nature portfolio

Peer Review File

Extracellular vesicles from the microalga Tetraselmis chuii are biocompatible and exhibit unique bone tropism along with antioxidant and anti-inflammatory properties

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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Wonderful and elegant paper showing the potential use of nanoalgosomes in relevant cell and animal models. Good data and results worth to publish.

Reviewer #2 (Remarks to the Author):

I thank the authors to share their results on the subject that are of interest to the community. In my opinion, many results, although of interest, are overstated (see detailed comments). If that was not the case, the paper would also be of interest to the community, and would not tend to bias other researchers.

In my opinion, and in the absence of new data, the major result of the paper is that nanoalgosome seems rather not toxic in vitro and in vivo ONLY if used in a single injection.
Additional data on biodistribution are more difficult to interprete.

I think that the discussion and conclusion should be rewritten without overstatements.

Here are my major comments :

introduction :
relatively "non scientific" and enthousiastic, it may be improved by focusing on facts instead of expectations and vague terms

Line 64 : probably not all plants have effects : precise "Some plants...", more generally, be more precise in the introduction regarding effects. For example : "[EVs] offer an interesting opportunity for therapeutic applications, particularly as vaccines, as they can carry a wide range of functional molecules and have unique bioactivity derived from their microbial origin that promotes enhanced immune activation". The phrase is long and is very general (interesting, therapeutic applications, functional, unique bioactivity, enhanced immune activation...)

line 77-80 is false : there are clinical trials in phase III with MSC EVs. It may be costly, but it is feasible

line 80 : probably too much enthousiasm "In this context, we are exploring a fascinating advance in the field, showcasing a new specific type of EVs that we call “nanoalgosomes” or “algosomes”"

what does "an original multi-branched approach" mean ?

l98-101 : probably not neeeded

results :
general comment : may also be focused on facts and results
Example : size by NTA is AA nm (+/- X nm), which is coherent with results obtained by Cryo (XX nm +/- X nm), showing a double layered ...

Figure 1 :
- see MISEV : you need negative controls (i.e. Alguae lysate) to state that these are EVs, enriched in ALix and H+ ATPase compared to lysate.
- see MISEV : provide wide field pictures for Cryo TEM

- stability is not demonstrated to my understanding (multiple timepoints and potency assay)
- proper EV protein/particle number ratio = what is proper ? or bad ? cut off ?
- consistency : no data provided to claim this
- "emphasize the importance of accurate quality control checking of nanoalgosome preparations to further exploit their potential as innovative therapeutic agents" to my understanding there are no demonstration of the importance of quality control, no "bad" EVs were tested ...

Figure 2 :
- provide data to support the fact that EV labeling was specific of EVs and not due to dye aggregation or aggregation with protein aggregates (for example see Pieter vader biodistribution paper with controls involving density gradient centrifugation)

Table 1 and biocompatibility :
i would guess that nanoalgosomes have a high chance to be recognized as a foreign body, and thus lead to the development of a specific immunity. The time needed to develop such immunity is about 2 weeks.
It has to be very clearly stated that the obtained results only allow to claim biocompatibility for single injections

Figure 6 e f : precise what is the dose injected
+ precise in the figure and text the fraction of dose in the femur : about 2-4% if I am correct, which does not support the claim that the dose was mostly present in the bones at 48h.

The sentence : "suggests that nanoalgosomes are stable in body fluids and that their circulating half-life is high" is not suported by results.
I would personally rather bet on another explaination :
1) the dye is aggregated during preparation and mostly stain proteins (hydrophobic pockets) and a fraction stains EVs.
2) once endocytosed in few minutes (short half life) in the liver and other cells these aggregates/protein/EVs are degraded rapidly (short stability in cellulo) and the fluorescence increase as dye aggregates are dispatched in the whole cell, and dye molecules do not quench each other anymore (explain increase in fluorescence)
3) once present in lipidic membranes, the dye (with a 100+ days half life for degrdation) allows to follow the lipid fluxes in the body from the liver to other organs, for example get in the bone marrow

via HDL/LDL/etc to produce hematopoietic cells.

In the absence of other results, you can only conclude that the dye is stable in the body and circulate from an organ to another

This sentence seems wrong :
"The results in Figure 7b and c show no significant differences in IL-6 induction following 24 hours of nanoalgosome treatment compared to untreated cells; this result is in line with the in vivo data, previously shown, and is indicative of the immune-compatibility of nanoalgosomes"

in figure 7 : you should also state that algosome on their own induce an increase in IL6 of about 5 fold, with a very small error bar that is probably significant (indicate n please), and even if not, the absence of significance does not mean that there are no differences (unless you have a statistical power calculation with 95% power to detect a 2-5 fold increase)

Reviewer #3 (Remarks to the Author):

Extracellular vesicles (EVs) showing high biocompatibility, low toxicity, and immunomodulatory properties are attracting attention as next-generation therapeutic modalities and delivery vesicles. In particular, interest in EVs from mesenchymal stem cells (MSCs) is growing due to their therapeutic potential, but sustainable production of well-characterized mammalian-cell-derived EVs has been challenging. Therefore, academic and industrial communities have been trying to find alternative approaches to obtain EVs from other resources that meet the requirements for therapeutic use.
In this manuscript, Adamo et al. reported the comprehensive characterization of extracellular vesicles (EVs) from microalgae, which they call nanoalgosomes, for future biomedical applications. The characterization includes toxicological analyses in C. elegans, hematological and immunological evaluations ex vivo and in vivo, analyses of uptake mechanisms, and biodistribution in mice. They also examined the anti-inflammatory bioactivities of the nanoalgosomes.
In their previous paper (J. Extracellular Vesicles. 2021, 10, e12081), they already introduced the basic properties of nanoalgosomes, so the reviewer needs to mention that the novelty of this work itself is somewhat limited. However, the reviewer agrees that comprehensive characterization of this kind of new class of EVs, especially in vivo, is quite important for real future applications. The data shown in this paper is solid and indeed supports the potential of nanoalgosomes for various applications, so it is worth publishing this work in a respectable biology journal. Considering the interesting bone tropism of the nanoalgosomes as well into consideration, the reviewer basically agrees that the manuscript meets the standard of Commun. Biol. However, the reviewer wants the authors to address the following points to improve the manuscript.

Major comments:
• Figure 1a, b: Please explain what the red and blue deviations stand for.
• Figure 1b: Please provide information in the figure legend about the dye used for membrane staining, as well as the conditions for excitation and emission.
• Line 117: It would be preferable to show the actual data on batch-to-batch consistency if the authors want to support this claim.
• Line 138: Please provide a rationale for the choice of cell line. Throughout the study, different cell lines are used depending on the experiments, but the reasons are sometimes unclear (this applies to other parts as well).
• Line 209 and Fig.3: If the nanoalgosomes are not destined for lysosomes after endosomal internalization, where do they go in the end? This information would be important to assess the potential of the nanoalgosomes for delivery purposes. It would be preferable to track the EVs for a longer period of time and include a discussion about their final fate.
• Line 258: The rationale for the choice of EV concentration should be already explained here.
• Figure 6: Since biodistribution is assayed by fluorescence, the signal is strongly influenced by the depth of each organ. To firmly demonstrate the bone tropism of the EVs (as well as to the other organs), the reviewer suggests evaluating the biodistribution after dissecting each organ.
• Line 406: The authors claim that the half-life of the nanoalgosomes is high. In contrast, EVs from mammalian cells reportedly have a short half-life in the body (typically on the order of minutes). The reviewer suggests presenting actual data on this point, which would further emphasize the uniqueness of nanoalgosomes.
• For cell culture conditions, please provide information about the media conditions. Additionally, for the endocytosis experiment, the presence of serum in the media and other important details should be included to enable replication of the data. Please provide more detailed information so that readers can follow the experimental procedure.
•
Minor comments:
• Put spaces between the number and unit (e.g., 60 uM in line 144 should be 60 uM). The presence of spaces is inconsistent throughout the manuscript.
• In the legend of Figure 7, "TPH-1 cells" should be "THP-1 cells."

