STRUCTURAL AND BIOLOGICAL CHARACTERISTICS OF SELF-ORGANISING CHITOSAN HYDROGELS

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Abstract

Creating innovative methods of treatment and regeneration of damaged tissues or organs is a key challenge of the twenty-first century. The aim of this study was to determine the possibility of producing and characterising the properties of self-organising chitosan hydrogels prepared with the use of chitosan lactate/chloride and disodium hydrogen phosphate dodecahydrate as a cross-linking agent. The structure and supramolecular architecture of the biomaterials were evaluated by Fourier-transform infrared spectroscopy and polarised optical microscopy. Biological studies assessed cytotoxicity by contact with a human colon adenocarcinoma cell line. The colourimetric resazurin assay showed that the obtained chitosan hydrogels are non-cytotoxic materials. Thus, self-organising biomaterials hold great promise for application in tissue engineering.

Keywords: *chitosan, biomaterials, self-organising hydrogels, structural properties, cytotoxicity, tissue engineering*

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1. Introduction

One of the fundamental directions of activities undertaken around the world, including the European Union, is the improvement of the health condition of societies by health risk prevention, early diagnosis of diseases, quick and effective implementation of medical procedures and ensuring the comprehensiveness and continuity of therapy [1-3]. Considering the above strategy, an important issue in contemporary clinical practice is the use of non-invasive/minimally invasive techniques for the treatment of traumatic or degenerative injuries – for example, orthopaedic lesions – as an alternative to conventional treatment methods and allowing the patient to return to full fitness. Until a few years ago, it was believed that human tissues could only be replaced by direct allogeneic transplants or the use of artificial implants [4-6].

Currently, great emphasis is placed on the implementation of solutions offered by tissue engineering, the main goal of which is to obtain biological materials that enable the replacement, restoration or maintenance of the basic functions of damaged tissues or entire organs [7-9]. The use of hydrogels based on natural polymers seems to be particularly promising, among which chitosan is one of the most fascinating candidates with a wide range of applications. The choice of this biopolymer is based on its biological, physical and chemical properties that can be controlled and designed under physiological conditions. Chitosan is non-toxic, biocompatible with living tissues, biodegradable and bacteriostatic. Additionally, it has antiviral and antifungal properties, and it does not cause allergies and skin irritations [10, 11]. Among hydrogels, particularly noteworthy biomaterials have the ability to gel in desired body tissues or cavities, assuming a shape that perfectly matches the site of damage, which eliminates the need for surgery. *In situ* gelling formulations under physiological conditions are formed in response to stimuli such as temperature, pH, ionic strength, electric potential or enzymes.

The aim of this study was to design a new form of chitosan matrix in the form of self-organising hydrogels intended for scaffolds for cell culture. The essence of the developed systems consists of obtaining a hydrogel form of a carrier from viscous chitosan salts with the use of disodium hydrogen phosphate dodecahydrate (DSHP) (Figure 1), enabling an increase in intermolecular interactions and the creation of a three-dimensional network structure at room temperature.

DSHP is an inorganic chemical compound in the form of colourless, highly hygroscopic crystals. It occurs in an anhydrous form and in the form of three hydrates: dihydrate, heptahydrate and dodecahydrate. This substance is mainly used in the food industry to regulate pH, as a stabiliser in the production of ultra-high temperature (UHT) milk and as an anti-caking additive in powdered products. In the water treatment process, it reduces the formation of limescale. On the other hand, in medicine, an oral solution of sodium phosphates (dibasic sodium phosphate and monobasic sodium phosphate) is administered to patients as a laxative in the case of constipation and for intestinal cleansing before surgery or colonoscopy [12-14].

