Univerzita Karlova

1. lékařská fakulta

Autoreferát dizertační práce

UNIVERZITA KARLOVA
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Targeted biocompatible nanoparticles for therapy and cancer diagnostics

(Cílené biokompatibilní nanočástice pro terapii a diagnostiku rakoviny)

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Dizertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

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SOUHRN

Nanočástice mají mnoho předpokladů k tomu, aby byly použity v cílené medicíně. Mohou zastávat více funkcí najednou a sloužit tak jako zobrazovací značky nebo nosiče účinných látek pro terapii. Po modifikaci vhodným cílícím ligandem mohou specificky interagovat s rakovinnými buňkami. Účinnost cílení nicméně závisí na poměru mezi specifickou a nespecifickou interakcí nanočástic s buňkami. Nespecifické interakce nanočástic s buňkami nesouvisí s cíleným receptorem a musí být odstraněny, aby bylo možné snížit pozadí zobrazování, případně snížit škodlivý efekt léků na zdravé tkáně.

V této práci byly povrchové modifikace nanočástic zkoumány především na biokompatibilních uhlíkových nanočásticích nanodiamantech, které vykazují výjimečné fluorescenční vlastnosti jako je dlouhá doba života fluorescence, fluorescence bez fotodestrukce („photobleaching“), bez blikání („photoblinking“) a citlivost fluorescence k elektromagnetickému poli. Hlavními nedostatky nanodiamantů, které jsou v této práci řešeny, jsou nízká koloidní stabilita částic v pufrech a médiích, jejich nespecifické interakce s proteiny a buňkami a omezené možnosti modifikací povrchu. Tyto nedostatky byly překonány pokrytím nanodiamantů vrstvou biokompatibilních, hydrofilních a elektroneutrálních polymerů poly(etylenglykolu) a poly[*N*-(2-hydroxypropyl) metakrylamidu]. Optimalizovaná polymerní vrstva poskytovala částicím stabilizaci v koncentrovaných pufrech, eliminovala nespecifické interakce s buňkami a umožnila bioortogonální modifikace cílícími ligandy. Pro cílení k rakovinným buňkám byly nanodiamanty nejprve modifikovány cyklickým peptidem RGD. Tyto částice vykazovaly výrazný cílící efekt díky eliminaci nespecifických interakcí nanočástic s buňkami. Specifická interakce nanodiamantů s rakovinnými buňkami byla dále vylepšena po modifikaci proteinem transferinem a nízkomolekulárním inhibitorem glutamát karboxypeptidasy II.

Díky vyvinutému biokompatibilnímu povrchu bylo možné nanodiamanty využít v biomedicínských aplikacích. Nejprve byly nanodiamanty s vrstvou zlata a polymeru použity pro účinné cílení a zabití rakovinných buněk pomocí fototermální ablace. Dále byly nanodiamanty

s polymerní vrstvou a komplexy s Gd^{3+} ionty použity pro vytvoření optických relaxometrických nanosenzorů pracujících ve fyziologických podmínkách. Povrchová struktura nanosenzorů umožnila optické čtení lokalizovaných chemických dějů s extrémní citlivostí (10^{-22}–10^{-20} mol).

Klíčová slova: nanočástice, nanodiamanty, povrchové modifikace, polymerní vrstva, cílení buněk, biomedicínské aplikace

SUMMARY

Nanoparticles (NPs) have considerable potential in targeted medicine. NPs can merge various functions and serve as labels for imaging or as nanocarriers in therapy. Modification of NPs with targeting ligands can lead to highly specific interactions with targeted cancer cells. However, the efficacy of targeting depends on the ratio between specific and non-specific interactions of a NP with the cell. Non-specific interactions of NPs are unrelated to targeted receptors and need to be eliminated in order to decrease background noise during imaging and adverse effect of drugs on healthy tissues.

In this thesis, surface modifications of NPs were explored mainly on biocompatible carbon NPs called nanodiamonds (NDs), which have exceptional fluorescent properties such as long fluorescence lifetime, no photobleaching and photoblinking and sensitivity of their fluorescence to electric and magnetic field. Main issues addressed in this thesis are low colloidal stability of NDs in buffers and media, their non-specific interactions with proteins and cells and limited approaches for ND surface modifications. These issues were solved by coating NDs with a layer of biocompatible, hydrophilic, and electroneutral poly(ethylene glycol) or poly[*N*-(2-hydroxypropyl) methacrylamide] polymers. Optimized polymer coating provided NDs steric stabilization in concentrated buffers, eliminated non-specific interactions with cells and enabled further bioorthogonal functionalization of NDs. Modification of NDs was demonstrated using various targeting ligands. First, NDs were modified with targeting peptide cyclic RGD. These conjugates showed reasonable targeting effect thanks to the elimination of non-specific interactions. The specific interactions of NDs with cancer cells were further improved upon surface modification with transferrin and small-molecule inhibitor of glutamate carboxypeptidase II.

The developed biocompatible interface of NDs enabled further biomedical applications. First, NDs with gold layer and polymer coating were shown to efficiently target and kill cancer cells using photothermal ablation. Second, optical relaxometric nanosensors working under physiological conditions were created from NDs with polymer layer containing Gd^{3+}complexes. The chemically programmable structure of the polymer enabled optical readout of localized chemical processes occurring on an extremely small scale (10^{-22}–10^{-20} mol).

