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# Peer Review File

# Inhibiting the Formation of Neutrophil Extracellular Traps to Prevent the Recurrence of Post-operative Glioblastoma

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have satisfactorily addressed the majority of my concerns. Nevertheless, a few remaining issues still necessitate further clarification.

1. I must emphasize that the authors appear to have either overlooked or misinterpreted my prior comments regarding the contradiction between the peroxidase (POD)-like activity and •OH depletion capability of CS nanozyme, as raised in my two previous reviews. To clarify, POD catalyzes the conversion of H2O2 into •OH, thereby increasing ROS levels. It is perplexing that the authors simultaneously claim CS nanozyme can both eliminate •OH and exhibit significant POD-mimicking activity. Therefore, the authors are required to provide a clear, rational, and logically consistent explanation for this apparent contradiction.
2. The high-resolution TEM image presented in Figure 3b clearly reveals that the CS nanozyme exhibits a well-defined crystal structure with distinct atomic lattice fringes. In contrast, the XRD pattern suggests a relatively weak crystalline state of the CS nanozyme. These inconsistent results necessitate a more comprehensive explanation or revision, supported by appropriately designed supplemental experiments.
3. The western blot bands of P38 and p-P38 in Figures S19 and S14 appear to lack significant differences. Therefore, the authors are required to carefully re-examine the corresponding raw images to ensure the accurate presentation of the western blot results and prevent any potential academic misconduct.
4. The authors are requested to provide a well-substantiated explanation for the comparable LDH release from neutrophils in the first two groups depicted in Figure S14c: the former, which received no treatment, and the latter, which was exposed to LPS. Furthermore, the authors are required to elucidate the distinctions in treatment between the second and fifth groups in Figure S14c, as well as the underlying cause of the wide difference in outcomes between these two groups.

Reviewer #3

(Remarks to the Author)
The authors have addressed all my concerns and questions by providing more data. This manuscript should be considered accept by Nature Communications.

Reviewer #4

(Remarks to the Author)
This reviewer assumes that NP were detected using a anti-IgG-AF647 antibody. This information is missing in the legend. Thank you for the information!

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I support the acceptance of this manuscript in its current form.

Reviewer #1:

The authors have satisfactorily addressed the majority of my concerns. Nevertheless, a few remaining issues still necessitate further clarification.

**Q1.** I must emphasize that the authors appear to have either overlooked or misinterpreted my prior comments regarding the contradiction between the peroxidase (POD)-like activity and •OH depletion capability of CS nanozyme, as raised in my two previous reviews. To clarify, POD catalyzes the conversion of H2O2 into •OH, thereby increasing ROS levels. It is perplexing that the authors simultaneously claim CS nanozyme can both eliminate •OH and exhibit significant POD-mimicking activity. Therefore, the authors are required to provide a clear, rational, and logically consistent explanation for this apparent contradiction.

**A1.** We sincerely appreciate the reviewer for keenly pointing out the potential contradiction in the description of POD-like activity of CS nanozyme. Therefore, we re-assayed the POD activity of CS nanozyme using a fresh POD assay kit (Beijing Solarbio Science & Technology Co., Ltd., BC0095), and compared results with that of horseradish peroxidase (HRP), which is a natural peroxidase (POD). The results revealed that CS nanozyme (Cu:Se = 0.57:1) did not show detectable POD activity, but HRP exhibited strong POD activity (Figure R1a).

