nature portfolio

Peer Review File

Reentrant DNA shells tune polyphosphate condensate size

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this well-written manuscript, the authors perform a bottom-up reductionistic study of PolyP-Mg2+ condensates in the presence and absence of a variety of DNA substrates in vitro using fluorescence and CryoEM imaging. First, the authors identify the [Mg] range that supports PolyP coacervation. Intriguingly, the lower end of the [Mg] range (10mM) can certainly be considered physiologically relevant for many bacteria. Furthermore, the condensates are reentrant at ~100mM Mg2+, suggesting PolyP condensation may be regulated by divalent cation concentration in cells. Given that polyP condensates are becoming appreciated as a fundamental feature of bacterial chromatin, and likely important for chromatin structure and function in all cells, the authors add DNA substrates of varying length and concentration to determine their influence on PolyP-Mg condensates in vitro. Excitingly, all DNA substrates coated the PolyP-Mg condensates, presumably due to favorable interactions between the negatively-charged phosphate backbone of DNA and Mg2+ at the surface of polyP-Mg2+ coacervates. CryoEM showed the coatings were very thin and did not grow with increased DNA length or concentration, with the exception of additional DNA filament radiations emanating from the surface. Despite the DNA coatings, the authors qualitatively show that the coatings do not influence the liquid-like nature of the core. The authors then quantify the effect of DNA concentration and length on droplet size, showing that with more DNA and/or longer length DNA, the droplets get smaller. The authors suggest the droplets also fuse less, although this data was not directly shown. Overall, this study establishes a fundamental interaction between DNA and polyP mediated by magnesium that determines the properties of PolyP-Mg condensates. DNA was shown to associate with the surface of polyP-Mg2+ coacervates, which affected the morphology of the DNA and tuned condensate size. The manuscript provides an important foundation for future cell-free reconstitutions including the addition of RNA and nucleoid associated proteins known to associate with polyP in vivo. The work is therefore an important step towards our understanding of how polyP affects chromatin structure and function in cells. Please see below my major comments that must be addressed prior to acceptance, as well as some minor issues for the authors' consideration:

Major Comments:

1) Given how thorough the authors were in quantifying the effects of DNA-concentration and -length, I was surprised that other obvious DNA properties weren't also probed. Specifically, (i) AT- versus GC-rich DNA substrates and (ii) DNA supercoiling (linear, relaxed, supercoiled).

(i) Sequence context, in my opinion, is likely less important as I agree with the authors that this interaction is largely via the phosphate backbone and not the bases. However, it would be best to show sequence influence even if the result is negative.

(ii) More importantly, the authors only briefly discuss supercoiling in the discussion, but it is not directly probed in the study. The authors appreciate the potential effects of supercoiling on this phenomenon, but the authors do not address it in their experiments. The authors should at the very least state what the supercoiling status is for each of the DNA substrates used. This can be provided as a supplementary gel showing what fraction of the substrates are supercoiled, nicked-relaxed circular, and linear.

2) Figure 1A and C – I assume the data in both graphs represent the mean and error bars represent std dev? Please provide this info in the legend.

3) Ln335: "… shell formation with longer DNA could result in slower droplet fusion and a consequent smaller droplet size." Why is "slower fusion" not tested directly here? It would be a valuable addition to show whether the droplets are smaller because (i) they grow and arrest at a smaller diameter with high [DNA] or longer length, (ii) the fusion events, when quantified, are indeed fewer within an FOV, or (iii) when fusion occurs, the relaxation rate to a sphere is significantly slower. This quantificaiton would provide useful data regarding whether the PolyP core composition/dynamics are affected.

Minor Comments:

1) Figure 1C: FRAP was only done at one experimental condition. It would be valuable to know if the exchange rate changes across the Mg2+ concentration regime that supports condensates. The findings further strengthen the suggested mechanisms for coacervation.

2) Ln 169: YOYO-1 causes supercoiling in closed circular DNA (Kundukad et al., 2014). It should be mentioned that your plasmid substrate here is therefore highly negatively supercoiled. More appropriately, the supercoiling status of all DNA substrates should be indicated. See major point 1(ii).

3) Ln183: "the pUC19 shells do not substantially restrict droplet fusion". Please show data by quantifying time to droplet relaxation after fusion +- DNA.

4) Figure 2: Why do many of the condensates have a punctate DNA signal squarely in the center of the droplet?

5) Ln 25: Delete “a” from “not a significantly different”.

6) Ln309: “rod-like filaments”. Have these ever been observed in vivo?

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors showed the establishing process of PolyP-Mg coacervate, and by introducing plasmid DNA, the reentrant DNA shell of this droplet was also realized.

However, due to the SHAB theory, divalent metal ions have a better affinity for polyP than DNA, which is self-evident. Recently, the work about PolyP-Mn droplets done by Tian et al., published in NComms, also showed that DNA cannot enter PolyP-divalent metal coacervate, so the conclusion was not fresh in the article.

The author controls the Mg2+ concentration range from 40 mM to 300 mM to ensure the formation of a PolyP-Mg droplet. But this concentration is several times higher than Mg2+ in cells？

Meanwhile, the charged proteins in cells show a much more important influence of polyP-droplets; why didn't the author take such a key role into consideration?

The evidence provided by the author in this article is not sufficient to support their conclusion, so I do not recommend publishing this article here

Reviewer #3 (Remarks to the Author):

This ms by Racki and co-workers investigates the interactions between polyP, Mg2+ and DNA. While the overall model is interesting, the study lacks biological relevance and has several flaws in the experimental design. The current ms does not provide any direct link between their in vitro data and previous in vivo observations, therefore it is entirely possible that the observed polyP-DNA coacervates might have no biological relevance at all.

Major issues:

1) Line 111: The authors seem to base this ms mainly on earlier studies in Pseudomonas aeruginosa, where the reported length of polyP chains range between 100-200 Pi units (see Neville et al., "A Dual-Specificity Inhibitor Targets Polyphosphate Kinase 1 and 2 Enzymes To Attenuate Virulence of Pseudomonas aeruginosa." mBio 2021 and Munevar et al., "Differential regulation of polyphosphate genes in Pseudomonas aeruginosa." Mol. Genet. Genomics 2016). It is therefore unclear why the authors use P700 particularly if they want to establish any connections between their current data and earlier work in P. aeruginosa.

2) Line 118: One of the most critical points are that the authors use unphysiologically high concentrations of polyP (12.5 mM in Pi units) and magnesium (10 mM-75 mM) to see polyP condensation and observe DNA shells. Such conditions are way beyond what can be observed in non-extremophiles (~2 mM) and can be considered as non-terrestrial. The authors may argue that bacterial polyP granules often contain higher levels of Mg compared with the surrounding cytoplasm. However, this argument is only valid if the authors can demonstrate that in their in vitro system, the majority of Mg is sequestered within the polyP condensates. Moreover, it is entirely possible that polyP directly interacts with the nucleic acid dye YoYo 1, which is positively charged. If this were the case, it would significantly increase the complexity of the measurements and interpretation, and makes one wonder why the authors used a dye in the first place.

