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Comparative Analysis of Bioinks in 3D Bioprinted Organoids of Colorectal Cancer

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Colorectal cancer (CRC) is the third most common and fourth deadliest cancer in Western countries. Despite treatment regimens, the number of deaths increases yearly, particularly among younger patients. Current therapies are insufficient due to the heterogenetic nature of CRC and demand new strategies for personalized treatment.

The aim of our pilot study was to develop 3D organoids utilizing bioinks suitable for extrusion 3D bioprinting of CRC cell lines. We focused on the characterization of 3D printed organoids based on bioink properties. We assessed cell viability and growth patterns by fluorescence microscopy. 3D CRC cells printed in different bioinks revealed 90 % viability up to seven days after printing showing various cell growth patterns.

In conclusion, our work demonstrates the immense opportunities of 3D bioprinting to generate tumor organoids. We proved that alginate and gelatin-based bioinks mixed with live cells are suitable for extrusion 3D bioprinting.

Key words: bioink, 3D bioprinting, colorectal cancer, tumor organoids

Introduction

3D bioprinting is defined as an innovative biofabrication strategy generating constructs through layer-by-layer deposition of cell-laden hydrogel materials, using computer-aided design. The technology allows easy reproducibility and ensures physiologically pertinent cell-cell and cell-matrix interactions by mimicking the 3D heterogeneity of real tumors. Major components of 3D bioprinting are multiple types of live cells, biopolymer gels (bioinks), and 3D design. Choosing the appropriate cell types and density helps to assess viability, growth pattern, integrity and morphology.

The term “bioink” describes the carrier material (biopolymer) where cells are embedded in and to which they attach. Bioinks are classified as natural or synthetic and differ in their ability to mimic cell microenvironments. They have mild cross-linking properties to preserve cells in the construct and to prevent degradation of the model [10]. Some bioinks require crosslinking with $CaCl_2$ while others need UV or blue radiation. All gels serve as 3D structural and mechanical support to the cells as they mimic extracellular matrix (ECM) and assist cell adhesion, differentiation and proliferation. The ideal bioink should possess biomechanical properties to allow easy extrusion during printing and to maintain construct shape after printing [9]. Natural and synthetic bioinks often combine to create the most functional model possible.

The choice of bioinks in 3D bioprinting is based on important characteristics. These include mechanical support (permeability, elasticity, degradation), gelation kinetics, bioprintability (encompassing viscosity) and biocompatibility with the chosen cell type supporting cell adhesion, migration, proliferation and ECM secretion [10].

In recent years, biomaterial engineering is improving to satisfy the need of mimicking *in vivo* tumor characteristics and preserving live cell functions. 3D bioprinted organoid models are equivalent of real tumors. They open new horizons in oncology, pharmaceutical screening, biological research and regenerative medicine [2, 3].

Organoids are three-dimensional novel model systems derived from numerous sources including differentiated pluripotent stem cells, adult primary tissue and primary or metastatic tumors [5]. The components are self-organized through cell-cell and cell-matrix interactions to recapitulate the architecture of real tumors *ex vivo* [6]. The ultimate goal of the 3D bioprinted organoid is to represent tumor structure closest to the actual *in vivo* tumor. They allow studying cancer processes including tumor cell behavior and testing of new personalized therapeutic combinations [7].

Our aim was to develop 3D bioprinting protocols and to create tumor organoids based on the utilization of different bioinks. We focused on identifying the mechanical integrity and cell viability of 3D bioprinted tumor models. In our algorithm, we applied extrusion-based 3D bioprinting technology and two different bioinks - RGD (composed of alginate with covalently bound RGD nanofibrillar cellulose) and GelMa (gelatin methacrylate). Previously, we successfully printed constructs using RGD bioink [8] and sought to compare it with other bioinks. GelMa was chosen because it is widely used in extrusion-based bioprinting [9]. We created cell-laden GelMa bioprints via a simple heating and cooling process. The constructs permit cell survival for over a week and enhance cells spreading.

