nature portfolio

Peer Review File

A dynamic biointerface in mussels mediated by a mechanoresponsive intermediate filament-based biopolymer

Corresponding Author: Professor Matthew Harrington

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Comments:
Youssef et al. in this manuscript focus on the mussel byssus stem root given its importance as a biointerface between living extracellular matrix comprising the soft tissue of the organism and a stiff non-living biopolymeric holdfast - the byssus. This is relevant and original, because the byssus stem root has received much less attention from the scientific community than byssus adhesive pads (the other biointerface between the byssus and the substrate) and because the presented results can contribute for designing innovative and nature-inspired multifunctional biointerfaces (e.g. implants, bioadhesives) capable of on-demand release. The authors support their conclusions with a multitude of robust data obtained with several complementary methodologies. The authors significantly advance the state of the art, demonstrating that the byssus stem root outer lamellar layer is composed by a newly identified protein, MSP-1, which exhibits structural similarities to intermediate filaments (IF) known for mechanically stabilizing intra- and extracellular structures. Interestingly, MSP-1 is shown to undergo a structural transition from an alpha helical coiled-coil to a beta-sheet conformation which the authors argue that provides mechanical toughness to the interface, but might also enable mechanosensing pathways at the interface, given the intimate connection to billions of motile cilia. Given the quality of the manuscript, I strongly support its publication at Nature Communications. I have just a few minor improvement suggestions, listed below.

Specific comments:
Put in italic the genus and species names
Lines 121-123: this sentence is quite confusing, and you should clarify if you used a publicly available transcriptome or produced a new transcriptome. From the M&M section it seems that you produced a new database. I would suggest “To identify the protein, the extract was digested with trypsin, followed by tandem mass spectrometry (MS/MS) analysis using a newly produced gland-specific transcriptomes from Mytilus edulis.”
Line 124: clarify if the mentioned “23 individual tryptic peptides” were all unique peptides or if not how many where. This is important to unequivocally demonstrate the correct protein ID
Lines 476-477: clarify which FDRs where considered and why genes differential expression is mentioned, if in the manuscript expression levels among the different mussel foot tissues is not mentioned nor discussed.

Reviewer #2

(Remarks to the Author)
Scope of this review: This review is limited in scope to the advanced characterization methods employed in the paper by Youssef et al. Namely, I have confidence in assessing the electron microscopy (STEM, FIB-SEM) and the WAXS experiments. Other analytical approaches are outside of my expertise and have not been commented on.

Key Findings: The manuscript utilizes a number of advanced microscopy approaches in correlative/complementary fashion such as, FIB-SEM, SIMS, WAXS, Raman, histology and proteomics, to support the claim that the structure of MSP-1 in the outer lamellar layer (OLL) experiences a transition from an alpha helical coiled-coil to a beta-sheet. In general, I find the quality of these analytical tools to be high, the data interpretation is sufficient, and data are presented clearly to support the conclusions in the work.

Analytical Approach: It is important to note that many of the advanced techniques are not routinely applied to large sample numbers in any literature, and the same is true for the work reported herein with 2 to 5 specimens appearing to be the most common. While this is standard for these advanced tools, it would be important to highlight if this is expected to present any limitations given the diversity of the species investigated (for which I am not aware). The authors often comment in the methods included herein, or referenced, that “over 100 images were taken”, and this is not the same as 100 specimens. It would be relevant to comment if 100 images were analyzed and if different regions reported the same/different findings, but otherwise, the most relevant number is the sample size.

Suggested improvements: I have minor comments for the authors to consider to improve the presentation of the data included below.

1. STEM Imaging:

1a. There is no information on how the STEM samples were prepared, both in terms of how the tissue was processed (e.g. embedded, stained) or how the samples were produced for TEM (e.g. ultramicrotome or FIB) – instead authors were referred to Ref 16, where the same text appears for the STEM imaging methods and the details on specimen preparation are given. Knowing the sample is osmium stained and cut by a microtome would significantly help interpretation by a reader, some limited duplication of preparation methods might be helpful, though I agree a complete duplication is not necessary.