To investigate the dependence of POD activity of CS nanozymes on their composition, we prepared different CS nanozymes by reducing the mass of κ-selenocarrageenan to change the initial Cu:Se molar ratio from 1:2 (reported ratio in the manuscript) to 1:1 and 1:0.5, respectively. As shown in Figure R1b, the resultant three kinds of CS nanozymes exhibited well-defined crystalline lattices in high-resolution TEM (HRTEM) images. Their actual Cu:Se ratios were 0.57:1, 1.04:1, and 1.71:1, respectively, as determined by ICP-OES analysis. We then evaluated their peroxidase (POD) activity using HRP as a positive control. The CS nanozymes (Cu:Se = 1.71:1, and 1.04:1) showed obviously POD activity, and the one (Cu:Se = 0.57:1)

exhibited no detectable activity (Figure R1c). Notably, after reaction for 4 h, the CS nanozyme (Cu:Se = 1.71:1) exhibited significantly higher POD activity than the other two counterparts (Figure R1d). Their activity difference can be attributed to their different Cu:Se ratio, *i.e.* the higher copper content in CS nanozyme, the stronger POD activity.

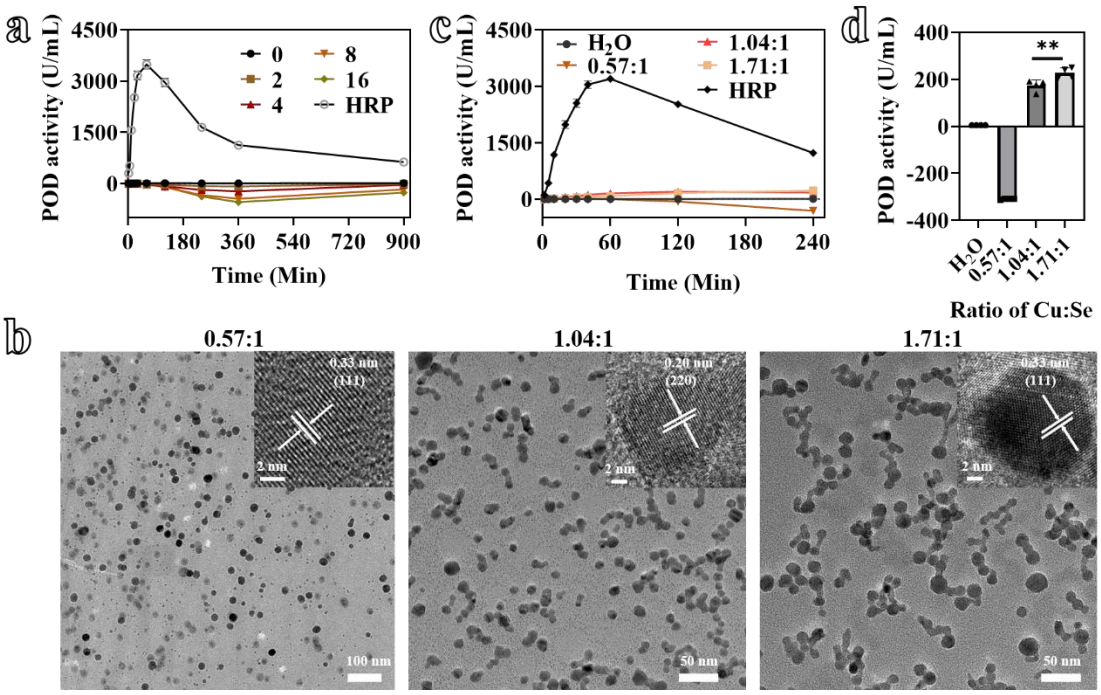


**Figure R1.** (a) Characterization of POD activity of CS nanozyme (Cu:Se = 0.57:1) with different concentrations (0, 2, 4, 8, 16 μg/mL). A diluted HRP solution (1:100) was used as a positive control. (b) TEM image of three different CS nanozymes with an inset of their high-resolution image. (c) Characterization of POD activity of CS nanozymes with varying Cu:Se ratios (1.71:1, 1.04:1, and 0.57:1). (d) Comparison of POD activity of three kinds of CS nanozymes after reaction for 4 h.