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Wednesday, March 25, 2024

POINT-BY-POINT RESPONSES

	Lanes
Reviewer #1 (Remarks to the Author): Wonderful and elegant paper showing the potential use of nanoalgosomes in relevant cell and animal models. Good data and results worth to publish.	
Our response 1 - Encouraged by the enthusiastic comment of the reviewer #1, we have now included additional results and amended the text to address the concerns of the other reviewers.	N.A.

	Lanes
Reviewer #2 (Remarks to the Author): I thank the authors to share their results on the subject that are of interest to the community. In my opinion, many results, although of interest, are overstated (see detailed comments). If that was not the case, the paper would also be of interest to the community, and would not tend to bias other researchers. In my opinion, and in the absence of new data, the major result of the paper is that nanoalgosome seems rather not toxic in vitro and in vivo ONLY if used in a single injection. Additional data on biodistribution are more difficult to interprete. I think that the discussion and conclusion should be rewritten without overstatements. Here are my major comments : introduction : relatively "non scientific" and enthousiastic, it may be improved by focusing on facts instead of expectations and vague terms Line 64 : probably not all plants have effects : precise "Some plants...", more generally, be more precise in the introduction regarding effects. For example : "[EVs] offer an interesting opportunity for therapeutic applications, particularly as vaccines, as they can carry a wide range of functional molecules and have unique bioactivity derived from their microbial origin that promotes enhanced immune activation". The phrase is long and is very general (interesting, therapeutic applications, functional, unique bioactivity, enhanced immune activation...) line 77-80 is false : there are clinical trials in phase III with MSC EVs. It may be costly, but it is feasible line 80 : probably too much enthousiasm "In this context, we are exploring a fascinating advance in the field, showcasing a new specific type of EVs that we call “nanoalgosomes” or “algosomes”" what does "an original multi-branched approach" mean ? l98-101 : probably not neeeded	

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results : general comment : may also be focused on facts and results	
Our response 2 - We thank the reviewer for her/his thorough revision of our manuscript. Regarding his/her concern about the potential overstatement of our results, we have carefully considered his/her comments, added additional results and revised the results and discussion section accordingly to ensure that the manuscript indeed reflects the scope and limitations of our study. **More specifically, we have revised the results and discussion section to clarify that our study aims to elucidate the biological properties of nanoalgosomes comprehensively, encompassing biocompatibility, tropism, and bioactivities. Furthermore, we think that the inclusion of data from two *in vivo* models, including the model organism *C. elegans* and mice, contributes to the robustness and relevance of our conclusions. We have also taken note of his/her comments regarding the interpretation of biodistribution data. We agree that this aspect requires careful consideration and have revised the relevant sections to provide clearer explanations, adding also additional data, as detailed below.** **The reviewer's major comments regarding the Introduction section has been revised in a way that, in our opinion, remains closely adherent to the description of our research.** **In particular:** **we added the sentence "some plant" as suggested;** **we modified the text relative to clinical trials in phase III with MSC EVs;** **we tone down the enthusiastic phrases removing the sentences: "through an original multi-branched approach starting with studies on living organisms and delving into molecular mechanisms at the subcellular level" and "Understanding and exploiting the potential of nanoalgosomes as natural bio-based nanoparticles with innate bioactivity could pave the way for safe, innovative, and effective formulations that would benefit various fields of nanomedicine, as well as cosmetics."** **For the result section we have amended the manuscript as follows:**	*64* *74-80* *91* *95*
Example : size by NTA is AA nm (+/- X nm), which is coherent with results obtained by Cryo (XX nm +/- X nm), showing a double layered ...	
Our response 3 – We thank the reviewer for bringing up this point. In our previous publication (Adamo et al., JEV 2021), we have extensively characterized nanoalgosomes, detailing both our experimental methodology and the interpretation of results. In the current manuscript, we aimed to streamline the methods and the presentation of results to enhance readability without compromising scientific rigor. However, we acknowledge the necessity of providing a more comprehensive characterization of nanoalgosome preparations also in this context. Consequently, we have revised the text to include a more detailed description of: **- methods relative to the separation and characterization of nanoalgosomes** **- results showed thoroughly in Figure 1.**	*611-716* *110-125*

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Figure 1: - see MISEV : you need negative controls (i.e. Alguae lysate) to state that these are EVs, enriched in ALix and H+ ATPase compared to lysate. - see MISEV : provide wide field pictures for Cryo TEM - stability is not demonstrated to my understanding (multiple timepoints and potency assay) - proper EV protein/particle number ratio = what is proper ? or bad ? cut off ? - consistency : no data provided to claim this - "emphasize the importance of accurate quality control checking of nanoalgosome preparations to further exploit their potential as innovative therapeutic agents" to my understanding there are no demonstration of the importance of quality control, no "bad" EVs were tested ...	
<u>Our response 4</u> – We thank the reviewer for providing detailed feedback on Figure 1. We have addressed these concerns as follows: **- We have now included a high- and low-magnification images for AFM (Fig. 1e) and the negative control (*T. chuii* lysate) in Fig. 1f, in line with MISEV guidelines. Regarding the wide-field pictures for cryo-TEM and in alignment with recent literature, as we are showcasing analyses to illustrate the morphology and presence of the lipid bilayer, rather than to assess the purity of the nanoalgosome preparations, we are confident that the images presented adequately fulfil the intended scope.** **Further:** **- We have better clarified what we meant by "proper" EV protein/particle number ratio to provide more context and understanding.** **- We have provided additional clarification regarding the consistency of our results by explaining that all nanoalgosome features were evaluated across multiple batches (n=6) and consistently yielded vesicles (~10^{12} nanoalgosomes/L of microalgal conditioned-medium, corresponding to ~10^{4} nanoalgosomes/microalgal cell).** **- We have removed the paragraph highlighted by the reviewer that was partly unclear and unnecessary.** **We have included a revised version of Figure 1 below:**	*Fig1e, f* *121-123* *121-126* *128* *Fig. 1e,f*

