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PREPARATION AND CHARACTERISATION OF COMPLEX NANOPARTICLES BY FIELD-FLOW FRACTIONATION AND ADVANCED SPECTROSCOPIC METHODS
PŘÍPRAVA A CHARAKTERIZACE KOMPLEXNÍCH NANOČÁSTIC S VYUŽITÍM ZEJMÉNA FRAKCIONACE V TOKOVÉM POLI A POKROČILÝCH SPEKTROSKOPICKÝCH METOD

SUMMARY OF DOCTORAL THESIS
SOUHRNNÁ TEZE K DIZERTAČNÍ PRÁCI

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ABSTRACT

Liposomes are versatile biocompatible and biodegradable carriers for a variety of medical applications. As the first nanoparticles, they have been approved for pharmaceutical use so far, and many liposome-based preparations are in clinical trials. Classical methods of liposome preparation represent potential limitations in technology transfer from laboratory to industrial scale. New, microfluidic techniques overcome these limitations and offer new possibilities for controlled, continuous preparation of liposomal particles in a laboratory and industrial scale. An important element in the development of new nanoparticle systems is their complex characterization and purification. In addition to the established chromatographic techniques, the Field flow fractionation technique, in particular the Asymmetrical flow Field-flow fractionation, is described. This relatively new technique in conjunction with the MALS/DLS/DAD-UV/dRI online detectors enables the purification and characterization of complex samples. The main advantage of this technique lies in the possibility of separation under native conditions, which plays an important role in the separation of biopolymers in particular. Separation in the "empty" channel then eliminates sample degradation due to unwanted interactions at the stationary phase-sample interface. The theoretical part of this thesis describes the possibilities of preparation, modification, and characterization of liposomal nanoparticles. For this purpose, optical methods based on dynamic light scattering, multi-angle dynamic light scattering and nanoparticle tracking analysis techniques are described, as well as a non-optical method using "particle by the particle" analysis, tunable resistive pulse sensing method. A separate chapter of the theoretical part is dedicated to the technique Asymmetrical flow Field-flow fractionation in connection with the above-mentioned detectors. Important results associated with this work are summarized in the attached scientific paper together with the result summaries and the author's contributions.

ABSTRAKT

Liposomy představují univerzální biokompatibilní a biodegradabilní nosiče pro celou řadu medicínských aplikací. Jako jediné z nanočástic byly doposud schváleny pro farmaceutické použití a celá řada přípravků na bázi liposomů je v klinickém testování. Klasické metody přípravy liposomů představují potenciální omezení v převádění technologie z laboratorního do průmyslového měřítka. Nové, mikrofluidní techniky tyto omezení do jisté míry překonávají a nabízejí nové možnosti kontrolované, kontinuální přípravy liposomálních částic. Důležitým prvkem při vývoji nových, nanočásticových systémů je jejich komplexní charakterizace a purifikace. Kromě ustálených chromatografických technik se dostává do popření technika Frakcionace v tokovém v poli, a to zejména Frakcionace tokem v asymetrickém tokovém poli (Asymmetrical flow Field-flow fractionation). Tato relativně nová technika ve spojení z online detektory MALS/DLS/DAD-UV/dRI umožnuje purifikaci a charakterizaci komplexních vzorků. Hlavní výhoda této techniky spočívá v možnosti separace za nativních podmínek, což hraje důležitou roli při separaci zejména biopolymerů. Separace v „prázdném" kanále poté eliminuje degradaci vzorku v důsledku nechtěných interakcí na rozhraní stacionární fáze. Teoretická část teto práce popisuje jednak možnosti přípravy, modifikace zejména liposomálních nanočástic. Sumarizuje jejich možnosti využití a charakterizaci. K tomu účelu jsou zde popsány optické metody na bázi dynamického rozptylu světla, Multi-angle dynamic light scattering a Nanoparticle tracking analysis a také neoptická metoda využívající průchodu

částic membránou tzv. „particle by particle" metoda Tunable resistive pulse sensing. Samostatnou kapitolou teoretické části je technika Frakcionace tokem v asymetrickém tokovém poli ve spojení z výše uvedenými detektory. Důležité výsledky spojené s touto prací jsou sumarizovány v přiložených vědeckých publikacích. V této práci jsou poté uvedeny krátké přehledy a autorovy přínosy k těmto přiloženým publikacím.

KEYWORDS

Liposomes, microfluidic mixing, dynamic and static light scattering, asymmetrical flow field-flow fractionation, recombinant proteins.

KLÍČOVÁ SLOVA

Liposomy, mikrofluidní směšování, dynamický a statický rozptyl světla, asymetrická frakcionace v tokovém poli, rekombinantní proteiny.

1 INTRODUCTION

The PhD thesis is consisting of two main topics; the first topic is focusing on preparation, modification, and characterization of nanoparticles, especially nanoliposomes. The second topic deals with the Asymmetrical flow Field-flow fractionation technique coupled with multiple detectors, such as Multi-angled light scattering detector, Dynamic light scattering detector, UV absorption, and Different refractive index detector.

Liposomes, the name of these vesicular particles, comes from the two Greek words *Lipos*, meaning Fat and *Soma*, the Body. Liposomes were first described by Dr Alec D. Bangham in 1965 and have since become a versatile, modular biodegradable and biocompatible platform for a variety of applications [1]. The first liposome-based pharmaceutical products were launched in the late 1980s (for example, Ambisome - liposomal amphotericin). Not only liposomes but other nanoparticles as well are subject of the development of modern vaccines, drug carriers, contrast agents or in general theranostics. A prerequisite for successful clinical applications is the production of nanoparticles on an industrial scale. The introduction of new techniques, especially in the field of microfluidics, allows the transition from laboratory to industrial scale, leading to extensive research in this field. The development of microfluidics is directing towards the design of integrated microfluidic chips enabling complex, controlled preparation of nanoparticle carriers [2,3].

Modularity/surface modification of nanoparticles is another prerequisite for their successful clinical application. The surface modification affects their biodistribution and biocompatibility. The new bioconjugation reactions have been developed, including the Click chemistry and orthogonal bioconjugation reactions [4]. Novel characterization and separation methods are associated with the development of the new nanoparticles. The most commonly used characterization techniques include light scattering techniques. In addition to established chromatographic techniques, a new Field flow fractionation technique finds its place and application in various fields.

The beginning of the Field-flow fractionation technique is dated back to the year 1966 when professor Giddings first introduced the concept [5]. Since then, multiple sub-techniques have been developed, including the most robust and hence the most used Asymmetrical flow Field-flow fractionation technique (AF4). Modern, versatility AF4 technology makes it possible to characterize a wide range of pharmaceutical substances, polymers, nanoparticles and recently found a place as a technique for quality control departments besides Size-exclusion chromatography. As a well-described theoretical technique, AF4 allows calculating the size of eluting particles based on retention time. Often, however, the AF4 is connected with light scattering detectors, both static and dynamic, concentration detectors such as UV absorption and Different refractive index detector. The platform AF4-MALS/DLS/DAD-UV/dRI allow the determination of the molecular weight, concentration, conformation, and size of the individual eluting fractions.