Key words: nanoparticles, nanodiamonds, surface modifications, polymer coating, anti-fouling, core-shell, cell targeting, biomedical applications

LIST OF ABBREVIATIONS

CCRF-CEM	human T lymphoblast
GCPII	glutamate carboxypeptidase II
GSH	glutathione
HeLa	human adenocarcinoma cell line
HPMA	*N*-(2-hydroxypropyl)methacrylamide

HUVEC	human umbilical vein endothelial cell line
ICP-MS	inductively-coupled plasma mass spectrometry
ND	nanodiamond
NP	nanoparticle
(N-V)	nitrogen vacancy
PBS	phosphate buffer saline
PEG	poly(ethyleneglycol)
PHPMA	poly[*N*-(2-hydroxypropyl)methacrylamide]
Qβ	bacteriophage virus-like particle
RGD	peptide with three amino acid sequence arginine-glycine-glutamic acid
SPR	surface plasmon resonance
U2OS	human osteosarcoma cancer cell line
MPyV	mouse polyomavirus-like particle
U-251 MG	glioblastoma cell line

1. INTRODUCTION

Optical imaging and drug delivery are key tools in contemporary biomedicine with emerging applications, such as cancer theranostics. The biggest drawback of the current theranostic techniques is their lack of specificity and resulting background noise and systemic toxicity. Compared with conventional small-molecule drugs and probes, nanoparticles (NPs) have a prolonged circulation time and increased tumor exposure. Uptake of NPs into tumors is enhanced through inherent nature of passive targeting (the enhanced permeation and retention – also known as EPR effect) and possibly using active targeting. (Prokop and Davidson, 2008) Active targeting is facilitated by targeting molecules (ligands), which recognize and bind to targeted moiety and helps NPs to be internalized in targeted cells (Fig. 1A). Targeting molecules can be attached on the surface of NPs in high concentration; therefore, avidity to a targeted moiety is greatly increased. Effect of targeting results in improved treatment effects and in lower systemic toxicity. (Prokop and Davidson, 2008) NPs can be used as contrast agents for MRI, for optical and photoacoustic imaging, as small drug delivery agents or nucleic acid carriers. However, their main disadvantage is their non-specific interactions and colloidal instability in biological media.

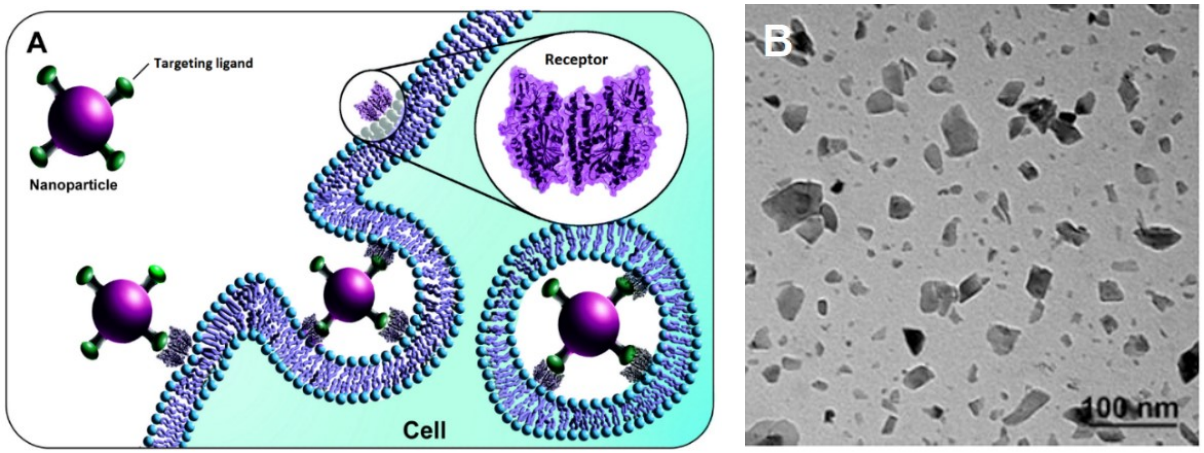

Fig. 1 (A) Scheme of targeting using NPs bearing polyvalent array of targeting ligands, which binds to receptor, anchored in cell membrane. (B) Transmission electron microscopy (TEM) image of pristine NDs.

Nanodiamonds (NDs, Fig. 1B) show low toxicity and are considered to be highly biocompatible carbon NPs. NDs host single crystal point defects in crystal lattice, so called nitrogen vacancy (N-V) centers, with extraordinary optical properties. NDs emit red fluorescence, have a long fluorescence lifetime (in few tens of nanoseconds) and are resistant to photobleaching and photoblinking. (Rehor et al., 2016) NDs are a promising alternative to fluorescent dyes utilizable in long-term *in vitro* tracking of single particle. (Chang et al., 2008; Zhang et al., 2009; Hui et al., 2017) The specific electronic structure of $(N\text{-}V)^-$ centers enables use of NDs as ultrasensitive magnetic and electric field sensors. (Balasubramanian et al., 2008; Hegyi and Yablonovitch, 2013; Rehor et al., 2016) NDs enter spontaneously into cells through endocytosis. The rate of the internalization depends on factors such as size, shape, charge and surface modifications. Small NPs enter to cells more freely than large aggregates; colloidal stability of NPs is therefore an important parameter. NDs are colloidaly stable in water, however without surface modifications not in buffers.

Majority of carbon atoms on NDs surface are oxidized and exist in form of alcohols or carboxylic acids. Biomolecules can be attached on NDs using non-covalent or covalent interactions. Covalent attachment enables to have the point of attachment fully under control, maintaining a proper biomolecule conformation and orientation. This is essential for retaining the activity of the attached biomolecules. Surface modification efficiency is generally low as the surface groups are sterically hindered. To retain biomolecule activity and enhance modification yield, spacers between surface and biomolecules are often used.

Various kinds of molecules ranging from small molecules (such as peptides) to large proteins are used to modify the ND surface for targeting applications. To successfully target, high specific interactions with cancer cells need to be ensured and inherent non-specific interactions with other cells need to be eliminated. Therefore, surface has to be first modified with polymers guaranteeing the improvement and protection of the surface including NPs stability, elimination of non-specific interaction and more efficient surface modifications. (Cigler et al., 2017; Neburkova et al., 2017)

Polymers for preparation of antifouling layers should meet certain criteria such as biocompatibility, hydrophilicity or optimally neutral charge. Usually, polyethylene(glycol) (PEG) and its derivatives or polymers based on polyglycerol (PG), polyoxazolines or methacrylate derivatives are covalently attached. (Amoozgar and Yeo, 2012) Preparation of the polymer first in the solution and then attachment by the end-functionality to the surface of NPs is called “grafting to” method and is used for PEG coatings. Density of the polymer chains on the surface is limited because of steric hindrance and limited diffusion of large polymer molecules to surface. Alternatively, polymers can grow from monomers directly on the surface of NPs in “grafting from” method. Without steric hindrance of the polymer chains, the efficiency of coating by this method is high and the prepared layer uniform. (Cigler et al., 2017; Neburkova et al., 2017).

