Report of the Max-Buchner research project

„Biohybrid hydrogels based on recombinant technology" (MBFSt-Kennziffer: 3806)

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1. Aims and motivation

Biohybrid hydrogels are materials, which contain a biological component and a synthetic constituent. The biological part of the polymer imparts the structure with the desired biological activity (e.g. cell adhesion, enzyme degradation site, growth factor binding), while the synthetic element usually promotes stable and controllable mechanical properties. For this reason, biohybrid hydrogels are popular materials in diverse applications such as drug and cell delivery, in vitro cell culture and tissue engineering. While the synthetic part of the polymer allows control of material properties, the natural component (protein or polysaccharide) is frequently responsible for batch-to-batch variation in final hydrogel properties due to the natural sourcing of the biopolymer [1]. One strategy to ensure uniform product quality and properties, as well as minimize contamination risks, is production using recombinant technology.

The current project combines material design, chemical synthesis and biotechnology. The objective was to produce ECM-inspired biohybrid hydrogels. The biological component is based on human collagen I and fibrinogen sequences, and is produced in simple microorganisms, thus ensuring high production titers and cost-efficient cultivation. The produced proteins were modified with synthetic polymers, creating biohybrid hydrogels with tunable properties like desired stiffness and controllable sol-gel transition through light or temperature cues.

2. Performed investigations

In the first stage of the project, the recombinant proteins were produced by microbial fermentation. The gelatin mimetic ColMP was produced in the yeast *Komagataella phaffii* (*Pichia pastoris*) by direct secretion into the media [2]. Purification was performed by cross-flow and freeze-drying or alternatively by ion exchange (IEX) and size-exclusion chromatography. The fibrinogen mimetic FGG was produced as inclusion bodies (IBs) in *Escherichia coli* [3]. FGG was then isolated from the cell culture by lysis, centrifugation, washing and solubilization steps. The isolated proteins were then conjugated to synthetic polymers to yield the biohybrid hydrogels. The chosen protocols were based on the available amino acid residues of the protein sequences (e.g. prevalence of primary amines or thiol groups in the polypeptide).

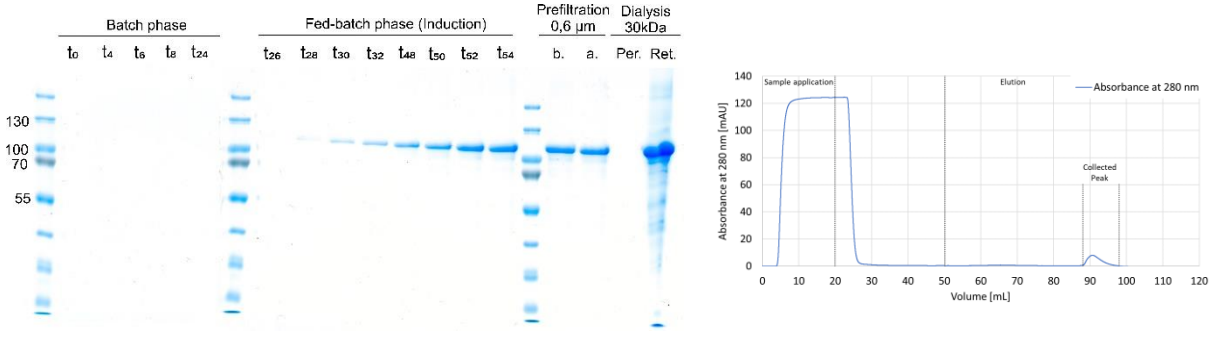

Fig. 1: Left: SDS-PAGE showing the production of ColMP in *P. pastoris* culture throughout the cultivation and purification. ColMP is expressed in the 100-130 kDa region after methanol induction (Fed-batch phase) and directly secreted into the media. Right: Chromatogram of the ion-exchange purification process with CaproS 10 ml column and acetate buffer system.

For the creation of photoactive structures, CoMP was subjected to methacrylation, in a manner similar for the creation of conventional semi-synthetic GelMA hydrogels [4]. In addition, modification of ColMP was performed with norbornene-thiol click chemistry, using norbornene-functionalized ColMP and dithiothreitol (DTT) or other di-thiols as a crosslinker [5]. The light mediated thiol-ene chemistry is not inhibited by oxygen and proceeds in a step-growth manner, leading to faster polymerization kinetics and enhanced cell viability [6]. FGG protein was modified with PEG-Diacrylate (PEG-DA) by Michael-type addition of the available thiol groups of the protein [7]. FGG was also modified with Pluronic F127-Diacrylate to create both: a temperature-tunable and a photo-crosslinkable biohybrid hydrogel [8].
The created biohybrid hydrogels were characterized by colorimetric assays and NMR for determination of functionalization efficacy. In addition, swelling behavior and long-term stability were also analyzed. The gelation was studied by small amplitude oscillatory shear rheology, using light illumination or temperature change to induce sol-gel transition. Biocompatibility of the created biohybrid hydrogels was studied in cell culture by evaluating indirect cell viability using the CellTiter-Blue® (CTB) assay.

