Supplementary Material

From Aggregates to Porous Three-Dimensional Scaffolds through a Mechanochemical Approach to Design Photosensitive Chitosan Derivatives

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Figure S1. Tissue reaction to the films based on allylchitosan (day 30). Note: the connective tissue formed the capsule (CAP) with blood vessels (V) around the implanted chitosan films (CHF); connective tissue grew into the film fractures (F) forming connective tissue septa (S); some macrophages (MPH) and giant cells (GC) adhered to the surface of scaffolds, the CHF material was oxyphilic, hematoxylin and eosin staining, simple microscopy, 100× (scale bar: 250 μm); (a) AC2 (b) AC3; and (c) AC4; (d) AC5.
Figure S2. Tissue reaction to the films based on allylchitosan (day 30).

Note: the connective tissue formed the capsule (CAP) with blood vessels (V) around the implanted chitosan films (CHF); CAP consisted of two layers: the inner layer (IL) was an immature connective tissue (granulation tissue) with macrophages (MPH) and giant cells (GC), the outer layer (OL) consisted of a more mature connective tissue; IL grew into the film fractures (F) forming connective tissue septa (S); some MPH and GC adhered to the surface of scaffolds: (a) the CHF material was oxyphilic, F with displacement of the film fragments, hematoxylin and eosin staining, simple microscopy, 100× (scale bar: 250 μm); (b–d) fragments of the previous sample, picrosirius red staining: (b) the CHF material was picrinophilic, with the periodic structure, simple microscopy, 100× (scale bar: 250 μm); (c) distinct periodic structure of CHF material, phase-contrast microscopy, 1000× (scale bar: 25 μm); (d) the CHF material was isotropic; the collagen fibers in the OL of the CAP carried of the most pronounced anisotropy (yellow and orange glow), while the collagen fibers in the IL and in the S carried of weak anisotropy (green glow), polarization microscopy, 100× (scale bar: 250 μm); (e and f) fragments of the previous sample: numerous MPH and single GC formed a lining of the IL of the capsule and adhered to the scaffold’s surface, hematoxylin and eosin staining, simple microscopy: (e) 400× (scale bar: 62.5 μm); and (f) 630× (scale bar: 39.7 μm).
Figure S3. Tissue reaction to the 3D scaffolds based on allylchitosan (day 30).

Note: the connective tissue formed the capsule (CAP) with blood vessels (V) around the implanted chitosan sponges (CHS); CAP consisted of two layers: the inner layer (IL) was a granulation tissue with macrophages (MPH) and giant cells (GC), the outer layer (OL) consisted of a more mature connective tissue; IL grew into the pores forming connective tissue septa (S); some MPH and GC adhered to the surface of scaffolds, picrosirius red staining: (a) the CHS material was picrinophilic, homogeneous; the CAP and S had a significant amount of collagen fibers and a moderate vascularization, simple microscopy, 200× (scale bar: 125 μm); b–d—fragments of the previous sample: (b) the scaffold was isotropic; the collagen fibers in the OL of the CAP carried of the most pronounced anisotropy (yellow and orange glow), while the collagen fibers in the IL and in the S carried of weak anisotropy (green glow), polarization microscopy, 200× (scale bar: 125 μm); (c) GC adhered to the surface of CHS material, simple microscopy, 1000× (scale bar: 25 μm); and (d) there was the weak transverse striation of the scaffold’s material in some septa of CHS, phase-contrast microscopy, 1000× (scale bar: 25 μm).
Figure S4. The data of connective tissue capsule thickness around the implanted films and 3D scaffolds based on allylchitosan. Note: (a) the connective tissue capsule thickness around the chitosan films (AC2–AC5), day 30, two-way ANOVA followed by Tukey’s test; (b) the connective tissue capsule thickness around the chitosan films and 3D scaffolds, day 30, two-way ANOVA followed by Sidak’s test; (c) the connective tissue capsule thickness around the chitosan 3D scaffolds on days 30, 60, and 90, two-way ANOVA followed by Tukey’s. Data are mean ± SD or median. n.s.: no significant differences.
X-axis: the minimum (min), average (mean) and maximum (max) thickness of the capsule.
Figure S5. Tissue reaction to the 3D scaffolds based on allylchitosan (day 60).

Note: unlike day 30 there were focuses of changes in tinctorial properties of scaffolds’ material (yellow arrows) in the chitosan sponge (CHS); also the inner layer (IL), the connective tissue septa (S), and the outer layer (OL) of the connective tissue capsule (CAP) consisted of a more mature connective tissue with larger blood vessels (V); giant cells (GC) were predominant, some of them adhered to the surface of scaffolds, simple microscopy: (a) numerous foci of red staining (yellow arrows), mainly in the surface areas of scaffold’s material; numerous V in the S, picrosirius red staining, 200× (scale bar: 125 μm); (b) basophilic foci in the surface of scaffold’s material (yellow arrow), GC on the scaffold’s surface; numerous V and areas of hemosiderosis in the large S, hematoxylin and eosin staining, 400× (scale bar: 62.5 μm); (c) a thin septum (lysis) of scaffold with a sharp basophilia of material (yellow arrow); numerous GC adhered to the surface of the scaffold; the large S with V and a small area of hemosiderosis, hematoxylin and eosin staining, 400× (scale bar: 62.5 μm); and (d) the area of red staining (yellow arrow); signs of scaffold’s material lysis and resorption in the place of GC adhesion, picrosirius red staining, 1000× (scale bar: 25 μm).
Figure S6. Tissue reaction to the 3D scaffolds based on allylchitosan (day 90). Note: focuses of changes in tinctorial properties (yellow arrows) and lysis of the chitosan sponge (CHS) material were more prominent, than on day 60, numerous giant cells (GC), simple microscopy, 1000× (scale bars 25 μm): (a) basophilia in the material of the scaffold septum, which was most pronounced in the surface areas (yellow arrow); the scaffold pore was filled with a GC, hematoxylin and eosin staining; (b) pronounced basophilia of CHS septs (yellow arrow), some lysis areas of the scaffold’s material, hematoxylin and eosin staining; (c) GC with a phagocytosed and partially lysed basophilic material of the CHS septum in the cytoplasm (yellow arrow), hematoxylin and eosin staining; and (d) areas of red staining (yellow arrows), septum lysis near the GC; some GC contained small fragments of red colored scaffold’s material, picrosirius red staining.
Table S1. Summary table of histological semiquantitative analysis results.

