Azido-functionalized gelatin via direct conversion of lysine amino groups by diazo transfer as a building block for biofunctional hydrogels

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Abstract
Gelatin is one of the most prominent biopolymers in biomedical material research and development. It is frequently used in hybrid hydrogels, which combine the advantageous properties of bio-based and synthetic polymers. To prevent the biological component from leaching out of the hydrogel, the biomolecules can be equipped with azides. Those groups can be used to immobilize gelatin covalently in hydrogels by the highly selective and specific azide–alkyne cycloaddition. In this contribution, we functionalized gelatin with azides at its lysine residues by diazo transfer, which offers the great advantage of only minimal side-chain extension. Approximately 84–90% of the amino groups are modified as shown by 1H-NMR spectroscopy, 2,4,6-trinitrobenzenesulfonic acid assay as well as Fourier-transform infrared spectroscopy, rheology, and the determination of the isoelectric point. Furthermore, the azido-functional gelatin is incorporated into hydrogels based on poly(ethylene glycol) diacrylate (PEG-DA) at different concentrations (0.6, 3.0, and 5.5%). All hydrogels were classified as noncyctotoxic with significantly enhanced cell adhesion of human fibroblasts on their surfaces compared to pure PEG-DA hydrogels. Thus, the new gelatin derivative is found to be a very promising building block for tailoring the bioactivity of materials.

KEYWORDS
biocompatibility, biopolymers, functional materials, hydrogels, tissue engineering

1 | INTRODUCTION

In recent years, the number of publications dealing with the development and characterization of biomaterials has risen steadily (Geckil, Xu, Zhang, Moon, & Demirci, 2010; Magin, Alge, & Anseth, 2016; Tibbitt & Anseth, 2009). Biomaterials are by definition of the European Society for Biomaterials “materials intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body.” In order to meet this definition, biomaterials have to mimic the tissue-specific extracellular matrix (ECM) as precisely as possible, as the ECM is the natural microenvironment of cells in the human body. Therefore, biomaterials have to fulfill a wide range of requirements. Besides being compatible with the body, they also have to meet certain mechanical requirements (O’Brien, 2011). The stimuli of materials toward cells are mainly given by stiffness (Engler, Sen, Sweeney, & Discher, 2006; Lo, Wang, Dembo, & Wang, 2000; Poveda-Reyes et al., 2016), surface topography (Dalby, Gadegaard, & Orefo, 2014; Greiner et al., 2016),
hydrophilicity (Wischerhoff et al., 2008), and the overall (bio)chemical composition (Jianhua et al., 2009; Kluger et al., 2009; Southan et al., 2014). The combination of these properties defines the tissue-specific material biofunctionality (Tibbitt & Anseth, 2009).

Up to now, the use of natural ECM such as decellularized tissues or in vitro-generated cell-derived ECM is highly promising, yet very challenging as it is quite difficult to isolate natural ECM in large amounts and to process the complex and insoluble organic material (Naba, Clauer, & Hynes, 2015). Therefore and due to their chemical, physical, and mechanical properties, hydrogels are an important class of materials to mimic the hydrated and highly cross-linked natural ECM (Nguyen, Hwang, Chen, Varghese, & Sah, 2012; Slaughter, Khurshid, Fisher, Khademhosseinii, & Peppas, 2009; Tuin et al., 2012; Van Vlierberge, Dubrueil, & Schacht, 2011).

Hydrogels are hydrophilic polymer networks, which are swollen in water and thus offer an aqueous, viscoelastic environment in which cells can survive and proliferate (Ahmed, 2015; Mouser et al., 2016; Peppas & Merrill, 1977). A major approach to combine the advantageous properties of synthetic and bio-based polymers is their combination in hybrid hydrogels (Hoang Thi, Lee, Lee, Park, & Park, 2016; Jia & Klick, 2009; Lau & Klick, 2015; Lutolf & Hubbell, 2003; Rizzi & Hubbell, 2005; Zhu, 2010). For this purpose, it is possible to simply mix bio-based polymers into synthetic hydrogel formulations which can be cross-linked afterward. However, in this case, the bio-based polymers can often not be bound tightly into the hydrogel network due to insufficient reactivity of the bio-based polymers (Jaiswal, Gupta, Dinda, & Koul, 2015). As a result, the bio-based polymers can leak out of the hybrid hydrogels upon washing, leading to a decreased bioactivity over time (Fu et al., 2012).

To overcome this issue, bio-based polymers are often chemically modified with functional groups, which can participate in the cross-linking reaction, resulting in a covalent immobilization of the bio-based polymer in the hydrogel (Hu, Li, Zhou, & Gao, 2011; Hutson et al., 2011). For instance, we recently described the preparation of an azido-functionalized decellularized cell-derived ECM and its use as potential biomaterial (Ruff et al., 2017; Keller et al., 2020). Although this material has an excellent biofunctionality, it is up to now very difficult to isolate it in large amounts and to use it for biofunctionalization of three-dimensional bulk materials. A bio-based material, which is a promising candidate for biofunctionalization and which is furthermore available in large quantities, is gelatin. This biofunctional polypeptide was modified in different ways in order to participate in different cross-linking reactions which resulted in gelatin derivatives like gelatin methacryloyl (Claassen et al., 2018; Hoch, Hirth, Tovar, & Borchers, 2013; Ramon-Azcon et al., 2012; Zhao et al., 2016), thiolated gelatin (Cao, Lee, Peled, & Venkatraman, 2016; Fu et al., 2012), and also azido-functional gelatin (AzGel, details see below; Heo et al., 2016; Tamura et al., 2015; Truong et al., 2015; Merrill & Unruh, 1963; Narayani & Panduranga, 1996). They undergo radical cross-linking, thiol-Michael cross-linking, and Huisgen cycloaddition cross-linking, respectively. They usually prove to be nontoxic and their bioactivity remained present also after modification (Egger, Tovar, Hoch, & Southan, 2016; Hoch, Tovar, & Borchers, 2016). In order to be able to couple gelatin covalently into hydrogels prepared with different cross-linking chemistry, it is of great interest to make gelatin derivatives available with different functional groups. Especially, the use of azido groups for chemical modification is attractive due to the nature and reactivity of the azides in the 1,3-dipolar Huisgen cycloaddition which generally is accepted as a click reaction (Kolb, Finn, & Sharpless, 2001). For gelatin, azido-functionalization was achieved in one-step or two-step polymer-analogous reactions either with the use of organic solvents (Truong et al., 2015) or in aqueous solution (Heo et al., 2016; Narayani & Panduranga, 1996; Tamura et al., 2015). In these cases, the nucleophilic lysine amino groups were utilized for this purpose by coupling of electrophilic, azido-functional small molecules to gelatin. As a result, rather hydrophobic side chains were introduced into the polymer.

