1. Introduction

Among the leading causes of death worldwide are cardiovascular diseases such as myocardial infarction.[1] Due to the shortage of donor organs and the limited regenerative capacity of the myocardium, myocardial tissue engineering represents a viable alternative strategy to support the regeneration of myocardial tissue and thus offers an improved quality of life for patients with myocardial disease. A major challenge in the field of biomaterials engineering, however, lies in the replication of a defined nonlinear elasticity observed in myocardial tissue.[2] An emerging field in tissue engineering to produce structures mimicking the complexity of natural tissues is 3D printing, where the reproduction of a defined hierarchical pattern is one of the main objectives. Consequently, a high shape fidelity must be ensured by the ink used.[3] Although 3D printing in cardiac tissue engineering has made progress over the past years, there are still fundamental problems that prevent the successful clinical application of cardiac-tissue-engineered scaffolds. Especially, the mechanical integration of scalable 3D scaffolds still creates bottlenecks. Thus, the relatively simple handling, as well as the complete process automation of extrusion printing, is being investigated as an attractive approach in the sector of tissue engineering, as it offers a reproducible control over the shape of a scaffold or its thickness. A promising material for soft tissue engineering in general, and cardiac tissue engineering in particular, is the cyclic polyester poly (glycerol sebacate) (PGS).[4] PGS was developed explicitly for cardiac tissue replacement therapies in 2002[5] and has since been used extensively in this field because of its suitable mechanical properties for this application.[2,5,6] It has already been shown in the literature that 3D-printed PGS scaffolds induced a thickening as well as expansion of the infarcted left ventricular myocardium close to non-infarcted control groups at 28 days post-implantation.[6] Moreover, it was demonstrated that a hierarchical porous structure led to stimulation of tissue growth and angiogenic effects, thereby limiting scar expansion and mending ischemic tissues.[6] However, a fundamental problem in the fabrication of defined, non-chemically modified PGS structures remains the high curing temperature of >120 °C, which is far above the glass transition temperature \( T_g \) of PGS, therefore leading to structural collapse during the crosslinking phase.[2,6] Thus, Lei et al.[6] developed a general strategy for 3D extrusion-based printing of thermosets using sacrificial sodium chloride particles as a removable thickening agent for printing and as an enhancer for curing. The ink developed demonstrated good printability and dimensional stability during heat curing for PGS: salt ratios above 1:2 (wt%), hence addressing the aforementioned limitation of 3D printing chemically unmodified PGS. In this study, we introduce a new approach to 3D print PGS-based cardiac patches, by a combination of PGS with the corn protein zein and incorporating sodium chloride particles to modify the printing
ink for achieving better printability and shape fidelity. The new 3D-printed PGS-based structures were comprehensively characterised.

2. Results and Discussion

2.1. Morphological Analysis

50PGS10Zein3Gly ink combinations (see Table 2) exhibited good printability and shape retention after curing when the PGS-zein/salt ratio (in wt%) was set between 1:2.0 and 1:3.0. For 50PGS30Zein10Gly (Table 2), in contrast, good printing and curing results were obtained for even lower wt% ratios of PGS-zein/salt, namely 1:1.5 and 1:2.5, indicating a stabilizing effect of zein in the ink formulations. Figure 1 shows the parametric pressure–time window indicating the shape fidelity of the printed constructs. The reduced shape fidelity and the required pressure increase over time can be explained by the salt particles removing water from the system and thus increasing its viscosity. In addition, ethanol, being used as solvent for PGS and zein, evaporates over time as well, leading to an increase in viscosity and drying up of the ink.

Figure 2 illustrates 3D-printed constructs after curing and salt-leaching using 50PGS10Zein5Gly (Figure 2A,B) and 50PGS30Zein10Gly (Figure 2C–F) as extrusion inks. To evaluate the printability of the various PGS-zein combinations, a semi-quantification of the extruded structure after crosslinking and curing was performed. Figure 2A,B shows the top view of hierarchical 50PGS10Zein3Gly constructs with a strand distance of: A,B) 1.75 mm as well as 50PGS30Zein10Gly with a strand distance of: C,D) 1.75 mm and E,F) 1.25 mm, respectively. (Scale bar: A, C, E: 2 mm, B, D, F: 1 mm).

Figure 1. Pressure–time relationship of poly (glycerol sebacate) (PGS)-zein composite inks with sodium chloride particles as a removable thickener for printing. With increasing printing time, the applied pressure had to be enhanced to achieve extrusion of the material. Starting from a printing time of 50 min, however, printing could not be conducted reliably.

Figure 2. Optical images of the morphology of various 3D-printed PGS-zein constructs after crosslinking and salt-leaching. Top view of hierarchical 50PGS10Zein3Gly constructs with a strand distance of: A,B) 1.75 mm as well as 50PGS30Zein10Gly with a strand distance of: C,D) 1.75 mm and E,F) 1.25 mm, respectively. (Scale bar: A, C, E: 2 mm, B, D, F: 1 mm).
leaching of the salt particles was performed. Thus, the shape fidelity ($P_s$) was defined by a combination of the spreading ratio of the filaments and the pore circumference. The filament spreading ratio ($P_s$) was determined as the width of the printed filament strut divided by the orifice diameter of the printing nozzle (Equation (1))

$$P_s = \frac{D_s}{D_N}$$

(1)

where $D_s$ is the diameter of the printed strut and $D_N$ is the nozzle diameter. The pore perimeter ($P_p$) was calculated according to Oussalt et al.,

$$P_p = \frac{L^2}{16 \times A}$$

(2)

where $L$ is the perimeter and $A$ the area of a square-shaped enclosed pore.