2. AIMS OF THE THESIS

The objective of the thesis was to improve surface properties of NPs and show their potential in biomedical applications, such as in targeting NPs specifically inside tumor cells. The central hypothesis was to reveal if proper surface design leads to principal and significant reduction of non-specific interactions and therefore improvement of specific interactions with cancer cells.
For confirmation of this hypothesis, these specific aims were proposed:

- Hypothesis: Does polymer coating enhance the colloidal stability of NDs? Experimental approach: To prepare ND particles, modify their surface with hydrophilic biocompatible polymer coating and characterize their colloidal behavior.
- Hypothesis: Does colloidal stability of NPs with polymer shell depend on polymerization method and type of the polymer?
  Experimental approach: To prepare polymer layer on NDs from various polymers by different approaches, measure colloidal stability and non-specific interactions of NDs with proteins and cells and optimize the density of polymer coating.
- Hypothesis: Does biorthogonal reactions increase surface modification yield, provide controllable way of attachment, and enhance the activity of molecules on the surface?
  Experimental approach: To examine surface biorthogonal modifications of various ligands from small molecules to proteins and find the most efficient reaction and conditions.
- Hypothesis: Is it possible to target efficiently cancer cells using NDs with attached targeting ligands?
  Experimental approach: To attach various targeting ligands on the surface of NDs and evaluate and compare targeting efficiency of individual systems.
- Hypothesis: Is targeting efficiency of the particles influenced by properties such as size, shape, composition and surface modification?
  Experimental approach: To prepare and modify diverse NPs (bioorganic monodisperse virus-like particles, polymeric nanoparticles, polymer-coated NDs) and compare their efficiency of targeting cancer cells with NDs.
- Hypothesis: Are NDs with optimized surface properties useful for bioapplications? Experimental approach: To explore applications of NDs such as photothermal ablation as therapeutic approach and preparation of ND-based nanosensors.

3. METHODS

This chapter summarizes methods used in this PhD thesis. Detailed information is described in related publications.

Synthetic methods: Modification of NP surface by silica, preparation of gold shell, polymerization of synthetic monomers on the surface of NPs, modification of NPs with various structures (e.g. fluorescent dyes, peptides, proteins), modification of glycosylic chains of protein transferrin.

Molecular biology methods: Transformation of *E.coli* cells with plasmid, recombinant expression of virus-like particles in *E.coli*, isolation of protein from the cell and purification.

Characterizations: Measuring of dynamic light scattering (DLS) and zeta potential of NPs, measuring of absorption and fluorescent spectra, transmission electron microscopy (TEM), thermogravimetric analysis (TGA), measurement of surface plasmon resonance (SPR), measurement of inhibition constants in enzymatic assay, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of modified transferrin, measurement of inductively-coupled plasma mass spectrometry (ICP-MS), measurement of T_1 relaxation time.

***In vitro* experiments:** Cell culturing, incubation of NPs in the cells, measuring of metabolic biocompatible assays, flow cytometry, confocal microscopy, laser ablation.

Statistical methods: For flow cytometry and viability experiments, statistical methods were utilized. Results were measured at least in triplicates or in monoplicates in three independent measurements. The results were evaluated by different statistic methods according to the suitability of the chosen measurements (student´s t-test, one-way ANOVA, two-way ANOVA).

4. SUMMARY OF THE RESULTS

During my PhD studies, I have co-authored ten publications in peer-reviewed journals and prepared three manuscripts waiting for either acceptance or submission. Seven of these publications are part of this thesis, chronological list of all of my publications (including book chapters) can be found at the end of the thesis. In this thesis, surface modifications and biological applications of NDs were thoroughly studied.

Publication 1: Fluorescent Nanodiamonds Embedded in Biocompatible Translucent Shells
Ivan Rehor, **Jitka Slegerova**, Jan Kucka, Vladimir Proks, Vladimira Petrakova, Marie-Pierre Adam, François Treussart, Stuart Turner, Sara Bals, Miroslav Ledvina, Amy M. Wen, Nicole F. Steinmetz, Petr Cigler, Small, *10*, 1029, **2014**.

ND, promising material for biological applications, is not colloidally stable in biological environment. This is the major disadvantage, which needs to be addressed. Low yields of surface direct modifications and high polydispersity of particles (in both size and shape) are other problematic features. We prepared multiple-layer structure on NDs, first, we coated NDs with cross-linked thick shell of silica. Silica shell modified with amino groups serves as a platform for further modifications and changes the shape of NDs from sharp NPs to monodisperse near-spherical NPs with ND core. Flexible hydrophilic polymer with activated carboxylic acid with *N*-hydroxysuccinimide (NHS-PEG-alkyne) was grafted to the surface. Alkyne moiety was further reacted using "click" reaction with either azide modified fluorogenic dye (coumarin) or radioactively labeled peptide (RGDS). PEG coated NDs were stable in phosphate buffered saline (PBS) and even in 1 M NaCl and in wide range of pH (2-10) and were internalized in human prostate adenocarcinoma cells (LNCaP).

Publication 2: Fluorescent Nanodiamonds with Bioorthogonally Reactive Protein-Resistant Polymeric Coatings
Ivan Rehor, Hana Mackova, Sergey K. Filippov, Jan Kucka, Vladimír Proks, **Jitka Slegerova**, Stuart Turner, Sara Bals, Miroslav Ledvina, Martin Hruby, Petr Cigler, ChemPlusChem, *79*, 21, **2014**.

