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Abstract

Traditional in vitro culture models are unable to fully reflect the organ microenvironment owing to difference in terms of cell morphology, protein expression, cell - cell and cell - matrix interactions, and drug response. By contrast, the flexibility of bioprinting modes allows deposition of cell-containing biomaterials in any free-form-inspired 3D structures on chip. The primary purpose of this study was to design and optimize 3D printing formulations based on commercially available Carbopol, because of its many advantages, such as low-cost, the ability to produce transparent and stable gels and the water thickening. For this purpose, three different Carbopol gels were tested (EDT 2020, Ultrez 10 and NF 980) in terms of printability and biocompatibility, using A549 and MRC-5 cell lines. This study demonstrates that Carbopol could be a promising candidate for the 3D printing of cell-laden constructs both in terms of rheology and printing performance, considering the maintenance of cell viability.

1. Introduction

Studying cells in 3D scaffolds may reveal aspects that can be lost in traditional 2D cultures. Indeed, matrix complexity and its mechanics, cell-matrix interactions and signal diffusion strongly influenced cell behavior [1]. 3D printing represents an opportunity to obtain devices, with tunable complex structures, that mimic tissue organization. In the last few years, several printing techniques have been used to reach this goal such as inkjet, extrusion or laser-assisted bioprinter [2]. Thanks to its low cost and flexibility, extrusion-based printing has been one of the most studied and improved in order to obtain scaffolds that mimic vasculature, bone or soft tissues [3]. In spite of the above mentioned benefits, extrusion also shows some disadvantages: setting of printing pressure, limited resolution, bio-printability strongly linked to material's crosslinking and lower cell viability compared to other printing techniques [4]. During years, several materials have been developed and adapted to be used as bio-ink, among all Gelatin methacryloyl (GelMA) [5], Alginate [6], Hyaluronic acid [7], Chitosan [8] and Carbopol [9]. Carbopol is a soft granular microgels, made from high molecular weight, hydrophilic, lightly cross-linked poly(acrylic acid) polymers. In the last decades, there has been growing interest in using this class of material as an excipient in a diverse range of pharmaceutical applications [10]. One of Carbopol interesting feature is that it reaches maximum swelling in water solution around neutral pH and present the possibility of reversible entanglements (thixotropic effect, a time dependent viscosity, depending on external stresses), avoiding the need for additional crosslinking step. In some previous studies, different kind of Carbopol microgels have been already investigated, analyzing their printability and the possibility to use them as 3D scaffold for subsequent cell cultures, obtaining promising results [9,11]. In this work, to further investigate the applicability of Carbopol as bioink, in which cells could be cultured, we compared three different Carbopol formulations (ETD 2020 NF, Ultrez 10 NF and NF-980), by using a commercial available extrusion-based bioprinter (BIO X, CELLLINK). In particular, the objectives of the present studies were to investigate microgel formulations that could fit all the parameters to be considered as a performing bio-ink, that means printability, printing resolution and not strong effects on cell viability. We also aimed, by selecting the right preparation protocol and printing setting, to understand if Carbopol formulations may improve cell viability which is generally quite low in extrusion-based bioprinting [12]

2. Methods

2.1 Carbopol preparation

Three types of Carbopol (Lubrizol Corporation, Wickliffe, OH, USA), were used: Carbopol ETD 2020 NF, Ultrez 10 NF and 980 NF. The starting materials are supplied as a white and dry powder of primary particles averaging 0.2 μm in diameter. All of them were dissolved in cell culture medium (EMEM or RPMI) at specific concentration: ETD 2020 and NF 980 at 1.2%w/v and Ultrez 10 at 1.5%w/v. Because of their anionic nature, the

polyelectrolyte polymer solutions obtained after dispersion, must be neutralized with some drops of NaOH 5M to achieve the maximum swelling and a high viscosity

2.2 Rheological characterization

Rheological measurements were performed using an Anton Paar rheometer (Physica MCR 302) at a constant temperature of 25 °C and in parallel-plate mode with a gap of 0.3 mm between two aluminum plates (both with a diameter of 20 mm). Oscillatory tests were performed at frequency of 1 Hz ranging from a shear stress of 0.01 to 100 Pa. For the analysis of shear thinning behavior, the shear rate range was set up from 1 to 2000 s^{-1}. To assess the recovery post printing, thixotropic curve was obtained by applying a shear rate of 1 s^{-1} for 25 s, of 100 for 50 s, and again 1 s^{-1} until the end of the measurement. All experiments were performed in triplicate.

