Immune-Informed Mucin Hydrogels Evade Fibrotic Foreign Body Response In Vivo

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The immune-mediated foreign body response to biomaterial implants can trigger the formation of insulating fibrotic capsules that can compromise implant function. To address this challenge, the intrinsic bioactivity of the mucin biopolymer, a heavily glycosylated protein that forms the protective mucus gel covering mucosal epithelia, is leveraged. By using a bioorthogonal inverse electron demand Diels–Alder reaction, mucins are crosslinked into implantable hydrogels. It is shown that mucin hydrogels (Muc-gels) modulate the immune response driving biomaterial-induced fibrosis. Muc-gels do not elicit fibrosis 21 days after implantation in the peritoneal cavity of C57Bl/6 mice, whereas medical-grade alginate hydrogels are covered by fibrous tissues. Further, Muc-gels dampen the recruitment of innate and adaptive immune cells to the gel and trigger a pattern of very mild activation marked by a noticeably low expression of the fibrosis-stimulating transforming growth factor beta 1 cytokine. Macrophages recruited to Muc-gels upregulate the gene expression of the protein inhibitor of activated STAT 1 (PIAS1) and SH2-containing phosphatase 1 (SHP-1) cytokine regulatory proteins, which likely contributes to their low cytokine expression profiles. With this advance in mucin materials, an essential tool is provided to better understand mucin bioactivities and to initiate the development of new mucin-based and mucin-inspired “immune-informed” materials for implantable devices subject to fibrotic encapsulation.

1. Introduction

A universal challenge for functional implants, such as encapsulated cells,[4] sensors,[2] electrodes,[3] and drug-eluting materials,[4] is their rapid isolation from the body by a fibrotic capsule mediated by a foreign body reaction (FBR). The FBR starts immediately after implantation with the passive adsorption of proteins onto the implant. This is followed by accumulation of monocytes and macrophages, that can further fuse to form multinucleated giant cells. The subsequent secretion of fibroblast-recruiting and proangiogenic factors by activated immune cells leads to the generation of a fibrous capsule around the biomaterial 2–4 weeks after implantation.[5,6] To prevent fibrosis, biomaterials can be designed to reduce the nonspecific binding of proteins and subsequent cell adhesion, or release anti-inflammatory drugs such as glucocorticoid dexamethasone[7,8] that dampen the immune response to implants. However, these strategies are relatively short-lived. An alternative approach is in the intelligent physical and molecular design of materials, which appears to be a promising strategy to modulate the immune response to implants; thus providing a more durable and resilient immune modulating signal. For instance, physical properties of materials such as size,[1] geometry,[9] roughness,[10] porosity,[11] hydrophobicity,[12] mechanical properties,[13] and chemistry[14] have all shown to influence immune activation.

Another strategy is to leverage mechanisms by which the immune system distinguishes foreign objects from the body’s own components. For instance, bacteria-derived LPS-presenting objects are marked as foreign and readily initiate immune responses, while cell surface markers such as CD47[15] and CD200[16] act as self-signaling molecules and contribute to lowering the immune activation state. Materials conjugated with CD47 could prevent macrophage attachment, activation, and phagocytosis[17,18] while polystyrene grafted with CD200 decreased the expression of TNF-α and IL 6 in macrophages.[19]

In addition to proteins, glycans are also known to regulate important immune functions through multivalent binding to cell surface protein receptors. Synthetic glycomaterials for immunomodulation are being developed[20] but the field is confronted with the monumental task of precisely reconstituting the “glycocluster” configurations of natural glycans, meaning finding the precise combination of sugar sequences,
valency, and spatial organization is required for the optimization of ligand binding.\textsuperscript{[20]} Naturally occurring glycoproteins offer the opportunity to create materials that contain complex bioactive glyoclusters to investigate their effect on the immune system. By doing so, we open the door to exploiting the potential biological information contained in the glycome.

The mucin glycoproteins have emerged as another important part of the immune regulatory arsenal in mammals. Mucins are either secreted to form the mucus gel that covers the mucosal epithelia, or expressed as cell transmembrane proteins as a main component of the cell glycocalyx.\textsuperscript{[21]} Every protein of the mucin family features densely glycosylated serine and threonine-rich regions. The mostly O-linked glycans are terminated by a wide variety of sugars, with sialic acids and fucose residues being the most common.\textsuperscript{[22]} Owing to this rich chemistry, the mucus gel is a multifunctional material that hydrates, lubricates and forms selective barriers that protect the epithelium.\textsuperscript{[21]} Secreted mucins have been shown to have an immunological role, although their exact activity is still unclear. For instance, in one study, intestinal MUC2 mucin glycans were shown to dampen inflammatory activation of dendritic cells as evidenced by the increased IL-10 secretion.\textsuperscript{[23]} While a more recent study suggested the same mucin can increase the expression of proinflammatory cytokine IL-8 in dendritic cells.\textsuperscript{[24]} Cancerous cells can escape the immune attack by expressing membrane-bound MUC1 mucins with altered glycosylation and by secreting substantial amounts of mucins that form a physical and biochemical protective encasing of the tumor.\textsuperscript{[25]} Mucins-containing materials have been suggested as potential building blocks for multifunctional biomaterials, but their immune modulating properties have not been investigated.\textsuperscript{[21,26,27]}