To clarify whether the CS nanozyme (Cu:Se = 0.57:1, molar ratio reported in the manuscript) produces or scavenges •OH radicals, we deliberately excluded the influence of $Fe^{2+}$ on the performance of CS nanozyme by two methods. The first method is that we utilized HRP to react with $H_2O_2$ for generation of •OH radicals, rather than the Fenton-reaction between $Fe^{2+}$ and $H_2O_2$. 3,5,3′,5′-Tetramethylbenzidine (TMB) as a POD substrate and an indicator was added into solution to result in a characteristic color

change from colorless to blue upon oxidation (Figure R2a-b). Measurement of absorbance at 652 nm revealed very low solution signals for the $H_2O$ + TMB, $H_2O_2$ + TMB, and $H_2O_2$ + CS + TMB groups. In contrast, the $H_2O_2$ + HRP + TMB group of solution exhibited a significant increase in absorbance at 652 nm accompanied by a distinct color change into deep blue, indicating generation of substantial •OH radicals by HRP. Importantly, after the addition of CS nanozyme (*i.e.* the $H_2O_2$ + CS + HRP + TMB group), the solution absorbance at 652 nm decreased by 29.1% compared to that of the $H_2O_2$ + HRP + TMB group. This result demonstrates the capability of CS nanozyme (Cu:Se = 0.57:1) scavenging •OH radicals.

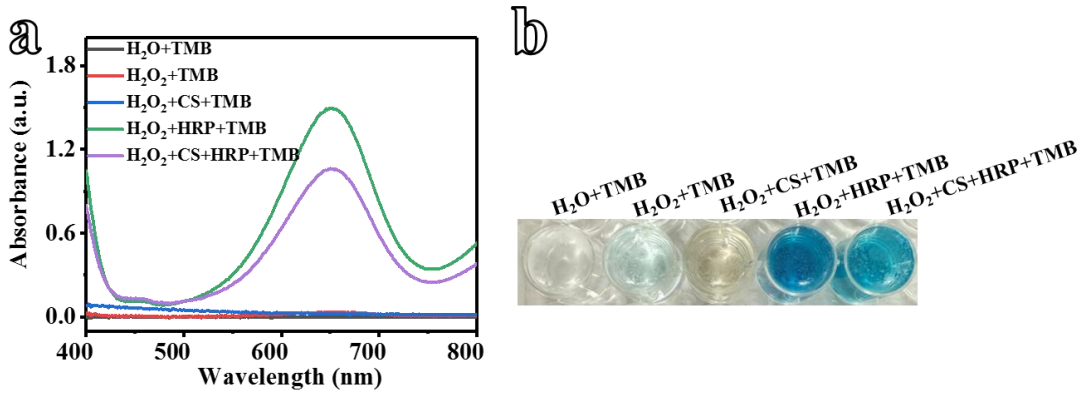


**Figure R2.** (a) Capability of CS nanozyme (Cu:Se = 0.57:1) scavenging •OH radicals assayed by measuring the UV–Vis–NIR spectra of different solutions (*i.e.* $H_2O$ + TMB, $H_2O_2$ + TMB, $H_2O_2$ + CS + TMB, $H_2O_2$ + HRP + TMB, and $H_2O_2$ + CS + HRP + TMB). •OH radicals were generated by the reaction between $H_2O_2$ and HRP, and indicated by TMB solution. The $H_2O_2$ + HRP + CS + TMB solution consisted of 3 mL PBS (pH 3.0), 31 μM $H_2O_2$, 1.2 mM TMB, 1:100 diluted HRP, and 1 μg/mL CS nanozyme. (b) Photograph of different solutions.

The second method is that we irradiated $H_2O_2$ with UV light (λ = 254 nm) to generate •OH radicals, and then detected them with TMB. We validated the generation of •OH radicals by UV irradiation under different pH conditions, and the capability of CS nanozyme scavenging them (Figure R3a-g). The results demonstrate that (1) •OH radicals can be easily generated by UV irradiation in a range of pH 1–5, especially under strong acidic condition (pH < 3); (2) CS nanozyme (Cu:Se = 0.57:1) exhibits

strong capability of scavenging •OH radicals. All the above results are consistent with our data shown in the manuscript (Figure R4a-b; Figure S11g-h), demonstrating that CS nanozyme (Cu:Se = 0.57:1) can effectively scavenge •OH radicals.