In this contribution, we introduce a novel synthesis strategy utilizing the azido transfer reagent imidazole-1-sulfonyl azide hydrochloride published by Goddard-Borger and Stick (2007). This innovative synthesis strategy offers the great advantage of only minimal side chain extension and takes place in a one-step polymer-analogous reaction in an aqueous environment. We describe the extensive characterization of the resulting AzGel in terms of its chemical, rheological/physicomechanical, and biological properties not only as raw material but also incorporated into hybrid hydrogels with synthetic poly(ethylene glycol) diacrylate (PEG-DA).

2 MATERIALS AND METHODS

2.1 Materials

The following materials were obtained from the commercial sources given in parentheses: tissue culture flasks, centrifugation tubes, 96-well plates, and polystyrene petri dishes (Greiner Bio One GmbH, Frickenhausen, Germany), trypsin (supplemented with Versene [Ethylenediaminetetraacetic acid, EDTA]), the aqueous mounting medium (Lab Vision™ PermaFluor™), the fluorophore Alexa Fluor® 546-Phalloidin, fetal calf serum (FCS; Gibco) and 1% penicillin/streptomycin (pen/strep; Thermo Fisher Scientific, Darmstadt, Germany), deuterium oxide, bovine serum albumin (BSA, dissolved in phosphate-buffered saline, PBS−), Triton X-100, PBS− (without calcium chloride and magnesium chloride), Tween 20, sodium dodecyl sulfate (SDS, 20% in ultrapure water, H2O), PEG-DA (number average molecular weight 700 g/mol), copper(II) sulfate pentahydrate (CuSO4 5H2O), Na-L-ascorbate, potassium carbonate, 2,4,6-trinitrobenzene-sulfonic acid (TNBS), glycine, hydrochloric acid (HCL), SDS solution and saponin, sodium hydrogen phosphate (Na2HPO4; Sigma Aldrich Chemie GmbH, Taufkirchen, Germany), the photoinitiator Irgacure® 2959 (BASF, Ludwigshafen, Germany), the formalin solution (Roti®-Histofix), sodium hydrogen carbonate and sodium chloride (Carl Roth, Karlsruhe, Germany), 4’,6-diamidino-2-phenylindole (DAPI), was dissolved in PBS+; Serva Electrophoresis GmbH, Heidelberg, Germany), Dulbecco’s modified Eagle medium (DMEM; Biochrom AG, Berlin, Germany), the Cell-Titer 96™ Aqueous One Solution Cell Proliferation Assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium [MTS] assay, inner salt; Promega, Madison, WI), filter papers (Macherey-Nagel, Düren, Germany), gelatin (type B, bovine bone, 232 g Bloom, viscosity 4.5 mPas, batch no. 635621; GELITA, Eberbach, Germany), glass slides (Polysine™).
cleaned) and glass cover slips (R. Langenbrinck, Emmendingen, Germany), dialysis tubes with a molecular weight cutoff of 12–14 kDa (Medicell Membranes, Greenich, UK), Amberlite® IRN150 and sodium 3-trimethylsilyl-propionate-2,2,3,3-d4 (TMSP; Merck, Darmstadt, Germany), and paraffilm (Bemis, Neenah, WI). All commercial reagents were used as received without further purification. For cell culture, DMEM was supplemented with 10% FCS and 1% pen/strep. The azido transfer reagent imidazole-1-sulfonyl azide hydrochloride was synthesized according to Goddard-Borger and Stick (2007) and the heterobifunctional linker N-propargylacrylamide according to Yilmaz, Kahveci, and Yagci (2011). Ultrapure water was withdrawn from a Barnstead GenPure xCAD water purification system (Thermo Scientific). Human primary fibroblasts were isolated from human foreskin, ages 2–12, obtained from six healthy volunteers under informed consent according to ethical approval granted by the ethical committee of the Landesärztekammer Baden-Württemberg (IGBZSF-2012-078). The fibroblast isolation was performed as previously described (Kluger et al., 2010). Fibroblasts were seeded in 175-cm² tissue culture flasks in supplemented DMEM. Cells were expanded until passage nine with 5% carbon dioxide, CO₂ at 37 °C. The amino acid composition published by Sewald et al. (2018) was changed for IEP calculation of AzGel by partly replacing lysine and hydroxylysine by glycine to simulate the derivatization of amino groups. Remaining amino groups after gelatin modification were determined using TNBS with Habeeb's method (Habeeb, 1966). Briefly, 25 μl of gelatin solutions (unmodified gelatin 5 mg/ml; gelatin derivatives 20 mg/ml) were pipetted into 96-well plates, 25 μl sodium hydrogen carbonate solution (4% wt/vol, pH 8.5), and 25 μl TNBS (0.1% vol/vol) were added. The plates were incubated (HERAsafe incubator, Thermo Fisher Scientific, Waltham, MA) for 2.5 h at 37 °C in the dark with gentle shaking. Then 25 μl SDS solution (10% wt/vol) and 12.5 μl HCl (1 mol/l) were added and absorption was measured at 330 nm using a fluorescence microplate reader (Tecan Reader Synergy 2) from BioTek (Bad Friedrichshall, Germany). Calculation of amino groups was done using a glycine standard curve (0.02–2.5 mmol/l). Size exclusion chromatography (SEC) was conducted as described before for gelatin methacryloyl derivatives (Sewald et al., 2018). A 1260 Infinity GPC-SEC Analysis System (Agilent Technologies, Waltham, MA) was used equipped with refractive index detector and the Suprema Linear S column (PSS, Berlin, Germany; nominal separation range 10⁻²–10⁻⁴ Da). Measurements were performed at 40 °C with 11.88 g/l Na₂HPO₄ (pH 9.2) as eluent (1 mg/ml sample concentration, 50 μl injection volume, 0.5 ml/min flow rate). For mechanical characterization of hydrogels, oscillatory rheology was performed on a Physica MCR 301 (Anton Paar) using a parallel plate geometry with a diameter of 20 mm. Amplitude sweeps (frequency = 1 Hz, amplitudes between 0.01 and 100%) and frequency sweeps (amplitude = 0.1%, frequencies between 0.1 Hz and 10 Hz) for the swollen chemical hydrogels were obtained with a normal force of 1 N at a temperature of 20 °C. Swelling behavior of hydrogels was determined by drying the surface of the swollen hydrogels on a filter paper and weighing them to determine the mass after swelling (mₛ). The hydrogels were then dried for 72 hr in a vacuum drying oven (Binder GmbH, Tuttingen, Germany) to determine the dry mass (m_d). The gravimetical equilibrium degree of swelling (Qₑ) was then calculated with:

\[ Qₑ = \frac{(mₛ - m_d)}{m_d} \times 100\% \]  

2.2 Analytical instrumentation and methods

Confocal laser scanning microscopy (LSM) was carried out using the device LSM 710 from Carl Zeiss AG (Oberkochen, Germany). Image processing was done with the software ImageJ downloaded from http://imagej.nih.gov/ij/. For absorbance measurements, the microplate reader Infinite M200pro from the Tecan Group Ltd (Männedorf, Switzerland) was used. Attenuated total reflection Fourier-transform infrared (ATR-FT-IR) spectra were recorded on a Bruker Vertex70 spectrometer equipped with a “Platinum ATR” single reflection unit with a diamond crystal. Measurements of freshly freeze-dried samples were performed with 60 scans from 4,000 to 400 cm⁻¹ with resolution of 4 cm⁻¹. Background measurements were performed before every sample measurement under the same conditions.