Given the strong indications that mucins hold immune-modulatory capacities, we aimed to develop a mucin material that can deliver immunological signals upon implantation. We show that bovine submaxillary mucin (BSM) can be assembled into a robust hydrogel material (Muc-gel) by functionalizing them with chemical groups that quickly react to form covalent bonds. Muc-gels implanted into the peritoneal cavity of mice showed no fibrosis after 21 days, while alginate hydrogels (Alg-gel) were covered by a fibrotic capsule. Analysis of the immune activity on and around the implant revealed large differences in cell composition and activation when compared to an Alg-gel, suggesting strong immune-modulating properties of this new class of material.

2. Results and Discussion

2.1. Click Mucins Can Be Assembled into Implantable Hydrogels

We successfully introduced either tetrazine (Tz) or norbornene (Nb) "clickable" functionality onto BSM molecules by reacting Tz and Nb amine derivatives with activated carboxylic groups of mucins (Figure 1A) as confirmed by \textsuperscript{1}H NMR (Figure S1, Supporting Information). The possible carboxylate residues targeted by the functionalization are located on the C-terminal of the mucin protein backbone, on the side chains of aspartic acid, glutamic acid, and on sialic acid residues. The resulting BSM-Tz and BSM-Nb form covalent bonds through an inverse electron demand Diels–Alder cycloaddition reaction when mixed in solution (Figure 1A) leading to the formation of hydrogels composed of an interconnected porous network of mucin molecules as evidenced by SEM (Figure 1B). Inverse electron-demand Diels–Alder reactions were successfully applied to crosslink biopolymer gels before and termed "click" chemistry because of its high yields and generation of inoffensive byproducts.\textsuperscript{[28–30]} This particular reaction between norbornene and tetrazine was chosen here because of its reactivity, relative stability of norbornene in solution,\textsuperscript{[31]} and no known reactivity with naturally occurring molecules, which makes it thus more suitable for cell encapsulation and in vivo implantation applications\textsuperscript{[32]} than other "clickable" reactions. Time-sweeping rheology analysis revealed that the sample transitioned from a viscous solution ($G'' > G'$) to a gel dominated by the elastic modulus after $\approx 4 \text{ min}$, then increased dramatically within $20 \text{ min}$ and slowly reached a plateau-like state after $\approx 40 \text{ min}$ (Figure 1C). It is known the reaction kinetics of inverse electron demand Diels–Alder cycloaddition reaction is quick\textsuperscript{[32]} but polymer crosslinking can be slowed down by steric hindrance and electrostatic repulsion. A frequency sweep performed after the viscoelastic moduli have reached plateaus demonstrated that the system appeared, indeed, to be efficiently and covalently crosslinked (Figure 1C). Based on the rheological data and rubber elasticity theory,\textsuperscript{[33,34]} we estimated the distance between two adjacent crosslinkers ($\xi$) to be $\approx 7.4 \text{ nm}$ (see methods in S1), which would allow the unrestricted diffusion of nutrients and cell secretomes through the gels.\textsuperscript{[35]} Indeed, THP-1 derived macrophage type 0 embedded in the Muc-gel maintained their viability over a 7 day culture (Figure S2, Supporting Information). Muc-gels were degraded by proteases as indicated by a gradual decrease of the Muc-gels' storage modulus when exposed to a high concentration of trypsin (Figure 1D). This indicates that the Muc-gel is primarily linked through the nonglycosylated protein backbone, which is sensitive to trypsin (Figure 1D). This is also supported by the unchanged sialic acid content measured before and after functionalizations of the mucin (Figure S3, Supporting Information). Such a versatile system allows for the rapid assembly of mucin molecules into robust hydrogels. Such gels are useful as model materials to study mucin bioactivity, as the local immune-isolation for cell transplantation, and as implantable materials or implant coatings.