3) Lines 120 & 394: To evaluate the viscoelastic properties of polyP condensates, the authors should, at a minimum, quantify the coalescence rate of polyP granules to obtain η/γ. It would be better if microrheology would be performed. To meet the standards of this journal these approaches should constitute an integral part of this MS and should not be left for future work.

4) Line 122: How does FRAP properties change with Mg concentration? Why not label DNA directly to test for FRAP properties.

5) Line 168: The bacterial nucleoid is a circular DNA of several Mbp and is usually present in one or two copies in a cell. Therefore, considering the local environment of an in vivo polyP granule, the DNA surrounding it should be considered as long linear strands rather than small circles. This makes pUC19 a less ideal mimic of in vivo conditions.

6) Line 182: FRAP analysis on entire condensates is also essential in understanding how DNA shell affects polyP dynamics. If the DNA shell really exists in vivo, then the fact that polyP granule fusion is never directly observed in vivo can lead to at least two explanations: 1) PPKs and nascent polyP are trapped within DNA shells while ATP/GTP can penetrate through; 2) PolyP chains are able to penetrate the DNA shell and Ostwald ripening might be at play. The latter might better explain the decline in polyP granule number during starvation in P. aeruginosa.

We thank the reviewers for their useful comments and feedback. In response, we have made a number of changes to the manuscript. Major changes include new data, analyses and several new SI Figures/text to address (1) the physiological relevance of our system, including key properties of the components, and (2) technical concerns about our experimental approach and strength of novel conclusions. Overall, we believe our experiments/analyses, revisions and responses not only effectively address all the reviewer concerns, but also have generated novel insights corroborating our original findings.

We believe that our revised manuscript is substantially improved, further strengthening our novel findings with important potential implications for cellular chromatin-polyP interactions and regulation, as well as new insight into the physical chemistry of the primordial polyP-Mg^{2+}-DNA system which will also be valuable in future industrial and biomedical applications. We have highlighted major changes in the manuscript in red. In addition, we made small changes throughout for clarification. Below, we provide our point-by-point responses to the reviewer comments.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this well-written manuscript, the authors perform a bottom-up reductionistic study of PolyP-Mg2+ condensates in the presence and absence of a variety of DNA substrates in vitro using fluorescence and CryoEM imaging. First, the authors identify the [Mg] range that supports PolyP coacervation. Intriguingly, the lower end of the [Mg] range (10mM) can certainly be considered physiologically relevant for many bacteria. Furthermore, the condensates are reentrant at ~100mM Mg2+, suggesting PolyP condensation may be regulated by divalent cation concentration in cells. Given that polyP condensates are becoming appreciated as a fundamental feature of bacterial chromatin, and likely important for chromatin structure and function in all cells, the authors add DNA substrates of varying length and concentration to determine their influence on PolyP-Mg condensates in vitro. Excitingly, all DNA substrates coated the PolyP-Mg condensates, presumably due to favorable interactions between the negatively-charged phosphate backbone of DNA and Mg2+ at the surface of polyP-Mg2+ coacervates. CryoEM showed the coatings were very thin and did not grow with increased DNA length or concentration, with the exception of additional DNA filament radiations emanating from the surface. Despite the DNA coatings, the authors qualitatively show that the coatings do not influence the liquid-like nature of the core. The authors then quantify the effect of DNA concentration and length on droplet size, showing that with more DNA and/or longer length DNA, the droplets get smaller. The authors suggest the droplets also fuse less, although this data was not directly shown. Overall, this study establishes a fundamental interaction between DNA and polyP mediated by magnesium that determines the properties of PolyP-Mg condensates. DNA was shown to associate with the surface of polyP-Mg2+ coacervates, which affected the morphology of the DNA and tuned condensate size. The manuscript provides an

important foundation for future cell-free reconstitutions including the addition of RNA and nucleoid associated proteins known to associate with polyP in vivo. The work is therefore an important step towards our understanding of how polyP affects chromatin structure and function in cells. Please see below my major comments that must be addressed prior to acceptance, as well as some minor issues for the authors' consideration:

Author Response. We thank the reviewer for the positive feedback about our work and for their constructive comments.

Major Comments:

1) Given how thorough the authors were in quantifying the effects of DNA-concentration and -length, I was surprised that other obvious DNA properties weren't also probed. Specifically, (i) AT- versus GC-rich DNA substrates and (ii) DNA supercoiling (linear, relaxed, supercoiled).

Author Response. We agree with the reviewer that these are pertinent questions, and address them with new experiments in Figs S10-S12, as well as S24-27.

(i) Sequence context, in my opinion, is likely less important as I agree with the authors that this interaction is largely via the phosphate backbone and not the bases. However, it would be best to show sequence influence even if the result is negative.

Author Response. To address the reviewer's question of sequence context, and specifically if GC content affects droplet size, we compared droplet size distributions for three 5.6kb plasmids spanning a range of GC contents (44%, 53%, 61%). Using our size quantification method, we were not able to detect differences in the size of droplets at 10min for these three plasmids. Given our limited sample size of one plasmid at each GC%, we cannot rule out the possibility for sequence or GC content to potentially play a finer tuning role than we can resolve.

To reflect these observations, we added the following comment in the "DNA concentration and length modulate droplet size" section of our results:

"We were also interested in the effects of DNA form(circular vs linear, Fig S11) and GC content (Fig S12), however, in both cases we were unable to resolve differences under the conditions we tested" (lines 291-93)

We also raise it as an open question in our discussion to acknowledge the potential for future work with either finer resolution or a larger experimental data set:

"Although we were not able to resolve differences in size distribution with GC content with our current data set (Fig S12), we cannot rule out the possibility for sequence or GC content to potentially play a finer tuning role than our method allows. Patterning of high GC regions, a wider range of GC content, among other sequence related properties, could all be relevant and will be an interesting area of future study." (lines

558-62)

(ii) More importantly, the authors only briefly discuss supercoiling in the discussion, but it is not directly probed in the study. The authors appreciate the potential effects of supercoiling on this phenomenon, but the authors do not address it in their experiments. The authors should at the very least state what the supercoiling status is for each of the DNA substrates used. This can be provided as a supplementary gel showing what fraction of the substrates are supercoiled, nicked-relaxed circular, and linear.

Author Response. The reviewer raises important questions about (a) characterizing the topological state of the circular DNAs used in our study and (b) experimentally interrogating the effect of DNA circular and linear form on the shell phenomenon. For the revision, we experimentally address both points, adding five SI Figures (Figs S11a, S24-7).