1H-NMR spectra were recorded in deuterium oxide (D₂O) using TMSP as internal standard on a Bruker Avance 500 spectrometer. The concentration of acrylamide groups in AzGel after conjugation with N-propargylacrylamide was determined similar to Claassen et al. (2018). The isoelectric points (IEP) of gelatin derivatives were measured by a deionization method according to the method recently published for methacryloylated gelatin (Sewald et al., 2018) being based on the deionization method of Janus, Kenchington, and Ward (1951). In short, gelatin and its derivatives were dissolved in ultrapure water (5% wt/vol) at 60 °C (unmodified gelatin) or 40 °C (azido-modified gelatin, AzGel) and shaking. Afterward, mixed-bed ion exchanger Amberlite® IRN150 was added and shaken at 60 or 40 °C, respectively, for additional 30 min. Deionization of gelatin was assumed in the case of a conductivity <50 μS/cm. As the isionic and IEP of gelatin are equal if deionized in this way, the pH of the deionized gelatin solution was equivalent to its IEP (Janus et al., 1951). The theoretical IEP was calculated using the ExPASY Proteomics tools site (Hattori et al., 1999). The amino acid composition published by Sewald et al. (2018) was changed for IEP calculation of AzGel by partly replacing lysine and hydroxylysine by glycine to simulate the derivatization of amino groups. Remaining amino groups after gelatin modification were determined using TNBS with Habeeb’s method (Habeeb, 1966). Briefly, 25 μl of gelatin solutions (unmodified gelatin 5 mg/ml; gelatin derivatives 20 mg/ml) were pipetted into 96-well plates, 25 μl sodium hydrogen carbonate solution (4% wt/vol, pH 8.5), and 25 μl TNBS (0.1% vol/vol) were added. The plates were incubated (HERAsafe incubator, Thermo Fisher Scientific, Waltham, MA) for 2.5 h at 37 °C in the dark with gentle shaking. Then 25 μl SDS solution (10% wt/vol) and 12.5 μl HCl (1 mol/l) were added and absorption was measured at 330 nm using a fluorescence microplate reader (Tecan Reader Synergy 2) from BioTek (Bad Friedrichshall, Germany). Calculation of amino groups was done using a glycine standard curve (0.02–2.5 mmol/l). Size exclusion chromatography (SEC) was conducted as described before for gelatin methacryloyl derivatives (Sewald et al., 2018). A 1260 Infinity GPC-SEC Analysis System (Agilent Technologies, Waltham, MA) was used equipped with refractive index detector and the Suprema Linear S column (PSS, Berlin, Germany; nominal separation range 10⁻²–10⁻⁴ Da). Measurements were performed at 40 °C with 11.88 g/l Na₂HPO₄ (pH 9.2) as eluent (1 mg/ml sample concentration, 50 μl injection volume, 0.5 ml/min flow rate). For mechanical characterization of hydrogels, oscillatory rheology was performed on a Physica MCR 301 (Anton Paar) using a parallel plate geometry with a diameter of 20 mm. Amplitude sweeps (frequency = 1 Hz, amplitudes between 0.01 and 100%) and frequency sweeps (amplitude = 0.1%, frequencies between 0.1 Hz and 10 Hz) for the swollen chemical hydrogels were obtained with a normal force of 1 N at a temperature of 20 °C. Swelling behavior of hydrogels was determined by drying the surface of the swollen hydrogels on a filter paper and weighing them to determine the mass after swelling (mₛ). The hydrogels were then dried for 72 hr in a vacuum drying oven (Binder GmbH, Tuttingen, Germany) to determine the dry mass (m_d). The gravimetical equilibrium degree of swelling (Qₑ) was then calculated with:

\[ Qₑ = \frac{(mₛ - m_d)}{m_d} \times 100\% \]  

2.3 Synthesis of AzGel

2.52 g of gelatin (containing approximately 0.88 mmol lysine residues, 1.0 eq.; Van Den Bulcke et al., 2000) was dissolved in a solution of 1.03 g potassium carbonate (7.5 mmol, 8.5 eq.) and 0.137 g CuSO₄ 5H₂O (0.55 mmol, 0.63 eq.) in 250 ml of water at 37 °C. After complete dissolution of the gelatin, 0.159 g imidazole-1-sulfonyl azide hydrochloride (0.76 mmol, 0.86 eq.) was added. The resulting purple solution was stirred overnight at 37 °C. The solution was then dialyzed against ultrapure water for 5 days, exchanging the water on a daily basis. The solution was lyophilized and 2.25 g of a pale blue powder were obtained (yield: 89%).

2.4 Preparation of AzGel-containing hydrogels

Hydrogel formulations containing AzGel were prepared with AzGel concentrations of 5.5% (wt/wt), 3.0% (wt/wt), and 0.6% (wt/wt). In
the following, the preparation of the formulation containing 5.5% (wt/wt) is explained. The other concentrations were prepared accordingly with reduced concentrations of N-propargylacrylamide, AzGel, CuSO\textsubscript{4} \(5\)H\textsubscript{2}O and Na-L-ascorbate. First, 1 g of a stock solution of the heterobifunctional linker N-propargylacrylamide was prepared in water with a concentration of 0.03 M, thus containing 30 μmol linker (1 eq.). In addition, stock solutions of CuSO\textsubscript{4} \(5\)H\textsubscript{2}O (0.01 M) and Na-L-ascorbate (0.1 M) were prepared. In the heterobifunctional linker solution, 100 mg of AzGel (containing approximately 30 μmol azido groups, 1 eq.) were dissolved and 60 mg of the CuSO\textsubscript{4} stock solution (0.6 μmol CuSO\textsubscript{4}, 0.02 eq.) together with 30 mg of the Na-L-ascorbate stock solution (3 μmol Na-L-ascorbate, 0.1 eq.) were added. The mixture was reacted for at least 16 hr at 37°C in a thermomixer (Grant Instruments, Cambridge, UK; 1,400 rpm). To prevent precipitation of the gelatin during the addition of the PEG-DA hydrogel solution, 34.8 mg NaCl were added, resulting in a total mass of the solution of 1.225 g and a concentration of AzGel in the solution of 8.2% (wt/wt). We then prepared a stock solution of the photoinitiator (Irgacure® 2959) with a concentration of 7 mg/g (wt/wt) by heating the appropriate amount of water and Irgacure® 2959 with a heat gun (Steinel America Inc., Bloomington, MN) to approximately 100°C and subsequent cooling to room temperature. Next, 0.128 g of the Irgacure® 2959 stock solution were added to 0.179 g PEG-DA so that the mass of the photoinitiator in the solution equaled 0.5% (wt/wt) relative to the PEG-DA mass. In order to reach the final concentration of 20% (wt/wt) PEG-DA in the hydrogel solution, we added 0.588 g of the above solution containing the linker-conjugated AzGel leading to a solution which in the end contained 5.5% (wt/wt) AzGel. The solution was then pipetted into a cylindrical aluminum mold (diameter: 3 cm, height: 1 mm), covered with a quartz glass pane (GVB GmbH Solutions in Glass, Herzogenrath, Deutschland) and cured under ultraviolet A (UVA) light (Dr. Hönle AG, Gräfelfing, Germany; 50 mW/cm\textsuperscript{2}) for 7.5 min.