2.3 Cell Culture

All the experiments are conducted using lung cancer epithelial cells A549, kindly provided by Valentina Monica, from the Department of Oncology, University of Torino, AOU San Luigi Gonzaga and normal lung fibroblast MRC-5, kindly provided by Chiara Tonda Turo, from Department of mechanical and aerospace engineering, Politecnico of Torino. A549 and MRC-5 were maintained in RPMI 1640 or EMEM medium, respectively, both supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (all from Sigma Aldrich) and 2 mM glutamine (Biowest).

2.4 3D printing

Printing was performed using BIOX pneumatic-driven extrusion bioprinter (CELLINK). All the experiments were performed with 3ml cartridge, using a sterile standard conical 25G nozzle. Cartridge temperature was set at 37°C. The material flow was controlled by pressure and speed regulators. Samples were printed subsequently to form a cylindrical design using droplet mode (5 s of extrusion).

2.5 Cell viability assay

For MTT assay, cells were suspended inside Carbopol at the density of $3x10^6$ cells/ml. Samples were plated into 96-multiwell plates and 84 µl of 1 mg/ml MTT (dissolved in PBS) were added at each time points. After 3h of incubation at 37°C, 250 µl of MTT solvent (10% SDS, 0.01 M HCl in H2O) was used to solubilize the formazan crystals. After an hour, the plate was read using the Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winoosky, Vermont, USA). The optical density was analyzed at wavelength of 570 and 650 nm. Carbopol without cells were used as negative control. The experiments were repeated at least three times. The results were expressed as mean ± standard deviation and significant differences were measured at a level $p < 0.05$

2.6 Fluorescence Microscopy

For microscopy, cells were stained with CYTO-ID® Red long-term cell tracer kit (ENZO life science) following manufacturer instruction. Then, cells were dispersed inside Carbopol and analyzed 24h after printing. Fluorescence images were obtained using a microscope (Eclipse Ti2 Nikon, Tokyo, Japan) equipped with a Crest X-Light spinning disk confocal microscope and a Lumencor SPECTRA X light engine. All images were displayed using the same scaling and were collected using a Plan Apo 20 _ 0.75 NA (Nikon, Tokyo, Japan).

3. Results

3.1 Mechanical characterization

The mechanical properties of a material are of vital importance in determining its extrusion based printability. A key requirement is to find the right balance between high viscosity, cell proliferation and migration possibility in it [2]. In this work, three different types of Carbopol were studied to determine their printability with extrusion-based bioprinter. Specifically, ETD 2020 NF and Ultrez 10 NF are copolymers of acrylic acid modified with polyethylene glycol and a long chain alkyl acid ester, whereas, 980 NF is a polyacrylic acid polymer (https://www.lubrizol.com/Health/Pharmaceuticals/Excipients/Carbopol-Polymer-Products). All the preparations were done at room temperature, Carbopol powder were dissolved under stirring into cell culture medium, RPMI, at specific concentration (1.2 w/v% for ETD 2020 and 980 NF, 1.5 w/v% for Ultrez 10). When powder was completely dissolved, solution neutralization was reached by adding 5M NaOH until maximum swelling. Hence, to test if our formulations were suitable for bioprinting, elastic (G^{I}) and viscous (G^{II}) moduli were obtained from oscillatory rheology as function of shear stress. Importantly, all the formulations showed elastic behavior, which suggests the feasibility to use these Carbopol formulations as bioinks (FIG. 1a). As reported by Paxton and colleagues, to determine the behavior during printing, the viscosity must be analyzed over an applied stress [13]. As reported in FIG. 1b, shear viscosity tests demonstrated that all the inks showed shear thinning behavior, which means that viscosity decreased over an increasing shear rate. Finally, to characterize the material's properties after printing, recovery tests were performed to assess the capability to return at rest condition after stress applying (FIG. 1c). All the performed tests suggest that the Carbopol formulations investigated in this study owned the mechanical properties typical of a bioink, in particular elastic behavior, shear thinning properties and capability to maintain its shape after printing.