We provide supplementary gels showing the supercoiling states of the DNA substrates used in our study (Fig S24-7). To approximate the fraction of supercoiled DNA, we ran 100 ng of our DNA substrates on a 1% agarose gel and post-stained the DNA after electrophoresis with SYBR Gold (1:4000 dilution). SYBR Gold labeling is less sensitive to supercoiling state than other DNA dyes, and exhibits a linear relationship between DNA amount and fluorescence intensity (PMID: 33905507, 38000393). For reference to other commonly-used DNA dyes, we compare a quantification of pUC19 using SYBR Gold to one using Ethidium Bromide, as well as the stain we used in our initial purification stages, APEX Safe (Fig S24). Signal intensity of the supercoiled state band (determined based on its position and disappearance when linearized by restriction digestion) was used to assess the percentage of supercoiled DNA relative to other bands. For pUC19, we compared uncut to both linearized plasmid, and nicked DNA, as suggested by the reviewer, by using the site-specific nicking enzyme Nt.BspQI, which has a single recognition site within pUC19 to relax the DNA (Fig S24). We also include a comparison of the different 5.6kb GC plasmids (Fig S25), in addition to quantification of the plasmids used in our DNA length experiments under different buffer conditions (Figs S26-27). In all cases, linearized DNA also includes a restriction digest buffer and enzyme.

We note that the percentage of supercoiled DNA decreases with increasing length, unsurprisingly given the sensitivity of long, supercoiled plasmids to non-specific nicking as a result of mechanical forces(PMID: 13874842, 16590544, doi.org/10.1007/S004490050552). To confirm the length effect is independent of DNA form, and to compare the effects of circular vs linear DNA, we quantified droplet size distributions for pUC19 and 15kb in both the circular state and their respective linearized forms (Fig S11a,c). We found that the phenomenon of longer DNAs resulting in smaller droplets for circular DNAs (Fig 4) holds for linear DNAs. We were not, however, able to resolve differences in size of droplets between circular and linear forms. Even still, cryo-ET data using these paired samples shows that the topology and organization of the linear DNA is distinct from circular DNA: linear DNA tends to lie flat along the surface compared to the more ‘hairy’ supercoiled/nicked forms. Thus, while

we are not able to resolve differences in droplet size due to circular vs linear form, supercoiling and DNA topology broadly could be an interesting future area of study, particularly in the context of DNA binding proteins that may affect DNA topology at the condensate surface.

The size quantification results comparing circular and linear DNA are acknowledged with the GC note described in the previous section (Major Comments 1(i)). However, given the additional information we have with Cryo-ET, we also made the following addition to the Cryo-ET section to reflect that, noting in the morphological observations:

"For reference, we also tested linearized pUC19 and 15kb and found that for plasmids of both lengths, DNA tends to lie flat along the surface compared to the more 'hairy' circular forms and result in a smoother surface texture that is more difficult to decouple from the polyP-Mg2+ condensate surface (Fig S10, and Fig S6e, f, Fig S7m-q). In the case of linearized 15kb plasmid, the thickness of the dense edge increases (Fig S8h)." (lines 280-84)

As with the GC experiment, we also agree with the reviewer that DNA topology is an interesting avenue of study. As such, we note in the open question section:

"Similarly, while we were not able to resolve differences in size distribution of linearized and circular plasmids (Fig S11), there are many unexplored facets of DNA topology including positive supercoiling and the distribution of topoisomers. Our cryo-ET suggests that DNA topological state does influence the orientation of DNA on the condensate surface (Fig S10). This could suggest that even if DNA topological state does not significantly alter size distribution, it could still affect the way DNA interacts with polyP-Mg^{2+} condensate surfaces, an important topic for further study." (lines 263-69)

We note that for the cryo-ET experiments and DNA form size quantifications, circular plasmids were put through Phenol-Chloroform extraction protocols to match the purification post-digest for linearized DNA. Every additional processing step has the potential to increase the ratio of nicked and cut DNA relative to supercoiled DNA. As such, when discussing our data comparing DNA topological state, we refer to our samples as "circular" and "linear."

2) Figure 1A and C – I assume the data in both graphs represent the mean and error bars represent std dev? Please provide this info in the legend.

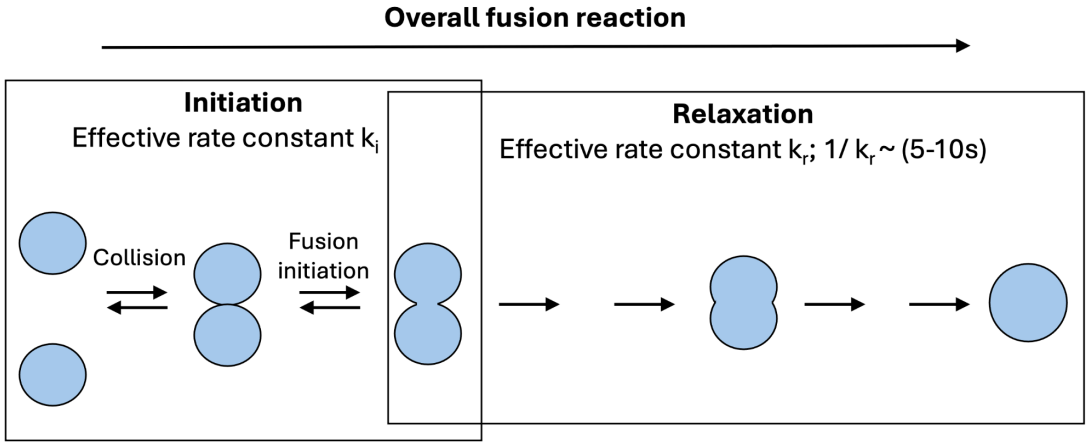
Author Response. We thank the reviewer for pointing this out. The points and error bars do represent mean and standard deviation respectively. We have updated the manuscript accordingly in line 125 of the manuscript with the following text:

"Individual points represent the mean of three replications, and error bars represent the standard deviation."

3) Ln335: "… shell formation with longer DNA could result in slower droplet fusion and a consequent smaller droplet size." Why is "slower fusion" not tested directly here? It would be a valuable addition to show whether the droplets are smaller because (i) they grow and arrest at a smaller diameter with high [DNA] or longer length, (ii) the fusion events, when quantified, are indeed fewer within an FOV, or (iii) when fusion occurs, the relaxation rate to a sphere is significantly slower. This quantification would provide useful data regarding whether the PolyP core composition/dynamics are affected.

Author Response. Like the reviewer, we are very curious about the potential mechanism(s) contributing to the differences in droplet sizes we observe and now experimentally address whether relaxation time changed under different DNA conditions by comparing relaxation once fusion is initiated for no DNA, pUC19 (1x), pUC19 (10x), and 15kb conditions (Fig S3).

