SUITABILITY OF LYOPHILIZED POLYMERS BASED ON CMC FOR CELL PROLIFERATION

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Abstract

The aim of the study was to develop a material suitable for wound dressing which would be also suitable for cell growth and cell proliferation. Such matrix should have the structure of interconnected pores and a high porosity to ensure the adequate penetration of the cells and the supply of nutrients to the cells during their formation, diffusion of waste products out of the matrix and exclusion the products of matrix degradation. The study is focused on optimization the method of preparation of 3D porous structures and subsequent testing of cytotoxicity of thus prepared structures. The cytotoxicity of the structers was tested on human skin fibroblast cells. The cytotoxicity was tested by measuring the cell viability and using XTT assay. Another performed test was the test of biocidity/biodegradability. XTT assay results demonstrated that the lyophilized sample of carboxymethylcellulose is suitable for cell proliferation. Better cell proliferation is also caused by the increase of porosity made by lyophilization.

Keywords: Lyophilization, carboxymethylcellulose, cells, fibroblasts, toxicity

1. INTRODUCTION

CMC is a typical anionic polysaccharide derived from cellulose, consisting of linear b-(1,4)-linked D-glucopyranose chains. CMC is prepared by swelling cellulose in an aqueous NaOH solution diluted with organic solvents, such as ethanol or isopropanol, followed by carboxymethylation with monochloroacetic acid or its sodium salt, aiming to convert the hydroxyl groups into the carboxymethyl groups. [1] Most CMCs dissolve rapidly in cold water and are mainly used for controlling viscosity without gelling. Because its viscosity drops during heating, it may be used to improve the volume yield during baking by encouraging gas bubble formation. Its adjustable viscosity allows it to be used as a thickener, phase and emulsion stabilizer (for example with milk casein) and suspending agent. [2] CMC can be also used for its water-absorption capacity, since this is high even at low viscosity. Thus, it is also used for retarding staling and reducing fat uptake into fried foods. Due to its good viscosity building, high shear stability, biocompatibility, wide availability and low price compared to other polysaccharides, CMC is used in many different ways. [3-5] CMC is non-toxic, biodegradable and water-soluble polymer material with a good biocompatibility and stable physical properties. These properties are used in engineering and medical applications, such as a tool to minimize blood loss and prevent postoperative adhesion. [6, 7] Carboxymethylcellulose is a macromolecule that has been used for drug delivery systems in many studies to controlled release of drugs such as microgel matrix for a new type of patches, thanks to its adhesive properties of CMC was also used as a bioadhesive material (for example: muco-adhesive polymer for nasal and oral drug delivery systems) [8]. Freeze drying (lyophilization) is widely used for pharmaceuticals to improve the stability and longterm storage stability of labile drugs, especially protein drugs. Lyophilization is the process we use to remove water from a formulation at low temperatures (prevents thermal degradation) through a process of sublimation.

2. MATERIALS AND METHODS

2.1. Materials

The sample used for testing was the textile form of sodium CMC (nonvowen fabric - areal density 60 $g.m^{-2}$, DS 0.345, pH 6.6).

2.2. Lyophlization

For this study a laboratory freeze dryer Telstar lyoQuest -85 was used. Conditions of lyophilization were 4 hours of freezing, 18 hours of vacuum (1.0 mbar) and 1 hour of heating shelves (0.5 mbar).

2.3. Structure and morphology

The structure was determined using scanning electron microscopy - Ultra-high-resolution field emission scanning electron microscope Zeiss Ultra Plus equipped with a microanalytic system EDS + WDS + EBSD, resolution: 1 nm @ 15 kV, 1.7 nm @ 1 kV, magnification 12x to 1,000,000x, possibility of 3D-imaging by the use of a four-quadrant AsB detector; Oxford). Samples were deposited with a thin layer of gold.

The porosity measurements and structure determination was also done using the computer tomography - Desk micro-tomograph device SkyScan 1272 is able to non-destructively analyze and visualize the structure of materials. (Image Pixel Size (μm)= 3.001061; Exposure (ms)=950; Source Voltage (kV)= 40)

2.4. Thermal insulation properties

Measurement of thermal absorbing capacity and thermal resistance was performed on device Alambeta with pressure of 200 Pa.

To measure thermal insulation properties the innovative device which uses the air-conditioning chamber (CTS) was used. Within the air-conditioning chamber a steel cylinder is placed which is heated to 35°C. The cylinder simulates the surface of human skin. Temperature of the material which is placed on the cylinder is measured using a digital infrared thermometer. The temperature in the chamber was set to - 20°C (+/ - 01°C) with 80% of humidity.