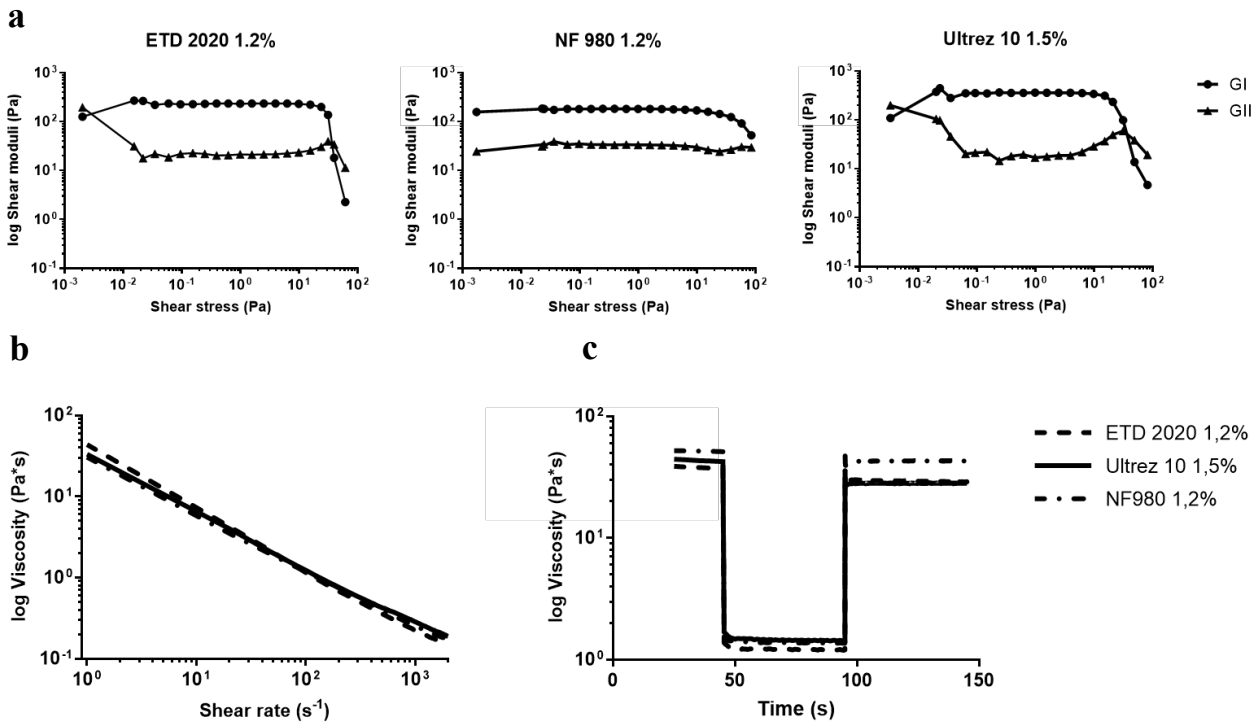

FIG.1 Rheological characterization of Carbopol formulations. a) Oscillatory rheology experiment at constant frequency of 1 Hz to measure the elastic modulus (G^{I}) and the viscous modulus (G^{II}). b) Shear viscosity test to assess viscosity during stress applying. c) Evaluation of thixotropic behavior to test the recovery post printing.

3.2 Printing test

To test the real printing feasibility of the three Carbopol formulations, we design a standard grid with BIO X bioprinter at different pressures, to assess the accuracy and choose the more suitable ones. We designed a 12x12 mm grid and lines were set at 3 mm each other. As can be seen in FIG.2, ETD 2020 and Ultrez 10 gave better results at lower pressure (13 kPa for ETD 2020 and 10, 13 kPa for Ultrez 10). Instead, NF 980 required higher pressures and the obtained grids did not show enough printing precision (FIG. 2). The printing test showed that ETD 2020 and Ultrez 10 were the best candidate for 3D bioprinting, because of their lower printing pressure, which may cause less cell damage, and the higher resolution obtained in the printed shape.