1b. The paper indicates that the “Using scanning transmission electron microscopy (STEM), we observed that the outer phase of lamellae exhibits a woven texture of thin fibrils, while the core consists of thicker fibrils that are presumably collagenous in nature…” . This is supported by the Raman and histological staining, but surprisingly, not by the STEM itself. There is no collagen banding clearly visible in this region – but in Ref 16, collagen banding is visible. Why is there no banding visible in this region if these are collagen fibrils? Did the authors not probe this region with higher resolution? Or by cutting the sample with an ultramicrotome, it can be difficult to maintain the sample integrity. Some comment on why the STEM does not support this is warranted. If one zooms in quite a bit, there is a faint hint of banding in 3b that goes unmentioned – investigating if this follows the periodicity for collagen (e.g. FFT) could provide an additional support to the composition of these filaments in the centre of the OLL.

1c. The authors state “several STEM images show locally aligned fibrillar domains within the OLL that are on the same scale as the vesicles (Figure 3d)” – it would be very helpful to demarcate this in the figure, since it is not immediately obvious.

2. FIB-SEM

2a. The article cites Ref 51 for prior FIB-SEM approaches, but it would be helpful to include some indication of sample preparation used herein, or more specifically confirm if samples were prepared in the exact same way or what differences were present, if any. The staining and embedding selected can have major implications for how one interprets the images.

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Reviewer #3

(Remarks to the Author)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all the points I have raised in my review and have improved the overall quality of the manuscript.

T recommend its publication in Nature Communications.

Reviewer #2

(Remarks to the Author)
All comments were well addressed by the authors

REVIEWER COMMENTS

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**We thank the reviewer kindly for their assessment and input.

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**We thank the reviewer for pointing this out. The change has been made.

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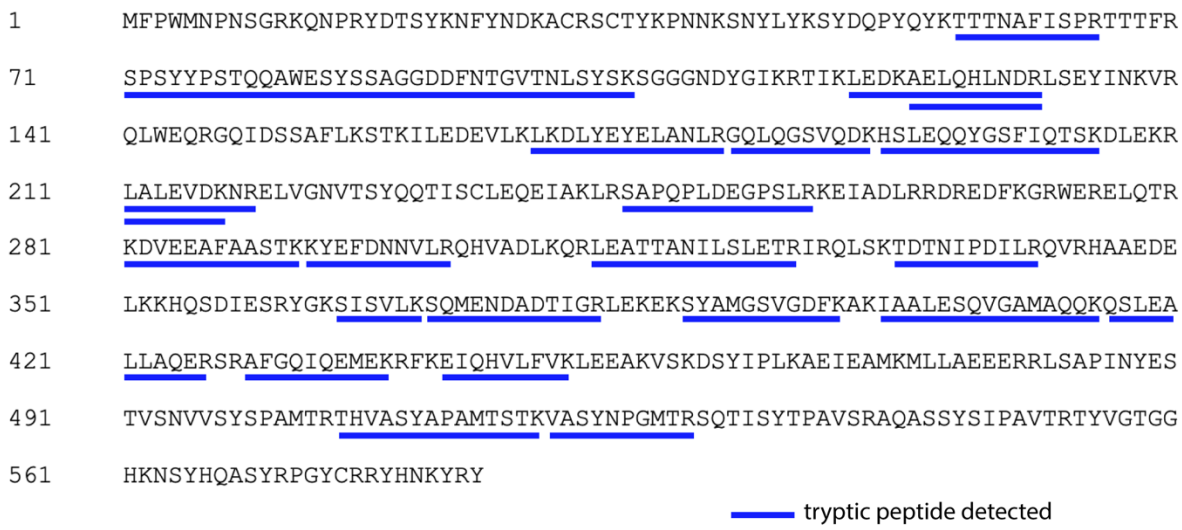
**We thank the reviewer for the opportunity to improve the clarity of the manuscript. We have changed the sentence as suggested.

