of available equipment and methods. The practical work with dilution of the suspensions and placement in the measurement cell, together with flow paths in the tubing, unfortunately makes the residence time longer than online PINQ conditions. We have remade all experiments, and decreased the time between addition of particles and measurements to a minimum (90 seconds). We are not aware how these measurements (which require manual sample preparation) could be made within the same time scale as online/real-time measurements with PINQ. During our experiments, we measured each suspension over a period of two minutes, to ensure a stable response, already this is longer than the time between mixing of the aerosol and probe liquid in PINQ, where the measurements are done on a continuous liquid flow. We clarify in the revised manuscript that 2 min is the shortest time we physically could do the measurements – due to manual work needed.

*"The measurements presented in Fig. 3 and 4 were performed within two minutes after adding Printex 90 to the probe solutions. This is comparable timescale to PINQ measurements, in which analysis is made within one minute after collection and mixing of the aerosol in the BPEAnit solution. The difference of two minutes in this study versus one minute in PINQ is due to practical issues, i.e. manual sample preparation and the duration of each measurement (two minutes to ensure a stable response). However, the observed effect and its underlying mechanisms remain relevant for PINQ measurements."*

As mentioned above, the recurring tests with particle-free solution would allow us to detect issues from particle deposition and the cleaning procedure described in the Method section is applied to both offline suspension tests and online PINQ measurements:

*"To prevent particle deposition, the tubing and the measurement cell are rinsed with solvent (DMSO) before and after each experiment. Additionally, the flow-through cell is manually cleaned, and the Teflon tubing is replaced at regular intervals."*

10. The hypothesized mechanism depends on particle surface area, aggregation, surface functional groups, and dispersion behavior, yet the manuscript reports only the manufacturer, stock mass concentration, bath sonication time, and temperature limitation. No characterization of Printex 90 in DMSO is provided.

Important information includes primary particle size, aggregate-size distribution after sonication, BET surface area, oxygen-containing surface groups, elemental composition, zeta potential where applicable, and suspension stability over the experimental timescale. Particle settling or progressive aggregation could change the accessible surface area and produce apparent temporal kinetics unrelated to probe chemistry. Preparing a new stock suspension for each replicate may also introduce substantial between-replicate variability if dispersion energy and aggregate breakup are not controlled quantitatively.

Unfortunately, we do not have the possibility to perform characterization tests on the suspension of Printex 90. However, we have added the available information on the Printex 90, provided by the supplier, and measured by our colleagues performing different experiments with Printex90 namely primary particle size and BET surface area. The lack of characterization of the suspension is mentioned as a limitation in the discussion section.

*" This study would also have benefited from measurements of the particle sizes in suspension, to ensure equal particle distribution and aggregation states of Printex 90 between experiments."*

If we were to keep the same stock suspension between all replicates, we would have lower variability. The same is true of the probe stock solutions. However, we did not aim for lowest variability, but decided to do new stock suspensions and solutions to capture differences between stocks and issues that could emerge in preparing the stock.