nature portfolio

Peer Review File

Crosslinking substrate regulates frictional properties of tissue-engineered cartilage and chondrocyte response to loading

Corresponding Author: Professor Travis Klein

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Version 0:

Decision Letter:

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Dear Professor Klein,

Thank you for submitting your manuscript, "Crosslinking substrate hydrophobicity regulates frictional properties of tissue-engineered cartilage and chondrocyte response to loading", to Communications Materials. It has now been seen by 3 referees, whose comments are appended below. You will see that while they find your work of interest, some important points are raised. We are interested in the possibility of publishing your study in Communications Materials, but would like to consider your response to these concerns in the form of a revised manuscript before we make a decision on publication.

In particular, the reviewers ask for clarification regarding some of the effects observed and request further discussions.

We therefore invite you to revise and resubmit your manuscript, taking into account the points raised.

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-A marked-up version of the manuscript with all changes to the text in a different colored font. Please do not include tracked changes or comments. Please select the file type ‘Revised Manuscript - Marked Up’ when uploading the manuscript file to our online system.

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Chemical and biomolecular materials: Characterization of chemical and biomolecular materials

Life sciences reporting summary: Reporting requirements for life sciences research

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Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further. We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Best regards,

Steven Caliari, PhD
Editorial Board Member
Communications Materials
orcid.org/0000-0002-7506-3079

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors explore a simple way to improve the performance of tissue-engineered cartilage by investigating hydrophobic surfaces (e.g. PTFE) as substrates during hydrogel crosslinking. They show that PTFE enhances surface lubrication of GelMA/HAMA hydrogels, which in turn supports better chondrocyte behaviour and tissue formation under mechanical loading. This is particularly exciting because it achieves a significant improvement—lower surface friction and better matrix deposition—without altering the chemical makeup of the hydrogels.
While previous studies have focused on tweaking the chemical properties of hydrogels to reduce friction, this study

introduces substrate hydrophobicity as an interestingly effective and underexplored factor. It's an elegant and practical finding that mimics the natural lubrication mechanisms seen in articular cartilage.
Strengths:
- The concept of using hydrophobic PTFE to fine-tune surface properties is interesting and relatively easy to implement without overcomplicating hydrogel design.
- The authors combine mechanical testing, shear strain mapping, and biochemical assays, to demonstrate that low-friction PTFE substrate for crosslinked hydrogels performs better.
- The simulated cartilage defect repair adds a translational angle to the study, bridging the gap between benchwork and potential patient applications.
- The findings are well-explained, connecting reduced crosslinking density and water exudation to self-lubrication—a biomimetic process that mirrors native cartilage function.
Suggestions for Improvement
- General comments on methodology: Some rationale on the experimental design is missing or not fully explained, if possible add further details (e.g. Why the selected sliding speeds and contact pressures (?) Why are the sliding tests just tested for 1 hour?). Similarly, on the mechanical stimulation, why 10% loading and the selected sliding motion as in discussion, time applied to the sliding motion (e.g. why 1h at 1Hz per day? Why 30% compressive strain? the constant interval over 28 days?)
- The reduced crosslinking density is attributed to PTFE's hydrophobicity, but oxygen diffusion through PTFE may also be at play. Since oxygen can inhibit hydrogel crosslinking, it would be helpful to disentangle these effects to strengthen the core argument.
- Also, regarding the differences in oxygen diffusion between the two surfaces, how does this effect facilitate the hypoxia conditions and therefore chondrocytes function?
- While the findings are supportive of GelMA/HAMA hydrogels, it's unclear if this effect applies to other hydrogel systems. It's anticipated that exploring different natural or synthetic hydrogels would make the study more broadly impactful.
- The study uses IC2959 as the photoinitiator, which works well but has known limitations under UV light, such as potential cell damage. So testing with visible-light alternatives, like LAP, could improve safety and clinical viability.
- Cartilage repair is a slow process. While the study looks at 28 days, we don't know how the hydrogels will perform under prolonged mechanical loading. A longer-term analysis would give a clearer picture of stability and durability.
- Some of the biological analyses, like gene expression, were performed on a relatively small number of donors and constructs. While the results are promising, increasing the sample size would add more weight to the conclusions.

Statistical Analysis and Reproducibility: The statistical analyses are appropriate, with clear reporting of significance levels. Experimental protocols, including hydrogel preparation, mechanical stimulation, and analytical methods, are detailed, ensuring reproducibility.

Reviewer #2 (Remarks to the Author):

This manuscript is clear, well-written, and thorough in their experiments and questions.

Major claims of the paper:
-The hydrophobicity of a material directly impacts the crosslinking efficacy of a hydrogel, and in turn this effects the properties of the material surface. In this paper, they show that crosslinking on PTFE vs on glass changes the hydrogel surface properties. The PTFE is hydrophobic, so it inhibits some crosslinking (they show strong supporting data this claim is true). The glass is hydrophilic and the crosslinking is more effective/complete. These differences result in showing that the PTFE/inhibition of crosslinking leads to surface properties advantageous to cartilage (lower friction coefficient, viscoelastic and time-dependent frictional properties under shear, cell viability, and correct ECM production from chondrocytes).

IMPACT: The surface of cartilage is critically important, and therefore the finding is important, especially for cartilage research. The team did a good job testing relevant loading as well to ensure it could hold up to multiple loading schemes and provide more strength as a cartilage specific solution.

POSITIVE NOTES BEYOND THE ABOVE:
- My big criticism of 'how would this actually work clinically as tissue is not PTFE' was answered with their prototype, so that was a very effective addition!

Critiques from reviewer:

1. The work is effective at showing the mechanism behind this phenomena, and demonstrating the changes in material properties on several length scales (under bulk shear, and with AFM). However, when addressing the question of 'Novelty' it is merely demonstrating a phenomena previously found by Gong et al. but showing it in a natural hydrogel. That being said, it is a very useful finding for hydrogel researchers and potentially for clinical translation.

2. The paper effectively shows imaging of collagen II vs collagen I production in the cells and quantifies this, but it is strange to me that at 28 days under loading this cellular production is not seen in an extensive ECM network AND gene expression differences are not seen. I think the language and implication of clinical efficacy claims may be a bit too strong given this.... The authors need to discuss this discrepancy and offer some potential explanations...

3. Do these effects partially depend on the opposite surface from PTFE being glass? Like if you do this between tissue and PTFE do you get the same effects on the PTFE surface? Would be good to understand whether the presence of the glass overstates the effect on opposite side of hydrogel…. Would thickness of the hydrogel matter?

4. The statistics are fairly rudimentary, and there is no discussion of whether the residuals were normalized, or details on the ANOVA model (one-way, two way?) . In the ANOVA were any co-factors considered related? Was cell donor considered a factor? Would be good to know more about the details of the model used for the ANOVA, and whether the 3 donors made a mixed cell population or whether donor might be a factor?

5. The shear loading was done at a given compression. With cartilage, there is also a very high level of direct compression loading under normal conditions (i.e. walking). I am unsure if this hydrogel can withstand that which is important in addition to the surface.

