SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Biocompatibility examinations of 3D printed implants

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

DEBRECEN, 2021

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Contents

1. Introduction and aim

Three-dimensional (3D) printing has become one of the major innovative technologies of recent years. It can be used in many areas, including the consumer society, mechanical engineering, the food industry and healthcare system. In the pharmaceutical industry it is used to manufacture various drug delivery systems. The first solid dosage form (Spritam®) of a medication printed by 3D technology was approved by the FDA in 2015, which is an orally disintegrating tablet and used for the therapy of epilepsy.

In the United States, 30 million compounded preparations were manufactured in the pharmacies considering the needs of patient. Personalized medication offers unique therapeutic advantages: the ability to raise the effectiveness, decrease the amount of the used active pharmaceutical ingredients (APIs), minimize adverse effects, and increase patient compliance. The application of 3D printing technology may be an alternative way to design effective, customized API combination for the patient immediately. Complex, personalized products can be prepared on-demand, through a low-cost manufacturing process, by healthcare providers. Nevertheless, some limitations should be taken into consideration. The APIs used have to be stable during printing, which means they must have high heat tolerance, stability in different solvents, compatibility with cross-linking polymers, and tolerance to UV light. Although it is a fast prototyping technique, it is not able to exceed the productivity of an industrial-sized tableting machine.

Our aim is to manufacture various drug delivery systems with FDM type 3D printing and to perform the necessary tests on the produced systems. In our 1st series of experiments, the samples were chemically modified by different chemical side chains. The aim was to determine the material structure and the biocompatibility of the samples.

In our 2nd series of experiments, samples were prepared from different polymers with diclofenac sodium powder content using an FDM 3D printer. Our aim is to study the material structure of the prepared samples, to assess the cytotoxicity of the samples, and to determine the dissolution profile of the samples prepared from different polymers with different diameters and infill percentages.

2. Literary overview

The 3D printing process is a summary name of different printing methods and has several synonyms, such as rapid prototyping and additive manufacturing.

The printing is a layer-by-layer process based on a digital design. The first step is to design the desired object in 3D designer programs (e.g. Rhinoceros, Google Sketchup). The digital design will result in a stl (standard tessellation language) file format, which can be handled by the software of the 3D printer. In these programs, the final printing parameters of the sample can be done, for example, size, infill percentage. The printing parameters can be modified in the software as well. If support parts are required during printing the program will recognize it automatically. These supports can be removed manually after printing. After this process, we receive a gcode file format what the 3D printer can interpret. The last step is to print the sample with the 3D printer itself.

With the use of the technique complex, personalized medications can be prepared on demand in a low-cost manufacturing process. As a personalized medication, the adherence, the drug safety and the patient compliance can be increased.

The pharmaceutical industry and a number of research groups are working to license 3D printed drug delivery systems to market. In 2015, the FDA approved the first 3D printed drug. Spritam® (levetiracetam) is an orodispersible tablet developed for the treatment of epileptic seizures. The manufacturing uses a so-called ZipDose® technology, which means that the homoganeus powder layers are fixed layer by layer with a liquid binder.

Tablets, capsules, implants, transdermal drug delivery systems (TDS) and orodispersible films are the most commonly formulated drug delivery systems with 3D printing.

Hollander et al. prepared prednisolone containing modified-release tablets from polydimethylsiloxane (PDMS), where UV light formed the crosslinks in the samples. Another research group developed the filaments for FDM printing with various cellulose modifications. The active pharmaceutical ingredient was 30% isoniazid and the manufactured drug dosage form was a tablet. Numerous researches are experimenting with chocolate-based 3D printing, where the binder will be the melted chocolate instead of different chemicals. One research team prepared ibuprofen and paracetamol containing tablets by this method. **Khaled and co-authors** produced a so-called ‘polypill’, which refers to different active pharmaceutical ingredient in one tablet. They used several print heads at the same time, which allowed that in the manufactured tablet five different active pharmaceutical ingredients were deposited in different compartments and their samples had two independent and well-defined dissolution profiles.

Maroni and co-authors had been involved in the production of capsules by 3D printing in several scientific publications. In one of their publications, two-compartment capsules with wall thicknesses of 600 and 1200 μm were produced by 3D printing, filled with yellow and blue inks as a model drug. In essence, the effect of wall thickness on the dissolution profile was investigated.

Implantable drug delivery systems have been developed by different methods. In one case, an implantable drug delivery system was made by 3D printing for the treatment of osteosarcoma. In another case, implantable nanogels were prepared to reach a long-term effect. Some researchers have used 3D printing to create a suitable frame that contains the active pharmaceutical ingredient in the polymeric base and as a result implantable drug delivery systems can be manufactured for bone surgery.

In my experiments every sample was manufactured by fused deposition modelling (FDM) or fused filament fabrication (FFF). The process is based on that the polymeric filament melts in the print head due to the heating, so that the polymer is melted, and the layers of the sample are formed on the bed. FDM technology is an off-patent, widespread and low-cost process that has inspired many research teams in the healthcare system and the pharmaceutical industry to manufacture drug delivery systems. Other benefits that mechanically stable systems can be produced in a high resolution printing process (30-200 μm) that do not require washing or cleaning steps after the printing is completed.

Many manufacturers offer commercially available filaments for FDM printing, commonly polylactic acid (PLA), polyethylene terephthalate (PET), polymethyl methacrylate (PMMA), nylon, polycaprolactone (PCL), acrylonitrile-butadiene-styrene (ABS) or thermoplastic polyurethane (TPU) filaments alone or modified (e.g. antibacterial poly lacticacid (AntiPLA), polyethylene terephthalate glycol (PETG)). During FDM printing, a number of parameters affect the quality of the printed sample, such as the physical and chemical properties of the base material and active pharmaceutical ingredients, the print head, the extruder, and the set printing parameters. It is very important to investigate the possible effects of the used parameters and to determine the critical printing parameters during the manufacturing of drug delivery systems. Two different research groups also investigated the relationship between wall thickness and dissolution profile for 3D printed capsules and modified-release tablets. It was found that wall thickness as a printing parameter has a very important effect on the dissolution profile.