2.2. Muc-Gels Implanted into the Peritoneal Cavity of Mice Averted Fibrotic Encapsulation

The foreign body reaction to Muc-gels were investigated by using immunocompetent C57Bl/6 mice in which the FBR is in many respects similar to that observed in humans.\textsuperscript{[1,36]} We implanted customized Alg-gel cylinders (diameter $= 4.78 \text{ mm}$, height $= 2.79 \text{ mm}$, 50 $\mu \text{L}$) or Muc-gel cylinders (Figure 1B) into the peritoneal cavity (i.p.) and compared the outcome on day 21, as Alg-gel cylinders are known to trigger fibrosis when implanted into C57Bl/6 mice.\textsuperscript{[36]} After 21 days of
implantation, we found Alg-gels were surrounded by clear fibrous capsules (Figure 2Aa,b), whereas there was no sign of fibrosis on the surface of Muc-gels, as illustrated by the few cells at the gel surface stained by H&E (Figure 2Bc,d). Masson’s trichrome staining indicated the presence of a collagen-rich matrix in the tissue formed around Alg-gels (Figure 2e,f, black arrows), whereas the Muc-gels showed no staining (Figure 2g,h). These results demonstrated that Muc-gels prevent the development of fibrosis in vivo. High-magnification images of the fibrous capsule around Alg-gels reveals the presence of fibroblasts and immune cells, including macrophages and neutrophils that play essential roles in FBR mediated fibrosis (Figure 2C).

2.3. Muc-Gel Implants Recruited Fewer Macrophages and B Cells

To investigate the cellular mechanism by which Muc-gels averted fibrotic capsule formation, we characterized the local immune response to the implanted materials. We examined both innate and adaptive immune cell compositions in the peritoneal cavity by performing lavages, and from Muc-gels and Alg-gels implants retrieved on days 14 and 21 (Figure 3A–C). We focused our attention on the macrophages (CD68^+CD11b^+), B lymphocytes (CD19^+IgM^+), neutrophils (CD11b^+Gr1^hi), and other myeloid cells (CD68^+Gr1^mixed^-CD11b^+) cell population since they have been shown to be the primary mediators of the FBR. Interestingly, the cell populations in the peritoneal...
cavity reflected rather closely the ones found associated with explanted hydrogels. Both on days 14 and 21, myeloid cells (CD68<sup>−</sup>Gr1<sup>low/−</sup>CD11b<sup>+</sup>) represented a majority of the cell population detected by FACS, followed by macrophages (CD68<sup>+</sup>CD11b<sup>+</sup>), B lymphocytes (CD19<sup>+</sup>IgM<sup>+</sup>) and neutrophils (CD11b<sup>+</sup>Gr1<sup>hi</sup>) (Figure 3B). Neutrophils, which can typically be found hours to weeks after implantation,[38–40] were found here in very low proportion at these later time points. The proportion of macrophages and B cells was significantly lower in Muc-gels compared to Alg-gels on day 14 (Figure 3C). On day 21, when the complete fibrotic isolation occurred, the proportion of macrophages and B cells dropped in mice with implanted Alg-gels to levels comparable to those of mice with Muc-gels. B cells represented a small but significant population of immune cells present in the i.p. space at 2 weeks following implantation of Alg-gels but not Muc-gels (Figure 3C). Although their contribution to the FBR is unclear, it has been recently shown that B cells could potentiate the biomaterial-induced fibrosis, perhaps by regulating macrophage phenotype and cytokine secretion.[37,41] The presence of immune cells on Muc-gels suggests that the antifibrotic effect is not only achieved by preventing cells from recognizing and interacting with the Muc-gels. Rather the Muc-gel dampened the immune cell recruitment.

2.4. Muc-Gel Implants Dampened the Macrophage and Myeloid Cell Activation

Chemokines and cytokines produced by activated immune cells play essential roles in the FBR cascades, i.e., inducing cell recruitment and differentiation of other immune cells and fibroblasts into myofibroblasts that participate in fibrotic capsule formation. We focused our attention on macrophages (CD68<sup>+</sup>CD11b<sup>+</sup>) and other myeloid cells (CD68<sup>+</sup>Gr1<sup>low</sup>/CD11b<sup>+</sup>) that were predominant fractions of immune cells sorted by FACS (Figure 4; Figure S4, Supporting Information). Macrophages
are especially relevant in this context since they display remarkable functional plasticity and participate in the host defense, immune regulation, and tissue repair, all aspects playing a pivotal role in mediating implant-triggered fibrosis.[3,37,42]

Strikingly, at 14 and 21 days’ postimplantation, the macrophages from Muc-gels and the corresponding peritoneal cavity showed nearly no expression of the analyzed proinflammatory and profibrotic markers[43–45] (Figure 4). This was in strong contrast with the macrophages from Alg-gels, which expressed significantly higher levels of M1 marker (proinflammatory cytokines and cell surface molecule) TNF-α and CD86 on day 21 and IL-1β on day 14, and of the M2 markers (anti-inflammatory cytokines and intracellular enzyme) IL-1RN on day 21, TGF-β1 on day 14, and Arg1 on day 14 and 21. The cytokine and cell-marker expression signature of the myeloid cells (CD68-Gr1low/CD11b+) were in many aspects similar to the pattern found for macrophages (Figure S4, Supporting Information). Exceptions to this were found for TNF-α and CD86 which were expressed at significantly higher levels on day 14 in cells from the Muc-gels and their i.p. space compared to Alg-gels.