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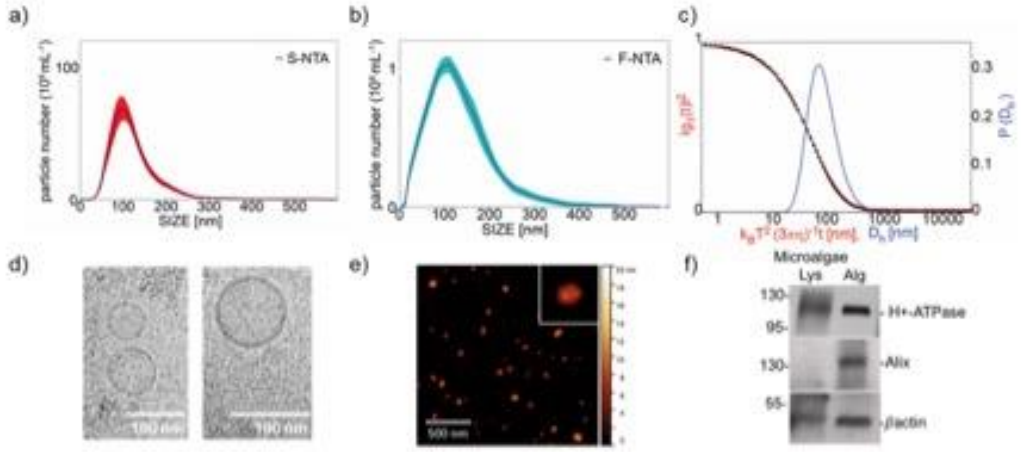

Figure 2:
- provide data to support the fact that EV labeling was specific of EVs and not due to dye aggregation or aggregation with protein aggregates (for example see Pieter vader biodistribution paper with controls involving density gradient centrifugation)

Our response 5 – We know that working with extracellular vesicles derived from biologic fluids or cell cultures requires the proper and rigorous controls to avoid artifacts (protein aggregates and lipoproteins). Microalgae have the advantage of growing in a medium consisting of seawater (we use certified sterile and ultrafiltered seawater), supplemented with salts and vitamins. In our previous study (Picciotto et al., 2022, Front. Bioeng. Biotechnol.), we meticulously designed and validated an experimental protocol to specifically label nanoalgosomes with various fluorescent lipid dyes (Di-8-ANEPPS, PKH26, or DiR) using rigorous unbound dye depletion methods such as pelleting by UC and washing twice in PBS, or dialysis, to eliminate any potential artifacts, including EV-unbound dye aggregates. By means of nanoparticle tracking analysis (NTA) of the labeled and unlabeled nanoalgosomes, we observed no variations in size or concentration compared to unstained nanoalgosome controls, underlining the specificity of our labeling approach. Importantly, nanoalgosomes were separated from microalgae conditioned media, which do not contain lipoproteins or exogenous proteins such as those found in sera, present in the conditioned media from mammalian cell culture.

To comprehensively address the reviewer's concern, we have:
- enhanced the description of the labelling method in Methods section and *748-789*

- included its workflow in the supplementary information (new Supplementary Figure 6). *Supp. Fig. 6a.d*

We have included a revised version of Supplementary Figure 6 below: *Supp. Fig.6a -l*

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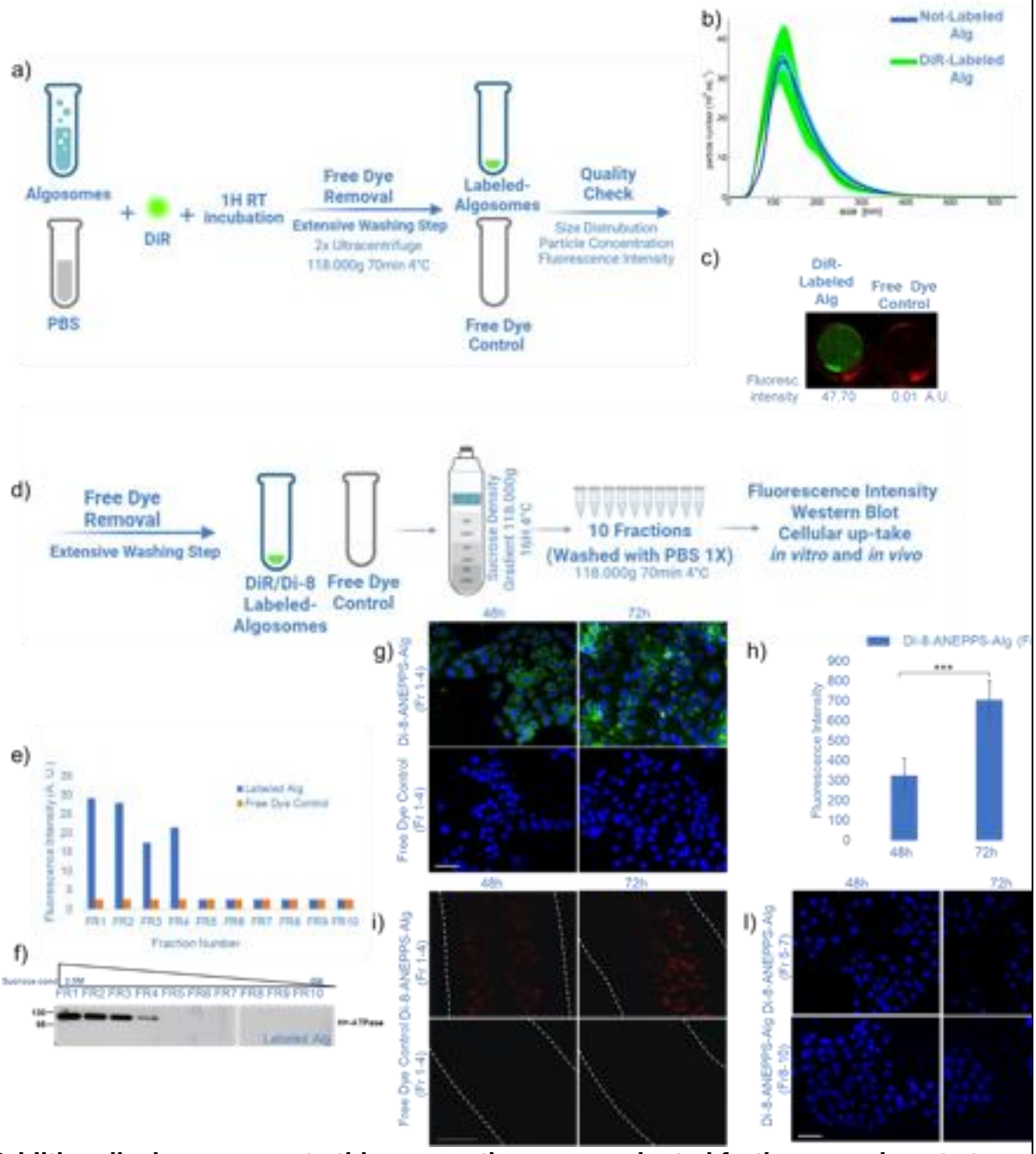

Additionally, in response to this suggestion, we conducted further experiments to reinforce the robustness of our results.
The new Supplementary Figure 6d-l includes analyses of density gradient fractions of labeled nanoalgosomes and a buffer/label-only negative control (*e.g.*, free dye control), inspired by the biodistribution study by Wiklander et al. (doi: 10.3402/jev.v4.26316) as the reviewer referenced. Based on these additional experiments and analyses, we can confidently assert the absence of non-specific labelling.