The implantable drug delivery systems (IDDS) are defined in Pharmacopoea Hungarica (Ph. Hg.) VIII. “Parenteral active pharmaceutical ingredient containing implants are solid, sterile preparations for parenteral implantation, of suitable size and shape, which release their

active pharmaceutical ingredient continuously over a long period of time. The implants are individually packaged in sterile containers. "

The IDDS's have a number of advantages, including the ability to achieve modified drug release compared to conventional drug dosage forms. Implants can provide continuous drug delivery for long periods of time, up to years. Depending on the site and route of administration, local or systemic effect may be provided. With the controlled drug release, constant plasma levels can be achieved, resulting in better patient compliance. At the same time, fewer amount of the active pharmaceutical ingredient is enough to achieve the same response, which also reduces the probability to side effect development.

On the other hand, there are also some disadvantages to consider during the application. The dosage form itself contains more than the maximum daily dose at one time, so in case of inappropriate drug release, it can cause toxicity. Redness, pain and, more severe cases, an anaphylactic reaction may occur at the site of implantation. Implantation and possible removal require minor surgery and should only be carried out by qualified personnel. The product is a complex structure that is more expensive to manufacture and authorize than a traditional dosage form.

Various methods can be used to manufacture implants, such as injection moulding, compression moulding, hot melt extrusion (HME), solvent casting and 3D printing.

3. Materials and methods

In our 1st series of experiments, the samples were 3D printed and then chemically modified. Different chemical side chains where attached to the pre-printed implants made of different polymers, so our aim was :

- examination of the material structure of the samples:
  - scanning electron microscopy and optical roughness measurement to determine surface properties
  - Fourier transformed infrared spectroscopy to study the functional groups related to the chemical modification
  - positron annihilation lifetime spectroscopy to determine the free volume on the surface of samples
  - contact angle measurement provides information on the wetting properties of the samples, which is important from the aspect of a possible rejection
- examination of the biocompatibility of the samples:
  - modified MTT is performed on 4th, 8th and 12th day and for 1, 2 and 3 month to determine the cytotoxic effect of the samples and if any kind of xenobiotic may be released from the samples
  - investigation of biofilm formation on the surface of samples by Candida albicans SC5314 reference isolate

In our 2nd series of experiments, the samples were manufactured by FDM 3D printer from different polymers with diclofenac sodium as the active pharmaceutical ingredient content:

- examination of the material structure of the samples:
  - scanning electron microscopy and microcomputed tomography analysis to determine surface properties and internal 3D structure
  - thermogravimetric and heat flow studies to map stability and decomposition
  - Raman spectroscopy to investigate the location of drug content in samples with different infill patterns
- cytotoxicity experiment of the samples by MTT test
- dissolution test of the samples to determine the dissolution profile of the samples with different diameter, infill percentage and polymer type

4. Results

4.1. 1st series of experiments

4.1.1. 3D printing and chemical modification

In our experiments, PLA filaments were pre-formed with a localized heating cylinder so the filaments were melted. Then, the filament was 3D printed at 215 °C through a 0.4 mm stainless steel nozzle, using a layer height of 50 μm or 75 μm. The infill used was 100%, ensuring that porosity arises from the small spaces between the extruded filaments and any cavities between individual polymer molecules in the extruded filament. An infill of 100% also ensures high mechanical strength and that the reactant solution does not enter the printed PVTP and break down inter-layer cohesion. These 3D printed PLA polymer PVTPs of 14 mm diameter and 3 mm height provided the base for chemical modification.

PLA PVTPs were chemically modified using different amines as side chains. This is unique to our study, because these 3D printed PLA PVTPs containing amine side chains have not been previously achieved. The concept of our modifications is based on the work of **Haddad et al**. PLA as a base polymer has advantageous mechanical properties and thermoplastic properties. Due to these favorable attributes, it has been widely used in FDM based 3D printing processes.

The amines were chosen to present hydrophilic side chains of different natures: PLA-ED presents a short spacer with a primary amine head group, PLA-NprN a spacer and a secondary amine head group, PLA-TET and PLA-BAPA oligo-amino chains, PLA-NPEGN a short PEG spacer, and finally PLA-Tris a ramified di-amine head group.

Measurement with a digital micrometer before and after treatment showed an isotropic decrease in diameter and height of 50 μm, showing that 25 μm etching occurs at each surface.

4.1.2. Material structure characterization

4.1.2.1. Scanning electron microscopy

The surface of the PLA PVTP and its modifications were tested using a scanning electron microscope. In our study, both the untreated surface and the deionized water washed surfaces had the general aspect of a stepped surface, with some small deviation from planarity for the untreated surface. After washing the surface appeared smoother, correlating with the change in the contact angle. For the six treated surfaces (PLA-BAPA, PLA-ED, PLA-Tris, and PLA-NPEGN), all appeared flattened with respect to the initial surface, however all showed a large number of indentations. PLA-Tris showed ca 1 μm pore diameters. In the case of the TET modified surface, the overall aspect was close to that of the untreated printed surface, and small indentations were visible with diameters of ca 100–200 nm. Finally, in the case of the MeNprN

modified surface, etching appeared to have occurred and the surface was separated into iceberg-like systems overlying a flat surface. This confirms that the surface of the 3D printed PLA PVTPs had been modified, which confirms the formation of separated plates on the printed surface.

4.1.2.2. Fourier transformed infrared spectroscopy

As expected, the spectrum of PLA is characterized by a weak C–H stretch band at 3000 cm^{-1} and a very strong C=O ester stretch at 1750 cm^{-1}. For PLA-ED, additional C=O amide bands are observed at 1550 cm^{-1} and 1650 cm^{-1}, and the presence of an amide stretch can only come from the formation of a covalent bond between the PLA polymer and the ethylene diamine. This is further confirmed by the presence of an N–H amide stretch band at 3300 cm^{-1}. As expected from the fact that only surface modification is occurring, the intensities are much lower than that of the ester C=O band.

