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Peer-Review Record:

Bending Elasticity Modulus of Giant Vesicles Composed of *Aeropyrum Pernix* K1 Archaeal Lipid

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Reviewer 1: Anonymous
Reviewer 2: Anonymous
Editor: Helga Stan-Lotter (Editor-in-chief of the Section "Life Sciences")

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First Round of Evaluation

Round 1: Reviewer 1 Report and Author Response

It is an interesting topic, however, the presentation seems not to be clear.

It would be better to have a short but clear description about what "bending elasticity modulus" is and its relevance to biological functions.

Response: Bending modulus is a material constant which does not depend on the size and shape of the object, but on the composition of the vesicle membrane and aqueous solution used (Lines 121–123 in the new version). We have inserted a definition of the membrane bending modulus (Lines 115–116) while the text regarding this issue extends between Lines 112–128.

The theory section could be in the Method section with both principle and procedures to get k_c values.

Response: As the referee suggested The Theory and Experimental section were organized into the Section "Materials and Methods". Other sections were renumbered accordingly.

However, not all results seem to be presented. For example, it is not clear what kind of images were recorded, which were used to calculate the k_c values, what the sizes/shapes of the fluctuating vesicles looked like. How these observations would be different or similar to other types of vesicles.

Response: As it was explained in the text our experimental method is developed for application to almost spherical vesicles. Vesicles with diameters between 20 and 40 micrometers were considered, whose deviations from spherical shape (fluctuation of the radius) were small in comparison with the mean sphere radius. We added the information concerning the shape and the size of the studied objects and added pictures of the equatorial cross section of a fluctuating vesicle (new Figure 3), as seen under the phase contrast microscope.

It is also not clear about the "thermally induced shape fluctuations", such as the temperatures and incubation length and *etc.*, especially this is a hyperthermophilic archaeal membrane vesicle, which would be expected to be more thermostable than those eukaryotic ones.

Response: The observed fluctuations of the membrane do not destabilize it. However, the comment of the reviewer is notable and we state in the conclusion of the manuscript (Lines 229–232) that at room temperature the thermophilicity is not necessarily related to the bending elasticity.

It would also be helpful to clarify/expand the description about the drug delivery system using the archaeal vesicle.

The first sentence in the introduction section should be corrected as cell should not be considered to be "the building block of life". It's better to re-write the "basic motivation" for the study.

Response: We have rewritten the introductory paragraph (Lines 46–60).

There are two reference lists, one for 1–31 and another from 1–34. There are repeated ones. It's not clear why the case was.

Response: Thank you for the comment, there was a mistake. We have tidied and updated the references.

Round 1: Reviewer 2 Report and Author Response

This manuscript described the measurement of bending elasticity of giant vesicles (GVs) prepared by archaea-derived lipids. Stroboscopic illumination measurements of GVs and theoretical models about shape fluctuation of GVs revealed that the archaeal lipid-based GVs showed the almost same value of bending elasticity modulus with synthetic lipid-based GVs. According to the authors, this is the first report for the measurement of bending elasticity modulus for archaeal lipid-based GVs. The measurement itself is novel, but same methods and theories have been reported in the authors' previous papers. In addition, the number of tables and figures are too low for article. If could, the author should add other experimental data.

Response: As the referee suggested, we have added data for the bending elasticity modulus of another lipid type—POPC lipid membrane. Also, we have added a new figure (Figure 3).

I also have questions and comments for acceptance. The list of comments is as follows.

In Page 1, Lines 27–28, the author described "the experimental set-up was improved …". However, the authors have already reported the measurements of bending elasticity using stroboscopic illumination in author's previous papers (such as Genova *et al.*, J. Optoelectro. *Adv. Mat.*, 2005). Describe how the experimental set-up was improved in this paper more precisely.

Response: A number of improvements of the experimental procedure for the thermally induced shape fluctuation method were used in this study: the stroboscopic illumination was improved on several steps to achieve a better experimental conditions; a thermostatic stage was used to set and control a constant temperature, the analysing procedure was improved by adding strict objective criteria for qualification of the vesicle as a whole as well as for acceptance or rejection of a given contour of the sequence of recorded images and the white noise contribution to the amplitudes of thermal shape fluctuations was

evaluated and taken into account. We have given short description of the improvements made and for each of them cited the corresponding articles for detailed explanation. (Lines 181–184; 187–193).