Of the mechanisms proposed by the reviewer, it could be possible that there exists some ideal packing that satisfies a particular surface area that arrests the droplets (i, thermodynamic control) or that the initiation of fusion via sterics or some other mechanism impedes initiation (ii, kinetic control) or alternatively that the material state means droplets relax at a different rates, thereby affecting overall growth (iii, kinetic control). Of the suggested mechanisms, (i) and (ii) would result in fewer apparent fusion initiations/events, while (iii) would be the relaxation part of that equation.



Overall growth quantification timescale ~ 500s, whereas relaxation timescale ~ 5-10s
→ Initiation processes but not relaxation must be rate limiting

The new analysis we performed quantitatively demonstrates that the last reason (iii, significant relaxation rate slowing) is not the primary reason for changes in droplet size. We analyzed passive droplet fusion of polyP-Mg^{2+} condensates in the presence of various DNA conditions in the absence of YOYO-1 (no DNA, + 10ng/uL pUC19, +100 ng/uL pUC19, and +10ng/uL 15kb plasmid), described in SI Methods. Our results suggest that a) the relaxation time scale for the four different conditions is very similar, likely within a factor of two difference, and b) that this occurs within a few seconds which is fast when compared to the time scale of size quantification (10 minutes). Thus,

we do not expect relaxation to be the rate determining step giving rise to our observed differences.

While decoupling whether arrest is from a thermodynamic origin (i) or a kinetic one (ii) is challenging, we do note that across many conditions, droplets are still in a state of growth (Figure 4) at 10 minutes, indicating the presence of a kinetic component (ii) throughout the time scale. Although quantifying the number of fusion events (as proposed by the reviewer) would be another way of testing droplet growth mechanism, we do not think it provides more insight into decoupling (i) and (ii) than our size distribution data already accomplish since growth of droplet size is coupled to fusion rate. We did make an attempt to quantify fusion events regardless, but this quickly became very complicated considering differences in the number of droplets and size distribution per FoV for each condition and how that affects the number of fusion events, as well as basic experimental difficulties such as handling fusion events that happen at skewed angles (not along the xy frame of imaging but incorporating some z component) and variance in the time it takes for condensates under different conditions to settle on the cover glass.

With our new experiments, we are more confident in assigning changes in droplet size distribution to a reduction in fusion events, assuming fusion is the predominant mechanism of droplet coarsening.

We have clarified our initial thoughts by changing the section of sentence that prompted this discussion in former line 335 to:

"we hypothesized that shell formation with longer DNA could also affect droplet growth by inhibiting the initiation of polyP-Mg^{2+} condensate fusion." (lines 357-59)

We also added the following two notes in the concentration and length tuning results sections respectively to address what we observe in our relaxation experiment:

"*Notably, once fusion starts, relaxation of fusing polyP-Mg^{2+} condensates in the presence of 100 μg/mL pUC19 occurs on a timescale of only a few seconds, similar to that of the no DNA conditions and 10 μg/mL pUC19 conditions (Fig S3). Assuming droplet growth is driven predominantly by fusion, this would suggest that DNA affects droplet growth in our* in vitro *system more through a reduction of fusion events rather than through slowing the coalescence and relaxation process." (lines 342-47)*

"Similar to what we observed in the higher DNA concentration system, aspect ratio-based fusion quantification demonstrates that condensates with 15kb plasmids take a similar amount of time to relax to a spherical shape after fusion starts as those in no DNA and 2.7kb DNA polyP-Mg^{2+} systems (Fig S3). The similar relaxation timescale again suggests that the reduction in size is a product of reduced fusion overall rather than a function of the coalescence and relaxation time provided that fusion is the predominant contributor to coarsening." (lines 377-83)

Minor Comments:

1) Figure 1C: FRAP was only done at one experimental condition. It would be valuable to know if the exchange rate changes across the Mg2+ concentration regime that supports condensates. The findings further strengthen the suggested mechanisms for coacervation.

Author Response. We agree that characterizing the exchange rates at different Mg^{2+} concentrates is worthwhile, and thus performed FRAP experiments with three additional Mg^{2+} conditions (12.5mM, 250mM, 500mM, and compared these to 100mM; Figure S1b). We observed that the internal exchange is slightly slower at higher concentrations of Mg^{2+}. All were in a similar regime of relatively slow recovery over the course of 30min.

These observations are noted in the text (lines 117-19),
"These observations were similar for condensates formed across multiple Mg^{2+} concentrations, with slightly slower recovery occurring as Mg^{2+} concentrations increased to the right of the peak (Fig S1b)."

2) Ln 169: YOYO-1 causes supercoiling in closed circular DNA (Kundukad et al., 2014). It should be mentioned that your plasmid substrate here is therefore highly negatively supercoiled. More appropriately, the supercoiling status of all DNA substrates should be indicated. See major point 1(ii).

Author Response. We agree with the reviewer that the effect of YOYO-1 on DNA state could be a confounding factor, and have both clarified in the text when YOYO-1 was used, and performed additional controls without YOYO-1 of our key phenomenon to ensure that these do not depend on the dye.

We repeated our droplet size quantification for linearized and circular pUC19 and 15kb in both presence and absence of YOYO-1 (Fig S11). These additional controls demonstrate that the longer 15kb DNA results in smaller droplets than 2.7kb pUC19, whether circular or linear, and with or without YOYO-1. We more clearly note in the text that the Cryo-ET experiments, as well as the DNA concentration and GC content size quantifications, were all performed in absence of YOYO-1 and should be unaffected by any potential YOYO-1 induced effects.

We explicitly reference the effect YOYO-1 can have on supercoiling while we talk about cryo-ET experimental conditions:
"Because YOYO-1 can both introduce supercoiling and, under long incubation times, induce nicking, the following experiments were performed in the absence of YOYO-1." (lines 238-40)

We discuss the new experimental control in lines 375-76:

"The shift from larger to smaller droplets from 2.7 to 15kb is also recapitulated in the absence of YOYO-1 (Fig S11)."

3) Ln183: "the pUC19 shells do not substantially restrict droplet fusion". Please show data by quantifying time to droplet relaxation after fusion +- DNA.

Author Response. We thank the reviewer for their comments. In our revision, we quantified this as described in our response to major concern 3, and have added the following sentence to reflect our new experiment in lines 186-89:

"*Further quantification supports this idea. Looking at various relaxation events suggest that, once fusion is initiated, the time scale of relaxation occurs on a few second timescale and is similar for no DNA and pUC19 conditions when adjusting for approximate droplet size (Fig S3).*"

4) Figure 2: Why do many of the condensates have a punctate DNA signal squarely in the center of the droplet?