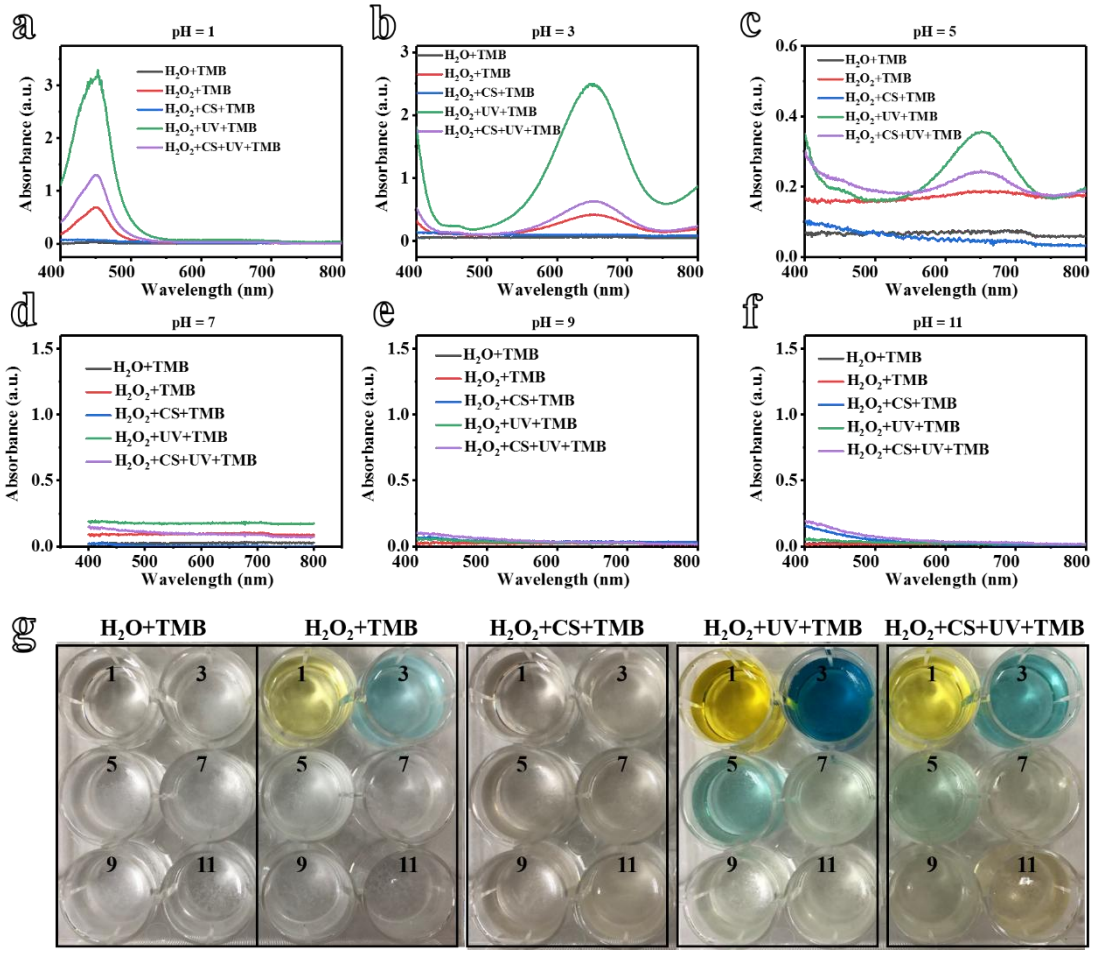


**Figure R3.** (a-f) Capability of CS nanozyme (Cu:Se = 0.57:1) scavenging •OH radicals assayed by measuring UV–Vis–NIR spectra of different solutions with different pH values (*i.e.* $H_2O$ + TMB, $H_2O_2$ + TMB, $H_2O_2$ + CS + TMB, $H_2O_2$ + UV + TMB, and $H_2O_2$ + CS + UV + TMB). •OH radicals were generated by irradiation of $H_2O_2$ with UV light (λ = 254 nm) and indicated by TMB solution. The $H_2O_2$ + CS + UV + TMB solutions with different pH values consisted of 3 mL PBS (pH 1, 3, 5, 7, 9, 11), 31 μM $H_2O_2$, 1.2 mM TMB, and 1 μg/mL CS nanozyme. (g) Photograph of different solutions.

We also used Cell Meter Mitochondrial Hydroxyl Radical Detection Kit (MHRD) to detect the •OH radicals produced in the mitochondria of neutrophils from different groups. Following LPS stimulation, an intense red fluorescence (indicating •OH

accumulation) was observed in the mitochondria of neutrophils. The red fluorescence was significantly weakened in the mitochondria of neutrophils co-cultured with LPS and CS nanozyme (LPS + CS group, Figure R4c). Collectively, these findings solidly demonstrate that CS nanozyme (Cu:Se = 0.57:1) can effectively scavenge •OH radicals.

An important finding of this study is that CS nanozyme acts as a potential anti-oxidative nanozyme to inhibit the formation of neutrophil extracellular traps (NETs) by scavenging reactive oxygen species (ROS), especially •OH radicals. The ROS scavenging mechanism might be explained by the favourable redox cycle between the oxidation of $HSeO_3^-$ in the surface ligand into $HSeO_4^-$ by ROS and the reduction of $HSeO_4^-$ into $HSeO_3^-$ by $Se^{2-}$ from the CS nanozyme (Figure R4d, Figure S11b in the manuscript), which was proved by the results of FTIR and XPS spectra in Figure R4e-f (Figure S11e-f in the manuscript).

We have revised the manuscript and deleted the related contents of POD-like activity of CS nanozyme (Cu:Se = 0.57:1). These modifications significantly improve the accuracy and scientific validity of the description of CS nanozyme. We very appreciate the reviewer for this valuable and constructive feedback.

