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RAMAN STUDY OF CELL ENCAPSULATING POLYMER MICROSPHERES

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Abstract

Microsphere encapsulation for cell therapy especially in type I diabetes treatment is well documented throughout scientific field showing some success even in clinical trials. Microspheres are composed of polymer held together by electrostatic interactions. Its matrix and membrane serves in mediating cellular signaling and at the same time as a barrier against host immune attacks. It is thus important to have knowledge about any potential changes to microsphere structure. In this work spatial distribution of polymer is studied using Raman confocal microscopy. Ability of this method in measuring absolute concentration with emphasis on monitoring distribution changes during *in vivo* exposure is presented. Two microsphere designs with differing spatial heterogeneity are monitored during multiple steps showing that in vivo exposure result in complete loss of initial heterogeneity. This can have direct impact on the immune protective abilities. At the same time quantitative information presented carry valuable message for cell microenvironment.

Keywords: encapsulation, spatial distribution of polymer, confocal Raman microscopy, alginate

1 Introduction

Biomaterial science is moving forward so that previously untreatable medical conditions are now treated as a part of standard medical praxis [1]. By the definition, biomaterial is a material which is used in devices targeted for interaction with biological systems [1]. Biomaterials science is thus multidisciplinary field which needs inputs from many research areas through biology, medicine, chemistry, physics up to informatics.

Before the biomaterial enters clinical praxis it needs to pass several phases of research [1]. These include setting the principal requirements for a specific application followed by testing *in vitro,* passing preclinical and clinical studies and if successful, finally ending up as a medical device. On this path, there have to be many criteria met and understanding of biomaterial nature is definitely one of the key aspects. This dissertation thesis is a confluence of a multidisciplinary work on specific biomaterial, which is the polymer microspheres aimed at immunoprotection of transplanted insulin-producing cells to provide the long-term control of the blood glucose levels of diabetic patients [2], [3], where the Raman spectroscopy has been implemented as a tool to enhance our understanding of this biomaterial properties and performance.

Polymer microspheres in bio-medical applications show promising results as therapeutic molecule carriers in challenging but long-term way. It is not just enclosing the therapeutic molecule but enclosing whole cells with its ability to sense and produce necessary levels of therapeutic molecule. By bringing living cells into a contact with body fluids, constant supply of therapeutic molecule with response to environment is accomplished. Transplanting insulin producing cells encapsulated in the polymer microspheres is one of such application. Microspheres separate cells from immune attack and at a same time promote interactions necessary for effective treatment [4]. Signaling has to be mediated through the membrane and matrix of microsphere. Spheres have to have sufficient diffusion of oxygen, glucose and support metabolic pathways. Knowing the performance-determining microsphere properties is thus important for having a system of expected behavior and function. Parameters as plasticity, molecular cut-off and density belong among those which are measured nowadays. In this dissertation thesis we propose

methodology for measuring the spatial distribution of polymer within a microsphere based on confocal Raman microscopy (CRM) [5].

Currently the spatial distribution of polymers has been measured using confocal laser fluorescence microscopy (CLSM) in a relative manner [6]–[8]. One drawback of CLSM is the necessity for tagging of molecules of interest. CRM gives us the chance to monitor microsphere density in native state without contaminating polymer with fluorescent markers. Monitoring microspheres in native form opens up the chance to study a polymer spatial distribution as a function of its environment, including the living organism.

2 Encapsulation technology for cell therapy in diabetes treatment

The type I diabetes is an autoimmune disease where the insulin-producing β-cells of pancreatic islets (islets of Langerhans) are destroyed by the attack of own immune system [9]. This renders patients with this disorder dependent on exogenous insulin. Diabetes is now manageable, nevertheless, even with available insulin therapy secondary complications on microvasculature occur to some degree depending on the level of glycemic control [10]. In some cases, unawareness to hypoglycemia may evolve which leads to life-threatening episodes of coma [11].