For the preparation of hydrogels containing unmodified gelatin, gelatin and water were added to the solution containing PEG-DA and Irgacure® 2959 so that identical concentrations as for the AzGel were obtained. For the preparation of hydrogels without AzGel, water was added to the solution containing PEG-DA and Irgacure® 2959 until a concentration of 20% (wt/wt) of PEG-DA was reached. With the help of a micro spatula the polymerized hydrogels were then removed from the mold and transferred to a petri dish filled with 20 ml of ultrapure water. Depending on the experiments, the obtained hydrogels were then washed in ultrapure water or sterile cell culture media, respectively.

### 2.5 Biological characterization of AzGel-containing hydrogels

Biological characterization of the AzGel-containing hydrogels included the assessment of the cytotoxicity as well as the cell response of primary human dermal fibroblasts to AzGel-containing hydrogels.

#### 2.5.1 Cytotoxicity assessment

Cytotoxicity of PEG-DA hydrogels, both with and without the addition of AzGel was evaluated in an MTS assay (Barltrop, Owen, Cory, & Cory, 1991; Goodwin, Holt, Downes, & Marshall, 1995). For this, the hydrogels were extracted with 1 ml of DMEM per 0.1 g of the previously washed and swollen hydrogels for 72 hr at 37°C, 95% humidity, and 5% CO\textsubscript{2}. Pure DMEM served as reference and was treated identically. The hydrogels were then removed and the resulting extracts were stored at 4°C until the cytotoxicity assay was performed (storage time not exceeding 24 hr).

Twenty-four hours before the extraction was completed, primary fibroblasts were seeded into the wells of a 96-well plate. For this, the concentration of the cell suspension was adjusted to \(10^5\) cells/ml of supplemented DMEM and 200 μl of this suspension was transferred to each of the wells of the 96-well plate (2 × 10^4 cells/well). For each hydrogel extract, six wells were seeded with fibroblasts. As reference, six wells were filled with 200 μl of DMEM without cells. Cells were then incubated overnight (37°C, 5% CO\textsubscript{2}, 95% humidity) in order to allow cell adhesion. After the complete adhesion, DMEM was removed from the cell layers and replaced by the respective extracts which were previously supplemented with 10% FCS. As a negative control, supplemented DMEM was added to the wells and as a positive, cytotoxic control supplemented DMEM including 1% SDS was added to the wells. The cells were again incubated for 24 hr under standard cell culture conditions. After that, the extracts or DMEM, respectively, were removed from the wells. Next, 120 μl of MTS working solution (20 μl MTS stock solution + 100 μl DMEM without any supplements) were added to each well. As soon as the absorption of the negative control reached values between 0.8 and 1 (usually after 30–45 min at 37°C) the absorption of the samples was measured at 492 nm.

For the quantification of the absorption values, mean values were calculated from the individual measurements and the mean absorption of the references was subtracted. The number of living cells in culture/cell viability was expressed relative to the absorption values of the positive control which was set to 0% and the negative control which was set to 100%. Cytotoxicity of the hydrogels was assessed using the international standard DIN EN ISO 10993-5, which defines relative cell proliferation rates >80% to be noncytotoxic.

#### 2.5.2 Cell response

Cell response to the AzGel-containing PEG-DA hydrogels was evaluated by seeding primary human dermal fibroblasts (up to passage 9) onto the hydrogel surfaces. For this purpose, cylindrical hydrogels with a diameter of 3.5 cm were prepared which had a cylindrical depression in the middle with a diameter of 2.4 cm and a depth of 0.5 mm. After preparation, the hydrogels were washed with water for 24 hr and then twice with supplemented DMEM, each washing step taking 24 hr. Next, a suspension containing primary human dermal fibroblasts was pipetted into the depression resulting in a cell density
of $6 \times 10^4$ cells/cm² of the depression surface. The cells were allowed to adhere for the first 3 hr under standard cell culture conditions (5% CO₂, 37°C, 95% humidity). After 3 hr, 3 ml supplemented DMEM was added carefully and the cells were incubated under static cell culture conditions (5% CO₂, 37°C, 95% humidity) for additional 93 hr. Afterwards, the cells were rinsed with PBS⁻, fixed with formalin solution (4%) for 10 min at room temperature (RT), permeabilized by saponin (0.2% in PBS⁻) for 10 min and incubated with blocking solution (PBS⁻ + 3% BSA + 0.1% Triton X-100) for 10 min.

Prior to the LSM analysis, cytoskeletons of the fibroblasts were stained with Alexa Fluor® 546-phalloidin (3 units/ml) and nuclei with DAPI (1 μg/ml) in blocking solution for at least 30 min at RT. Cells were then washed two times with 0.1% Tween 20 in PBS⁻ and thereafter, once in PBS⁻. In order to image the stained cells by confocal LSM, the outer ring of the hydrogel was removed with a scalpel so that the hydrogel could be covered with one drop of mounting media and a glass cover slip. To prevent desiccation, another glass cover slip was used to cover the bottom side of the hydrogel.

For quantification of the cells present on the hydrogel surfaces, we performed three independent experiments with cells of three different donors on three independently prepared hydrogels for each concentration level. Confocal LSM images were generated from three positions (top, middle, bottom) of each hydrogel sample. The number of cells was then analyzed using the digital image processing software ImageJ. Cell nuclei were identified in a threshold analysis and quantified using the "analyze particles" function (cutoff size: <20 μm).

2.6 | Data and statistical analysis

Statistical significance was assessed by a one-tailed analysis of variance combined with a post hoc test using Bonferroni correction. Data were expressed as mean values ± standard deviation (SD). As labeled in the graphs, p values lower than $\alpha = 0.05$ (*), $\alpha = 0.01$ (**), or $\alpha = 0.001$ (****) were defined as statistically significant.