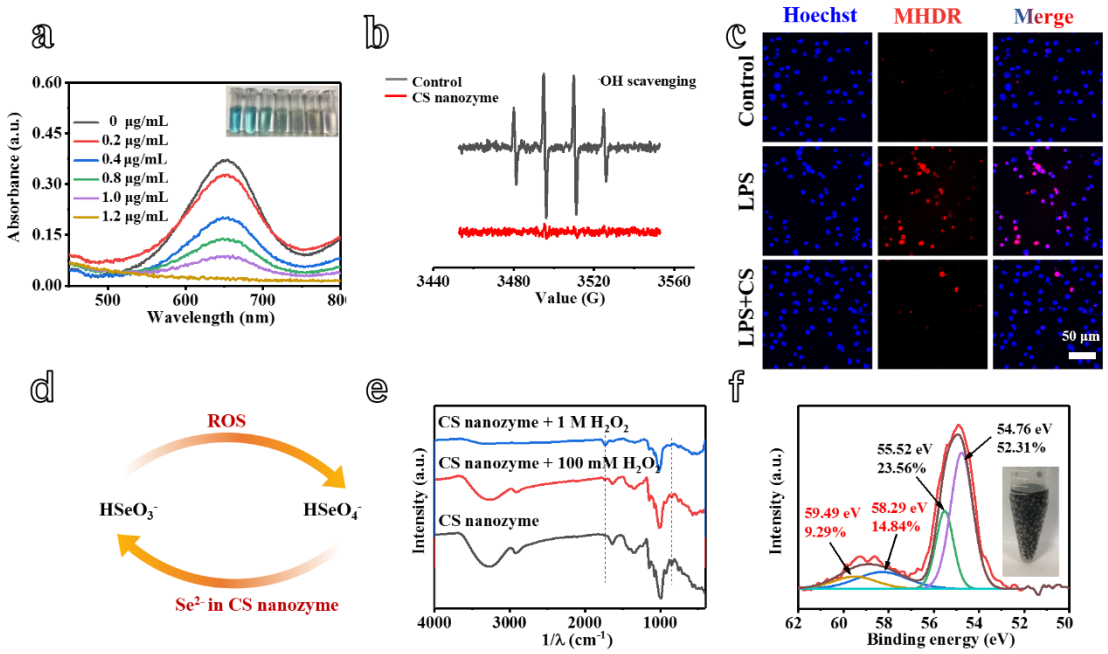


**Figure R4.** Capability of CS nanozyme (Cu:Se = 0.57:1) scavenging •OH radicals. (a–b) Depletion of •OH radicals assayed by measurements of UV–Vis–NIR absorbance and ESR. (c) CLSM images of intracellular •OH radicals in neutrophils detected by

using Cell Meter Mitochondrial Hydroxyl Radical Detection Kit (MHRD). (d) Proposed ROS scavenging mechanism. (e) FTIR spectra of CS nanozyme before and after reaction with different concentrations of $H_2O_2$. (f) XPS spectra of CS nanozyme after reaction with 1 M $H_2O_2$ (insert: photograph of solution showing the generation of bubbles during the reaction)

**Q2.** The high-resolution TEM image presented in Figure 3b clearly reveals that the CS nanozyme exhibits a well-defined crystal structure with distinct atomic lattice fringes. In contrast, the XRD pattern suggests a relatively weak crystalline state of the CS nanozyme. These inconsistent results necessitate a more comprehensive explanation or revision, supported by appropriately designed supplemental experiments.

**A2.** Thank you very much for the comment. The inconsistence between the XRD pattern of nanoparticle powder (indicating weak crystalline characteristics) and the well-defined crystal structure of individual nanoparticles may be attributed to excessive kappa-selenocarrageenan in the powder. We removed kappa-selenocarrageenan through centrifugation (20,000 × g, 20 min × 2 cycles) and re-examined the XRD pattern of the purified sample (Figure R5; Figure S10d in the manuscript). The diffraction peaks were well-matched to those of cubic berzelianite ($Cu_{2-x}Se$, JCPDS Card No. 06–0680).

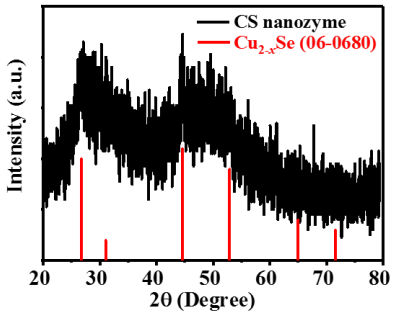


**Figure R5.** XRD pattern of CS nanozyme in comparison with that of cubic berzelianite ($Cu_{2-x}Se$, JCPDS Card No. 06–0680).

**Q3.** The western blot bands of P38 and p-P38 in Figures S19 and S14 appear to lack significant differences. Therefore, the authors are required to carefully re-examine the corresponding raw images to ensure the accurate presentation of the western blot results and prevent any potential academic misconduct.

**A3.** Thank you very much for the comment. We repeated experiments to investigate the influence of LPS and CS treatments on the expression of p38. The results showed that the phosphorylation of p38 proteins increased after the addition of LPS to neutrophils but decreased after co-culturing with the CS nanozyme (Figure R6). We have corrected and added new results in Figure S14 and Figure S19.

