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Inducing mesenchymal stem cell attachment on non-cell adhesive hydrogels through click chemistry

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We introduce an innovative approach to adhere mesenchymal stem cells (MSCs) to a hydrogel scaffold by nucleating adhesion through strain-promoted click chemistry. This method yields a significant increase in cell viability compared to non-functionalized and RGD peptide functionalized hydrogels, providing a promising alternative to traditional biomaterials cell attachment approaches.

Hydrogels can be precisely designed to mimic the physicochemical properties of the extracellular matrix (ECM). However, several promising polymeric hydrogels are non-cell adhesive, hindering their use in regenerative medicine without modification.[1] These materials are commonly functionalized or coated with ECM proteins such as fibronectin, vitronectin, laminin, collagen, and elastin or peptides derived from these proteins (e.g., RGD, KQAGDV, IKLLI, DGEA, VAPG) to promote cell adhesion.[2] Although widely adopted, these modification strategies are limited due to factors including coating instability, random protein folding during adsorption, and potential lack of control in ligand presentation and surface density, requiring significant prior optimization of the material.[3] In order to address these limitations, we report an alternative strategy to render non-cell adhesive hydrogel biomaterials cell-adhesive utilizing covalent cell-hydrogel interactions promoted via bioorthogonal click chemistry.

Since the strain-promoted [3+2] cycloaddition of cyclooctyne and azide was first reported as a useful tool in living cells,[4] it has been used primarily to attach small molecules, including fluorophores, to cell surfaces.[5] Due to the selectivity of this linkage and lack of necessary additional reagents, this chemistry has more recently been adopted to crosslink polymers, even utilizing cells as crosslinker units in forming hydrogels.[6] In this work, we specifically exploited the well-known reactivity between azide and dibenzocyclooctyne (DBCO) occurring at conditions benign to live cells to covalently attach bone marrow-derived mesenchymal stem cells (MSCs) to non-cell adhesive gellan hydrogels (**Figure 1A**). We suggest that covalent binding of azide expressing MSCs and DBCO modified gellan is needed to initiate cell attachment to the surface of the material, while subsequent protein adsorption from the culture media and cell-produced ECM on the DBCO modified gellan (promoted by DBCO hydrophobicity) enables these MSCs to form natural integrin binding interactions with this nascent ECM. To the best of our knowledge, this is the first report of utilizing strain-promoted click chemistry to enable cell adhesion on a two-dimensional biomaterial surface.

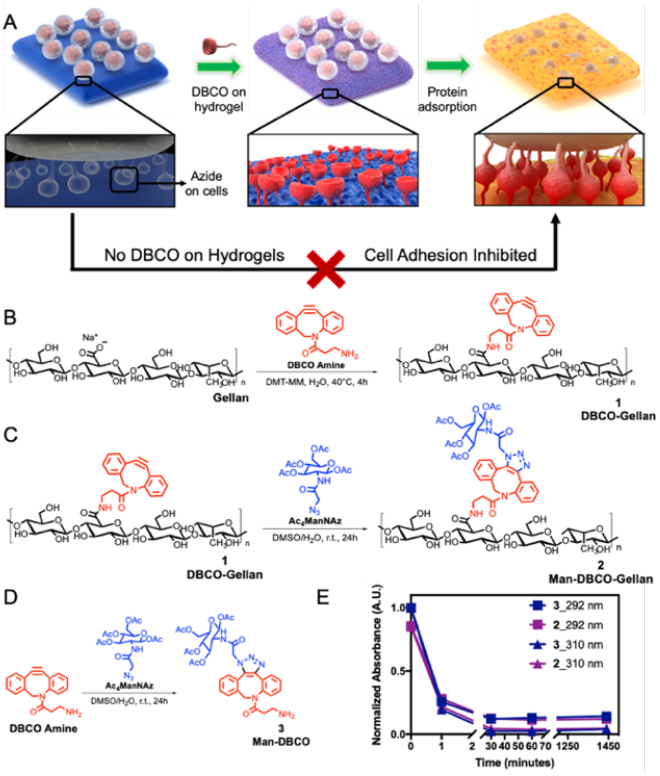

Figure 1. A) Proposed cell adhesion mechanism on non-cell adhesive gellan hydrogels. B) Synthetic scheme of gellan functionalization with DBCO. 1,3-dipolar cycloaddition reaction between (C) DBCO-gellan or (D) DBCO amine and Ac4ManNAz. E) DBCO absorbance at 292 nm and 310 nm (normalized to the reaction solvent, DMSO/H2O) over time, confirming the conjugation reaction between Ac4ManNAz and DBCO-gellan (purple) or DBCO amine (blue).

We first conjugated DBCO to deacetylated gellan, a hydrogel-forming polysaccharide.[7] Gellan is a United States

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Food and Drug Administration-approved food additive and has more recently gained interest as a tissue engineering scaffold.[8] Gellan, like many other hydrogel forming polymers, is known to be relatively non-cell adhesive due to its hydrophilicity and negative charge.[9-10] Briefly, gellan was purified (**Figure S1A**) and the carboxyl groups on the glucuronic acid monosaccharide of the polymer were activated using 4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) as a coupling agent; these activated carboxylic acids were then conjugated to a DBCO amine (**Figure 1B**). Proton NMR (^{1}H-NMR) confirmed successful synthesis of DBCO modified gellan **1** (DBCO-gellan) showing that 45% of the total carboxylate groups were functionalized (**Figure S1B**). We compared the reactivity of the DBCO-gellan conjugate to the reactivity of DBCO amine with an azide-functionalized sugar, specifically *N*-azidoacetylmannosamine-tetraacylated (Ac_4ManNAz) (**Figure 1C-D**). DBCO-gellan and DBCO amine were separately mixed with Ac_4ManNAz in 50% v/v dimethyl sulfoxide in water and the reaction was monitored over time via UV-visible spectroscopy (UV-vis). We monitored the characteristic absorbance maximums of DBCO at 292 nm and 310 nm over time in both mixtures (**Figure S2A**). As the reaction proceeded, the normalized absorbance at both wavelengths decreased for the DBCO-gellan and DBCO amine mixtures at similar rates (**Figure 1E**). Complete disappearance of these absorbance peaks was seen at 30 minutes, indicating successful 1,3-dipolar cycloaddition between cyclooctyne and azide and the formation of mannose-DBCO-gellan **2** and mannose-DBCO **3** (the triazole formation was also confirmed in the control reaction leading to product **3** via mass spectrometry, **Figure S2B**). The successful cycloaddition indicated that the reactivity of the cyclooctyne was not affected by conjugation to gellan.

We also confirmed that DBCO-gellan was capable of forming hydrogels when mixed with non-functionalized gellan (**Figure S3**). As expected, we found that non-modified gellan was required to promote gelation, given the importance of the carboxyl groups (used for the conjugation of DBCO) in the gellan gelation process[11] along with the increased hydrophobicity of the DBCO modified polymer. We investigated hydrogel physical properties including viscosity, shear (G'') and elastic (G') modulus, and mass in MSC culture media, indicating increased stiffness upon media incubation (**Figure S4 – S7**).

Next, we investigated the conditions required to glycoengineer MSCs to express the azide on their surface for subsequent click reaction with the DBCO-gellan hydrogel surfaces. Culture with azide modified sugars, like Ac_4ManNAz, yields cell surface glycoproteins bearing azide groups.[12] Consistent with previous studies on MSCs,[13] we found that culture with 50 µM Ac_4ManNAz for 72 hours yielded optimal azide expression over at least 6 days (**Figure S8**). Initial viability tests were then performed upon treating MSCs with the DBCO-gellan polymer in solution. Azide-modified cells showed a moderate decrease in viability (~75 ± 5.0% normalized to untreated MSC controls) when exposed to high DBCO-gellan concentrations (greater than or equal to 10 µM for 24 hours at 37 °C) (**Figure 2A**), while non-azide expressing cells behaved similar to control MSCs. These results suggest effective interactions only between azide modified MSCs and DBCO-gellan.

In order to further investigate the nature of the interaction between azide modified cells and DBCO-gellan, azide and non-azide modified MSCs were incubated with a rhodamine tagged DBCO-gellan (10% functionalization, **Figure S9**, **Figure 2B-G top**). Additionally, a two-step approach, in which MSCs were first incubated with DBCO-gellan for 24 hours and subsequently treated with an azide-functionalized rhodamine (carboxyrhodamine 110 azide) for two hours (**Figure 2B-G bottom**) was also investigated. For both approaches, we observed rhodamine fluorescence for the azide modified MSCs and the non-azide modified cells using fluorescence microscopy (**Figure 2C and E**), suggesting that along with the 1,3-dipolar cycloaddition reaction of DBCO and azide, non-specific interactions (*e.g.*, hydrophobic interactions) can occur between the DBCO-gellan and MSCs. In fact, flow cytometry showed a separate population of what is likely DBCO-gellan aggregates and/or dead cells (the non-gated population shown to the left of **Figure 2D and 2F**) not observed in MSCs that were not incubated with DBCO-gellan (**Figure S10**). Comparing cell fluorescence quantified via flow cytometry for azide modified and non-azide modified MSCs incubated with fluorescent DBCO-gellan, ~94% of azide-modified cells were positive for

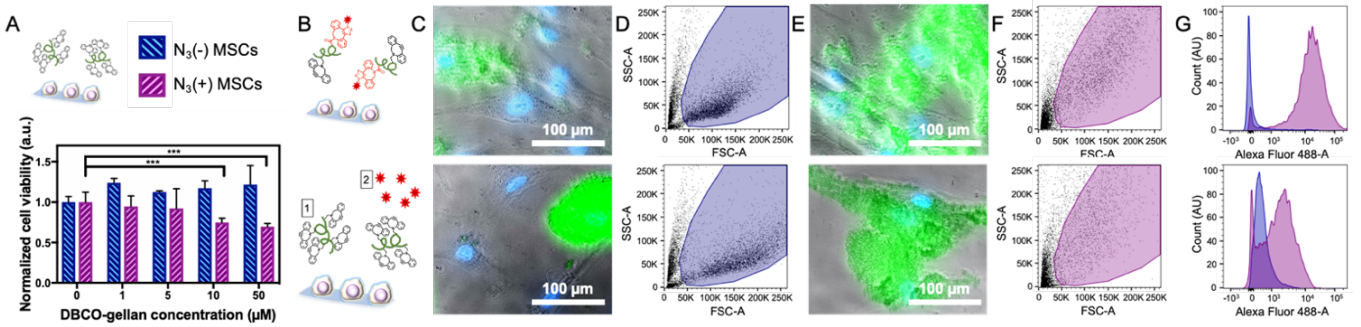

Figure 2. A) Normalized MSC viability for azide (N_3(+)) and non-azide (N_3(-)) modified MSCs after treatment with DBCO-gellan (supplemented in the culture media) for 24 hours. B) Schematic of the two approaches investigated to verify the interaction between MSCs and gellan. Top: MSCs were treated with carboxyrhodamine 110-labeled-DBCO-gellan and results corresponding to this approach are shown in C-G (top row). Bottom: MSCs were first treated with DBCO-gellan **1** and then with carboxyrhodamine 110 azide and results corresponding to this approach are shown in C-G (bottom row). Representative (C and E) fluorescence microscopy images (green for rhodamine and blue for nuclei) and (D and F) flow cytometry dot plots of non-azide (C and D) and azide (E and F) modified MSCs. G) Flow cytometry fluorescence intensity histograms showing overlay of the gated populations highlighted in (D) and (F). Note, blue indicates non-azide modified MSCs and purple indicates azide modified MSCs. Statistical significance was examined using unpaired t-test (n = 3, *** p <0.001).

rhodamine fluorescence with only ~22% of non-azide modified cells positive for rhodamine fluorescence, indicating interactions primarily between only azide modified MSCs with the DBCO-gellan (**Figure 2G top**). Interestingly, when incubating MSCs first with DBCO-gellan followed by the azide-functionalized rhodamine (**Figure 2G bottom**), a reduced separation for azide and non-azide modified MSCs was observed compared to the first method (**Figure 2G top**). Nonetheless, also here a marked distinction in fluorescence intensity between the two groups remained, with ~86% of azide-modified MSCs positive for rhodamine fluorescence. We attribute this reduced separation between the two conditions to the experimental protocol used in these studies; in the second method, during the first 24 hours of polymer incubation, presumably all DBCO groups are available to interact with the cell-surface azide moieties, leaving few remaining functional groups available for reaction with the azide-functionalized rhodamine during subsequent incubation with this fluorophore. These findings suggest that the interactions between DBCO-gellan and azide modified MSCs are dominated by specific click-mediated covalent interactions.

Upon successfully confirming DBCO-gellan click reaction with azide modified MSCs, we next investigated whether this covalent interaction could be used to promote MSC adhesion to gellan hydrogel surfaces. **Figure 3A** shows the morphology of azide and non-azide modified MSCs seeded on gellan hydrogels with 0.1% DBCO-gellan 24 hours after seeding. On this functional material, non-azide modified MSCs formed large, weakly attached, three-dimensional multi-cellular aggregates. In contrast, azide modified MSCs cultured on DBCO-gellan hydrogels exhibited elongated morphologies, even when existing as part of a multi-cellular structure, suggesting interaction with the DBCO-gellan hydrogel surface. Furthermore, azide modified MSCs exhibited a 100% increase in metabolic activity when cultured on gellan hydrogels containing DBCO-gellan (0.1 and 0.25% w/v) for 24 hours compared with gellan hydrogels not containing DBCO (**Figure 3B**). For non-azide modified MSCs, no change in metabolic activity was observed between all test conditions (with and without DBCO-gellan). Thus, the unnatural click-mediated reaction of azide modified MSCs and DBCO-gellan hydrogels improved cell viability on these materials.

We then compared MSC attachment on DBCO-gellan hydrogels to attachment mediated via the more traditional approach of utilizing RGD peptide modified gellan (**Figure S1C**).[9] As expected, we observed increasing viability of non-azide modified MSCs seeded on RGD modified hydrogels with increasing RGD functionalization on the hydrogels (**Figure S11**). We analyzed the presence of focal adhesions in MSCs seeded on these hydrogels by fluorescently labeling and imaging vinculin, a focal adhesion protein (**Figure 3C**). MSCs on both gellan hydrogels with either 1% w/v RGD-gellan or 0.1% w/v DBCO-gellan displayed typical punctate vinculin staining, characteristic of focal adhesion formation during integrin-mediated ECM interactions. These results suggest that for both DBCO and RGD modified hydrogels, MSCs form natural integrin mediated interactions.

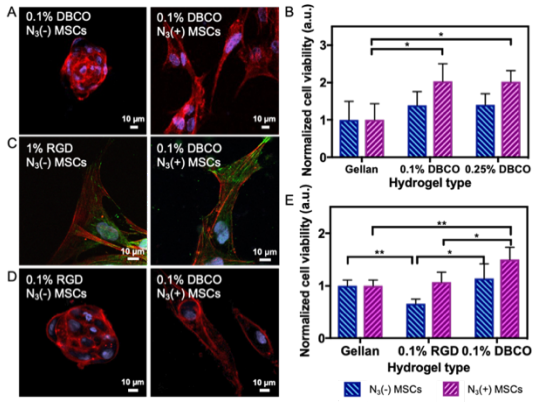

Figure 3. Interaction of MSCs with DBCO and RGD modified gellan hydrogels. A) Confocal microscopy images 24 hours post-seeding N_3(-) (left) or N_3(+), (right) MSCs seeded on DBCO-gellan 1. B) Normalized MSC viability for N_3(+) MSCs and N_3(-) MSCs 24 hours post-seeding on gellan and gellan-DBCO hydrogel surfaces. C) Confocal microscopy images of MSCs 24 hours post-seeding on hydrogels highlighting their focal adhesions. N_3(-) MSCs on unmodified gellan with 1% w/v gellan-RGD hydrogels (left) and N_3(+) MSCs on unmodified gellan with 0.1% w/v DBCO-gellan hydrogels (right). D) Confocal microscopy images 24 hours post-seeding N_3(-) MSCs on unmodified gellan with 0.1% w/v RGD-gellan (left) and N_3(+) MSCs on unmodified gellan hydrogels with 0.1% w/v DBCO-gellan (right). (E) Normalized MSC viability for N_3(+) MSCs and N_3(-) MSCs 24 hours post-seeding on gellan hydrogel surfaces with or without RGD or DBCO gellan. F-actin is indicated in red, nuclei in blue and vinculin in green. Statistical significance was examined using unpaired t-test (n = 3, * $p < 0.05$, ** $p < 0.01$).

The morphology and viability of MSCs on gellan hydrogels with 0.1% DBCO-gellan was then compared to cells on gellan hydrogels with the same RGD functionalization (0.1% RGD-gellan). **Figure 3D** (right) shows a spread morphology of azide modified MSCs on DBCO hydrogels; in contrast, non-azide modified MSCs on RGD-gellan hydrogels formed large cell aggregates (**Figure 3D left**) similar to what was observed for non-azide modified MSCs on DBCO hydrogels (**Figure 3A left**). For non-azide modified MSCs, there was no significant difference in cell viability between unmodified gellan hydrogels and DBCO-gellan hydrogels. However, a significant decrease in viability was observed for cells seeded on RGD-gellan, compared to both unmodified and DBCO-gellan hydrogels (**Figure 3E**). Previous studies have shown that a minimum threshold of RGD surface modification is needed to ensure cell adhesion and viability,[14] suggesting that 0.1% w/v RGD-gellan does not meet this threshold concentration. For cells expressing azide, viability significantly increased on DBCO-gellan hydrogels compared to both unmodified gellan hydrogels and RGD-gellan hydrogels. Thus, DBCO functionalization of gellan hydrogels enhances MSC spreading and viability compared with a similar level of RGD functionalization. Taken together, these results suggest that the DBCO modification of gellan may promote protein adsorption on the hydrogel surface, which can enable integrin-mediated interactions with the MSCs only once cell attachment is initiated by the strain-promoted click reaction between azide modified cells and DBCO-gellan hydrogels. In order to investigate this hypothesis, we incubated HiLyte Fluor™ 488 fluorescently labeled fibronectin with gellan hydrogels formulated both with and without 0.1% w/v DBCO-gellan. Indeed, we observed an increased fluorescence signal on DBCO-gellan hydrogels compared with gellan hydrogels exposed to the protein (**Figure 4A-B**), confirming the protein

adhesive nature of DBCO-gellan hydrogels. A heterogeneous distribution of islets of higher fibronectin fluorescence intensity was observed on DBCO-gellan hydrogels (**Figure 4A-B**), likely due to protein adsorption on DBCO-gellan aggregates on the hydrogel surface promoted by DBCO-gellan hydrophobicity (**Figure S12**). We further investigated the importance of protein adsorption on promoting cell spreading by culturing MSCs in serum free conditions on gellan hydrogels with 0.1% w/v DBCO-gellan and on glass controls (**Figure 4C**). At these conditions, both azide and non-azide modified cells on DBCO-gellan hydrogels formed round multicellular aggregates, in contrast with experiments in serum-containing media (**Figure 3A**). These observations confirmed that interactions with adsorbed proteins are necessary but not sufficient to promote MSC attachment on DBCO-gellan hydrogels, as cells lacking azide groups in serum containing media are unable to spread on this material (**Figure 3A left**).

In summary, we utilized the well-known azide-DBCO bioorthogonal click reaction to develop a unique approach to covalently attach MSCs to a 2D, traditionally non-cell adhesive hydrogel surface. We confirmed that azide modified MSCs were able to attach to and spread on the surface of these DBCO-gellan hydrogels exhibiting enhanced metabolic activity compared to non-azide modified cells, specifically in serum containing conditions. These results support the proposed two-step MSC adhesion mechanism for DBCO functionalized gellan hydrogels (**Figure 1A**): first, cells covalently attach to the polymer via a 1,3-dipolar cycloaddition between azide moieties on the cell surface and the cyclooctyne groups on the gellan hydrogel surface and next, the cells spread on the material enabled by protein adsorption and focal adhesion-mediated interactions. Although it is possible that the azide-cyclooctyne cycloaddition reaction and protein adsorption may occur together, our results indicate that the covalent reaction must occur first to promote MSC adhesion. Cell adhesion to hydrogel scaffolds is of fundamental importance in the design of biocompatible biomaterials. Thus, the ability to induce cell adhesion to a material using the methods described herein, which incorporate a low-cost, facile synthetic approach and can be readily adapted to other materials for applications in cellular engineering and regenerative medicine, is highly promising. Future studies may expand the use of such materials to injectable hydrogels and the study of MSC differentiation.

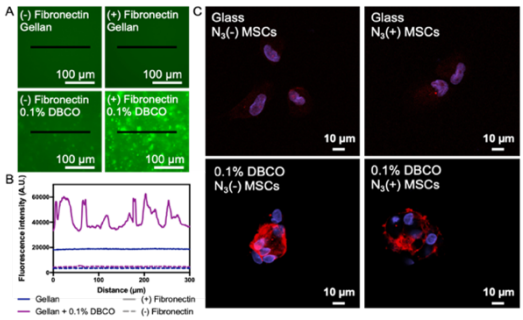

Figure 4. Investigating the importance of protein interactions with gellan-DBCO in promoting cell adhesion. A) Fluorescence microscopy images of unmodified gellan hydrogels formulated without (top) and with (bottom) 0.1% w/v DBCO-gellan incubated without (left) or with (right) fluorescent fibronectin (HiLyte Fluor™ 488). Green indicates fibronectin fluorescence. B) Representative fluorescence intensity profiles of the images in (A), corresponding to the black lines indicated on the images. C) Confocal microscopy images of N3(-) (left) and N3(+) MSCs (right) on glass (top) and on gellan hydrogels with 0.1% w/v DBCO-gellan (bottom) cultured in serum-free media. F-actin is indicated in red and nuclei in blue.

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Conflicts of interest

There are no conflicts to declare.

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Table of Contents

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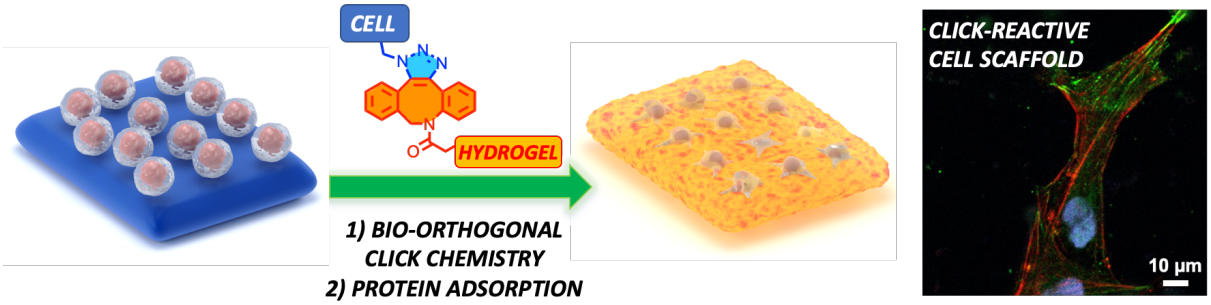

Strain-promote bio-orthogonal click chemistry is used to promote cell attachment and viability to non-cell adhesive materials.