6. Do these effects partially depend on the opposite surface from PTFE being glass? Like if you do this between tissue and PTFE do you get the same effects on the PTFE surface? Would be good to understand whether the presence of the glass overstates the effect on opposite side of hydrogel…. Would thickness of the hydrogel matter?

MINOR CRITIQUES
1. Clarify this: “We also observed a further reduction of μ_kin in cell-laden gels crosslinked on both PTFE and glass as a result of increased culture duration (Figure 2A,C). This reduction was more emphasized in PTFE-exposed construct surfaces where μ_kin values were reduced by 35–70% relative to day 1 values, depending on sliding velocity and contact pressure.”

“more-emphasized” is not strong enough or clear. Please give detail here of the reduction percentage on glass (i.e. summarize that data as well as the PTFE reduction

2. how long were cells expanded? On what substrate? At what passage were they encapsulated? This has been shown to dramatically change chondrocytes so is information that is critical to share.

Reviewer #3 (Remarks to the Author):

This is a detailed study of the surface properties of hydrogels prepared via methods commonly used in the field. This study highlights the importance of tribological properties of hydrogels, which are often overlooked in the assessment of mechanical properties of biomaterials, and are of particular importance in relation to cartilage tissue engineering. Overall, I found the study interesting and highly relevant to the field.
1. In discussing Figure 2A/C authors mention a “reduction” of the kinetic frictional coefficient due to culture duration – is the trend significant? Statistical analyses should be performed/discussed to contextualise these results.
2. Also in relation to Figure 2A/C, the following sentence mentions values were “reduced by 35-70%”. This is quite a wide range and it could be useful to also state the percentage reduction for glass to make the difference clearer to the reader.
3. Is it possible there is directionality to the crosslinking that would explain some of the differences between the hydrogel surfaces crosslinked in contact with PTFE vs glass? Could the authors discuss this and how it was ruled out? E.g. if the light was always shone through the glass slide on top, what might happen if light was shone through a sufficiently transparent PTFE surface first, hitting the glass side last?
4. Similarly, did the authors do any analysis of surface properties of gels crosslinked in contact with the PTFE membrane as in the Figure 6 set-up? Would they expect any differences to the gels crosslinked in contact with the PTFE molds?

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Version 1:

Decision Letter:

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Dear Professor Klein,

Your manuscript titled "Crosslinking substrate regulates frictional properties of tissue-engineered cartilage and chondrocyte response to loading" has now been seen again by our referees, whose comments appear below. In light of their advice I am delighted to say that we are happy, in principle, to publish a suitably revised version in Communications Materials.

We therefore invite you to edit your manuscript to comply with our journal policies and formatting style in order to maximise the accessibility and therefore the impact of your work.

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We hope to hear from you within two weeks; please let us know if the process may take longer.

Best regards,

Steven Caliari, PhD
Editorial Board Member
Communications Materials
orcid.org/0000-0002-7506-3079

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The reviewer would like to thank the authors for taking into consideration the comments and suggestions made. I understand

that some of the suggested revisions are challenging due to limitations such as the availability of tissue donors, or access to in vivo studies. Since the authors have addressed these limitations in the manuscript and acknowledged criticisms, space for improvements or future work, this demonstrates self-awareness of the limitations of the study -The reviewer would like to thank the authors for considering the comments and suggestions provided. I acknowledge that some of the proposed revisions are challenging due to constraints such as the availability of tissue donors and access to in vivo studies. However, the authors have addressed these limitations in the manuscript and recognized the criticisms, highlighting areas for improvement and future work. This demonstrates their awareness of the study's limitations. Therefore, the reviewer is satisfied with the changes and accepts the manuscript in its current form.

Wishing you all the best

Reviewer #2 (Remarks to the Author):

I think the authors addressed all my comments and well and I am happy to recommend the paper for publishing.

Reviewer #3 (Remarks to the Author):

I am satisfied the authors have addressed the points in their revised manuscript and letter.

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AUTHOR RESPONSE LETTER

Manuscript Number: **COMMSMAT-24-0737-T**

Crosslinking substrate regulates frictional properties of tissue-engineered cartilage and chondrocyte response to loading

To the Editorial Board of *Communications Materials*,

We appreciate the feedback and comments provided by the reviewers, and thank you for the opportunity to submit our revised manuscript. We have thoroughly addressed the reviewer's suggestions, incorporating the necessary changes enhance the quality of the manuscript for publication. Every change made in response to the comments has been outlined within the revised manuscript, with responses addressing each comment provided below.

Kind Regards,

Prof Travis Klein

REVIEWER #1

Reviewer #1 (Remarks to the Author): The authors explore a simple way to improve the performance of tissue-engineered cartilage by investigating hydrophobic surfaces (e.g. PTFE) as substrates during hydrogel crosslinking. They show that PTFE enhances surface lubrication of GelMA/HAMA hydrogels, which in turn supports better chondrocyte behaviour and tissue formation under mechanical loading. This is particularly exciting because it achieves a significant improvement—lower surface friction and better matrix deposition—without altering the chemical makeup of the hydrogels.

While previous studies have focused on tweaking the chemical properties of hydrogels to reduce friction, this study introduces substrate hydrophobicity as an interestingly effective and underexplored factor. It's an elegant and practical finding that mimics the natural lubrication mechanisms seen in articular cartilage.

Strengths:

- **The concept of using hydrophobic PTFE to fine-tune surface properties is interesting and relatively easy to implement without overcomplicating hydrogel design.**
- **The authors combine mechanical testing, shear strain mapping, and biochemical assays, to demonstrate that low-friction PTFE substrate for crosslinked hydrogels performs better.**
- **The simulated cartilage defect repair adds a translational angle to the study, bridging the gap between benchwork and potential patient applications.**
- **The findings are well-explained, connecting reduced crosslinking density and water exudation to self-lubrication—a biomimetic process that mirrors native cartilage function.**

Suggestions for Improvement:

General comments on methodology: Some rationale on the experimental design is missing or not fully explained, if possible add further details (e.g. Why the selected sliding speeds and contact pressures (?) Why are the sliding tests just tested for 1 hour?). Similarly, on the mechanical stimulation, why 10% loading and the selected sliding motion as in discussion, time applied to the sliding motion (e.g. why 1h at 1Hz per day? Why 30% compressive strain? the constant interval over 28 days?)

Firstly, we would like to thank Reviewer #1 for their detailed feedback and recommendations. We have made a concerted effort to improve the manuscript based on each comment, with all changes outlined throughout the revised document also included here in addition to our comment responses.