Line 124: clarify if the mentioned “23 individual tryptic peptides” were all unique peptides or if not how many where. This is important to unequivocally demonstrate the correct protein ID

**We thank the reviewer for this question. Yes, these are 23 unique peptides that cover 45% of the protein sequence. Please see the figure below which has been added as a new Fig. S1 to the SI to help visualize this point. This coverage map shows regions of msp-1 corresponding to specific tryptic peptide sequences determined from mass spectrometric analysis.



Lines 476-477: clarify which FDRs where considered and why genes differential expression is mentioned, if in the manuscript expression levels among the different mussel foot tissues is not mentioned nor discussed.

**We thank the reviewer for this question. These sentences are a remnant from an earlier version of the manuscript. We performed differential gene expression analyses as a parallel line of evidence to identify likely target protein candidates but did not end up explicitly using this data in the paper as it did not bring any new insights. Therefore, we have just removed this text from the manuscript.

Reviewer #2 (Remarks to the Author):

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**We appreciate the reviewer’s suggestion to improve the clarity of our description of the STEM sample preparation. While we referred to Ref 16, which itself cites the original publication[6] where the full preparation protocols are detailed, we understand the importance of including key information directly in our manuscript for clarification. In response, we have included further information describing tissue processing, fixation, staining, embedding, and sectioning in the

Methods section, following the methodology outlined previously[6]. Below is the revised text that has been added.

“Generator tissue was dissected from mussel feet following a previously established protocol[6], rinsed in cold water, and blotted to remove excess mucus. Samples were pre-fixed at 4 °C for 30 min in 3% glutaraldehyde, 1.5% paraformaldehyde, and 650 mM sucrose in 0.1 M cacodylate buffer (pH 7.2), then cut into thin cross-sections and fixed again for 2 h at 4 °C in the same buffer. After five rinses in cacodylate buffer, specimens were post-fixed with 1% osmium tetroxide (OsO□) for 1 h at 4 °C. Osmium-free specimens were used for EDS analysis. Dehydration was performed in a graded ethanol series (50%, 70%, 90%, 3×100%, 10 min each at RT), and samples were embedded in epoxy resin (Epon 812 or Hard Plus 812, EMS #14115). Polymerization was performed at 65–70 °C for 48 h. Ultrathin sections (~100 nm) were cut using a PowerTome XL ultramicrotome and mounted on carbon-coated copper grids (200 mesh) for imaging.”

1b. The paper indicates that the “Using scanning transmission electron microscopy (STEM), we observed that the outer phase of lamellae exhibits a woven texture of thin fibrils, while the core consists of thicker fibrils that are presumably collagenous in nature…” . This is supported by the Raman and histological staining, but surprisingly, not by the STEM itself. There is no collagen banding clearly visible in this region – but in Ref 16, collagen banding is visible. Why is there no banding visible in this region if these are collagen fibrils? Did the authors not probe this region with higher resolution? Or by cutting the sample with an ultramicrotome, it can be difficult to maintain the sample integrity. Some comment on why the STEM does not support this is warranted. If one zooms in quite a bit, there is a faint hint of banding in 3b that goes unmentioned – investigating if this follows the periodicity for collagen (e.g. FFT) could provide an additional support to the composition of these filaments in the centre of the OLL.
**We thank the reviewer for raising this point. Indeed, collagen banding is a well-established ultrastructural feature in fibrillar materials comprised of type I collagen due to characteristic 64-67 nm stagger of collagen triple helices. As mentioned by the reviewer, previous TEM images from the stem generator living tissue in reference 16[5] do exhibit such banding patterns. This is because the living tissue (into which the stem root lamellae are interdigitated) is largely comprised of fibrillar, presumably type I collagen in the extracellular matrix (ECM). However, in this same reference and in our current study, the characteristic banding pattern was never observed in the fibrils at the center of the byssus stem root lamellae in our STEM images, even

at high resolution. This is because these ILL fibrils are not composed of type I collagen, but rather of specialized collagenous proteins specific to mussels called preCols that contain non-collagenous flanking domains and histidine-rich regions at the N- and C-termini as has been determined through decades of investigation[3].