Results within the PhD thesis had been published in a total of five published, and three submitted scientific papers. These scientific papers are presented together with a brief commentary and evaluation of the author's contribution.

The thesis Preparation and characterization of complex nanoparticles by Field-flow fractionation and advanced spectroscopic methods is constructed as a thematically arranged set of published works and works accepted for publication according to article 42 of paragraph 1b, of The study and examination rules of Brno University of Technology.

2 GOALS OF THE THESIS

1. Preparation and characterization of nanoparticles with the use of advance spectrophotometric methods.
    - The thesis is mainly focused on the preparation of liposomal nanoparticles using microfluidic mixing technique, lipid film hydration, detergent dilution, and high-pressure extrusion. Other nanoparticles are be obtained by biotechnological procedures within the department or are be obtained from cooperating laboratories within the OPVVV projects.
    - The characterization of nanoparticles is performed using advanced physicochemical and spectrophotometric methods. Especially dynamic light scattering/multi-angled dynamic light scattering, Nanoparticle tracking analysis for measurement of size distribution, concentration and their ζ-potential. And technique Tunable resistive pulse sensing for particle by particle analysis to obtain particle size and number distributions.
2. Surface modification on the nanoparticles with the use of bioconjugation orthogonal reactions.
3. Optimization and implementation of the separation techniques for nanoparticles and biopolymers.
    - The thesis deals mainly with the Asymmetrical flow Field-flow fractionation for separation various types of nanoparticles and biopolymers coupled with multiple detectors.
    - Furthermore, the thesis deals with additional online analytical techniques such as online dynamic light scattering/multiangled light scattering, UV-VIS absorption detector and different refractive index detector.

3 RESULTS

The data measured within the author's dissertation thesis were published in the bellow mentioned scientific papers. The results include the implementation of nanoparticle characterization techniques, where the author participated in the optimization of measurements and data evaluation. The techniques used by the author are given in the theoretical particles of the dissertation thesis. The results further include the development of the nanoparticle itself. Here, the author focused on liposomes and their preparation using mainly microfluidic mixing technique. The preparation of the liposomes using microfluidic mixing technique is dealt with within the author's first article [P1]. In the following scientific papers, the author also deals with the characterization of carbon-based materials and bacterial ghost. Other publications focus on the preparation of liposomes and their post-modification and subsequent application. The author's second first article [S1] deals exclusively with the A4F technique. Here, the author focus on the development of purification and characterization methods of pentameric protein particles. With his work, the author contributed to the broader topic of the development of recombinant, mRNA vaccines, targeted cytostatics, and theranostics.

Nanoparticles characterization play a crucial role during platform development and biomedicinal application. The important physical properties of the nanoparticles are particle size, size distribution and surface characterization. The size of nanoparticle carriers determines their biodistribution *in vivo*, and their passive targeting to the desired site. The particle size distribution then indicates the nature of the sample as a whole, the presence of the aggregate and the overall homogeneity of the suspension. Homogeneity is an important parameter for the stability of the nanoparticle platform. The size or its change makes it possible to monitor the success of nanoparticle modifications. An important criterion is also the value of the zeta potential, where the “golden rule ± 30 mV” represents an important stability factor. The DLS sizing has its limitations, especially for inhomogeneous suspensions. The NTA provides greater resolution, especially for heterogeneous samples. The NTA also allows the differentiation of fluorescently labelled populations and can provide the concentration of the particles. A complementary technique for sizing as well as concentration is the non-optical "particles by particles" TRPS method. Allowing a more detailed view of the distribution and concentration of particles. The online light scattering detector collects and evaluated data of the individual species which promotes better resolutions and eliminates batch measuring limits. Combination of MALS and DLS detector enables to determine the conformation from R_h/R_g ratio.

3.1 Liposomes

The scientific paper [P1] aimed to clarify the mechanism of liposomes formulation, by microfluidic mixing technique, via the vesiculation of the phospholipid bilayer fragment. The liposomes were formulated using “herring bone” mixing channel. The liposome composition included unsaturated 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) or saturated 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EPC) phospholipids with the different cholesterol content. After the formulation, fluorescence membrane probe DPH-TMA (N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)) was incorporated into the phospholipid bilayer, and the steady-state fluorescence anisotropy was measured. Liposomes prepared under the same

process parameters such as the Flow Rate Ratio (3:1) and Total Flow Rate (7 ml/min) exhibits various sizes based on the cholesterol content and saturation or unsaturation of the acyl chain. In the case of the liposomes with unsaturated phospholipid (**Fig. 1**) with the increasing cholesterol contend (from 0 mol% to 50 mol%) the size of the resulting liposomes increased from 25 nm to 153 nm. Increasing size correlates with decreased fluidity of the phospholipid bilayer expressed as anisotropy. In the non-organized system, such as *n*-heptane the steady-state fluorescence anisotropy reached a value of 0.001. In phospholipid bilayer with increased cholesterol, the anisotropy values increased from 0.195 to 0.265. The plot **Fig. 1** B shows good polydispersity and thus the overall homogeneity of the formed vesicles across the composition.

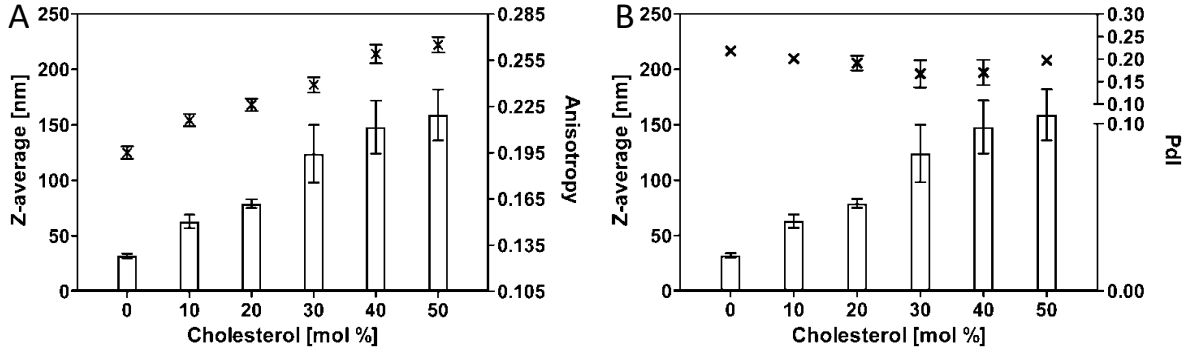

Fig. 1 – *(A) The plot of cholesterol concentration (mol %) versus Z-average diameter (nm) on the major axis and steady-state fluorescence anisotropy of the DPH-TMA on the minor axis. (B) The plot of cholesterol concentration (mol%) versus Z-average diameter (nm) on the major axis and polydispersity index (PDI) and the minor axis* [7].