In regard to the comments and queries on experimental design and rationale, additional details have been provided within the Methods section of the revised manuscript as requested:

> Frictional forces were assessed against PTFE (same material as the loading pistons in the mechanical stimulation bioreactor) over a sliding distance of 10 mm for 5 reciprocating cycles at constant sliding velocities ranging from 0.1 mm/s to 3 mm/s in high-D-glucose basal chondrocyte medium with ITS-G (100 × dilution) and 1.25 mg/mL BSA. Determination of these low sliding speeds was intended to provide data regarding the tribological properties of the hydrogels while maintaining the system within a mixed or boundary lubrication regime, in line with the work of Katta *et al.* [82], as described in their early study of cartilage frictional properties…
>
> …The long-term reciprocal sliding friction tests, performed on cell-free constructs to ascertain frictional kinematics prior to dynamic cell culture studies, were performed for 1 hour to match the daily dynamic loading duration implemented in dynamic culture, as applied in our previous dynamic culture studies [9]. This established cyclic compression protocol was determined to provide controlled, reproducible and stable

mechanical stimulation for dynamic culture of hydrogel constructs in our bioreactor system.

A custom microscope-mounted mechanical loading system capable of applying precise shear stimulation mimicking physiological joint articulation, was employed for the application of sliding shear motion to the hydrogels, with lower gel surface movement restricted. Gels were compressed to ~10% strain to stabilise samples for frictional imaging without altering bulk morphology and subjected to 1 mm amplitude sliding shear motion at 1 mm/s, mimicking dynamic cell culture shear and physiological loading. Digital image correlation (DIC) software (Vic-2D DIC; Correlated Solutions, Columbia, SC, USA) was used to track particle displacement within sequential microscope images obtained for calculation of the local E_{xz} throughout the cross-sectional area of the constructs.

…Constructs were either cultured under free swelling conditions for 28 days (static controls), or precultured for 14 days, followed by daily biaxial dynamic loading in a custom bioreactor [9] for 1 hour at 1 Hz per day with a compressive strain of 30% of construct height and shear amplitude of 1 mm for another 14 days (3 donors, 2 constructs per donor for DNA and GAG analysis; 3 donors, 1 construct per donor for viability and immunofluorescence analysis; 3 donors, 2 constructs per donor for gene expression analysis). For all *in vitro* loading experiments, the compression setting of 30% replicated our previous studies indicating superior ECM production by cultured chondrocytes in comparison to 10% or 50% compression protocols [9].

We appreciate these recommendations from Reviewer 1, and believe the additional details provide clarity for the reader in terms of methods and rationale.

The reduced crosslinking density is attributed to PTFE's hydrophobicity, but oxygen diffusion through PTFE may also be at play. Since oxygen can inhibit hydrogel crosslinking, it would be helpful to disentangle these effects to strengthen the core argument. Also, regarding the differences in oxygen diffusion between the two surfaces, how does this effect facilitate the hypoxia conditions and therefore chondrocytes function?

We thank Reviewer 1 for raising this important point. We agree that oxygen diffusion can inhibit radical polymerization as it has been described previously in scientific literature. To address this directly in the revised manuscript, the following has been added to the Results & Discussion to expand upon the brief mention of oxygenation in the original manuscript:

While the molds used represent a fully enclosed system, with the substrate-induced effect the primary mechanism being studied here, differences in oxygen diffusivity between glass and PTFE substrates may have contributed to the observed local effects on hydrogel crosslinking and mechanical/tribological properties. Work by Spencer, et al., clearly demonstrates that oxygen available from the substrate inhibits crosslinking near the surface for several hydrogel-substrate systems, resulting in differences in compressive modulus and friction coefficient [40,41]. Future studies with controlled oxygen availability from the substrates are warranted to determine if these crosslinking conditions have long-term effects on chondrocyte differentiation and ECM production. Further, a similar approach could be used to induce an oxygen gradient to mimic the *in vivo* hypoxic environment of cartilage through careful design of the culture vessel. Ultimately, if localized oxygen control could be achieved by altering the substrates upon which a hydrogel is prepared and cultured, tuneable internal environments could be induced without the use of oxygen-scavenging or oxygen-releasing microparticles such as those described in literature [40,41].

While the findings are supportive of GelMA/HAMA hydrogels, it's unclear if this effect applies to other hydrogel systems. It's anticipated that exploring different natural or synthetic hydrogels would make the study more broadly impactful.

We appreciate the reviewer's suggestion. As noted in our Discussion of existing literature, Gong *et al.* have studied this phenomenon in various synthetic hydrogel systems, attributing the lower coefficients of friction dangling polymer chains at the hydrophobic substrate-crosslinked hydrogel surface, with these systems crosslinked via radical polymerization. They also mention that this substrate effect has been shown for a broad range of synthetic hydrogels formed on a variety of substrates. Hence, we agree that investigating other natural or semi-synthetic hydrogel systems similar to ours here could increase the broader applicability of our findings. To address this in the revised manuscript, we have included description of these future directions to highlight how our findings may be generalized across different materials:

> While this study solely investigates GelMA/HAMA hydrogels and their use in cartilage neo-tissue generation, the underlying principles associated with the observed tribological effects on hydrogel mechanics and chondrocyte behavior are likely to extend to other hydrogel systems, both natural and synthetic, albeit at different degrees of influence. Alternative hydrogel systems which may be investigated in our future work, to assess crosslinking substrate induced effects on tribology, include GelMA, GelMA/alginate, GelMA/chitosan, as well as collagen, or synthetic polymer-based systems such as PEG and PVA materials, of which each possess advantageous properties for cartilage tissue engineering. Gong *et al.,* [24,25,30] have investigated a range of synthetic hydrogel systems crosslinked on various substrates, with these hydrogels crosslinked via radical polymerization.
>
> We hypothesize that if surface tribology can be improved in various natural hydrogel systems, similar effects such as the promotion of chondrocyte differentiation and ECM production may be observed in vitro. To compare such systems to the results reported herein, future studies would require matched mechanical conditioning and testing protocols, while consideration must be given to the degree of crosslinking, hydrogel stiffness, and the ability to modulate oxygen availability within these distinct systems. These studies would provide better understanding of the general applicability of the observed results, and allow us to identify key factors that govern cellular behavior in response to loading across a broader range of hydrogel systems.

We look forward to expanding on these findings in future work, which will strengthen the broader applicability of our conclusions and provide further insights into the use of hydrogels with tuneable tribological properties in regenerative medicine.

The study uses IC2959 as the photoinitiator, which works well but has known limitations under UV light, such as potential cell damage. So testing with visible-light alternatives, like LAP, could improve safety and clinical viability.

We thank Reviewer 1 for this suggestion. We acknowledge the potential limitations of IC2959 under UV light, particularly regarding cell viability, despite negligible negative results observed here in this regard, and in work we have published previously comparing UV and visible light photocrosslinking in cartilage tissue engineering using the same hydrogels, with the UV crosslinked gels exhibiting somewhat better cartilage formation (Pahoff *et al.*, 2019). Nevertheless, exploring visible-light photoinitiators, such as LAP, could indeed enhance the clinical relevance of our approach. We will incorporate this suggestion into our future experiments and will test LAP as an alternative photoinitiator to improve clinical applicability.