Although chemokines and cell-surface markers can, in principle, discriminate between macrophages of inflammatory (M1) or alternatively activated (M2) phenotype, no distinct signature could be found in our study. This is exemplified by the simultaneous expression of typical M1 and M2 markers in macrophages on and around Alg-gels. It is now understood that rather than being at distinct activation states, macrophages in vivo are found on a continuum between the classic M1 versus alternative M2 phenotypes.[46] Our results reinforce the observation that biomaterials implanted in the peritoneal cavity of mice lead to macrophages with both M1 and M2 characteristics.[47,48] Importantly, and regardless of the

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**Figure 3.** The host immune response to implanted Alg-gels and Muc-gels in vivo. A representative FACS profile showing the gating strategy A) used for the analysis of host macrophages (CD68-CD11b+), neutrophils (CD11b+Gr1+), B cells (CD19-IgM+), and myeloid cells (CD68-Gr1low/CD11b+). Cell frequency of host immune cells to mononuclear cells retrieved from the i.p. space (lavage), or from gels on days 14 and 21 postimplantation in the mice B, C). Data are from four or five mice. Differences were determined using one-way ANOVA with Bonferroni correction.
Figure 4. Muc-gels dampened the macrophage activation. Gene expression in freshly sorted macrophages (CD68+CD11b+) from the explant and i.p. space on days 14 and 21 was analyzed by RT-PCR. The data represent the mean from independent RT-PCR measurements done in duplicate from pool-cell samples harvested from four mice. Differences were determined using a nonparametric one-way ANOVA test. Asterisks without comparison bars indicate the significant difference can be applied to the rest of the groups.
nomenclature, several cytokines known for their roles in non-biomaterial-induced fibrosis were expressed by immune cells on and around Alg-gels. For instance, TNF-α is a critical factor in the liver and pulmonary fibrosis,[49] IL-1β was also found to potentiate the pulmonary fibrosis,[50,51] and the ratio of IL-1β to IL-1RN expression determines its extent.[52] In addition, Arg1, an enzyme expressed in M2 macrophages promotes collagen deposition, which has also been associated with fibrosis.[53] Finally, TGF-β1 is strongly linked to tissue fibrotic cascades[54,55] and biomaterial-induced fibrosis.[56,57] At high concentrations, TGF-β1 can increase the extracellular matrix deposition by stimulating the differentiation of quiescent fibroblasts to collagen type-I producing myofibroblasts. Remarkably, none of these fibrosis-related cytokines and chemokines were expressed by macrophages and myeloid cells in contact with Muc-gels. It could thus be that the Muc-gels provide the necessary signals for macrophages and other immune cells to adopt a “fibrosis-repressing” phenotype.

We hypothesized that cytokine regulatory proteins that are essential to ensure an appropriate and controlled cellular response of immune cells could be, in part, responsible for the low cytokine expression profile of immune cells recruited to Muc-gels.[58] We found that both protein inhibitor of activated STAT 1 (PIAS1) and SH2-containing phosphatase 1 (SHP-1) were significantly upregulated in macrophages collected from Muc-gels on day 14 but not in macrophages collected from Alg-gels (Figure 4). PIAS1 is a negative regulator of STAT1, which is a transcription factor activated by cytokines including IFN-α and IFN-γ, epidermal growth factor, and IL-6.[59] PIAS1 is important downregulator of innate immunity, a deficiency in PIAS1 results in increased protection against viral and bacterial infection.[60] SHP-1 is known to down regulate signaling from toll-like receptors, which in turn reduces the production of pro-inflammatory cytokines. This role has been extensively shown in macrophages[61] and SHP-1 deficiencies linked with inflammatory diseases.[62,63] Interestingly, in mice, SHP-1 is recruited by the sialic acid-binding mSiglec-E receptor, which could very well interact with the mucin-bound sialic acid residues found in Muc-gels.[64] Put together, these results suggest that Muc-gels upregulate cytokine inhibitory proteins expression which is likely contributing to the overall low cytokine expression.