Besides insulin therapy other treatments exist. It can be either transplantation of whole pancreas or transplantation of isolated islets. Any of these options have one disadvantage in common that is the life-long immunosuppression. The reason is that the tissue transplanted to the body is recognized as non-native by immune system and would be destroyed if the immune response is not suppressed. The immune system attack is the reason, why cell encapsulation is researched as one of the option in cell therapy to prevent this attack by immunoisolation of cell behind a semipermeable membrane.

Encapsulation will put a barrier between immune system response and cells encapsulated within. There are various options how to tackle this task. One of the ways to make a division of different strategies is based on the size and form of the semipermeable membrane (Figure 2.1).

Macroencapsulation devices are in the range of several centimeters and provide numerous ways for material preparation controlling its porosity or immunoisolating membrane thickness [4]. They are easily retrievable, which makes them good candidates for initial testing in case undesirable effects occur. On the other hand, the size is limiting factor for molecular diffusion which limits speed of interaction [12]. Microencapsulation devices are within hundreds of micrometers large in the form of microspheres in which cells reside. Their size offers medium effectiveness in diffusion processes and adaptation to various transplant sites. Retrieval of microencapsulation devices is limited because large quantities are needed for meaningful effect. Conformal encapsulation is the smallest in size which can be practically of the size of islets. It is more or less a coating in the nano to micrometer range representing only small diffusion barrier which is the biggest advantage and also the challenge for manufacturing [13], [14]. Conformal encapsulation or coatings have even more options for transplant sites because of the little restrictions in volume.