Author Response. This is a really interesting question that we've also thought about quite often. More careful study of the puncta specifically might be needed to further parse this. In some of our videos (Mov S3), there are some that look like they are left behind immediately after fusion as a residual 'scar' of sorts, but it's unclear given that we do not see them in all instances of fusion nor do we necessarily see fusion before the appearance of all puncta.

5) Ln 25: Delete "a" from "not a significantly different".

Author Response. Thank you! We made this correction.

6) Ln309: "rod-like filaments". Have these ever been observed in vivo?

Author Response. To our knowledge, these have not been observed *in vivo*.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors showed the establishing process of PolyP-Mg coacervate, and by introducing plasmid DNA, the reentrant DNA shell of this droplet was also realized.

However, due to the SHAB theory, divalent metal ions have a better affinity for polyP than DNA, which is self-evident. Recently, the work about PolyP-Mn droplets done by

Tian et al., published in NComms, also showed that DNA cannot enter PolyP-divalent metal coacervate, so the conclusion was not fresh in the article.

Author Response. We disagree with the reviewer that our work is a recapitulation of the work by Tian et al. (PMID: 38052788). Shell formation and the tuning of condensate size given different DNA properties represent a novel system distinct from that explored in the Tian et al. publication.

DNA shell formation (and clear DNA-polyP/Mg^{2+} condensate interactions via a novel morphology) is very different from DNA exclusion (no significant interaction) brought up by Reviewer 2 and observed previously in many cases including by us. Hence, rather than being a weakness, Reviewer 2's point (and the lack of such DNA shell observations in the Tian et al. study which was published while our manuscript was under review at Nature Communications) instead only strengthens the novelty of our study.

Furthermore, the Tian et al. 2023 study predominantly focuses on polyP-Mn^{2+} systems which have the potential to behave differently given the different divalent cation focus. That specific paper even acknowledges differences in radiotolerance/recruited protein protection from polyP-Mn^{2+} and polyP-Mg^{2+} based systems, again suggesting that even amongst systems of polyP + cations, different properties can be conveyed based on the cation identity and physical properties. Given the context of Mg^{2+} enrichment in polyP granules as well as the granule localization in the nucleoid, there is a need for further characterization of polyP-Mg^{2+}-DNA studies that our work fulfills which is not covered by that of Tian et al., 2023.

Our work contributes in a complementary space to the work of Tian et al. (2023) rather than a redundant one.

The author controls the Mg^{2+} concentration range from 40 mM to 300 mM to ensure the formation of a PolyP-Mg droplet. But this concentration is several times higher than Mg^{2+} in cells？

Author Response. We thank the reviewer for sharing their concerns over our Mg^{2+} concentration range and its applicability in cells. In our work, we do not claim that a certain Mg^{2+} concentration exists in the cell in these exact conditions. Rather, we use an *in vitro* model system to demonstrate general principles, including that of re-entrance, which suggests the existence of optimal windows and an environmental sensitivity governed by the chemical and physical properties of the overall system. The existence of these windows could be something complex systems such as cells could modulate and leverage given the intricate machinery and mechanism cells have evolved, for example in analogy to reentrance-related transcriptional feedback mechanisms proposed by us (PMID: 29569441 and doi.org/10.1016/j.cocis.2021.101488) and tested by Henninger & Young et al. in cells (PMID: 33333019). Furthermore, we demonstrate that a simplified *in vitro* system, even in the absence of a crowder, can provide insights about DNAs surface association and the ability for relatively small concentrations of

DNA to modulate condensate size and growth. The *in vivo* implications of these observations remain to be tested which will depend on reliable and robust measurements of actual concentration of cations involved, crowding, as well as the bona fide protein partners.

Meanwhile, the charged proteins in cells show a much more important influence of polyP-droplets; why didn't the author take such a key role into consideration?

Author Response. We agree with the reviewer that proteins are likely an important component in modulating droplet states. However, an understanding of the system consisting of the minimal set of conserved elements observed in polyphosphate condensates *in vivo* provides an essential baseline for future work incorporating different proteins into the mix. As our study demonstrates, even with this apparently simple system, we observe complex and novel behaviors. We also note that the biophysical observations in our study contribute to our understanding of different tuning effects components can have in multicomponent systems, especially for those with potential surface effects.

The evidence provided by the author in this article is not sufficient to support their conclusion, so I do not recommend publishing this article here

Author response. We respectfully strongly disagree. As we have noted in our responses above and clearly discussed in the paper, we have reported on multiple novel findings related to the condensation architecture and dynamics of the polyP-Mg^{2+}-DNA system backed by strong evidence. Our exciting findings have important implications for cellular chromatin-polyP interactions and regulation, as well as new insight into the physical chemistry of this system which will also be valuable in future industrial and biomedical applications.

Reviewer #3 (Remarks to the Author):

This ms by Racki and co-workers investigates the interactions between polyP, Mg2+ and DNA. While the overall model is interesting, the study lacks biological relevance and has several flaws in the experimental design. The current ms does not provide any direct link between their in vitro data and previous in vivo observations, therefore it is entirely possible that the observed polyP-DNA coacervates might have no biological relevance at all.

Author Response. We thank the reviewer for their comments. While we agree with Reviewer 3 that our *in vitro* system is very simplified, it recapitulates important in-cell observation and establishes a mechanistic baseline, enabling extension towards a complex cellular environment where other factors also play key roles.

As we note in the introduction of our manuscript (lines 49-64), there is ample evidence

to suggest that DNA-polyP interactions are a relevant facet of biology. It has been widely observed across different organisms and labs spanning decades of study that polyP granules are associated with the nucleoid and, significantly, that these are enriched in Mg^{2+}. Despite differences in protein partners across different species, DNA, polyP, and Mg^{2+} are consistent, and thus are the focus of this study. Therefore, we believe that our study offers novel findings and general principles, which also opens doors for future work regarding polyP-chromatin biology and organization as discussed extensively in the manuscript (lines 589-604).

Major issues:
1) Line 111: The authors seem to base this ms mainly on earlier studies in Pseudomonas aeruginosa, where the reported length of polyP chains range between 100-200 Pi units (see Neville et al., "A Dual-Specificity Inhibitor Targets Polyphosphate Kinase 1 and 2 Enzymes To Attenuate Virulence of Pseudomonas aeruginosa." mBio 2021 and Munevar et al., "Differential regulation of polyphosphate genes in Pseudomonas aeruginosa." Mol. Genet. Genomics 2016). It is therefore unclear why the authors use P700 particularly if they want to establish any connections between their current data and earlier work in P. aeruginosa.