2.5. Muc-Gel Implants Showed no TGF-β1 and α-Smooth Muscle Actin Protein Expression

Next, we studied protein expression of TGF-β1 and α-smooth muscle actin, two markers of biomaterial-induced fibrosis,[57] by immunohistological staining and confocal microscopy imaging of day 21 explants (Figure 5; Figures S6–S9, Supporting Information). The Muc-gel surface had very few cells as indicated by DAPI staining, whereas Alg-gels were surrounded by a few layers of cells, which is in agreement with the H&E staining (Figure 2A,B). The strong expression of α-smooth muscle actin in the tissue surrounding of Alg-gels confirmed its fibrotic nature (Figure 5, red). In addition, TGF-β1 expression was induced by Alg-gels (Figure 5, green), whereas it was not expressed around or on the Muc-gel. This is in agreement with the low TGF-β1 gene expression levels measured in macrophages and myeloid cells (CD68+Gr1low/CD11b+) (Figure 4; Figure S4, Supporting Information). The absence of TGF-β1 is likely to contribute to the absence of fibrotic capsule around Muc-gels.

The chemical and physical properties of implanted materials can determine the immune response in several ways. For instance, initial protein adsorption to implanted materials are known to mediate subsequent immunological events.[65] Indeed, both Alg-gels and Muc-gels likely adsorb and absorb proteins,[66,67] as suggested by their colonization by cells in vivo. Thus, differences in the quantity and conformation of adsorbed protein coronas between Alg-gels and Muc-gels could be responsible for the observed differences in immune cell recruitment and activity. However, it is also likely that two highly hydrated and negatively charged materials such as Alg-gels and
Muc-gels will lead to rather similar protein coronas. Another possibility is from the direct interaction of mucin O-glycans with cell surface receptors. Indeed, we chose BSM as the model mucin for this study because it is well-characterized with known amino acids[60] and carbohydrate components. The main O-linked glycan on the BSM is the sialyl Tn antigen,[69] which is composed of N-acetylgalactosamine linked to a terminal sialic acid residue via a α2-6 glycosidic bond. The sialic acid residue of BSM binds to several sialic acid-binding immunoglobulin superfamily lectins (siglec) 2, 3, 5, 6, and 9,[70,71] which regulate the function of macrophages, B cells, neutrophils, monocytes and other immune cells,[72] and could therefore explain some of the effects observed here. Sialylated glycans are recognized as “self” ligands by siglec and tend to dampen an excessive innate immune response.[73] However, protein fragments on the mucin backbone could also be important to elicit such bioactivities.

3. Conclusion

We have developed the first “clickable” mucin material that is capable of modulating the immune response to avert biomaterial-induced fibrosis. Our results highlight the strong bioactivity of mucin molecules and their potential value as building blocks of immune-modulatory materials. The mucin hydrogel is bio-compatible and can be assembled into various forms (macrogel, droplets, thin films) opening a variety of applications including cell transplantations and biomaterial surface modifications. These results also highlight the value in considering extracellular mucins and mucus not only as passive barriers but also as matrices rich in biochemical signals with interesting possibilities for biomedical applications.

4. Experimental Section

Materials: Amine-derivatives of tetrazine (Tz) and norbornene (Nb) were purchased from Bioconjugate Technology Company and TCI Europe N.V., respectively. All other chemicals were purchased from Sigma Aldrich. PCR-related reagents were purchased from Thermofisher Scientific. RNA extraction micro or mini kits were purchased from Qiagen.

Synthesis of BSM Tz and Nb: Bovine submaxillary mucin (BSM) was predissolved in MES buffer (0.1 M MES, 0.3 M NaCl, and pH 6.5) at a concentration of 10 mg mL⁻¹. To this reaction mixture, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 4 mmol per gram of dry mucin) and N-hydroxysuccinimide (NHS; 4 mmol per gram of dry mucin) were added and stirred for 15 min. Thereafter, 1 mmol of tetrazine-amine (Tz) and 2 mmol of norbornene-amine (Nb) were added individually to generate the BSM tetrazine (BSM-Tz) or BSM norbornene (BSM-Nb). The reaction mixtures were stirred overnight at 4 °C and then dialyzed for 2 days (100 kDa cutoff) against 300 × 10⁻³ M NaCl and then MilliQ (MQ) H₂O for 2 days. The samples were freeze-dried and stored at −20 °C.

NMR Analysis of the Tz and Nb Functionalities: BSM derivatives were dissolved in deuterium oxide (Sigma-Aldrich). After solubilization, 600 µL of the solutions were transferred into a 5 mm NMR tube (Norel, USA). ¹H-NMR spectra were obtained on a Bruker Ultrasound plus 500 MHz spectrometer (Bruker Corporation, USA). The processing for the spectra was carried out by using the MestReNova Software (version 12.0.1-20560).