Supp. Fig 6d-l;

397-419

Table 1 and biocompatibility:
I would guess that nanoalgosomes have a high chance to be recognized as a foreign body, and thus lead to the development of a specific immunity. The time needed to

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develop such immunity is about 2 weeks. It has to be very clearly stated that the obtained results only allow to claim biocompatibility for single injections	
Our response 6 – We have now further specified that our study relative to biocompatibility in mice were performed after single dose injection.	356
Figure 6 e f: precise what is the dose injected + precise in the figure and text the fraction of dose in the femur : about 2-4% if I am correct, which does not support the claim that the dose was mostly present in the bones at 48h.	
Our response 7 – We thank the reviewer to bring this point to our attention; we have now specified the dose injected and the fraction of dose in the femur (~2.5% of total radiance signal), that indeed show that nanoalgosomes are not mainly present in femur but unexpectedly have a specific bone tropism.	443-444
The sentence : "suggests that nanoalgosomes are stable in body fluids and that their circulating half-life is high" is not suported by results.I would personally rather bet on another explaination : 1) the dye is aggregated during preparation and mostly stain proteins (hydrophobic pockets) and a fraction stains EVs. 2) once endocytosed in few minutes (short half life) in the liver and other cells these aggregates/protein/EVs are degraded rapidly (short stability in cellulo) and the fluorescence increase as dye aggregates are dispatched in the whole cell, and dye molecules do not quench each other anymore (explain increase in fluorescence) 3) once present in lipidic membranes, the dye (with a 100+ days half life for degrdation) allows to follow the lipid fluxes in the body from the liver to other organs, for example get in the bone marrow via HDL/LDL/etc to produce hematopoietic cells. In the absence of other results, you can only conclude that the dye is stable in the body and circulate from an organ to another	
Our response 8 – We thank the reviewer for these insightful comments regarding our interpretation of the results concerning nanoalgosome biodistribution and stability in mice. However, we respectfully disagree with the alternative explanation proposed for the following reasons: **- As addressed in our response 5, we have taken measures to address the presence of potential artifacts due to free/self-aggregate dye not incorporated into EVs, in our labeled-nanoalgosome preparations. To evaluate the presence of artifacts, we have included the evaluation of a buffer/label-only control (*e.g.*, free dye control) in all experiments, which is the buffer containing the same amount of fluorescent dye, after the step to deplete unbound dye.** **- We have supplemented our findings with additional evidence from experiments in cell line and *C. elegans* (new supplementary Figure 6), demonstrating that labeled-nanoalgosomes (specifically a pull of fractions 1-4 of the sucrose density gradient separation of Di-8ANEPPS-labeled nanoalgosomes) are indeed taken up by cells and *C. elegans* in a time-dependent manner up to 72 hours. Conversely, pull of fractions 5-6 and fractions 7-10 of the density gradient separation of Di-8ANEPPS only, containing the remaining fluorescent dye after extensive removal steps, show no fluorescence when incubated with cells. These additional results support the specificity of the fluorescent signal as well as the stability of nanoalgosomes in biological fluids *in vitro*, such as fetal calf serum**	Supp. Fig. 6 397-419 Supp. Fig. 6 450-459

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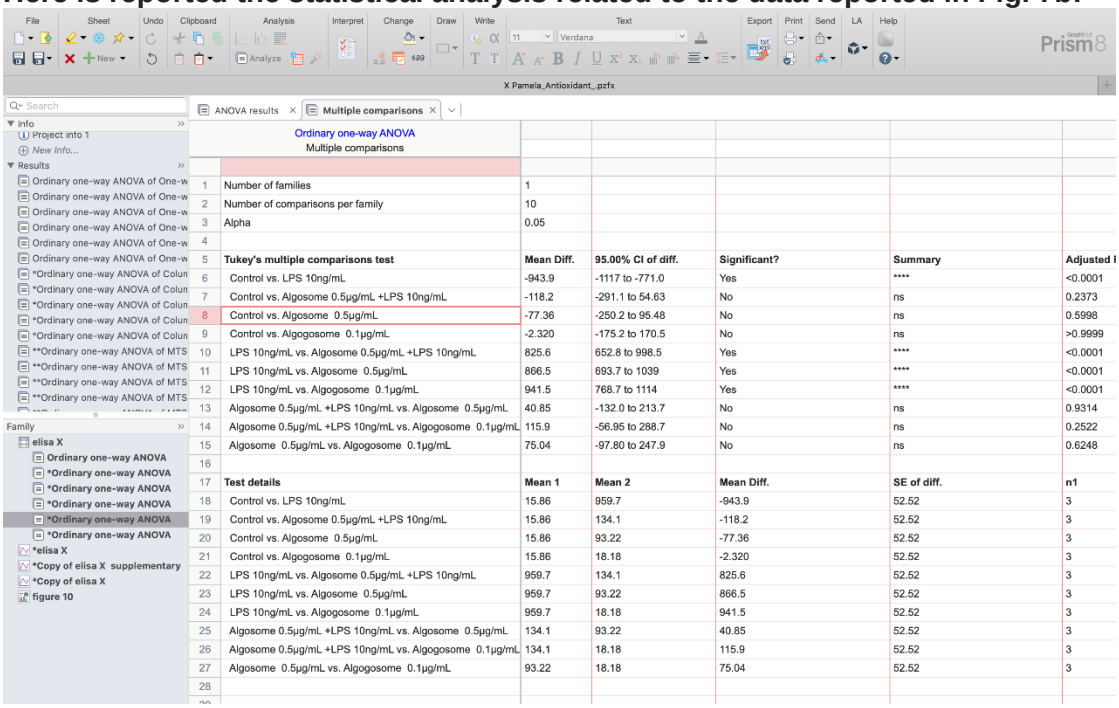
present in tissue culture media (10%) as evidenced by the sustained increase in fluorescence over time up to 72 hours *and in vivo* given the persistency of the fluorescent signal in the body of the animals up to 72 hours (see new Supplementary Figure 6 in "Our response- 5")

This sentence seems wrong :
"The results in Figure 7b and c show no significant differences in IL-6 induction following 24 hours of nanoalgosome treatment compared to untreated cells; this result is in line with the in vivo data, previously shown, and is indicative of the immune-compatibility of nanoalgosomes"
in figure 7 : you should also state that algosome on their own induce an increase in IL6 of about 5 fold, with a very small error bar that is probably significant (indicate n please), and even if not, the absence of significance does not mean that there are no differences (unless you have a statistical power calculation with 95% power to detect a 2-5 fold increase)

Our response 9 – We agree with the reviewer that nanoalgosomes seem to cause an induction of IL-6 upon exposure at the concentration of 0.5 ug/mL, nevertheless this result is not significantly different from control using the software Graphpad Prism for statistical analysis. We used One-way ANOVA with the recommended Tukey's multiple comparison (family-wise alfa threshold 0.05 and 95% confidence interval). Statistical results are shown below in the picture. We also modified the figure legends (Fig. 2, 4, 7, 8) adding the precise adjusted p value also for non-significant results.