Improvement of colloidal stability and reactivity of NDs is key requirement, however also non-specific interactions of NDs with proteins and cells need to be reduced, which was solved by a new polymerization method on NDs. We coated NDs with ultrathin silica layer, which had less than 1 nm. Methacrylate groups attached to the silica layer further reacted with monomer *N*-(2-hydroxypropyl)methacrylamide (HPMA) in radical polymerization. This radical polymerization proceeding directly on the surface ("grafting from" procedure) leads to the growth of dense polymer layer of poly[*N*-(2-hydroxypropyl)methacrylamide] (PHPMA) (Fig. 2)

In this work, either propargylacrylamide or 3-(azidopropyl)methacrylamide were mixed with the HPMA monomer and alkyne or azide moieties, respectively, were introduced to the polymer structure (Fig. 2A). Possibility of NDs modification using "click" reaction was confirmed with fluorogenic probe coumarin-azide, which becomes fluorescent only after reaction. Colloidal stability of NDs was verified in typical cell biology buffers. NDs were stable in a wide range of pH. Adsorption of bovine serum albumin (BSA) was reduced four-fold in comparison to non-coated NDs.

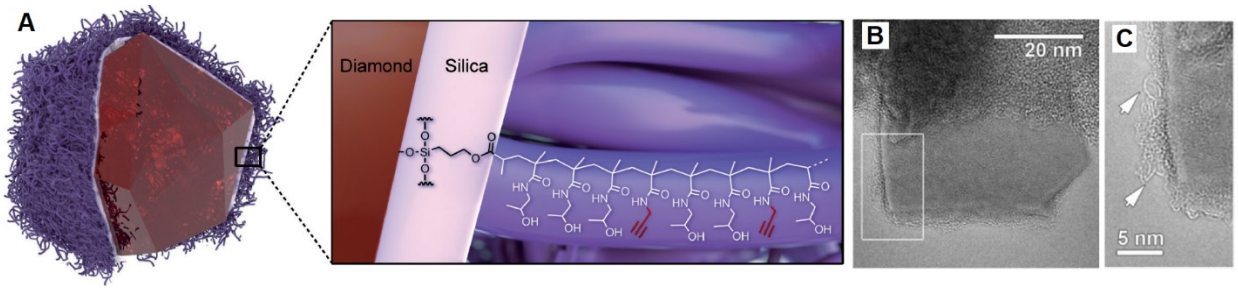

Fig. 2 (A) Scheme of polymer-coated NDs. NDs are first modified with thin silica layer, from which PHPMA with chains grow. Small portion of HPMA monomer was replaced by propargylacrylamide introducing alkyne moieties in the structure. (B) High resolution TEM image of polymer-coated NDs with few nanometers thick polymer layer. (C) area indicated in (B) in white rectangle.

Publication 3: Designing the nanobiointerface of fluorescent nanodiamonds: highly selective targeting of glioma cancer cells
Jitka Slegerova, Miroslav Hajek, Ivan Rehor, Frantisek Sedlak, Jan Stursa, Martin Hruby, Petr Cigler, Nanoscale, *7*, 415, **2015**.

Coating NDs in hydrophilic dense polymer layer and optimization of the surface are crucial conditions for successful biological applications such as cancer cells targeting. In this publication, PHPMA-polymer layer was optimized to completely eliminate non-specific interactions. NDs were further modified with cyclic peptide RGD, ligand targeting integrin receptors on cancer cells. Interaction of ND particles with cells was measured by flow cytometry and confocal microscopy.

We observed no toxicity for any ND particle. ND-cRGD conjugates interacted highly with human glioblastoma cell line U-87 MG cells, which overexpress integrin receptors on the cell membrane. The interaction of ND-cRGD was 8-fold higher than of three performed control experiments. NDs without RGD did not bind to the cells. Pre-incubation of the cells with free cyclic RGD peptide also prevent binding of ND-cRGD to the cells, confirming specific interaction of ND-cRGD with integrin receptor. Internalization of ND-cRGD in the cells was shown by confocal microscope.

Publication 4: Polyvalent display of ligand combined with antifouling bionanointerface enables extremely selective targeting of NPs to human T lymphoblast cells
Jitka Neburkova, Miroslav Hajek, Frantisek Sedlak, Stuart Turner, Jan Stursa, Petr Cigler, submitted

Elimination of non-specific interactions was already shown in previous publication. However, the difference between specific and non-specific interactions of targeted NDs with cancer and normal cells is the crucial aspect of satisfactory targeted carrier.

In this work, we used transferrin as a targeting ligand. Transferrin has one recognizing epitope interacting with transferrin receptor. Therefore, a method of attachment needs to be considered to enhance interaction with transferrin receptor. There are two glycosylic sites present in transferrin (each with two sialic acids) at the distant place far from the recognizing epitope, which were modified with azide groups using bio-orthogonal reactions. Transferrin-modified with azide was coupled to alkyne modified NDs, prepared by the same optimized procedure as in previous publication.

We observed no toxicity for any ND particle (Fig. 3A). Efficiency of targeting of NDs with transferrin (ND-Tf) was measured by flow cytometry. ND-Tf conjugate has significantly higher interaction with all three tested cell lines (human umbilical vein endothelial cell line (HUVEC), human osteosarcoma cancer cell line (U2OS) and human T lymphoblast (CCRF-CEM)) than negative controls (Fig. 3B). Pre-incubation with free transferrin blocks the interaction of ND-Tf conjugate suggesting specific uptake of ND-Tf through transferrin receptor. To mimic cancer environment, we cultivated cells in the co-culture of two cell types (normal and cancer cell line). We used simple method how to distinguish between cell types using cell-specific antibodies with different fluorescent dyes. The interaction of ND-Tf conjugate with cell lines is proportional to the amount of transferrin receptor present on the cells (cancer cells express more transferrin receptors). ND-Tf particles are localized inside the cells as observed with confocal microscope.