Examining Sialic Acid (Neu5Ac) Content in BSM and BSM Derivatives: The conjugation of Tz and Nb used in this study were achieved by targeting the activated carboxylic groups on BSM. To locate whether the functionalities were on the BSM protein backbone or the tips of O-glycans sialic acid, we examined the sialic acid content in BSM and BSM-Tz and BSM-Nb via high-performance anion-exchange chromatography (HP-AEC) based method. In brief, we solubilized the BSM, BSM-Tz, and BSM-Nb (2.5 mg in 500 µL MQ H₂O), then released the sialic acid from the BSM by adding 500 µL of sulfuric acid (0.1 N) and incubating at 80 °C for 1 h.[74] We then added 500 µL of sodium hydroxide (0.1 N) to the solutions to neutralize the pH. Neu5Ac (Sigma Aldrich) dissolved in MQ H₂O was used as the standard. The samples were filtered and injected (10 µL) into the CarboPac PA1 column (4 × 250 mm, Dionex). Neu5Ac was eluted by a solution containing 33% (w/v) NaOH 10⁻¹ µM and 15% (w/v) of 1 µM sodium acetate in H₂O and detected by pulsed amperometric detection (HPAEC-PAD) with an ICS-3000 system (Dionex).[75] The Neu5Ac content was calculated via the integration of sialic acid elution peak in comparison to a sialic acid standard curve.

Field Emission Scanning Electron Microscopy (FE-SEM): Muc-gels sample preparation for imaging was performed as described before.[74] In brief, samples were dehydrated in an ascending series of ethanol solutions from 10%, 25%, 50%, 70% to 100%. Specimens were further dried in a critical point dryer and then coated with a thin layer of gold by Agar HR sputter coater (Cressington Scientific Instruments Ltd, UK).

Rheological Characterization of BSM Hydrogels: Rheological measurements were performed using a commercial shear rheometer (MCR302, Anton Paar) equipped with a plate–plate measuring geometry (measuring head: PP25, Anton Paar, Graz, Austria). The gap between the measuring head and the bottom plate (P-PTD200/Air, Anton Paar) was set to d = 150 µm for all measurements. Immediately before measurement, the two components of the Muc-gel (BSM-Tz and BSM-Nb) were diluted in PBS (pH 7.4) to a concentration of 25 mg mL⁻¹ each. The two components were thoroughly mixed and centrifuged to remove bubbles before 100 µL of the sample were pipetted onto the rheometer plate. First, gel formation was analyzed for a total time span of t = 100 min. Both, the storage (G') and loss modulus (G'') were determined by a torque controlled (M = 5 μN m) oscillatory (f = 1 Hz) measurement. Secondly, a strain-controlled frequency sweep (from fstart = 10 Hz to fend = 0.01 Hz) was performed to determine the frequency dependent viscoelasticity of the crosslinked sample. For this frequency sweep, a constant strain was used which was chosen as the average of the five last values determined from the prior torque-controlled measurement.

To test the stability of the mucin gel toward enzymatic degradation, we introduced a custom-made rheological setup, which allowed for exposing the sample to an enzyme solution in situ. This setup consists of a commercial PP25 measuring head (Anton Paar) and an in-house developed bottom plate—from now on referred to as holey plate—that can be mounted onto the bottom plate of the rheometer (P-PTD200/80-I, Anton Paar). This holey plate comprises 19 regularly orientated holes with a diameter of 1.5 mm each. This design was chosen to allow fluid to diffuse from a reservoir chamber located below into the actual sample located above the holey plate. The sample and the holey plate were separated by a polycarbonate membrane (Whatman Nuclepore 50 nm, Sigma Aldrich) to prevent the sample from leaking into the fluid chamber while allowing small molecules (such as trypsin) to penetrate the membrane and to enter the sample. An inlet and outlet to the fluid chamber allowed for a continuous renewal of fluid during the measurement (see schematic in Figure S5 in the Supporting Information), e.g., to maintain a constant concentration of enzyme in the fluid chamber.

The sample was prepared as described above; however, the measuring gap was adjusted to d = 300 µm to ensure that the measuring head did not interfere with the membrane. Before each measurement, the membrane was wetted and the reservoir was filled with PBS to avoid air bubbles in the system. The inlet was connected to a syringe, which was filled with PBS and then fixed to a syringe pump (LA 100, Landgraf Laborsysteme HLL GmbH, Langenhagen, Germany). Afterward, 200 µL of the sample was placed onto the membrane, and the measuring
head was lowered to the measuring position. The measurement was performed in torque-controlled mode (M = 10 µN m) and started after a delay of $t_{\text{delay}} = 30$ min to allow the sample to crosslink. After the storage modulus had reached a steady state, the syringe pump was set to a continuous flow rate of $Q = 25$ µL min$^{-1}$ to equilibrate the system. After another 30 min, the buffer syringe was exchanged by a syringe filled with a trypsin solution (100 µg mL$^{-1}$ trypsin in PBS).

