Bioprinting for osteosarcoma model: Methodological aspects and experimental applications

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Abstract

The study aims at using the bioprinting technique to create an *in vitro* 3D construct of osteosarcoma, as an alternative model for studies related to Boron Neutron Capture Therapy (BNCT).

Introduction

Osteosarcoma is the most common primary malignant bone tumor affecting children and adolescents. Despite the introduction of several therapeutic options, the treatment and cure of osteosarcoma still remains an open challenge, due to its infiltrative growth that leads to a high incidence of metastasis and, therefore, to low survival rates. For this reason, Boron Neutron Capture Therapy (BNCT) has been investigated as an alternative or integrative treatment. BNCT is an experimental binary radiotherapy based on the irradiation with low energy neutron of neoplastic cells previously enriched with atoms of 10-boron (^{10}B).[1] This alternative technique selectively destroys neoplastic cells while sparing normal ones. To date, experimentation has been conducted on both *in vitro* and *in vivo* models, but animal testing has several disadvantages. In recent years, 3D bioprinting has led to rapid progress towards the modelling of pathological tissues. In particular, this technique is attracting for the engineering of tumor tissues, as it allows to mimic *in vitro* the tumor microenvironment thus improving the ability in modelling cancer compared to the existing methods.[2] In this context, the goal of proposed work is to create 3D *in vitro* tumor models for BNCT studies. In particular, this study focuses on the optimization of the technical protocol for bioprinting the *in vitro* model in terms of construct geometry, hydrogel composition, cell density, crosslinking frequency and boron uptake.

Materials and Methods

Rat osteosarcoma cell line (UMR-106) grow adherent in DMEM medium (Dulbecco's modified Eagles Medium, Lonza) supplemented with 10% of fetal bovine serum (FBS) (Euroclone) and 1% gentamicin (Euroclone), at 37°C in a humidified 5% CO_2 atmosphere.

The following printing protocol is the result of a series of tests carried out with the parameters summarized in Table 1. At about 80% of confluence cells were encapsulated into a sodium alginate-SA 8% and gelatin-GL 4% (Sigma Aldrich) hydrogel and 3D bioprinted with the Cellink INKREDIBLE+® (Cellink AB) in order to obtain constructs with 2x10^6 cells. After printing, 3D constructs were crosslinked for 5 minutes with 2% Calcium Chloride-$CaCl_2$ (Sigma Aldrich) and cultured in DMEM.

Construct colonization - At 0, 5, 7, 14, 21, 28, and 35 days constructs were fixed with ethanol 70%, followed by Hoechst staining (HO) by observation with a fluorescence microscope.

Intracellular boron evaluation - To quantify the intracellular levels of boron, constructs with a concentration of 4x10^6 cells were printed, to mimic the quantity of cells contained in samples usually used in 2D studies for the evaluation of the boron concentration by neutron autoradiography.

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Two different treatments were tested: constructs printed with cells previously exposed to Boronophenylanine (BPA) 80 ppm for 4 hours (pre-printing treatment) and constructs printed with cells not previously enriched with BPA, but exposed after printing to BPA at a concentration of 80 ppm/4h (post-printing treatment). The pre-printing treatment was intended to evaluate the gel interference with the ^{10}B quantification. The post-printing one was aimed to verify the gel influence on the intracellular boron uptake and to define a method suitable to remove the residual BPA trapped in the gel matrix at the end of the contact time. To this end constructs were washed three times with PBS or DMEM solutions for 10', 15' or 30' (Table 1).

PRINTING PARAMETERS			
Hydrogel	**Cell concentration**	**Geometry**	**Crosslinking**
SA 6%-GL 4%	1x10^6 cells/construct	full circumference	$CaCl_2$ for 5 minutes every week
SA 8%-GL 4%	2x10^6 cells/construct	circumference with grid	$CaCl_2$ for 5 minutes every two weeks

BORON UPTAKE			
PRE-PRINTING TREATMENT			
Cell concentration	**BPA treatment**	**Washing treatment**	
4x10^6 cells/construct	80 ppm/4h	Three times with PBS	
POST- PRINTING TREATMENT			
Cell concentration	**BPA treatment**	**Washing treatment**	
4x10^6 cells/construct	80 ppm/4h	PBS	3x10'
			3x15'
			3x30'
		DMEM	3x10'
			3x15'
			3x30'

Table 1. Summary of the experiments for the optimization of the technical protocol and the boron uptake.