Within the original manuscript we did describe these limitations, though upon recommendation this paragraph has been updated within the revised manuscript as copied below:

Throughout this study, the photoinitiator used for photocrosslinking of methacrylated hydrogels was IC2959; which, despite its proven cytocompatibility and extensive use in cartilage tissue engineering [75–78], possesses some limitations. Namely, given the molar extinction coefficient of IC2959 at 365 nm, the wavelength typically used for hydrogel photopolymerization, is very low and trails off almost entirely before 370 nm [8], hydrogel crosslinking efficiency is quite limited. Further, UV irradiation of hydrogels may lead to the formation of reactive oxygen species and subsequent cell damage [9].

More recently, visible-light photoinitiators such as lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) have been increasingly applied for photopolymerization of cell-laden hydrogels with very good cytocompatibility and short gelation times [81,82]. As such, exploring visible-light photoinitiators, such as LAP, could indeed enhance the safety and clinical relevance of our approach. Future work would therefore be required to characterize the tribological properties of photocrosslinked hydrogels prepared with alternate systems.

However, we hypothesize that differences in frictional properties will be negligible with respect to photoinitiator used. This is based on the inference that the primary mechanism by which hydrogel surface friction is reduced herein is predominantly substrate related, where future work may be suitable to investigate the observed phenomena in various alternative hydrogel systems. To test these hypotheses, manufacture of hydrogel constructs with reproducible bulk mechanical properties, manufactured with alternative photoinitiators is required, with replication of the mechanical tests performed to directly compare performance. While minimal differences in tribological properties would be expected, modification of our system to improve safety and efficacy would be beneficial in the context of clinical translation.

Cartilage repair is a slow process. While the study looks at 28 days, we don't know how the hydrogels will perform under prolonged mechanical loading. A longer-term analysis would give a clearer picture of stability and durability.

We appreciate the Reviewer's consideration of this. Indeed, cartilage repair is a long-term process, and we agree that additional time points beyond 28 days would be valuable for assessing the stability and durability of our hydrogels under prolonged mechanical loading. As such, the following has been added to the revised manuscript, within the Discussion:

> While the 28-day dynamic *in vitro* cell culture study reported herein has provided promising results, cartilage repair is a particularly slow process [57]. Thus, long-term studies with additional timepoints would be valuable for assessing the stability and durability of our low-friction hydrogels under prolonged mechanical loading. Thus, in the planning of future experimental work, long-term follow-up studies are required to evaluate the performance of these low-friction hydrogels over extended periods, which may enable enhanced ECM accumulation thus providing determination of specific differences in gene expression results. Additionally, with appropriate data and resources, there exists potential for future preclinical animal studies for extended performance assessment *in vivo*. Both extended *in vitro* and potential *in vivo* work would further develop our understanding of construct longevity, cellular behavior long-term, and maintenance of tribological properties, to ascertain suitability for clinical translation.

Thank you again for these recommendations, we look forward to sharing the results of long-term studies in future work.

Some of the biological analyses, like gene expression, were performed on a relatively small number of donors and constructs. While the results are promising, increasing the sample size would add more weight to the conclusions.

We agree with the Reviewer that increased sample sizes would enhance the robustness of our findings. For gene expression analysis for example, 3 individual donors were used. Unfortunately, access to donor cartilage samples is limited, prohibiting us to perform larger scale studies. This has been stated in the revised manuscript:

> Additionally, we acknowledge that increasing the sample size, donor number, and number of constructs would enhance the robustness and reproducibility of our findings. Future work will incorporate larger sample sizes to further investigate the gene expression results and strengthen the translational potential of our hydrogel models with tuneable tribological properties.

These considerations will be included in future work, and we thank Reviewer 1 for the recommendation.

Statistical Analysis and Reproducibility: The statistical analyses are appropriate, with clear reporting of significance levels. Experimental protocols, including hydrogel preparation, mechanical stimulation, and analytical methods, are detailed, ensuring reproducibility.

We are grateful for the positive feedback from Reviewer 1 on the statistical analysis and reproducibility aspects of our study. Our experimental design and reported methods ensure transparency, and we appreciate acknowledgment of this.

REVIEWER #2

Reviewer #2 (Remarks to the Author): This manuscript is clear, well-written, and thorough in their experiments and questions.

Major claims of the paper: The hydrophobicity of a material directly impacts the crosslinking efficacy of a hydrogel, and in turn this effects the properties of the material surface. In this paper, they show that crosslinking on PTFE vs on glass changes the hydrogel surface properties. The PTFE is hydrophobic, so it inhibits some crosslinking (they show strong supporting data this claim is true). The glass is hydrophilic and the crosslinking is more effective/complete. These differences result in showing that the PTFE/inhibition of crosslinking leads to surface properties advantageous to cartilage (lower friction coefficient, viscoelastic and time-dependent frictional properties under shear, cell viability, and correct ECM production from chondrocytes).

IMPACT: The surface of cartilage is critically important, and therefore the finding is important, especially for cartilage research. The team did a good job testing relevant loading as well to ensure it could hold up to multiple loading schemes and provide more strength as a cartilage specific solution.

POSITIVE NOTES: My big criticism of 'how would this actually work clinically as tissue is not PTFE' was answered with their prototype, so that was a very effective addition!

We would like to sincerely thank Reviewer 2 for the positive and constructive feedback. We are pleased to hear that the manuscript was found to be clear, well-written, and thorough, and that the experimental approach and major claims were appreciated. We also greatly value the recognition of our work's potential impact, and appreciate the recommendations of the Reviewer. We are glad that the addition of the prototype was effective in addressing the application of this research. Thank you again for your comments and recommendations, with each particularly helpful in strengthening our manuscript. Please find below our responses and modifications to the manuscript in response to each query.

Critiques from reviewer:

1. The work is effective at showing the mechanism behind this phenomena, and demonstrating the changes in material properties on several length scales (under bulk shear, and with AFM). However, when addressing the question of 'Novelty' it is merely demonstrating a phenomena previously found by Gong et al. but showing it in a natural hydrogel. That being said, it is a very useful finding for hydrogel researchers and potentially for clinical translation.

We thank Reviewer 2 for these comments. We acknowledge both here and within the manuscript that the phenomenon being studied throughout this study was initially described by Gong *et al.* with synthetic polymers, and this is highlighted by our effort to reference a number of their publications in this area. Our study extends our general understanding of this phenomena within the field by investigating the intentional application of this phenomenon to *in situ* photocrosslinking of our hydrogel materials, while further characterising the influence of substate-induced material properties in terms of both surface and bulk performance. We believe this study represents a significant contribution to the field which may inform the biofabrication of tissue-engineered hydrogel constructs for clinical applications, as demonstrated by our *ex vivo* demonstration.