**Hydrogel Preparation for Implantation:** All the reagents for the hydrogels’ preparation were sterile-filtered using 0.45 µm pore filters. Hydrogels were prepared inside a cell culture laminar hood. Alginate hydrogels (Alg-gel) were formed by mixing 2.5% (w/v) solution of clinical grade alginate (PRONOVA SLG20, NovaMatrix) dissolved in 0.9% (w/v) saline solution (pH 7.4), and crosslinked with $\text{BaCl}_2$ gelling solution ($2 \times 10^{-3}$ M $\text{BaCl}_2$, 250 $\times 10^{-3}$ M $\text{MgCl}_2$, 25 $\times 10^{-3}$ M HEPES, $n$ (Ca$^{2+}$) = $n$ (monomer of guluronate)). BSM hydrogels were prepared by mixing equal volumes of 2.5% (w/v) BSM-Tz and BSM-Nb dissolved in PBS. The hydrogels were allowed to reach gelling equilibrium then immersed in a saline solution. For each hydrogel, 50 µL disks were prepared using a 1 mL syringe with the tip cut off (BD Bioscience). After formation, the alginate disks were washed with HEPES buffer ($2 \times 10^{-3}$ M HEPES, 1.2 $\times 10^{-3}$ M $\text{MgCl}_2$, $4.7 \times 10^{-3}$ M KCl, and $132 \times 10^{-3}$ M NaCl). Alginate disks were then kept at 4 °C before implantation in a saline solution, while the BSM disks were stored in saline solution. All the solutions used for alginate hydrogel formation have a pH of 7.4 and an osmotic pressure of 290 mOsm. We performed two independent experiments in four or five different mice. We implanted four gels in each mouse.

**Biocompatibility Assay:** The biocompatibility of Muc-gel was investigated by Alamar blue assay. In brief, a monocyte cell line; THP-1, was maintained in a complete RPMI 1640 culture medium containing 10% (v/v) FBS, and 1% (v/v) penicillin/streptomycin and incubated in a humidified incubator with 5% (v/v) CO$_2$, at 37 °C. THP-1 cells were then incubated with the complete culture medium supplemented with 150 $\times 10^{-3}$ M phorbol-12-myristate-13-acetate (PM, Sigma, P8139) and 4% (v/v) heat-inactivated fetal bovine serum (FBS) for 3 days followed by 24 h incubation in the complete cell culture medium to obtain THP1-derived macrophage type 0 (M0). M0 cells in 3D Muc-gel, cells were resuspended in 2.5% (w/v) BSM-T/N solution which was presolubilized in the complete culture medium. The Muc-gel cylinder was then prepared as described above. M0 cells in 3D Muc-gels were cultured for 7 days in a humidified incubator with 5% CO$_2$, at 37 °C. Alamar blue assay was performed on days 0, 1, 3, and 7. Cells were incubated with 10% (v/v) resazurin in the complete culture medium for 1 h, and the fluorescence intensity was measured by CLARIOStar microplate reader at the excitation of 530 nm and emission of 590 nm. The fluorescence intensities measured on day 1, 3, and 7 were normalized to the fluorescence intensity measured on day 0. Three independent experiments were performed.

**Hydrogel Implantation:** Mice were housed under standardized conditions (21–22 °C, 12 h light, 12 h dark cycle) in a SPF facility and were used at 10–12 weeks old. The animal experiments were performed following the guidelines of the Swedish National Board for Laboratory Animals and were approved by the Swedish Laboratory Animal Ethical Committee in Uppsala (approval number C116/14). Mice were allowed to acclimatize for one week prior to the surgery. For peritoneal cavity (i.p.) implantation of the hydrogels, the mice were anesthetized by 2.5% (v/v) isoflurane and the fur on the abdomen was shaved and sprayed with 70% (v/v) ethanol. An incision (1 cm length) was made to implant the hydrogels into the peritoneal cavity which was then sutured.

**Isolation of Peritoneal and Gel-Infiltrated Cells:** After 14 and 21 days, the mice were euthanized by isoflurane followed by cervical dislocation. Cells were obtained by lavage from the peritoneal cavity and by isolation from the harvested hydrogels. 5 mL of ice-cold PBS solution (1X PBS, pH 7.4, 2 $\times 10^{-3}$ M EDTA, and 0.5% (w/v) BSA) was injected into the peritoneal cavity in order to collect the floating cells. The implanted hydrogels were harvested and placed into a 5 mL PBS solution and single-cell suspension was prepared using a gentleMACS Dissociator (Miltenyi Biotec) following the manufacturer’s instructions. Cells were then filtered through 70 µm cell strainer (Fisher Scientific) and the red blood cells were lysed by hypotonic shock (0.2% (v/v) NaCl, 25 s, v/v 1.6% (w/v) NaCl).