Author Response. The reviewer's concern about making sure that we are working in a polyP length regime that is relevant *in vivo* is important, and relates to a larger point about probing the effects of different polyP qualities, including the effect of length and polydispersity. We performed additional experiments with P300 (see below) to address generalizability. However, Reviewer 3 is incorrect that polyP chains range between 100-200 Pi units *in vivo* in *Pseudomonas aeruginosa*. Such shorter polyP chains are observed in eukaryotes, but decades of previous work has extensively established that in bacteria: (1) The two classes of polyP kinases are very processive, producing chains from 200–800 Pi residues (Ppk2 family) to 500-800 residues (Ppk1 family) in length (PMID: 33420907 for review) (2) Gel-based measurements of polyP extracted from bacterial cells demonstrate longer polyPs are prevalent.

Of the two studies the reviewer cites regarding polyP length in *P. aeruginosa*, only one has a gel where length could be assessed. This gel shows polyP chain lengths are greater than 130, but due to the absence of polyP standards longer than 130, and non-linear gel migration, one cannot say more from these gels. In studies where standards spanning a wider range of lengths are included, bacterial polyP chain lengths from cells have been shown to be much longer than P130, and similar to P700, used in our study (PMID: 33113373).

To address the important question of the potential generality of our findings, we include additional work completed with P300, which recapitulates key behaviors (DNA shells with DNA length dependent and concentration dependent size) observed with P700 with regards to the DNA shell phenomenon. P300 also serves as a point of reference to another recent study of bacterial protein-mediated polyP condensates (PMID: 34936433). These observations are included in the text:

"As a further control for the potential generality of the DNA shell phenomenon and a point of reference to another recent study of bacterial protein-mediated polyP condensates[25], we tested polyP with a different size distribution, P300 (Fig S23). For P300, we not only observe DNA shells both with YOYO-1-labeled pUC19, and with the end-labeled, 400bp PCR fragment (Fig S23b-c), but also see that droplet size can also be modulated by DNA concentration and length. Like that of the P700 system, the droplets with higher concentration pUC19 (50 µg/mL) are reduced in size compared to at 10 µg/mL, and the longer 15kb plasmid similarly tends to have smaller droplets compared to the shorter pUC19 case (Fig S23d-e). While it is possible that the exact relationship between concentration and length differs in P300 compared to P700, our results demonstrate that these two parameters have the potential to also tune droplet size for polyP of different length distributions." (lines 398-408)

2) Line 118: One of the most critical points are that the authors use unphysiologically high concentrations of polyP (12.5 mM in Pi units) and magnesium (10 mM-75 mM) to see polyP condensation and observe DNA shells. Such conditions are way beyond what can be observed in non-extremophiles (~2 mM) and can be considered as non-terrestrial. The authors may argue that bacterial polyP granules often contain higher levels of Mg compared with the surrounding cytoplasm. However, this argument is only valid if the authors can demonstrate that in their in vitro system, the majority of Mg is sequestered within the polyP condensates.

Author Response. We agree with the reviewer that it is important to describe the parameter space of our work in the context of the physiological relevant concentration ranges. We designed our study using a fixed polyP concentration near physiological concentrations. We disagree with Reviewer 3's claim that our polyP concentrations of 12.5mM (in Pi units) are significantly beyond the range of those found *in vivo*. Studies in *E. coli*, *P. aeruginosa*, and other species show that cells accumulate > 100 nmol polyP per mg protein, which corresponds to 20-50 mM polyP in terms of Pi units, which is within an order of magnitude of the concentration used in this work (PMIDs: 24560923, 9537383, 9515698, 9555903).

In our revision, we more clearly frame the polyP concentration issue:

"We used 1mg/mL P700, corresponding to 12.5 mM polyP in terms of Pi units. Studies in E. coli, P. aeruginosa, and other species show that cells accumulate > 100 nmol polyP per mg protein, which corresponds to 20-50 mM polyP in terms of Pi units[41-44]." (lines 104-106)

Regarding the Mg^{2+} concentrations chosen in the study, we would like to clarify that we are not claiming that free Mg^{2+} concentrations in the cell reach 100mM. Instead, we seek to demonstrate the general principles underlying a set of phenomena (DNA/polyP-Mg^{2+} condensate surface interaction + condensate size modulation based on DNA properties) rooted in a biologically based system. We agree with the reviewer that there are elements that make our system an imperfect replica, as would be the case in many simplified model systems. With respect to phase boundaries, we could

hypothesize that, in the cellular environment, proteins and other cationic species would likely shift the phase boundary. Additionally, we emphasize that by focusing on the most abundant divalent cation, shown to be enriched in polyP condensates in all three domains of life, our work presents a general mechanistic framework critical in enabling extension towards multi-component studies that more globally capture the complex cellular environment. We note that the protein players may not be conserved, but this system of nucleic acids, polyP, and Mg^{2+}, is universal.

We have explicitly acknowledged this important point and the importance of future *in vivo* work in lines 588-91 of the open question section in our revised manuscript:

"How this system plays out in a biological context merits further study, particularly balancing considerations of lower free Mg^{2+} concentrations observed in cells (1-2 mM), and the potential impacts of compensatory networking species, including proteins, metabolites, and other metals *in vivo*."

Moreover, it is entirely possible that polyP directly interacts with the nucleic acid dye YoYo 1, which is positively charged. If this were the case, it would significantly increase the complexity of the measurements and interpretation, and makes one wonder why the authors used a dye in the first place.

Author Response. We agree with the reviewer that YOYO-1 could have confounding effects on our measurements. This is something we thought about frequently in preparing our manuscript, and we therefore included controls without YOYO-1 in the original submission (Figs S2b(left), 3, 4d-e). However, prompted by both Reviewer 3 and Reviewer 1's suggestions, we performed additional controls in the revision, described below, to rule out potential YOYO-1 artifacts for our major conclusions (Figs S2b(right), S10). These additional orthogonal controls promoted by the Reviewer, strengthen our conclusions.

Regarding the question of why we chose YOYO-1, we specifically used an intercalating dye to overcome low signal-to-noise in shells for higher DNA lengths, which was not possible with end-labeled DNAs. Even with pUC19, the signal intensity of an end-labeled plasmid with Cy5 was relatively faint and required high LED power to observe by widefield microscopy (Fig S2b, left). We anticipated that this problem would be exacerbated at longer lengths as we held the mass concentration constant. YOYO-1 enables us to visualize and confirm the presence of shells at varied lengths spanning different orders of magnitude, and to get much brighter signal, given the low overall concentration of DNA applied.

However, as the reviewer points out, the potential for non-specific interaction is one to be wary of, and thus, we included several controls and orthogonal approaches in our study.

(a) In our supplementary data, we recapitulate DNA shells using covalently bound, Cy5 end-labeled, linearized pUC19 (Figure S2b, left).

(b) Our quantification of condensate size as a function of DNA concentration was also performed in absence of YOYO-1 (Fig 4d-e). For the revision, we added a direct comparison of circular and linear forms of pUC19 and the 15kb plasmid with and without YOYO-1 which recapitulates our size distribution observations for two extreme DNA length conditions (Fig S11).