4.1.2.3. Optical microscopy roughness measurement

The line section of all modification were obtained using a Keyence VHX 6000 optical microscope. Using the internal image processing program, Rz values of 29 μm for PLA, 3 μm for PLA-ED, and 6 μm for PLA-NPEGN were observed. The values of the PLA results from the presence of a void of about 20 μm between two extruded polymer filaments, and small depressions at 400 μm intervals arising from other filaments, were less than 5 μm in depth. The surfaces of modified PVTPs were generally quite flat with small pits and protrusions present in the modified samples, which was in agreement with the morphology observed using SEM. No systematically repeated depressions at 400 μm distances were observed. This was in agreement with the observation that 25 μm was etched from the PVTP during modification.

4.1.2.4. Positron annihilation lifetime spectroscopy

PALS experiment determines the average discrete o-Ps lifetime values of various samples. The higher lifetime values indicate higher free volume holes between the polymeric chains, and also residual space between filaments. The results showed that the modified polymeric bases (BAPA through NPEGN) were of similar o-Ps lifetimes, thus were free volume holes, while the PLA PVTP represented significantly higher o-Ps lifetimes. Based on our results, after the modification, PLA-ED and PLA-Tris contained the most free volume in their structures. PLA-MeNprN had the least free volume in its structure. The latter could be explained by the fact that through modification, the amines could be built into the polymeric chains, resulting in smaller free volumes between chains and especially between polymer molecules in the filaments.

4.1.2.5. Contact angle measurement

The contact angle measurement results can be sorted based on a decreasing volume: PLA PVTP without water wash = PLA PVTP with water wash > PLA-MeNprN > PLA-ED > PLA-NPEGN > PLA-BAPA > PLA-Tris > PLA-TET. The contact angle values of our PVTPs were between 26.5 ± 3.0° and 69 ± 0.1°, showing that all the PVTPs are hydrophilic and that the chemical modification by introduction of polar oligo-amine functions results in increasing hydrophilicity. As expected, the unmodified PVTPs show the highest contact angles.

4.1.3. Biocompatibility

4.1.3.1. MTT assay

A prolonged cell viability test was utilized in our work to gain information about the cytocompatibility of our PLA PVTPs. The samples were incubated in the cell culture medium for four, eight, and 12 days, and the Caco-2 cells were treated by this medium. This method differs from the original MTT assay because the inhibitory concentration (IC_{50}) was not measured, but the cell viability was calculated in comparison with a negative (DMME medium). In the works of **Kinnari and Chessa et al**., Caco-2 cell lines were the gold standard for the cytotoxicity examinations of orthopedic and breast implants. Caco-2 cell lines are a model of the intestinal barrier, because numerous attributes are the same as in the mature enterocyte.

The results are expressed as the percentage of negative or untreated control (Co−). As a positive control (Co+) Triton-X 100 (10% *w/v*) solubilizing agent was used, which resulted in significant differences from the other examined samples. We compared cytotoxic values and found none of the samples decreased significantly in cell viability compared to the untreated control. Based on our MTT assay, PLA-TET cytotoxicity did not decrease under 100% in comparison with the negative control. PLA-ED, PLA-BAPA, PLA-Tris, and PLA-NPEGN cell viability values were higher than 90% in the case of prolonged exposure. Based on the ISO 10993-5:2009(E) standard, if the relative cell viability is higher than 70% in comparison with the control group (100%), the materials can be considered non-cytotoxic. According to this regulation, all our PLA PVTPs qualified as cytocompatible.

4.1.3.2. Biofilm formation

Our experiments were based on the fact that the formed biofilm can be visualized with a crystal violet (CV) stain, and the absorbance of the dye can be measured. The measured absorbance values were in correlation with the amount of biofilm in the implants. All of the results of biofilm formation were under a 0.24 absorbance value. PLA PVTP and BAPA modification result in significantly higher absorbance results than other modifications. MeNprN

has higher absorbance results than 0.05, but TET, Tris, NPEGN, and ED have lower absorbance results than 0.05. In comparison with the other PVTPs, ED resulted in the smallest absorbance value. According to the classification from **Marcos-Zambrano et al**., low biofilm forming ability occurs with absorbance values under 0.44, moderate biofilm forming ability occurs between absorbance values of 0.44–1.17, and high absorbance forming ability occurs with absorbance values above 1.17. Based on this classification, all of our implants showed low biofilm forming ability. There are some significant differences among the chemically modified PLA PVTPs because PLA-ED resulted in the lowest biofilm formation, however PLA-BAPA and PLA PVTP represented the highest biofilm formation.

4.2. 2nd series of experiments

4.2.1. 3D printing

In our experiments, four different polymers, polylactic acid (PLA), antibacterial PLA (Anti), polyethylene terephthalate glycol (PETG), and poly(methyl methacrylate) (PMMA), were used for the 3D printing. The sample thickness was 2.4 mm and the diameter was 16, 19, or 22 mm. The infill percentage rates were 0%, 5%, 10%, and 15%.

First, the filaments were melted to the desired temperature. In the case of PLA and antibacterial PLA, the printing temperature was 215 °C; PETG was printed at 250 °C, and PMMA at 270 °C. Our research group developed an easy method to fill the samples without the loss of API, so an extra covering part is printed separately. The sample printing took place and at the appropriate moment the process was stopped; then the API was poured and the preprinted extra covering part was placed in the top of the API. Finally, the top two layers were printed. In this way, neither the extrusion temperature nor the ventilation disturbed the FDM process.

The samples were labeled with the polymer used (e.g., PLA), the diameter of the sample (e.g., 16), finally the sample's infill percentage (e.g., 0); in this case, the sample name is PLA_16_0.