For the saturated phospholipid (DSPC) the effect on the size is opposite but also correlates with the anisotropy trend. The cholesterol in the bilayer is increasing the fluidity of the lipid bilayer fragment during the formulation which allows the formation of smaller vesicles (**Fig. 2**). Steady-state anisotropy is gradually decreasing with increasing cholesterol content from 0.288 to 0.187. Size of the vesicles follows the same trend, with diameter from 190 nm to 62 nm with the good polydispersity.

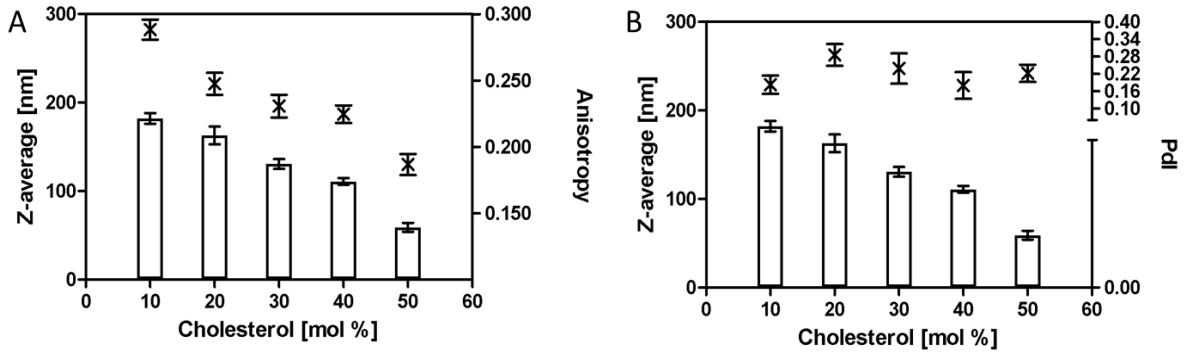

Fig. 2 – *(A) The plot of cholesterol concentration (mol %) versus Z-average diameter (nm) on the major axis and steady-state fluorescence anisotropy of the DPH-TMA on the minor axis. (B) The plot of cholesterol concentration (mol %) versus Z-average diameter (nm) on the major axis and polydispersity index (PdI) and the minor axis* [7].

An example of the use of microfluidic mixing and subsequent post liposomes modification is described in the paper [P4]. In the study, aminooxy lipid was incorporated into the surface of the liposome, and then modified by orthogonal binding of cellular mannan. Different techniques were used for binding confirmations as is shown in **Fig. *3***.

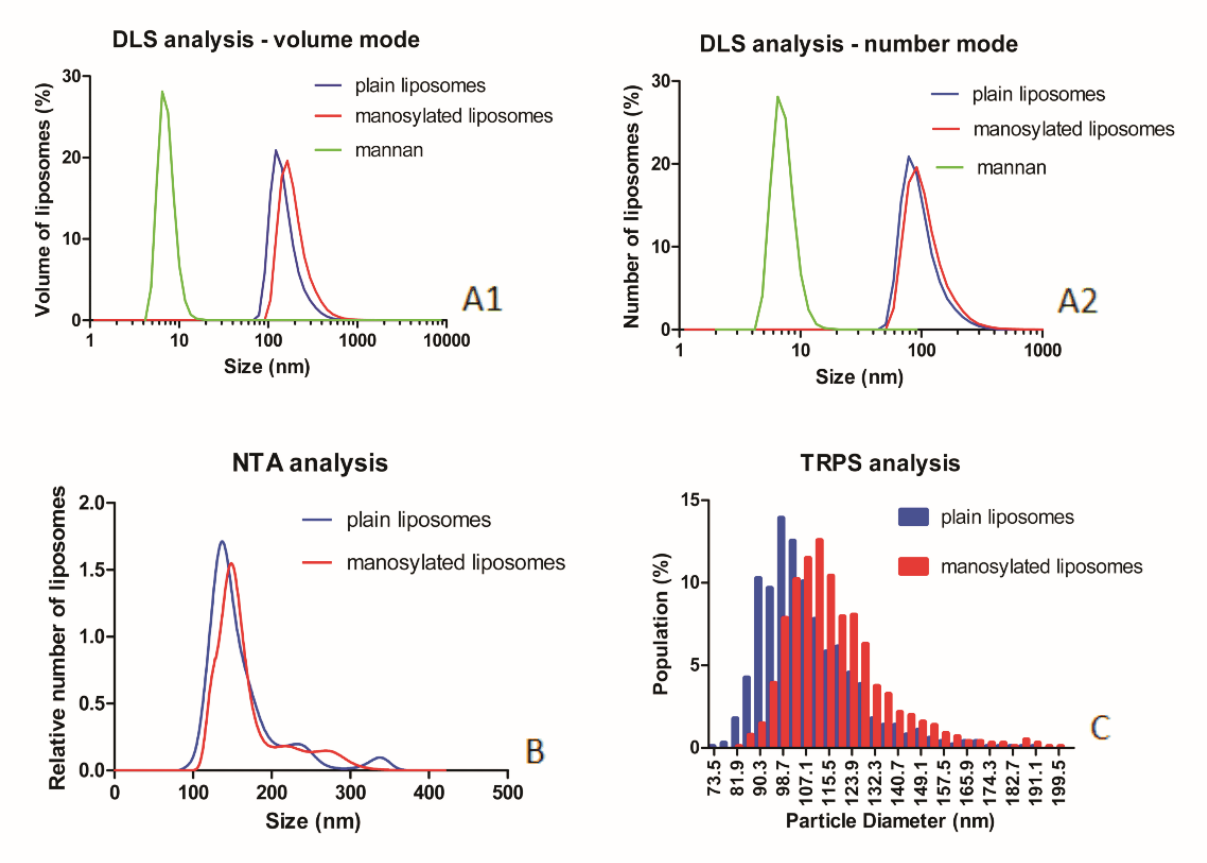

Fig. 3 – *A1) Size distribution by volume A2) Size distribution by number. B) Size distribution determined using NTA. C) Size distribution obtains from TRPS [8].*

The size of the liposomes obtains from DLS increased from 102 nm to 114 m after the modification while maintaining good polydispersity (0.118 for plain liposomes and 0.128 for mannosylated liposomes). With different complementary techniques, the size increased as well from 137 nm to 149 nm for NTA and from 108 nm to 126 nm for TRPS. The zeta potential decreased from −4.8 mV to −10.9 mV after the modification. The negative zeta potential of the mannosylated liposomes well corresponds to the presence of residual phosphate groups in isolated mannan. Individual characterization techniques, using different principles, offer a comprehensive view of the nanoparticle system.