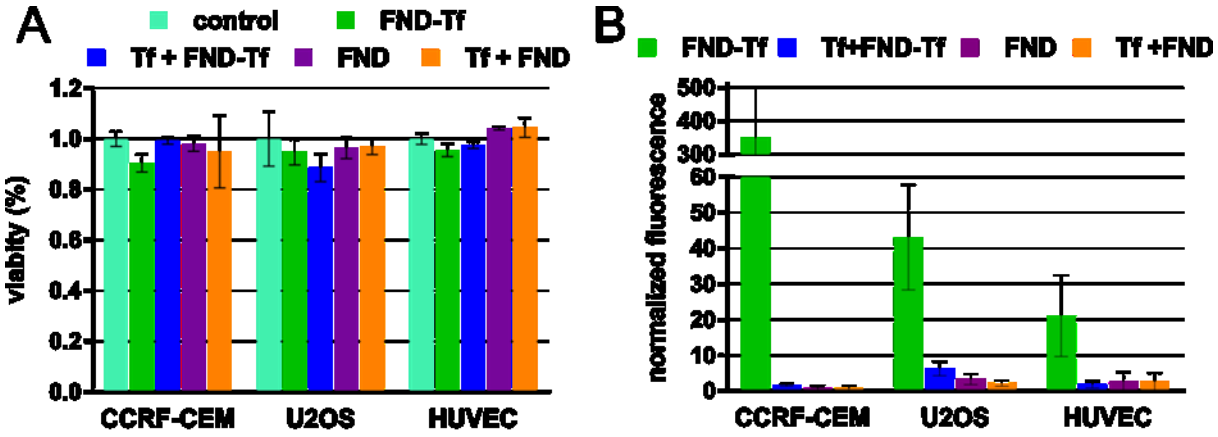

Fig. 3 (A) Viability assay (XTT) of fluorescent ND conjugates (with transferrin, FND-Tf; without transferrin, FND) with all three cell lines used in targeting experiment. No apparent toxicity was observed. (B) Fluorescence intensity measurements of three cell lines with ND conjugates measured by flow cytometry. Only FND-Tf conjugate interacts significantly with all three cell types with the interaction dependent on the amount of transferrin receptors on the surface. All negative controls for each cell lines are statistically not distinguishable among each other.

Publication 5: Inhibitor−GCPII Interaction: Selective and Robust System for Targeting Cancer Cells with Structurally Diverse NPs

Jitka Neburkova[#], Frantisek Sedlak[#], Jirina Zackova Suchanova[#], Libor Kostka, Pavel Sacha, Vladimir Subr, Tomas Etrych, Petr Simon, Jitka Barinkova, Robin Krystufek, Hana Spanielova, Jitka Forstova, Jan Konvalinka, Petr Cigler, Molecular Pharmaceutics, doi: 10.1021/acs.molpharmaceut.7b00889, **2018.** [#]***Equal contribution.***

Specific interaction is dependent on the selection of targeting ligand-receptor system. Receptors for cancer targeting (such as receptors for transferrin, folate or RGD) are usually overexpressed on cancer cells, but present also on non-cancerous cells. More tissue-specific receptors are needed, ideally only receptors present on cancer cells.

In this work, we chose to target a transmembrane protease glutamate carboxypeptidase (GCPII), primarily expressed in the prostate, central nervous system, small intestine and kidney. Expression in other tissues is much lower. GCPII is overexpressed by prostate cancer cells and in neovasculature of most solid tumors. GCPII can be target either by large antibody or small-molecule inhibitor of GCPII. We modified NPs with inhibitor, which is stable, easy-to-handle and has high affinity to GCPII (in nanomolar range).

Apart from NDs, other NPs were studied to cover a broad range of representatives: polydisperse inorganic NDs, two types of hollow monodisperse protein virus-like particles based on either bacteriophage Qβ or mouse polyomavirus (MPyV) and polymeric NPs as small, hydrophilic particles. Polymeric NPs are the only flexible ones and able to change their conformation or size. Different surface functionalization was utilized for NPs (PHPMA-, PEG- or none coating, inhibitor attachment using amidic coupling or "click" reaction).

First, we evaluated the interaction of NPs with GCPII *in vitro*. All NP-inh conjugates interacted specifically with GCPII (SPR). NPs without inhibitor did not bind to any surface. Inhibition constants (K_i) were calculated from interaction of NP-inh with GCPII in the solution. K_i for small-molecule inhibitor is in nanomolar range. K_i decreases to subnanomolar range for pol-inh NPs and to picomolar range for other NP-inh conjugates.

Second, interaction of NPs with cells was evaluated using flow cytometry and confocal microscopy. NP-inh conjugates showed high interaction with GCPII-expressing cells (Fig. 4A). For NDs, polymer-coated and uncoated Qβs and polymeric particles, no non-specific interactions were observed (either of NP-inh with cells without GCPII expression or NPs without inhibitor with both types of the cells). However, MPyV particles exhibit high non-specific interactions toward the cells (unrelated to GCPII receptor). PHPMA polymer reduce the non-specific interaction, although, the coverage of few tens of PHPMA polymers seems to be insufficient. Elimination of non-specific interactions was observed for PEG-coated MPyV (Fig. 4B).

