

Referee's report to the dissertation Thesis of Kateřina Štěpánková
Chemical Modification of Polysaccharides for Biomedical Applications

Doctoral Thesis deals on one of the hot topics of current biomedicine where new smart biocompatible materials are introduced. The furcellaran was selected for the current study. Under the first step, it was successfully immobilized onto polyethylene terephthalate surfaces via different chemical and plasma chemical + chemical multistep processes. Later, the surfaces were characterized by relevant analytical techniques to visualize the surface structure in nanometer scale, to obtain chemical composition of surface layers and to characterize the surface free energy with its components that are related to the cells (proteins) adhesion. Finally, the prepared samples with immobilized furcellaran were tested to their antibacterial and anticoagulant properties, as well as to cytocompatibility with fibroblasts and stem cells. The results showed that PET films coated with furcellaran exhibited a significant increase in the proliferation of embryonic stem cells. The second part of results focused on the furcellaran sulfatation using four different methods to improve its hemocompatibility. The modified furcellaran increased its anticoagulant activity depending on the sulfatation procedure. All tested samples were non-cytotoxic. These results significantly improve knowledge in the field and open new perspective in the application of furcellaran in biomedicine.

The short Introduction presents the motivation for the current research. After that, an extensive review of the state of art in the field of biomaterials and their functionalization is given. This is followed by review of seaweed polysaccharides, their chemical modifications and immobilization. This theoretical part is presented on over 30 pages of Thesis. Based on this review, the Thesis aims are briefly but clearly presented.

The experimental part consists of two papers where Kateřina is the first author. Here, I have the principal problem with whole work. The information that two sections of results are based (generally copied papers) is not given and the author's contribution is fully missing. Additionally, the copies of papers are not included in the Thesis that is really non-standard. It is not obligatory for referee to search for additional materials; all must be included in Thesis.

The first paper presents results of furcellaran immobilization on the PET surface. The native PET surface was activated by air plasma and after that furcellaran was applied immediately or with intermediate step of N-allylmethylamine application. The final samples properties were characterized by contact angle measurements of three liquids (allowing detail calculation of the surface energy and its components) and XPS. Here, the more detailed analysis using high resolution of the selected peaks will be very beneficial, because XPS use for the simple elementary composition is rather expensive (there are other methods allowing the same much cheaper). The detail information about real chemical composition will be useful for the understanding results obtained by contact angle measurement. The surface nanostructures visualization was examined by SEM. The antibacterial properties were tested by *Escherichia coli* and *Staphylococcus aureus*. Standard procedures were applied to study anticoagulation properties. Finally, the selected cell lines were used for the surface proliferation and cytocompatibility studies. Obtained results showed that furcellaran is promising for use in biomedical applications and has a great potential for applications in the biomedicine.

The second paper is focused on the chemical modification of furcellaran for enhanced blood compatibility. Four different procedures of furcellaran sulfation are presented. Based on Fig. 21, the resulting molecules seem be the same but they have different properties as it is shown in results. This should be clearly explained. The FTIR, gel permeation chromatography, organic elemental analyser, and XPS were used for the modified molecules characterization. *In vitro* fibroblast cytocompatibility and blood compatibility test were used to characterize all four derivatives bio-properties. The obtained results showed that furcellaran sulfation has the potential to improve its hemocompatibility. Thus this material can be a promising candidate for anticoagulant therapy but further optimization is needed.

Finally, a brief Conclusion summarizes Thesis achievements. Unfortunately, there are presented no ideas for the future research. So, also this point is important to include into presentation/discussion at defence.

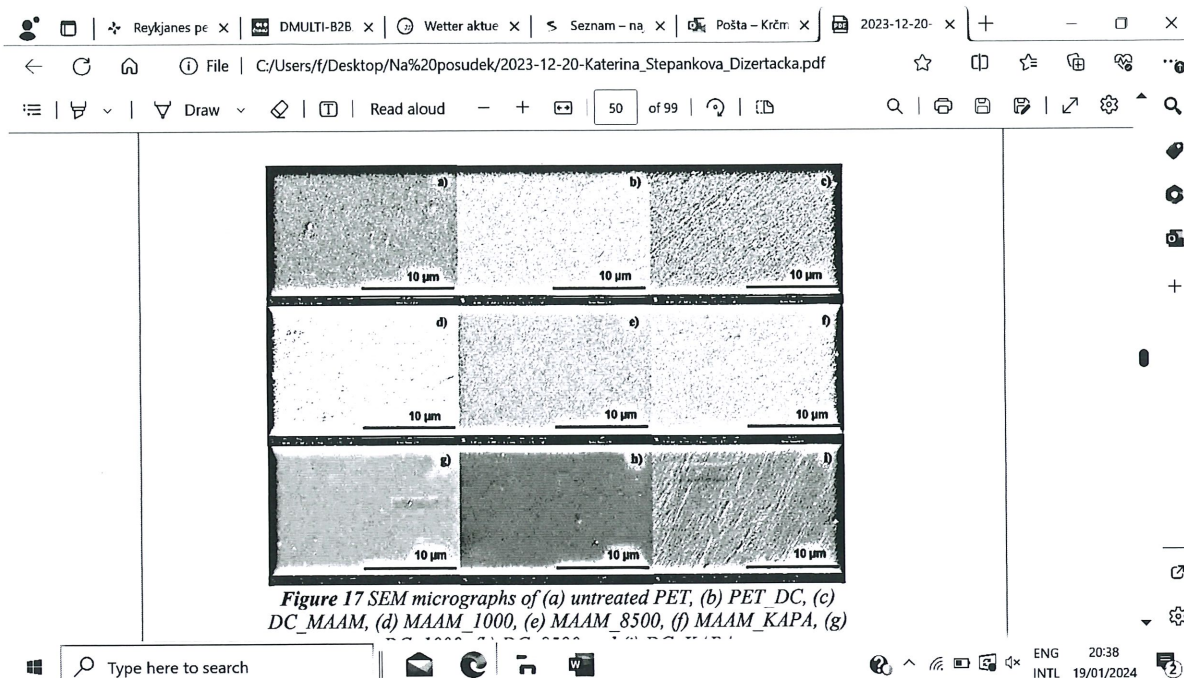
I have besides the main notes following comments and questions for the discussion at the defence:

General comments:

1. Section 8.1.1 – what PET was used? Properties can differ by manufacturer. What air was used? Synthetized, dried, ambient (with what humidity)? This is very critical point for the plasma effects. Plasma was operating in continuous or pulsed mode? If pulsed mode, what was the duty cycle? Why some words are in bold font? There is no reason for this.
2. 8.1.2, surface energy – square roots in equations are not clearly depicted, so reader is confused without knowledge of them. 3rd equation is not correct. Droplets of 5 μL are rather big, commonly 2 μL droplets are used. 5 droplets per liquid is sufficient for the rough estimation, standard is at least 10 droplets to minimize experimental uncertainty. Commonly, some droplets must be excluded from the processing because of very different measured contact angles.
3. 8.1.2, XPS – spot size should be area because it is not linear. Was spot really circular? And what was XPS binding energy resolution?
4. Tables 2 and 3 – what is uncertainty of the presented data? Symbols of samples are not too clear. How many samples were averaged?
5. Table 4 – what are experimental uncertainties?
6. Fig. 18- why there is a horizontal line at 70%? There is no explanation in text.
7. Fig. 20 should be supported by some quantitative analysis. Presented pictures can be from the same sample, only from different place.
8. p. 57 – sulfation procedure of fulcellaran was prepared according to some literature or it is your own contribution. In the second case, much detail description is needed (why steps are used), in the first case, reference is missing.
9. p. 60 – what is origin of the factor 2.25 in the degree of substitution equation?
10. Table 5 – what technique was used for the elemental composition? XPS or some other? And what was the rest of samples, sum of masses is very far from 100%. How the uncertainties were obtained? And what are uncertainties of the last two columns?
11. More detailed analysis of data presented in Fig. 23 is needed. What differences in the spectra say? Is sulfate peak the same for all derivatives?
12. Table 6 – what are the uncertainties? The information about differences is not presented in table, so why it is in the note?

Technical comments:

1. p. 20 “One study reported that amine groups may be lost from the surface of aminolyzed polymers when stored at 37°C.” – reference is missing.
2. p. 20 “Aminolysis is a versatile technique that finds application not only in tissue engineering but also in various other fields such as the textile industry, environmental remediation, material science” – not supported by references.
3. p.27 “Numerous sulfated polysaccharides have been identified for their cytotoxic properties and have been employed as antiviral agents against viruses such as respiratory syncytial virus (RSV), herpes simplex virus (HSV) types 1 and 2, and human immunodeficiency virus (HIV).”- missing reference.
4. p. 29 – Fig. 10 – missing reference.
5. Some figures (like Fig. 11) are not important and there is no link on them in the text.
6. p.33 – link to Fig. 4 is incorrect. Correct figure is 12.
7. Fig. 16 – missing reference.
8. 8.1.2, SEM – the second sentence is not complete.
9. According to nomenclature, all variables should be in italic font, not roman.
10. 8.1.3, Antibacterial tests – what nutrient broth was used? There are many different kinds. The subscript index is used instead superscript in the last not least unit. There is no common logarithm in terminology. It is decadic logarithm.
11. Fig. 17 is not too clear. I tried to change pictures properties and information is much better visible (see below). Why this not done before?



12. p. 62 “The elemental analysis data presented in Table 7 confirmed” – information is non-correct. Data are presented in Table 5.
13. p. 64 “As depicted in Figure 3b,” – correct figure number is 23b.
14. p. 67-68 – non-correct link to the figure just above.

Formal comments:

1. English needs revisions.
2. Definition of plasma is not general. Used form is definition of so-called cold plasma.
3. p. 16 – direct current does not mean continuous. There are many applications where different power supplies are operating in pulsed mode with general aim to keep high reactivity at low mean energy. AC plasmas are plasmas using any AC power supplies (i.e. typically any frequency above in electricity network). What is pointed in text (RF and MW) are specific types of AC plasmas.
4. p. 17 – “In cold plasma, collisions between electrons and neutrals are most common due to the low degree of ionization.” Statement is not correct. If electron concentration is low (i.e. low ionization degree), the collisions among neutral species are dominant.
5. p. 17 “Plasma treatment refers to a specific method of hydrophilic surface functionalization.” – Also this sentence is non correct. Term plasma treatment is much broader than simple hydrophilic surface functionalization.
6. There are many abbreviations in the text but not all of them are explained at their first use. List of them is also non-completed.

Finally, I’m recommending with reservation to the author share this doctoral Thesis for the defence where the main questions will be successfully answered. Thereafter, I’m recommending getting title Doctor to Kateřina Štěpánková.

In Brno, 20. 1. 2024

prof. RNDr. František Krčma, Ph.D.