In this research, the fabricated chitosan hydrogels with DSHP were evaluated by determining their structural properties using Fourier-transform infrared spectroscopy

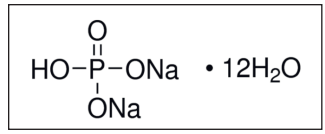

Figure 1. Structural formula of disodium hydrogen phosphate dodecahydrate

(FTIR). Moreover, the degree of ordering of the samples at a supramolecular level was investigated by using polarised optical microscopy. As part of biological studies, the cytotoxicity of the gels was assessed using HT-29 cells, a human colon adenocarcinoma cell line.

2. Materials and Methods

2.1. Materials

The following chemical reagents of analytical grade supplied by Sigma-Aldrich (Poznan, Poland) were used in the experiments: chitosan from crab shells (CH), product no. 50494-100GF; lactic acid (LA), product no. L6661-100ML; and hydrochloric acid (HCl), product no. H1758-100ML. The cross-linking agent was DSHP, product no. 799280115, purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland). Deionised water treated by a water purification system (Elga Purelab, High Wycombe, UK) was used in the preparation of hydrogels.

2.2. Fabrication of Hydrogels

Chitosan lactate and chitosan chloride hydrogels (the CH/LA/DSHP system and the CH/HCl/DSHP system) was prepared by dissolved 0.4 g of CH in 16 ml of LA or HCl (0.1 mol/l). The thoroughly stirred solutions were left at room temperature for 24 h to allow the polysaccharide to dissolve completely. Then, 1 g of DSHP distributed in 2 ml of deionised water (the cross-linking agent was dissolved in the hot water bath) was added dropwise to the samples. The prepared formulations gelled at 24°C.

2.3. Structural Studies

The structural characteristic of chitosan hydrogels was based on the analysis of FTIR spectra generated using a Nicolet™ iS50 FT-IR apparatus, equipped with a monolithic diamond ATR crystal (Thermo Fisher Scientific Inc., Waltham, MA, USA), from 4000 to 500 cm^{-1}. One hundred scans were recorded for all the spectra at a resolution of 4 cm^{-1}.

Moreover, a polarised optical microscope was used to obtain visual insight into the ordering of the materials. Measurements were made by placing the samples between two slides. The microscopic images were captured at room temperature.

2.4. Biological Studies

2.4.1. Cell Culture

All investigations were performed in an *in vitro* experimental model using a commercially available human colon adenocarcinoma cell line (HT-29) purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cell line was cultured in McCoy's 5A medium (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 10% (*v*/*v*) foetal bovine serum (FBS; Sigma-Aldrich Corp., St. Louis, MO, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (both from GIBCO-BRL, Life Technologies Ltd., Paisley, Scotland). After exposure to Accutase solution, the cells were passaged at 85%-95% confluency.

2.4.2. Cytotoxicity Analysis

The cytotoxicity of the chitosan hydrogels was measured using the colorimetric resazurin assay, In Vitro Toxicology Assay Kit, Resazurin based (Sigma-Aldrich Corp., St. Louis, MO, USA). Experiments were performed in triplicate with similar results. The cells were seeded in 12-well plates at a density of 2×10^5 cells per well and cultured in 1 ml of complete McCoy's 5A medium for 24 h. The positive control constituted the cells treated with 100% dimethyl sulphoxide (DMSO; Sigma-Aldrich Corp., St. Louis,

MO, USA), whereas the cells suspended in 1 ml of complete culture medium were used as a negative control. After the cells had adhered, they were incubated for 48 h with small pieces of hydrogels that had previously been prepared under aseptic conditions. Following incubation, the well contents were removed, and the cells were rinsed twice with 1X Dulbecco's phosphate-buffered saline (DPBS). Subsequently, 100 µl of the resazurin solution was added to each well. After incubation for 2 h, absorbance was measured at 600 nm with a Synergy HT spectrophotometer (BioTek, Winooski, VT, USA).

2.4.3. Statistical Analysis

The data collected were analysed using the statistical program Sigma Plot (Systat Software, Inc., San Jose, CA, USA) and the Mann–Whitney test. Each of the analyses in individual experiments were based on the results of three independent tests. Significant statistical differences are presented as follows: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ versus the negative control.

3. Results and Discussion

3.1. FTIR Spectroscopy

FTIR spectroscopy is an analytical technique employed to identify the functional groups or bonds in organic or inorganic samples by using the beam of infrared radiation [15]. Figure 2 shows the spectra of the lyophilised systems: the chitosan lactate hydrogel (the CH/LA/DSHP system) and chitosan chloride (the CH/HCL/DSHP system) with DSHP and, for comparison, the spectra of CH powder and DSHP powder.

Absorption bands in the 4000 to 1450 cm^{-1} region are usually due to stretching vibrations of diatomic units, and therefore this is sometimes called the group frequency region.

In the spectra of the CH/LA/DSHP and the CH/HCL/DSHP systems, there is a broad spectrum at 3600-3100 cm^{-1} corresponding to the oscillations of O-H. The asymmetric shape of this band, which is shifted towards lower wavenumbers compared with the spectrum of CH, indicates the presence of strong hydroxyl bonds, which overlap the asymmetric stretching vibrations of NH_2 groups and the N-H stretching vibrations between molecules N-H…O=C in this region. On the other hand, the spectrum of DSHP has a clear peak at 3225 cm^{-1}, characteristic of O-H stretching due to water of crystallisation, which is not observed in the spectra of hydrogels.

For 2950-2850 cm^{-1}, the spectra of both types of gels, as well as the spectrum of CH, have a band at 2874 cm^{-1}, which consists of two overlapping bands representing the $-CH_2$ and $-CH_3$ aliphatic groups.

Analysing the range of 1680-1500 cm^{-1}, it is possible to observe typical peaks of chitosan, which are assigned to the C=O bond in the amide group (amide I vibration) and the amide II band coming from NH_2. On the other hand, in the spectrum of DSHP there is a distinct band at 1670 cm^{-1}, which represents the O=P-OH deformation vibration. As a result, the spectra of hydrogels have a band at 1575 cm^{-1} (the CH/LA/DSHP system) and 1588 cm^{-1} (the CH/HCL/DSHP system).

In turn, in the frequency range of 1500-1200 cm^{-1}, the chitosan molecule shows four peaks at 1420, 1375, 1315 and 1260 cm^{-1}, associated with oscillations characteristic of the O-H and C-H bending of CH_2 groups and representing the C-O stretching of the primary alcoholic group $-CH_2$-OH. For both types of biomaterials, the band at 1420 cm^{-1} moves towards higher frequencies (1455 cm^{-1}), and the band at 1375 cm^{-1} moves towards lower frequencies (1370 cm^{-1}). On the other hand, the peak at 1315 cm^{-1} is shifted to 1320 cm^{-1} for the CH/LA/DSHP system and remains unchanged for the CH/HCL/DSHP system. The peak at 1260 cm^{-1} for both gels also remains unchanged.

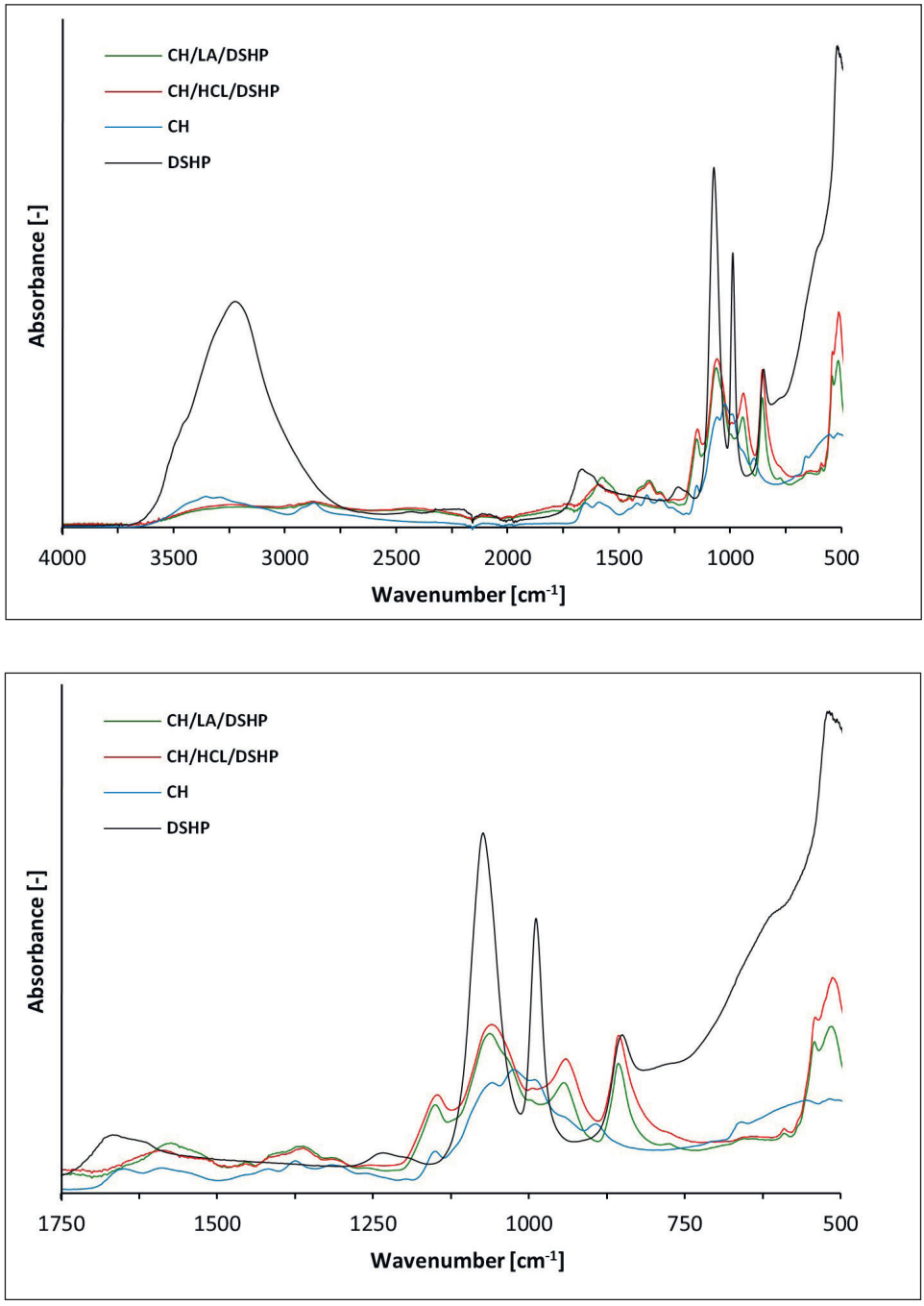

Figure 2. Fourier-transform infrared spectra of the CH/LA/DSHP system, the CH/HCL/DSHP system, the CH powder and the DSHP powder (upper), with a zoom of the region 1750-500 cm-1 (lower)

In the range of 1200-800 cm^{-1}, the spectrum of CH demonstrates bands at 1151, 1060, 1020, 988 and 891 cm^{-1}. In turn, in the spectra of both hydrogels there are four major peaks at 1150 cm^{-1}, 1062 cm^{-1} (the CH/LA/DSHP system) or 1070 cm^{-1} (the CH/HCL/DSHP system), 938 cm^{-1} (the CH/LA/DSHP system) or 944 cm^{-1} (the CH/HCL/DSHP system) and a distinct band at 855 cm^{-1}. These bands are probably related to the shift in bands characteristic of the saccharide structure or the presence of DSHP in the structure of hydrogels (P=O stretching mode, P-O stretching mode and PO-H bending mode). Additionally, for both variants of gels there is a characteristic band at 520 cm^{-1} with an arm for the wavenumber around 540 cm^{-1} (out of plane P-OH bending mode).

Interpretation of the FTIR spectra was based on previous studies [16-19].

3.2. Supramolecular Characterisation by Polarised Optical Microscopy

Polarised optical microscopy is an important tool for identifying and imaging birefringent and optically active materials such as liquid crystals, minerals, and crystals. Photomicrographs are used to demonstrate the observed textures, typically using polarised light. Under polarised light plane, the sample is anisotropic if it can divert the plane of incident light that is isotropic and does not deflect light [20, 21].

Figure 3 shows images obtained by polarised optical microscopy for both types of hydrogels (the CH/LA/DSHP system and the CH/HCL/DSHP system) and DSHP as a control. DHSP (Figure 3A) exhibits birefringence. It appeared as a beautiful texture that shimmers with different colours. On the other hand, the images for both types of chitosan hydrogels (Figure 3B and C) did not differ much from each other: the photographs clearly show crystalline light spots.

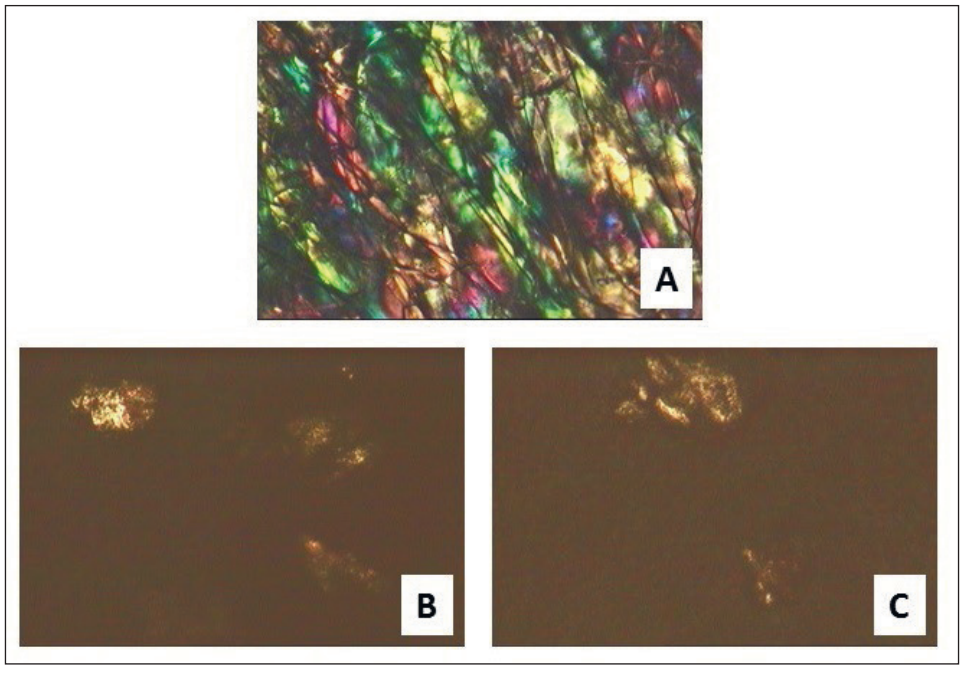

Figure 3. Polarised optical micrographs of disodium hydrogen phosphate dodecahydrate DSHP (A), the chitosan(CH)/lactic acid (LA)/DSHP system (B) and the CH/hydrochloric acid (HCl)/DSHP system (C)

3.3. Evaluation of Cytotoxicity of Hydrogels

The lack of toxic effect on cells is one of the most important properties of biomaterials for application in tissue engineering. The colourimetric resazurin assay was used to screen the cytotoxic range of the CH/LA/DSHP and CH/HCL/DSHP systems. The results presented in Figure 4 summarise the viability of HT-29 cells incubated with the hydrogels for 48 h. Both variants of hydrogels with DSHP did not cause any negative effect on human colon adenocarcinoma cells (HT-29). These biomaterials are characterised by exceptionally good cytocompatibility. Indeed, there was increased cell proliferation in relation to the negative control (median of cell viability: 106.7% for the CH/LA/DSHP system and 106.2% and the CH/HCL/DSHP system).

4. Conclusions

In summary, the progressive integration of the chemical, biological and engineering sciences has enabled the creation of bioactive hydrogels for specific applications. It should be mentioned that engineering of complex tissues or organs is still a big challenge, but it allows for solving medical problems that societies have been struggling with for years. Modification and modernisation of all materials, as well as the development of their practical use, has made it possible to save the lives of many people.

This study has presented the self-organising chitosan lactate and chitosan chloride hydrogels obtained with the use of DSHP as a cross-linking agent. Based on structural studies, the changes in the FTIR spectra of hydrogels with DSHP were mainly in the following ranges: 1680-1500 cm^{-1}, 1500-1200 cm^{-1} and 1200-800 cm^{-1}. In addition, changes in the supramolecular architecture of the hydrogels in relation to DSHP were confirmed by polarised optical microscopy. On the other hand, the biological studies

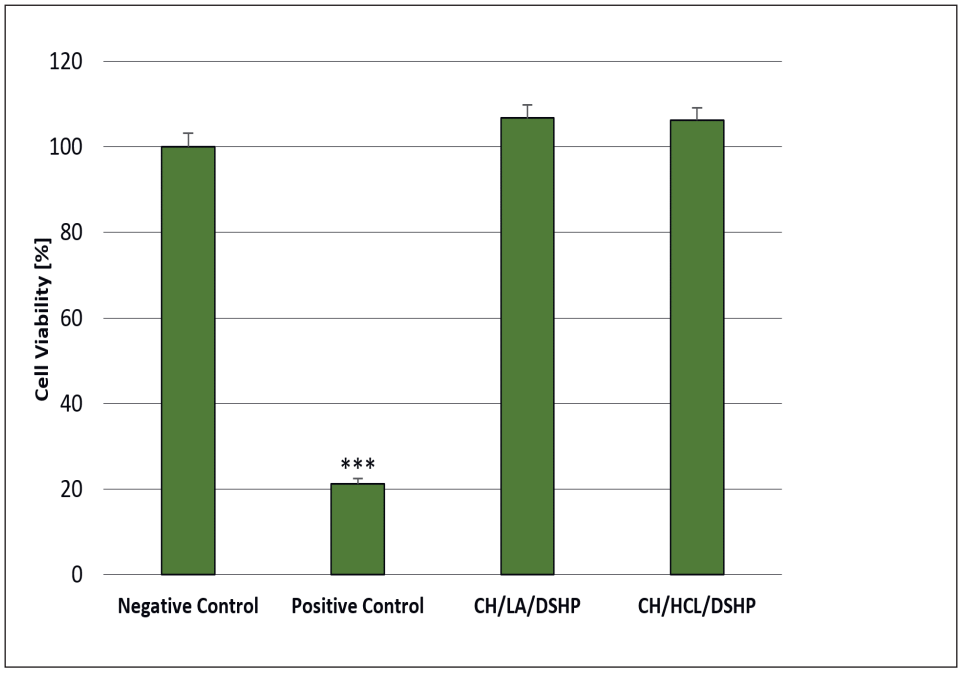

Figure 4. *In vitro* cytotoxicity of the chitosan hydrogels toward HT-29 cells by the resazurin assay. *** $p < 0.001$ versus negative control

showed no cytotoxic effect of the developed hydrogels on the HT-29 cell line, and even revealed an increase the cell proliferation in relation to the negative control. Thus, self-organising chitosan hydrogels could be good candidates for scaffolds in tissue engineering applications. We expect that this work could provide much inspiration for the development and evolution of a new generation of materials for use in clinical practice.

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