Wiebke Kempin, Anna Baden, Werner Weitschies and Anne Seidlitz*

Glycerol gelatin for 3D-printing of implants using a paste extrusion technique

Abstract: Fused deposition modeling as an additive manufacturing technique has gained great popularity for the fabrication of medical devices as well as pharmaceutical dosage forms over the last years. Particularly the variety of geometries that can be printed determines the attractiveness of this technique enabling a shape adaption of e.g. implants. In the presented work the soft hydrogel material glycerol gelatin was investigated towards its applicability in 3D-printing as an alternative to the commonly applied and mostly rigid polyesters. Model implants loaded with the model drug quinine and with the shape of a hollow cylinder were printed via an extrusion based technique utilizing the piston feed in a hydrogel filled heatable syringe. Glycerol gelatin hydrogels need to be crosslinked to avoid gel-sol-transition at body temperature. For this purpose three different crosslinking methods (insertion, dipping, spraying) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were evaluated regarding their crosslinking efficiency and drug losses during the crosslinking process. Dipping of the implant into an aqueous solution with at least 50 mM EDC and 10 mM NHS was found to be the most efficient crosslinking technique in conjunction with a smaller drug loss during processing compared to inserting. However, the use of hydrogels also causes problems as an intense and highly variable swelling of the printed structures during crosslinking (120.7 % ± 11.9 % for 10 times dipping in 50mM EDC/10 mM NHS) and a great dependency of the volume on storage conditions complicate the preparation of tailor-made implants. The release of the model drug quinine from printed and crosslinked implants was fast and nearly completed within 6 hours.

Keywords: glycerol gelatin, 3D-printing, EDC, NHS, paste extrusion, crosslinking

https://doi.org/10.1515/cdbme-2017-0081

1 Introduction

In recent years 3D-printing and in particular Fused Deposition Modeling (FDM) has gained increasing interest for the preparation of pharmaceutical dosage forms [1,2]. The attractiveness of those additive manufacturing techniques especially for the production of implants lies in its nearly infinite variety of shapes that can be printed, enabling an individual shape adaption of the implant for every patient. Dosage forms printed via FDM are mostly solid and rigid as the current research is focussing on classical thermoplastic feedstock material like polyesters. The testing of hydrogels for their use in printing of implants is thus interesting as a higher flexibility allows a temporary folding or compression of the implant on its way to the application site e.g. during the endoscopic placement in the paranasal sinuses. Another benefit of hydrogels with their high water content is their similarity to the native extracellular matrix and their biocompatibility. That is why hydrogels are often used in the field of tissue engineering and bioprinting [3]. In this work glycerol gelatin that is traditionally used for the preparation of vaginal suppositories was examined regarding its suitability in extrusion-based printing of drug loaded implants and their subsequent crosslinking with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxy-succinimide (NHS).

***Corresponding author: Anne Seidlitz:** Institute of Pharmacy, Center of Drug Absorption and Transport, University of Greifswald, Felix-Hausdorff-Str. 3, 17487 Greifswald, Germany, e-mail: anne.seidlitz@uni-greifswald.de

Wiebke Kempin, Anna Baden, Werner Weitschies: Institute of Pharmacy, Center of Drug Absorption and Transport, University of Greifswald, Felix-Hausdorff-Str. 3, 17487 Greifswald, Germany, e-mail: wiebke.kempin@uni-greifswald.de, mail@anna-baden.de, werner.weitschies@uni-greifswald.de

2 Material and methods

2.1 Hydrogel preparation and extrusion-based printing

The glycerol gelatin gel was prepared as described in German Pharmacopoeia (DAB 1996). In short 1.19 g gelatin type A (bloom value 300, Sigma Aldrich, Germany) were added to a mixture of 2.38 g deionised water and 5.94 g glycerol 85 % (Caelo GmbH, Germany), left 20 minutes for swelling and dissolved completely at 80 °C. An ethanolic solution of 0.5 g of the model drug quinine (anhydrous, ≥ 98.0 %, Sigma Aldrich, Germany) was added to the glycerol gelatin, ethanol evaporated, the water loss was replaced and the mixture was subsequently transferred to a 50 mL-syringe (Omnifix® Luer Lock Solo, B. Braun Melsungen AG, Germany).