Equation (3) combines the two quantitative parameters $P_s$ and $P_p$ and provides information about the printability as well as print quality

$$P_R = \frac{1}{2} \times (P_s + P_p) = \frac{1}{2} \times \left( \frac{D_s}{D_N} + \frac{L^2}{16 \times A} \right)$$

(3)

Since both parameters ideally approximate the value one, the overall result was also normalized to one. The measured strand widths were thus in close proximity to the inner diameter of the high viscosity nozzle used (pink tip 20 gauge, 580 μm) with values of 557 ± 90 μm for 50PGS30Zein10Gly and 589 ± 19 μm for 50PGS10Zein3Gly, respectively, indicating an excellent spreading ratio as well as good dimensional stability. The final designs showed remarkable printing compliance and an ordered hierarchical structures and the primary, 3D multilayer structure showed excellent shape fidelity. The printed struts had a distinct cylindrical shape with no noticeable deflection along the length of the strand spacing. In addition, the filaments were stacked vertically and did not fuse to the parallel struts placed underneath. The coalescence of the struts that can be observed in the cross-sectional view (Figure 3C) occurred due to the cutting process of the scaffolds to prepare samples for SEM imaging and not due to the printing process itself.

By leaching salt particles, an additional secondary structure of numerous interconnected micropores, homogeneously distributed within the primary filament network, was achieved, resulting in higher porosities and specific surface area of the struts (Figure 3E,F). The pore sizes were defined by the dimension of the salt particles, which had previously been ground and sieved to obtain pores in the range of tens of micrometers (Figure 3E). The resulting unique hierarchical porous structures with extended micrometer-sized pores are highly desirable in tissue engineering but are uncommon in typical 3D printing strategies without damaging the respective layers (Figure 3F). Microscopic structural analysis revealed an average apparent porosity of the individual struts of 48 ± 3% with a mean pore diameter of 15 ± 7 μm, closely approximating the values reported by Lei et al.,[8] who reported porosities between 51.0 ± 3.5% and 61.3 ± 6.6% with pore sizes of 17.0 ± 4.6 μm. In contrast to Lei et al.,[8] however, a high number of submicron pores was present in the scaffolds, further enhancing the interconnectedness of the overall structure. This “void fusing” can be attributed to the partial dissolution of sodium chloride in ethanol, which was used to dissolve PGS and zein. Since only between 0.55 and 0.65 g of sodium chloride dissolves in one liter of ethanol (equivalent to less than 3 mg in the ink combination used),[10] complete dissolution of the salt particles during the printing process and thus disintegration of the printed structure can be excluded. In general, high porosities and adequate pore sizes are essential for tissue engineering applications, but the target values vary depending on the size and origin of the transplanted cells and the surrounding tissue.[11]

In addition to the morphology of scaffolds, their physicochemical properties play an essential role as they affect both protein adsorption and cell adhesion on the material surface.[12] The wettability of a material is therefore of great importance. Since zein is an amphiphilic protein, consisting of a relatively equal amount of hydrophilic and hydrophobic amino acids,[13] a decrease in contact angle from 52 ± 10° for pure, dense PGS films to 26 ± 9° for cast 50PGS30Zein10Gly samples was observed. Due to the conformational change of the secondary structure of zein caused by exposed methyl or carboxyl groups, zein exhibits either a hydrophilic or a hydrophobic character.[13,14] The assembly behavior of the α-helix and β-sheet structure is determined by the concentration of zein and the specific equilibrium between the polar and nonpolar groups of zein molecules, as well as the polarity of the solvent.[14] Thus, protein folding was also affected by the introduction of the polar and highly hydrophilic osmolyte glycerol.[15] The increased hydration of the zein surface induced by glycerol leads to an inward folding effect of the hydrophobic chains on the surface of the unfolded protein structure to avoid direct contact with water. In this way, glycerol physically interacted with zein, resulting in hydrophilic properties.[13]
In a previous study, no significant influence of zein on the measured contact angle of electrospun PGS-zein fibers has been found when acetic acid was used as solvent.[16] In contrast, when ethanol was used, the contact angles were reduced, which is likely due to the increased amount of polar functional groups on the surface of zein.[17] Consequently, the pronounced hydrophilic effect of 50PGS30Zein10Gly samples can be attributed to a combination of the osmolyte glycerol and the use of ethanol as a solvent.

2.2. Chemical Analysis

To investigate possible chemical crosslinking reactions between carboxylic and hydroxyl side groups of PGS with amine and carboxyl groups of zein, respectively, Fourier transform infrared (FTIR) measurements were performed. Figure 4A shows representative FTIR results of the printed PGS-zein combination before and after thermal crosslinking in comparison to PGS pre-polymer, crosslinked PGS, and neat zein as reference samples.