**Flow Cytometry Cell Sorting:** Cells were incubated with mouse BD Fc-block (BD biosciences, 2.5 µg per 1 $\times 10^8$ cells in 100 µL) at 4 °C for 15 min to avoid nonspecific antibody binding prior to staining with an antibody cocktail in the dark at 4 °C for 30 min. The antibody cocktail contained the following fluorescent conjugated monoclonal antibodies: CD68 (1 µL per 1 million cells in 100 µL staining volume, CD68-Alexa47, Clone: FA-11, Cat. No. 137004, BioLegend); CD11b (1.25 µL per 1 million cells in 100 µL staining volume, CD11b-PE, Clone: M1/70, Cat. No. 101207, BioLegend); Gr-1 (0.5 µL per 1 million cells in 100 µL staining volume, CD11b-Alexa488, Clone: RB6-8C5, Cat. No. 108417, BioLegend); CD19 (1.25 µL per 1 million cells in 100 µL staining volume, CD19-PE-Cy7, Clone: 6D5, Cat. No. 115520, BioLegend); IgM (5 µL per 1 million cells in 100 µL staining volume, IgM-BV421, Clone: RMM-1, Cat. No. 406532, BioLegend). Cells were washed with 3 mL PBS twice before to be resuspended in PBS with 1% (w/v) BSA and to be sorted (BD FACSAria, BD Biosciences). Data were analyzed with Flowjo 10.5.2 Software (Tree Star, Inc.).

**Gene Expression Analysis by Real-Time PCR:** The total RNA of cells was extracted by using either Qiagen RNeasy mini kit or Qiagen RNeasy kit method depending on cell numbers sorted. The extracted mRNA was diluted to a concentration of 0.67 ng µL$^{-1}$ and synthesized into cDNA using Superscript III Polymerase (Invitrogen). Real-time PCR was then performed to analyze the gene expression by using a TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) together with TaqMan probes. A list of TaqMan Assay ID is available in Table S1 (Supporting Information). The RT-PCR was carried out in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min, and then go to step 3 for 50 cycles. Target gene relative expression to housekeeping gene Actβ were performed. The validation of housekeeping gene was performed for both RL37 and Actβ and revealed that Actβ showed better consistency among tested samples.

**Histological Staining:** Muc-gel and Alg-gel explants were fixed by 4% (w/v) paraformaldehyde overnight and then washed with 70% (v/v) ethanol, before being processed for paraffin embedding. Samples were cut into 5 µm sections, and then deparaffinized, and rehydrated for hematoxylin and eosin (H&E) and Masson’s trichrome staining. The slides were then dehydrated and mounted with cover slides. The images were scanned by using automated image scanner (Aperio Scanscope AT, Leica Biosystems Imaging, Inc.) and analyzed by Aperio ImageScope Software (Leica Biosystems Imaging, Inc.).

**Confocal Immuno-histofluorescence** Immuno-fluorescent imaging was performed to examine the expression of TGFβ1 and α-smooth muscle actin. In brief, deparaffinized and rehydrated paraffin sections of Muc-gels and Alg-gels were first washed with PBS buffer. Sections were permeabilized for 30 min with 0.1% (v/v) Triton X-100 solution followed by three times wash with PBS. Sections were then blocked by incubation with 1% (w/v) bovine serum albumin (BSA) solution for 1 h followed by three times wash with PBS buffer. We added 200 µL of primary antibodies (primary anti-TGFβ1 antibody (Novus Biologicals, Cat. No. AB-246-NA, 5 µg mL$^{-1}$) and anti-α-smooth muscle actin-Cy3 conjugated antibody (Sigma Aldrich, Cat. No. C6198, 1:2000) onto the specimen and incubated at 4 °C overnight in a humidified dark chamber before washing three times with PBS. 200 µL of secondary antibody was then added (Alexa Fluor 488, Jackson ImmunoResearch, Cat. No. 705-485-147, 1:400) onto the specimens and incubated at room temperature for 2 h to label the primary anti-TGFβ1 antibody. Specimens were mounted with cover slides with mounting medium fluoroshield containing DAPI (Sigma Aldrich). Confocal images were acquired using an LSM 700 Laser Scanning Microscope with ZEN 2011 Software (Carl Zeiss AG, Germany) equipped with 40x and 63x objectives. Image analysis was undertaken on ImageJ 1.52k (National Institutes of Health, USA).

**Statistical Analysis:** Data are shown as mean of implants from four mice per time point and per experimental group. The significance was analyzed via one-way ANOVA with Bonferroni correction or nonparametric one-way ANOVA test using the GraphPad Prism 7.0; "*", "**", "***", and "****" indicate p value <0.05, 0.01, 0.0005, and 0.0001, respectively.
Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

B cells, bovine submammary mucin, fibrosis, foreign body response, hydrogels, macrophages, TGF-$\beta$

Acknowledgements

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