We have clarified this distinction in the revised manuscript to emphasise the novelty of our approach and its potential impact on hydrogel research and clinical applications:

> …This is based on the inference that the primary mechanism by which hydrogel surface friction is reduced herein is predominantly substrate related, as originally described by Gong *et al.*, [24,25]. In conjunction with existing literature surrounding this phenomenon in synthetic polymer materials, the results of our study aim to develop our general understanding of this mechanism, while presenting a clinical application of this using our natural hydrogel material and *in situ* photocrosslinking. Further, our study aimed to delve deeper into the mechanisms by which substate-induced material properties at both the surface and overall, are influenced. We believe this study represents a significant contribution to the field which may inform the

biofabrication of tissue-engineered hydrogel constructs for clinical applications, with potential for further work in future. Thus, future work may be suitable to investigate the observed phenomena in various alternative hydrogel systems.

We appreciate these comments and believe the contribution of this study is more effectively communicated within the revised manuscript.

2. The paper effectively shows imaging of collagen II vs collagen I production in the cells and quantifies this, but it is strange to me that at 28 days under loading this cellular production is not seen in an extensive ECM network AND gene expression differences are not seen. I think the language and implication of clinical efficacy claims may be a bit too strong given this.... The authors need to discuss this discrepancy and offer some potential explanations...

We appreciate the reviewer's observation. While gene expression differences were not significant at 28 days under loading, extensive ECM accumulation was evident in PTFE-crosslinked constructs, as shown by interterritorial and pericellular staining for collagen II. This suggests that protein-level changes and matrix deposition occurred, but gene expression may have stabilized by this stage, reflecting a more mature chondrocyte phenotype rather than an ongoing transcriptional response.

Importantly, transcriptional activity does not always directly correlate with protein production, as gene expression represents an early regulatory step, while post-transcriptional modifications, translation efficiency, and protein turnover influence final ECM deposition (Vogel *et al.,* 2012; Maier *et al.,* 2009; Liu *et al.,* 2016). Previous studies have shown that cartilage ECM accumulation can be heavily regulated at the post-translational level, with enzymatic processing and matrix incorporation occurring independently of steady-state mRNA levels (Guilak *et al.,* 2018 & Little *et al.,* 2010).

To address this, we have refined our discussion to highlight that clinical efficacy claims should be framed as promising but not conclusive, while emphasizing the strong ECM formation observed in PTFE-exposed constructs. Future studies with longer timepoints and post-transcriptional analyses would further clarify the mechanistic link between gene expression and ECM deposition in these constructs.

> While the 28-day dynamic *in vitro* cell culture study reported herein has provided promising results, cartilage repair is a particularly slow process. Thus, long-term studies with additional timepoints would be valuable for assessing the stability and durability of our low-friction hydrogels under prolonged mechanical loading. Thus, in the planning of future experimental work, long-term follow-up studies are required to evaluate the performance of these low-friction hydrogels over extended periods, which may result in enhanced ECM accumulation thus providing determination of specific differences in gene expression results. While gene expression differences were not significant at 28 days under loading, extensive ECM accumulation was evident in PTFE-crosslinked constructs, as indicated by strong interterritorial and pericellular staining for collagen II. This suggests that protein-level changes and matrix deposition occurred, but gene expression may have stabilized by this stage, reflecting a more mature chondrocyte phenotype rather than an ongoing transcriptional response.
>
> Importantly, transcriptional activity does not always directly correlate with protein production, as gene expression represents an early regulatory step, while post-transcriptional modifications, translation efficiency, and protein turnover influence final ECM deposition [58–60]. Previous studies have shown that cartilage ECM accumulation can be heavily regulated at the post-translational level, with enzymatic processing and matrix incorporation occurring independently of steady-state mRNA levels [61,62].
>
> …Additionally, we acknowledge that increasing the sample size, donor number, and number of constructs would enhance the robustness and reproducibility of our findings. Future work will incorporate larger sample sizes to further investigate the limitations associated with the gene expression results and strengthen the

translational potential of our findings pertaining to hydrogels with tuneable tribological properties.

In terms of the Reviewer comment regarding the presence of observable differences in collagen production despite a lack of an extensive ECM network, this is an important observation that warrants further discussion. In our GelMA-based hydrogels used herein, we expect to observe extensive proliferation of the encapsulated human articular chondrocytes via the formation of collagen-dense spheroids, rather than formation of a branched ECM network. This spheroid formation is clearly represented in our collagen I and II immunostained images in Figure 5, with clear differences in collagen production between groups. These results align with our previous studies using various hydrogels in the 3D cell culture of cartilage neo-tissues, as referenced throughout. Based on this, and as recommended by the Reviewer, this has been described in more detail in the revised manuscript:

> Given the high relative stiffness of the GelMA/HAMA hydrogels within which the human articular chondrocytes were encapsulated herein, spheroid formation, rather than a branched ECM network, was expected across the 28-day study, coinciding with cellular proliferation under mechanical stimulation. This spheroid formation was observed via fluorescence microscopy analyses of samples stained for both cellular viability (Figure 4D) and collagen components (Figure 5D). These results align with our previously published studies in the 3D cell culture of cartilage neo-tissues, whereby chondrocyte spheroid formation in both natural [51,52] and synthetic [53] hydrogels was observed with negligible branching despite the production and accumulation of essential collagen components of the cartilage ECM.

In addition to these modifications to the manuscript, the clinical efficacy claims have been updated generally throughout to better align with the results and limitations of the studies.

3. Do these effects partially depend on the opposite surface from PTFE being glass? Like if you do this between tissue and PTFE do you get the same effects on the PTFE surface? Would be good to understand whether the presence of the glass overstates the effect on opposite side of hydrogel…. Would thickness of the hydrogel matter?

The queries from Reviewer 2 regarding the potential influence of substrate combinations on surface tribology are interesting and warrant further consideration, though investigation of various surfaces will require substantial studies to be performed in future work. Specifically, the Reviewer raises an interesting point as to whether the opposite surface (eg. glass) influences the observed effects on the primary surface (eg. PTFE). This may also vary depending on different material options or combinations of these, and would require a range of studies to be performed to determine the effects. As such, in the revised manuscript, we have included some discussion of this:

> Furthermore, given this study investigates only two materials as crosslinking substrates, namely PTFE and glass, future work may benefit from incorporating alternative materials and combinations of these to determine effects on tribology. While different materials would be expected to influence surface crosslinking and tribology, additional testing would be necessary to determine if the combination of substrates affect each other and the resultant hydrogel properties. Thus, experimental planning would need to consider the application of such studies given the extensive number of potential materials and combinations.

We also acknowledge that the thickness of the hydrogel could potentially influence these outcomes and while we expect this is unlikely future work could address this. The thickness of our gels is consistent with human cartilage, hence clinically relevant for partial and full thickness defect repair. Given the focus on of this paper is to describe an observed phenomenon with useful outcomes specific to the surface properties of our hydrogels for cartilage tissue-engineering, where low-friction surfaces mimic the properties of cartilage, we have not included experiments to assess whether thickness plays a role. The Gong group have investigated thickness and observed greatest effects in the first couple of mm.