Fig. 2, 4, 7, 8 legends

Here is reported the statistical analysis related to the data reported in Fig. 7b:



ANOVA results | Multiple comparisons

Ordinary one-way ANOVA
Multiple comparisons

1	Number of families	1				
2	Number of comparisons per family	10				
3	Alpha	0.05				
4						
5	**Tukey's multiple comparisons test**	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted I**
6	Control vs. LPS 10ng/mL	-943.9	-1117 to -771.0	Yes	****	<0.0001
7	Control vs. Algosome 0.5µg/mL +LPS 10ng/mL	-118.2	-291.1 to 54.63	No	ns	0.2373
8	Control vs. Algosome 0.5µg/mL	-77.36	-250.2 to 95.48	No	ns	0.5998
9	Control vs. Algogosome 0.1µg/mL	-2.320	-175.2 to 170.5	No	ns	>0.9999
10	LPS 10ng/mL vs. Algosome 0.5µg/mL +LPS 10ng/mL	825.6	652.8 to 998.5	Yes	****	<0.0001
11	LPS 10ng/mL vs. Algosome 0.5µg/mL	866.5	693.7 to 1039	Yes	****	<0.0001
12	LPS 10ng/mL vs. Algogosome 0.1µg/mL	941.5	768.7 to 1114	Yes	****	<0.0001
13	Algosome 0.5µg/mL +LPS 10ng/mL vs. Algosome 0.5µg/mL	40.85	-132.0 to 213.7	No	ns	0.9314
14	Algosome 0.5µg/mL +LPS 10ng/mL vs. Algogosome 0.1µg/mL	115.9	-56.95 to 288.7	No	ns	0.2522
15	Algosome 0.5µg/mL vs. Algogosome 0.1µg/mL	75.04	-97.80 to 247.9	No	ns	0.6248
16						
17	**Test details**	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**
18	Control vs. LPS 10ng/mL	15.86	959.7	-943.9	52.52	3
19	Control vs. Algosome 0.5µg/mL +LPS 10ng/mL	15.86	134.1	-118.2	52.52	3
20	Control vs. Algosome 0.5µg/mL	15.86	93.22	-77.36	52.52	3
21	Control vs. Algogosome 0.1µg/mL	15.86	18.18	-2.320	52.52	3
22	LPS 10ng/mL vs. Algosome 0.5µg/mL +LPS 10ng/mL	959.7	134.1	825.6	52.52	3
23	LPS 10ng/mL vs. Algosome 0.5µg/mL	959.7	93.22	866.5	52.52	3
24	LPS 10ng/mL vs. Algogosome 0.1µg/mL	959.7	18.18	941.5	52.52	3
25	Algosome 0.5µg/mL +LPS 10ng/mL vs. Algosome 0.5µg/mL	134.1	93.22	40.85	52.52	3
26	Algosome 0.5µg/mL +LPS 10ng/mL vs. Algogosome 0.1µg/mL	134.1	18.18	115.9	52.52	3
27	Algosome 0.5µg/mL vs. Algogosome 0.1µg/mL	93.22	18.18	75.04	52.52	3
28						

We also added the explicitly available raw data presented in all the figures of the main text and in the supplementary figures, now available on figshare (https://figshare.com/s/3359ba02cdeea1d9d881).

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We are confident that the revised version addresses the reviewer concerns adequately and enhances the overall quality of the paper.

Reviewer #3 (Remarks to the Author): Extracellular vesicles (EVs) showing high biocompatibility, low toxicity, and immunomodulatory properties are attracting attention as next-generation therapeutic modalities and delivery vesicles. In particular, interest in EVs from mesenchymal stem cells (MSCs) is growing due to their therapeutic potential, but sustainable production of well-characterized mammalian-cell-derived EVs has been challenging. Therefore, academic and industrial communities have been trying to find alternative approaches to obtain EVs from other resources that meet the requirements for therapeutic use. In this manuscript, Adamo et al. reported the comprehensive characterization of extracellular vesicles (EVs) from microalgae, which they call nanoalgosomes, for future biomedical applications. The characterization includes toxicological analyses in C. elegans, hematological and immunological evaluations ex vivo and in vivo, analyses of uptake mechanisms, and biodistribution in mice. They also examined the anti-inflammatory bioactivities of the nanoalgosomes. In their previous paper (J. Extracellular Vesicles. 2021, 10, e12081), they already introduced the basic properties of nanoalgosomes, so the reviewer needs to mention that the novelty of this work itself is somewhat limited. However, the reviewer agrees that comprehensive characterization of this kind of new class of EVs, especially in vivo, is quite important for real future applications. The data shown in this paper is solid and indeed supports the potential of nanoalgosomes for various applications, so it is worth publishing this work in a respectable biology journal. Considering the interesting bone tropism of the nanoalgosomes as well into consideration, the reviewer basically agrees that the manuscript meets the standard of Commun. Biol. However, the reviewer wants the authors to address the following points to improve the manuscript.	
Our response 10 – We thank the reviewer for his/her encouraging comments and we have now included additional results and amended the text to address his/her major and minor comments.	
Major comments: • Figure 1a, b: Please explain what the red and blue deviations stand for. • Figure 1b: Please provide information in the figure legend about the dye used for membrane staining, as well as the conditions for excitation and emission.	
Our response 11 – Following the reviewer suggestion, we have now amended the legend of Figure 1	*130-137*
• Line 117: It would be preferable to show the actual data on batch-to-batch consistency if the authors want to support this claim.	
Our response 12 – We thank the reviewer for pointing out this oversight. We have now modified the text, to specify that "all the nanoalgosome features were evaluated in different nanoalgosome batches (n=3) and repeatedly	*123-126*

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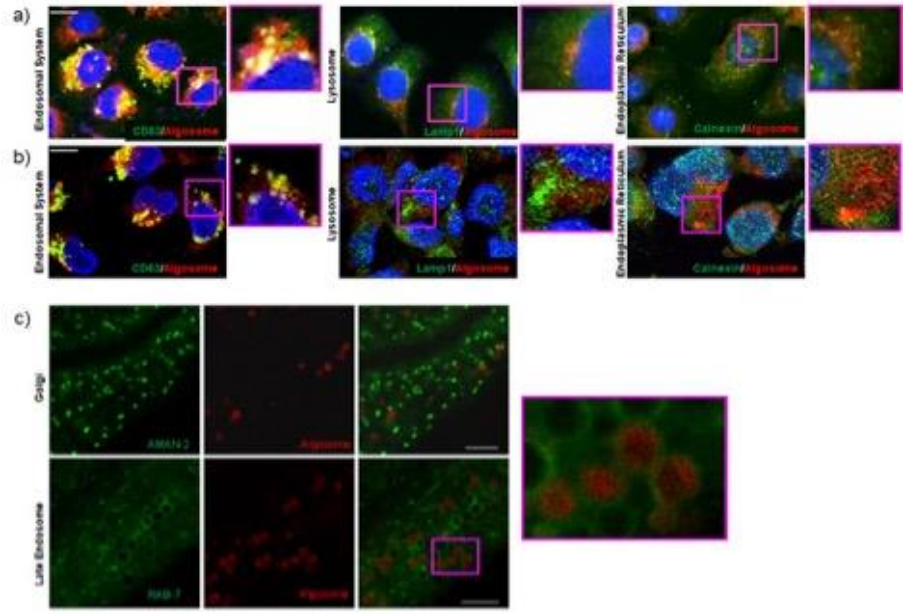
showed yielding vesicles in several (n=6) preparations. These results on nanoalgosomes are consistent with our previous studies.	
• Line 138: Please provide a rationale for the choice of cell line. Throughout the study, different cell lines are used depending on the experiments, but the reasons are sometimes unclear (this applies to other parts as well).	
Our response 13 – We thank the reviewer for bringing up this important point. Throughout the study, we utilized the human mammary epithelial cell line (1-7 HB2) and its tumorigenic counterpart, the MDA MB 231 mammary epithelial cell line. **More specifically, for genotoxicity studies and for the inhibition of endocytosis mechanisms, we chose to use the non-tumorigenic cell line (1-7 HB2), which has greater genetic stability and a more physiological condition (i.e., unaltered membrane plasticity). For intracellular localization studies, we now included 1-7 HB2 cell line in our investigation of nanoalgosome intracellular fate, demonstrating that both cell lines employ the same endosomal localization. To provide clarity and rationale for our choice of cell lines, we have included Figure 3a in the manuscript, which specifically addresses the study conducted on 1-7 HB2 cells.** **We have included a revised version of Figure 3a:**  	*149-152* *Fig. 3a*
• Line 209 and Fig.3: If the nanoalgosomes are not destined for lysosomes after endosomal internalization, where do they go in the end? This information would be important to assess the potential of the nanoalgosomes for delivery purposes. It would be preferable to track the EVs for a longer period of time and include a discussion about their final fate.	
Our response 14 – Different pathways can concurrently operate, resulting in diverse effects. On the basis of our results, following clathrin-mediated endocytosis, internalized nanoalgosomes undergo trafficking within the endosomal pathway. The precise role or fate within this pathway remains	