Materials and Methods

Cell cultures

The human colorectal cell line Caco-2 was grown in DMEM/F12 supplemented with 10% FBS and 5% Pen/Strep (P04-41250, P40-37500, and P06-07100, PAN-Biotech, Germany) under standard cell culture conditions 5 % CO_2 and high humidity 37°C. 3D bioprinted organoids were cultured in the media described above for 7 days.

Bioinks

Two types of bioinks were applied. The CELLINK RGD bioink contains the ECM peptide motif RGD (R-arginine, G-glycine, and D-aspartic acid), (catalog # IK1020100301, CELLINK, Sweden). It is composed of alginate with covalently bound RGD and nanofibrillar cellulose with viscosity of 3–20,000 Pa/s and shear rate 0.002–500 1/sec. The second bioink -GelMa A (gelatin methacrylate), (catalog # IK352102, CELLINK, Sweden) is with 30-150 Pa/s and shear rate from 0.002 $^{s-1}$ to 500 $^{s-1}$, 22°C (parameters from the manufacturer's specification sheet).

3D bioprinting

Mini tumor organoids from the human colorectal cell line Caco-2 were printed. The process began by detaching Caco-2 cells using Accutase® (catalog # 423201, BioLegend, USA). Cells were then counted (30 milion cells/ml), centrifuged and mixed with the CELLINK RGD or GelMaA bioink respectively. The cell-bioink mixture was placed in 3 ml cartridges (catalog # CSC010311101, CELLINK, Sweden) and extruded. We used a computer-generated disk-shape model with 2 mm of diameter and 0.82 mm of height. To preserve the tumor model, it was cross-linked for 1 min with $CaCl_2$, when using the RGD ink. Cross-linking agent was not applied to the GelMa model. We employed the extrusion technique to print CRC organoid models through a 410 µM (0.41 mm), high-precision nozzle (catalog # NZ3220005001, CELLINK, Sweden) at a pressure of 8-10 kPa, speed 10 ms in standard 24-well plates on an extrusion printer BioX Cellink, Sweden. The printed tumors were cultured in DMEM/F12 with 10% FBS and 0.5% Pen/Strep under standard condition at 37ºC, 5% CO_2. The medium was replaced every 2 days over a period of 1 week.

Calcein AM/PI viability assay

To assess cell viability after printing we used calcein AM (cat. # 56,496, Sigma-Aldrich, USA) and propidium iodide (PI), (cat. #P4170, Sigma-Aldrich, USA). In short, 3D biopints were incubated with calcein AM (at 5 ng/ml final concentration) for >10 min at 37°, 5% CO_2, staining metabolically active cells in green. Then PI (at 2 µg/ml final concentration) was added staining cells with destroyed membrane in red. Microscopic images were acquired and analyzed by fluorescent microscope (Nikon Eclipse Ni, Japan) at 10x magnification.

Morphological assay

3D bioprinted organoids using both bioinks RGD and GeLMa were fixed in 10% neutral buffered formaldehyde. Further processes include embedding the organoid models in paraffin and slicing in 4 µm thick sections. Pathomorphological assessment was performed with standard hematoxyline and eosin (H&E) staining, which revealed different cell growth patterns.