While future work is required to assess this, we do not anticipate that thickness would have a major effect on tribology, given the results of surface topography and crosslinking density as presented in Figure 3. The microscopy images obtained to visualize unreacted methacryloyl groups in hydrogel sections indicate that crosslinking is consistent throughout the bulk of the hydrogel section, with only the very edges expressing the presence of unreacted groups. This would become more of a concern with very thick samples, if visible light crosslinking was unable to penetrate throughout the entire sample. Discussion of this has been added to the revised manuscript:

> One limitation of this study was that the influence of hydrogel thickness on crosslinking efficacy or tribology was not studied. Based on our findings from surface topography and crosslinking density (Figure 3), we do not anticipate significant effects of hydrogel thickness on tribological properties, given the microscopy images of unreacted methacryloyl groups indicate consistent crosslinking throughout the bulk of the hydrogel, with unreacted groups observed only at the edges exposed to PTFE. Thickness-related concerns would become more relevant for very thick samples, where limited penetration of visible light crosslinking might occur. Ultimately, future work will be necessary to investigate the role of hydrogel thickness in this regard.

We thank you Reviewer 2 for these considered comments and questions, and look forward to investigating such factors in future work.

4. The statistics are fairly rudimentary, and there is no discussion of whether the residuals were normalized, or details on the ANOVA model (one-way, two way?) . In the ANOVA were any co-factors considered related? Was cell donor considered a factor? Would be good to know more about the details of the model used for the ANOVA, and whether the 3 donors made a mixed cell population or whether donor might be a factor?

We appreciate the Reviewer's queries regarding the statistical analyses performed. Regarding the three cell donors used, these cell populations were kept distinct, however given low cell numbers, for the *in vitro* study analyses, 2 constructs per donor were used for DNA and GAG analysis, 1 construct per donor for viability and immunofluorescence analyses, and 2 constructs per donor for gene expression analyses. Hence, cell donor was not considered a factor in statistical analyses. These details have been added to the Methods section on cell encapsulation and copied below, as well as in the description of statistics:

> Following isolation, cells were expanded for 1–2 weeks to passage 1 on standard treated tissue culture plastic flasks (Nunc, ThermoFisher Scientific, QLD, Australia) in low-D-glucose chondrocyte basal medium…
>
> … For all cell culture experiments performed, three donor populations of human articular chondrocytes were isolated and expanded, with 2 constructs per donor used for DNA and GAG analysis, 1 construct per donor for viability and immunofluorescence analyses, and 2 constructs per donor for gene expression analyses. Thus, given the low number of samples per donor, cell donor was not considered a factor in statistical analyses, with these grouped together.

In future work, we will aim to perform reproducibility experiments with a range of donor cells independently, to further assess the performance of our hydrogel-based biofabrication of cartilage neo-tissues and incorporate cell donor as a factor in our analyses of tests where appropriate. This would be expected to provide complementary data to support the findings determined herein.

Additionally, further details have been added to the revised manuscript to provide greater description of statistical analyses performed:

> Statistical analysis was performed using GraphPad Prism software (version 7–10; GraphPad, CA, USA) with a significance level of 0.05. <u>One-way analysis of variance analyses (ANOVAs), with Tukey's multiple comparisons post-hoc tests with single pooled variance,</u> were performed to investigate the effects of culture duration and culture condition on chondrocyte viability and gene expression, as well as total DNA

and GAG content, wet weight, and Young's moduli of cell-laden constructs. For all ANOVAs, a confidence interval of 95% was used, with residuals assessed for normality with Shapiro-Wilk tests and Q-Q plots. Unpaired Student's t-tests were performed to investigate differences in AFM roughness and compression data… Statistical differences are indicated in figures using symbols (****p <0.0001, *** p <0.001, **p <0.01, *p <0.05).

We thank Reviewer 2 for these comments toward improving description of the analyses performed.

5. The shear loading was done at a given compression. With cartilage, there is also a very high level of direct compression loading under normal conditions (i.e. walking). I am unsure if this hydrogel can withstand that which is important in addition to the surface.

We agree with the Reviewer that direct compression loading is a critical factor for the performance of hydrogels in cartilage-related applications, and we appreciate the concerns raised. To address this directly within the revised manuscript the following discussion has been added:

> While this study focused primarily on shear and frictional testing to assess surface-related kinematics, cyclic compression was included in the dynamic culture studies to mimic physiological loading patterns experienced *in vivo*. While our hydrogel materials possess compressive moduli in the low physiological range of native cartilage, these hydrogels exhibit advantageous frictional properties which may be particularly relevant in the ongoing development of *in vitro* cartilage models and cartilage repair therapies.
>
> To improve the compressive performance and failure properties of these low-friction hydrogels, application or combination of these manufacturing methods with alternative materials may achieve low-friction hydrogels while maintaining the beneficial frictional kinematics observed here. Specifically, these low-friction manufacturing methods may be combined with our previously published work where the addition of glycol chitosan to GelMA increased the compressive modulus and adhesive crosslinking properties significantly, while maintaining high cell viability [54,55].
>
> In small defects, the hydrogel alone may be suitable to handle the loading that is shared with the surrounding cartilage as we have shown *in vitro*. However, for larger defects, where the gel is bearing the load independent to surrounding tissue, failure may occur. Thus, we have developed fibre-reinforcement techniques using melt electrowritten (MEW) scaffolds, increasing the load-bearing capacity of our constructs substantially, replicating cartilage-like mechanical properties and robustness [56–58]. Thus, as previously noted, future work is required to ascertain the degree to which tribological properties of other hydrogel systems may be influenced by crosslinking substrate hydrophobicity. However, translation of the methods reported here, we anticipate similar effects on surface friction to be achievable in other hydrogel systems, particularly our GelMA-based hydrogel systems.

Thank you again for the recommendation to address these concerns in the revised manuscript.

MINOR CRITIQUES

1. Clarify this: "We also observed a further reduction of μ_kin in cell-laden gels crosslinked on both PTFE and glass as a result of increased culture duration (Figure 2A,C). This reduction was more emphasized in PTFE-exposed construct surfaces where μ_kin values were reduced by 35–70% relative to day 1 values, depending on sliding velocity and contact pressure."

"more-emphasized" is not strong enough or clear. Please give detail here of the reduction percentage on glass (i.e. summarize that data as well as the PTFE reduction

We thank Reviewer 2 for also identifying these statements as requiring clarification alongside Reviewer 3. Statistically significant reductions were observed in frictional kinematics of PTFE exposed cell-cultured hydrogels from day 1 to day 14 when subject to 10 kPa compression during dynamic culture, however at 20 kPa these were reductions were observed but not statistically significant. This has been clarified directly within the text, with additional representation of the cell-laden frictional testing data across the cell-culture period provided within the Supplementary Data.