As can be seen from the FTIR spectral analysis, zein exhibits a secondary structure typical of proteins, dominated by an α-helical backbone with β-sheet as well as turn and random coil sections.[18] Thus, the prevailing peak at 1645 cm⁻¹ relates to amide I (C=O) stretching vibrations of the α-helical component.[18] Further distinct protein peaks are found at 1530 and 1516 cm⁻¹ as N–H bending vibration and C–N stretching vibration of C–N–H groups, respectively, as well as at 1447 cm⁻¹ as C–N stretching vibration and at 1238 cm⁻¹, identified as amide III.[18]

Absorption maxima related to the cyclic polyester PGS before and after heat crosslinking can also be seen in Figure 4A. The most striking peaks at 1734 and 1516 cm⁻¹ can be interpreted as stretching vibrations of carbonyl groups (C=O) and as C–O stretching of hydroxyl groups, respectively.[19] In contrast, the two peaks at 2928 and 2855 cm⁻¹ can be explained by alkane groups (C–C) and C–H stretching vibrations of the polymer backbone, which do not change significantly during the crosslinking reaction.[20,21]

After mixing zein with PGS, printed structures showed characteristic peaks of both polymers. Thus, the vibrations of alkane groups (2928 and 2855 cm⁻¹) and carbonyl groups (1734 cm⁻¹) of...
PGS and the protein peaks of amide I (1645 cm$^{-1}$) and amide II (1530 cm$^{-1}$) of zein can be observed. During the thermal crosslinking process of the pre-condensed PGS and zein combinations, hydroxyl and carboxyl groups were able to generate ester bonds, while amide bonds between amino and carboxyl groups of both polymers could develop via polycondensation reactions.$^{[22]}$

The proposed chemical structure of a possible PGS-zein complex is depicted in Figure 4C. The underlying chemical reaction was first reported by Yoon et al.$^{[22]}$ who crosslinked PGS similarly with the polypeptide gelatin.

Cured PGS-zein structures exhibited increased amide peaks at 1645, 1530 and 1516 cm$^{-1}$ and decreased absorptions of the ester bonds at 1734 and 1161 cm$^{-1}$ when compared to neat zein and the unmodified crosslinked PGS (Figure 4B). This behavior can be related to zein containing predominantly amide groups in its polymer backbone, leading to an increase in the amide content of the copolymer.$^{[22]}$ Thus induced amide groups likely react with carboxylic acid groups in competition with hydroxyl groups of PGS, leading to elevated amounts of amide bonds and reduced ester bonds in the copolymer.$^{[22]}$ Another modification can be seen in the wavenumber range between 3000 and 3600 cm$^{-1}$. A broad peak centered at 3290 cm$^{-1}$ is evident in both neat zein and the printed structure before and after crosslinking. The 3D-printed samples exhibit a pronounced shoulder at around 3400 cm$^{-1}$ before crosslinking, which can be ascribed to hydroxyl bond stretching in water and alcoholic groups of non-crosslinked PGS.$^{[20,21]}$ Thermal crosslinking entails the reduction of alcoholic groups and the formation of amide bonds between zein and PGS.$^{[22]}$ However, the release of water during polycondensation lowers the intensity of the peak in the crosslinked 3D printed structures. The changes in the respective peaks in the spectral analysis thus indicate the successful copolymerization of PGS and zein.
2.3. Mechanical Analysis

To investigate the mechanical adaptability of 3D printed PGS-zein constructs, tensile tests were performed. The human ventricular myocardium is a densely vascularized, quasi-lamellar tissue wherein myocardial fibers consisting of cardiomyocytes are embedded in a honeycomb structure of collagen.\(^{[23]}\) Auxetic honeycomb patterns result in anisotropy with specific directional electrical and mechanical properties of the heart, whose mimicking remains a challenge in the development of cardiac tissue scaffolds.\(^{[23]}\) To evaluate the bulk mechanical properties of the 3D-printed scaffolds, specimens were tested under uniaxial loads after thermal curing and leaching of the salt particles. Figure 5 illustrates typical tensile stress–strain curves of the untreated, salt leached reference samples (Figure 5A), as well as the 50PGS10Zein3Gly (Figure 5B) and 50PGS30Zein10Gly (Figure 5C) samples, respectively. Measurements were performed in the dry state and after swelling of the samples for 12 h in 37 °C warm Roswell Park Memorial Institute (RPMI) cell culture medium.

As indicated in Figure 5A–C, samples prepared with the highest percentage of zein showed a significant increase in mechanical properties in the dry state. Non-modified, salt-leached reference samples reached elastic moduli of 60 ± 9 kPa and ultimate tensile strength (UTS) values of 38 ± 8 kPa. The incorporation of 10 wt% zein and 3 wt% glycerol caused only a slight change in Young’s modulus and UTS values of 66 ± 6 kPa and 38 ± 6 kPa, respectively. However, the elongation at failure

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Figure 5. Indicative uniaxial tensile stress–strain curves for salt-leached reference sample, B) 3D-printed 50PGS10Zein3Gly, and C) 50PGS30Zein10Gly specimens in dry condition and after swelling for 12 h in 37 °C warm RPMI cell culture medium. D) Visualized illustration of the applied vertical tractive force in relation to the strand alignment. E) Stress–strain curves of salt-leached PGS substrates (red) as well as 50PGS30Zein10Gly samples in linear (green) and transverse direction (blue) after swelling for three days in 37 °C warm water. F) Detailed curve of the progression of the samples in a physiologically relevant strain regime compared to native human myocardium (in this figure values of the passive human myocardium were adapted from the literature data\(^{[33,38]}\)).
reduced slightly from 67 ± 5% for salt-leached samples to 49 ± 3% for the dry 50PGS10Zein3Gly printed structures. For extrusion-processed 50PGS30Zein10Gly samples, a considerable gain in specimen stiffness to 29 ± 2 MPa as well as the maximum tensile strength of 1400 ± 14 kPa can be observed. Concomitantly, the elongation at break in the dry state diminishes to 21 ± 3%. Both printed PSG specimens were tested in transverse strut direction (Figure 5D) and featured the same strand spacing and scaffold thickness, thus the observed difference between the specimens resulted solely from a different zein content. 50PGS30Zein10Gly constructs experienced considerable softening with a reduction in elastic moduli to 342 ± 87 kPa, ultimate strength to 272 ± 83 kPa, and a twofold increase in elongation at break to values of 51 ± 7%. The increased softening of the samples could be explained by a shift of the glass transition of zein to lower temperatures with an increasing equilibrium water content between the dry state and the swollen polymer network, as well as by an increased amount of the plasticizer glycerol.[24]