Another liposomal modification is presented in publication [P3] where the liposomes were prepared using DOGS NTA-Ni (1,2-di-(9Z-octadecenoyl)-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)). DOGS NTA-Ni creates an anchor in the phospholipid bilayer which enables functionalization of the liposomes with the anti-insoluble fibre protein binder via the metallochelating bond for direct thrombus targeting (**Fig. 4** A). Protein binder attachment was reflected as an increase of the hydrodynamic radius

from 102 nm to 112 nm. The difference in zeta potential was −6 mV after surface modification, see **Fig. 4** B.

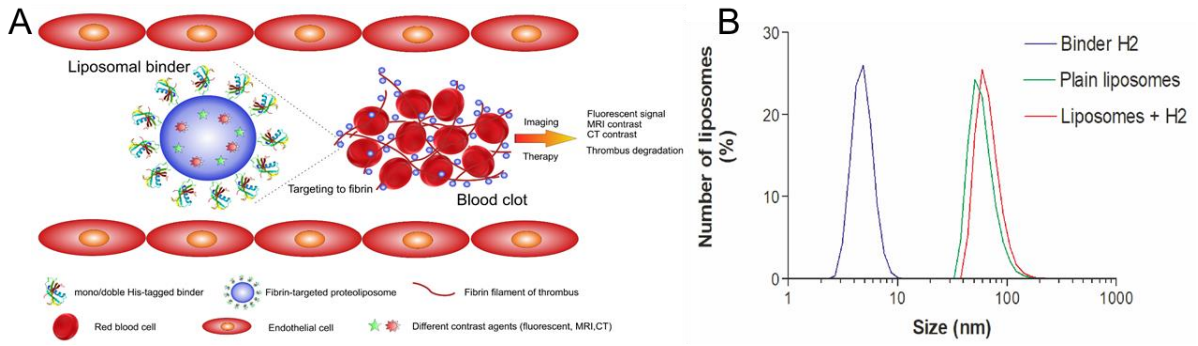

Fig. 4 – *A) The schematic presentation of the thrombus fibrin fibres targeting via the modified liposomes under flow conditions B) Number distribution of protein binder, plain and modified liposomes [9].*

3.2 Carbon-based nanoparticles, nanodiamonds

Nanodiamonds were characterized in the paper [P5]. The measured pH dependence on zeta potential shows that the acidification of the aqueous medium reduces the zeta potential of the carboxylated ND, resulting in aggregation, which occurs near pH 6 **(Fig. 5)**.

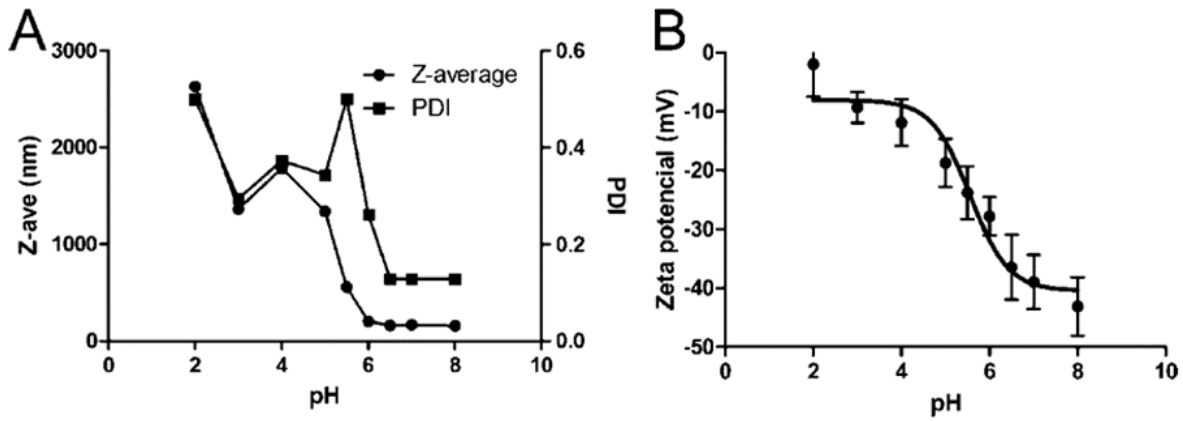

Fig. 5 – *Nanodiamond, Z-average (A) and zeta potential (B) under different pH conditions. The sample was dispersed in 10 mM sodium phosphate buffer and titrated with a pH ranging from 2 to 8 [10].*

The NTA analysis (**Fig. 6** B) further shows that even due to the relatively high negative zeta potential at neutral pH, the suspension contains an aggregate fraction. To compare, the DLS data are shown (**Fig. 6** A) with a different distribution (intensity, volume and number).

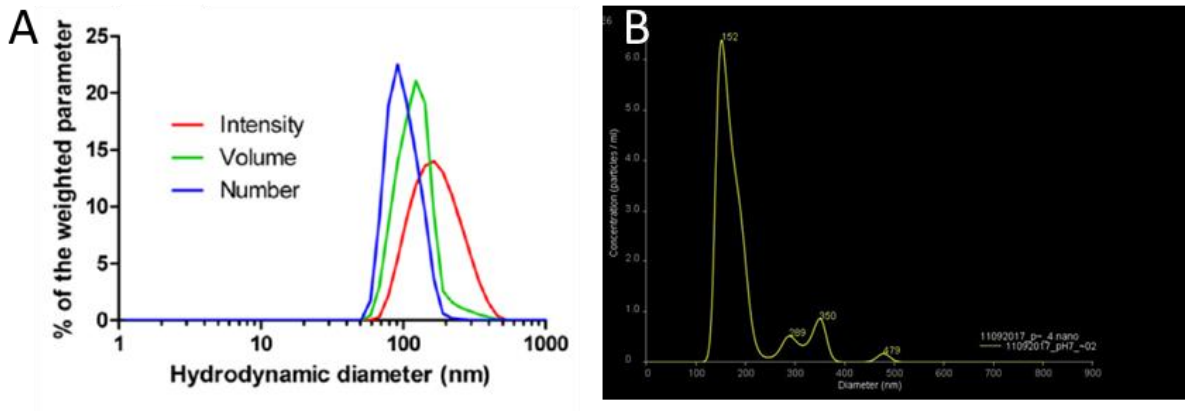

Fig. 6 – *A) DLS size distribution with z-average 182 nm and polydispersity of 0.127. B) The NTA size distribution of ND with a mean diameter of 140 nm [10].*

Different carbon-based nanoparticles were characterized in scientific paper [P2] such as multi-walled carbon nanotubes (MWCNT) and two types of graphene platelets (GP1 and GP2). Both of the GP types formed aggregates with different morphology, GP2 aggregates were larger with sharp edges. Example of pH influence on the zeta potential and size is shown in **Fig. *7*** A and B respectively.