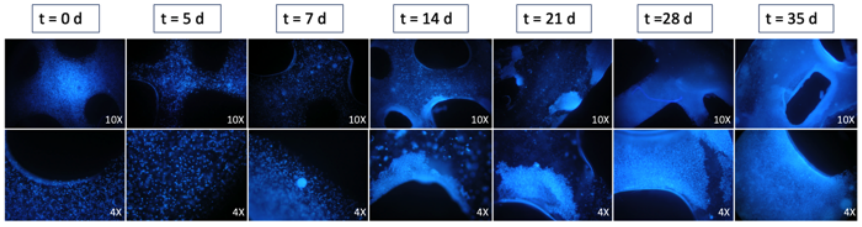

Figure 1. Microscopical observations at different end points of the 3D structures printed with an initial concentration of 2x10^6 cells/constructs. Nuclei stained by Hoechst 33258, blue fluorescence.

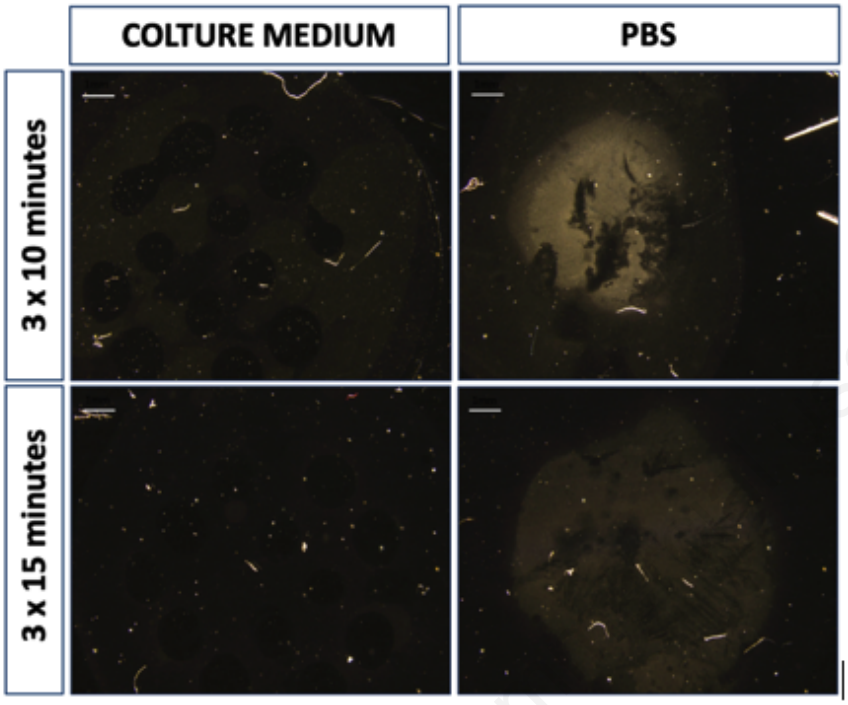

Figure 2. Qualitative neutron autoradiography images of boron biodistribution in 3D cellular constructs. Comparison between PBS and DMEM washed samples.

Results

Construct colonization - Microscopic observations at different end points of the 3D printed structures evidenced the presence of the first cellular clones just 7 days after printing. The size of the clones increased over time, leading to a complete three-dimensional colonization of the constructs 35 days after printing (Figure 1).

Intracellular boron evaluation - Preliminary results obtained from the pre-printing condition showed that the hydrogel interferes with the intracellular boron measurement. In fact, traces generated by the neutron capture reaction are dispersed in the thickness of the gel, limiting the actual quantification of the signal by about 70%, compared to values obtained in the correspondent 2D samples. With regard to the construct washing procedure 15' wash with DMEM was found to be suitable for removing the boron background (Figure 2).

Discussion and Conclusions

3D bioprinted osteosarcoma constructs should represent an effective alternative model in the context of the 3Rs principle (*Replacement, Reduction, Refinement*) to reduce the use of animal models for BNCT studies. The intracellular boron evaluation is a crucial point for BNCT studies. The method for its quantification in cells within constructs needs to be further refined. These preliminary studies have allowed us to obtain important information for its development. We expect that by improving the method it will be possible to use this newly 3D osteosarcoma model for BNCT studies.

References

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