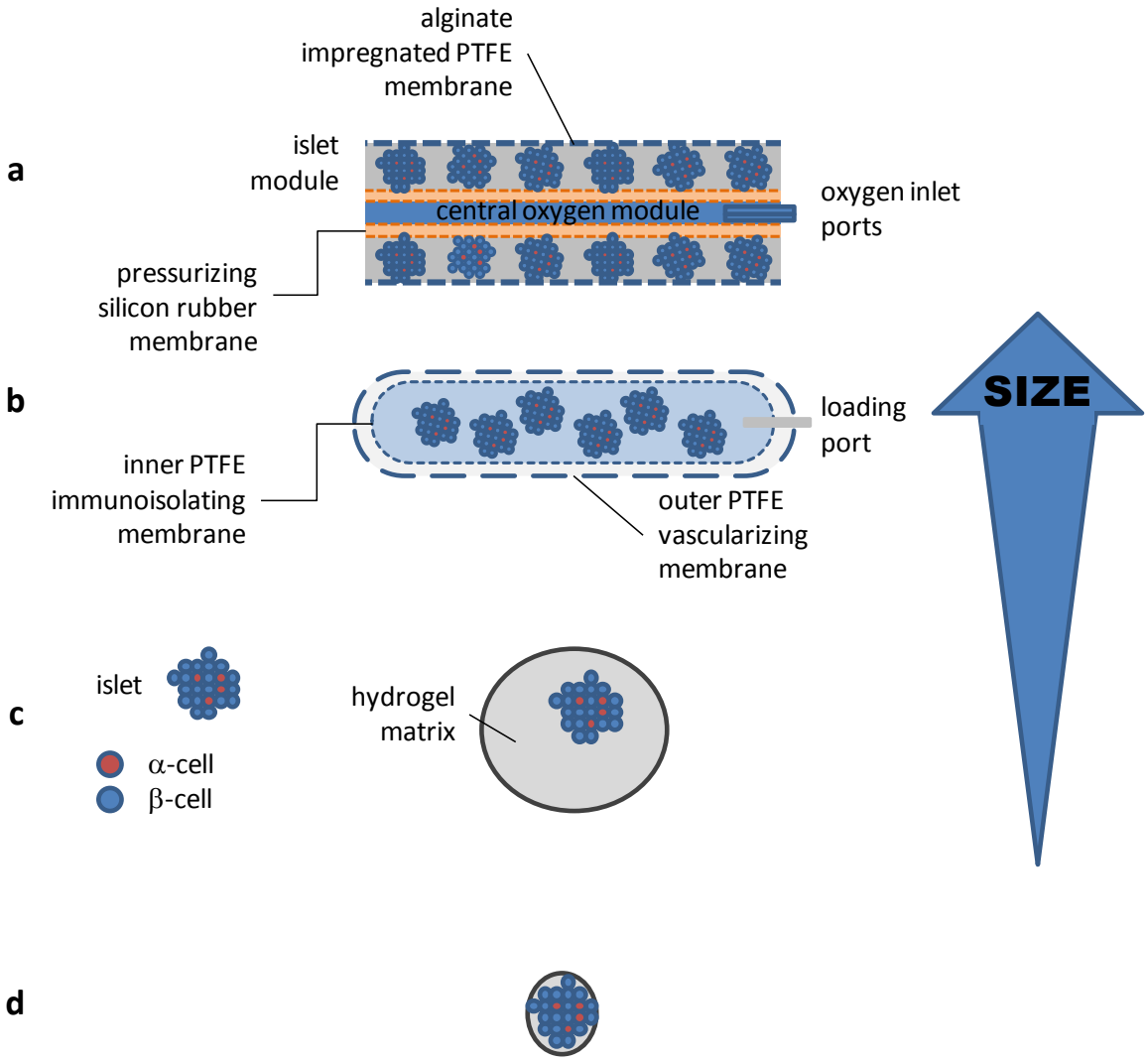

Figure 2.1 Schematic representation of different strategies for cell encapsulation with basic characteristics. a-b) examples of macroencapsulation devices a) Beta-O2 device b) Encaptra device c) hydrogel microspheres d) conformal coating.

All of these strategies are targeted to accomplish key aspects of successful device that is to provide (i) response to blood glucose levels mimicking normal body action, (ii) efficient nutrient and waste exchange, and (iii) prevent immune system reaction which would influence viability and function of cells encapsulated within a device [4]. Immmunoisolation of encapsulated cells, nutrient and waste removal is established via semi-permeable membrane.

Overall, encapsulation of islets within microspheres has still some drawbacks, but it proved some functionality in most stringent models, including human trials. Studies showed some basics, which needs to be addressed as necessity to understand why some

capsules aggregate [15], [16] and form clusters, even when same system can effectively induce normoglycemia in small animal models [15]. In some cases, islets encapsulated in microspheres did not trigger any antibody formation, which is considered as a good result in light of type I diabetes being an autoimmune model with ready-to-act immune system. On the other hand, this does not prevent innate immune system to interact with the implants also no cytokine levels were reported. In fact, bioinvisibility of islet grafts provided by microspheres as defined by U.S. Food and Drug Administration and mentioned in [17], did not prevent slow decline in graft function. Other parameters impacting the results in clinical trials might be overstimulation of inadequate amount of functional islets implanted with the respect to transplantation site [17] which leads to apoptosis and cellular damage [18]

3 Objectives

Non-covalent crosslinked polymers have proved its applicability as immunoprotective devices in cell therapy for next generation treatment of type I diabetes mellitus but there are still some flaws of the system which needs to be addressed [13]. Understanding functional properties of microspheres is necessary step for mechanistic understanding of these systems. Polymer spatial distribution and structure is one of the aspects which defines stability and immunoprotective quality of microspheres [6], [28], [29]. Even when spatial distribution of polymer is known at the beginning of its *in vivo* testing, any changes during this *in vivo* path are not being evaluated because straightforward, non-invasive method is not available [20].

Confocal Raman microscopy is proposed as the method which posses these qualities. To be able to use such method several objectives are needed to fulfill.

a) Adaptation of microsphere measurement for Raman spectroscopy
b) refinement of experimental data for data analysis
c) investigate possibilities for defining polymer structure with Raman spectroscopy
d) evaluate possible transition changes before and after microspheres implantation to living organism

4 Results

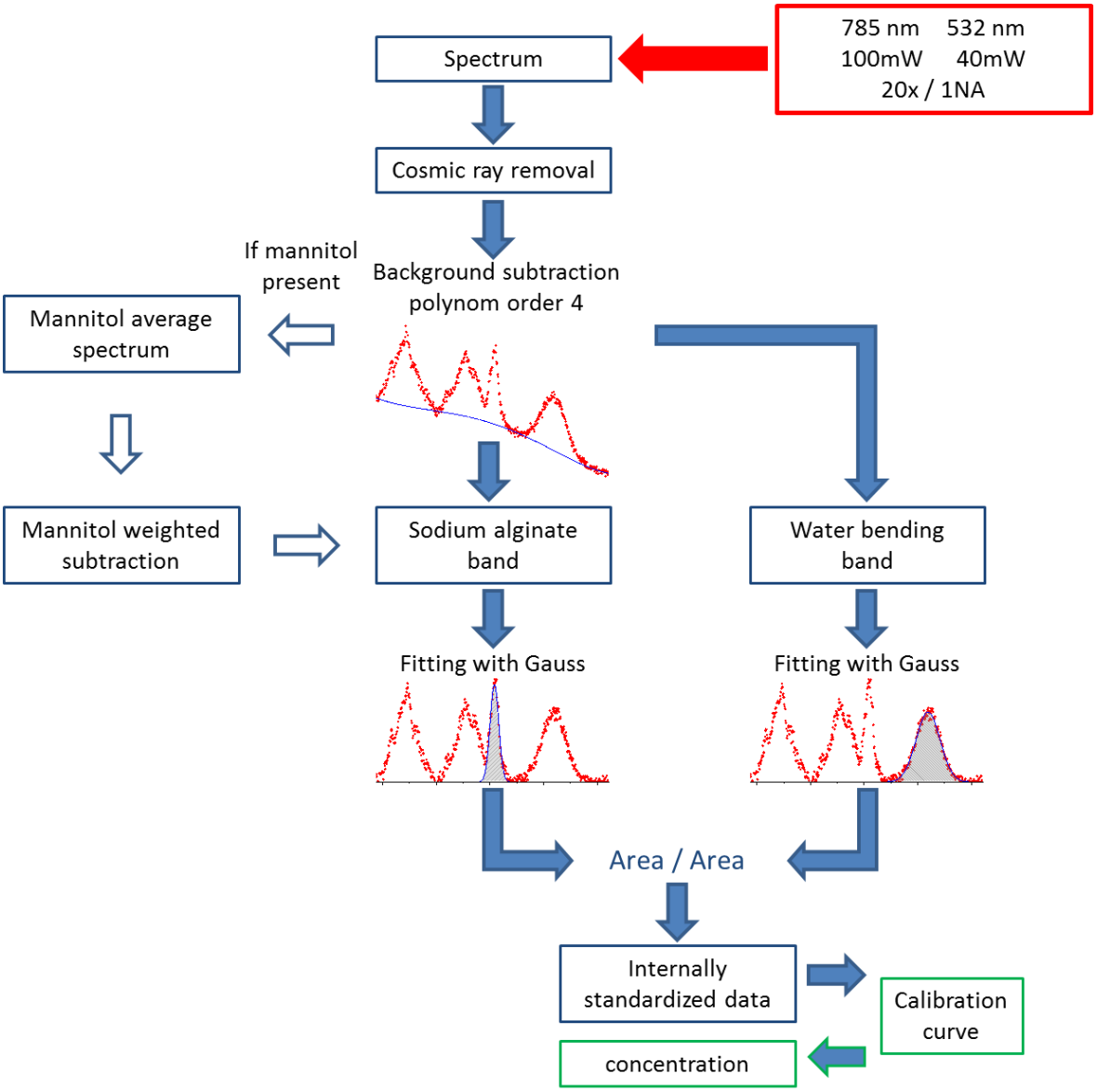

Figure 4.1 Workflow diagram depicting whole process of data acquisition and evaluation with optional mannitol spectrum subtraction.

4.1 Environmental impact on microsphere structure analyzed by CRM

CRM imaging can be used in monitoring changes of spatial distribution of polymer upon exposure to different environments. Most important is to understand what happens after *in vivo* exposure as microspheres are used in clinical trials. We already mentioned that spatial distribution of polymer have effect on immunoprotective properties of microsphere. Changes which would lead to significant redistribution may directly influence survival or function of encapsulated microspheres. Currently whether such changes are present is not known.

Microspheres with markedly different heterogeneity were prepared in similar conditions as microspheres already tested in clinical trials. Higher degree heterogeneity microspheres [16] and lower degree of heterogeneity [15]. Microspheres were prepared and stored in D-mannitol as this solution lacked anti-gelling ions, preservation of structure is ensured. Before implantation to mice, microspheres were transferred to saline because they have to be introduced in physiological solution. At this point some microspheres were separated and stored at 37°C for 24 hours to mimic potential temperature effects. The rest of microspheres were implanted into a peritoneal cavity of nude mice for a time period of 4 weeks. All storage solutions contained 2mM $CaCl_2$ to match and simulate physiological concentration of Ca^{2+}. With this treatment also attained state of microspheres after explantation were preserved. Environmental impact on microspheres was analyzed on three different time points: after preparation (0.3M mannitol + 2mM $CaCl_2$), after 24h at 37°C (0.9 % NaCl + 2 mM $CaCl_2$) and after explantation.

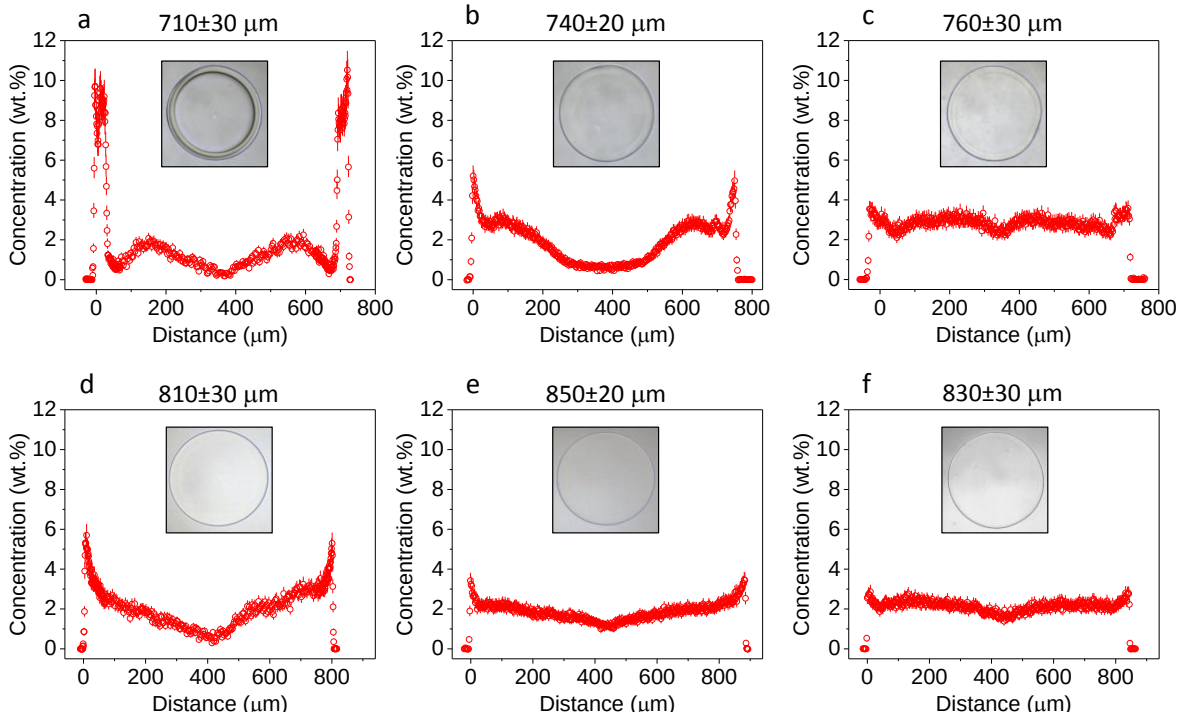

Figure 4.2 Representation of microspheres with different heterogeneities presented as equatorial plane cross-section plots of polymer distribution throughout different time points. Upper row) higher heterogeneity alginate microspheres, lower row) lower heterogeneity alginate microspheres. First column microspheres after preparation, second column microspheres after 24h storage in saline at 37°C, last column explanted microspheres 4 weeks after implantation.

High heterogeneity microspheres after preparation in mannitol (Figure 4.2a) show in optical microscope distinct shell and it can be expected this involves higher density layer of alginate localized at the surface. Size of the shell is 40 μm as measured by optical microscopy. CRM analysis confirms this size and indicates about 10 wt.% of polymer in the shell with rapid decay down to 0.6 wt.%. Moreover, CRM visualize local variations of polymer towards the core where minimum of concentration is measured which is around 0.5 wt.%. Profile has expected radial symmetry with only minor variations. High heterogeneity is attained by high diffusion rate of alginate towards microsphere surface what manages more alginate to be displaced before being crosslinked [25]. After microsphere storage for 24h at 37°C lower heterogeneity is gained and concentration at sphere surface is about 5 wt.% with much less steep gradient in concentration into the core where around 1 wt.% of alginate is present. Change is obvious also in light microscopy with much less distinct shell present at microsphere surface. The diameter of sphere has changed after this step pointing to the swelling of microsphere. This is present because of partial dissolution of dimer crosslinks due to exchange of gelling ions to non-gelling sodium ions [6]. Upon explantation of microspheres from mice we see significant decrease of heterogeneity with nearly constant spatial distribution of polymer varying only 1 wt.% across microsphere. Local concentration is at surface 3.6 wt.% to 2.2 wt.% in the core. Optical microscopy is in this case not helpful and change cannot be followed. Such change is surprising because prolonged in vitro storage in 0.9% NaCl + 2 mM $CaCl_2$, of up to 10 months did not result in any significant change of polymer distribution (see appendix).

Lower heterogeneity microspheres were treated in same manner and are showed in lower row of the figure (Figure 4.2 d-f). Optical microscopy is in this case quite similar and any differences are hard to depict. CRM analysis is on the other hand is able to follow variations through different measurement steps. Concentration span from microsphere

surface to microsphere core varies varies from 5 wt.% to 0.7 wt.%, from 3.5 wt.% to 1.2wt.% and from 2.8 wt.% to 1.6 wt.% for microsphere after preparation, after storage and after explantation, respectively. This trend is similar but less pronounced than in high heterogeneity microsphere.

Resulting trend of alginate spatial profile homogenization is definite. Both alginate types of microspheres are non-covalently crosslinked hydrogels stabilized predominantly by electrostatic interactions [34], [35]. Number of crosslinks is dynamic system and can fluctuate in response to environmental characteristics such as presence of gelling and non-gelling ions or proteins [6], [36]. Partial dissolution of ionotropically stabilized network of alginate upon exposure to *in vivo* conditions is at present not known. But we can hypothesize that this effect was present also in microspheres used in clinical trials using simple alginate microspheres. Thus any control of initial gelling conditions seems to be not necessary. With redistribution of polymer and its lower concentration at the microsphere surface it is also expected that permeability of microspheres will be affected [6]. Because number of crossslinks is proportional to concentration of polymer [36] and these are affecting permeability properties [14]. Lower MWCO than that which was expected will directly compromise immunoprotective properties of microspheres. Therefore, other preparation methods than just simple ionotropic gellation should be used in order to preserve initial parameters of prepared microspheres. Polyelectrolyte complexation [8] or covalent crosslinking [27], [37] might be other options. This study shows it is important to know characteristics of microspheres so that conclusions on why some microspheres fail and other not are based on valid knowledge.