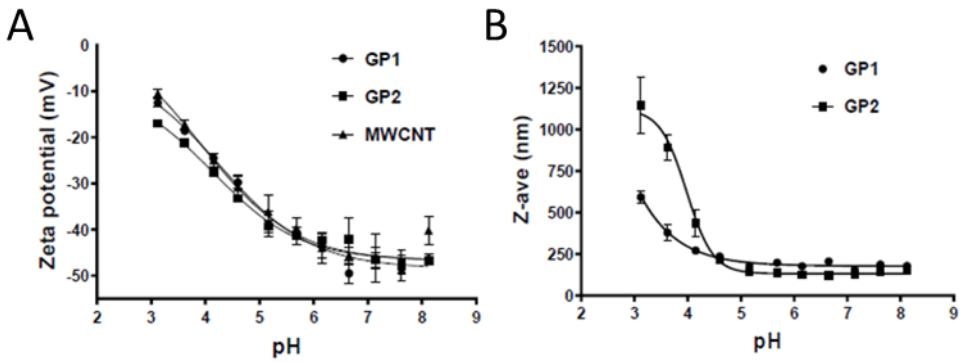

Fig. 7 – *Effect of pH titration on the zeta potential value (A) and Z average (B) on C-BNM [11].*

3.3 Protein pentameric particles

In the scientific paper [S1] the purification of lysate of the pentameric protein particles is described. In the publication, different elution profiles were tested (constant, linear and exponential decreasing cross-flow). The best resolution was obtained from the elution under elution profile combining exponential cross-flow followed by constant cross-flow. Using the combined elution profile, the individual pentameric units were separate from the aggregates. The comparison of hydrodynamic radius obtain from online DLS detector (**Fig. 8** A) is in good correlation with hydrodynamic radius obtain from the full retention equation using FFF theory (**Fig. 8** B). Good correlation suggesting no or minimal sample-membrane interaction. The plot **Fig. 8** C show gyration radius which was used for conformation characterization as a ratio of gyration radius (R_g) to the hydrodynamic radius (R_h) (**Fig. 8** D). According to the R_g/R_h ratio measurement, the aggregates with ratio 1.3 correspond to the branched structure. The individual pentameric particles are spherical with a ratio of 0.8.

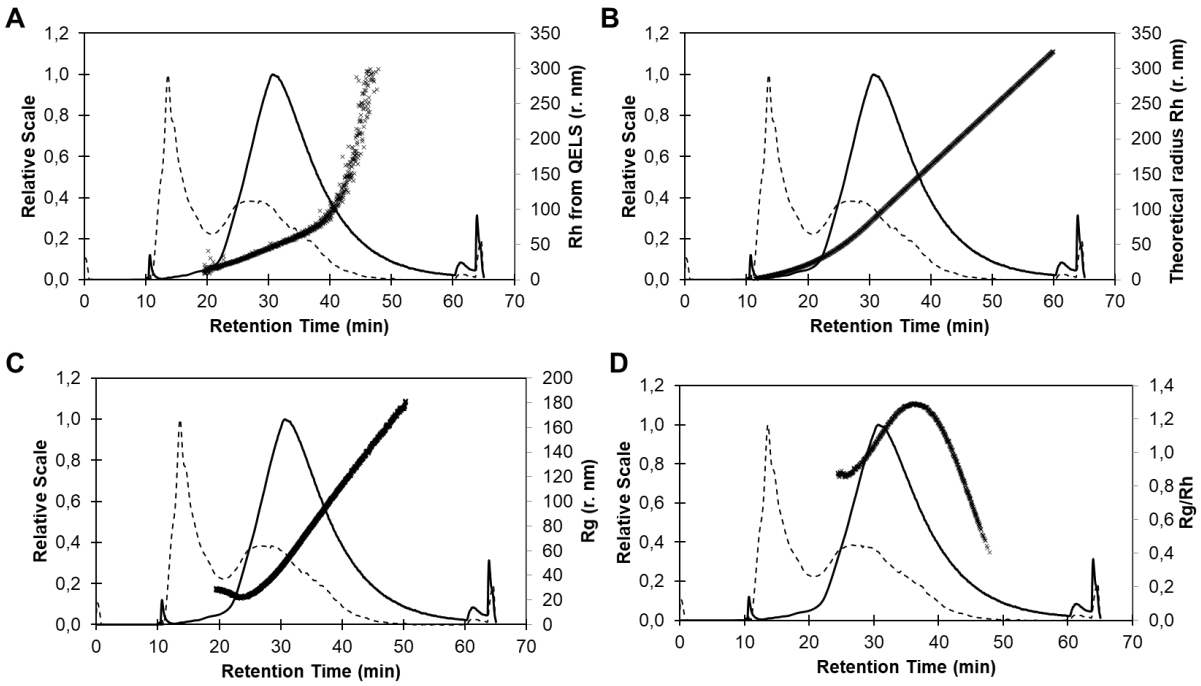

Fig. 8 – *Chromatogram of final elution profile: Left axis, the relative scale shows normalized data where the full line represents Rayleigh ratio; dashed line represents UV absorbance at 280 nm, dots represent different radiuses on the right axis: (A) hydrodynamic radius based on online DLS measurement. (B) hydrodynamic radius based on the retention equation calculation. (C) the radius of gyration based on the MALS measurement. (D) the radius of gyration and hydrodynamic radius ratio based on the MALS/DLS measurement.*

The pentameric protein lysate and individual fractions were measured using batch DLS. The comparison of the batch DLS measurement of the lysate and the measurement of individual fractions showed that no additional aggregates are generated due to AF4 separation. The immunogold labelling of individual fraction together with SDS-PAGE analysis showed preservation of the native structure of the pentameric antigen. Thus, AF4 working at native conditions proved themselves to be a technique suitable for the separation and analysis of the large recombinant protein complex. The ability to induce an immune response of purified pentameric protein samples was tested, on mice animal model in manuscript [M1].

3.4 Bacterial ghosts

The example of the application of the different technique for characterization on nanoparticles is shown in the manuscript [M2]. The release of the bacterial ghosts was tested on the mucoadhesive platform and measured using the TRPS technique (**Fig. 9**). The time 0 is referring to the particles which were put into the fibres pool, 100% concentration. At the 3rd minute, 30% of the particles are released. At 15th minute 53% of the particles are released but the concentration was in the same order of magnitude as the original concentration.

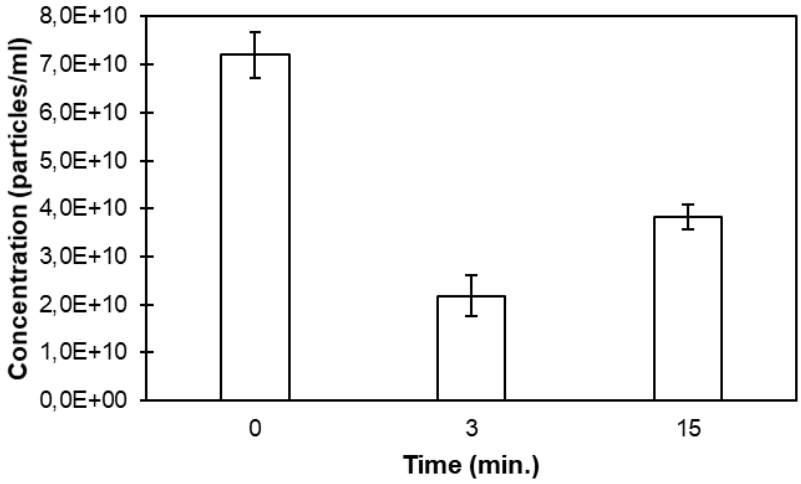

Fig. 9 – *Plot of particle release in time. Time 0 is referring to the initial particle concentration.*

An example of a comparison of the two techniques, TRPS and MADLS, is in **Table 1** below. In general, MADLS results show lower concentration and lover mean z-average then TRPS technique. The negative zeta potential around −27 mV in phosphate buffer maintains well-dispersed suspension. The concentration measurements were crucial for dose optimization during the next mucosa platform testing and proving of bacterial ghost released.

Table 1 – *Table of concentration and size measurement base on different techniques. * time 0 represents the concentration of particles in the fibres before release.*

t (min.)	TRPS results		MADLS results		
	Mean Size (d. nm)	Mean concentration (particles/ml)	Mean z-Average (d. nm)	Mean concentration (particles/ml)	Mean PDI
0*	731±8	$7.20\pm0.48\cdot10^{10}$	950±36	$6.73\pm2{,}38\cdot10^{10}$	0.261±0.051
3	712±6	$2.18\pm0.42\cdot10^{10}$	588±75	$0.10\pm0.08\cdot10^{10}$	0.523±0.047
15	714±5	$3.82\pm0.25\cdot10^{10}$	546±16	$0.20\pm0.07\cdot10^{10}$	0.378±0.022

4 MANUSCRIPTS LISTED IN DOCTORAL THESIS

[P1] **Kotoucek J**., Hubatka F., Masek J., Kulich P., Velinska K., Bezdekova J., Fojtikova M., Bartheldyova E., Tomeckova A., Straska J., Hrebik D., Macaulay S., Kratochvilova I., Raska M., Turanek J. **Preparation of nanoliposomes by microfluidic mixing in herring-bone channel and the role of membrane fluidity in liposomes formation**. *Scientific Reports* (Q1, IF 4.1). *2020, 10(1).* DOI: 10.1038/s41598-020-62500-2. ISSN 2045-2322.

[P2] Svadlakova T., Hubatka F., Turanek Knotigova P., Kulich P., Masek J., **Kotoucek J**., Macak J., Motola M., Kalbac M., Kolackova M., Vankova R., Vicherkova P., Malkova A., Simeckova P., Volkov Y., Prina-Mello A., Kratochvilova I., Fiala Z., Raska M., Krejsek J., Turanek J. **Proinflammatory Effect of Carbon-Based Nanomaterials: In**

Vitro Study on Stimulation of Inflammasome NLRP3 via Destabilisation of Lysosomes. *Nanomaterials* (Q1, IF 4.0) *2020, 10(3)*. DOI: 10.3390/nano10030418. ISSN 2079-4991.

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[P4] Bartheldyova, E.; Turanek Knotigova, P.; Zachova, K.; Masek, J.; Kulich, P.; Effenberg, R.; Zyka, D.; Hubatka, F.; **Kotoucek, J**.; Celechovska, H.; Hezova, R.; Tomeckova, A.; Maskova, E.; Fojtikova, M.; Macaulay, S.; Bystricky, P.; Paulovicova, L.; Paulovicova, E.; Droz, L.; Ledvina, M.; Raska, M.; Turánek, J. **N-Oxy lipid-based click chemistry for orthogonal coupling of mannan onto nanoliposomes prepared by microfluidic mixing: Synthesis of lipids, characterisation of mannan-coated nanoliposomes and in vitro stimulation of dendritic cells**. *Carbohydrate Polymers* (Q1, IF 6.0). *2019, 207(12), 521-532*. DOI: 10.1016/j.carbpol.2018.10.121. ISSN 01448617.

[P5] Knötigová, P.; Mašek, J.; Hubatka, F.; **Kotouček, J.**; Kulich, P.; Šimečková, P.; Bartheldyová, E.; Machala, M.; Švadláková, T.; Krejsek, J.; Vaškovicová, N.; Skoupý, R.; Krzyžánek, V.; MacAulay, S.; Katzuba, M.; Fekete, L.; Ashcheulov, P.; Raška, M.; Kratochvílová, I.; Turánek J. **Application of Advanced Microscopic Methods to Study the Interaction of Carboxylated Fluorescent Nanodiamonds with Membrane Structures in THP-1 Cells: Activation of Inflammasome NLRP3 as the Result of Lysosome Destabilization**. *Molecular Pharmaceutics* (Q1, IF 4.4) *2019, 16(8), 3441-3451*. DOI: 10.1021/acs.molpharmaceut.9b00225. ISSN 1543-8384.

[S1] **Kotoucek J**., Hezova R., Vrablikova A., Hubatka F., Kulich P., Roessner D., Macaulay S., Raska M., Psikal I., Turanek J. **Characterization and purification of the pentameric chimeric protein particles using Asymmetrical Flow Field-Flow Fractionation coupled with MALS/DLS/UV detector**. (*The Journal of Chromatography A* (Q1, IF 3.9); submitted 2020).

[M1] Hezova R., Vrablikova A., **Kotoucek J**., Hubatka F., Kulich P., Turanek-Knotigova P., Gebauer J., Macaulay S., Roessner D., Raska M., Psikal I., Turanek J. **Expression, purification and characterization of chimeric VP1-PCV2bCap protein as antigen for construction of recombinant vaccine against porcine circovirus.**

[M2] Turanek J., Masek J., Knotigova P., Kulich P., Hubatka F., **Kotoucek J.**, Lubasova D., Maskova E., Goepfert E., Raska M., Sorensen P., Leenhouts K. **Allergen-specific immunotherapy delivered by sublingual mucoadhesive nanofiber film in a pig model.**

5 COMMENTS AND AUTHOR CONTRIBUTION TO THE LISTED MANUSCRIPTS

5.1 [P1] Preparation of nanoliposomes by microfluidic mixing in herring-bone channel and the role of membrane fluidity in liposomes formation

This work aimed to clarify the mechanism of liposomes formulation, by microfluidic mixing technique, via the vesiculation of the phospholipid bilayer fragment. During the liposomal formulation by microfluidic mixing the phospholipid bilayer fragment is formed as a metastable structure. The instability cause bending resulting in vesiculation and liposome formulation. The critical parameter for bending is the fluidity of the phospholipid bilayer fragment expressed as the steady-state fluorescence anisotropy of the DPH-TMA. With the decrease of the fluidity, the lipid bilayer fragment can form larger structures and vesiculation results in larger liposomes. An opposite effect is observed, when the fluidity is larger, due to the composition. The flexible fragment may vesiculate faster and the resulting liposomes are smaller. Clarification of the mechanism of liposome formation by microfluidic mixing is important for optimizing the process of formulating liposomes of different sizes for specific applications. Microfluidic mixing also minimizing potential problems with chemical and morphological stability and enables in situ preparation of therapeutics and contrast agents (as is shown in the study on the Ga-liposomal contrast formulation).