PreCols are very different from type I collagen and previous studies have similarly reported the absence of D-periodic banding in TEM images of mature byssal thread fibers, despite the presence of highly aligned fibrillar structure determined by X-ray diffraction [7-9]. It has been proposed that, during thread formation, the flanking and His-rich domains—which are presumed to be unfolded in secretory vesicles—undergo conformational changes that disrupt the banding pattern observed in the liquid crystalline storage phase of the preCols [2,9]. In contrast, the collagen banding shown in Ref 16[5] originates from extracellular matrix (ECM) collagen (presumably type I) in the septal region of the generator, rather than from the stem root lamellae.

1c. The authors state “several STEM images show locally aligned fibrillar domains within the OLL that are on the same scale as the vesicles (Figure 3d)” – it would be very helpful to demarcate this in the figure, since it is not immediately obvious.
**The reviewer’s comment on the clarity of Figure 3 has been addressed by adding dashed pink markings in figure 3a,b to guide the reader to the aligned fibrillar regions discussed. In our interpretation, this region of consistent fibril orientation could represent the contents of a single vesicle that has become elongate by shear forces. This is of course just a hypothesis, and we tried to treat it as such. The figure legend has been adjusted accordingly as shown below.

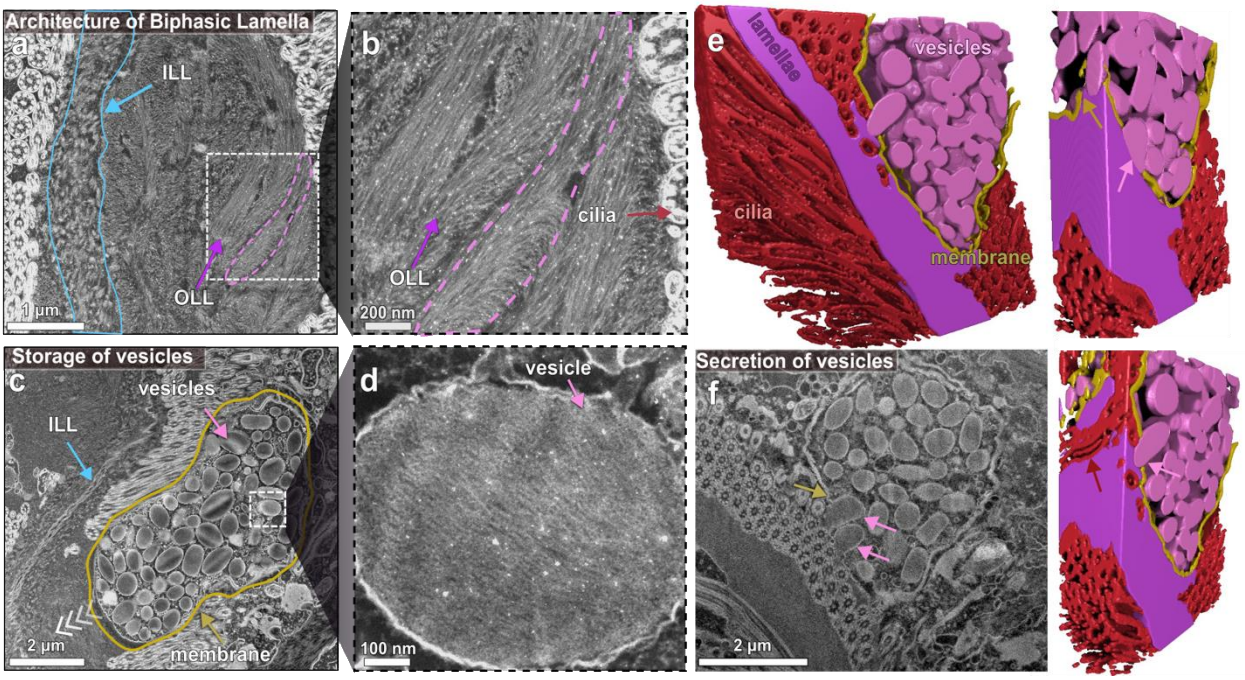

Figure 3. Electron microscopic characterization of stem root biointerface. (a) STEM image indicating the biphasic nature of the lamella in contact with the cilia. (b) Zoomed-in image of the region highlighted in (a), showing the OLL fibrils reorienting almost parallel to the cilia surface. The dashed pink outline in (a) and (b) indicate a region of the stem OLL that possibly originated from the contents of a single vesicle. (c) STEM image showing the concentration of vesicles at the interfaces prior to release. The yellow outline encloses a cluster of vesicles. (d) Zoomed-in image from (c) of a single vesicle consisting of IF fibrils. (e) False-colored 3D model reconstructed from FIB-SEM image stack acquired from the generator. The colored arrows indicate the individual components in the 3D model. (f) Image from the FIB-SEM stack showing the fusion of the vesicles (pink arrow) with the membrane (yellow arrow)

2. FIB-SEM

2a. The article cites Ref 51 for prior FIB-SEM approaches, but it would be helpful to include some indication of sample preparation used herein, or more specifically confirm if samples were prepared in the exact same way or what differences were present, if any. The staining and embedding selected can have major implications for how one interprets the images.