Measurement of thickness was performed on device Uni-thickenss-meter (Computex) with area of jaw 20 mm^2 and pressure of 1 kPa.

Permeability measurement was performed by FX 3300 Air Permeability Tester III from TexTest company. The entire test procedure was in accordance with EN/ISO 9.237 (used pressure 100 Pa).

2.5. The Cell Viability/Cytotoxicity Test

In order to determine the toxicity of tested samples, the Viability/Cytotoxicity Test was performed. Human dermal fibroblasts (ATCC, No. PCS-201-012) were seeded onto 96-wells plates at density of 2×10^3 cells per well and cultured for 24h at 37°C in a 5% CO_2 humidified incubator. In all experimental was also done the control (non-treated cells). The conditioned medium was replaced and the cells were exposed to the samples containing medium for 24 h. The medium was then removed, and the cells were rinsed with D-PBS and incubated for 30 minutes at calcein-AM (Santa Cruz Biotechnology, cat. No. sc-203865) at the final concentration of 0.5 μM and ethidium homodimer-1 (Molecular Probes, No. L3224) at the final concentration of 1 μM . Live cells were distinguished by the presence of intracellular esterase activity, determined by the enzymatic conversion of the nonfluorescent cell-permeant calcein-AM to the green fluorescent calcein well-retained within live cells. Dead cells were discriminated by staining with red-fluorescent ethidium homodimer-1 to indicate loss of cellular membrane integrity. The tests were performed according to standard PN -EN ISO 10993-5.

2.6. The XTT/Cytotoxicity Test

The XTT reduction assay for cell survival assessment, a sensitive marker of mitochondrial activity was used to evaluate a cytotoxicity of provided emulsions on the human dermal fibroblast cell culture. The cells were seeded onto 96-wells plates in the number of 8×10^3 cells per well in 100 μL of grow medium and cultured for 24h at 37°C in a 5% CO_2 humidified incubator. The tested samples were diluted completely in FBM medium to make the extracts of samples. The extracts were prepared according to standard PN -EN ISO 10993-12. In all experimental was also done the control (non-treated cells). The conditioned medium was replaced with the

medium containing extracts and the cells were incubated for next 48h. The test was conducted in accordance with the manual. [9] For all the performed tests the reaction time in the incubator was 3 hours and 40 minutes. Subsequently the absorbance was measured for each well.

3. RESULTS AND DISCUSSION

3.1. Vizualization using SEM and CT

A) B)

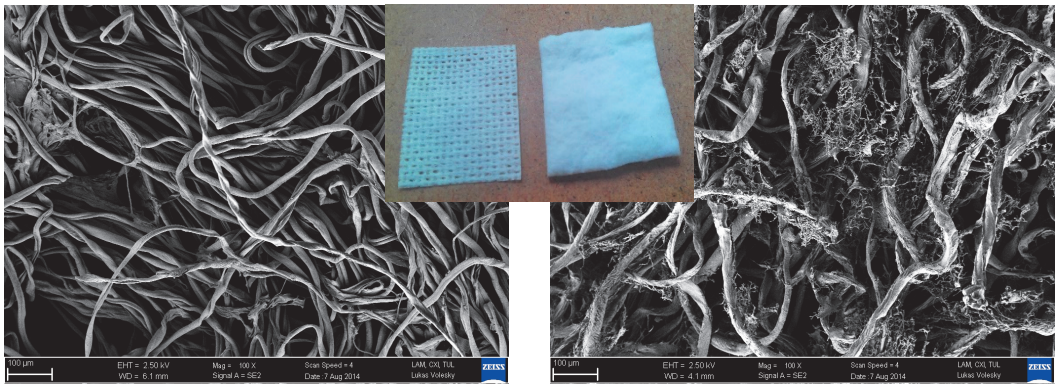

Fig. 1 CMC structure of the nonwoven fabric before (A) and after lyophilization (B) taken by SEM

From **Fig. 1** it is evident a significant change in structure after the lyophilization. Tested sample have a gelling character. Gelling parts in material created a „nanostructure“ under the low pressure. The resulting structure was fixed by drying at low temperature.

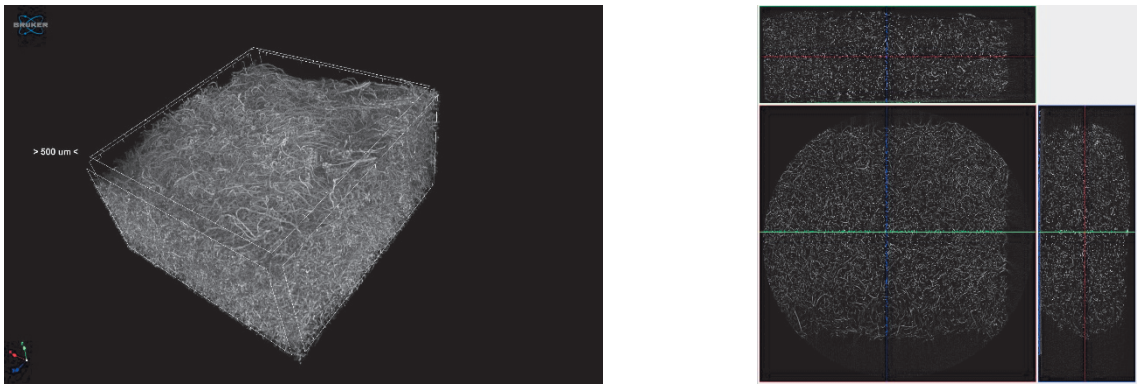

Fig. 2 CMC structure of the nonwoven fabric after the lyophilization taken by CT

The images of cross-sections of samples taken by CT (see **Fig. 2**) shows that the sample of nonwoven fabric after the lyophilization kept the fiber structure.

3.2. Porosity of lyophilized CMC

Table 1 Porosity of lyophilized carboxymethylcellulose sample

Total porosity [%]	87.95
Open porosity [%]	87.95
Closed porosity [%]	0.00055
Volume of the sample [mm^3]	20.23
Total volume of pore space [mm^3]	17.80

The results of measurement of porosity shows that a sample after the lyophilization process has a highly porous structure of interconnected open pores.

3.3. Thermal insulation properties

Table 2 Thermal insulation properties

	Thermal conductivity	Thermal absorbtivity	Thermal resistivity	Sample thickness	CTS (-20°C)	Permeability
	λ (.10^{-3})	b	r (.10^{-3})	h		
Sample	[$W.m^{-1}.K^{-1}$]	[$W.s^{1/2}.m^{-2}.K^{-1}$]	[$W^{-1}.K.m^2$]	[mm]	[°C]	[$l/m^2/s$]
A	34.06 ± 0.9	78.23 ± 0.9	36.00 ± 0.2	1.21 ± 0.1	18.90 ± 0.6	1660.00 ± 5.4
B	41.66 ± 0.4	56.63 ± 0.7	112.00 ± 0.6	3.37 ± 0.2	-0.40 ± 0.1	29.60 ± 3.2

In the case of high value of thermal absorbing capacity the material has a cold feel touch and conversely if this value is low, the material has a warm feel. The higher value of the resistance of the textile means that the material is less able to dissipate heat. That shows a good ability of material to keep the warmth. Higher temperature measured by digital infrared thermometer means worse thermal insulation properties. The results of both tested methods for evaluation of thermal insulation properties shows that sample after lyophilization obtained an excellent thermal insulation properties.

3.4. Suitability of lyophilized CMC for cell proliferation

Both tests were performed 5 times.

3.4.1. Live/Dead (Viability tests using esterase activity)

Due to the strong fluorescence of sample was not possible to take enough of pictures of dead cells to count.

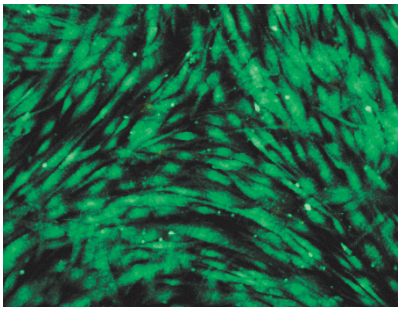

Fig. 3 Control sample - live cells

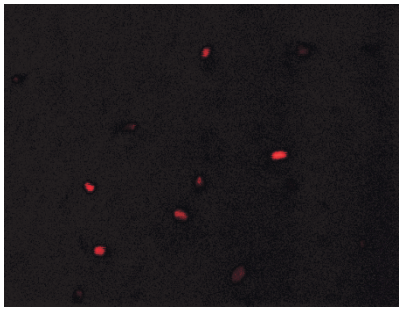

Fig. 4 Control sample - dead cells

From images of control sample is evident that cells are viable because green calcein is well retained within the cytoplasm and they show appropriate morphology. Due to the interest whether the cells are viable in the vicinity of the sample were also scanned wells under the samples.

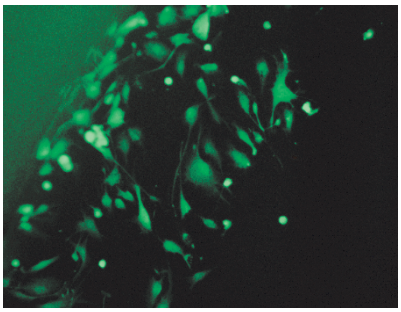

Fig. 5 Lyophilized sample - live cells

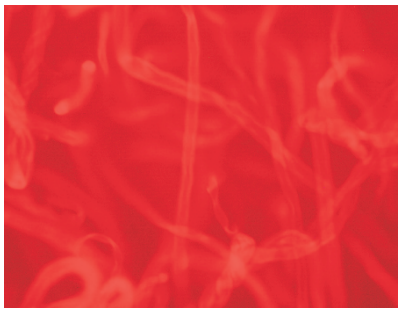

Fig. 6 Lyophilized sample - dead cells

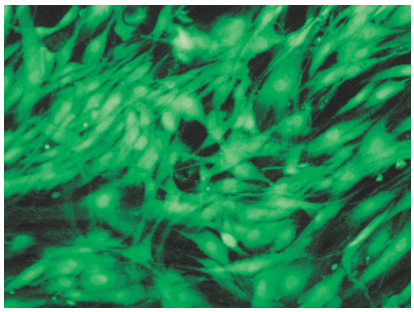

Fig. 7 Lyophilized sample - well under the sample - live cells

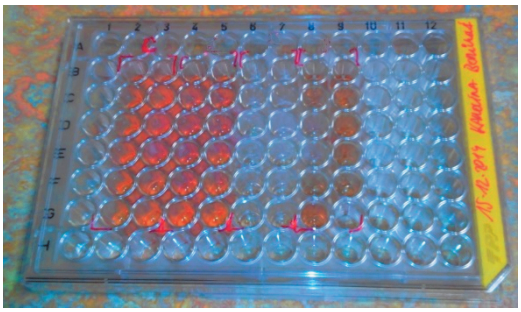

Fig. 8 Illustration of XTT reaction

The images 10-12 reveals a higher incidence of viable cells on the sample and also in the well under the sample. There was not find any dead cell.

3.4.2. XTT (cytotoxicity test)

The higher measured value of absorbance means the higher number of viable cells. The cytotoxicity test clearly points to the fact that the freeze-dried sample is suitable for cell growth and proliferation.

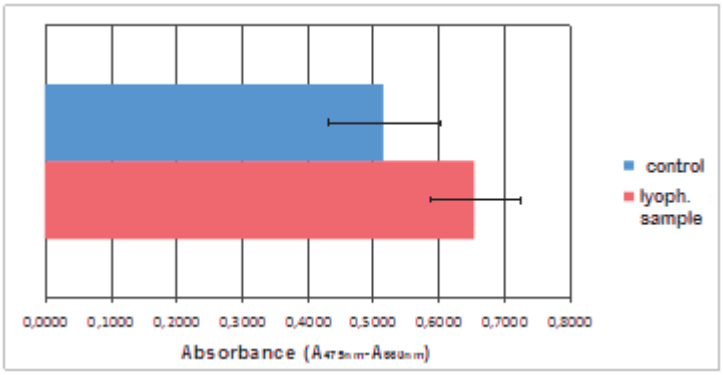

Fig. 9 Measured value of absorbance

4. CONCLUSION

This work dealt with the preparation and testing of samples of modified CMC, which were subsequently lyophilized. The aim of this study was to create the structure of interconnected pores. Lyophilization led to increase in thikness of sample up to ten 3 times higher values. The tested sample after the lyofilization had lower value of thermal absorption capacity and higher value of thermal resistance to retain the heat as compared to sample which was not freeze dried. It means that sample after the lyofilization have a warm feel and it is more efficient to keep the warmth. Measurements of porosity showed the structure of open pores which is suitable for cell proliferation. The cytotoxicity tests also proved the suitability of lyophilized CMC for cell growth and proliferation.

ACKNOWLEDGEMENTS

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