As a comparison of archaeal lipids, SOPC was used in this study. SOPC contains an unsaturated bond in its alkyl chains and its transition temperature seems to be quite low compared with archaeal lipids. What is the reason why the authors selected the SOPC for the representative of eukaryotic lipids?

Response: SOPC was chosen as both lipid types have transition temperature far below the temperature at experiments. In the revised version, we measured the bending modulus for another lipid type (POPC), measured and analysed by the same method for the same experimental conditions.

In page 4, between line 108 and 109, this equation should be numbered as (6).

Response: We have corrected the wrong number.

Describe the temperature condition for measurement of fluctuation of GVs. Are there any effects of temperature on the fluctuation of GVs?

Response: The aim of the present study was to obtain the bending elasticity modulus of the archaeal membrane at fixed temperature (27 degrees C) far above the phase transition temperature. We have not measured the temperature dependence of the bending constant.

In this paper, bending elasticity modulus was measured in pure water. Are there any effects of ionic strength or composition of solution on the fluctuation of GVs?

Response: In this work we present the bending elasticity modulus of archaeal lipid membrane in pure water environment. We added the details concerning the water purification (Lines 173–175).
In other works the influence of different admixtures in the aqueous solution around the membrane was studied and the values depending on the type and the concentration of it for given lipid were reported. As the referee suggested we added such information with the corresponding references in the introduction section (Lines 57–60).

Second Round of Evaluation

Round 2: Reviewer 1 Report and Author Response

It is also not clear about the “thermally induced shape fluctuations”, such as the temperatures and incubation length and *etc.*, especially this is a hyperthermophilic archaeal membrane vesicle, which would be expected to be more thermostable than those eukaryotic ones.

The observed fluctuations of the membrane do not destabilize it. However, the comment of the reviewer is notable and we state in the conclusion of the manuscript (Lines 229–232) that at room temperature the thermophilicity is not necessarily related to the bending elasticity.

Response: We thank the reviewer for further comments and hope that we can clarify the issues as given below. We have made changes in the manuscript (marked red) and added one new reference (ref. [35]).

If only measured at one temperature, how would it be possible to draw a conclusion with “thermally induced shape fluctuations”?

Response: In principal in order to obtain elastic constants you need to apply force and measure the deformation that this force causes. If we want to obtain the bending elasticity of lipid vesicle we need a force with a very small power. In the case of thermally induced shape fluctuations as a force we use the Brownian (thermal) motion of water molecules, bombarding the membrane. This force is stochastic (we do not know its instant value), but the mean value of it is proportional to the temperature. As a result of this stochastic bombardment the lipid vesicle deforms (changes its shape or fluctuates). We acquire a big amount of pictures of fluctuating vesicle in order to get the mean value of the deformation that our force causes. To extract mean we need to have stationary conditions over the time, so the mean is taken, that is why it is really important to have constant temperature throughout the experiment.

Is it possible to do the measurement at elevated temperatures?

Response: Using our experimental system we can measure bending elasticity also at higher temperatures (up to approximately 40 degrees C).

Would k_c be temperature dependent? If yes, what would be the valid range?

Response: The bending elasticity modulus depends on the temperature below and near the phase transition temperature, but far above the phase transition temperature (this is the case in our experiment) the bending elasticity modulus is practically constant. See: Temperature and Chain Length Effects on Bending Elasticity of Phosphatidylcholine Bilayers. Fernandez-Puente, I. Bivas, M. D. Mitov and P. Meleard, Europhys. Lett., 28, 181 (1994). We have added this text to the Discussion (Lines 234–237) and also added the reference to the reference list (ref. [35]).

The photo added merely shows the shape, but is it possible to show a few time related changes of the shape at different temperatures?

Response: It is not a problem for us to make pictures of a fluctuating vesicle at different temperatures, but these photos would not be related to the manuscript.

The numbering from Lines 306–307 may not be correct as there are two 2.2.

Response: We thank the referee for pointing to negligent mistakes in numbering. We have corrected wrong numbering of subsections.

Round 2: Reviewer 2 Report and Author Response

The manuscript was thoroughly revised according to reviewer's suggestions and comments. Now this revised manuscript can be acceptable for publication.

Response: As I understand the referee had no further comments.