The author participated in the design of the study and experiments, was responsible for the preparation of liposomal samples with different cholesterol content using the NanoAssemblr Benchtop (Precision NanoSystems, Canada). The author characterized the size, polydispersity and distributions of the resulting liposomes using Multi-angle DLS technique, Zetasizer Ultra (Malvern Panalytical, UK). The author also characterized the steady-state anisotropy of the incorporated membrane probe using a fluorescence spectrometer Chronos DFD Fluorescence spectrometer (ISS, USA). The author evaluated the measured data and participated in manuscript writing and editing during the review process.

5.2 [P2] Proinflammatory Effect of Carbon-Based Nanomaterials: In Vitro Study on Stimulation of Inflammasome NLRP3 via Destabilisation of Lysosomes

This work is a follow-up to previous work [P5] in which the disruption of the lysosome membrane due to the accumulation of nanodiamonds was observed. The disruption leads to the induction of inflammation via the inflammasome pathway. In this study, the same cell model (THP1) was exposed to the different carbon-based nanomaterials such as multi-walled carbon nanotubes (MWCNT) and two types of graphene platelets (GP1 and GP2). Both of the GP types formed aggregates with different morphology, GP2 aggregates were larger with sharp edges indicates potential larger cellular damage. The study shows an extensive accumulation of carbon-based nanomaterials in the cytoplasm but also demonstrates that the increased dose does not lead to cell death. The carbon-based nanomaterial particles did not exert actuate toxicity, but they are not degradable, and the accumulation in different tissues may lead to chronic inflammation. The possible proinflammatory and immunomodulatory effects of above mention particles were confirmed in *in vitro* model. The bio-resistance and proinflammatory effect of the carbon-based materials are limiting for their medicinal applications

The author was responsible for the characterization of carbon-based nanomaterials by application of the DLS method for measuring the size distribution and zeta potential, and for evaluating partial results using Zetasizer Ultra (Malvern Panalytical, UK). The author participated in the writing and editing of the manuscript during the review process.

5.3 [P3] Targeting Human Thrombus by Liposomes Modified with Anti-Fibrin Protein Binders

The publication presents the platform for surface modification of the liposomes by the selective fibrin binders for direct thrombus imaging and therapy. The ability of the resulting proteo-liposomal platform to bind to the fibrin fibres of the thrombus was tested and proven *in vitro* on the coronary artery flow model. The proteo-liposomal platform is a first step for developing of the theranostic for thrombus imaging. Gadolinium-lipid complexes appear to be a suitable contrast agent for in vivo MRI imaging. The liposomes with protein binders surface modification represent a potential platform for MRI imaging of the thrombus and delivery of the specific thrombolytic drugs.

The author was responsible for data evaluation, and characterization of various liposomal preparations. The characterisation includes measurement of the size, size distributions and zeta potential using Multi-angle DLS technique Zetasizer Ultra (Malvern Panalytical, UK). The author also participated in the manuscript writing and editing in the review process.

5.4 [P4] N-Oxy lipid-based click chemistry for orthogonal coupling of mannan onto nanoliposomes prepared by microfluidic mixing: Synthesis of lipids, characterisation of mannan-coated nanoliposomes and in vitro stimulation of dendritic cells.

The study describes a new drug delivery platform based on the mannan coated liposomes. The liposomal composition includes aminooxy lipid responsible for the orthogonal binding of the low molecular weight mannan onto the surface via oxime ligation. The orthogonal reaction enables the formulation of a specific well-orientated structure. The structure was confirmed with the DLS, NTA and TRPS measurements. The functionality of the platform was tested in vitro on human and mouse dendritic cells. The results show selective dendritic cell stimulations. There were no changes in the mannan to recombinant mannan receptor binding affinity upon the surface modification, the mannan on the surface stimulated the murine dendritic cells. Described liposomes can be potential self-adjuvant drug delivery platform, targeting on the mannan receptor.

The author was responsible for the design of methods for characterization of the size distribution and zeta potential of the plain and modified liposomes using batch DLS Zetasizer ZSP (Malvern Panalytical, UK) and TRPS technique qNano instrument (iZON Science, UK). The author participated in the liposome preparation using NanoAssemblr Benchtop (Precision NanoSystems, Canada). The author also participated in the manuscript writing and editing in the review process.

5.5 [P5] Application of Advanced Microscopic Methods to Study the Interaction of Carboxylated Fluorescent Nanodiamonds with Membrane Structures in THP-1 Cells: Activation of Inflammasome NLRP3 as the Result of Lysosome Destabilization.

The publication is focused on the study of the physical-chemical properties of the fluorescence nanodiamonds and their potential application as the drug carrier and *in vitro* and *in vivo* imaging agents. Furthermore, the work deals with the intracellular distribution of nanodiamonds, the effect on cell membranes and cell immunity activation of the inflammasome of the THP-1 cell line model. The specific, irregular shape of the NB is responsible for mechanical penetration of the cell membrane, and NDs are further accumulated in lysosomes in immune cells. The lysosome's destabilization leads to the activation of inflammasome NLRP3. The lysosome destabilization is probably induced by aggregation od ND due to the different pH values of the lysosome environment.

The author developed and performed methods for characterization of ND by measuring the size distribution, zeta potential and the effect of the pH on zeta potential using batch DLS Zetasizer Nano ZSP (Malvern Panalytical, UK) and NTA, NanoSight NS500 (Malvern Panalytical, UK) techniques. The author also participated in data evaluation, and on the manuscript writing and editing in the review process.