**We thank the reviewer for giving us the opportunity to clarify this point. The FIB-SEM sample preparation in this study was performed following the protocol described previously, including

fixation, staining, and embedding procedures. As shown below, we have added more details in the current manuscript to clarify this point.

"*FIB-SEM.* This approach was adapted from previous studies on mussel foot and generator tissue FIB-SEM analysis[2,5]. Briefly, the generator tissue was dissected from the mussel and excess water was removed by dabbing with Kimwipes. The sample was prefixed for 30 minutes at 4°C in a solution of 3 % glutaraldehyde, 1.5 % paraformaldehyde and 0.1 M cacodylate pH 7.2 buffer. After pre-fixation, the generator was cut into thin slices using a scalpel and fixed again in the solution described above for 2 hours at 4°C. The fixed sections were rinsed 5 times using the cacodylate buffer at 4°C and then stained using 1 % OsO4 for 1 hour at 4°C. Afterwards, the stained sections were rinsed again 3 times for 5 minutes each using the same solution as before, and then were dehydrated stepwise in different concentrations of acetone (50%, 70%, 90%, and 3×100%) for 1 hour total. The dehydrated samples were then embedded in Epoxy (Epon 812 substitute Sigma-Aldrich, no 45359) and polymerized at 70°C for approximately 2 days."

2b. Some of the FIB-SEM imaging reported in this work appeared previously in Ref 16 with new regions investigated herein regarding the fusion of vesicles with the membrane. The images presented in 3e (3D renderings) and f (2D slice) would benefit from a scale bar, though approximate for the 3d projections. The video is superb to demonstrate in 3D the intersection of the vesicles with the membrane. This type of conclusion could not be drawn from the 2D STEM images alone but is well supported with this rendering.

**We thank the reviewer for raising this point regarding the inclusion of scale bars. We agree that providing scale is important for accurate interpretation. However, we did not include a scale bar in the 3D renderings due to the limitations of perspective projection. In 3D views, depth distortion causes objects at different distances from the viewer to appear at different sizes, making a single scale bar potentially misleading. This stems from the fact that, in 3D perspective views, there is no single pixel-to-distance ratio and the scale changes depending on depth, i.e., the same object can appear at many different sizes depending on where it sits in the 3D volume. This occurs because scale bars assume a flat, orthographic view without depth-related distortion. To address this, we have included a scale bar in the corresponding 2D slice view from the same dataset in panel Fig. 3f (see above), where spatial dimensions are accurately represented. We believe this provides a more reliable reference for scale.

3. WAXS

3a. The WAXS clearly shows the alpha helix at q=11.7. But I'm wondering the size of the probe used, thickness of the sample, and if other regions of interest than the OLL are also overlapped on these spectra? Can the authors identify the features in the WAXS signal at q=6 in the meridian? And 4.5 in the equatorial? Was it not possible to detect the beta sheets with WAXS after mechanical loading?

**The reviewer raises important points regarding the WAXS data and the interpretation of specific peaks. Measurements were carried out using an Anton Paar SAXSpoint 2.0 system with a beam size of 580 µm × 360 µm focused on the root region of the stem where the composition is known to consist of the (OLL) and the (ILL). This information has been added to the manuscript. Because these layers are closely integrated, it is not possible to isolate a diffraction pattern from just the ILL, so the WAXS signal includes contributions from both the inner and outer layers of the stem root lamellae.

In terms of interpreting peaks, we erred on the side of caution since this material is comprised of several different protein components, and we did not want to overinterpret and possibly misinterpret the peaks. Nonetheless, we feel quite confident in our assignment of the alpha helical peak at q = 11.7 nm-1 based on the AlphaFold prediction, immunostaing, and supporting FTIR and Raman spectroscopic data. In addition, we did speculate in the manuscript a bit on possible identities of equatorial peak at lower q. In the literature, it has been observed that an equatorial peak at q ≈ 4.5 nm^{-1} in coiled coil materials corresponds to the lateral spacing between the coils[10]. However, given the presence of collagen fibrils in the ILL, the equatorial peaks at q ≈ 4.5 nm^{-1}could also correspond to the lateral spacing of the preCol triple helices in the ILL, which is also in this same range and fits well to previous studies on the byssal thread collagen X-ray diffraction studies[8]. We have added a bit more information on this in order clarify this point better in the manuscript.

The meridional peak at 6 nm^{-1} is more challenging to assign at this point. Technically, this would correspond to an axial d-spacing of ~1 nm along the stem long axis. At this point, we do not have a good inclination of what this might correspond to within the higher order structure of the material but plan to explore this in future studies. Thus, we choose not to discuss this further in the manuscript. Regarding beta-sheet detection with X-ray diffraction following mechanical loading – we did attempt these investigations. Unfortunately, however, the broad background scattering from disordered proteins overlapping the beta-sheet region (around q = 13.7 nm^{-1}),

combined with relatively weak signal from the root samples to start with, made it challenging to detect beta-sheet features definitively in the WAXS data under stretched conditions.

1 Simmons, M. *et al. Proceedings of the National Academy of Sciences* **120**, e2311901120, (2023).
2 Jehle, F. *et al. ACS Nano* **15**, 6829-6838, (2021).
3 Waite, J. H. & Harrington, M. J. *Canadian Journal of Chemistry* **100**, 197-211, (2021).
4 Miserez, A. & Guerette, P. A. *Chemical Society Reviews* **42**, 1973-1995, (2013).
5 Sivasundarampillai, J. *et al. Science* **382**, 829-834, (2023).
6 Jehle, F. *et al. Nature Communications* **11**, 862, (2020).
7 Bairati, A. & Zuccarello, L. V. *Cell and Tissue Research* **166**, 219-234, (1976).
8 Krauss, S., Metzger, T. H., Fratzl, P. & Harrington, M. J. *Biomacromolecules* **14**, 1520-1528, (2013).
9 Vitellaro-Zuccarello, L. *Journal of Ultrastructure Research* **73**, 135-147, (1980).
10 Er Rafik, M., Doucet, J. & Briki, F. *Biophysical Journal* **86**, 3893-3904, (2004).