3 | RESULTS

3.1 | Synthesis and characterization of AzGel

Gelatin was modified by a diazo transfer reaction (Figure 1, first reaction step) using the diazo transfer reagent imidazole-1-sulfonyl azide hydrochloride first described by Goddard-Borger and Stick (2007). The reaction outcome was assessed by ¹H-NMR spectroscopy and ATR-FT-IR spectroscopy, the photometric TNBS amino group assay as well as by comparing properties like the IEP or the thermogelling properties. The results of AzGel characterization compared to unmodified gelatin are summarized in Table 1.

In the ¹H-NMR spectrum of gelatin, the ε-CH₂ group of lysine was described to give a relatively isolated signal around 3 ppm (Rodin & Izmailova, 1996). In fact, we observed such a signal in the unmodified gelatin, which was reduced to an intensity of approx. 10% after the diazo transfer reaction in AzGel (Figure 2). Concomitantly, a new signal around 3.3 ppm appeared in the AzGel spectrum. In

![FIGURE 1](image-url) Reaction scheme for the incorporation of azido-functionalized gelatin into PEG-DA hydrogels. (a) In the first step, gelatin is azido-functionalized by diazo transfer to the lysine amino groups. (b) The azide groups are then reacted with the heterobifunctional linker N-propargylacrylamide. (c) The thus functionalized gelatin is then covalently bound into PEG-DA hydrogels by photocuring with ultraviolet A (UVA) light. PEG-DA, poly(ethylene glycol) diacrylate.
addition, in the 1H-NMR spectrum of AzGel, no residues of the diazo transfer reagent or of any other impurity were observed.

In order to further quantify the conversion of the amino groups during diazo transfer, the TNBS assay was applied (Habeeb, 1966). This photometric assay gives an absorbance proportional to the amount of primary amino groups present in the sample and was used for analyzing the degree of modification in methacryloylated gelatins before (Hoch, Schuh, Hirth, Tovar, & Borchers, 2012). By this method, we found a decrease of the amino group concentration after diazo transfer of 84%. To further prove that this reduction of amino groups is accompanied by the introduction of azido groups into gelatin, the presence of azido groups in AzGel was probed by ATR-FT-IR spectroscopy of unmodified gelatin and AzGel (Figure 3). In the AzGel ATR-FT-IR spectrum, a new characteristic absorption band, which is not present in the gelatin spectrum, was observed at 2102 cm$^{-1}$.

Apart from that, the spectra were practically identical for unmodified gelatin and AzGel.

It can be expected that chemical modification of gelatin also has an impact on its ability to form physical hydrogels (Wang et al., 2017). Therefore, we measured storage moduli $G_0$ and loss moduli $G_00$ of solutions containing 10% (wt/wt) gelatin or AzGel at different temperatures (Figure 4). At high temperatures, both the AzGel and gelatin solutions were mobile liquids as indicated by the low $G_00$ values and the non-measurable $G_0$ values. Upon cooling, an increase of both, $G_0$ and $G_00$, was observed for both solutions until $G_0$ finally was larger than $G_00$. At this temperature $T_{gel,down}$, a physical hydrogel was formed. Also in the heating curves, an intersection of $G_0$ and $G_00$ was observed at the temperature $T_{gel,up}$ above which the gels were mobile liquids again. As shown in Table 1, the value for $T_{gel,down}$ decreased after diazo transfer from 24.7 to 18.6°C. Also the value for $T_{gel,up}$ decreased from 31.4 to 25.3°C. Due to the conversion of amino groups during diazo transfer, also the IEP of AzGel should be different from unmodified gelatin. In fact, we measured an IEP of 4.9 for the unmodified gelatin, and for AzGel we found an IEP of 4.2.

### Table 1

| IEP   | $T_{gel,down}$ a (°C) | $T_{gel,up}$ b (°C) | $Y_{NMR}$ c (%) | $Y_{TNBS}$ d (%) |
|-------|------------------------|---------------------|-----------------|-----------------|
| Gelatin | 4.9 | 24.7 | 31.4 | – | – |
| AzGel | 4.2 | 18.6 | 25.3 | 90 | 84 |

Abbreviations: AzGel, azido-functional gelatin; IEP, isoelectric point; TNBS, 2,4,6-trinitrobenzene-sulfonic acid.

a $T_{gel,down}$ = gel transition temperature measured by lowering the temperature from 40 to 10°C.

b $T_{gel,up}$ = gel transition temperature measured by increasing the temperature from 10°C to 40°C.

$Y_{NMR}$ = amino group conversion determined by 1H-NMR spectroscopy.

$Y_{TNBS}$ = amino group conversion determined by the TNBS assay.
Another important property of polymers is their molecular weight distribution which we analyzed by SEC. The elugrams of SEC analysis showed that AzGel was eluted at higher elution volumes compared to unmodified gelatin, indicative of smaller hydrodynamic radii of AzGel compared to unmodified gelatin (Figure 5). Apart from the shift to higher elution volumes, no additional shoulders or low-molecular-weight fractions were visible, meaning the shapes of the elugrams were very similar.

### 3.2 Preparation and physical characterization of AzGel-containing hydrogels

In the next step, we aimed to make use of the azido groups in AzGel by immobilizing AzGel covalently into hydrogels. This was achieved in two steps as depicted in Figure 1 (second and third reaction steps). First, the heterobifunctional linker N-propargylacrylamide was bound to AzGel in a Huisgen cycloaddition. The outcome of this reaction was assessed by $^1$H-NMR spectroscopy where after the conjugation reaction new signals appear in the typical region for acrylic protons around 5.8 ppm (Figure 2). Concomitantly, the signal around 3.3 ppm vanished, and also in the ATR-FT-IR spectrum, the absorption band at 2102 cm$^{-1}$ disappeared.

After coupling of the linker, the newly formed AzGel-linker derivative was reacted with PEG-DA in aqueous solution by radical photopolymerization of the double bonds. This way, three different initial concentrations (0.6, 3.0, and 5.5%) of AzGel were introduced into PEG-DA hydrogels. In all cases, macroscopic hydrogels, which could be handled easily, were obtained. In addition, hydrogel controls were prepared where the cycloaddition step was omitted and either AzGel or unmodified gelatin was mixed directly into the PEG-DA solutions. Interestingly under such circumstances, with AzGel no macroscopic hydrogels were obtained, whereas with unmodified gelatin hydrogel curing proceeded without difficulties. Photos of the corresponding hydrogels containing either AzGel including the cycloaddition step or unmodified gelatin are depicted in Figure S1. Depending on the concentration of immobilized AzGel or unmodified gelatin, the hydrogels exhibited differences in their appearance. The higher the amount of immobilized AzGel or unmodified gelatin, the higher the opacity of the resulting hydrogels. With higher AzGel concentration, the hydrogels also retained and increasing amount of the greenish-blue color, which could not be washed out of the hydrogels. This color was not observed for the hydrogels containing unmodified gelatin.