| Histological findings | Score | Films, Day 30 | 3D Scaffolds |
|------------------------|-------|---------------|--------------|
|                        |       | Day 30 | Day 60 | Day 90 |
| Changes in tinctorial properties of scaffolds | 0 | 0 | 1 | 1–2 |
| Scaffolds’ lysis | 0 | 0 | 0–1 | 0–1 |
| A maturity of a connective tissue capsule | 2–3 | 2–3 | 3 | 3 |
| Connective tissue ingrowth in pores | - | 0–1 | 1–2 | 1–2 |
| Vascularization in pores | - | 0–1 | 1–2 | 1–2 |
| The macrophage reaction | 1–2 | 1–2 | 0–1 | 0–1 |
| Foreign-body giant cell reaction | 0–1 | 2 | 2–3 | 2 |

Table S2. Correlation analysis: correlations between the time after implantation and the histological findings in samples of 3D-scaffold implantations.

| Histological Findings | Coefficients | p value |
|-----------------------|--------------|---------|
| The capsule thickness | minimum -0.53 | 0.04285 |
|                       | maximum -0.54 | 0.03985 |
| The degree of maturity of the connective tissue capsule | 0.74 | 0.00166 |
| The degree of ingrowth in scaffold pores | 0.55 | 0.03455 |
| The degree of vascularization in scaffold pores | 0.55 | 0.03455 |
| The degree of changes of the scaffold tinctorial properties | 0.88 | 0.00002 |
| The degree of scaffold lysis | 0.52 | 0.04712 |

1Only significant correlations are shown
**Table S3.** A histological semiquantitative scoring system for the evaluation of macrophage and foreign-body giant cell reactions to the scaffolds.

| Points | The Mean Number of Macrophages/Giant Cells on a Scaffold’s Surface in 10 Random Fields of View (400×) |
|--------|-------------------------------------------------------------------------------------------------|
| 0      | Not more than 1 cell                                                                             |
| 1      | More than 1, but not more than 5 cells                                                          |
| 2      | More than 5, but not more than 11 cells                                                          |
| 3      | More than 11 cells                                                                               |

*The score system was based on an algorithm for semiquantitative evaluation of inflammatory infiltration around the implantation of nanocomposites [69].*

**Table S4.** A histological semiquantitative scoring system for the evaluation of changes in tinctorial properties of scaffolds and scaffolds’ lysis.

| Points | Changes in a scaffold (changes in tinctorial properties/lysis)                                      |
|--------|------------------------------------------------------------------------------------------------------|
| 0      | No change or weak focal changes in less than 25% of the scaffold area                                |
| 1      | Weak focal changes in more than 25% of the scaffold area                                              |
| 2      | Pronounced focal or weak diffuse changes in more than 25% of the scaffold area                       |
| 3      | Pronounced diffuse changes in more than 25% of the scaffold area                                     |

**Table S5.** A histological semiquantitative scoring system for the evaluation of a maturity of connective tissue capsules around scaffolds.

| Points | Characteristics of a Capsule Around the Scaffold                                                   |
|--------|---------------------------------------------------------------------------------------------------|
| 0      | The capsule in all areas is immature (represented exclusively by granulation tissue) or mild focal fibrosis of granulation tissue in less than 25% of the capsule area |
| 1      | Mild focal fibrosis of granulation tissue in more than 25% of the capsule area                     |
| 2      | Pronounced focal or weak diffuse fibrosis of granulation tissue over 25% of the capsule area       |
| 3      | Pronounced diffuse fibrosis of granulation tissue in more than 25% of the capsule area             |

**Table S6.** A histological semiquantitative scoring system for the evaluation of a connective tissue ingrowth and vascularization in pores of 3D scaffolds.

| Points | Signs of a Connective Tissue Ingrowth/Vascularization                                            |
|--------|--------------------------------------------------------------------------------------------------|
| 0      | All pores of the scaffold are empty or less than 25% of the surface pores contain connective tissue/blood vessels |
| 1      | More than 25% of the surface pores of a scaffold contain connective tissue/blood vessels, deep pores of a scaffold are empty |
| 2      | More than 25% of surface pores and less than 10% of deep pores of a scaffold contain connective tissue/blood vessels |
| 3      | More than 25% of surface pores and more than 10% of deep pores of a scaffold contain connective tissue/blood vessels |

*The pores adjacent to the outer surface were attributed to the surface pores of the 3D scaffold; the remaining pores were attributed to the deep ones.*