(c) Furthermore, we include an orthogonal approach to imaging shells through cryo-ET, which showed differential DNA surface organization of select length and concentration conditions (Fig 3). All cryo-ET experiments were conducted in the absence of YOYO-1.

(d) Due to the high noise in SI Fig 2b, for the revision, we performed a new experiment using a shorter DNA which increased dye density given a constant mass concentration and used a brighter dye, ATTO488 (Fig S2b, right). After coupling, ATTO488 carries a -1 net charge.

Although we acknowledge that the use of YOYO-1 does add complications, our controls without the dye and those recapitulating observations using orthogonal labeling approaches demonstrate that the shell properties we do observe, as well as the differences we see in size due to changes in length and concentration, are likely to be real and not artifacts of the system.

3) Lines 120 & 394: To evaluate the viscoelastic properties of polyP condensates, the authors should, at a minimum, quantify the coalescence rate of polyP granules to obtain η/γ. It would be better if microrheology would be performed. To meet the standards of this journal these approaches should constitute an integral part of this MS and should not be left for future work.

Author Response. We acknowledge that careful quantification of rheological properties of condensates in our work would be an interesting avenue of study. However, we believe that this is outside the scope of this specific paper, which highlights the novelty of a) an observed surface interaction of DNA and polyP-Mg^{2+} condensates and b) the potential for DNA properties to modulate droplet properties and size. While the bulk material properties and viscoelasticity of condensates raise interesting questions, these are distinct from the observations of shells and the way that DNA properties tune overall condensate size distributions.

In addition, we note that while coalescence rate measurements have been used in several studies, the standard analysis/interpretation is technically applicable only for Newtonian fluids (e.g., see PMID: 34645832). Given that the condensates we are studying in this work are quite likely viscoelastic materials/complex fluids (based on current emerging understanding in the biomolecular condensate field, a number of detailed studies of biomolecular condensate rheology, and related work from the polymer physics field - PMID: 34645832, 33453924, and 36881934), as well as the experimental limitations in interpreting passive droplet fusion for accurate material property quantification (ie: potential for surface effects, limits in statistics and sample size, complications in time resolution through microscopy based techniques, etc), we

feel that a quantitative study of the kind that the reviewer brings up would require dual-trap optical-tweezers or other similar method that can directly test rheological properties for droplets.

Thus, even though in our revision, we do perform calculations for analyzing fusion to estimate the timescale at which relaxation occurs (Figure S3), we are careful to not interpret our data too broadly or to draw conclusions of viscoelasticity or lack thereof based on the data we have on hand. We provide the slope of our pUC19 tau vs length plot, which—if we assume that our system is a Newtonian fluid and other experimental limitations have a relatively small effect—would be our experimentally calculated η/γ, but also include the note in our SI with Figure S3 explaining experimental caveats.

We also add that the specifics of how to make and interpret such measurements are still an active area of development in the field (e.g., see PMID: 37066350; PMID: 36881934). This point again adds to our consideration that such studies, while important, are outside the scope of our current work. Thus, because we agree with the reviewer that the question is interesting and also believe that claims of viscoelasticity and material state require careful study, we were careful to not make claims about the exact material state of our observed system and instead leave them open to further investigation.

4) Line 122: How does FRAP properties change with Mg^{2+} concentration?
Author Response. We agree with the reviewer that this is an important question, which was also raised by Reviewer 1. We thus performed FRAP experiments with three additional Mg^{2+} conditions (12.5mM 250mM, 500mM, and compared these to 100mM; Figure S1b). We observed that the internal exchange is slightly slower at higher concentrations of Mg^{2+}. All were in a similar regime of relatively slow recovery over the course of 30min.

These observations are noted in the text (lines 117-119),
"These observations were similar for condensates formed across multiple Mg^{2+} concentrations, with slightly slower recovery occurring as Mg^{2+} concentrations increased to the right of the peak (Fig S1b)."

Why not label DNA directly to test for FRAP properties.
Author Response. We believe that getting at the dynamics of shell properties is a more difficult question than FRAP could address. Given the nature of the shell, any recovery observed would be a potential product of (a) shell lateral/surface fluidity and/or (b) exchange with dilute phase unbleached fluorophore. Decoupling internal exchange/fluidity and external exchange or recovery for condensates has typically been done by bleaching a small area within a droplet. However, since the shell is notably not enclosed, it is not possible to set the experiment up in this way, which limits our ability to decouple surface fluidity and molecular exchange.

In this sense, even were we able to overcome the technical limitations based on the signal/noise of fluorophores, it would be difficult to draw definitive conclusions regarding the material state/surface fluidity alone using a FRAP based method.

5) Line 168: The bacterial nucleoid is a circular DNA of several Mbp and is usually present in one or two copies in a cell. Therefore, considering the local environment of an in vivo polyP granule, the DNA surrounding it should be considered as long linear strands rather than small circles. This makes pUC19 a less ideal mimic of in vivo conditions.

Author Response. We agree with the reviewer that drawing conclusions just from circular pUC19 would not test properties of DNA relevant to the bacterial nucleoid. However, our study uses a range of DNA lengths and topologies, from supercoiled plasmids of 2.7 to 30 kb, to linear phage DNAs of 48 and 166 kb, not just pUC19 (2.7kb). Additionally, we strongly disagree with the assertion that 'long linear strands' of DNA would be more physiologically relevant than supercoiled plasmid DNA. Supercoiling is vital to recapitulate *in vivo* conditions, and we picked a range of DNA lengths that span below and above the length of plectonemes observed *in vivo* in bacteria, as noted in lines 265-67. Nevertheless, we still see the basic phenomenon of DNA shells with long, linear DNAs (Figs S18-9).

6) Line 182: FRAP analysis on entire condensates is also essential in understanding how DNA shell affects polyP dynamics. If the DNA shell really exists *in vivo*, then the fact that polyP granule fusion is never directly observed *in vivo* can lead to at least two explanations: 1) PPKs and nascent polyP are trapped within DNA shells while ATP/GTP can penetrate through; 2) PolyP chains are able to penetrate the DNA shell and Ostwald ripening might be at play. The latter might better explain the decline in polyP granule number during starvation in P. aeruginosa.

Author Response. We believe that Reviewer 3's interpretation regarding what is known about fusion *in vivo* might have stemmed from our note about incomplete cellular granule coalescence (former lines 180-182). We note that the premise for fusion not occurring *in vivo* is incorrect and apologize for this unintentional confusion due to our phrasing. We meant to convey that all visible granules do not completely coalesce to a single larger granule under these conditions as the mechanism of coarsening of polyP granules in bacteria has yet to be determined. To our knowledge, labs have not yet attempted/published imaging of polyP granule fusion events in live bacterial cells using the super-resolution imaging techniques that would be required for these diffraction limited foci.