In order to investigate the effect of gelatin and AzGel addition into the PEG-DA hydrogels on the physical hydrogel properties, we measured the equilibrium degree of swelling $Q_g$ as well as $G'$ and $G''$ of the resulting hydrogels. The results of the mechanical characterization of gelatin- and AzGel-containing hydrogels are shown in Figure 6. In all cases, $G'$ was one to two orders of magnitude larger than $G''$, showing that mainly elastic hydrogels were obtained. The highest value for $G'$ with 231 kPa was found for PEG-DA hydrogels without AzGel or gelatin. Upon gelatin addition, $G'$ decreased to 130 kPa with 5.5% gelatin. With AzGel addition, $G'$ decreased even further to 95 kPa at 5.5% AzGel concentration. The results for the equilibrium degree of swelling $Q_g$ of PEG-DA hydrogels with the three different concentrations of unmodified gelatin and AzGel, respectively, are shown in Figure 7. Interestingly with 436%, the highest value for $Q_g$ was found for PEG-DA hydrogels containing no gelatin derivate. By adding either 0.6% unmodified gelatin or AzGel, $Q_g$ decreased to 353 and 400%, respectively. Further addition of unmodified gelatin and AzGel had no further great impact on $Q_g$, resulting in values of 333 and 409% with 5.5% gelatin derivatives.
3.3 Biological response to AzGel-containing hydrogels

Because AzGel is a new compound, no toxicity data exist on AzGel-containing materials. Therefore, a cytotoxicity assay of AzGel-containing hydrogels compared to pure PEG-DA hydrogels was performed with primary human fibroblasts. The cytotoxicity assay was based on the reduction of the MTS tetrazolium reagent by viable cells. The results are shown in Figure 8. For pure PEG-DA hydrogels, cell proliferation rates of 95% were found without any washing protocol and of 97% after washing. PEG-DA hydrogels containing AzGel concentrations between 0.6% and 5.5% showed similarly high values between 82% and 113%.

The objective of the covalent integration of AzGel into otherwise bioinert PEG-DA hydrogels was to increase the bioactivity of the material and therefore, to allow cell adhesion onto the hydrogel surface. Furthermore, a suitable amount of AzGel should be identified which allows the adhesion and proliferation of cells on the materials surface. Therefore, we examined the response of primary human fibroblasts to the either AzGel-modified or unmodified PEG-DA hydrogels by determining the cell number present on the hydrogel surfaces after 4 days of static cell culture. Imaging was done by the fluorescence staining of the cytoskeleton and the nuclei followed by a confocal LSM analysis (Figure 9). The LSM images on the left-hand side of Figure 9 as well as in the middle row revealed that an increasing AzGel content also increased the number of cells adherent on the hydrogel surfaces. While there were almost no cells detectable on the unmodified PEG-DA hydrogels, the density of the cell layer increased through the incorporation of AzGel. Even the addition of as little as 0.6% AzGel resulted in an obvious increase in adherent cells. Morphologic effects were also fairly evident in the images with a higher magnification (Figure 9, right column). Here it can be seen that cells adherent on pure PEG-DA hydrogels as well as on PEG-DA hydrogels with a low amount of AzGel exhibited a spherical shape. Generally, the cells tended to form cell clusters, which indicate that these cells strongly avoid contact with the material. With increasing AzGel content, fibroblasts elongated to their typical spindle-like shape and confluent cell layers were formed.

In order to quantify the number of adherent cells, the LSM images were processed with ImageJ. A significantly higher number of adhered cells was detected on the AzGel-containing PEG-DA hydrogels when compared to the unmodified hydrogels after 4 days of culture.
For gels containing 0.6% AzGel, cell number was approximately 18 times higher, for 3.0% AzGel 33 times higher, and for 5.5% AzGel 47 times higher.

4.1 Synthesis and characterization of AzGel

In the first part of this study, we introduced a novel synthesis strategy utilizing the azido transfer reagent imidazole-1-sulfonyl azide hydrochloride published by Goddard-Borger and Stick (2007). This azido transfer reagent was already successfully applied for diazo transfer to proteins like horseradish peroxidase and the red fluorescent protein DsRed, resulting in azido-functionalized proteins (van Dongen et al., 2009). Although the reaction was described to be quite effective, little is known about possible side reactions when used in excess. Therefore, we utilized 0.86 eq. of the diazo transfer reagent relative to gelatin amino groups in order to avoid unnecessary side reactions of unreacted diazo transfer reagent. We slightly modified the existing literature procedures in order to optimize the reaction with gelatin. We verified the successful azido modification of gelatin indirectly by detecting the depletion of amino groups within gelatin affected by the performed diazo transfer. The reason for this is that the main site for modification can be expected to be the lysine amino groups. Using $^1$H-NMR spectroscopy, amino group depletion was 90% according to the $^1$H-NMR experiment, evidenced by the corresponding loss of intensity of the $\varepsilon$-CH$_2$ signal of the lysine residues around 3.0 ppm.
of the (Figure 2), pointing to a successful reaction. However, the reduction

**FIGURE 10** Cell number on PEG-DA hydrogels with different AzGel contents after 4 days of static culture. Cell number was measured by identifying and quantifying cell nuclei in a threshold analysis. The incorporation of AzGel resulted (regardless of the concentration) in a significantly higher cell number as on unmodified PEG-DA hydrogels. AzGel, azido-functional gelatin; PEG-DA, poly(ethylene glycol) diacrylate

(Figure 2), pointing to a successful reaction. However, the reduction of the ε-CH₂ signal in the ¹H-NMR spectrum should be considered as a rough estimate for the conversion of the amino groups during the reaction because of the multitude of signals in the ¹H-NMR spectrum and the fact that the signals are not completely baseline-separated. Therefore, with the TNBS assay another method was used, and indeed the observed amino group reduction of 84% is quite close to the ¹H-NMR results and in very good agreement with the applied amount of diazo transfer reagent, again pointing to a successful and clean reaction outcome.

In addition to the indirect evidence of amino group depletion during the reaction, the new characteristic absorption band at 2102 cm⁻¹ observed in the ATR-FT-IR spectrum (Figure 3), which was not present in the gelatin spectrum before diazo transfer, can be considered as a direct proof of the presence azido groups in AzGel; it can clearly be assigned to the asymmetric stretching vibration of the azido groups (Gai, Coutifaris, Brewer, & Fenlon, 2011). Additional spectroscopic evidence for the presence of azido groups is the new signal in the ¹H-NMR spectrum after diazo transfer at 3.3 ppm (Figure 2). This signal is very close to the reported chemical shift of the ε-CH₂ signal of 6-azido-L-lysine of 3.35 (Wang et al., 2017), which is the expected amino group residue after diazo transfer to lysines in gelatin.