The z-axis of the used FDM-3D-printer (Multirap M420, Multec GmbH, Germany) was modified for printing with a syringe. The new construction mainly consists of a heatable aluminium cylinder exactly fitting the syringe and the cannula (Sterican® 0.6x80 mm, shortened to 15 mm, B. Braun, Germany) and a threaded rod, that is connected to the syringe piston and driven by a stepper motor (NEMA17 2.5 A, Nanotec Electronic GmbH & Co. KG, Germany). The extrusion speed and steps per unit settings in the firmware were adapted to the new extrusion technique. The model implant geometry ($Ø_{outer}$ = 5 mm, $Ø_{inner}$ = 3 mm, h = 3 mm) was designed using FreeCAD 0.14 and sliced with Simplify3D® (version 2.2.2, Simplify3D, USA).

2.2 Evaluation of crosslinking-methods

Printed imlants were crosslinked with EDC and intermediate products were stabilised with NHS in a ratio of 5:1 (EDC: NHS) that was described as most efficient in literature [4]. Three crosslinking methods were compared: insertion, dipping, spraying. Implants were either inserted (10 min, 30 min, 3 h) in 1 mL or dipped (3 times, 10 times) in 0.8 mL of aqueous crosslinker-solution or were finely sprayed (6 sides, 4 times) with an ethanolic crosslinker solution. After every dip and every spray, implants were dried under a constant air flow. The tested concentrations of the crosslinker solutions were 5 mM EDC/1 mM NHS, 10 mM EDC/2 mM NHS, 50 mM EDC/10 mM NHS and 100 mM EDC/ 20 mM NHS.

Quinine contents for both, implants directly after printing and implants after crosslinking procedure, were determined via ethanolic extraction as described previously [5]. Quinine contents in the crosslinker solution were directly measured by fluorescence spectrometry as explained in 2.3. The effectiveness of crosslinking was evaluated by slowly heating of the implants in a water bath and observing gel-sol-transitions or changes in consistency.

Imaging and the measurements of bottom and top areas and the height of the implant for lumen loss and volume calculations were performed with a reflected-light microscope (Zeiss Stemi 2000-C, camera Zeiss AxioCam and AxioVision software, Carl Zeiss Microscopy GmbH, Germany).

2.3 Drug release investigation

Quinine release from printed and crosslinked (10 dips, 50 mM EDC/10 mM NHS) implants was investigated in 15 mL-Falcon® tubes filled with 10 mL phosphate buffered saline (PBS) pH 7.4 under motion and at an incubation temperature of 37 °C (150 rpm, incubator Titramax 1000, Heidolph Instruments GmbH & Co.KG, Germany). Implants were transferred to fresh media at each sampling point. Detected amounts of quinine were summed up and related to the total released amount for the cumulative presentation. The quinine quantification was performed by fluorescence spectrometry (VarioSkan Flash λ_{ex} = 326 nm, λ_{ex} = 382 nm, measurement time 1000 ms, bandwidth 12 nm, Thermo Fisher Scientific Inc., USA) in black 96-well plates along with a calibration of quinine in the same solvent.

3 Results and discussion

3.1 3D-printing of model implants

In this work extrusion-based printing utilized the temperature dependent sol-gel-transition of gelatin during layerwise deposition of the strands. To prevent solidifications of the glycerol gelatin due to a temperature decrease before deposition on the printing bed, the used cannulas were shortened to 15 mm.

The sliced G-code was changed to a spirally pathway of the printing head to avoid long dwell times at the turning points in every layer. Best printing results for the quinine loaded glycerol gelatin hydrogels were observed for a printing temperature of 40 °C, a bed temperature of 20 °C, a printing speed of 80 mm/min and a layer height of 300 µm, without the use of the fan and without a piston retraction in order to avoid air bubbles rising in the syringe. Implants consecutively printed with this settings (figure 1) show an average mass of 32.1 ± 4.3 mg (n=30).

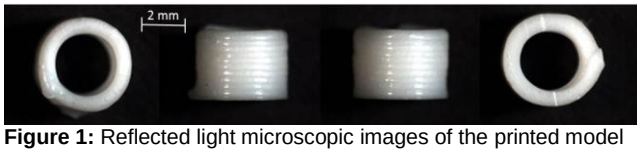

Figure 1: Reflected light microscopic images of the printed model implant in top and side view.