4.2.2. Content uniformity and weight variation

The samples' average weight (mg) depended on the polymer and increased with the increase in diameter and infill percentage. The samples were filled with ~30 mg of diclofenac sodium salt.

4.2.3. PLA degradation

As PLA and antibacterial PLA are biodegradable polymers, the degradation amount of the samples was determined at pH = 7.4, 37 °C. The degradation was determined by weight measurement, but during the examination period the samples did not degrade at all.

4.2.4. Characterization

4.2.4.1. Scanning electron microscopy

The surfaces of the samples were examined with scanning electron microscopy. The SEM images of the PLA, Antibacterial PLA, PETG and PMMA samples were determined at 50x magnification. The surface adequateness was measured, and the layers were properly structured on the surface of the 3D-printed samples. The distance between two printed filaments next to each other were determined. In the case of PLA, the average was 713.67 μm (±17.72); for antibacterial PLA it was 712.86 μm (±2.26), for PETG it was 704.33 μm (±12.07), and for PMMA it was 700.16 μm (±7.73). PLA, antibacterial PLA, and PETG had flat but PMMA had a stepped surface, with some small deviations from planarity.

The surface morphology and pore structure of the 3D-printed samples are compared right after the 3D printing and after the dissolution test with 20x magnification. After the dissolution test, pores were formed, which supported our assumption that API liberation happened with diffusion through the API dissolving holes.

4.2.4.2. Micro computed tomography

The samples were examined by micro computed tomography (microCT) before and after the dissolution test to determine their morphology. The localization of the diclofenac sodium was well defined before the dissolution test and after the dissolution test no remaining can be seen. The dissolution test did not affect the location of the lateral filament layers.

The upper surface of the PLA_16_0 sample before and after dissolution were compered. It can be clearly seen that pores formed on the surface of the dissolved sample.

The exact difference was calculated from three parallel sliced surfaces of 5 μm image pixel size. The percentage of the filament infill was examined. The PLA_16_0 sample before dissolution showed 94.06% area and after dissolution 87.95%. There were 6.11% more pores on the surface of the sample after dissolution.

4.2.4.3. Thermogravimetric and heatflow analysis

The TG curve of diclofenac sodium showed thermal stability until 280 °C, so we can state that the API used is stable at the applied printing temperatures. Below 100 °C, a less than 1% mass loss was observed, probably due to absorbed water evaporation from the surface of the

API. In the diclofenac powder samples poured out from the polymeric frames, this did not occur. Based on the TG curve of the API, it is very likely that melting was associated with thermal decomposition. The API decomposition was around 39% until 500 °C. The DSC curve showed an endothermic peak at 289.97 °C, which was immediately followed by an exothermic peak where the diclofenac decomposed.

The thermogravimetric analysis of the PLA, antibacterial PLA, PETG, and PMMA samples was performed on filaments, polymeric samples without diclofenac, and polymeric samples with diclofenac, but the API was poured out before the TG analysis (polymer frame). The curves proved that all the APIs and the filaments were stable at the printing temperature of the experiment; no sign of chemical decompositions or changes was detected below 270 °C. All four polymers can be considered thermally stable, but the polymers can be grouped based on the temperature range: PLA and antibacterial PLA had a stable maximum of 280 °C, PETG was stable until 400 °C, and PMMA was stable until 340 °C. We can state that, at the applied printing temperatures (PLA—215 °C, PETG—250 °C, and PMMA—270 °C), all the polymers were stable.

In the case of PLA and antibacterial PLA polymer frames the thermal stability was smaller at 50 °C in comparison with the samples without diclofenac; these samples can still be considered stable because the printing temperature was 215 °C. The PETG and PMMA polymer samples did not show a similar alteration; the thermal stabilities were not altered, with or without diclofenac sodium.

4.2.4.4. Contact angle measurement

The contact angle values were measured on four types of polymer (PLA, PLA antibacterial, PETG, and PMMA), without and with diclofenac sodium. The different polymers had different contact angle values: PLA around 63°, antibacterial PLA around 22°, PETG around 74°, and PMMA around 84°. Interestingly, the contact angle values of antibacterial PLA filaments decreased nearly to third compared to the PLA contact angle values due to the antibacterial nanoparticles in the filament.

4.2.4.5. Raman spectroscopy

Raman spectroscopy was performed on a Wasatch Photonics WP-785-R-SR_l-50 equipment (Morrisville, NC, USA) to determine the distribution of the diclofenac sodium salt within the six cavities of the 5%, 10%, and 15% infill samples. The detector was a charge-coupled device (CCD), the detector temperature was 10 °C, the laser beam wavelength was 785 nm, the integration time was 500 ms, and the scan time was 32 s. The data were analyzed in

Unscrambler X version 10.5.1. software (Camo Analytics, Montclair, NJ, USA). For these measurements, polylactic acid samples with 16 mm diameter and 0%, 5%, 10%, or 15% infill were used. The sample with 0% infill had only one cavity, so in this case the whole amount of API was determined. For the PLA_16_5, PLA_16_10, and PLA_16_15 samples, the diclofenac amount in every cavity was determined with the use of the calibration curve. All samples contained approximately 30 mg of diclofenac sodium salt; these results were in accordance with the content uniformity measurements. Based on our results, around 75% of the whole API amount was in cavities 1, 2, and 3 in sample PLA_16_5, PLA_16_10, and PLA_16_15.

4.2.5. MTT assay

A prolonged cell viability test was performed to gain information about the cytocompatibility of the 3D-printed samples. The samples were incubated in the cell culture medium for four, eight, and 12 days, and the monolayer formed by Caco-2 cells was treated with this medium to determine if any kind of xenobiotic was dissolved from the sample. This method differed from the original MTT assay because the inhibitory concentration (IC_{50}) was not measured, but the cell viability was calculated in comparison with the untreated, negative control (DMME medium). This method was harmonized with the ISO standard.