In addition, it can be expected that the modification of the lysine amino groups in AzGel also has a direct impact on physical properties compared to unmodified gelatin. A typical property of gelatin solutions is the formation of physical hydrogels in aqueous solution above certain concentrations and below certain temperatures. It was reported before that depletion of amino groups due to chemical modification leads to less effective formation of physical hydrogels from the solutions (Hoch et al., 2013; Van Hoorick et al., 2017). The rheological characterization of AzGel is in line with these findings by giving decreased $T_{gel,up}$ and $T_{gel,down}$ upon modification (Figure 4).

Another important property of gelatin is its IEP. The IEP of unmodified gelatin type B as used in this study was measured to be 4.9. This acidic IEP is due to hydrolysis of the amino acids asparagine and glutamine into their corresponding acids during gelatin preparation (Schrieber & Gareis, 2007). The observed shift of the IEP from 4.9 to 4.2 upon diazo transfer can be fully explained with the loss of amino groups during synthesis, as it has been recently described for gelatin methacryloyls prepared out of the same batch of raw material as our AzGel (Sewald et al., 2018). In that recent study, the IEPs of all gelatin type B methacryloyls were 4.1. Due to the fact that all accessible amino groups were converted in the case of the investigated gelatin methacryloyls, 4.2 as IEP of our AzGel seems to be comprehensible, since 84–90% of amino groups were converted. To furthermore verify our experimental data, we calculated IEP values with the use of ExPASy proteomics tools site for 84 and 90% conversion of amino groups. The result for both was an IEP of 4.3, which supported our experimental results.

Another method to detect changes of biopolymers upon chemical modification is the investigation of the molecular weight distribution by SEC (Figure 5). The observed shift to higher elution volumes for AzGel compared to unmodified gelatin is similar to what was observed for gelatin methacryloyls before (Sewald et al., 2018) caused by increasing degree of modification. While Van Hoorick et al. (2017) hypothesized that this shift is due to hydrolysis occurring during modification, Sewald et al. (2018) proposed a change in the hydrodynamic radii due to less pronounced formation of secondary, tertiary, and supramolecular structures of the protein. We tend to adhere to the second reasoning, especially because gelatin backbone hydrolysis would result in an increase of amino groups that are found by the TNBS assay, a reasoning already used by Zheng, Zhu, Ferracci, Cho, and Lee (2018) for basic hydrolysis of methacrylate groups present in gelatin methacryloyl. Therefore, we conclude that no pronounced hydrolysis during AzGel synthesis took place and the derivatization was successful. However, further analysis of molecular weight distributions of AzGel by absolute methods would be necessary to settle this question.

Thus, all results show a successful and clean introduction of azido groups into gelatin by diazo transfer, yielding AzGel in sufficient purity. The azido group concentration in AzGel can be estimated using the concentration of amino groups in gelatin of 0.35 mmol/g reported by Van Den Bulcke et al. (2000). Using the amino group conversions as summarized in Table 1, this would result in a concentration range of azido groups in AzGel between 0.315 mmol/g (determined by ¹H-NMR by reference to lysine groups) and 0.294 mmol/g (determined by TNBS amino group assay). This translates into an average molecular weight between azido groups of 3,175 g/mol (¹H-NMR, lysines) and 3,401 g/mol (TNBS assay). Taken together, this innovative synthesis strategy offers the great advantage of only minimal side chain extension and takes place in a one-step polymer-analogous reaction in an aqueous environment.
4.2 Preparation and physical characterization of AzGel-containing hydrogels

To equip the newly synthesized AzGel with photopolymerizable groups, AzGel was conjugated with the alkyne-functional linker N-propargylacrylamide in a Huisgen 1,3-dipolar cycloaddition between azides and alkynes. The success of this reaction could be directly seen in the \(^1\)H-NMR spectrum (Figure 2) through the appearance of the double bond protons from the coupled linker molecules around 5.8 ppm. Based on these double bond protons, we calculated an acrylamide concentration of 0.34 mmol/g after the conjugation reaction which translates into an average molecular weight between acrylamide groups of 3,175 g/mol. These values are in a similar range as the azido group concentrations and average molecular weights between azido groups in AzGel. With respect to the accuracy of \(^1\)H-NMR determination, these results suggest a complete conversion of azido groups. Moreover, the azide band in the ATR-FT-IR spectra (Figure 3) at 2102 cm\(^{-1}\) disappeared after conjugation reaction with the alkyne-functional linker N-propargylacrylamide.

To prepare AzGel-containing PEG-DA hybrid hydrogels, the AzGel-linker conjugate was reacted with PEG-DA in aqueous solution by radical photopolymerization of the double bonds. AzGel was thereby introduced to PEG-DA hydrogels in three different initial concentrations (0.6%, 3.0%, 5.5%). PEG-DA with unmodified gelatin served as control. It is well known that azido groups are often not stable under the conditions of free radical polymerizations and participate in side reactions making the polymerization impossible (Hua et al., 2005; Sumerlin, Tsarevsky, Louche, Lee, & Matyjaszewski, 2005). Therefore, the hydrogel formation was only possible after conversion of the azido groups to the corresponding triazoles, also giving an indication towards a successful cycloaddition.

Opacity of the prepared hydrogels correlated with the amount of incorporated AzGel (Figure S1). The higher the AzGel concentration, the higher the opacity of the resulting hybrid hydrogel. The fact that higher AzGel concentrations also lead to an increasing greenish-blueish color of the gel is probably caused by the complexation of the copper(II) ions used for coupling of N-propargylacrylamide to the AzGel, an effect which is used, for example, in the biuret reaction for protein quantification (Gornall, Bardawill, & David, 1949). This color was not observed for the hydrogels containing unmodified gelatin because no copper(II) ions were used for preparing these samples. However, a similar trend in opacity was detectable.

To characterize the physicomechanical properties of the prepared hybrid hydrogels, \(G'\), \(G''\), and \(Q_e\) were analyzed (Figures 6 and 7). It turned out that the integration of AzGel into the PEG-DA hydrogel network compared to pure PEG-DA hydrogels caused a reduction of \(G'\). This effect was not expected. By adding AzGel, both the solid content of the hydrogels as well as the cross-link density in the hydrogels were expected to increase which should result in an increase of \(G'\). This does not seem to be the case. However, our findings are in accordance with a recent literature report given by Berkovich and Seliktar (2017), who integrated gelatin covalently into PEG-DA networks by thiol-Michael addition and also found a decrease of \(G'\). They speculated that gelatin self-assembly interferes with PEG-DA cross-linking. From our perspective, the reason for this behavior is not entirely clear at this point. In any case, a similar effect for both, AzGel and gelatin was observed, so that the azido transfer to gelatin did not change the underlying property fundamentally. However, the effect of decreasing \(G'\) was more pronounced for AzGel which might be explained with trace amounts of unreacted azido groups prior to curing which interfere with cross-linking as described above.