3.2 Evaluation of crosslinking-methods

Unlike their use as suppositories, implants made of glycerol gelatin ideally should not show the characteristic gel-sol-transition at body temperature and therefore need to be crosslinked. In contrast to the often used aldehydes, the crosslinkers in this work - EDC and NHS - are generally known as zero-length crosslinker as they do not integrate new structures to the existing hydrogel-network, but link carboxylic acid groups with amine groups to new amide bonds within the gelatin [4].

The absence of signs of liquefaction of the implant while heated to 50 °C in a water bath was used to indicate a successful crosslinking. A concentration of 5 mM EDC/1 mM NHS could not crosslink the implants sufficiently neither for insertion for 10 minutes, 30 minutes nor 3 hours since a considerably decrease of consistency could be observed at temperatures higher than 40°C. Implants inserted in higher concentrated crosslinker-solutions (10 mM EDC/2 mM NHS and 50mM EDC/10 mM NHS) did not show gel-sol-transition or consistency changes. Implants also demonstrated a sufficient crosslinking after dipping for 10 times in 10 mM EDC/2 mM NHS or higher concentrations (3x, 10x, 50mM EDC/10 mM NHS). The method of spraying the implants with an ethanolic EDC/NHS-solution was more error-prone, because a complete wetting especially in the inside of the hollow cylinder was more difficult to achieve also with regard to the thin metal wire placed through the lumen to hold the implant during spraying. Thus higher crosslinker-concentrations of at least 100 mM EDC/20 mM NHS were required for a successful crosslinking. For some of the tested implants crosslinked with a less concentrated EDC solution an intact outer implant surface and a liquefication originated from the luminal part of the implant at temperatures above 40°C was observed confirming the complete lumen wetting as the weakness of this method.

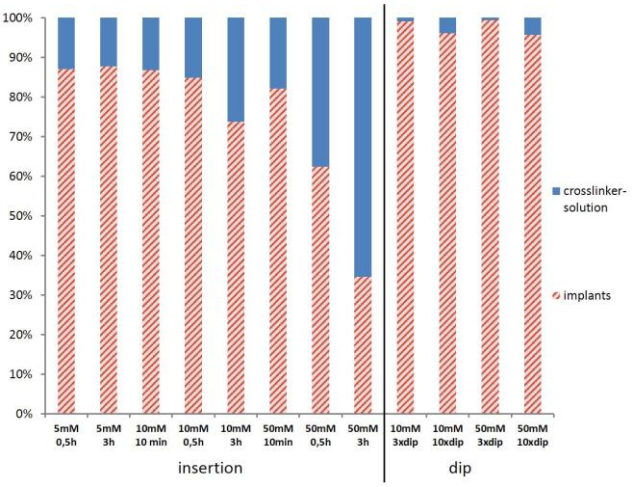

Figure 2: Percentage distribution of quinine in crosslinker-solution and implants after crosslinking, given concentrations refer to EDC (n=1).

Another crucial point for the evaluation of crosslinking methods for drug loaded dosage forms is the drug loss during crosslinking. In figure 2 quinine amounts that were lost to the aqueous crosslinking-medium related to the total detected amount of quinine are depicted as the percentage of drug loss. An increase in drug loss with growing insertion time, as expected, but also with growing concentration of the crosslinker solution for the insertion method is apparent. The highest drug loss of approximately 65 % could be observed for the longest insertion time of 3 hours and the highest tested crosslinker concentration (50mM EDC/10 mM NHS). Due to the shorter contact time dipping demonstrated smaller quinine losses than inserting the implants. Taking the wetting problems of the spray method and the high drug losses of the insertion method into account, 10 times dipping of the implant in 50mM EDC/10 mM NHS solution was found to be the best method and used for following experiments.

A critical view has to be taken on the absence of a washing step after crosslinking, as residual crosslinkers can be harmful at the application site. However an additional drug loss through washing needs to be considered. For this reason a drug loading process downstream of printing, crosslinking and washing will be considered in future tests.

The investigation of implant-swelling during the crosslinking process clearly shows a volume growth with a high variability between all measured implants (table 1). If the crosslinked implants were stored in a closed container afterwards volumes stayed nearly constant, whereas implants stored in an open container desiccated and the waterloss lead to volume shrinkage. The lumenloss during swelling in the crosslinker solution followed the trend described for the

volume growth. This severe changes in the implant geometry and its strong dependency on the storage conditions must be taken into account for the design of the printing shape and certainly complicate the preparation of tailor-made implants.