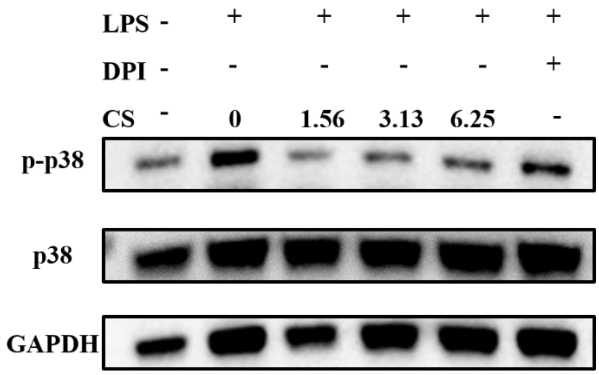


**Figure R6.** Western blot analysis of p-p38 in different groups of neutrophils. Neutrophils were treated with LPS and co-cultured with different concentrations of CS nanozyme (1.56, 3.13, 6.25 μg/mL). DPI was used as the negative control.

**Q4.** The authors are requested to provide a well-substantiated explanation for the comparable LDH release from neutrophils in the first two groups depicted in Figure S14c: the former, which received no treatment, and the latter, which was exposed to LPS. Furthermore, the authors are required to elucidate the distinctions in treatment between the second and fifth groups in Figure S14c, as well as the underlying cause of the wide difference in outcomes between these two groups.

**A4.** Thank you very much for the comment. The comparable LDH release observed in the first two groups (Figure S14c in the original manuscript) might be due to insufficient rupture of the neutrophil cell membrane. Consequently, we increased both the LPS

concentration (to 60 μg/mL) and culture time (to 24 hours) to assess the release of LDH again (Figure R7). The results showed that the LDH release ratio in the LPS group was twice that of the untreated group, and CS nanozyme showed excellent inhibitory effect.

The fifth group in Figure S14c in our previous version of manuscript should be the group of neutrophils co-cultured with LPS and CS nanozyme (6.25 μg/mL). We are sorry for this mistake and have corrected it.

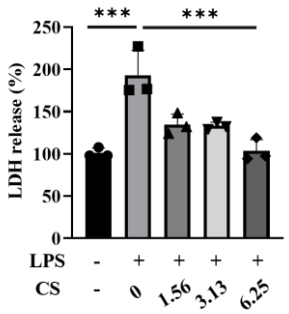


**Figure R7.** LDH release in different groups of neutrophils. Neutrophils were treated with LPS and co-cultured with different concentrations of CS nanozyme (1.56, 3.13, 6.25 μg/mL). n = 3 in each group. The data were presented as the means ± s.d. One-way ANOVA with Tukey's post hoc test was used for comparisons among multiple groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, *ns,* not significant.

Reviewer #4:

**Q1.** This reviewer assumes that NP were detected using a anti-IgG-AF647 antibody. This information is missing in the legend. Thank you for the information!

**A1.** Thank you very much for the good suggestion. We functionalized nanoparticles with Alexa Fluor 647-conjugated Ly6G antibody to specifically target neutrophils. The results clearly indicate that Ly6G antibody-functionalized nanoparticles exhibited excellent targeting capability, and enhanced uptake by neutrophils compared to non-functionalized nanoparticles (Figure R8). We have added this information in the legend.

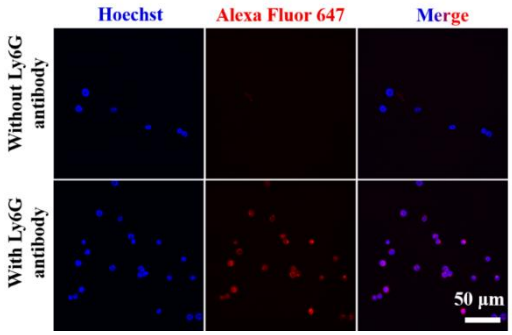


**Figure R8.** *In vitro* assay of neutrophil uptake of nanoparticles functionalized with or without Alexa Fluor 647-conjugated Ly6G antibody.