5.6 [S1] Characterization and purification of the pentameric chimeric protein particles using Asymmetrical Flow Field-Flow Fractionation coupled with MALS/DLS/UV detector

This manuscript deals with the influence of AF4 separation conditions on the separation of cell lysate with recombinant vaccination protein particles assembled from identical subunits into pentamer structure. The chimeric fusion proteins were derived from circovirus capsid antigen Cap and the multimerizing subunit of mouse polyomavirus capsid protein VP1 (VP1-PCV2bCap protein). Different elution profiles (constant, linear, and exponential cross-flow) were tested. A combination of the exponential decrease, followed by the constant cross-flow profile reduced separation time with good separation efficiency. The results show that the AF4 separation process doesn't promote the aggregation of the individual pentameric particles and preserves their native state. The R_g/R_h ration obtain from online MALS/DLS shows a soft sphere structure on the individual pentameric particles, which can be separated from larger, super unit pentameric clusters with a branched structure. The theoretical hydrodynamic radius obtained from the FFF theory using the full retention equation is in correlation with the measured radius showing minimal sample to membrane interaction. Individual fractions were collected and subjected to offline DLS, TEM, and SDS-PAGE analysis. The results show maintaining the native structure of the pentameric antigen during the AF4 separation.

The author designs the experiments and performed the analysis of the lysate sample using AF4-MALS/DLS/UV (Wyatt Technology Europe GmbH, GE). The author developed the FFF analytical method, confirmed the theoretical model, measured the size distribution and denaturation temperature using batch Multi-angle DLS technique using Zetasizer Ultra

(Malvern Panalytical, UK). The contribution of the author is in the writing of the manuscript and additional editing during the review process.

5.7 [M1] Expression, purification and characterization of chimeric VP1-PCV2bCap protein as antigen for construction of recombinant vaccine against porcine circovirus

This work describes the expression, purification, and immunity testing of the chimeric VP1-PCV2bCap protein sequences based on the mouse polyomavirus (MPyV). The chimeric protein based on the mouse polyomavirus was expressed in a baculovirus expression system. The circovirus is a small non-enveloped DNA virus, PCV2 is one of the four types representing the pathogenic form causing the post-weaning multi-systemic wasting syndrome. The chimeric protein forms super-unit structures, pentamers which have been characterized elsewhere [S1].

The aggregates and residual proteins were separated from the lysate using A4F. Fractions containing pentamers of VP1-PCV2bCap protein were collected. The humoral immune response of the mice immunized by purified pentameric protein was tested. The strong immunity response was detected upon first mice vaccination. The response gradually increased with the second and third immunization.

The author was responsible for the design and purification of the lysate sample using the AF4-MALS/DLS/UV (Wyatt Technology Europe GmbH, GE) system. The author also participated in the writing of the manuscript.

5.8 [M2] Allergen-specific immunotherapy delivered by sublingual mucoadhesive nanofiber film in a pig model.

This study proves the concept of sublingual anti-allergic vaccination using non-invasive mucoadhesive films with bacteria ghost as adjuvants. The vaccination was performed *in vivo* on piglet animal model of allergy against egg albumin. Results showed rapid penetration of antigens into animal model submucosa. Prior to the vaccination, release test from the reservoir layer of the mucoadhesive film were performed *in-vitro*. An almost complete release of the particles was measured in 15 minutes. With the know concentration of the particles and area of the film, the results show sufficient adjacent mucosal surface covering with bacterial ghosts.

The author performed DLS (Malvern Panalytical, UK), NTA, NanoSight NS500 (Malvern Panalytical, UK) analysis and TRPS analysis using qNano instrument (iZON Science, UK) of different bacterial ghost samples. The author evaluates the data and participates in manuscript writing and editing.

6 CONCLUSION

In my PhD thesis "Preparation and characterization of complex nanoparticles by Field-flow fractionation and advanced spectroscopic methods" I summarize modern methodological approaches and instrumentation for the preparation and characterization of nanoparticles. My work is part of wider research aiming at the development of modern recombinant and mRNA vaccines, targeted cytostatics, and theranostics.

The first section of the PhD thesis is dedicated to the liposomes. The novel method of preparation is presented. The results describe the mechanism of liposomal formulation via the vesiculation of the lipid bilayer fragment. The size of the resulting vesicle is given by the fluidity of the fragment, which can be tuned using cholesterol with respect to the saturated/ unsaturated nature of the phospholipid acyl chains. The application of liposomal nanoparticles with different surface modification are presented as well. The application, including orthogonal binding of the mannan on the surface of the liposome. The mannan coated liposomes stimulating the murine dendritic cells represent the self-adjuvants platform for targeting the mannan receptor. Another application includes nanoliposomes with surface binder domain for targeting the fibrin fibres. The second part of the thesis describes different characterization techniques. By determination of size, size distribution, polydispersity, zeta potential and concentration, this thesis contributes to the characterization of the different carbon-based nanoparticles in the study of proinflammatory effects. Another participation is described in the study of the anti-allergic vaccination using bacterial ghosts. The last part is focused on the Asymmetrical flow Field-flow fractionation technique coupled with multiple detectors. The results show the possible application in the field of vaccine development, especially for purification and characterization of the pentameric chimeric protein particles VP1-PCV2bCap. Maintaining the native state of the protein particles is crucial for the purification process. The series of additional post purification analysis show maintaining the native structure of the pentameric antigen during the AF4 purification. In another study, the immunity response of purified pentameric protein was tested *in vivo* on mice animal models.

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8 CURRICULUM VITAE

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Education	08/2018	**Light Scattering University, Dernbach** One-week training of separation techniques (SEC/FFF-MALS/QELS)
	since 2016	**Brno University of Technology, Faculty of Chemistry** PhD. Program of Physical Chemistry (expected end date 2020)
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	08/2019	**Wyatt Technology Europe GmbH** One-month internship, development of FFF-MALS methods
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	10/2015 – 11/2015	**Teva Czech Industries s.r.o.** One-month internship at the Research and Development Department of Analytical Chemistry with a focus on gas chromatography
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Teaching practice	2019/2020	**Preparation of the study materials** for seminars and practical laboratory exercise of the Immunopharmacotherapy study program (Accreditation 2020).
	2018/2019	**Laboratory of the Molecular biotechnology**
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Conference	2018	**The 11th International Conference Drug Delivery Systems, Nanotechnology for Healthcare** Progress in Recombinant Vaccines, Molecular Adjuvants, Modern Drug Delivery Systems and Cell Therapy Talk: Mechanism of liposome formation by microfluidic mixing technology **The 10th International Virology summit and 4th International conference on Influenza, Vienna** **6. Česká lipidomická konference v Praze ve Ústavu organické chemie a biochemie AV ČR (2018)** Poster: Mechanism of liposome formation by microfluidic mixing: the concept based on lipid bilayer fragments vesiculation
	2017	**The 5th Nano Today 2017** Poster: Mechanism of liposome formation by microfluidic mixing: the concept based on lipid bilayer fragments vesiculation **10th International Conference, Nanotechnology for Healthcare (201**7): Progress in Recombinant Vaccines, Molecular Adjuvants and Modern Drug Delivery Systems. Jesuit College, Telč, Czech Republic. **Student Conference Chemistry is Life, Faculty of Chemistry, Brno University of Technology (2017)** Talk: Characterization of colloid particles by excited-state proton transfer with advanced fluorescence techniques