Results

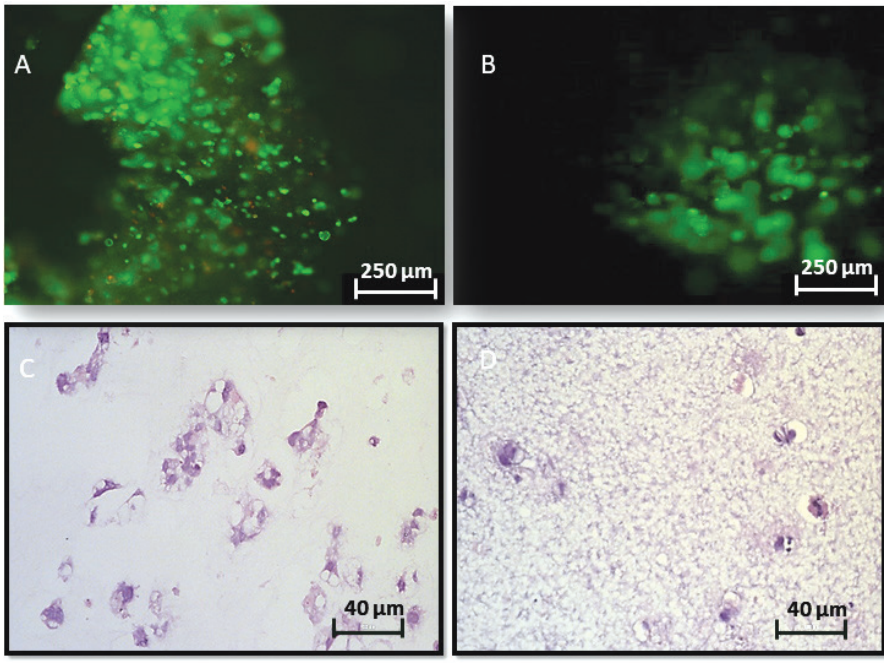
A simple two-layer cylindrical 3D organoid model was created using the human colorectal cell line Caco-2 embedded in two different commercially available bioinks. We employed extrusion-based technique and observed good extrudability and

biocompatibility with Caco-2 cells in both RGD and GelMa bioinks. We assessed the viability and distribution of cells in both prints at day 7. Post-printing cell viability of Caco-2 cells is demonstrated on **Fig. 1.** Both RGD and GelMA bioinks showed 90% viability as analyzed by the ImageJ software.

No significant cell death was registered during the 7day follow-up period (**Fig. 1A, B**).

The parallel morphological analysis revealed Caco-2 cells growing in large clusters. This started on day 3 (data not shown). Clusters resembled glandular-like structures as in the *in vivo* tumor when the RGD bioink was used. We observed no cluster formation in 3D organoids in GelMa ink but a wider distribution of cells throughout the print was noted.

Fig. 1. 3D organoids of Caco-2 cells using RGD and GelMa bioinks at day 7. (A)



Live/dead cell assay using Calcein AM/PI, (C) H&E in RGD ink prints. (B) Live/dead cell assay using Calcein AM/PI, (D) H&E in GelMA ink prints.

Discussion

3D preclinical models closely mimic the dynamics of the *in vivo* environment and serve as a powerful tool for studying different cell behavior particularly when bioinks possessing varying properties are being used. These innovative tumor organoids offer significant potential in oncology, pharmacology testing and biological research. 3D bioprinting enables the fabrication of a “bottom up” physiologically-relevant organoid, using live cells mixed with bioinks. 3D models closely mimicking native ECM environment with interconnected pores are ideal for nutrient and oxygen

delivery and intracellular communication [1, 4]. Overall, the usage of different bioinks revealed high cell viability exhibiting different growth patterns. Given the complexity of the 3D organoid architecture, it is important to analyze the correlation between characteristics and properties of the bioinks and cell behavior. Diverse bioinks provide useful information in the development of complex organoids and in tissue modeling. Creating organoid models using various bioinks can be applied as screening platforms to identify novel antitumor drug candidates. The present 3D CRC model utilizing these specific bioinks (RGD and GelMa) provides unconventional drug testing strategy for personalized medicines.

In conclusion, we present a novel, simple 3D bioprinted construct of Caco-2 colorectal cancer cells incapsulated in RGD and GelMa bioinks. We validated our algorithm as a platform for studying cell viability and morphology. Therefore, 3D bioprinted organoid models have potential in preclinical studies as a platform for identifying new therapeutic regimens.

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