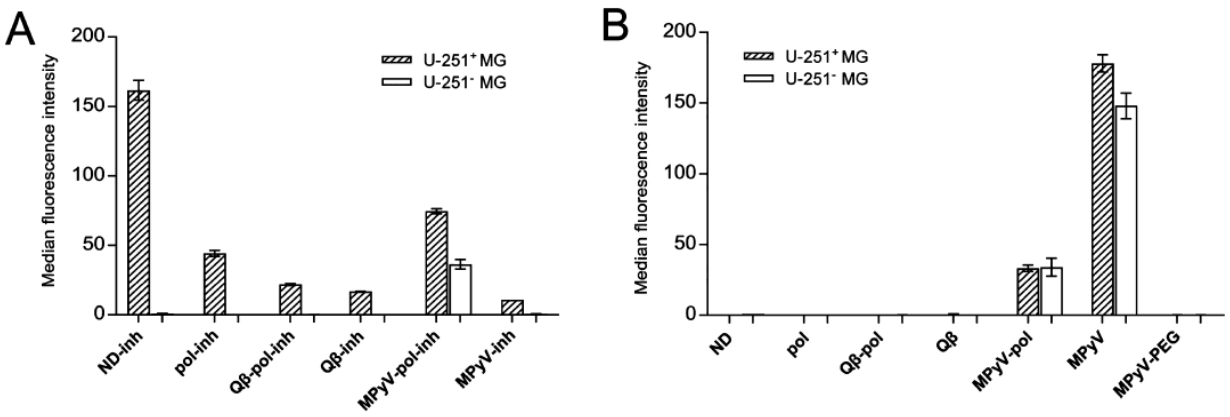

Fig. 4 Flow cytometry measurement of NPs (A) with inhibitor and (B) NPs without inhibitor on either glioblastoma cells expressing GCPII (U-251$^+$ MG) or without GCPII expression (U-251$^-$ MG). Fluorescence is normalized to autofluorescence of negative cells and adjusted to the relative fluorescence of NPs (to overcome the problem of differently fluorescent NPs). Significant difference (on significance level of $\alpha = 0.001$) between NP-inhibitor particles on U-251$^+$ MG cell and all negative controls.

Confocal microscopy revealed that NP-inh particles are internalized inside the cells into perinuclear region. MPyV particles non-specifically interacting with cells seem to be located near cell membrane.

Publication 6: Plasmonic Nanodiamonds: Targeted Core–Shell Type NPs for Cancer Cell Thermoablation

Ivan Rehor, Karin L. Lee, Kevin Chen, Miroslav Hajek, Jan Havlik, Jana Lokajova, Milan Masat, **Jitka Slegerova**, Sourabh Shukla, Hamed Heidari, Sara Bals, Nicole F. Steinmetz, Petr Cigler, Advanced Healthcare Materials, *4*, 460, **2015**.

Once we master ND surface and properties, NDs can be used for broad spectrum of biomedical applications. For therapy, special features of NPs such as photothermal ablation can be utilized. Certain NPs, for example gold nanoshells, transform light to heat. Cells that contain NPs are illuminated, heated up and killed. Once NPs are targeted only to cancer cells, light can be applied to whole tissue using laser with harmful effect only on cells internalizing NPs.

We prepared multiple-layer structure on NDs consisting of silica layer and gold nanoshell with polymer coating. ND@Au particles had absorption maximum at 675 nm, they were stable in buffered solution (PBS and media with serum) and were functionalized on alkyne groups of PEG with transferrin-azide using "click" reaction.

Transferrin interacted with transferrin receptors on targeted cells (human breast cancer cell line SKBR3 or human adenocarcinoma cell line HeLa cells) and resulted in higher internalization of ND@Au-Tf particles than non-targeted particles to cells. We demonstrated the possibility of killing cancer cell upon red laser irradiation. Cells without NPs did not change their viability upon laser irradiation, similarly to non-radiated cells with NPs. Only HeLa cells exposed to both ND@Au-Tf and laser irradiation were completely killed after one minute of irradiation (power laser 37 W/cm^2).

Publication 7: Optical imaging of localized chemical events using programmable diamond quantum nanosensors

Torsten Rendler[#], **Jitka Neburkova[#]**, Ondrej Zemek, Jan Kotek, Andrea Zappe, Zhiqin Chu, Petr Cigler, Joerg Wrachtrup, Nature Communications *8*, 14701, **2017**. [#]***Equal contribution.***

Optical properties of NDs are sensitive to electric and magnetic field. Paramagnetic ions, such as Gd^{3+}, create a fluctuating magnetic field that can be sensed by $(N\text{-}V)^-$ relaxometry. T_1 electronic relaxation time of $(N\text{-}V)^-$ center is influenced by the number of spins (Gd^{3+} complexes) within effective $(N\text{-}V)^-$ sensing radius, therefore their detachment from NDs can be measured.

In this publication, we attached Gd^{3+} complexes to the surface of PHPMA-coated NDs. Gd^{3+} complexes with non-cleavable linkage, cleavable linkage sensitive to either acidic pH (hydrazone linker) or increased redox potential (disulfide linker) were prepared.

The release of Gd^{3+} complexes was evaluated by ICP-MS. Detachment of Gd^{3+} complexes in both acidic pH and increased concentration of glutathione (GSH) occurs in a physiologically relevant time (within 1 hour) under physiological conditions, which correspond to condition change after NDs entering the cells.

Perfect stability of polymer-coated NDs in buffers enabled T_1 measurement to be done in a microfluidic channel that mimics cellular environments. Measurement of T_1 relaxation time of $(N\text{-}V)^-$ centers corresponds to ICP-MS measurements. The pH sensing system operates in quite a broad pH range (pH 2.0-7.4) with accuracy ± 0.7 pH unit (Fig. 5A). We were able to distinguish under confocal microscope NPs of various T_1 relaxation time, for example, NPs incubated in acidic pH and newcomers from neutral pH (Fig. 5B). These sensors working under physiological

conditions can enable further monitoring of intracellular processes that are important for various bioapplications.

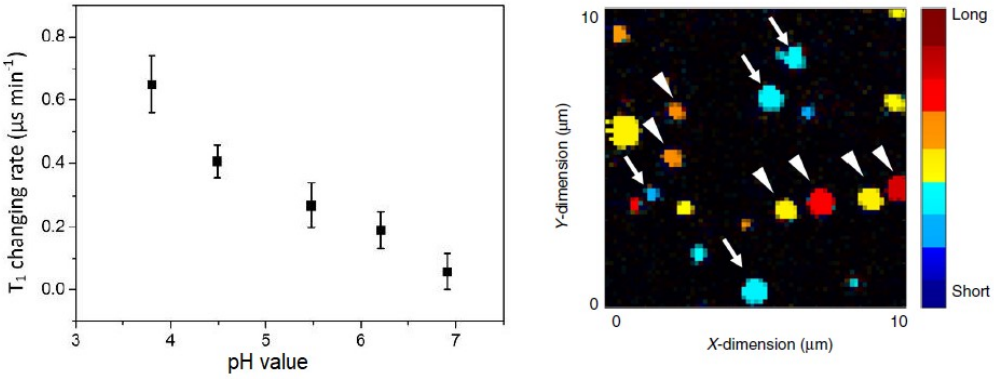

Fig. 5 (A) Dependence of the fitted T_1 changing rate of acidic-sensing NPs on pH. T_1 points were measured for 120 seconds. (B) T_1 contrast image of NPs incubated in pH 2.0 and freshly added NPs from pH 7.4 buffer. White arrows point to newly added ones. The color bar indicates the T_1 value ranging from short (blue) to long (red).