Figure 5E,F shows representative uniaxial static tensile stress–strain curves of salt-leached PGS and 50PGS30Zein10Gly samples with measured vertical tensile forces in transverse and longitudinal strand directions, respectively, after three days of incubation at 37 °C in ultrapure water. As can be seen in Figure 5E, 3D-printed structures had a considerably increased elongation at break relative to salt-leached samples, with overall improved mechanical properties. Accordingly, salt-leached samples achieved rather low values (26 ± 5%) of tensile elongation, elastic moduli of 100 ± 19 kPa, and tensile strength of 9 ± 3 kPa. 50PGS30Zein10Gly specimens, in contrast, revealed an anisotropic behavior at uniaxial tension in the corresponding linear or transversal strand direction. Thus, Young’s moduli of 42 ± 13 kPa in the linear direction and 21 ± 3 kPa in the transversal direction were obtained with an associated anisotropy ratio $E_{\text{linear}}/E_{\text{transverse}}$ of 2.0 ± 0.4. With the aim of evaluating whether such stiffness values are generally appropriate, Engelmayr et al.[23] examined samples of right (RV) and left (LV) adult rat ventricular myocardium in circumferential (CIRC) and longitudinal (LONG) directions with effective stiffness values ($E_{\text{CIRC}}$, $E_{\text{LONG}}$) assessed in a linear physiological strain regime. They found that the RV myocardium exhibited an anisotropic $E_{\text{CIRC}}/E_{\text{LONG}}$ ratio of 2.8 ± 0.5 with Young’s moduli $E_{\text{CIRC}} = 54 ± 8$ kPa and $E_{\text{LONG}} = 20 ± 4$ kPa.[23] The LV counterpart showed a reduced anisotropy ratio of 2.1 ± 0.4, but obtained higher stiffness values of $E_{\text{CIRC}} = 157 ± 14$ kPa and $E_{\text{LONG}} = 84 ± 8$ kPa.[23]

In addition to the anisotropic stiffnesses, the 3D-printed designs yielded negligible differences in elongation at break and tensile strength. Elongation at break values of 61 ± 3% and maximum tensile strengths of 21 ± 4 kPa were measured in the linear direction while values of 63 ± 10% and 20 ± 4 kPa were determined in the transversal direction. The direction-dependent deformation and stress generation in the physiologically relevant strain regime are additionally visualized in Figure 5F. Besides salt-leached and 3D-printed samples, a typical tensile curve of passive human myocardium is illustrated in Figure 5F. Stress–strain curves of 50PGS30Zein10Gly samples resulted in slightly higher values in both strand directions compared to the nonlinear curve shape of the native myocardium. Salt-leached samples demonstrated a corresponding curve progression in this loading regime as well, but had no directional dependence, with a strain of 20% already close to the tensile strain limit of these specimens. Fatigue eventually occurs in the linear direction of the 3D printed struts due to a lower connectivity of the struts and a higher ductility in the transverse direction (Figure 5E). Such physiological adaptability of extrusion-printed specimens increases the extensibility and stability of PGS-based patches and could enable them to absorb and release energy against the force of cardiac contraction.[25]

Table 1 summarises the measured elongations at break, tensile strength values, and elastic moduli of the respective specimens in the dry and wet states.

### 2.4. In Vitro Studies and Degradation Behavior

Water uptake, pH change, and weight loss of the produced samples were investigated to assess the behavior of porous salt-leached and 3D-printed structures in a hydrolytic environment. In addition, cell viability studies were conducted using an indirect cell assay with the C2C12 mouse myoblast cell line.
For swelling and degradation studies, PGS samples were incubated in 37°C warm RPMI for up to 28 days. Results of dynamic degradation of salt-leached PGS, 50PGS10Zein3Gly, and 50PGS30Zein10Gly samples in terms of pH change, water uptake, and weight loss as a function of immersion time in RPMI for different time points are presented in Figure 6.

The time-dependent swelling ratio of thermally crosslinked PGS-based samples is shown in Figure 6A. As can be noted, initial swelling within the first 24 h differs significantly with increasing zein content. A reduced water uptake was observed from 162 ± 7% for salt-leached PGS to 121 ± 2% for 50PGS10Zein3Gly and 52 ± 20% for 50PGS30Zein10Gly, respectively. After three days of incubation, consistent water uptake was achieved in all samples. The sponge-like structure of the salt-leached reference samples facilitated water incorporation into the large pore size structure, resulting, however, in an overall increased standard deviation of the data. Principally, hydration processes depend on crosslinking density, hydrophilic groups, and the chemical structure of polymers. As indicated in Section 2.2, (Figure 4), zein could form amide and ester bonds with the PGS pre-polymer during thermal crosslinking, increasing the degree of crosslinking of zein-containing samples. According to Yoon et al. and Aghajan et al., having incorporated gelatin as a protein in the PGS network, the introduction of amine and carboxyl groups by the protein not only causes increased chemical crosslinking, but also improves the physical entanglement of the molecular chains. Despite a reduced hydrophobicity caused by zein, densely crosslinked and entangled networks emerge, making them more resistant to the penetration of water molecules and thus reducing the swelling of the samples. Since crosslinked PGS networks swell only in polar organic protic solvents such as ethanol or tetrahydrofuran (THF), but not in water or RPMI, the high swelling rates are likely due to the high porosity and the sponge-like structure of the samples. Consequently, the samples do not change their dimensions significantly and are therefore suitable for in vivo applications. A further influence on the hydration rate and swelling capacity of zein-containing samples could be related to the pH dependency of the protein structure. Reference samples of salt-leached, pure PGS showed a swelling plateau when reaching a water uptake equilibrium after the first three days, indicating no further swelling of the samples. In contrast, 3D-printed structures containing zein exhibited an adverse behavior after two weeks of incubation. Thus, the observed swelling ratio doubled for 50PGS30Zein10Gly samples, but was halved for 50PGS10Zein3Gly structures compared to the first week, both