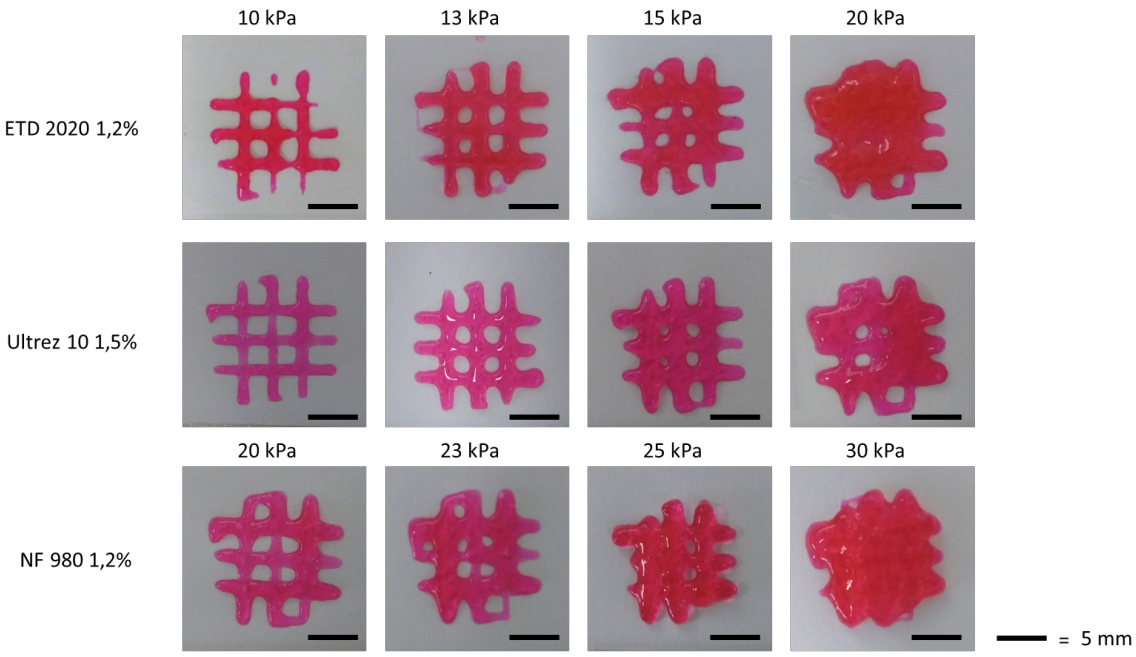

FIG.2 Printing tests with BIO X bioprinted (CELLINK) at different pressures depending on Carbopol formulations used as bioink, using 3 ml cartridge with 25G conical needle.

3.3 Cell viability and microscopy images

Our aim is to assess the effects of extrusion-based printing on cell viability, when cultured on Carbopol material, in particular on ETD 2020 and Ultrez 10, that were the selected ones, after the printing tests. For this purpose, printing pressure was set at 13 kPa and 3 ml cartridges with 25G conical needle and cell lines (A549, lung cancer epithelial cells, or MRC-5, normal lung fibroblasts) were dispersed into, to produce the starting bioink. Cell viability was analyzed instantly to assess printing-related cell death and 24h later. As expected, for both materials printing is associated to viability decrease. Specifically, ETD 2020 did not show significant difference at 24h for both A549 and MRC-5, but the latter seems to suffer less the culturing into the material. In both the cell lines, printing reduced cell viability (FIG. 3a). Ultrez 10 strongly decreased A549 viability also without printing, suggesting some toxic effects towards this cell line. On the other hand, MRC-5 tolerated better the material, and slight viability decrease was observed only at 24h in printed samples (FIG. 3b). Considering the results, ETD 2020 it is more suitable for cell culturing compared to Ultrez 10.

To further confirm the presence of cells inside the bioink, cells were stained with a red fluorescent dye and visualized at fluorescent microscopy. As shown in FIG. 3c, cells appeared alive and well dispersed at 24h, further confirming MTT assay results.

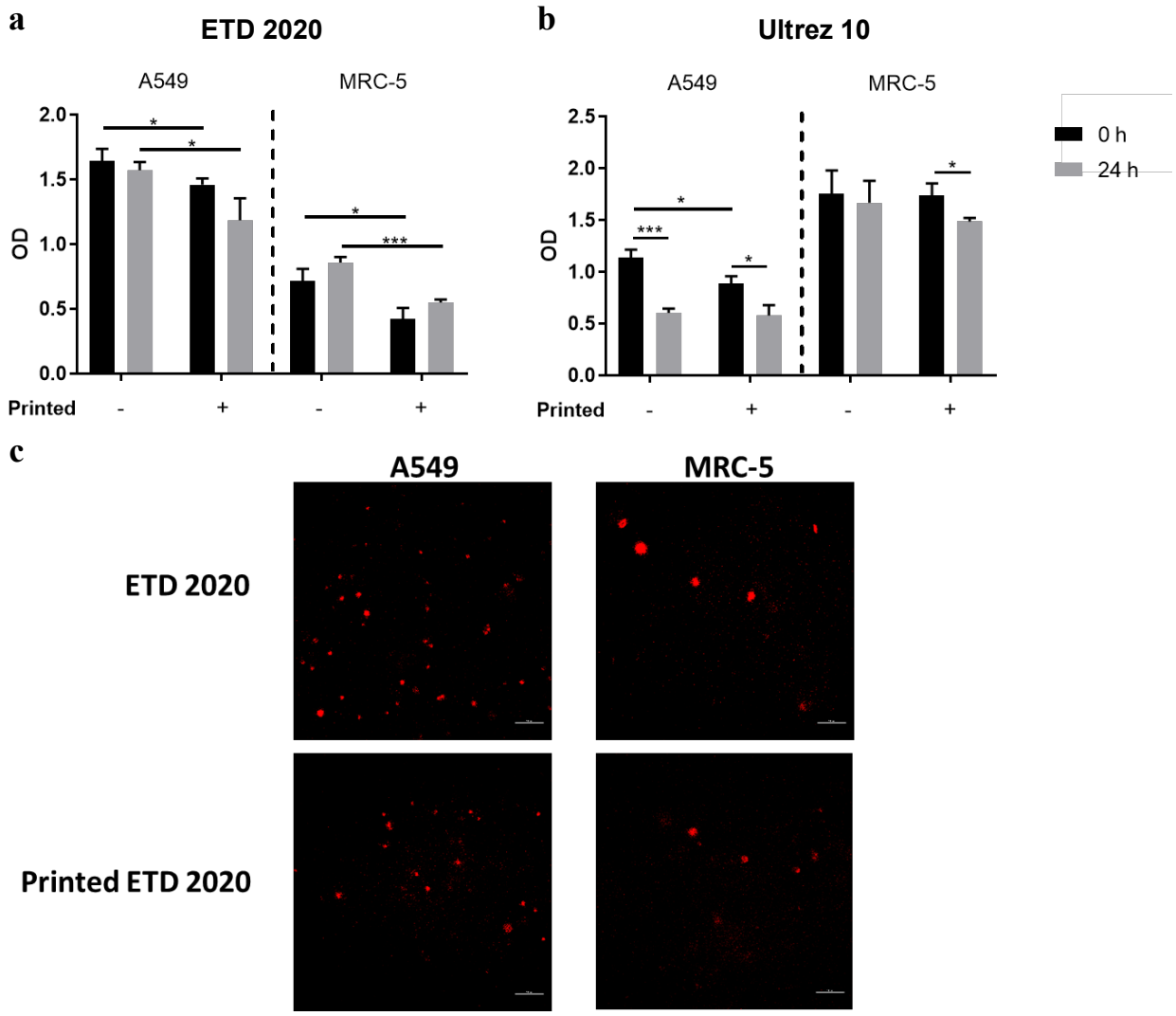

FIG 3. MTT assay. a) A549 and MRC-5 were dispersed into ETD 2020 1,2% prepared in cell culture medium, RPMI or EMEM, respectively. Signal was analysed immediately or 24h after printing. Printed (+) or not printed (-) samples were tested. b) Cells were dispersed into Ultrez 10 1,5%. Tests were performed at least three times and results are presented as the means ± SD. *P < 0.05, ***P < 0.001. c) Fluorescence images of A549 and MRC-5 suspended inside ETD 2020 24h after printing (Bar scale = 100 μm).

Conclusion

In the present study, Carbopol-based formulations were prepared as cell-laden bioinks through a bioprinter. In particular, the printability of three different Carbopol gels were tested (EDT 2020, Ultrez 10 and NF 980), showing that only ETD 2020 and Ultrez 10 can be used, because of their lower printing pressure value, which may cause less cell damage, and the higher resolution that can be obtained. Additional cell imaging and viability

test, confirm the presence of cell inside the material, showing no great harm on them after short-term studies of cells cultured.

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Author Contribution

Formulation development: DB, FF; Mechanical, printing and viability tests: DB; Ideation: DB, FF; Manuscript: DB, FF; Research funding support: CFP

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