3. Results

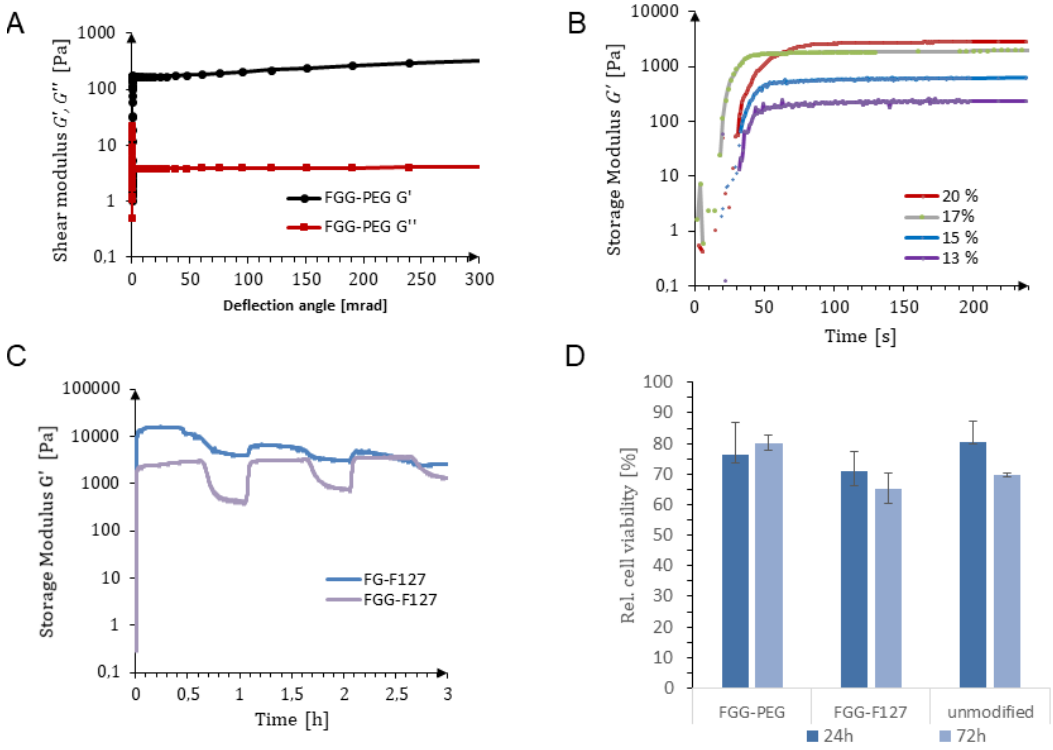

Fig. 2: Rheological characterization and biocompatibility studies of the recombinant biohybrid hydrogels A) All recombinant biohybrid hydrogels based on fibrinogen could be polymerized by the chosen method, example here shows an amplitude sweep of FGG-PEG indicating a stable gel structure (G'>G'') achieved after photo-polymerization; B) All biohybrid hydrogels showed tunable stiffness, related to e.g. protein concentration; Example here shows a time sweep experiment of different concentrations of FGG-F127 after 120 sec of light polymerization C) Reverse thermo-gelation of recombinant FGG-F127 in comparison to native fibrinogen (FG) conjugated to F127, cycles were performed on crosslinked hydrogels by alternating the temperature between 37°C and 4°C, D) CTB viability assay of the fibrinogen mimetic biohybrid hydrogels after 24h and 72h in relation to cell media alone and by comparison to conventional gelatin (unmodified)

The produced recombinant proteins could all by modified by the selected protocols. The gelatin mimetic ColMP was successfully modified by both norbornene functionalization and methacrylation as indicated by NMR studies (data not shown). However, the created biohybrid ColMP

hydrogels did not yield stable gels. SDS-PAGE studies indicated that the modification reaction leads to fragmentation of the polypeptide chain. Current studies are focusing on applying milder reaction conditions (pH and temperature, alternative dialysis times) for successful modification of the sensitive recombinant gelatin-mimetic proteins.
In contrast, the fibrinogen-mimetic protein FGG could be conjugated to both PEG-diacrylate and Pluronic F127 diacrlylate. The modification was confirmed by SDS-PAGE. The resulting recombinant biohybrids yielded stable gels upon light illumination, and in the case of FGG-F127, displayed the intended reversible thermo-gelation (Fig. 2A & 2C). The biohybrid hydrogel properties can be tuned by varying the hydrogel concentration (Fig. 2B), alteration of the synthetic component (e .g. PEG MW) polymerization conditions, or the starting polymerization temperature in the case of the temperature-sensitive FGG-F127. The biocompatibility of the fibrinogen-mimetic biohybrid hydrogels was investigated in their unpolymerized state by using the indirect CTB viability assay and hAD-MSCs. Unmodified gelatin in the same concentration was used as a control. Viability values were related to cell culture media alone without any hydrogel addition. FGG-PEG showed slightly higher cell viability values than FGG-F127, but both biohybrids yielded cell viabilities equal to or exceeding 70%, classifying them as biocompatible. Moreover, there was no significant differences in comparison to unmodified gelatin alone, indicating that the modification reactions introducing the synthetic polymer did not affect cell viability in a negative way.

4. Conclusion

Biohybrid hydrogels with different functionalities (photo activity and thermo-responsiveness) could be created from recombinant ECM-based proteins, produced in simple microorganisms. The hydrogels exhibit uniform properties, but lower stiffness in comparison to conventional reference proteins at the same concentration level (See Fig. 2C: Comparison between biohybrid from whole fibrinogen molecule (FG-F127) and biohybrid from fibrinogen gamma chain mimetic FGG-F127). This is to be expected, as the chosen ECM sequences represent shorter segments of the proteins of interest. Studies are currently focusing on continued characterization in cell culture, including 3D cell cultivation. The project represents an important foundational stone in showing the potential of recombinant technology for the sourcing/design of biohybrid hydrogels for biomedical applications.

5. Literature

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