5. DISCUSSION

NPs can be used for various biological applications in both diagnostics and therapy. Especially, targeted nanomedicine has remarkable potential in cancer related applications. Superiority of NPs emerges from their proper size, ability of acquire various functions and extreme local concentration of ligands achieved around the NPs resulting in increased affinity (phenomenon called avidity). Mastering surface properties and preparation of controllable interface are key requirements for all applications. In this PhD thesis, I focused on preparation of such surfaces on NDs with low non-specific interactions and high specific interactions toward cancer cells. Many researchers previously published polymer coatings on NDs, although the quality of the coating was not sufficient. Usually, "grafting to" procedures introducing PEG on the surface were established. (Dong et al., 2015; Wang et al., 2014; Zhang et al., 2015)

We coated NDs with polymer using two approaches, PEG and "grafting to" approach (Rehor et al., 2014a) and also PHPMA copolymer "grafted from" the surface of NDs. (Rehor et al., 2014b) We were able to ensure stability of both types of polymer-coated NDs in buffers, to further modify them with different molecules and therefore to fulfill fundamental condition for further applications. However, ND-PEG conjugates were internalized spontaneously inside the cells, so their non-specific interactions with protein and cells were not eliminated. "Grafting from" coating with PHPMA, a hydrophilic biocompatible non-immunogenic polymer, on the other hand resulted in four-fold reduction of protein adsorption. (Rehor et al., 2014b) Furthermore, after optimization, (Slegerova et al., 2015) we achieved NDs with dense polymeric coating and no measurable non-specific interaction with proteins and cells, useful for further applications.

As a next step, we focused on targeting efficiency of NDs after attachment of targeting ligand. Usually, poor targeting efficiency is an outcome of an incomplete polymer layer resulting in non-specific interactions. As we are able to eliminate the non-specific interactions of cells, potential of our NDs is high. We modified PHPMA-coated NDs with three different targeting ligands and evaluated the results using flow cytometry (quantitative data) or confocal microscopy (localization of NPs). All ligands were targeting overexpressed receptors on cancer cells. First, we used cyclic RGD peptide, ligand for targeting integrin $\alpha_v\beta_3$. (Slegerova et al., 2015) Cyclization of

RGD improves binding efficiency and integrin selectivity by enhancing stability and structural rigidity of the peptide. We observed 8-times higher interaction of ND-cRGD than interactions of negative controls with glioma cancer cells. As negative controls, NDs without peptide and pre-incubation with free cyclic RGD were used. Free cyclic RGD binds to the receptor and blocks the receptor from further interactions. If the subsequent interaction of ND-cRGD is blocked, as in this case, the interaction is specific and requires the receptor. We observed no significant difference between non-treated cells and cells treated with negative controls. Therefore, we were successful in our goal of eliminating non-specific interactions.

Further, we focused on increasing the amount of specific interactions. For more complexed ligands (such as proteins), conformation and exposition on the surface is important. Proteins contain in their structure many potential moieties for attachment using either amidic coupling or disulfide bond formation. However, this attachment is not controllable. Site-specific attachment of transferrin on far end from the reaction site with receptor is needed. We modified the sialic acid in glycosylic chains in the structure (four in every transferrin molecule) with clickable moiety. Introduction of maximally four azide groups is therefore ensured. (Neburkova et al., submitted) Transferrin-azide reacted in "click" reaction with alkyne-modified PHPMA-NDs labeled with Alexa Fluor 488.

We examined interaction of NDs with three different cell-lines: two cancer cell lines (U2OS, CCRF-CEM) and one non-cancerous cell line HUVEC. We performed the experiments in co-culture to imitate cancer environment. Cancer cell line was always mixed with endothelial cell line and incubation with NDs was done in this mixture of two lines. We proposed the method how to distinguish between co-cultured cell lines by labeling them for flow cytometry analysis after experiment without having any influence on cell surface structures. Before analysis, specific antibodies towards cell line labeled with different fluorescent dyes were introduced. Flow cytometry on three different fluorescent channels was measured without overlapping of the fluorescence. For all three cell lines, ND-Tf conjugate signal was significantly higher than signal of controls. However, ND-Tf selectively chose cancer cells over endothelial cells from the mixture, the highest interaction, 175-times, was observed with CCRF-CEM lymphoblast cells. Difference between cell lines is dependent on amount of transferrin receptors on the cell surface. Cancer cells overexpress transferrin receptors as their iron(III) uptake is higher. Expression of transferrin receptors on non-cancerous cells results in specific internalization of certain amount of ND-Tf, which nevertheless decrease the difference between cancer and non-cancerous cells. Therefore, more tissue-specific targeting system (receptor-ligand) is needed.

