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Repeatability and reproducibility of hydrogel 3D bioprinting

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Abstract: The study presents the basic terms of three-dimensional bioprinting and physical, chemical, and biological properties affecting the printability of hydrogel biomaterials. It deals with the principle of evaluating the quality of three-dimensional bioprinting. The goal was to design a procedural algorithm to analyze prints produced by extrusion 3D bioprinting critically. Forty-one cylindrical scaffolds were created experimentally from the same material and under varying printing parameters. The settings of the most plausible sample compared to the CAD design were used to 3D bioprint ten cylindrical samples. Analysis of measurement system (ASM) with three operators was used for evaluation. The results showed that the printability measurement system is conditionally suitable. At the same time, the methodology for evaluating the shape similarity of samples through macroscopic pore classification requires re-evaluation and further experiments.

1 Introduction

Three-dimensional (3D) bioprinting is a multidisciplinary field of science, enabling the creation of heterogeneous objects and complex biological structures based on a digital CAD model through additive manufacturing processes [1]. Thanks to the high level of structure and composition control, it has the potential to solve a diverse demand in medical research and practice, including applications in testing cosmetics, drugs, or therapy. At the same time, its primary and long-term goal is the development of fully functional organ and tissue substitutes. Current applications include bioprinting of skin, ear, and cartilage, research in the cardiovascular and gastrointestinal areas, nephrology, etc. [1,2].

We divide 3D bioprinting into cellular and non-cellular based on the printing material. [1, 3] In the cellular method, the cells are always incorporated into the printing material, i.e., bio-ink. The printing of hydrogel scaffolds replacing intercellular mass (ECM) imitating organic microstructure is mainly used here. The advantage is the spatially controlled placement of cells in a defined 3D microenvironment [4]. Cells can be, e.g., encapsulated and printed simultaneously with the scaffold. [5] By the term scaffold, we mean supporting structures made of synthetic or natural materials on which cells with the potential to replace damaged tissue can be grown [4-6]. In bio-ink, supervision over factors threatening cell viability is essential. There is another approach, without a scaffold, mimicking embryonic development. In acellular printing, the term biomaterial is used to produce objects, e.g., scaffolds. The most common are hydrogels.

Much attention is focused on formulating knowledge and principles regarding hydrogel bioprinting, and a consensus is being sought between print quality assessment criteria. The terms discussed are printability, continuous extrudability, print accuracy, precision, microstructure, and structural integrity [5].

The fundamental factor is printability, which characterizes the term evaluating the difference between the designed (CAD) and the printed construction [3]. It includes the mechanical properties of the ink, allowing it to pass through the nozzle (rheology) [4] and the application of individual layers (extrudability) to each other, according to a pre-planned code (g-code). This term includes the entire process from the programming phase (CAD design, choice of ink, slicing, g-code) through creating the construct (printing parameters, cross-linking, etc.). At the same time, the shape fidelity of the object is also influenced by the type of bioprinter and, in addition to the material's rheological properties, the shape and diameter of the nozzles [4]. The difference between the design and the created object may be due to the printed bio-inks extrudability and the printed constructs' structural malleability and stability [5]. Shape fidelity is the degree

to which the 3D printed structure matches the size and spatial location of the original CAD model in terms of geometry.

Additive manufacturing based on hydrogels is challenging in terms of the variable physicochemical behavior of the printing material. Considering the problematic 3D bioprinting, the presented study aims to propose a general procedural algorithm applicable when introducing a new biomaterial for the critical analysis of prints.

2 Methodology

Tools: Cellink BIO X bioprinter; biomaterial Start Cellink; cartridge 3ml; conical nozzle 22G; SolidWorks (CAD); Slic3r (G-code); SW CorelDraw (vector graphics editor), ruler; camera (iPhone 14 Pro) with tripod, laboratory slides.

The measurement was carried out in one day at the average temperature of the environment in the laboratory (T≈23.5°C). The used Start Cellink biomaterial has a storage temperature of up to 25°C.

2.1 3D bioprinter and biomaterial

Cellink BIO X is a bioprinter based on pneumatic extrusion with an integrated compressor, UV-C germicidal lamps, a HEPA H14 double filter system, three print heads (hotends) with integrated heating elements with a thermistor, proximity sensor, cooling, and others.

CELLINK START Biomaterial* (Polybutylene succinate (PBS; polytetramethylene succinate; Polypropylene oxide) is a thermoplastic polymer resin (polyester). Biodegradable resin. It is a water-soluble gel that supports cell-laden constructs, bio-inks with poor shape fidelity and constructs with porosity along all three axes. For use as bioink in 3D Bioprinting, cell encapsulation and delivery, tissue engineering and regenerative medicine, biomedical devices, drug delivery for research. Not for human use, for research only.

3D model, a simple cylindrical scaffold with a diameter of 10 mm, a height of 3 mm, 20% rectilinear filling pattern.

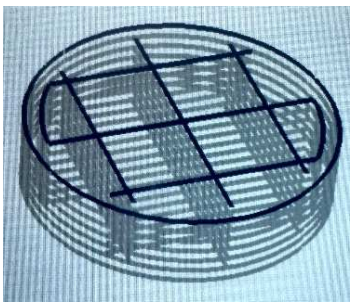

Figure 1 Visualization of cutting a 3D model in SW Cellink BioX

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2.2 Procedure Algorithm of Print Evaluation

Goal: Creation of a print protocol [7,8] for a specified sample 3D model and biomaterial. (1) Forty-one cylindrical scaffolds were printed. Each with different print settings. Experimentally, pressure (25-45 kPa), temperature (30-32 °C), and speed (16-20 mm/s) were varied. (2) Selection of the best print based on macroscopic observation of extrudability and conformation. (3) Repeatability: 3D bioprinting of ten samples with the same print settings. (4) Imaging of the created scaffolds (n=10) from a predefined perpendicular distance to the top surface of the object (TOP). (5) Measurement System Analysis (MSA).

2.3 Procedure Algorithm of Print Evaluation

The aim is to assess the extrusion and acceptability of the measurement system in terms of repeatability and reproducibility (R&R). The samples were evaluated by three operators (h=3). Ten models (j = 10) were produced for testing, all under the same printing parameters. Each operator evaluated each piece twice (k=2) using pre-agreed measurement templates for the assessed indicators. The data obtained during the patient survey were first processed using descriptive statistics. In the tables, the absolute abundance (n) is always indicated, which indicates the number of samples from the total number of samples in the examined set, and the relative abundance (%), which indicates the relative number of samples and the total extent of the set represents 100% [9,10]. Descriptive data analysis was followed by data analysis using inductive statistics methods. Working hypotheses were established, the validity of which was verified by the Shapiro-Wilk Test. Both tests verify the null hypothesis H0, which states that there is no statistically significant dependence or difference between the obtained and expected values. For each null hypothesis H0, there is an alternative hypothesis H1, which, on the contrary, claims that there is some statistically significant (significant) dependence, or difference, between the obtained and expected values. The result of both tests is the p value, the so-called p-value. If the p-value is less than the significance level α, i.e. $p < \alpha$, we say that it is possible to reject the null hypothesis H0 [9,10]. This means that there is some statistically significant dependence between the obtained and expected values, or a difference that could not be caused by chance. In case $p > \alpha$, we say that we cannot reject the null hypothesis H0. This means that there is no statistically significant (significant) dependence or difference between the obtained and expected values. The significance level α in these tests represents the probability (error rate) with which we reject the null hypothesis even if it is true. The α

value was set at 0.05, i.e. 5%. Data pieces of information obtained from the prints are quantitative.The measurement system characteristics were calculated based on the obtained data, such as linearity, accuracy, stability, R&R, and discrimination.

Acceptability of the measurement system:

- R&R < 10% is fully compliant
- R&R = 10%– 30% conditionally acceptable
- R&R > 30% is not satisfactory and needs improvement.

3 Results and discussion

Printability, see Figure 1. Using the B-spline in the CorelDRAW graphics software, the outline of the first and bottom layers was made, which were then dimensionally compared with the CAD design. A six-level rating scale in the interval <0 was used to evaluate the printability of the print; 6>, where 0-unusable, 1-very bad, 2-bad, 3- average, 4-good, 5- very good, 6- consistent with CAD design.

The Shapiro-Wilk test showed a significant departure from normality, W(30) = .91, p = .015, see Figure 3.

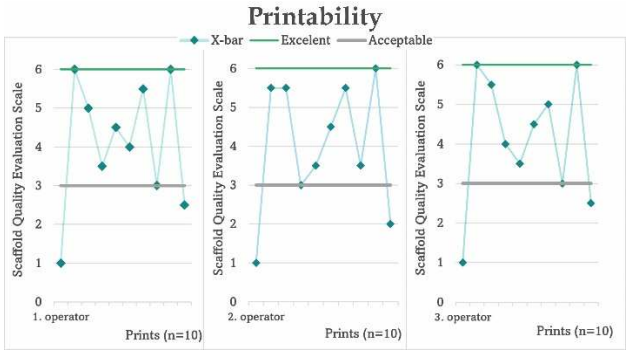

Figure 2 Printability Rating

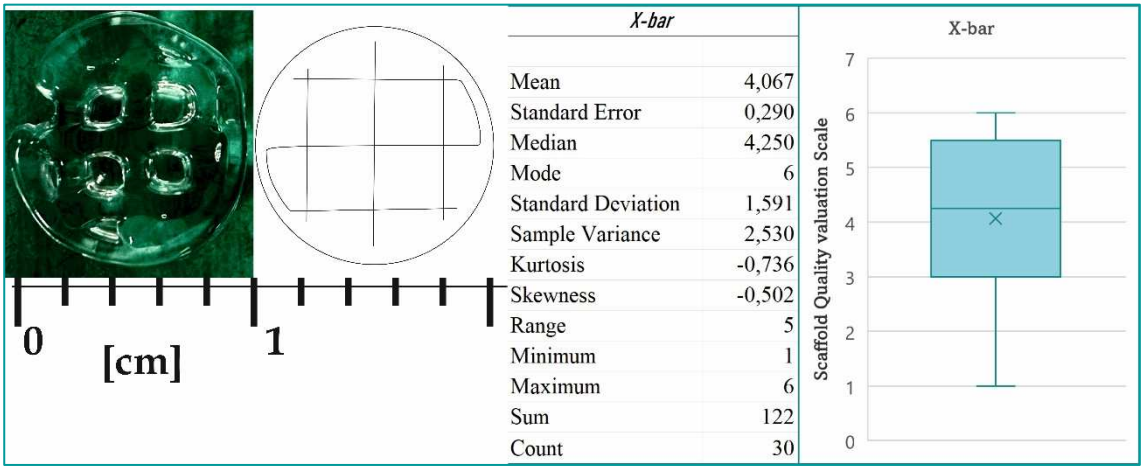

Figure 3 Printability rating

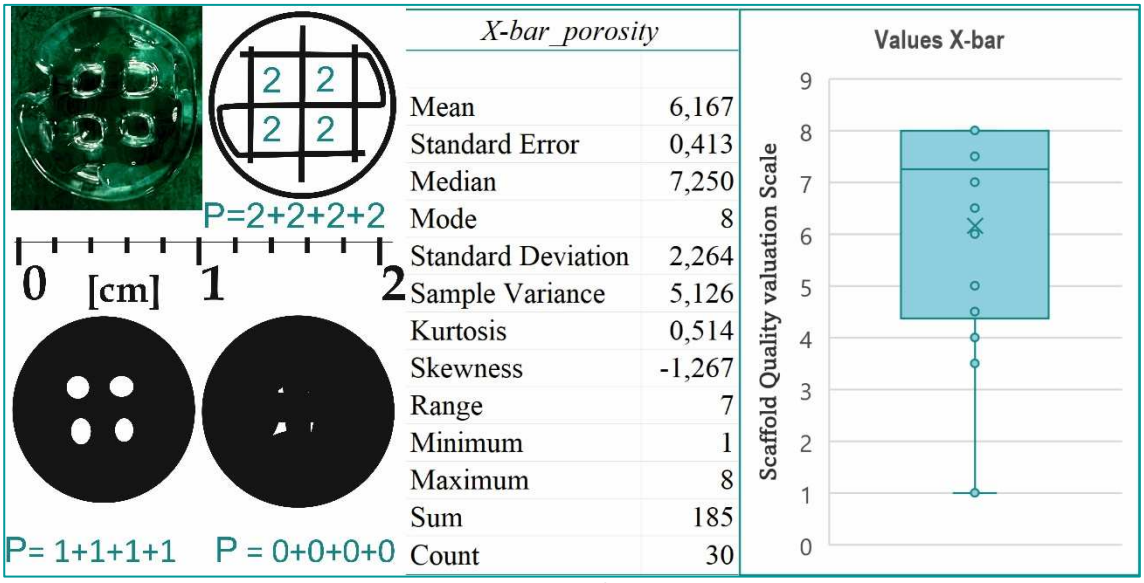

Figure 4: Evaluation of shape similarity

Evaluation of the shape similarity, see Figure 4-5. A three-level evaluation scale in the interval <0 was used to evaluate the shape of the printout with the CAD design; 2>, where 0-unusable, 1-average, 2-very good. The evaluation of one pore was defined by the difference of the bottom surface and the last top surface of the pore. Each was traced with the b-spline vector function in SW CorelDRAW. Four central pores were scored on each print.

The Shapiro-Wilk test showed a significant departure from normality, $W(30) = .78$, $p < .001$, see Figure 4.

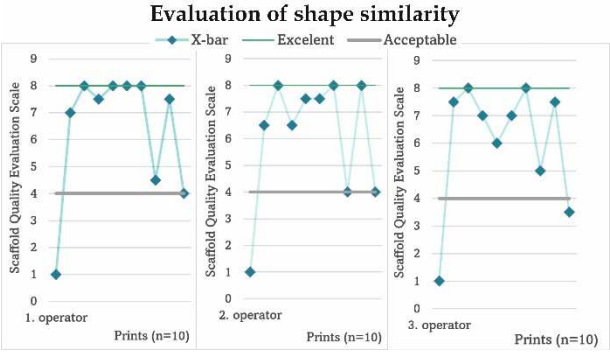

Figure 5 Evaluation of shape similarity

4 Discussion

Macroscopic observation of the internal structure, focusing on the spatial shape and the presence of visible pores, pointed out the problematic reading of the result. According to previous studies, the printability of hydrogel materials depends on rheological properties such as viscosity, shear stress or slip limit, as well as on the crosslinking process. Viscosity [3,11,12], i.e. the resistance of the fluid to flow during the application of stresses, or as the ratio of shear stress to shear rate, can be identified as the main factor affecting printability, print accuracy and shape fidelity. The viscosity of hydrogel inks can be influenced by temperature, molecular interactions and the molecular weight or concentration of the polymer [3]. In order to determine the exact parameters of 3D bioprinting and crosslinking of hydrogels, it would be interesting in the future to continuously monitor the temperature in the room and at the same time verify the viscosity of the biomaterial. Alternatively, look for other connections causing deformation of the structure of the printout.

5 Conclusions

The main goal of the study was the compilation of a procedure algorithm for the introduction of new materials and the evaluation of the set measurement system. The results pointed to the fact that, despite the same conditions, 20% of the prints did not reach the required external dimensions according to the set pattern (uniform cylinder 10x3 mm). Statistical analysis calculated the percentage of total variability for R&R extrudability of 29.5%, i.e., the measurement system is conditionally suitable. The R&R shape similarity evaluation was 41.4%, which means that it is necessary to revise the methodology for evaluating the similarity of samples.

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