Table 1: Volume growth and lumen loss, both related to measures after printing, of printed implants after crosslinking (10 dips, 50 mM EDC/10mM NHS) and after 5 days storage in closed or opened 2 mL Eppendorf-tubes® at 20°C.

	after cross-linking (n=12)	**After 5 days in closed tubes** (n=6)	**After 5 days in open tubes** (n=6)
Volume growth	120.7 % ± 11.9 %	118.6 % ± 12.9 %	71.3 % ± 12.4 %
Lumen loss	109.7 % ± 5.3 %	104.9 % ± 5.0 %	93.8 % ± 4.4 %

3.3 Drug release investigation

The release of the model drug quinine from the printed and crosslinked implants in PBS pH 7.4 is illustrated in figure 3. The drug release occurred very fast as expected for hydrogels with high water content. Within 6 hours the containing quinine was almost completely released.

The drug delivery from hydrogels can be extended applying various strategies [6] including ionic charge interactions between the polymer and the drug as also described for a modified gelatin [7]. The short period of drug release observed in this work appears reasonable for acute treatments and therefore can only justify the application of less-invasive implants e.g. implants in the ears-nose-throat (ENT) region placed via endoscope.

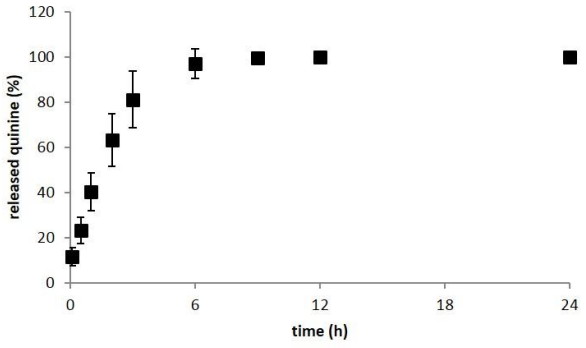

Figure 3: Quinine release from printed implants after crosslinking (10 dips, 50 mM EDC/10mM NHS) in phosphate buffered saline pH 7.4, n = 3 ± SD.

4 Conclusion

This study showed that drug loaded glycerol gelatin can successfully be printed with the used extrusion-based technique. The crosslinking process of 10 times dipping of the implant in 50mM EDC/10 mM NHS solution was identified to be the best of the tested methods concerning the crosslinking outcome and the drug loss. However, the very fast drug release, the pronounced swelling of the implants during processing and its variability pose problems for the approach of printing shape adapted implants.

Author's Statement

Research funding: Financial support by the German Ministry of Education and Research (BMBF) within "RESPONSE" is gratefully acknowledged. Conflict of interest: Authors state no conflict of interest. Informed consent: Informed consent is not applicable. Ethical approval: The conducted research is not related to either human or animals use.

References

[1] G. Jonathan, A. Karim, 3D printing in pharmaceutics: A new tool for designing customized drug delivery systems, International Journal of Pharmaceutics 499 (2016) 376–394.
[2] M.A. Alhnan, T.C. Okwuosa, M. Sadia, K.-W. Wan, W. Ahmed, B. Arafat, Emergence of 3D Printed Dosage Forms: Opportunities and Challenges, Pharmaceutical Research 33 (2016) 1817–1832.
[3] I.T. Ozbolat, M. Hospodiuk, Current advances and future perspectives in extrusion-based bioprinting, Biomaterials 76 (2016) 321–343.
[4] A.J. Kuijpers, Engbers, Gerard H. M., J. Krijgsveld, Zaat, Sebastian A. J., J. Dankert, J. Feijen, Cross-linking and characterisation of gelatin matrices for biomedical applications, Journal of Biomaterials Science, Polymer Edition 11 (2000) 225–243.
[5] W. Kempin, C. Franz, L.-C. Koster, F. Schneider, M. Bogdahn, W. Weitschies, A. Seidlitz, Assessment of different polymers and drug loads for fused deposition modeling of drug loaded implants, European Journal of Pharmaceutics and Biopharmaceutics (2017).
[6] T.R. Hoare, D.S. Kohane, Hydrogels in drug delivery: Progress and challenges, Polymer 49 (2008) 1993–2007.
[7] M. Sutter, J. Siepmann, W.E. Hennink, W. Jiskoot, Recombinant gelatin hydrogels for the sustained release of proteins, Journal of Controlled Release 119 (2007) 301–312