The results were expressed as the percentage of negative or untreated control (Co−). As a positive control (Co+), Triton-X 100 (10% *w/v*) solubilizing agent was used, which has significant differences from the other examined samples. Based on the ISO 10993-5:2009(E) standard, if the relative cell viability was higher than 70% in comparison with the control group (100%), the materials could be considered non-cytotoxic. According to this regulation, all 3D-printed PLA, antibacterial PLA, PETG, and PMMA samples qualified as cytocompatible.

4.2.6. In vitro dissolution test

The dissolution profiles of the samples were determined by a USP type I in vitro dissolution apparatus to gain information about the effect of the polymers, diameters, and infill percentages on the dissolution profiles.

Pairwise comparison results of the dissolution profiles were determined: f1 value of the difference factor calculation and f2 value of the similarity value calculation. We found that all of our sample dissolution profile can be considered non-similar. Based on this comparison, all samples had different dissolution curves.

Drug release data were fitted to zero-order and first-order models. Determination coefficients were used to determine the best fit. The PLA_16_0 sample fitted to the zero-order model, which confirms the linear curve shape. The Anti_16_0 sample showed first-order

kinetics, with a correlation coefficient higher than 0.93. The calculations revealed that other samples can be fitted neither to the zero-order nor the first-order model since the correlation coefficients were all smaller than 0.80 if we compared the results from 0–24 h. The dissolved API amounts were fitted to the same kinetic models, but only at 0–2 h. PLA_19_0, PLA_22_0, PLA_22_5, PLA_22_10, PLA_22_15, PMMA_16_0, PMMA_19_0, and PMMA_22_0 can be considered zero-order kinetics in the first 2 h, while PETG_16_0 and PETG_22_0 fitted more to the first-order kinetic model at 0–2 h. The Anti_19_0, Anti_22_0, and PETG_19_0 samples were examined based on their dissolution curves. First, we determined the last time point before the curve reached the plateau phase and the kinetic models were fitted only before that time point. Anti_19_0 and PETG_19_0 until 30 min and Anti_22_0 until 45 min were fitted to a first-order kinetic model.

The diameter and infill percentage have a similar effect to what was expected. The dissolution was faster with the increase in the diameter due to the larger surface area and the formation of more API-dissolving pores. The API dissolution from the samples can be slowed down with the alteration of the infill percentage from 0% to higher amounts, e.g., 5%, 10%, or 15%.

5. Discussion

5.1. 1st series of experiments

Nowadays, many studies have been published on 3D printed implants and dosage forms, however, the connections between the chemical structures, structural parameters, and cytocompatibility results have not been well characterized yet. The objectives of our study were to find connections between the above mentioned parameters in case of chemically modified PLA based 3D printed polyvalent test plates platforms, or PVTPs. Various amines were used for the post fabrication chemistry to form active amide functionalities along the surface of 3D printed PVTP structures.

Polyvalent test plates were designed for investigation of the physical, chemical, and in-vitro biological properties of FDM printed materials, thus the term polyvalent in this article refers to the use of a single structure for testing of a wide range of properties. The advantage of such a PVTP was the exclusion of effects due to differing geometries, and enabling of the direct comparison between test results. A polyvalent can be used in many situations—chemistry, physics, mechanics, biology, and cell growth, and even biofilm formation.

The structural parameters of the samples were evaluated using Fourier transform infrared (FTIR) spectroscopy, contact angle measurements, scanning electron microscopy (SEM), surface roughness measurement, and positron annihilation lifetime spectrometry (PALS). A cytotoxicity test based on an MTT assay on Caco-2 cells and investigation of biofilm formation with *Candida albicans* were performed to certify the cytocompatibility of the PVTPs.

Of the available methods to characterize printed and modified PLA objects, FT-IR will definitively show the presence of new chemical functions, but intensities of the IR bands may be weak in the case of surface changes. Other methods are not suitable to prove the formation of covalent bonds on the surface, so we can state that the chosen test method was suitable for our purposes. For all modified samples, additional C=O amide bands are observed at 1550 cm^{-1} and 1650 cm^{-1}, and the presence of an amide stretch can only come from the formation of a covalent bond between the PLA polymer and the ethylene diamine.

The results of contact angle measurement can provide information about the hydrophilic and hydrophobic characteristic of the surface. There is clear evidence for the influence of surface topographical and wetting characteristics on macromolecular and cellular levels at implant interfaces. The polymer ester backbone was surface modified by a series of ramified and linear oligoamines to increase the hydrophilicity of the PVTP surfaces and introduce positive charge. However, as ethylene diamine is a small and highly water-soluble molecule, it would be expected to be washed off the surface. This is not the case, as the contact angle

remains constant after washing vigorously which proves that covalent bond was formed. Wettability is modulated by surface chemistry, as was shown in our work. The surface wettability of biomaterials determines the biological events at the biomaterial/host interface.

Scanning electron microscopy is an appropriate method for characterization of porosity and pore size of the surface of implants, even though the results may depend on several parameters (e.g., the filters applied, the areas evaluated). The evaluation of the surface of the samples in the micro, submicro and nano size ranges is a mandatory test to predict the behaviour of different implants in the human body.

Positron annihilation spectroscopy (PALS) can give correct information about the pore sizes and the porosity of the surfaces. The supramolecular structure of polymeric-based implants can be determined approximately in the upper 100 μm of the surface and deviations can be seen in a nano-dimensional range.

Comparing the results of PALS and SEM experiments, PLA-ED, PLA-TET, and PLA-Tris all showed high free volumes and the presence of numerous pits. For PLA-ED and PLA-Tris these were ca 1 μm in diameter, and for PLA-TET these pits were ca 100–200 nm in diameter. However, for MeNprN, few pits were present and the free volume was much reduced. Thus, there appeared to be a direct relationship between the free volume measured by PALS and the presence of large numbers of pits observed by SEM.

Biocompatibility investigation is a compulsory test of implantable devices. The ISO 10993-5 standard determines the parameters of the required cytotoxicity test. The main requirement is the time of contact duration: In the case of limited exposure the duration time is less than 24 h, and for prolonged exposure 1–30 days of investigation is required. Contact periods of longer than 30 days is regulated as requiring long-term studies. MTT assay is a broadly used, rapid colorimetric method to measure the in-vitro cytotoxicity of certain compounds on cell lines or primary cells. MTT assay is a highly efficient screening test because only the viable cells are able to convert the dye through their mitochondrial enzymes. A research group revealed that the MTT dye alteration to formazan salt depends on the cells' metabolic rate and number of mitochondria. In spite of this limitation of MTT assay, it is still the most reliable and quickest test for the assessment of cytocompatibility.

Our samples were stored in DMEM medium at 37 °C until the end of the 12^{th} day, and these extracts were measured on days four, eight, and 12 on the Caco-2 cells. The incubation period was 30 min. The cytotoxicity test was harmonized with the ISO 10993-5 standard, but the incubation period was shorter. There is clear evidence that PLA degrades into lactic acid and glycolic acid during storage, and different factors (pH, molecular weight, etc.) can influence

this degradation. Many studies have dealt with the degradation of PLA polymers, so the main focus of our study was not the investigation of PLA degradation, but the cytotoxic and biofilm formation effects of the modified PLA samples stored in DMEM medium, where the samples may degrade. In-vitro cytotoxicity methods can be the first filter in the assessment of cytocompatibility, because the assay is sensitive, efficiently adaptable, and well reproducible. Based on the MTT assay results all our samples can be considered as cytocompatible.

Microbial biofilm formation could be a risk factor of implanted devices, potentially resulting in unpredictable complications which can lead to infections and antimicrobial resistance. These microbial biofilm formations may contribute to causing an inflammatory response and result in subsequent operations, such as dental operations. The ability of *Candida albicans* to form biofilms on medical devices could be a key property that enhances its ability to cause disease in humans. Direct chemical modification of the polymers forming 3D printed objects would seem to be of interest, as the actual surface of the object is changed and not coated. Here, the use of chemical modification may allow inhibition of microbial biofilm formation, or may enhance such surface colonization. Interestingly, both growth inhibition and enhancement can be of considerable use to the researcher. A key point in such a modification is the presence of reactive ester groups in many of the polymers used in 3D printing.

Biofilm formation is also a compulsory element of cytocompatibility examination. However, biofilm formation can alter the structure of PLA and may modify the liberation of different APIs in the case of 3D printed medicine, for example. In our study, biofilm formation was performed with *Candida albicans* SC5314 reference isolate, because one of the most common biofilm forming fungi are *Candida* spp. and other species of *Candida* can cause serious invasive candidiasis, with a mortality rate of about 45%. Ideally, the measured absorbance is zero, which means that no kind of biofilm is formed on the implant by the reference isolate. According to the evaluation of biofilm formation, the chemically modified PLA-PVTPs are biocompatible. In our research, coupling of oligoamine functional groups to the polylactic acid backbone resulted in more favourable antimicrobial properties without altering the properties of polylactic acid. One research team used an antimicrobial agent in their experiments, which, while producing adequate properties, reduced the mechanical suitability of the polylactic acid sample.

In our paper, we demonstrated the importance of surface characterization and cytocompatibility investigations of 3D printed PLA- PVTPs and chemically modified PLA-based PVTPs. The amines as side chains can favorably alter the surface properties, wettability,

and biofilm formation of PLA PVTP. Low biofilm formation ability and favorable cytocompatibility profiles were also presented in the case of prolonged exposure. There are connections between the type and the other properties of PLA PVTPs. Overall, the properties of 3D printing materials can be dramatically enhanced by the modification of base polymers. These implants may ensure a high possibility for the incorporation of different antimicrobial APIs. According to these results, PLA-ED, PLA-Tris, and PLA-TET PVTPs can show favorable surface and anti-infective properties. Therefore, these samples were selected for further in-vivo and/or human studies.

5.2. 2nd series of experiments

In our study, different samples of PLA, antibacterial PLA, PETG and PMMA polymers with 16, 19, or 22 mm diameters and 0%, 5%, 10%, or 15% infill were printed using FDM technology to manufacture an implantable drug delivery system that enables fast manufacturing from commercially available filaments. The reason for these investigations was to certify the applicability of the FDM method in the field of personalized medication in surgeries. Some authors reported that personalized medication was an excellent response to the need for appropriate and adequate healthcare. The APIs can be delivered at the right dose and at the right time. 3D printing significantly speeds up the design cycle, both in the development and in the industrial manufacturing.

Tailoring the performance of the 3D printing process to the infilling of the API may be difficult. Our research group developed an easy method to fill the samples with the API. First a two-layer cover is printed for each sample in advance, which is then placed on the top of the API-containing lower part; then, the 3D printing is finished with the printing of the upper part. The degradation of the API due to the high printing temperature (210–270 °C) and the loss of API due to ventilation may also be eliminated. The APIs' weight loss was around 5% after the FDM printing from polyvinyl alcohol filament. In the pharmaceutical industry, only a maximum of 1% API deviation can be accepted. In a study, the filament contained 50% of the API but it was stated that by increasing the dispersal of the API content, the properties of the filaments for 3D printing could be altered, which would have effects on the printability and the product properties. The interplay through the printing is not well understood yet, and further investigations are required. In our work, the developed FDM process is applicable because the API amount and stability are not affected. As it is an implantable drug delivery system, API can be directly applied to the immediately printed implants and the drug delivery system may be applied during surgical interventions.

The TGA results of diclofenac sodium confirmed that API was stable up to 280 °C and our samples with diclofenac were stable after the 3D printing. Based on our results, no chemical decompositions or changes were detected below 270 °C in the polymers. All polymers can be considered thermally stable, which enables the filament sterilization. The sterilized filaments can be directly printed in the operating room.

Contact angle results showed that PLA, antibacterial PLA, PETG, and PMMA had different wettability, which can affect the samples' rejection in the human body. In the case of antibacterial PLA, which is a PLA modification, the contact angle results decreased but based on research, the surface hydrophilicity increased. The low contact angle indicated that the interaction of material and water molecules was high, resulting in the high hydrophilicity of the surfaces.

Based on a study, Raman spectroscopy can be used in different ways throughout the characterization. The distribution of different APIs can be determined, and the particle size and shape can be examined [37]. We found that around 70% of the whole API amount was in cavities 1, 2, and 3 in samples PLA_16_5, PLA_16_10, and PLA_16_15. Based on the results, all samples contain 30 mg diclofenac sodium salt; these results are in accordance with the content uniformity measurements.

The samples' surfaces were examined via scanning electron microscopy (SEM) to determine the surface morphology, the porosity and the pore size before and after the dissolution test. After 3D printing, the samples' surfaces looked flattened, without pores, but after the dissolution tests, pores had formed on the surface, so the dissolution can be described as a free diffusion from a reservoir system.

The micro computed tomography (micro CT) method can be widely used to display samples in three dimensions, which also allows the determination of porosity and internal structure. The microCT results confirmed the SEM results and the pore amount was determined; a 6.11% increase was found.

The impacts of the polymer and the geometry (diameter and infill percentage) on the dissolution profile of diclofenac sodium were considered. It is expected that, if the diameter increases, the number of pores on the lower and upper surfaces will increase as well, and the dissolution of the API will be faster. In the PLA_16_0, PLA_19_0, and PLA_22_0 samples, with the increase in diameter, the dissolution was faster because of the larger surface area. If the infill percentage increases within the structure, it hinders the dissolution. In our study, we found that the sample with 0% infill had the fastest dissolution due to the lack of contact points, but the increase in the infill percentage (5%, 10%, or 15%) did not constantly influence the

dissolution. The horizontal surfaces manufactured by FDM-based 3D printers did not hermetically seal the inner volume and may affect the diffusion of APIs. These two parameters influence the dissolution in an expected way. The polymer type does not have a significant impact on the dissolution profiles, but the results show that PMMA has the highest drug release after 24 h.

It was determined that oligomers and solubilized drug molecules are able to diffuse through the polymeric reservoir system. The dissolution of drugs from drug delivery systems depends on various physical and chemical rules, which results in difficulties in ascribing proper mathematical models to the processes occurring, so our samples were examined with first-order and zero-order kinetics at different time intervals. In our research, the PLA_16_0, PETG_16_0, and PETG_22_0 samples could be described with a zero-order kinetic, but all the other samples presented first-order kinetics. The findings suggested that changes in the implant microstructure could significantly alter the drug release. Therefore, researchers have to design implants that reduce these changes so a correlation between in vitro/in vivo drug release profiles can be achieved.

As PLA and antibacterial PLA are biodegradable polymers, the degradation was investigated at pH = 7.4, 37 °C by weight measurement, but within these eight weeks the samples did not degrade at all. Based on a study in aqueous solutions, the hydrolytic degradation depends on different factors. It is well known that the pKa of lactic acid is 3.84 and the hydrolysis is fast at a low or high pH. Some authors investigated the degradation at varying pH (1.5, 4.5, or 7.4) at 65 °C. It was found that the degradation rate depends on the molecular weight, the temperature, and the pH. Based on these studies, we can state that our samples did not degrade within eight weeks at 37 °C and pH = 7.4.

The in vitro cytotoxicity profile of the samples was determined with a long-term MTT assay. The theoretical knowledge of the MTT study are the same as described in 1st series of experiments. The method was harmonized with the ISO 10993 standard but with a shorter incubation period. The sterile samples were stored in DMEM medium at 37 °C and the potentially dissolved xenobiotics were measured by MTT assay on days 4, 8, and 12. Based on the prolonged MTT assay, results were in accordance with the ISO 10993:5 standard, and all our samples can be considered cytocompatible.

In conclusion, twelwe 3D-printed samples with different diameters, infill percentages, and four types of polymers were printed adequately in our work. This special design ensures that all kinds of API can be utilized during printing without heat damage or API loss, confirmed by TG and Raman spectroscopy results. The diameter and infill percentage have the expected

effect on the dissolution profile. SEM and microCT images confirmed that the dissolution takes place through pores on the surface by diffusion. Based on the MTT assay results, our samples can be considered cytocompatible. Contact angle values determined that antibacterial PLA had the most hydrophilic surface. Based on the overall project, PLA and antibacterial PLA have been suggested for the preparation of an implantable drug delivery system.

6. Summary

In conclusion it is stated that we have successfully printed 3D printed samples. One batch consists of 50 samples. The 3D printed samples have been chemically modified at the surface by various amine functional groups. The material structure experiments confirm the modification on the surface. The FT-IR graphics certify that the samples connected to the polylactic acid through a covalent bond. The samples can be considered as biocompatible. The biofilm formation of the *Candida albicans* is reduced by the chemical modification. Based on the biocompatibility tests (MTT and biofilm formation tests), it can be concluded that more than one assay should be used to determine cytotoxicity. Cytotoxicity data alone are not necessarily predictive of the in vivo response but can be completed with other experiments (contact angle, PALS and SEM experiments) and the in vivo compatibility data may be estimated. This work is being extended to other modifications and other polymers such as PET or PMMA.

In the second experiments the implantable drug delivery systems have been manufactured by FDM 3D printing. The active pharmaceutical ingredients (APIs) can be directly printed. Neither the high printing temperature (215–270 °C) nor the ventilation do not affect the amount of the API, which is confirmed by the TG/DSC investigations. FDM technology can be used for the manufacturing of 3D printed implantable drug delivery systems with the required dissolution profile. The design of the sample and the printing parameters have an important role to optimize the dissolution profile. The API is dissolved through the pores in the upper and lower surfaces of the samples, as confirmed by the SEM and micro CT images. If the size is increased, the surface area is higher and the dissolution is faster due to the higher number of pores. The dissolution profile can be modified by the infill percentage as well. Cytotoxicity measurements are compulsory experiments through the manufacturing of the implantable drug delivery systems. All samples are considered cytocompatible based on the ISO 10:993 standard and these results give a good prediction of the in vivo data. Therefore, these samples can be selected for further in vivo and/or human studies. PLA, antibacterial PLA, PETG, and PMMA polymers are all applicable for the manufacture of drug delivery systems, but PLA and antibacterial PLA are selected as the most appropriate implantable systems. The printing technique is developed by us and can be easily applied during surgical interventions. Personalized medications can be immediately manufactured in the operating room during the surgery and the most appropriate combination of APIs (for example anti-inflammatories or antibiotics) can be prepared right at the patient’s bedside.

7. List of publications

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Registry number: DEENK/18/2021.PL
Subject: PhD Publication List

Candidate: Petra Arany
Doctoral School: Doctoral School of Pharmacy

List of publications related to the dissertation

1. **Arany, P.**, Papp, I., Bodroginé Zichar, M., Csontos, M., Elek, J., Regdon, G., Budai, I., Béres, M., Gesztelyi, R., Fehér, P., Ujhelyi, Z., Vasvári, G., Haimhoffer, Á., Fenyvesi, F., Váradi, J., Vecsernyés, M., Bácskay, I.: In Vitro Tests of FDM 3D-Printed Diclofenac Sodium-Containing Implants.
   Molecules. 25 (24), 1-31, 2020.
   DOI: http://dx.doi.org/10.3390/molecules25245889
   IF: 3.267 (2019)
2. **Arany, P.**, Róka, E., Mollet, L., Coleman, A. W., Perret, F., Kim, B., Kovács, R. L., Kazsoki, A., Zelkó, R., Gesztelyi, R., Ujhelyi, Z., Fehér, P., Váradi, J., Fenyvesi, F., Vecsernyés, M., Bácskay, I.: Fused Deposition Modeling 3D Printing: Test Platforms for Evaluating Post-Fabrication Chemical Modifications and In-Vitro Biological Properties.
   Pharmaceutics. 11 (6), 1-23, 2019.
   DOI: http://dx.doi.org/10.3390/pharmaceutics11060277
   IF: 4.421

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List of other publications

3. Nemes, D., Ujhelyi, Z., **Arany, P.**, Pető, Á., Fehér, P., Váradi, J., Fenyvesi, F., Vecsernyés, M., Bácskay, I.: Biocompatibility investigation of different pharmaceutical excipients used in liquid dosage forms.
   Pharmazie. 73 (1), 16-18, 2018.
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   Drug Discovery Today: Technologies. 27, 81-86, 2018.
   DOI: http://dx.doi.org/10.1016/j.ddtec.2018.06.005

Total IF of journals (all publications): 8,508
Total IF of journals (publications related to the dissertation): 7,688

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

12 January, 2021

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

8. Acknowledgement

I am very grateful to my supervisor Ildikó Bácskay PharmD, PhD who provided me the opportunity to start my research work at the department in 2015 as a pharmacy student. Her attitude, devotion and altruist help guided me through my studies. Under her supervision, I could always count on her advices, ideas and professional knowledge.

I would like to thank the Head of the Department, Miklós Vecsernyés PharmD, PhD for providing the opportunity to start my experiments at the Department of Pharmaceutical Technology. He made it possible to collaborate with pharmaceutical scientists and to present our novel results to the scientific community.

I express my kindest thanks all my co-authors for their collaboration in this work. They were open minded and highly cooperative during the experiments and data evaluations, as well.

I would like to thank to all members of the Department of Pharmaceutical Technology: Ferenc Fenyvesi PharmD, Judit Váradi PharmD, Zoltán Ujhelyi PharmD, Pálma Fehér PharmD Katalin Réti-Nagy PharmD, Dániel Nemes PharmD, Dávid Sinka PharmD, Ágnes Rusznyák PharmD, Ádám Haimhoffer PharmD, Ágota Pető PharmD, Dóra Kósa PharmD, Liza Józsa PharmD, Mrs. Anikó Pardi, Mrs. Mária Körei Horányi, Ms. Erika Szilágyi, Mrs. Mária Vaszily, Mrs. Szilvia Lakatos, Mrs. Szilvia Sipos, Miss Tünde Nagy and Mrs. Brigitta Pataki for their excellent assistance and support.

I am very grateful to Csaba Székelyhídi for his honest, caring support and encouragement.

I owe my thanks to my family for their loving support, encouragement and understanding attitude in my whole life.

Funding

The research was financed by the Higher Education Institutional Excellence Program (NKFIH-1150-6/2019) of the Ministry of Innovation and Technology in Hungary, within the framework of the Therapeutic purposes thematic program of the University of Debrecen.

The project was supported by EFOP-3.6.1-16-2016-00022 and EFOP-3.6.3-VEKOP-16-2017-00009. The project was co-financed by the European Union and the European Social Fund.

The project was supported by the Gedeon Richter's Talentum Foundation (1103 Budapest, Gyömrői út 19-21, Hungary).

The work/publication is supported by the GINOP-2.3.3-15-2016-00021.Developing Pharmaceutical Technology R&D Infrastructure on the University of Debrecen" project. The project is co-financed by the European Union and the European Regional Development Fund. The research was supported by the Thematic Excellence Programme (TKP2020-IKA-04) of the Ministry for Innovation and Technology in Hungary.

The work/publication is supported by the GINOP-2.3.4-15-2020-00008 project. The project is co-financed by the European Union and the European Regional Development Fund.