GCPII is expressed only in few tissues such as prostate, central nervous system, small intestine and kidney. GCPII is overexpressed on prostate tissue-cancer cells. We compared the targeting efficiency of one ligand (GCPII inhibitor) within the same experimental setup dependent on various types and characteristics of NPs, surface ligand density, surface functionalization and modification method. (Neburkova et al., 2018) The interaction of NP-inh particles with GCPII was studied in solution, on the artificial surface or on the cell membrane. Inhibition efficiency of NP-inh in solution was sustained. Inhibition constants (K_i) were for NP-inh conjugates in picomolar range, three orders of magnitude lower than for free inhibitors. This decrease is probably caused by avidity effect of inhibitors on the surface of NPs. NP-inh selectively interacted only with GCPII bound to the gold surface of a chip in SPR measurement. Regardless the difference in inhibitor loading, surface chemistry and type and size of the NPs, all NPs were able to interact with GCPII on gold chip in a similar manner. We observed similar result also concerning NP-inh interaction with GCPII on cell membrane measured by flow cytometry. Only interaction of ND-inh was

notably higher (75-fold higher than controls), probably because of the combination of optimized bionanointerface with high loading of inhibitors. For NDs, polymer-coated and uncoated Qβs and polymeric particles, no non-specific interaction were observed. High non-specific interactions of MPyV (unrelated to GCPII receptor) were observed because of MPyV interactions with its primary target (certain receptors and glycosylated surfaces) on the cells. Elimination of non-specific interactions was the only requirement for the efficient targeting, achieved by PEG-coating of MPyV. Although the polymer was attached by "grafting to" approach, the yield was sufficient for protection. Qβ do not show non-specific interactions with cells. However, polymer coating would be needed for Qβs for *in vivo* applications, as Qβs are immunogenic.

NPs with gold nanoshells transform very efficiently light (from the laser) to the local heat and kill the cell, in which they are internalized. Rounded NDs coated with thick silica layer served as a good platform for gold shell layer growth. (Rehor et al., 2015) Seeded growth of gold resulted in a fairly compact layer of thickness around 10 nm. Absorption properties of gold shells depend on the size of the core and thickness of the shell. Prepared ND@Au had maximum of the absorption peak in near-infrared region at 675 nm, where the light penetration through tissues is easier because of the lower tissue absorbance, lower auto-fluorescence and lower near infrared light scattering. HeLa cells with NDs inside were completely killed after a one-minute irradiation with a pulse 750-nm pulse laser (37 W/cm^2). These irradiation conditions did not cause any adverse effect to cells without ND@Au.

PHPMA-coated NDs were used also as a proof-of-concept for further biological applications of NDs as sensors of physiological relevant parameters. (Rendler et al., 2017) NDs with PHPMA coating were modified with Gd^{3+} complexes. Presence of paramagnetic Gd^{3+} ion shortens T_1 relaxation time of $(N\text{-}V)^-$ center. Three complexes were attached, non-cleavable one, sensitive to lower pH, and responsive to higher concentration of reduction agents. Kinetics of the cleavage was studied using ICP-MS method, in which only Gd^{3+} released from the surface was measured. It was shown that the cleavage of Gd^{3+} occurs in reasonable fast conditions. Too fast or slow cleavage would prevent the use of this system as a sensor inside of the cells. All cleavable Gd^{3+} complexes were cleaved within an hour in biologically relevant intracellular conditions (in pH 4.5 or GSH concentration 5 mM). The T_1 relaxation time and its change can be measured because of the colloidal stability of NDs in microfluidic channel. The change of T_1 relaxation time was caused only by the Gd^{3+} cleavage, no shrinking or swelling of the polymer layer was observed. No change in T_1 was observed for controls (NDs with non-cleavable Gd^{3+} complex). The kinetics of T_1 relaxation time change (relaxation rate) was in agreement with release measurement from ICP-MS and theoretical model. The T_1 relaxation time measurement is fast and the T_1 relaxation rate can be fitted with high precision from two-minute measurement. For pH sensor, value of pH can be extracted from the relaxation rate. NDs with different T_1 relaxation time can be distinguished under confocal microscope thanks to the fast T_1 measurement.

6. CONCLUSIONS

In this thesis, a superior nanobiointerface on NPs was proposed, prepared and studied. NPs with optimized biocompatible surfaces were shown to have potential in biomedical applications. The particular hypotheses listed within aims of the thesis were successfully answered.

- NDs were coated with multilayer structure consisting of silica and polymeric shell. They were stable in buffer solutions.
- Different polymerization methods ("grafting to" and "grafting from") with different types of polymers (PEG or PHPMA) provided diverse ability of surface protection. Ability of

polymer shell to increase colloidal stability, to decrease non-specific interactions with proteins and cells and to enable high yield surface modifications was studied. Dense polymeric shells from PHPMA "grafted from" the surface of NDs showed the best results in eliminating non-specific interactions.

- PHPMA-NDs were modified with biorthogonal "click" reactions with various ligands including fluorescent dyes and targeting ligands (peptide RGD, protein transferrin and small-molecule inhibitor of protease). The controlled way of attachment and environmental exposition was important especially for transferrin.
- Successful targeting was shown for all PHPMA-coated ND conjugates with increasing targeting effect from peptide, small-molecule inhibitor to protein.
- Differences in targeting of various types of NPs were studied on NDs, virus-like NPs and polymeric NPs modified with small-molecule GCPII inhibitor. The highest targeting effect was observed for NDs, although other types of the NPs were also successfully targeted to cancer cells overexpressing GCPII. Requirement of proper polymerization methods for preparation of dense polymeric shells was again confirmed.
- Apart from targeting, two other biological applications were studied. First, NDs were modified with plasmonic gold nanoshell and their ability to kill the cancer cells using photothermal ablation was investigated. The cells with internalized NDs were effectively killed upon a short exposition to near-infrared laser. Second, pH and redox potential was measured by selective release of Gd^{3+}-complexes from NDs. The release resulted in change of T_1 relaxation time of NDs enabling optical readout of localized chemical processes occurring on an extremely small scale (10^{-22}–10^{-20} mol) using confocal microscopy.

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8. LIST OF MY PUBLICATIONS (including book chapters)

a. Publications, which are part of the thesis

Jitka Neburkova[#], Frantisek Sedlak[#], Jirina Zackova Suchanova[#], Libor Kostka, Pavel Sacha, Vladimir Subr, Tomas Etrych, Petr Simon, Jitka Barinkova, Robin Krystufek, Hana Spanielova, Jitka Forstova, Jan Konvalinka, Petr Cigler: Inhibitor−GCPII Interaction: Selective and Robust System for Targeting Cancer Cells with Structurally Diverse NPs, