An Advanced 'clickECM' That Can be Modified by the Inverse-Electron-Demand Diels-Alder Reaction

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The extracellular matrix (ECM) represents the natural environment of cells in tissue and therefore is a promising biomaterial in a variety of applications. Depending on the purpose, it is necessary to equip the ECM with specific addressable functional groups for further modification with bioactive molecules, for controllable cross-linking and/or covalent binding to surfaces. Metabolic glycoengineering (MGE) enables the specific modification of the ECM with such functional groups without affecting the native structure of the ECM. In a previous approach (S. M. Ruff, S. Keller, D. E. Wieland, V. Wittmann, G. E. M. Tovar, M. Bach, P. J. Kluger, *Acta Biomater.* **2017**, *52*, 159–170), we demonstrated the modification of an ECM with azido groups, which can be addressed by bioorthogonal copper-catalyzed azide-alkyne cycloaddition (CuAAC). Here, we demonstrate the modification of an ECM with dienophiles (terminal alkenes, cyclopropene), which can be addressed by an inverse-electron-demand Diels-Alder (IEDDA) reaction. This reaction is cell friendly as there are no cytotoxic catalysts needed. We show the equipment of the ECM with a bioactive molecule (enzyme) and prove that the functional groups do not influence cellular behavior. Thus, this new material has great potential for use as a biomaterial, which can be individually modified in a wide range of applications.

Introduction

The extracellular matrix (ECM) is a complex network of various macromolecules, which is synthesized and assembled by the residing cells of a tissue. The main components of ECM are fibrous and non-fibrous collagens and various glycosaminoglycans and proteoglycans as well as adhesion proteins and enzymes.[1] As the ECM is the natural environment of the cells, it represents a promising biomaterial for regenerative medicine and tissue engineering approaches. Through cell-ECM-interaction, mainly mediated by the interaction of anchor proteins (e.g. integrins) and specific adhesion peptides (e.g. those containing the RGD sequence), a variety of cellular mechanisms are regulated related to the characteristics (e.g. stiffness, pore size) of the surrounding of the cell.[2] Also, different proteins (collagens, glycosaminoglycans (GAGs), proteoglycans) within the ECM and consequently the composition of the ECM have extensive impact on cellular behavior. Although individual ECM proteins are used as coating, scaffolds, and hydrogels in tissue engineering and regenerative medicine approaches, to date the complexity of the natural ECM cannot be rebuilt.

Next to the decellularization of native tissues, *in vitro* generated cell-derived ECM represents a promising source for natural ECM. Among others, fibroblasts and mesenchymal stem cells were shown to produce relevant amounts of this cell-derived ECM.[3] Adipose-derived stem cells (ASCs), a subgroup of the mesenchymal stem cells, represent a promising cell type as they can be obtained with minimal invasiveness and adipose tissue is permanently available. Previous studies demonstrated the high impact of natural ECM as coating or scaffold material on stem cell fate concerning adhesion, proliferation, and differentiation.[4] These cell-derived ECMs resemble the tissue specific ECM more closely than individual proteins and are used in a variety of applications.[3,5]

Depending on the application, it is desirable to covalently modify the ECM to achieve, for example, covalent linking on surfaces or cross-linking without affecting the structure or functionalization with molecules providing specific characteristics. One method to functionalize ECM is the application of amine-targeting strategies including *N*-hydroxysuccinimide (NHS) chemistry which ends up in unspecific conjugation to different amines. However, this unspecific conjugation can lead to a partial or full loss of the (bio)activity of the protein by the blockade of the active site.[6] Metabolic glycoengineering (MGE)[7] is a successful approach to introduce unnatural functional groups (so-called chemical reporter groups) into the glycan structures of glycoconjugates on the cell surface and within the ECM enabling site-directed conjugation of molecules. For MGE, cultured cells are treated with chemically modified monosaccharides which are metabolized by the cell and

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incorporated into the intra- and extracellular glycan structures. The incorporated chemical reporter group can now be reacted in a biorthogonal ligation reaction[8] which allows a chemoselective modification of the ECM without touching protein side chains. Previously, we[9] and others[10] reported the preparation of a functionalized ECM by MGE. For example, we demonstrated the incorporation of azide functionalities into the ECM of human fibroblasts[9a] and ASCs,[9b] which can be addressed via bioorthogonal copper-catalyzed azide-alkyne cycloaddition (CuAAC).[11] We demonstrated different possible applications of the azide-modified 'clickECM' and showed, for example, the positive effect of a clickECM coating on the fibroblast culture and demonstrated the clickECM as a bioconjugation platform using biotin-streptavidin interaction.[12]

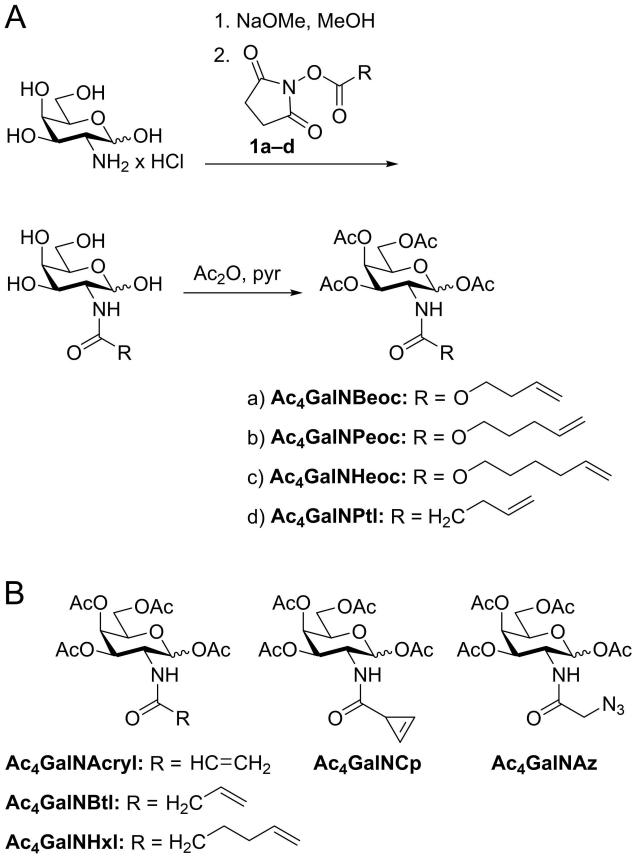
One disadvantage of this azide-modified clickECM is the need for copper as a catalyst for CuAAC. It is known that copper is cytotoxic, and it can be assumed that even after extensive washing significant amounts of copper remain in the ECM.[13] Alternative bioorthogonal ligation reactions that do not need any toxic catalyst include the strain-promoted azide-alkyne cycloaddition (SPAAC)[14] and the inverse-electron-demand Diels-Alder (IEDDA) reaction.[15] The latter has found widespread application as a bioorthogonal ligation reaction in various applications including MGE.[16] Dienophiles that undergo an IEDDA reaction with tetrazines and that have been used for MGE include terminal alkenes[17] and strained cyclic alkenes, such as cyclopropenes,[18] bicyclononynes,[19] and norbornenes.[20] These dienophiles can have markedly different reaction kinetics enabling various applications including sequential modifications with different tetrazines.[21] Furthermore, the IEDDA reaction can be orthogonal to other bioorthogonal ligation reactions, such as the strain-promoted azide-alkyne cycloaddition (SPAAC) and the light-induced nitrile imine-alkene cycloaddition (photoclick reaction) enabling dual-[17a,18a] and even triple-orthogonal labeling[22] after incorporation of two or three differently modified monosaccharides. This opens future opportunities to modify the ECM simultaneously in two (or three) ways. These opportunities include but are not limited to the incorporation of bioactive molecules (e.g., growth factors or cell growth inhibiting molecules), enzymes, antibacterial substances, and cross-linking using specific linkers.

Here we present the investigation of a series of alkene-modified sugar derivatives and their suitability for the preparation of a new material, an advanced clickECM that can be modified by the IEDDA reaction. We demonstrate the incorporation of the dienophile functional group via the activity of a linked enzyme. Further, we show that the modification has no impact on the physical characteristics of gellan gum-ECM-hybrid hydrogels and the cellular behavior of encapsulated ASCs. These results highlight the possibilities of this new modified ECM material for different applications as the modification itself does not interfere with possible functionalization by, e.g., bioactive molecules.

Results and Discussion

Synthesis of modified monosaccharides

In previous experiments, Ac_4GalNAz was demonstrated to be a suitable monosaccharide derivative for efficient incorporation into the ECM.[9a] For the preparation of a clickECM that can be modified by the IEDDA reaction, we thus synthesized a series of new dienophile-modified GalNAc derivatives (Scheme 1A). Galactosamine hydrochloride was neutralized with sodium methoxide and subsequently reacted with the respective alkene derivative activated either as succinimidyl carbonate (**1 a**–**c**) or succinimidyl ester (**1 d**). Acetylation with acetic anhydride in pyridine gave Ac_4GalNBeoc, Ac_4GalNPeoc, Ac_4GalNHeoc, or Ac_4GalNPtl. In addition, we synthesized the GalNAc derivatives depicted in Scheme 1B according to previously reported procedures (Ac_4GalNAcryl,[23] Ac_4GalNBtl,[17e] Ac_4GalNHxl,[24] Ac_4GalNCp[18g]). The GalNAc derivatives with terminal alkenes feature a lower reactivity in the IEDDA reaction than the cyclopropenyl derivative. However, they have a higher chemical stability which might be advantageous during the ECM preparation.



Scheme 1. A) Synthesis of dienophile-modified GalNAc derivatives. B) Investigated GalNAc derivatives that have been synthesized following published procedures.

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Cytotoxicity of investigated modified monosaccharides

Good biocompatibility of the used monosaccharide derivates is needed to ensure the ECM-production capacity of the cells and to prevent the accumulation of unwanted cytokines in relevant concentrations within the produced ECM, which might exhibit a negative impact on the cells in further application of the ECM material. For example, the secretion and accumulation of tumor necrosis factor in the ECM might induce pro-inflammatory or even apoptotic pathways in cells that are in contact with the ECM material in further applications.[25]

To exclude any cytotoxic effects of the used monosaccharides, cell death (lactate dehydrogenase (LDH) assay) and metabolic activity (resazurin assay) after supplementation of the monosaccharides were determined (Figure 1). The LDH assay is based on the release of this enzyme during cell death, which can be colorimetrically quantified in the cell culture supernatant. The relative amount of LDH in the supernatant can be used to quantify cell death. The colorimetric resazurin assay is based on the metabolic turnover of resazurin in the mitochondria of cells which reflects the metabolic activity of the cells.

The LDH release revealed no cytotoxic effects of any of the modified monosaccharides at the concentration used during MGE (100 μM) (Ac_4GalNBeoc: (94.5 ± 11.8) %; Ac_4GalNPeoc: (111.1 ± 19.1) %; Ac_4GalNHeoc: (100.7 ± 14.0) %, Ac_4GalNAcryl: (110.2 ± 9.1) %, Ac_4GalNBtl: (103.6 ± 7.9) %, Ac_4GalNPtl: (117.7 ± 35.2) %, Ac_4GalNHxl: (109.5 ± 10.1) %, Ac_4GalNCp: (60.2 ± 4.3) %) compared to the negative control ((100.0 ± 12.0) %) (Figure 1). Interestingly, the LDH release of ASCs treated with Ac_4GalNCp was significantly lower compared to the other monosaccharides and the control, indicating an enhancing effect on cellular survival. However, the underlying mechanism of this effect is not known. The resazurin assay revealed that the metabolic activity of the ASCs treated with the modified monosaccharides (Ac_4GalNBeoc: (87.1 ± 16.9) %; Ac_4GalNPeoc: (89.8 ± 13.4) %; Ac_4GalNHeoc: (90.4 ± 10.2) %; Ac_4GalNAcryl: (79.7 ± 15.4) %; Ac_4GalNBtl: (95.7 ± 8.4) %, Ac_4GalNPtl: (89.9 ± 9.9) %; Ac_4GalNHxl: (84.0 ± 12.3) %; Ac_4GalNCp: (79.6 ± 14.8) %) was comparable to the negative control ((100.0 ± 2.2) %) (Figure 1). According to DIN EN ISO 10993-5, cytotoxic effects of a tested substance are indicated by a reduction of metabolic activity by 30% or more following substance incubation (indicated by a red line). Thus, the results of the resazurin assay are in line with the LDH release and showed no cytotoxicity of the tested monosaccharides.

Incorporation of alkene-modified monosaccharides into the ECM

To prove the incorporation of the modified monosaccharides into the ECM by MGE, the presence of the functional groups was detected by tagging them with an enzyme and subsequent measurement of the enzymatic activity. This method was previously applied[12] and therefore considered as suitable for this purpose. By this method, we simultaneously demonstrated the incorporation of the functional groups and the possibility to covalently bind bioactive molecules such as enzymes to the ECM via the dienophile functional groups. Since all tested alkene-modified monosaccharides had a comparable low cytotoxicity, we focused on the carbamate-linked terminal alkenes because carbamate derivatives had higher incorporation efficiencies in previous studies on sialic acid labeling.[17c] In addition, we investigated Ac_4GalNCp because of its much higher reactivity in the IEDDA reaction. The functional groups (terminal alkenes and cyclopropene) were ligated with a tetrazine-biotin conjugate by an IEDDA reaction (Figure 2A). Subsequently, the biotin was labeled with horseradish peroxidase (HRP)-linked streptavidin. The unmodified negative control ECM (coECM) was treated in the same way as the samples. Addition of the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) allowed the quantification of HRP-tagged reporter groups by colorimetric detection.

Figure 2B shows the relative TMB turnover as an indicator for the incorporated functional groups. The value of the unmodified coECM was set to 100% and values of the modified ECMs were normalized to the coECM. As expected, modified ECM produced by the use of dienophile-modified monosaccharides

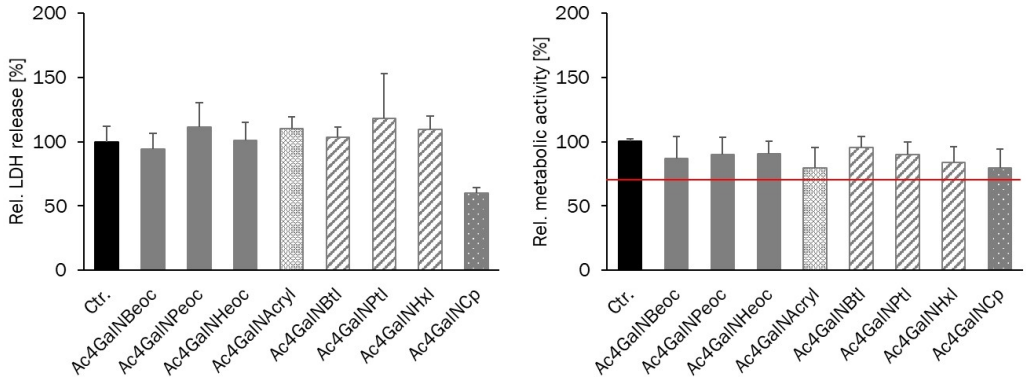

Figure 1. Cytotoxicity of monosaccharide derivatives. ASCs were seeded in growth medium at a density of 50,000 cells cm^{-2}. Cytotoxicity of the monosaccharides was determined by measurement of LDH release (cell death) and resazurin turnover (metabolic activity) of ASCs treated with 100 μM of the compounds for 24 h. Negative control (Ctr.) was treated with sterile water. Values are the means of 3 independent experiments using cells from different donors (each 2 technical replicates) and normalized to the negative control, which was set as 100%.

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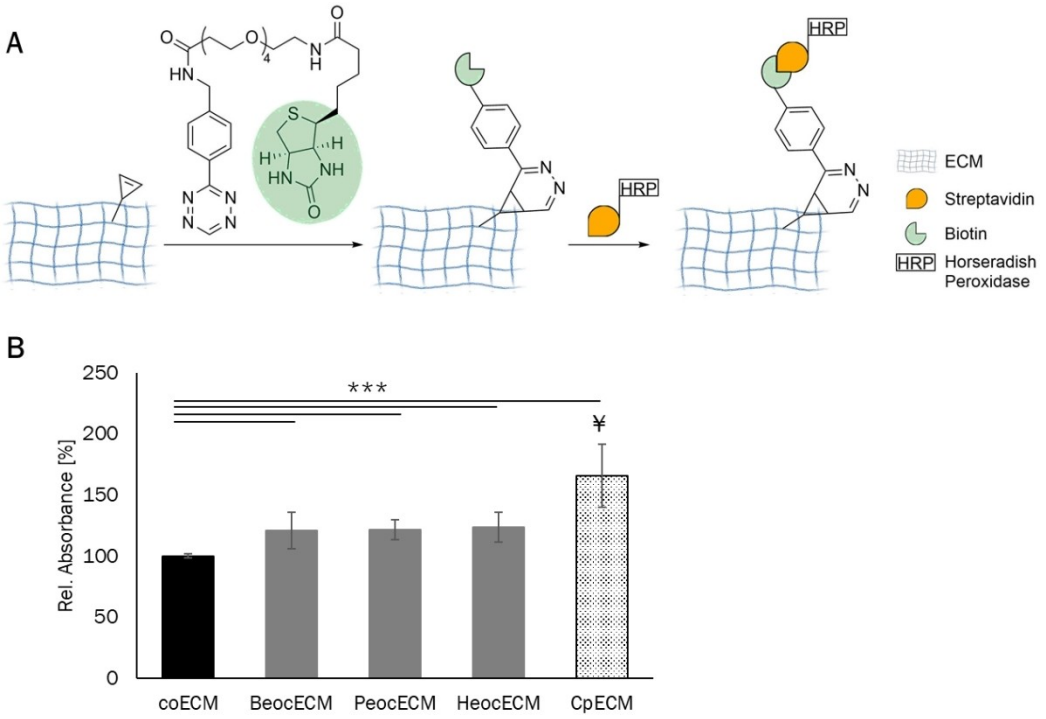

Figure 2. Detection of functional groups incorporated into the ECM. A) Schematic overview of the detection mechanism. Control ECM (coECM) and dienophile-modified ECM were concentrated, homogenized, and dried into a well plate, and incubated with a tetrazine-biotin conjugate (50 μM). After IEDDA reaction, biotin residues were labeled with a streptavidin-HRP conjugate (6.6 μg/mL). Subsequent addition of HRP substrate TMB allowed the quantification of HRP by colorimetric detection of TMB turnover. B) Relative TMB turnover from 3 independent experiments using cells from different donors (each 2 technical replicates) normalized to the unmodified coECM (set to 100 %). *** $p \leq 0.001$, ¥ ≤ 0.001 to all other samples; partly created in https://biorender.com.

ides resulted in a significantly higher TMB turnover demonstrating successful functional group incorporation by this approach (BeocECM: (120.6 ± 14.8) %; PeocECM: (121.6 ± 8.1) %; HeocECM: (123.5 ± 12.2) %; CpECM: (165.6 ± 25.7) %). These results are in the same range as the results with an azide-modified clickECM that revealed a twofold turnover rate of the substrate compared to unmodified ECM.[12] The highest TMB turnover was found for the Cp modification. The labeling efficiency in MGE experiments depends on both the amount of reporter group incorporated and the chemical reactivity in the bioorthogonal ligation reaction.[16,17c] In case of the terminal alkenes, an increasing length of the side chain leads to higher reactivity in the IEDDA reaction. At the same time, it can be expected that the metabolic acceptance of the GalNAc derivative is lower with increasing length of the side chain. This might explain similar TMB turnover of the BeocECM, PeocECM, and HeocECM. Ac_4GalNCp has a small reporter group and a high IEDDA reactivity which is in line with a higher TMB turnover of the CpECM. Since the Cp-modified ECM gave the highest TMB turnover, this material was used in all following experiments.

Impact of the advanced clickECM in 3D gellan gum-ECM-hybrid hydrogels

We showed that the incorporated dienophiles can be addressed by the IEDDA reaction. However, it cannot be ensured that all functional groups within the clickECM reacted with the tetrazine derivative. This also applies to a future modification of the clickECM, e.g., with growth factors using this system. Thus, we wanted to ensure that the functional group itself has no negative impact on cellular behavior. To investigate the influence of the functional group within the clickECM as biomaterial on cellular behavior, ASCs (300,000 cells/100 μL hydrogel) were encapsulated into ECM-gellan gum-hybrid hydrogels consisting of 1 wt% gellan gum and 0.25 wt% homogenized ECM (non-functionalized: coECM; azide-functionalized: AzECM; cyclopropene-functionalized: CpECM). Gellan gum is a mostly bioinert bacterial polysaccharide that is used in different tissue engineering approaches.[26] Gellan gum itself does not influence ASCs behavior and thus possible changes can be traced back to the ECM. Next to (bio)chemical characteristics, it is well known that the stiffness of a matrix has a high impact on cellular behavior.[2a,27] To exclude differences in stiffness as a source for different behavior, rheological measurements were performed on hydrogels without ASCs (Figure 3). As a control, gellan gum hydrogels without ECM were measured (w/o ECM). The storage and loss modulus of the different hydrogels exhibit no significant differences. Thus, in this study, possible changes in cellular behavior of encapsulated ASCs can be traced back to the ECM and/or the modification with functional groups and not to differences in the stiffness of the hydrogel.

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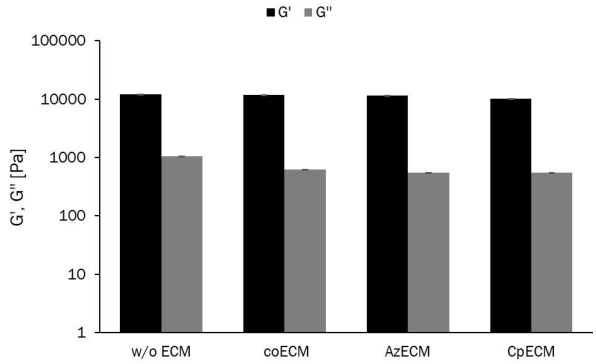

Figure 3. Stiffness of the hydrogels. For the determination of the stiffness of the hydrogels, ECM-gellan gum-hybrid hydrogels without ASCs were used (mean values of 3 independent experiments using cells from different donors). Determination of stiffness showed no significant difference between the different hybrid hydrogels.

The distribution of the ECM particles is a critical parameter for cell experiments that impacts the extent of possible interaction of cells with ECM and, therefore, the possible positive impact on cellular survival, proliferation, and differentiation.[4] To evaluate the distribution of the ECM particles within the hydrogels, we used ECM-gellan gum-hybrid hydrogels with encapsulated ASCs and performed hematoxylin eosin (HE) staining on histological slices (Figure 4). HE staining is a standard overview staining for the visualization of ECM structures in red and nuclei in blue. As expected, in the hydrogel without ECM no staining of ECM was found. In the hydrogels with ECM, a homogeneous distribution of ECM particles was observed. This ensures the proximity of ASCs and ECM throughout the hydrogel.

For the determination of the cellular response to the different functional groups within the ECM materials in a 3D environment, ASCs were encapsulated into gellan gum-ECM-hybrid hydrogels (Figure 5). As controls, hydrogels without ECM and with azide-modified AzECM were used. After three days of culture in the growth medium, the impact of ECM and their functionalization was determined by the analysis of supernatant and live/dead staining of the cells. Using the supernatant, LDH release and resazurin assays were performed to determine cell death and metabolic activity of the ASCs in contact with the different modified ECM materials (Figure 5A). We were not able to detect a significant difference in LDH release between the different hydrogels, which indicates that the functional groups do not have a negative impact on the ASCs regarding cellular survival. Analogously, no difference in metabolic activity was found between the ASCs cultured with the different functionalized ECMs and the negative control. These results are in line with previous experiments in which no cytotoxic effects of the coating with azide modified ECM on human dermal fibroblasts were found.[12]

On day three of cell culture, live/dead staining was performed and analyzed concerning cellular survival and proliferation of the encapsulated ASCs by image analysis software (Figure 5B). As expected, we observed that ASCs in hydrogels with ECM exhibited a higher survival rate and proliferation compared to the control without ECM. This effect was independent of the functionalization of the ECM. Previously, several studies demonstrated that ECM and ECM components enhance cellular adhesion, survival, and proliferation.[28] Within the samples with ECM, no differences could be observed between the differently modified ECMs or unmodified ECM. The results of the counting of viable and dead cells (Figure 5C) do not coincide with the results of the LDH release, as a higher percentage of dead cells was found in the live/dead staining whereas no differences in LDH release were found. One possible explanation might be an irregular diffusion of the LDH protein within the hydrogel. LDH released from the encapsulated cells might be caught in the hydrogel and consequently cannot be measured in the supernatant. This further might explain the relatively high standard deviations found in the values of LDH determination. Against this background, results from the image analysis seem to be more reliable. The results of the proliferation were given as the

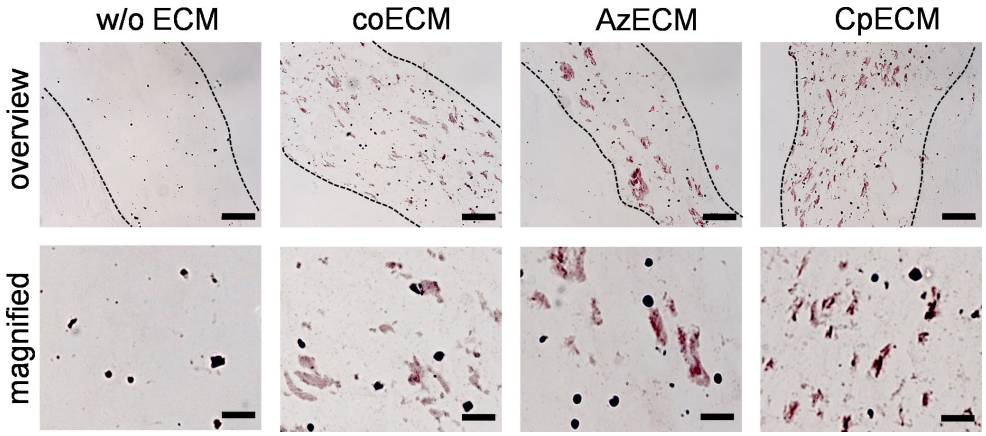

Figure 4. HE staining of gellan gum-ECM-hybrid hydrogels. For the histological staining, ECM-gellan gum-hybrid hydrogels with encapsulated ASCs (300,000 cells/100 μL hydrogel) were used. HE staining showed the presence and homogeneous distribution of the ECM particles within the gellan gum hydrogels for all samples. ECM particles are stained in rose/red and nuclei are stained in dark blue/black. Scale bar: overview: 200 μm; magnified: 50 μm.

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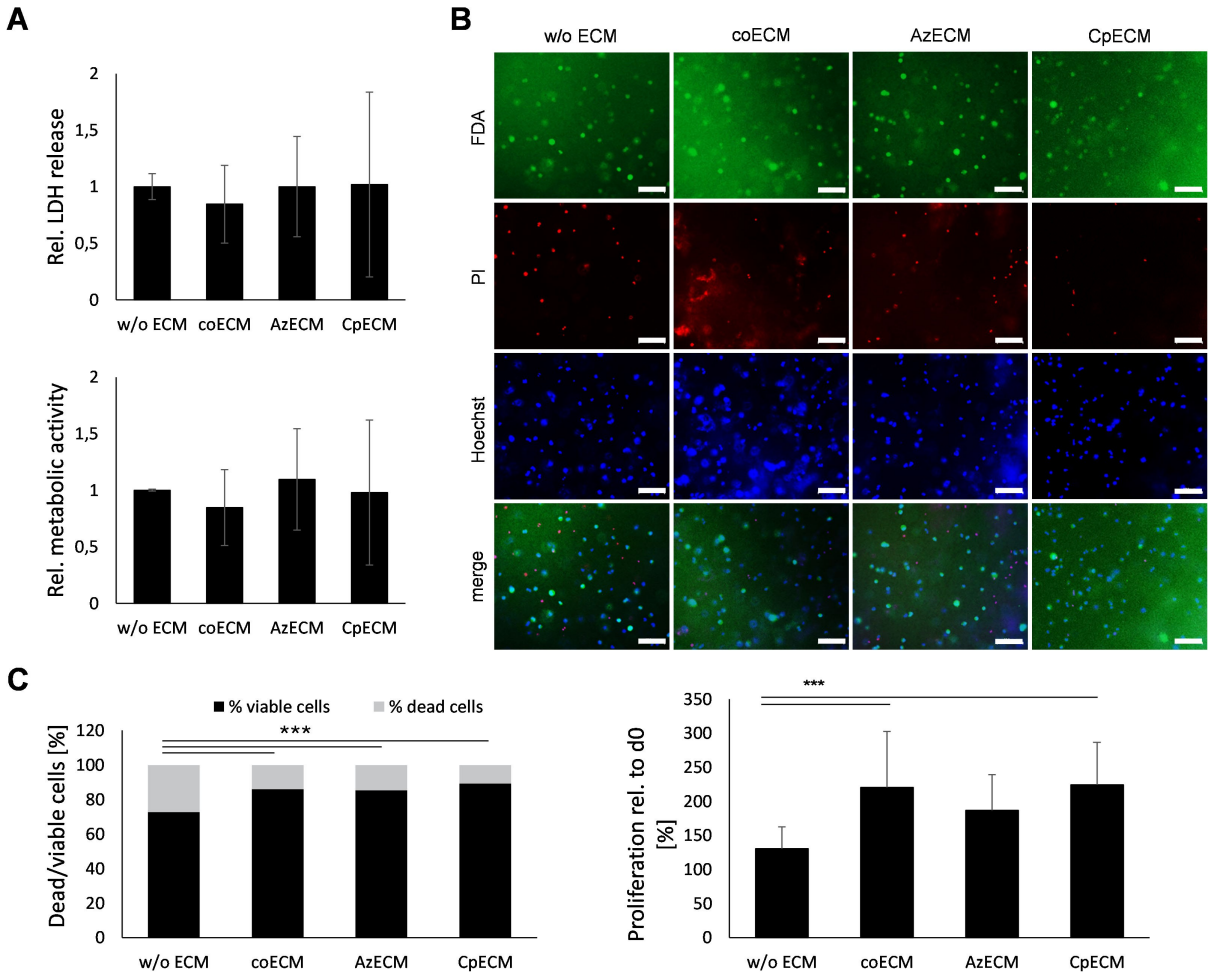

Figure 5. Impact of azide- and cyclopropene-modified ECM on cellular behavior of ASCs encapsulated in gellan gum hybrid hydrogels. Cells (300,000) were encapsulated into gellan gum hybrid hydrogel containing different modified or unmodified types of ECM and cultured in growth medium for three days. A) To determine the apoptosis rate, an LDH assay was performed from the cell culture supernatant. Mean values of 3 independent experiments using cells from different donors (each 2 technical replicates) were normalized to the gellan gum without ECM. The metabolic activity of the encapsulated ASCs was determined using a resazurin assay, which is based on the metabolic turnover of the resazurin salt and a resulting color change. B) Representative figure of live/dead staining of the encapsulated ASCs. After three days culture, viable cells were stained with fluorescein diacetate (FDA, green), and dead cells were stained with propidium iodide (PI, red). For an overview of the total cell number nuclei were stained with Hoechst (blue). C) Quantitative evaluation of the live/dead staining. Based on the images of the live/dead staining, cellular survival and relative proliferation were determined. For cellular survival, the number of viable and dead cells was determined and shown as a percentage. Proliferation was determined by counting of total cell numbers on day zero and day three and relative proliferation is shown as the increase of total cell number on day three relative to day zero (mean values of 3 independent experiments using cells from different donors (each 2 technical replicates)). Scale bar: 200 μm; *** $p \leq 0.001$.

percentage of total cell number on day three relative to day zero. Next to the interaction with ECM, bioactive molecules such as growth factors are known to be bound to ECM and might enhance proliferation. We previously demonstrated the presence of different growth factors in ASC-derived ECM.[29] As there are no differences between the approaches with coECM and the functionalized ECMs (AzECM and CpECM), the functionalization exhibits no effect on the parameters shown in Figure 5 (cellular survival, proliferation, and metabolic activity) and therefore represents a promising method for ECM modification without unintentionally affecting cellular behavior.

In general, these results demonstrate that the incorporated functional groups in the ECM themselves have no negative impact on encapsulated ASCs. Therefore, this new cell-derived ECM-based material provides a variety of possible applications including the equipment with bioactive molecules exhibiting desired effects and the possibility of cross-linking of the ECM itself by using corresponding linker molecules.[10,12] The great advantage over the previously reported functionalization of a clickECM by CuAAC is the independence of any catalyst which might exhibit cytotoxic effects or have any other impact on the ECM producing cells.

Conclusions

In this study, we demonstrated for the first time that it is possible to modify a cell-derived ECM with dienophiles as chemical reporter groups (functionalization) using MGE. The used monosaccharide derivatives exhibited no cytotoxic effects.

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A good cytocompatibility is important for monosaccharide derivates used in MGE to prevent cell death and the accumulation of unwanted cytokines with possible negative effects within the ECM. We were further able to show that the incorporated functional groups were addressable by an IEDDA reaction without the need for cytotoxic catalysts. In this way, it was possible to incorporate a bioactive enzyme. This feature opens numerous future applications, such as equipment of the ECM with desired growth factors, cross-linkers, and other molecules. Importantly, we demonstrated that the functional groups themselves have no impact on basic cellular behavior such as survival, metabolic activity, and proliferation. Thus, this new material provides great potential as a biomaterial in a variety of tissue engineering and regenerative medicine approaches as it allows the linking of molecules with desired effects without the need for cytotoxic catalysts.

Experimental Section

General methods: Reactions were monitored by TLC on silica gel 60 F254 (*Merck*) with detection under UV light ($\lambda = 254$ nm). Additionally, acidic ethanolic *p*-anisaldehyde solution or basic $KMnO_4$ solution followed by gentle heating were used for visualization. Preparative flash column chromatography (FC) was performed with an MPLC-Reveleris X2 system from *Büchi*. NMR spectra were recorded at room temperature with Avance III 400 and Avance III 600 instruments from *Bruker*. Chemical shifts are reported relative to solvent signals ($CDCl_3$: $\delta_H = 7.26$ ppm, $\delta_C = 77.16$ ppm; CD_3OD: $\delta_H = 4.87$ ppm, $\delta_C = 49.00$ ppm; D_2O: $\delta_H = 4.73$ ppm). The numbering of compounds is given in the supporting information. High-resolution mass spectra (HRMS) were obtained with a micrOTOF II instrument from *Bruker Daltonics*.

General procedure for the synthesis of dienophile-modified GalNAc derivatives: Galactosamine hydrochloride (3.3 g, 15.3 mmol, 1 equiv.) was dissolved in dry MeOH (90 mL), and NaOMe (0.5 M in MeOH, 31 mL, 1 equiv.) was added. The reaction mixture was stirred for 1.5 h at rt and a solution of the alkene derivative **1 a**–**d**) (1.04 equiv) in dry MeOH (90 mL) was added. After having been stirred at rt for 18 h, the solvent was removed under vacuum and the residual brown syrup was dissolved in pyridine (40 mL). Acetic anhydride (14 mL, 150 mmol) was added, and the mixture was stirred for 18 h. The solvent was removed under vacuum, the residue was dissolved in DCM and washed with aqueous $KHSO_4$ (3×), sodium bicarbonate (2×), and brine (1×). The organic layer was dried over $MgSO_4$ and concentrated resulting in a dark brown solid which was purified by FC on silica (petroleum ether/ethyl acetate) yielding the corresponding GalNAc derivative.

Ac_4GalNBeoc: The title compound was synthesized with but-3-en-1-yl succinimidyl carbonate (**1 a**) according to the general procedure and obtained as a colorless solid (63%) as a mixture of anomers ($\alpha/\beta = 3.2/1$). TLC: $R_f = 0.33$ (petroleum ether/ethyl acetate 1:1); α-anomer: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.23$ (d, $J = 3.5$ Hz, 1H, C^1H), 5.70–5.80 (m, 1H, C^9H), 5.42 (d, $J = 2.3$ Hz, 1H, C^4H), 5.19–5.03 (m, 3H, C^3H, $C^{10}H_2$), 4.64 (d, $J = 9.7$ Hz, 1H, NH), 4.41 (dt, $J = 3.4$, 11,4 Hz, 1H, C^2H), 4.22 (m, 1H, C^5H), 4.04-4.15 (m, 4H, C^7H_2, C^6H_2), 2.35–5.33 (m, 2H, C^8H_2), 2.16 (s, 2×3H, CH_3), 2.02 (s, 2×3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.8$, 170.3, 168.8, 155.9 (C=O), 133.9 (C^9), 117.2 (C^{10}), 91.5 (C^1), 68.5 (C^5), 68.0 (C^3), 66.7 (C^4), 61.2, 64.5 (C^6, C^7), 48.6 (C^2), 33.3 (C^8), 20.9, 20.6 (CH_3) ppm; β-anomer: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.17$ (d, $J = 4.7$ Hz, 1H, C^1H), 5.69–5.80 (m, 1H, C^9H), 5.32–5.28 (m, 1H, C^3H), 5.18–5.03 (m, 4H, NH, C^5H, $C^{10}H_2$), 4.52–5.47 (m, 1H, C^2H), 4.18–4.12 (m, 5H, C^4H, C^7H_2, C^6H_2), 2.35 (m, 2H, C^8H_2), 2.12 (s, 3H, CH_3), 2.07 (s. 2×3H, CH_3), 2.02 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.5$, 169.9, 169.3, 168.9, 155.9 (C=O), 133.9 (C^9), 117.2 (C^{10}), 93.9 (C^1) 78.9 (C^5), 73.9 (C^3), 68.6 (C^4), 64.5 (C^7), 62.0 (C^6), 57.8 (C^2), 33.2 (C^8), 20.6, 20.7, 20.8, 21.1 (CH_3) ppm; HRMS (ESI-MS): *m/z* calcd. for $C_{19}H_{27}NO_{11}$: 446.1657 $[M+H]^+$; found: 468.1471 $[M+Na]^+$.

Ac_4GalNPeoc: The title compound was synthesized with pent-4-en-1-yl succinimidyl carbonate (**1 b**) according to the general procedure and obtained as a colorless solid (49%) as a mixture of anomers ($\alpha/\beta = 2.2/1$). TLC: $R_f = 0.5$ (petroleum ether/ethyl acetate 1:1); α-anomer: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.21$ (d, $J = 3.5$ Hz, 1H, C^1H), 5.71–5.81 (m, 2H, $C^{10}H_2$), 5.40 (d, $J = 2.9$ Hz 1H, C^4H), 5.21–5.17 (m, 1H, C^3H), 5.05–4.96 (m, 2H, $C^{11}H_2$), 4.70 (d, $J = 9.5$ Hz, 1H, NH), 4.44–4.40 (m, 1H, C^2H), 4.24–4.19 (m, 1H, C^5H), 4.10–4.01 (m, 4H, C^6H_2, C^7H_2), 2.14 (s, 6H, CH_3), 2.06–2.07 (m, 2H, C^9H_2), 1.99 (s, 3H, CH_3) 2.00 (s, 3H, CH_3), 1.64–1.71 (m, 2H, C^8H_2) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.8$, 170.3, 170.1, 168.8, 155.9 (C=O), 137.3 (C^{10}), 114.9 (C^{11}), 91.3 (C^1), 68.4 (C^5), 68.1 (C^3), 66.8 (C^4), 64.9, 61.2 (C^6, C^7), 48.6 (C^2), 29.9 (C^9), 28.1 (C^8), 20.8, 20.7, 20.6 (CH_3) ppm; β-anomer: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.19$ (d, $J = 4.7$ Hz, 1H, C^1H), 5.84–5.74 (m, 1H, $C^{10}H$), 5.34–5.30 (m, 1H, C^3H), 5.12–4.97 (m, 3H, NH, C^5H, $C^{11}H_2$), 4.54–4.49 (m, C^2H), 4.25–4.04 (m, 5H, C^4H, C^6H_2, C^7H_2), 2.17 (s, 3H, CH_3), 2.11–2.08 (m, 8H, CH_3, CH_3, C^9H_2), 2.04 (s, 3H, CH_3), 1.74–1.67 (m, 2H, C^8H_2) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta =$ 170.7, 170.3, 170.1, 168.9, 156.2 (C=O), 137.8 (C^{10}), 115.2 (C^{11}), 93.3 (C^1), 78.8 (C^5), 73.9 (C^3), 68.6 (C^4), 65.3 (C^7), 61.5 (C^6), 57.6 (C^2), 29.6 (C^9), 27.8 (C^8), 21.1, 21.2, 21.0, 20.8, 20.7 (CH_3) ppm; HRMS (ESI-MS): *m/z* calcd. for $C_{20}H_{29}NO_{11}$: 460.1813 $[M+H]^+$; found: 482.1628 $[M+Na]^+$.

Ac_4GalNHeoc: The title compound was synthesized with hex-5-en-1-yl succinimidyl carbonate (**1 c**) according to the general procedure and obtained as a colorless solid (76%) as a mixture of anomers ($\alpha/\beta = 2.6/1$). TLC: $R_f = 0.45$ (petroleum ether/ethyl acetate 1:1); α-anomer: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.21$ (d, $J = 3.5$ Hz, 1H, C^1H), 5.81–5.70 (m, 1H, $C^{11}H$), 5.40 (d, $J = 2.3$ Hz, 1H, C^4H), 5.21–5.11 (m, 1H, C^3H), 5.00–4.92 (m, 2H, $C^{12}H_2$), 4.68 (d, $J = 9.6$ Hz, 1H, NH), 4.42–4.36 (m, 1H, C^2H), 4.23–4.18 (m, 1H, C^5H), 4.12–4.01 (m, 4H, C^6H_2, C^7H_2), 2.14 (s, 2×3H, CH_3), 2.06–2.03 (m, 2H, $C^{10}H_2$), 2.00 (s, 2×3H, CH_3), 1.59 (m, 2H, C^8H_2), 1.45–1.37 (m, 2H, C^9H_2) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.9$, 170.4, 169.9, 168.9 (C=O), 156.1 (C^{13}), 138.3 (C^{11}), 114.9 (C^{12}), 91.6 (C^1), 68.6 (C^5), 68.1 (C^3), 66.9 (C^4), 65.5 (C^7), 61.4 (C^6), 48.7 (C^2), 33.3 (C^{10}), 28.4 (C^8), 25.1 (C^9), 20.8, 20.7 (CH_3) ppm; β-anomer: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.16$ (d, $J = 4.7$ Hz, 1H, C^1H), 5.81–5.70 (m, 1H, $C^{11}H$), 5.32–5.28 (m, 1H, C^3H), 5.21–5.11 (m, 2H, C^5H, NH), 4.95 (m, 2H, $C^{12}H_2$), 4.52–4.47 (m, 1H, C^2H), 4.20–4.01 (m, 5H, C^4H, C^6H_2, C^7H_2), 2.06–2.03 (m, 2H, $C^{10}H_2$), 2.11 (s, 3H, CH_3), 2.07 (s, 2×3H, CH_3), 2.02 (s, 3H, CH_3), 1.59 (m, 2H, C^8H_2), 1.45–1.37 (m, 2H, C^9H_2) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.9$ 170.4, 169.9, 168.9 (C=O), 156.1 (C^{13}), 138.3 (C^{11}), 114.9 (C^{12}), 94.1 (C^1), 79.0 (C^5), 74.1 (C^3), 70.4 (C^4), 68.0 (C^4), 66.8 (C^7), 62.2, 61.3 (C^6), 57.9 (C^2), 33.3 (C^{10}), 28.4 (C^8), 25.1 (C^9), 21.2, 21.1, 21.0, 20.8, 20.7 (CH_3) ppm; HRMS (ESI-MS): *m/z* calcd. for $C_{21}H_{31}NO_{11}$: 474.1970 $[M+H]^+$; found: 496.1786 $[M+Na]^+$.

Ac_4GalNPtl: The title compound was synthesized with succinimidyl pent-4-enoate (**1 d**) according to the general procedure and obtained as a colorless solid (70%) as a mixture of anomers ($\alpha/\beta =$ 2.4/1). TLC: $R_f = 0.2$ (petroleum ether/ethyl acetate 1:1); α-anomer: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.21$ (d, $J = 3.6$ Hz, 1H, C^1H), 5.79–5.69 (m, 2H, $C^{10}H_2$), 5.55 (m, 1H, NH), 5.39 (m, 1H, C^4H), 5.19–5.16 (m, 1H, C^3H), 5.04–4.95 (m, 2H, C^9H_2), 4.73–4.68 (m, 1H, C^2H), 4.23–4.20 (m, 1H, C^5H), 4.10–4.01 (m, 2H, C^6H_2), 2.33–2.28 (m, 2H, C^8H_2), 2.23–2.19 (m, 2H, C^7H_2), 2.14 (s, 2×3H, CH_3), 1.99 (s, 3H, CH_3) 2.00 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 172.3$, 171.0, 170.3, 170.2, 158.8 (C=O), 136.6 (C^{10}), 115.7 (C^9), 91.3 (C^1), 68.6 (C^5), 68.5 (C^5), 67.8 (C^3),

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66.7 (C^4), 61.3 (C^6), 46.8 (C^2), 35.5 (C^7), 29.2 (C^8), 20.9 (CH_3), 20.6 (CH_3) ppm; β-anomer: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.70$ (d, $J = 8.7$ Hz, 1H, C^1H), 5.81–5.73 (m, 1H, $C^{10}H$), 5.38 (m, 2H, NH, C^4H), 5.10–4.98 (m, 3H, C^3H, C^9H_2), 4.50–4.43 (m, 1H, C^2H), 4.20–4.09 (m, 1H, C^6H_2), 4.02–4.00 (m, 1H, C^5H), 2.36–2.31 (m, 2H, C^8H_2), 2.24–2.21 (m, 2H, C^7H_2), 2.17 (s, 3H, CH_3), 2.12 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 2.01 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 172.4$, 170.7, 170.1, 164.6 (C=O), 136.5 (C^{10}), 115.7 (C^9), 93.0 (C^1), 71.9 (C^5), 70.3 (C^3), 70.3 (C^3), 66.3 (C^4), 61.3 (C^6), 49.6 (C^2), 35.8 (C^7), 29.2 (C^8), 21.0, 20.8, 20.6, 20.5 (CH_3) ppm; HRMS (ESI-MS): *m/z* calcd. for $C_{19}H_{27}NO_{10}$: 430.1708 $[M+H]^+$; found: 452.1524 $[M+Na]^+$.

Adipose-derived stem cell isolation: All used media contained 1% penicillin/streptomycin. ASCs were isolated from human tissue samples obtained from patients undergoing plastic surgery (Dr. Ziegler; Klinik Charlottenhaus, Stuttgart, Germany) as described before.[30] Briefly, tissue was cut into small pieces and digested in Dulbecco's modified eagle medium (DMEM, BioChrom, Germany) containing 0.1% collagenase NB4 (Serva Electrophoresis, Germany) and 1% bovine serum albumin (BSA; Sigma, Germany) for 5 h at 37 °C under constant shaking. The suspension was filtered through a 500 μm sieve and centrifuged for 5 min at 200×g. To remove erythrocytes, the pellet was suspended in erythrocyte lysis buffer and incubated for 10 min at room temperature. The suspension was centrifuged for 5 min at 200×g, the remaining pellet was suspended in phosphate-buffered saline (PBS; Biochrom, Germany) and filtered through a 100 μm meshed sieve. ASCs were initially seeded at a density of 5×10^3 cells/cm^2 in a serum-free MSC growth medium (MSCGM; PELOBiotech, Germany) containing 5% human platelet lysate (hPL). The phenotype of the ASCs was previously characterized, and it was shown that the cells exhibit the typical surface proteins.[31] All research was carried out following the rules for the investigation of human subjects as defined in the Declaration of Helsinki. Patients provided written agreement in compliance with the Landesärztekammer Baden-Württemberg (F-2012-078, for normal skin from elective surgeries). ASCs were used up to passage three.

Cytotoxicity of the modified monosaccharides: The biocompatibility of functionalized monosaccharides was evaluated by a lactate dehydrogenase (LDH) assay (TaKaRa Bio Inc.) and a resazurin assay (Sigma Aldrich, Germany). ASCs were seeded in growth medium (DMEM + 10% FCS) at a density of 50,000 cells/ cm^2. After 24 h cells were treated with 100 μM monosaccharide or sterile water and incubated for another 24 h. LDH assay was performed according to the manufacturer's protocol with cell culture supernatant. For the resazurin assay, the culture medium was changed to a medium with resazurin salt (11 μg/mL) and incubated for 3 h at 37 °C and 5% CO_2. The untreated negative control was set as 100% and values were normalized to control.

Metabolic glycoengineering and isolation of functionalized extracellular matrix: For the generation of functionalized ECM, ASCs were seeded into Petri dishes ($d = 14.5$ cm) at a density of 25,000 cells/cm^2 in DMEM containing 10% FCS. The next day, 50 μg/mL sodium ascorbate was added to the medium. The medium was changed every second day and removed sodium ascorbate was replaced. On day 4 100 μM modified monosaccharides were added to the cell culture medium for MGE. After 72 h incubation cells were lysed using hypotonic 0.7% ammonium hydroxide solution and isolated ECM was washed with ultrapure water. After isolation, ECM was concentrated using ultracentrifugation tubes (Amicon, Merck, Germany) with a molecular cut-off of 10 kDa. Concentrated ECM was recovered and homogenized using lysis tubes and homogenizer FastPrep-24™ 5G (MP Biomedicals™, Germany).[12] The dry weight of ECM samples was determined by freeze-drying.

Detection of incorporated functional groups: For detection of functional groups, homogenized ECM was dried on TCPS. ECM was incubated with 50 μM biotinylated tetrazine for detection of dienophile groups for 1 h at RT. Samples were washed with PBS and incubated with 6.6 μg/mL streptavidin linked with horseradish peroxidase. Subsequently, TMB was added to the samples and after color change reaction was stopped with 1 M HCl. The supernatant was measured at 450 nm using the plate reader Tecan Saphire II (Tecan, Switzerland). The reference wavelength was set as 620 nm. Unmodified ECM was used as negative control and results were normalized to it.

Preparation of gellan gum-ECM hybrid hydrogels with encapsulated ASC and evaluation of cellular behavior: Hybrid hydrogels were prepared of 1 wt% gellan gum and 0.25 wt% ECM. As a negative control, 1% gellan gum hydrogels without ECM supplementation were prepared. Liquid hydrogel solution (100 μL) was filled in a plastic ring with 0.6 cm in diameter and covered with PBS with magnesium and calcium (PBS+) to induce cross-linking. Before rheological analysis, hydrogels were swollen for 72 h in PBS+ at RT. For evaluation of stiffness, storage modulus and loss modulus were measured. Oscillatory rheology was performed on a Physica MCR 301 rheometer (Anton Paar) using a parallel plate geometry with a diameter of 8 mm at a temperature of 20 °C. Amplitude sweeps (frequency = 1 Hz, amplitudes between 0.01% and 10%) were performed to estimate the linear viscoelastic range, resulting in a comparison of hydrogel stiffness via storage modulus G′ and loss modulus G″ at a deformation of 0.1% and a frequency of 1 Hz.

To determine the effect of functionalized ECM on cellular behavior, ASCs were encapsulated into ECM-gellan gum-hybrid hydrogels. Therefore, hydrogels with 1 wt% gellan gum, 0.25 wt% ECM, and 300,000 ASCs per 100 μL were prepared. Liquid hydrogel solution (100 μL) was filled in a plastic ring with 0.6 cm in diameter and covered with PBS with magnesium and calcium (PBS+) to induce cross-linking. After 30 min incubation at 37 °C PBS+ was changed to DMEM containing 10% FCS and 1% P/S. On day 0 and day 3 after hydrogel preparation, live/dead staining and resazurin assay were performed. For live/dead staining gels were rinsed two times with PBS+ followed by incubation with staining solution, consisting of 200 ng/mL fluorescein diacetate (FDA, Sigma Aldrich, Germany) and 20 μg/mL propidium iodide (PI, Sigma Aldrich, Germany) in DMEM, for 15 min at 37 °C. Nuclei were counterstained with Hoechst33342 (5 μg/mL). Subsequently, gels were rinsed with PBS + and placed onto a slide for microscopic analysis. Images were taken with an Axio Observer microscope and Axiocam color using the software ZENblue (Carl Zeiss, Germany). Nuclei, viable cells, and dead cells were counted using the software ImageJ.

To investigate the metabolic activity of the encapsulated ASCs, a resazurin assay was performed on day 3 after hydrogel preparation. Hydrogels were incubated with resazurin solution (11μg/mL) at 37 °C. The absorbance of the supernatant was measured at 570 nm with a correction wavelength set to 595 nm (Tecan Safire 2, multimode microplate reader, Tecan Trading AG, Switzerland). Results were calculated based on the number of viable cells per hydrogel and values were normalized to the control hydrogel without ECM.

Statistics: All experiments were performed using samples from three different biological donors. Data were analyzed by one-way analysis of variance (ANOVA) with a Bonferroni posthoc test using Origin 2018b. Statistical significances were stated as $p < 0.05$ (*), very significant as $p < 0.01$ (**), and highly significant as $p < 0.001$ (***).

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: bioorthogonal chemistry · carbohydrates · extracellular matrix · inverse-electron-demand Diels-Alder reaction · metabolic engineering

[1] C. Frantz, K. M. Stewart, V. M. Weaver, *J. Cell Sci.* **2010**, *123*, 4195–4200.
[2] a) A. J. Engler, S. Sen, H. L. Sweeney, D. E. Discher, *Cell* **2006**, *126*, 677–689; b) G. C. Reilly, A. J. Engler, *J. Biomech. Eng.* **2010**, *43*, 55–62.
[3] L. E. Fitzpatrick, T. C. McDevitt, *Biomater. Sci.* **2015**, *3*, 12–24.
[4] V. Guneta, Z. Zhou, N. S. Tan, S. Sugii, M. T. C. Wong, C. Choong, *Biomater. Sci.* **2017**, *6*, 168–178.
[5] a) N. L'Heureux, N. Dusserre, G. Konig, B. Victor, P. Keire, T. N. Wight, N. A. F. Chronos, A. E. Kyles, C. R. Gregory, G. Hoyt, R. C. Robbins, T. N. McAllister, *Nat. Med.* **2006**, *12*, 361–365; b) G. M. Cunniffe, T. Vinardell, J. M. Murphy, E. M. Thompson, A. Matsiko, F. J. O'Brien, D. J. Kelly, *Acta Biomater.* **2015**, *23*, 82–90.
[6] A. C. Braun, M. Gutmann, T. Lühmann, L. Meinel, *J. Controlled Release* **2018**, *273*, 68–85.
[7] a) O. T. Keppler, R. Horstkorte, M. Pawlita, C. Schmidt, W. Reutter, *Glycobiology* **2001**, *11*, 11R-18R; b) D. H. Dube, C. R. Bertozzi, *Curr. Opin. Chem. Biol.* **2003**, *7*, 616–625; c) C. Agatemor, M. J. Buettner, R. Ariss, K. Muthiah, C. T. Saeui, K. J. Yarema, *Nat. Chem. Rev.* **2019**, *3*, 605–620.
[8] E. M. Sletten, C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2009**, *48*, 6974–6998; *Angew. Chem.* **2009**, *121*, 7108–7133.
[9] a) S. M. Ruff, S. Keller, D. E. Wieland, V. Wittmann, G. E. M. Tovar, M. Bach, P. J. Kluger, *Acta Biomater.* **2017**, *52*, 159–170; b) S. Nellinger, S. Keller, A. Southan, V. Wittmann, A.-C. Volz, P. J. Kluger, *Curr. Dir. Biomed. Eng.* **2019**, *5*, 393–395.
[10] M. Gutmann, A. Braun, J. Seibel, T. Lühmann, *ACS Biomater. Sci. Eng.* **2018**, *4*, 1300–1306.
[11] a) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057–3064; b) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599; *Angew. Chem.* **2002**, *114*, 2708–2711.
[12] S. Keller, K. Wörgötter, A. Liedek, P. J. Kluger, M. Bach, G. E. M. Tovar, A. Southan, *ACS Appl. Mater. Interfaces* **2020**, *12*, 26868–26879.
[13] M. van Dijk, D. T. S. Rijkers, R. M. J. Liskamp, C. F. van Nostrum, W. E. Hennink, *Bioconjugate Chem.* **2009**, *20*, 2001–2016.
[14] a) N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046–15047; b) X. Ning, J. Guo, Margreet A. Wolfert, G.-J. Boons, *Angew. Chem. Int. Ed.* **2008**, *47*, 2253–2255; *Angew. Chem.* **2008**, *120*, 2285–2287.
[15] a) M. L. Blackman, M. Royzen, J. M. Fox, *J. Am. Chem. Soc.* **2008**, *130*, 13518–13519; b) K. Braun, M. Wiessler, V. Ehemann, R. Pipkorn, H. Spring, J. Debus, B. Didinger, M. Koch, G. Muller, W. Waldeck, *Drug Des. Dev. Ther.* **2008**, *2*, 289–301; c) N. K. Devaraj, R. Weissleder, S. A. Hilderbrand, *Bioconjugate Chem.* **2008**, *19*, 2297–2299.
[16] L. M. Haiber, M. Kufleitner, V. Wittmann, *Front. Chem.* **2021**, *9*, 654932.
[17] a) A. Niederwieser, A.-K. Späte, L. D. Nguyen, C. Jüngst, W. Reutter, V. Wittmann, *Angew. Chem. Int. Ed.* **2013**, *52*, 4265–4268; *Angew. Chem.* **2013**, *125*, 4359–4363; b) A.-K. Späte, V. F. Schart, S. Schöllkopf, A. Niederwieser, V. Wittmann, *Chem. Eur. J.* **2014**, *20*, 16502–16508; c) J. E. G. A. Dold, J. Pfotzer, A.-K. Späte, V. Wittmann, *ChemBioChem* **2017**, *18*, 1242–1250; d) A. Kitowski, G. J. L. Bernardes, *ChemBioChem* **2020**, *21*, 2696–2700; e) J. E. G. A. Dold, V. Wittmann, *ChemBioChem* **2021**, *22*, 1243–1251.
[18] a) D. M. Patterson, L. A. Nazarova, B. Xie, D. N. Kamber, J. A. Prescher, *J. Am. Chem. Soc.* **2012**, *134*, 18638–18643; b) A.-K. Späte, H. Bußkamp, A. Niederwieser, V. F. Schart, A. Marx, V. Wittmann, *Bioconjugate Chem.* **2014**, *25*, 147–154; c) D. M. Patterson, K. A. Jones, J. A. Prescher, *Mol. BioSyst.* **2014**, *10*, 1693–1697; d) A.-K. Späte, V. F. Schart, J. Häfner, A. Niederwieser, T. U. Mayer, V. Wittmann, *Beilstein J. Org. Chem.* **2014**, *10*, 2235–2242; e) D.-C. Xiong, J. Zhu, M.-J. Han, H.-X. Luo, C. Wang, Y. Yu, Y. Ye, G. Tai, X.-S. Ye, *Org. Biomol. Chem.* **2015**, *13*, 3911–3917; f) F. Doll, A. Buntz, A.-K. Späte, V. F. Schart, A. Timper, W. Schrimpf, C. R. Hauck, A. Zumbusch, V. Wittmann, *Angew. Chem. Int. Ed.* **2016**, *55*, 2262–2266; *Angew. Chem.* **2016**, *128*, 2303–2308; g) J. Hassenrück, V. Wittmann, *Beilstein J. Org. Chem.* **2019**, *15*, 584–601.
[19] P. Agarwal, B. J. Beahm, P. Shieh, C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2015**, *54*, 11504–11510; *Angew. Chem.* **2015**, *127*, 11666–11672.
[20] A.-K. Späte, J. E. G. A. Dold, E. Batroff, V. F. Schart, D. E. Wieland, O. R. Baudendistel, V. Wittmann, *ChemBioChem* **2016**, *17*, 1374–1383.
[21] M. Wiessler, W. Waldeck, R. Pipkorn, C. Kliem, P. Lorenz, H. Fleischhacker, M. Hafner, K. Braun, *Int. J. Med. Sci.* **2010**, *7*, 213–223.
[22] V. F. Schart, J. Hassenrück, A.-K. Späte, J. E. G. A. Dold, R. Fahrner, V. Wittmann, *ChemBioChem* **2019**, *20*, 166–171.
[23] Y.-J. Lee, Y. Kurra, W. R. Liu, *ChemBioChem* **2016**, *17*, 456–461.
[24] I. Jeon, D. Lee, I. J. Krauss, S. J. Danishefsky, *J. Am. Chem. Soc.* **2009**, *131*, 14337–14344.
[25] D. E. Place, T. D. Kanneganti, *J. Exp. Med.* **2019**, *216*, 1474–1486.
[26] a) M. E. L. Lago, L. P. da Silva, C. Henriques, A. F. Carvalho, R. L. Reis, A. P. Marques, *BioEngineering* **2018**, *5*, 52; b) L. R. Stevens, K. J. Gilmore, G. G. Wallace, M. I. H. Panhuis, *Biomater. Sci.* **2016**, *4*, 1276–1290; c) C. J. Ferris, K. J. Gilmore, G. G. Wallace, M. I. H. Panhuis, *Soft Matter* **2013**, *9*, 3705–3711; d) M. B. Oliveira, C. A. Custodio, L. Gasperini, R. L. Reis, J. F. Mano, *Acta Biomater.* **2016**, *41*, 119–132.
[27] a) Kshitiz, J. Park, P. Kim, W. Helen, A. J. Engler, A. Levchenko, D. H. Kim, *Integr. Biol.* **2012**, *4*, 1008–1018; b) Y. N. Wu, Z. Yang, J. B. K. Law, A. Y. He, A. A. Abbas, V. Denslin, T. Kamarul, J. H. P. Hui, E. H. Lee, *Tissue Eng.* **2017**, *23*, 43–54.
[28] a) G. D. Kusuma, M. C. Yang, S. P. Brennecke, A. J. O'Connor, B. Kalionis, D. E. Heath, *ACS Biomater. Sci. Eng.* **2018**, *4*, 1760–1769; b) R. Rakian, T. J. Block, S. M. Johnson, M. Marinkovic, J. Wu, Q. Dai, D. D. Dean, X.-D. Chen, *Stem Cell Rev.* **2015**, *6*, 235; c) A. I. Hoch, V. Mittal, D. Mitra, N. Vollmer, C. A. Zikry, J. K. Leach, *Biomaterials* **2016**, *74*, 178–187; d) H. Lin, G. Yang, J. Tan, R. S. Tuan, *Biomaterials* **2012**, *33*, 4480–4489.
[29] S. Nellinger, I. Schmidt, S. Heine, A. C. Volz, P. J. Kluger, *Biotechnol. Bioeng.* **2020**, *117*, 3160–3172.
[30] A.-C. Volz, B. Huber, A. M. Schwandt, P. J. Kluger, *Differentiation* **2017**, *95*, 21–30.
[31] A.-C. Volz, P. J. Kluger, *Cytotherapy* **2018**, *20*, 576–588.

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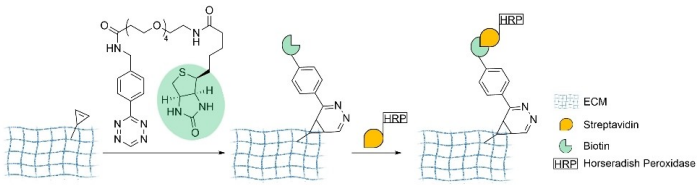

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An Advanced 'clickECM' That Can be Modified by the Inverse-Electron-Demand Diels-Alder Reaction

Metabolic glycoengineering was employed to incorporate dienophiles (terminal alkenes and a cyclopropene) into the extracellular matrix (ECM) of adipose-derived stem cells (ASCs). A subsequent inverse-electron-demand Diels-Alder (IEDDA) reaction allowed the equipment of the ECM with bioactive molecules, opening numerous applications.