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poorly understood (Margolis, L.; Sadovsky, Y. The biology of extracellular vesicles: The known unknowns. PLoS Biol. 2019, 17, e3000363). Should fusion occur with the endosomal membrane, their soluble cargo would access the cytoplasm, while EV-associated membrane proteins might potentially traverse to the trans-Golgi network, Golgi complex, and endoplasmic reticulum (ER) via retrograde transport. Additionally, they could reach the plasma membrane through recycling endosomes . Our preliminary findings (not shown in the present manuscript) regarding doxorubicin- and siRNA-loaded nanoalgosomes indicate their capability to release the drug intracellularly, leading to nuclear localization and induction of apoptosis in the case of doxorubicin, and to silencing of the expression of target gene for siRNA. We agree with the reviewer that showing the potential of the nanoalgosomes for delivery purposes is also of high interest; this nanoalgosome feature towards different exogenous cargos (hydrophobic molecules, siRNA, peptides) is currently under investigation, but it is beyond the scope of the present manuscript.	
• Line 258: The rationale for the choice of EV concentration should be already explained here.	
Our response 15 – We thank the reviewer for her/his suggestion. We added the rationale for EV concentration choice in the text where requested.	*276-277*
• Figure 6: Since biodistribution is assayed by fluorescence, the signal is strongly influenced by the depth of each organ. To firmly demonstrate the bone tropism of the EVs (as well as to the other organs), the reviewer suggests evaluating the biodistribution after dissecting each organ.	
<u>Our response 16</u> – Although IVIS analysis on organs post-autopsy can be considered useful and is reported in many studies, we deemed that *in vivo* analysis through techniques like IVIS, coupled with high-performance software (Living Image software), allows for the precise selection of a specific body region (such as the femur) with high accuracy. This approach can provide a dynamic and detailed 3D perspective on nanoalgosome localization within the organ *in vivo*, offering several advantages over the traditional approach involving autopsy and post-mortem analysis. This helps avoid post-mortem artifacts; indeed, post-mortem analysis can introduce artifacts due to sample preparation and the loss of biological dynamics. **In this context, a recent study reported a side-by-side comparison of three different bioimaging modalities using the fluorescent tracers DiR dye and mCherry fluorescent protein, the bioluminescent tracers Firefly (Fluc) and NanoLuc (Nluc) luciferases, as well as nuclear imaging using the [111In] radioisotope (Elisa Lázaro-Ibáñez et al., 2021 - https://dx.doi.org/10.1021/acsnano.0c09873). In line with our findings, the Authors demonstrated that DiR (unlike mCherry protein or other non-near-infrared probes) allowed detection of the vesicles *in vivo* with better sensitivity, signal-to-noise ratio, and no background fluorescence at expected tissue locations. Similarly to other reports on DiR-labelled EV biodistribution in nude mice, these Authors also reported that DiR-labelled EVs tend to accumulate mostly in the liver, followed by the spleen, and to a lesser extent the lungs and kidney, but not in bones as is the case for nanoalgosome in our study. To our knowledge, this specific organotropism in bones has been never reported in literature for DiR-labelled EVs and thus we believe is directly related to nanoalgosomes. As support of this finding, the control group**	

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administered I.V. with a corresponding amount of unbound dye negative control demonstrates that even small amounts of free probe exhibit behaviour entirely distinct from the nanoalgosomes. As suggested by the reviewer, having a more comprehensive evaluation of the bone tropism of nanoalgosome is very important and is indeed the logic follow up of the present study, but it goes beyond the scope of the present manuscript.	
• Line 406: The authors claim that the half-life of the nanoalgosomes is high. In contrast, EVs from mammalian cells reportedly have a short half-life in the body (typically on the order of minutes). The reviewer suggests presenting actual data on this point, which would further emphasize the uniqueness of nanoalgosomes.	
Our response 17 – We appreciate the reviewer's suggestion to provide additional evidence regarding the stability of nanoalgosomes. In response, we have conducted further experiments and the results are now included in supplementary figure 6 g-l. Our findings, supported by cellular and *C. elegans* experiments, demonstrate that labeled nanoalgosomes, particularly nanoalgosome positive fractions 1-4 of the sucrose density gradient separation of Di-8ANEPPS-labeled nanoalgosomes, are efficiently taken up by cells in a time-dependent manner. Remarkably, the fluorescent signal persists for up to 72 hours also in *C. elegans* intestinal cells, indicating the sustained presence of nanoalgosomes within biological systems. This persistence is evident even in the presence of fetal calf serum, a common component of tissue culture media, at a concentration of 10% (see new Supplementary Figure 6 as in "Our response 5")	*Supp. Fig. 6g-l* *450-459*
• For cell culture conditions, please provide information about the media conditions. Additionally, for the endocytosis experiment, the presence of serum in the media and other important details should be included to enable replication of the data. Please provide more detailed information so that readers can follow the experimental procedure.	
Our response 18 – We thank the reviewer for this feedback regarding the cell culture conditions and the endocytosis experiment. We acknowledge the importance of providing detailed information to ensure the replicability of our experiments. In response to this comment, we have updated the manuscript to include comprehensive information about the media conditions used for all cell culture described. Additionally, for the endocytosis experiment, we have provided specific details about the presence of serum in the media and any other pertinent information necessary for replicating the experiment.	*720-727* *792-794*
Minor comments: • Put spaces between the number and unit (e.g., 60 uM in line 144 should be 60 uM). The presence of spaces is inconsistent throughout the manuscript. • In the legend of Figure 7, "TPH-1 cells" should be "THP-1 cells."	
Our response 18 – We thank the reviewer for the attention in providing these minor comments. We have addressed them accordingly.	

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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

My queries have been correctly addressed by the authors

Reviewer #2 (Remarks to the Author):

The new version of the manuscript is greatly improved.

Thre are yet remaining major comments, especially regarding the interpretation of the results :

- "Furthermore, the increasing fluorescent signals for up to 48 hours in mice and in individual areas (i.e., backbone and femur)
suggests that nanoalgosomes are stable in body fluids and that their circulating half-life is high. "
=> this remains to be demonstrated in my opinion. Would it be possible to perform serial blood sampling to calculate the blood half life ? this would allow to clearly decipher between a long half life and a recirculation of EVs

The absence of significativity in figure 7c (nanoalgosomes versus control) does not mean that there is no effect. The p value is the chance of concluding to an effect by error in the absence of effect. The correct conclusion would be that "nanoalgosomes have a limited, if any, inflamatory effect".

in the conclusion :
The conclusion does not state that the biocompatibility is only demonstrated after a single injection, this is major.

"endowed with unparalleled biocompatibility in living organism"
=> overstated and not clear

'Indeed, nanoalgosomes are immune-tolerated in vivo and ex vivo"
=> only after a single injection

"where they counteract aging" => Precise that it is in C elegans

"an ideal situation to tap into the enormous and still underexploited potential of EVs as novel biological therapeutics"
=> overstated

Reviewer #3 (Remarks to the Author):

I feel that the authors sufficiently addressed the majority of the points raised by the reviewers. I am up for accepting this manuscript.

Just a couple of minor points:
1. New Fig.7b, there should be a line connecting “control” and “Algosome 0.1 ug/mL”(no line below ns)
2. New Fig.7c, ”Algogosome 0.1 ug/ml" should be ”Algosome 0.1 ug/ml"
3. New Supplementary Fig.6, the resolution of the images of (g) is too low. It would be preferable to show the figure such that the readers can confirm endosomal localization of the labeled nanoalgosome in both time points because the authors are discussing the stability of the nanoalgosomes in cellulo.

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Wednesday, May 15, 2024

POINT-BY-POINT RESPONSES

	Lanes
Reviewer #1 (Remarks to the Author): My queries have been correctly addressed by the authors	
Our response 1 - We thank the reviewer.	N.A.

	Lanes
Reviewer #2(Remarks to the Author): The new version of the manuscript is greatly improved. Thre are yet remaining major comments, especially regarding the interpretation of the results : - "Furthermore, the increasing fluorescent signals for up to 48 hours in mice and in individual areas (i.e., backbone and femur) suggests that nanoalgosomes are stable in body fluids and that their circulating half-life is high. " => this remains to be demonstrated in my opinion. Would it be possible to perform serial blood sampling to calculate the blood half life ? this would allow to clearly decipher between a long half life and a recirculation of EVs	
Our response 2 – We thank the reviewer for his/her appreciation of the revised version of the manuscript, and we are addressing his/her major comments in this second revision of our manuscript (novel modifications are highlighted in green). Specifically, as suggested by reviewer 2, we have now performed an additional experiment to address his/her comment, relative to the durable retention of DiR-labeled nanoalgosomes in the tissues with detectable levels of the fluorescent tracer up to 48 h. Unfortunately, we found that the additional experiment suggested by reviewer 2 in this second revision (e.g., "…to perform serial blood sampling to calculate the blood half life") is technically and temporally unfeasible as, from data reported in literature, blood EV analysis from DiR EV-treated small animals (e.g., mice treated I.V. with DiR-EV at 1-5x10^{11}/mouse) is not possible as the recorded signals would be below the limit of detection (Lázaro-Ibáñez et al., ACS Nano 2021, 15, 3212−3227 - https://dx.doi.org/10.1021/acsnano.0c09873). As alternative, we performed an experiment to determine the stability of DiR-labeled nanoalgosomes in biofluids ex vivo by characterizing their features in 50% serum (e.g., fetal calf serum, FCS) over 48 hours.	

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For that, equal numbers of DiR-labeled nanoalgosomes (1.5x10^{11}particles/ml, corresponding to 30% of the in vivo injected nanoalgosome dose in our in vivo experiments) were spiked in 50% FCS in a test tube, incubated at different time points at 37° C, and analysed using: i) the Odyssey IR scanner to measure the intensity of DiR infrared fluorescent emissions (λ 800 nm) (**Figure a-b**); ii) nanoparticle tracking analysis (NTA, Nanosight N300) to measure size distribution and concentration (**Figure c**); iii) flow cytometric analysis with CytoFLEX SRT and CytExpert software to evaluate the fluorescence relative to the DiR-labeled nanoalgosomes (**Figure d-e**). Also, to exclude the presence of artifacts, we have included the evaluation of a buffer/label-only control (e.g., free dye control) in all experiments, which is the buffer containing the same amount of fluorescent dye, undergoing the step to deplete unbound dye.

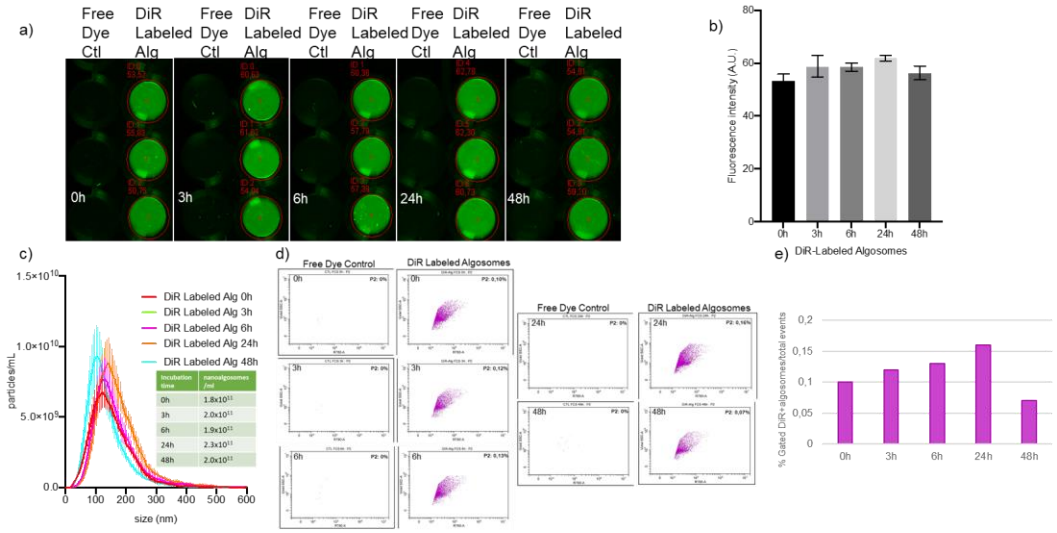

Figure a-e. In vitro *stability of DiR-labeled nanoalgosomes in serum. Equal numbers of DiR-labeled nanoalgosomes (1.5x10^{11}/ml final concentration) were spiked in 50% serum (e.g., FCS) and either directly subjected to the assays (0h) or incubated at 37°C for 3, 6, 24, or 48 hours before the characterization assays.* ***a-b)*** *Intensity of DiR infrared fluorescent emissions of DiR-labeled nanoalgosomes in 50% FCS;* ***c)*** *Size distribution and concentration of DiR-labeled nanoalgosomes in 50% FCS, measured by nanoparticle tracking analysis;* ***d)*** *Representative dot plots of DiR-positive nanoalgosomes at different time-points post-incubation with FCS;* ***e)*** *Percentage of gated DiR-positive nanoalgosomes out of the total events.*

Our results show that the fluorescent signals of DiR-labeled nanoalgosomes in 50% FCS are stable during time at 37°C, up to 48 hours (**Figure a-b**). No detectable fluorescence signal was present in the free dye controls. This result is in line with our *in vivo* results in mice and are also consistent with those reported previously for other types of EVs incubated up to 24 hours in FCS suggesting that serum helps maintain EV integrity, by conferring protection from dissociation of the tracer for labeled EVs (Lázaro-Ibáñez et al., ACS Nano 2021, 15, 3212−3227 - https://dx.doi.org/10.1021/acsnano.0c09873). Furthermore, the size distribution and concentration of DiR-labeled nanoalgosomes, incubated for 3, 6, 24, and 48 hours in 50% FCS at 37°C, remain relatively constant (**Figure c**). The free dye controls were below the detection level for this analysis. **Figure d and e** display the outcomes of the direct flow cytometric analysis of the DiR-labeled nanoalgosomes incubated in 50% FCS at various time points. In this analysis, after calibrating fluorescent and light scatter parameters using MegamixPlus beads

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(100, 160, 200, 240, 300, 500, 900 nm), we compared the percentage of DiR-labeled nanoalgosomes among total events in all samples, including the free dye controls. Unlike the DiR-only solutions devoid of labeled objects, solid populations of DiR-labeled objects were observed in DiR-labeled nanoalgosomes (**Figure d**). The percentages of gated DiR-positive nanoalgosomes among total events were consistently similar across all samples tested, with only a slight deviation observed after longer incubation periods (48 hours), aligning with it the fluorescence and NTA results. Based on these findings, we can deduce that the DiR-labeled nanoalgosomes demonstrate high stability in serum for up to 24 hours. This observation rules out the possibility that the degradation or lysis of DiR-labeled nanoalgosomes contributes to a decline in the nanoalgosome-relative DiR signal in serum. For the sake of readability and to streamline the manuscript, we would prefer to refrain from incorporating this additional result. In light of the results showed in the manuscript and of this additional result, and to address the concern of the reviewer, we have now modified the text to specify that our result "suggests that nanoalgosomes are stable in body fluids and have sustained retention and potential accumulation from the bloodstream in mouse tissue (*e.g.*, bone), as evidenced by detectable levels of the DiR-nanoalgosomes up to 48 hours in mouse tissues". As suggested by the reviewer, having a more comprehensive evaluation of the pharmacokinetics of nanoalgosome (including clearance and circulating half-life also after repeated injection) is very important and is indeed the logic follow up of the present study, but it goes beyond the scope of the present manuscript.	449-451
Reviewer #2(Remarks to the Author): The absence of significativity in figure 7c (nanoalgosomes versus control) does not mean that there is no effect. The p value is the chance of concluding to an effect by error in the absence of effect. The correct conclusion would be that "nanoalgosomes have a limited, if any, inflamatory effect".	
Our response 3 – We thank the reviewer for bringing up this important point. We have now corrected as suggested.	487-488
Reviewer #2(Remarks to the Author): in the conclusion : The conclusion does not state that the biocompatibility is only demonstrated after a single injection, this is major.	
Our response 4 – We thank the reviewer for pointing out this omission in the conclusion, we have now modified the text.	603

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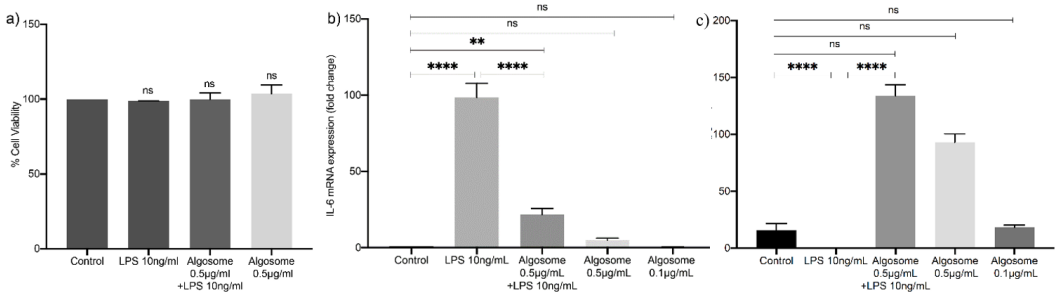
Reviewer #2(Remarks to the Author): "endowed with unparalleled biocompatibility in living organism" => overstated and not clear 'Indeed, nanoalgosomes are immune-tolerated in vivo and ex vivo" => only after a single injection "where they counteract aging" => Precise that it is in C elegans "an ideal situation to tap into the enormous and still underexploited potential of EVs as novel biological therapeutics" => overstated	
Our response 5 – We thank the reviewer for pointing out this oversight. We have now modified the text, to specify that nanoalgosomes have "anti-inflammatory and antioxidant bioactivities and show also biocompatibility and unique tropism in living organisms, after a single I.V. administration	601-605

	Lanes
Reviewer #3 (Remarks to the Author): I feel that the authors sufficiently addressed the majority of the points raised by the reviewers. I am up for acc epting this manuscript. Just a couple of minor points: 1. New Fig.7b, there should be a line connecting "control" and "Algosome 0.1 ug/mL"(no line below ns) 2. New Fig.7c, "Algogosome 0.1 ug/ml" should be "Algosome 0.1 ug/ml" 3. New Supplementary Fig.6, the resolution of the images of (g) is too low. It would be preferable to show the figure such that the readers can confirm endosomal localization of the labeled nanoalgosome in both time points because the authors are discussing the stability of the nanoalgosomes in cellulo.	
Our response 6 - We appreciate the reviewer's acknowledgment of the revised manuscript, and in this second iteration, we are addressing his/her minor feedback points relative to Fig. 7b, Fig.7c and Supplementary Fig. 6 (we are now including a high-resolution figure and submitting the word file of supplementary materials).  	Fig 7b Fig 7c Supp. Fig. 7g

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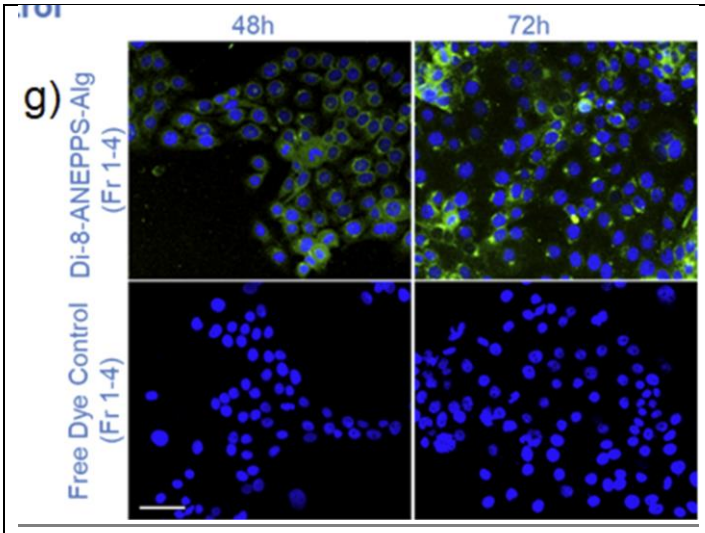

REVIEWERS' COMMENTS:

Reviewer #2 (Remarks to the Author):

I feel that the authors responded to most comments, although not completely.

Reviewer #3 (Remarks to the Author):

My comments have been correctly addressed by the authors.