5 Conclusion

In this work confocal Raman microscopy as non-invasive method has been used to quantitatively and qualitatively analyze spatial distribution of polymer inside microspheres used in cell therapy. Whole analysis proved to be manageable as a part of standard characterization methodology at the research level. Scientific as well as technical problems were addressed during this work.

Intitial choice of representative Raman band for measuring spatial distribution has been based on literature. Carboxylate symmetric vibration was out of several possible options most satisfactory choice. Its stretching vibration is well isolated where only carboxylate atoms move substantially. It is well isolated in spectra without any significant overlap from other Raman bands which permits good basis for further analysis. Its choice for Raman analysis was validated in multiple steps.

Our workflow was compared side by side with current state-of-the-art method which is confocal laser scanning microscopy. Results pointed to important aspect of CRM analysis which proves to be comparable with CLSM but with much better adaptability to optical errors present in specific environment of microspheres measurement.

Quantitative analysis was made possible by construction of calibration curve for two laser lines based on alginate solutions. Quantitative analyis was validated for extrapolation ability with overall good performance. Validation of quantitative analysis for transferability to gelled state of alginate which is present in microspheres has been performed. CRM analysis of several planar gels showed inclusion of their real concentration within predicted range of quantitative model. By following microenvironment changes it was hypothesized that no further deviation is expected for microsphere measurements.

Whole workflow was tested for invariability of the preparation of calibration curve and independency on laser line used. These multiple stages of validation underlines that proposed CRM analysis is flexible and results in robust estimates.

Quantitative analysis limits were proposed by quantifying limit of detection and some considerations were laid out for possible errors in high concentration region.

Technical details have been also addressed as fixation of microspheres which has been solved by creating custom made silicon holder. In production emphasis was put on artifact free measurement.

Quantitative analysis by CRM was applied in two designs of empty microspheres with different initial heterogeneity of polymer distribution. Structure of microsphere was analyzed by mapping alginate concentration at equatorial plane cross-sections. Gained concentration profiles provide invaluable information with respect to cell environment which experience upon encapsulation. CRM imaging proved to be effective in visualizing structural changes within microsphere triggered by environmental change. It was found that irrespective of initial heterogeneity of microspheres upon preparation and storage, exposure to *in vivo* environment resulted in heterogeneity loss. This might have direct consequences for intended immunoprotective properties of microspheres in diabetes treatment. For preparation of microspheres we followed protocols also used in clinical trials, thus it is hypothesized that the same level of change happened.

Lastly CRM can deliver performance sensitive data of different microspheres designs and help at increasing critical knowledge for further development.

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AFH Abstrakty príspevkov z domácich vedeckých konferencií

AFH01 Eliášová Sohová, Marianna [UKOMFKJFBd] (50%) - Pelach, Michal [UKOMFKJFBd] (25%) - Šiffalovič, Peter (15%) - Hianik, Tibor [UKOMFKJFB] (10%): Možnosti prípravy SERS substrátov na detekciu onkologických markerov
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AFH02 Pelach, Michal [UKOMFKJFBd] (20%) - Eliášová Sohová, Marianna [UKOMFKJFBd] (20%) - Weiss, Martin (20%) - Ivanov, Ilia N. (10%) - Šiffalovič, Peter (15%) - Hianik, Tibor [UKOMFKJFB] (15%): SERS surfaces for bio applications
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BEF Odborné práce v domácich zborníkoch (konferenčných aj nekonferenčných)

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