To clarify this point in our revision, we have changed that sentence to:
"This question is especially relevant given our prior observations that in P. aeruginosa under nitrogen starvation conditions, initial coarsening of smaller granules results in fewer larger polyP granules that are transiently evenly spaced in the nucleoid, but do not coalesce to a single larger droplet" (lines 181-84)

However, we think Reviewer 3's question about Ostwald ripening is an interesting one. While we do think in our *in vitro* system growth is largely driven by fusion, it is interesting to consider the potential effects the DNA shell has on polyP exchange across the condensate interface, especially given the potential for suppressed fusion relative to our *in vitro* system in potentially more crowded *in vivo* environments. We do note that previous work has suggested that Ostwald Ripening may contribute less to coarsening than fusion in the nuclei of U2OS cells (doi.org/10.1038/s41567-020-01125-8). However, this has not been demonstrated to be the case for polyP condensates.

As suggested, we conducted an experiment comparing whole droplet FRAP of polyP-Mg^{2+} condensates in the presence and absence of DNA (no YOYO-1 added). The recovery of these curves were similar for the two cases, with both showing about 50% recovery by 10 minutes (Fig S4). As expected, recovery occurred radially into the droplet through the boundary of the condensate (Fig S4). The similarity would suggest that the DNA shells are not substantially inhibiting interfacial exchange of polyP. We have added sections in our main text to include this new insight:

"We also conducted FRAP on whole droplets in the presence and absence of DNA to test whether DNA shells significantly restricted polyP exchange between dense and dilute phases. The recovery curves were similar for both cases, suggesting that DNA shells do not substantially prohibit exchange (Fig S4)." (Results, lines 189-192)

While we think this likely indicates that Ostwald ripening is not a major contributor to the observed differences in droplet size/growth, the FRAP results do not provide information about Ostwald ripening directly. More specifically, our experiments show that polyP diffusion across droplet interfaces occurs which is required for Ostwald ripening. They do not, however, directly address whether Ostwald ripening is kinetically competitive with fusion. As such, a more comprehensive study of Ostwald ripening, using more direct approaches (PMID: 34155210 & doi.org/10.1038/s41567-020-01125-8) and assessing the relative contributions of different coarsening mechanisms would be needed to fully answer this question. Thus, we think further analysis beyond what we have done is outside the scope of this work and better suited for future experiments. Accordingly, we have added the following note in the main text discussion.

"Our work opens questions regarding the material state of DNA shells and their influence on condensate dynamics. As noted above, the mobility and packing of the DNA shells likely impact various condensate dynamics, hence a detailed understanding of this aspect will be an important future direction. In addition, while our data suggest that exchange of polyP across the condensate interface appears similar without and with pUC19 shells, it is possible that DNA affects exchange to a finer degree than we resolve if we take into account potential uneven shell morphology and limitation of experimental methods. Therefore, interesting future directions will be a more extensive

characterization of this effect across different DNA properties, as well as directly testing the related question of shell influence on Ostwald ripening." (lines 547-55)

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

All of my concerns have been addressed.

- Anthony Vecchiarelli

Reviewer #2 (Remarks to the Author):

In the new manuscript, author largely improved completeness of the research. Here we still have some questions that need answer.

1, The authors used the P700（Kerafast） and P300（RegeneTiss）. It is necessary to show the exact chain length and chain-length distribution. Here, we suggest P-NMR and urea-PAGE.

Author can refer to the reported work (Electrophoresis 2018, 39, 2454–2459 and Anal. Chem. 2019, 91, 7654−7661).

2, The authors claimed "We used 1mg/mL P700, corresponding to 12.5 mM polyP in terms of Pi unit". In our knowledge, polyP 1 mg/mL equals 9.7~9.8 mM Pi (1/102≈0.0098） with Na as its counter ion.

Why here it equals to 12.5 mM polyP？

3, High molecular weight polyP is always separated from cells. There may be Mg2+ contained in the polyP species as counter ion, ICP-MS test of polyP should be done since the system is related with Mg concentration.

Reviewer #3 (Remarks to the Author):

The reviewers have addressed the comments adequately. The main issue still stands, however, whether their in vitro findings have any in vivo relevance and/or significance.

We thank the reviewer for their comments. Below, we provide our point-by-point responses (blue) to the reviewer comments (black), and have made the corresponding noted changes in the revised manuscript.

Reviewer Comment: 1, The authors used the P700（Kerafast） and P300（RegeneTiss）. It is necessary to show the exact chain length and chain-length distribution. Here, we suggest P-NMR and urea-PAGE.
Author can refer to the reported work (Electrophoresis 2018, 39, 2454–2459 and Anal. Chem. 2019, 91, 7654−7661).

Author Response: We agree with the reviewer that assessing the relative length distributions is important, given that they come from different commercial sources. We have therefore performed PAGE analysis according to PMID=30009536 as suggested by the reviewer and included the gel (Fig. S23a). We have updated the text (Lines 372-375) accordingly:

As a further control for the potential generality of the DNA shell phenomenon, we tested polyP from a different commercial source which, using previously established gel methods, are more narrowly dispersed with shorter and longer size distributions compared to our P700 samples, P130 and P300 (Fig S23a).

Because we observed that P300 is less polydispersed and on the longer end of the P700 distribution, we further included a control with P130, which is shorter (PMID: 33113373). These are commercial sources of polyP and we therefore believe the gel is sufficient for qualitatively assessing length and polydispersity.

Reviewer Comment: 2, The authors claimed "We used 1mg/mL P700, corresponding to 12.5 mM polyP in terms of Pi unit". In our knowledge, polyP 1 mg/mL equals 9.7~9.8 mM Pi (1/102≈0.0098）with Na as its counter ion.
Why here it equals to 12.5 mM polyP？

Author Response: Thank you for pointing this out and we have adjusted the number in the text accordingly (Line 108).

Reviewer Comment: 3, High molecular weight polyP is always separated from cells.

Author Response: We disagree with the claim that high molecular weight polyP always is separated from cells. Polyphosphate can also be made from phosphate glasses, which are typically prepared through dehydration of inorganic phosphate salts at high temperature to form glass melts (PMID: 33721178 for a recent historical summary).

Reviewer Comment: There may be Mg2+ contained in the polyP species as counter ion, ICP-MS test of polyP should be done since the system is related with Mg concentration.

Author Response: We acknowledge that for polyP prepared from cells, Mg^{2+} could be expected to be present.

Kerafast (https://www.kerafast.com/productgroup/330/polyphosphate-long-chain-p700) states that Na^{+} is the dominant cation and estimates that Li^{+} may comprise up to 10% of the total cation content, consistent with polyphosphate made through limited alkaline hydrolysis of sodium phosphate glass (see PMID: 20709905).