The following has been modified within the revised manuscript to describe this more effectively, with the new Supplementary Figure S3 included below:

> Our results confirmed that in comparison to glass-exposed construct surfaces, μ_{kin} values of PTFE-exposed construct surfaces were significantly reduced over a range of sliding speeds (0.1–3 mm/s) and contact pressures (10 and 20 kPa) (Figure 2A,C). We also observed lower μ_{kin} in cell-laden gels crosslinked on PTFE as a result of increased culture duration, though only the 10 kPa compression sliding tests of PTFE-exposed gels exhibited statistically significant decreases in μ_{kin} across timepoints, with no statistically significant reductions at 20 kPa compression for either PTFE or glass (Figure 2A A,C and Figure S3, Supplementary Data). From day 1 to day 14 of culture, PTFE samples subject to 10 kPa compression during sliding, exhibited significant reductions in μ_{kin} of 61%, 71%, 62%, 54% and 53% at sliding velocities of 0.1, 0.5, 1, 2, and 3 mm/s, respectively; while for glass at 10 kPa, significant reductions were only observed at sliding velocities of 0.5, 1 and 3 mm/s, namely 34%, 31% and 19%. For samples subject to 20 kPa compression during dynamic culture, slight reductions were observed across sliding velocities, though these were non-significant due to large standard deviations. For samples prepared on glass, only very minimal reductions were observed in the 10 kPa groups from day 1 to day 14 of culture, while negligible differences were observed at 20 kPa compression across timepoints. Thus, the crosslinking substrate may have a long-lasting effect on construct friction, with PTFE showing more promising results in this regard.

We appreciate these suggestions and recommendations to improve the clarity of the results.

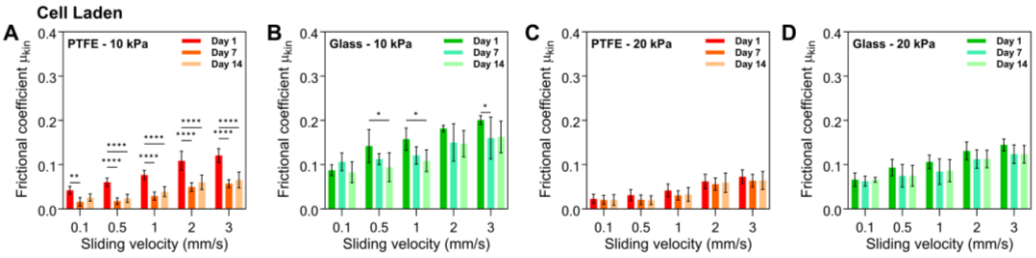

Figure S3. Tribological properties of cell-cultured GelMA/HAMA hydrogels photocrosslinked on glass or PTFE substrates. Kinetic friction coefficients μ_{kin} of 9.5% w/v GelMA / 0.5% w/v HAMA constructs photocrosslinked on glass (green) or PTFE (red) substrates at day 1, 7, and 14 incubation, with chondrocyte differentiation media used as lubricant, at contact pressures of (A,B) 10 kPa and (C,D) 20 kPa, respectively. Values reported as mean ± SD, where symbols indicate significance (****p <0.0001, ***p <0.001, **p <0.01, *p <0.05).

Again we thank the Reviewers for their recommendations to clarify these statements, with the modified section of the manuscript provided a clearer and more detailed explanation, while the claims have been reduced given they require further investigation to make direct conclusions.

2. How long were cells expanded? On what substrate? At what passage were they encapsulated? This has been shown to dramatically change chondrocytes so is information that is critical to share.

Thank you for your queries regarding the cell culture methods. In accordance with our established and referenced protocols for the isolation and expansion of human articular chondrocytes, cells were isolated and then expanded for up to 2 weeks in standard tissue culture flasks, with these encapsulated in hydrogel precursors at passage 1.

We have added this information to the revised manuscript to ensure clarity, as we agree it is crucial for understanding the potential effects on chondrocyte behavior:

> Human articular chondrocytes were isolated from macroscopically normal cartilage of the lateral femoral condyles of knee samples obtained from three donors (66 and 67 year old males, 56 year old female) undergoing total knee arthroplasty for osteoarthritis, as described elsewhere [9,51,81]. Following isolation, cells were expanded for 1–2 weeks to passage 1 in standard treated tissue culture plastic flasks (Nunc, ThermoFisher Scientific, QLD, Australia) in low-D-glucose chondrocyte basal medium (Dulbecco's modified Eagle's medium (DMEM) with 2 mM GlutaMAX™, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1 mM nonessential amino acids, 50 U/mL penicillin, 50 µg/mL streptomycin, 0.5 µg/mL amphotericin B (Fungizone®) (all Invitrogen, CA, USA), 0.4 mM L-proline and 0.1 mM L-ascorbic acid (both Sigma Aldrich, St. Louis, MO, USA)) supplemented with 10% foetal bovine serum (FBS) (Hyclone, Logan, UT, USA). All cell cultures/hydrogel constructs were incubated at 37 °C in a humidified 5% CO_2/95% air CO_2 incubator with the medium replaced every 3–4 days.
>
> GelMA/HAMA hydrogel precursor solutions were prepared as described above. Passage 1 human articular chondrocytes were suspended in the hydrogel precursor solutions at 10 million cells/mL and photocrosslinked by exposure to 365 nm light within the aforementioned PTFE molds, as described above, in sterile conditions.

We thank Reviewer 2 for recommending this additional detail and trust this sufficiently describes the relevant information regarding cell isolation, expansion and encapsulation.

REVIEWER #3

Reviewer #3 (Remarks to the Author):

This is a detailed study of the surface properties of hydrogels prepared via methods commonly used in the field. This study highlights the importance of tribological properties of hydrogels, which are often overlooked in the assessment of mechanical properties of biomaterials, and are of particular importance in relation to cartilage tissue engineering. Overall, I found the study interesting and highly relevant to the field.

The authors thank Reviewer 3 you for their encouraging comments as well as the queries below. We are pleased the study was interesting, as we believe our results are quite relevant to the field of cartilage tissue engineering. The manuscript has been improved with input from the Reviewer's queries, as outlined in response to each below.

1. In discussing Figure 2A/C authors mention a "reduction" of the kinetic frictional coefficient due to culture duration – is the trend significant? Statistical analyses should be performed/discussed to contextualise these results.

We thank Reviewer 3 for identifying this alongside Reviewer 2. Statistically significant reductions were observed in frictional kinematics of PTFE exposed cell-cultured hydrogels from day 1 to day 14 when subject to 10 kPa compression during dynamic culture, however at 20 kPa these reductions were observed but not statistically significant.

This section of the Results & Discussion required clarification, and we have endeavoured to provide this within the modified manuscript as copied below, as supported by the new Supplementary Figure S3 included below:

> Our results confirmed that in comparison to glass-exposed construct surfaces, μ_{kin} values of PTFE-exposed construct surfaces were significantly reduced over a range of sliding speeds (0.1–3 mm/s) and contact pressures (10 and 20 kPa) (Figure 2A,C). We also observed lower μ_{kin} in cell-laden gels crosslinked on PTFE as a result of increased culture duration, though only the 10 kPa compression sliding tests of PTFE-exposed gels exhibited statistically significant decreases in μ_{kin} across timepoints, with no statistically significant reductions at 20 kPa compression for either PTFE or glass (Figure 2A A,C and Figure S3, Supplementary Data).