![Swelling ratio vs. Incubation time](image1)

![Weight loss vs. Incubation time](image2)

![pH vs. Incubation time](image3)

Figure 6. A) Assessment of the hydrolytic degradation behavior of salt-leached PGS as well as 3D-printed 50PGS10Zein3Gly and 50PGS30Zein10Gly structures, respectively. B) Percentage swelling ratio as well as weight loss measurements of the respective samples for an incubation period of 28 days in RPMI at 37°C. C) Progression of the pH variation for up to four weeks of in vitro degradation.
reaching further constant values as incubation progressed. Figure 6B shows the corresponding in vitro degradation profiles of the respective samples for up to 28 days. All samples demonstrated a relatively low mass loss of PGS in a non-enzymatic culture medium. Accordingly, the only degradation mechanism involved was likely hydrolytic surface erosion without enzymatic cleavage of the ester bonds nor enzymatic degradation of the protein zein. Pure PGS reference samples showed the highest degradation rate of 11 ± 8% after four weeks of incubation. Although the degradation value of the two zein-containing samples increased at the beginning of the incubation period, the samples reached a reduced weight loss of only 4 ± 2% for 50PGS10Zein3Gly and 5 ± 1% for 50PGS30Zein10Gly at the end of in vitro degradation. After one, three, and seven days, salt-leached PGS samples even exhibited a negligible weight gain, which can be explained by salt residues of the RPMI medium in the pore structure that were not removed despite thorough washing of the samples. The increased initial mass loss of 4 ± 2% for 50PGS10Zein3Gly and 7 ± 4% for 50PGS30Zein10Gly in the first 24 h is most likely due to leaching of unbound zein molecules and glycerol from the polymer network. Nonetheless, a copolymerization of PGS with zein could delay hydrolysis of the elastomeric polyester, as -(CH2)n=CH3 side chains of zein could sterically inhibit hydrolysis of ester bonds and thus significantly slow down the mediation of enzyme molecules in vivo. The pH curve over the entire incubation period of 28 days for all samples is displayed in Figure 6C. This behavior reveals a slightly elevated pH of 8.1 at the beginning of the incubation period as well as a rather rapid adjustment of the pH to the physiological range within the first three days of incubation.

All samples showed an overall stable pH trend with values between 7.4 and 7.6, indicating no pronounced degradation of the samples during the first 21 day period (Figure 6C). The relatively sharp increase in pH after 28 days to values of 8.7 ± 0.1 for the salt-leached PGS samples, 7.8 ± 0.4 for 50PGS10Zein3Gly and 8.0 ± 0.4 for 50PGS30Zein10Gly, respectively, could be explained by the fact that the samples had to be transferred from the CO2-controlled cell incubator to a 37 °C warm-shake incubator without CO2 exchange for the last week of the degradation study. Due to the now reduced bicarbonate buffered system, alkaline bicarbonate ions likely accumulate in the cell culture medium thus causing an increase in pH. Pomerantseva et al. reported that the pH of a degradation solution does not directly contribute to the degradation of PGS. Thus, samples degraded in a comparable manner whether they were incubated in a phosphate-buffered lipase solution at neutral pH or in a lipase solution with an acidic pH of 1.34. Nevertheless, maintaining the pH in the physiological range during degradation limits a negative cellular response, as intracellular pH responds to changes in the extracellular pH.

To investigate any physicochemical changes in the samples, FTIR measurements were carried out after selected time points of the incubation period. Figure 7 illustrates FTIR results of salt-leached PGS, 50PGS10Zein3Gly and 50PGS30Zein10Gly scaffolds, respectively, after one and 28 days of in vitro degradation. For 50PGS30Zein10Gly samples, the absorbance after six months is additionally displayed. All graphs were normalized according to the amide I peak of pure zein powder.

Figure 7. Direct comparison of distinctive absorption maxima of salt-leached PGS, 50PGS10Zein3Gly and 50PGS30Zein10Gly scaffolds, respectively, after one and 28 days of in vitro degradation. For 50PGS30Zein10Gly samples, the absorbance after six months is additionally displayed. All graphs were normalized according to the amide I peak of pure zein powder.

in PGS at 1732 cm$^{-1}$ could be detected over the entire time of in vitro incubation. Moreover, in samples containing zein, primary peaks of amide I and II are still clearly visible, indicating the remaining presence of zein in the structure after 28 days of degradation. However, a hypochromic shift of the two characteristic protein peaks is evident as soon as the samples are immersed in RPMI. Thus, amide I shifts from 1645 to 1651 cm$^{-1}$ and amide II from 1530 to 1541 cm$^{-1}$ (N–H bending) and 1515 to 1520 (C–N stretching) cm$^{-1}$, respectively. This shifting could indicate reduced intramolecular hydrogen bonding between carbonyl (C=O) and carboxyl (-COOH) groups in zein.

Furthermore, a relatively weak shift in the ester peak of the PGS samples containing zein is visible with the absorption maximum converting from 1732 to 1735 cm$^{-1}$ for 50PGS10Zein3Gly and to 1736 cm$^{-1}$ for 50PGS30Zein10Gly, respectively. In particular, for 50PGS30Zein10Gly samples incubated in RPMI for more than half a year, a hypochromic shift of all three main peaks is detectable, indicating a reduced presence of excited molecular
bonds and thus providing semiquantitative information about the degradation of the material.\textsuperscript{[12]}

The mechanical properties of the degrading structure play a crucial role in the intended applications of scaffolds, as the mechanical resistance of a cardiac patch needs to adapt according to the recovery of the myocardium.\textsuperscript{[34,35]} Figure 8 shows stress-strain curves of 3D-printed 50PGS30Zein10Gly constructs after 12 h of incubation in 37 °C RPMI as well as after six months of in vitro degradation in RPMI.

Figure 8A shows that Young’s moduli and tensile strength values decreased from 342 ± 87 kPa and 272 ± 83 kPa for specimens immersed in RPMI for 12 h to 133 ± 15 kPa and 110 ± 7 kPa after six months of incubation, respectively. Thus, 50PGS30Zein10Gly samples achieved a 61 % reduction in structural stiffness after six months of degradation, converging progressively toward the values of passive human myocardium (Figure 8B).

It is important to mention that in artificial-technical systems, other aspects have to be taken into account besides purely the mechanical properties. It has been shown that maximal contractile work of embryonic chick cardiac myocytes occurred for soft (10 kPa) native-like substrates, whereas increased material stiffness of 144 kPa resulted in both increased maximal force and an improved force-area ratio in neonatal rat cardiomyocytes.\textsuperscript{[34]} Moreover, ventricular pressure in vivo, rather than the stiffness of the tissue itself, was observed to dominate the biomechanics of the cardiac contractile cycle.\textsuperscript{[34]} Moreover, experimental simulations of cardiac contraction cycles showed beneficial effects of higher stiffness of cardiac patches leading to better function of an injured heart, including a reduction in infarct dilation as well as an overall remodeling of the left ventricle due to reduced wall stress.\textsuperscript{[34,35]} Hence, the degradation rate and mechanical behavior of PGS could be adjusted to match the possible recovery kinetics of human myocardium with the incorporation of zein into the structure. As evidenced by tensile tests of the incubated samples, a cardiac patch made from PGS-zein combinations would still perform its primary function of cell delivery and biomechanical support even after six months of in vitro degradation, as the scaffold structure remained intact even though its stiffness had decreased to 39 % of the original value.

2.5. Cell Culture Studies

To assess possible cytotoxic behavior due to the leaching of non-crosslinked substances from the PGS constructs, an indirect cell assay was performed using the murine C2C12 myoblast cell line. To differentiate an effect of the individual PGS scaffolds, neat, crosslinked PGS, salt-leached PGS, and 50PGS30Zein10Gly samples were stored in RPMI cell culture medium for 24 h prior to cell seeding. Figure 9A shows the absorbance values of untreated cells as a positive control (PC), cells treated with 6 v% DMSO as a negative control (NC), and RPMI medium supplemented with supernatant from pure PGS, PGS after salt leaching, and 50PGS30Zein10Gly samples. Potential cytotoxicity was determined according to Kasparkova et al.\textsuperscript{[16]} following EN ISO 10 993-5, where the viability of the reference is 1 and corresponds to 100% survival of the cells in the absence of any tested substance in the culture medium (well plate without sample).

Briefly, values above 0.8 are classified as “non-cytotoxic,” values from 0.6 to 0.8 as “mildly cytotoxic,” values between 0.4 and 0.6 as “moderately toxic” and values below 0.4 as “severely cytotoxic.”\textsuperscript{[16]} As indicated by absorption values of the WST-8 formazan color change at 24 h post-seeding, cells cultured in pure PGS, salt-leached, and zein-containing PGS extracts showed fairly similar absorbance values above 0.8, signaling that there was no burst release of cytotoxic substances.

In contrast, the extracted medium from dense films of pure PGS showed significantly greater variance in the measurements, suggesting inhomogeneous washing behavior of the samples as well as leaching of potentially toxic components. A comparison of cell number per unit area, defined by the nuclear density (colored blue DAPI, Figure 10) of living cells, showed that salt-leached PGS samples had a significantly reduced cell number compared to pure PGS and 50PGS30Zein10Gly. Nevertheless, analysis of indirect cell viability, based on measurements of mitochondrial activity at 24 h after seeding, did not reveal reduced cell activity.

Cell viability was further assessed by live/dead staining of C2C12 cells at 24 h post-seeding (Figure 10). Fluorescence micrographs confirmed that live cells (stained green with calcein AM, Figure 10) were predominantly present in all samples examined. Dead cells (red stained with propidium iodide, Figure 10) could

![Figure 8](https://www.aem-journal.com/)

**Figure 8.** A) Uniaxial stress–strain curves of 50PGS30Zein10Gly samples in transverse direction after incubation for 12 h (purple) and six months (green), respectively, in 37 °C warm RPMI. B) More detailed illustration of the stress–strain progression compared to native human myocardium in a physiologically relevant strain regime (in this figure values of the passive human myocardium were adapted from literature data\textsuperscript{[13,38]}).
not be detected in any sample. One reason for this result is that diseased cells detached from the bottom of the Petri dish and were pipetted away during successive staining and washing steps of the samples. However, there was a clear difference in cell density between samples. Thus, cells used in the NC showed a greatly reduced proliferation. Pure PGS as well as
50PGS30Zein10Gly extracts, in contrast, showed no significant differences compared to the PC, suggesting unhindered cell growth in the extraction medium. In contrast, the cell density of the salt-leached PGS samples showed larger unpopulated areas but no reduced mitochondrial activity and thus cytotoxic activity according to WST-8 measurements. Indirect viability assessments using water-soluble tetrazolium salt assays (WST-8) in combination with fluorescence microscopy data confirmed an overall increased viability and proliferation of C2C12 cells in all extraction media. Thus, the 50PGS30Zein10Gly sample shows high potential for 3D printing of challenging and biocompatible structures and provides a basis for further studies for application in myocardial tissue engineering.

3. Conclusions

As demonstrated in this work, highly porous and interconnected PGS patches could be successfully fabricated using a novel extrusion-based 3D printing approach with PGS-zein inks. Accordingly, 50PGS10Zein3Gly and 50PGS30Zein10Gly compositions produced multilayered structures with clinically relevant dimensions of 20 mm × 20 mm and well-defined macro- and micropores. Both ink formulations resulted in high shape fidelity during the printing process as well as sufficient structural stability to withstand the relatively harsh thermal crosslinking conditions. Contact angle measurements showed improved hydrophilicity with increasing levels of zein. FTIR studies indicated the copolymerization of zein in the PGS network. Furthermore, degradation studies in the RPMI cell culture medium showed the increased stability to hydrolytic degradation of the scaffolds for up to six months. Evaluations by uniaxial tensile strength tests indicated an anisotropic characteristic of the 3D-printed structures for vertical tensile forces in linear and transversal directions, respectively. Indirect cell culture studies indicated no negative effect of neat PGS films, salt-leached PGS, or 50PGS30Zein10Gly samples on the viability and proliferation of C2C12 cells. As demonstrated in this work, the 3D printing of unique sophisticated and biocompatible structures using PGS-zein combinations was successful in providing a platform for their further investigation for myocardial tissue engineering applications.

4. Experimental Section

Materials: For the polycondensation of poly (glycerol sebacate) (PGS), glycerol (purity ≥99%, BioXtra, Sigma-Aldrich, Germany) and sebacic acid (purity ≥99%, Sigma-Aldrich, Germany) were used. Commercially available sodium chloride (NaCl, purity ≥99.5%, BioXtra) and the corn protein zein (Z3625) were purchased from Sigma-Aldrich, Germany. All experiments were carried out using the same batch of zein powder to avoid lot-related variations.

Preparation and Crosslinking of PGS-zein: PGS was synthesized using a polycondensation reaction, as described by Wang et al.[9] with a minor adaptation of the reaction parameters, according to Rai et al.[37] For the pre-polycondensation step of the synthesis, an equimolar mixture (0.1 m) of glycerol and sebacic acid was heated at 120 °C under an inert nitrogen atmosphere for 24 h in a three-necked round bottom flask to obtain PGS pre-polymer. The pre-polycondensed form had an approximate molecular weight of $M_w = 1.11 \times 10^7$ g mol$^{-1}$ and was stored in closed plastic beakers until further use.

For 3D-printing, 50 wt% of PGS pre-polymer was dissolved in 90 v% ethanol under continuous stirring at room temperature. Subsequently, 3 or 10 wt% of glycerol was added, respectively. Eventually, 10 or 30 wt% of zein was included in the solution and homogenized for 24 h under stirring. Before printing, 14 g of finely ground and sieved (38 μm) salt (NaCl) was added until a highly viscous paste was formed. Samples consistent of 50 wt% PGS, 30 wt% zein, and 10 wt% glycerol are further referred to as 50PGS30Zein10Gly and samples composed of 50 wt% PGS, 10 wt% zein, and 3 wt% glycerol as 50PGS10Zein3Gly, as summarized in Table 2.

For the processing of PGS-zein-NaCl combinations, an extrusion-based, three axes bioplotter (BioScaffold 3.1, GeSim mbH, Großerkmannsdorf, Germany) was used. The printer was equipped with a media control system (F-box) and temperature-controlled pneumatic extruders compatible with a syringe micro-nozzle system (Nordson EFD, Germany). The design and the dimensions of the plotting geometries were defined over the scaffold generator function using the GeSim Robotics software of the bioplotter. Simple grid-like square structures with an edge length of 20 mm and a height (in the z-direction) of 1 mm were generated. Each printed layer was rotated 90° as well as several strand distances between 1 and 2 mm were investigated to generate a possible anisotropic effect of the fabricated structure. A 20G conical smooth-flow nozzle (SmoothFlow 7 005 009, Nordson EFD, Oberhaching, Germany) with an inner diameter of 580 μm was used for all printing experiments. The plotting speed varied between 8 to 12.5 mm s$^{-1}$ and the pressure between 50 and 300 kPa for PGS-zein-NaCl compositions. Processing took place at room temperature. Table 3 summarizes the used printing and scaffold design parameters.

After 3D printing, the scaffolds were transferred to a vacuum oven and crosslinked at 125 °C and a vacuum level of 1.1 to 2.5 × 10$^{-2}$ mBar for four days.

Characterization – Material Properties: To assess morphological properties, SEM analysis (FE-SEM, Auriga, Carl-Zeiss, Jena, Germany) was performed. Before SEM observations, specimens were sputtered with gold. Fiji ImageJ (version 1.52n) analysis software (NIH, Bethesda, MD, USA) was used to determine the printed scaffolds’ average strut diameter.

The molecular structure (chemical bonds present) of PGS-zein was investigated with (FTIR) attenuated total reflectance spectroscopy (iRAfinity-1S, Shimadzu Corporation, Kyoto, Japan). 32 spectral scans were averaged across the spectral range 4000–400 cm$^{-1}$ with a resolution of 4 cm$^{-1}$ at room temperature.

Table 2. Identification and composition of PGS-zein combinations used for 3D printing (the rest of 100 wt% was ethanol).

| Sample ID     | PGS [wt%] | Zein [wt%] | Glycerol [wt%] |
|---------------|-----------|------------|----------------|
| 50PGS10Zein3Gly | 50        | 10         | 3              |
| 50PGS30Zein10Gly | 50        | 30         | 10             |

Table 3. Compilation of the printing and scaffold parameters used for PGS-zein-NaCl combinations.

| Printing parameters | Scaffold design parameters |
|---------------------|----------------------------|
| Speed               | Radius                      |
| Pressure             | Corners                     |
| Temperature          | Height                      |
| z-Offset             | Strain distance             |
| Strand height        | Minimal distance            |
| Strand width         | Minimal length              |
| Tear off             | Angle/change after layer    |

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Static contact angles were measured using a DSA30 (sessile drop method, Krüss GmbH, Hamburg, Germany). Five measurements were performed for each specimen 10 s after placing drops of deionized water (3 µL) onto the individual samples. The generated data was evaluated by the corresponding DSA software (DSA4 2.0, Krüss GmbH, Hamburg, Germany).

A standard uniaxial tensile test (Instron 5967, Darmstadt, Germany) was used to evaluate the mechanical properties of the produced structures. Scaffolds were cut into rectangular strips with a length of 10 mm and a width of 5 mm, and their thickness was measured using a precision micrometer (Schut Geometrical Metrology, Germany). Mechanical tests were conducted using a crosshead speed of 1 mm min\(^{-1}\). All tests were executed at room temperature in dry condition and after being immersed in RPMI cell culture medium (RPMI 1640, Gibco, ThermoFisher Scientific Inc., USA) for 12 h, respectively. Young’s moduli, UTS, and FS were obtained from stress–strain curves. Mean values and standard deviations were defined after testing at least five specimens of each composition.

Characterization – Biological Properties: Indirect cell tests according to EN ISO 10993-5 were carried out using the mouse myoblast cell line C2C12, purchased from PromoCell GmbH, Heidelberg, Germany.\(^{[9]}\)

Cells were cultured in T75 cell culture flasks (Nunc. EasYFlask. Cell Culture Flasks, T75, filter, ThermoFisher Scientific GmbH, USA) by using RPMI growth medium, supplemented with 10 % fetal bovine serum (FBS) (FBS Gibco, ThermoFisher Scientific Inc., USA) and 1 % penicillin-streptomycin (Penicillin - Streptomycin 5000 U mL\(^{-1}\) Gibco, ThermoFisher Scientific Inc., USA), in a 37 °C warm incubator, humidified with 5% CO₂. The cell culture medium was changed twice weekly. For all experimental protocols, cells at passage 10 were used. Each well was seeded with 10 × 10⁵ cells re-suspended in their standard culture medium and cultured for 24 h.

Mitochondrial activity was quantitatively assessed by applying a water-soluble tetrazolium salt assay (WST-8, Sigma-Aldrich, Doiding, Japan). To compare a negative result, RPMI was supplemented with 5 % DMSO and added to the cells as an NC. Samples to be examined were stored in RPMI cell culture medium for 24 h prior to cell seeding. Subsequently, cells were immersed for another 24 h in the supernatant of the individual specimen to study the overall initial cell behavior. After the time point of interest, the culture medium was removed from the respective well and cells were washed with PBS. After the addition of 1 % WST-8 reagents in each well, the culture plates were incubated for an additional three hours. Subsequently, the supernatant of all samples was transferred to a 96-well plate (100 µL in each well) to measure the absorbance at 450 nm with an ELISA-Reader (Perkin Elmer, Multilabel Reader Enspire 2300, Germany). Results were expressed as absorbance at 450 nm normalized to the PC.

Cell viability, as well as nuclei of C2C12 cells, were analyzed using calcein acetoxymethyl ester (Calcein AM, Invitrogen, Life Technologies, Germany) by using a fluorescence microscope (Axio Observer, Zeiss, Germany). The number of live cells was quantified using n = 4 biological replicates with a minimum of three images measuring the nucleus (DAPI, blue) of calcein-AM 488 green fluorescent stained cells on identically sized images (1388 × 1038 pixel) using the Fiji ImageJ (version 1.52w) plugin.

For statistical analysis of mean value differences, one-way analysis of variance (ANOVA) was used, implemented in Origin 2020 (OriginLab Corporation, Northampton, USA) software. The number of samples was N = 5 for all studies with a significance level set as p < 0.01(9K). For the comparison of the mean values with ANOVA, the Tukey posthoc test was used.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

3D printing, cardiac tissue engineering, poly (glycerol sebacate), self-regulating properties, zein

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