Dear editor and reviewers,

We want to thank the reviewers for their helpful comments. Based on their suggestions, we could improve the manuscript substantially.

**Point-by-point response**

**Editorial comments:**

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. done

2. Please revise lines 284-286, 289-291 to avoid previously published text. done

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].” Done; <http://www.rsc.org/journals-books-databases/journal-authors-reviewers/licences-copyright-permissions/>

4. Please remove the embedded table(s) from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text. done

5. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …” done

6. Please spell out each abbreviation the first time it is used. done

7. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. done

8. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc. done

9. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Milli-Q, Nanodrop, Amicon, etc. done

10. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations. done

11. Please revise the *protocol text* to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). done

12. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. done

13. Lines 55-67: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. done

14. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. done

Lines 70, 176, etc.: What container is used? Please specify throughout. done

Line 76: Please specify the buffer used in this step. done

Line 112: Please specify how to measure concentration by Nanodrop. For instance, what wavelengths are measured? done

Line 120: Please describe how AGE analysis or nsTEM imaging is performed. done

Line 147: Please specify the amount/volume/concentration of NaOH added in this step. done

Lines 186-187: Please describe how this is done. done

Line 222: Please describe how to perform PEG purification. done

Lines 250-251: Please describe how this is done. done

15. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section. done

16. Lines 111-113, 115-117, 122-134: Please note that calculations are not appropriate for filming. Please un-highlight the calculation steps. done

17. Please include single-line spaces between all paragraphs, headings, steps, etc. done

18. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. done

19. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. done

20. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted. done

21. As we are a methods journal, please add a Discussion section to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

all addressed in discussion

22. JoVE article does not have a Conclusion section. Please move information in the Conclusion section to Results or Discussion section. done

23. References: Please do not abbreviate journal titles. done

24. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. done

Reviewers' comments:

**Reviewer #1:**

Manuscript Summary:

In this method article, Barišić and co-workers employed LPEI and chitosan coating to protect the DNA origami in the Mg depleted and nuclease-rich media. The coated DNA origami still remained the addressability. This protocol is well-written and detailed procedures have been provided. I would like to recommend it for publication in JoVE after minor revisions.

Minor Concerns:

1. In the step of PEG purification of DNA origami, the excess PEG has been fully removed or not. The PEG residual will whether affect the following coating process…. Based on the original paper (cited in the manuscript), residual amount of PEG will be left. However, this residual PEG doesn’t interfere with polyplex formation nor with DNA hybridization and super-assembly of DNA origami.

2. Other DNA origamis such as triangle and square have been widely used in the drug delivery system. protection of triangle and square origami should be also discussed …In our work, we used 3 different types of DNA nanostructures for the polyplex formation; square-lattice, honeycomb lattice and wireframe DNA origami. Other structures such as triangular and square DNA origamis could have been also used to demonstrate the stabilisation concept. We added additional studies that discuss the stabilisation of DNA origamis.

**Reviewer #2:**

Manuscript Summary:

In their manuscript 'Gene-therapy inspired polycation coating for protection of DNA origami nanostructures', Barisic and Ahmadi describe a protocol for encapsulating DNA nanostructures with polymers and thereby protecting them from nuclease degradation.

Major Concerns:

The introduction omits the three papers closest to the work presented herein:

1) Kiviaho, Nanoscale 2016, DOI: 10.1039/C5NR08355A

2) Agarwal, Angewandte Chemie 2017, DOI: 10.1002/anie.201608873 and

3) Ponnuswamy, Nature Communications 2017, DOI: 10.1038/ncomms15654.

All suggested references were added.

**Reviewer #3:**

The authors have introduced a method for the protection of DNA origami nanostructures through coating them with natural cationic polysaccharide chitosan and the synthetic linear polyethyleneimine (LPEI). This is informative protocol for users who are looking for further application of the DNA origami with higher stability. The protocol is well-written and should be followed by others with relative ease.

I have the following minor comments:

1. Previously, protocol papers describing DNA origami (or nanostructure) synthesis, and characterization were published through the JOVE.

Ben-Ishay, E., Abu-Horowitz, A., Bachelet, I. Designing a Bio-responsive Robot from DNA Origami. /J. Vis. Exp./ (77), e50268, doi:10.3791/50268 (2013).

Amir, Y., Abu-Horowitz, A., Bachelet, I. Folding and Characterization of a Bio-responsive Robot from DNA Origami. /Journal of Visualized Experiments/. (106), e51272, doi: 10.3791/51272 (2015).

Wei, B., Vhudzijena, M.K., Robaszewski, J., Yin, P. Self-assembly of Complex Two-dimensional Shapes from Single-stranded DNA Tiles. /Journal of Visualized Experiments/. (99), e52486, doi: 10.3791/52486 (2015).

Since the protocol shows detailed protocol with the step by step movie, this will help the reader's understand if the authors add the reference on the manuscript.

2. From line 29 to 31 of page 1, the authors described current development of DNA origami field, including functionalization and conformational changes. We recommend the authors to add more reference on the part, to help readers understand of the field, such as;

for functionalization :

Kuzyk, A., Schreiber, R., Zhang, H., Govorov, A.O., Liedl, T., Liu, N. Reconfigurable 3D plasmonic metamolecules. /Nature Materials/. \*13\* (9), 862-866, doi: 10.1038/nmat4031 (2014).

for conformational change :

Choi, Y., Choi, H., Lee, A.C., Lee, H., Kwon, S. A Reconfigurable DNA Accordion Rack. /Angewandte Chemie International Edition/. \*57\* (11), 2811-2815, doi: 10.1002/anie.201709362 (2018).

All references were added.

3. In the materials part, author described the gel staining dye as "SYBER safe DNA gel stain". However, from our knowledge, the proper term for the dye is "SYBR safe". Also, the proper name for "Freeze squeeze gel extraction" is \*Freeze 'N Squeeze™ DNA Gel Extraction Spin Columns\*. To help the reader's understanding and direct use of the protocol, please double check the name of the materials.

The "SYBER safe DNA gel stain" is the actual name used by the supplier company. "Freeze squeeze gel extraction" was corrected to \*Freeze 'N Squeeze DNA Gel Extraction Spin Columns\*.

**Reviewer #4:**

Manuscript Summary:

This manuscript describes a protocol to stabilize DNA nanostructures with cationic polymers. As DNA origami structures are destabilized in low Mg++ concentrations and are susceptible to nuclease degradation, techniques which can stabilize and protect nanostructures will be useful for future applications involving DNA nanotechnology.

Major Concerns:

The only major concerns were the lack of some relevant citations:

The instability of DNA origami in low Mg++ conditions has been documented before. The first major description was described in "Addressing the Instability of DNA Nanostructures in Tissue Culture" DOI: 10.1021/nn503513p and more recently in "On the Stability of DNA Origami Nanostructures in Low-Magnesium Buffers" https://doi.org/10.1002/anie.201802890.

Also work has been done on stabilizing nanostructure. Two notable efforts are "Virus-Inspired Membrane Encapsulation of DNA Nanostructures To Achieve In Vivo Stability" DOI: 10.1021/nn5011914 and "Oligolysine-based coating protects DNA nanostructures from low-salt denaturation and nuclease degradation" https://doi-org.ezp-prod1.hul.harvard.edu/10.1038/ncomms15654

These articles should be cited

All references were added.

Minor Concerns:

There are several minor concerns.

-Section 1 Note 4

The protocol states to resuspend the pellet in a desired buffer; it should be specified that the desired buffer must be stabilizing towards the structure. Added

-Section 1 Note 5

The protocol states to "Incubate the sample for one day at r.t. at 650 rpm" but it is unclear what that refers to. Does this mean to vortex? Incubation in a thermomixer was meant, which was corrected in the manuscript.

-Section 8 Note 1

please state centrifugation conditions added

-Section 12

Is it not possible to visualize nanostructure-protein complexes via TEM?

Many publications have been able to capture this. For example, see "Light-Triggered Release of Bioactive Molecules from DNA Nanostructures" DOI: 10.1021/acs.nanolett.6b00530

Yes, it is possible to image nanostructure-protein by TEM. The purpose of the step described in section 12 was to evaluate if the functionality remains intact upon the polycation coating.

-Fig 3c caption

reiterate that decomplexation of chitosan polyplexes was performed prior to AGE

done

-Figure 4a,b

What is the chitosan control for these graphs? Should it be LPEI for 4a?

As chitosan has also a slight oxidative effect, it was used as the control itself. All the samples including chitosan were blanked (water was used instead of polyplex or polycation solution).

We hope that we could successfully clarify all raised points.

Kind regards,

Ivan Barisic