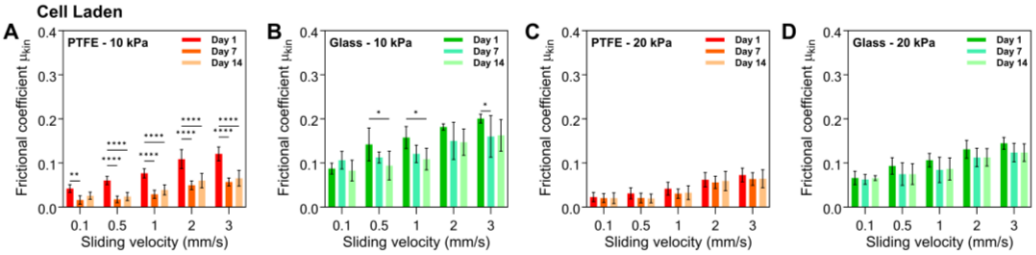

Figure S3. Tribological properties of cell-cultured GelMA/HAMA hydrogels photocrosslinked on glass or PTFE substrates. Kinetic friction coefficients μ_{kin} of 9.5% w/v GelMA / 0.5% w/v HAMA constructs photocrosslinked on glass (green) or PTFE (red) substrates at day 1, 7, and 14 incubation, with chondrocyte differentiation media used as lubricant, at contact pressures of (A,B) 10 kPa and (C,D) 20 kPa, respectively. Values reported as mean ± SD, where symbols indicate significance (****p <0.0001, ***p <0.001, **p <0.01, *p <0.05).

Further discussion of this has been included in response to the second question by Reviewer 3 below, and we appreciate both comments and questions relating to this section of the manuscript.

2. Also in relation to Figure 2A/C, the following sentence mentions values were "reduced by 35-70%". This is quite a wide range and it could be useful to also state the percentage reduction for glass to make the difference clearer to the reader.

As noted above, we appreciate these comments pertaining to the statements made regarding the reductions in frictional kinematics of the constructs during cell culture. As recommended by the Reviewer, the following has been updated within the revised manuscript to clarify the reduction %'s within groups across timepoints, with values reported with discussion of statistical significance provided and supported by the new supplementary Figure S3 as copied above.

> From day 1 to day 14 of culture, PTFE samples subject to 10 kPa compression during sliding, exhibited significant reductions in μ_{kin} of 61%, 71%, 62%, 54% and 53% at sliding velocities of 0.1, 0.5, 1, 2, and 3 mm/s, respectively; while for glass at 10 kPa, significant reductions were only observed at sliding velocities of 0.5, 1 and 3 mm/s, namely 34%, 31% and 19%. For samples subject to 20 kPa compression during dynamic culture, slight reductions were observed across sliding velocities, though these were non-significant due to large standard deviations. For samples prepared on glass, only very minimal reductions were observed in the 10 kPa groups from day 1 to day 14 of culture, while negligible differences were observed at 20 kPa compression across timepoints. Thus, further tests are required to elaborate upon these results, however it may be inferred that the crosslinking substrate may have a long-lasting effect on tissue construct friction, with PTFE showing more promising results in this regard.

Once again we thank Reviewer 3 for identifying this section of the manuscript which required elaboration and clarification of the statements made. We believe this portion is now better supported by reported values and the data within the figures, while the claims made have been adjusted to reflect the need for future work to be performed to fully ascertain whether cell-culture influences the frictional properties over time.

3. Is it possible there is directionality to the crosslinking that would explain some of the differences between the hydrogel surfaces crosslinked in contact with PTFE vs glass? Could the authors discuss this and how it was ruled out? E.g. if the light was always shone through the glass slide on top, what might happen if light was shone through a sufficiently transparent PTFE surface first, hitting the glass side last?

We appreciate the Reviewer's comment regarding the potential directionality of the crosslinking process. We acknowledge this must be studied in future, however the results would not be anticipated to differ substantially from those observed herein, given the consistent surface and hydrophobic properties of PTFE. Our current experimental setup of crosslinking through glass into the PTFE mold is primarily due to the mold design and material manufacturing limitations: since the mold has depth requirements to create our 3D hydrogel models, thick PTFE sheets are machined to our mold specifications, and these thick PTFE sheets are only supplied in virgin white PTFE.

To rule out the possibility of crosslinking directionality, we could consider conducting experiments where the orientation of substrates was varied, though custom manufacturing of specifically transparent PTFE molds would be required to achieve the correct hydrogel geometries, which at this stage is not a possibility. Alternatively, as noted in response to Reviewer 2's third comment, both hydrogel thickness and alternative materials or combinations of materials, and as outlined in our response to the next comment from Reviewer 3, crosslinking though the transparent PTFE film used in the *ex vivo* demonstration would also not be expected to alter the results substantially given the consistent properties of PTFE, though tests in future *ex vivo* or *in vivo* studies will be required to confirm this.

Unfortunately, as described in our response to the below comment, removal of the injected hydrogels crosslinked *in situ* in the *ex vivo* joint defect model was not possible without damaging the integrity of the hydrogel. Hence, frictional testing and other analyses could not be performed on *in situ* crosslinked hydrogels, though future studies could be designed to assess these factors. Discussion of these points has been included in the revised manuscript, as copied in response to the following Reviewer comment.

4. Similarly, did the authors do any analysis of surface properties of gels crosslinked in contact with the PTFE membrane as in the Figure 6 set-up? Would they expect any differences to the gels crosslinked in contact with the PTFE molds?

In the current study, we did not specifically assess the surface properties of gels crosslinked in contact through the PTFE membrane within the *ex vivo* joint defects shown in Figure 6. While we acknowledge that the surface characteristics of the hydrogel could vary depending on whether it is crosslinked in situ through a PTFE membrane. While replication of the experiments performed herein would be required to assess this directly, with such studies to be potentially included in further animal studies, we do not anticipate significant differences in frictional performance given the consistent material properties of PTFE. This has been noted in the Results & Discussion of the revised manuscript:

> Furthermore, while this *ex vivo* study clearly demonstrates the potential for clinical application of our low-friction hydrogel system, the properties of the hydrogels injected and crosslinked *in situ* were not specifically assessed. Primarily, this was a physical limitation whereby the crosslinked materials could not be removed from the joint defect without damaging the integrity of the implant. While we do not anticipate significant differences in frictional performance given the consistent material properties of PTFE, it must be considered that the surface properties of these gels crosslinked through the PTFE membrane and in contact with native tissue may exhibit slightly variations to our *in vitro* models. Replication and extension of the experiments performed herein would be required to assess this directly in future work, with this to be potentially assessed in future animal studies.

We once again thank Reviewer 3 for the consideration of our manuscript and greatly appreciate the comments and recommendations made toward improving the quality and suitability of our paper for publication in *Communications Materials.*

Revier Response References

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