The higher \(Q_e\) value for the AzGel-containing hydrogels compared to the gelatin-containing hydrogels turned out to be consistent with the measurements of \(G'\) (Figure 6). Both, a lower value for \(G'\) as well as a higher value of \(Q_e\) point to a less densely cross-linked polymer network for the AzGel-containing hydrogels. However, the lower \(Q_e\) values both, for AzGel and gelatin containing hydrogels compared to pure PEG-DA hydrogels cannot be explained in this way because also \(G'\) of PEG-DA hydrogels was larger. For understanding this effect, it is helpful to look at the Flory-Huggins interaction parameters \(\chi_{PEG}\) of PEG (0.426) (Merrill, Dennison, & Sung, 1993) and \(\chi_{gel}\) of gelatin (0.49) (Bohdar & Jena, 1993). The larger value for \(\chi_{gel}\) compared to \(\chi_{PEG}\) indicates that water is a poorer solvent for gelatin than it is for PEG. Thus, the hydrogels containing both, gelatin and AzGel can absorb less water than their gelatin-free counterparts, although \(G'\) is also smaller. In summary, these results proof that it is possible to couple the AzGel synthesized in this contribution in different concentrations covalently into PEG-DA hydrogels.

4.3 Biological response to AzGel-containing hydrogels

Equipping gelatin with azido groups is of great interest, as these groups are well known to be able to participate in highly selective and specific azide-alkyne cycloadditions. Azide-alkyne cycloaddition is a desirable tool for (bio)conjugation such as the coupling with the alkyne-functional linker N-propargylacrylamide used in this contribution. In the case of the hydrogels prepared above, especially the copper(I)-catalyzed Huisgen cycloaddition for reacting AzGel with N-propargylacrylamide could have an effect on the toxicity of the hydrogels because copper(I) ions are known to be cytotoxic (Gutteridge & Wilkins, 1983).

Therefore, we first assessed the cytotoxicity of extracts made from PEG-DA hydrogels with and without different concentrations of AzGel using the MTS assay (Figure 8). The international standard DIN EN ISO 10993-5 defines relative cell proliferation rates \(>80\%\) to be noncytotoxic. As expected, neither PEG-DA hydrogels directly after preparation (not washed) nor PEG-DA hydrogels after washing showed any cytotoxic effect. This is in accordance with the findings of García-Soto, Lillienstern, Murphy, and Nealey (2013) and Cucuianu et al. (2016) that synthetic hydrogels based on PEG-DA are cytocompatible and furthermore the findings of Williams, Malik, Kim, Manson, and Eliseeff (2005), who showed that the photoinitiator Irgacure® 2959 is well suited for polymerization reactions in the presence of cells. Next to the pure washed and unwashed PEG-DA hydrogels we also analyzed the cytotoxicity level of PEG-DA hybrid hydrogels containing the varying amounts of AzGel (0.6, 3.0, and...
5.5%). All AzGel-containing hydrogels were washed before the cytotoxicity assay. All of the investigated AzGel-PEG-DA hybrid hydrogels could be classified as noncytotoxic. Summarized, these findings suggest that the simple washing procedure is sufficient to eliminate all possibly cytotoxic components in the hydrogel formulations, such as N-propargylglycylamide or copper(I) ions.

In the last experiment of this contribution, we examined the response of primary human fibroblasts to both, the AzGel-modified as well as the unmodified PEG-DA hydrogels by determining the number of cells present on the hydrogel surfaces after 4 days of static cell culture (Figures 9 and 10). Regardless of the concentration, the incorporation of AzGel resulted in a significantly higher cell number compared to the unmodified PEG-DA hydrogels. This highlights the exceptional bioactivity of AzGel addition to otherwise bioinert PEG-DA hydrogels. Even the addition of as little as 0.6% AzGel resulted in a significant increase of adherent cells compared to pure PEG-DA hydrogels.

Assessment of the cell morphology emphasized these findings (Figure 10). On pure PEG-DA hydrogels, almost no cells were found. The little number of cells present on these surfaces displayed a spherical morphology, giving rise to the suspicion that they try to avoid contact with the material. They rather formed clusters with themselves than adhering to the surface. With increasing AzGel content, fibroblasts elongated to their typical spindle-like shape and confluent cell layers were formed, resulting in the significantly enhanced numbers adherent on the material surfaces mentioned above (Pellegrin & Mellor, 2007).

These results indicate that the incorporation of AzGel into the PEG-DA hydrogels increased the bioactivity of the material, although the stiffness of the hydrogels was decreased simultaneously. We found $G'$ to be in the range of 2.1 kPa (pure PEG-DA) to 130 kPa (5.5% gelatin) or 95 kPa (5.5 %AzGel). This is remarkable, since other investigations showed that 2D-fibroblast proliferation and adhesion increased with increasing stiffness of the hydrogels (Wells, 2008; Sunyer, Jin, Nossal, and Sackett (2012) compared matrices with a Young’s modulus of ~1–240 kPa and Wang, Chung, and Kurisawa (2012) examined the storage moduli of materials which they found to be in the range of ~0.3–8.2 kPa. In both studies they found that with increasing stiffness of their substrates cell spreading also increased.

Furthermore, the increased bioactivity proves that cells are not impaired by potentially remaining free azido groups within the AzGel. This finding is consistent with the findings of Ruff et al. who modified cell-derived ECM with azido groups (Ruff et al., 2017). Together with the cytotoxicity assay (Figure 8), the cell response of the fibroblasts seeded onto the AzGel-containing hydrogels proves that the material is cytocompatible and offers very promising bioactive properties.

5 | CONCLUSIONS

We could show that AzGel can be synthesized in a straightforward way by diazo transfer to the native amino groups in gelatin. This one-step reaction resulted in highly bioactive building block for the modification of biomaterials without unnecessary side-chain extension or introduction of hydrophobic groups. By incorporating AzGel into PEG-DA hydrogels, biofunctionality was added to the otherwise bioinert hydrogels. This effect is generally comparable to adding unmodified gelatin to PEG hydrogels (Jingjing et al., 2016) yet, expanding this favorable bioactivity by the feature of the covalent incorporation of the bio-based component into the synthetic component. The described AzGel can therefore be considered as a promising building block for biomaterials, where covalent immobilization of gelatin or additional functionalization of gelatin by the azide-alkyne cycloaddition is of advantage.

In view of the general need of biorthogonal functional groups in polymers used for biomaterials science and of the properties reported for the AzGel, the latter can be regarded as a polymer with great prospects for various applications, for example, tissue engineering or drug delivery.

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CONFLICT OF INTERESTS

The authors state no conflict of interest. No benefit of any kind will be received either directly or indirectly by the author(s).

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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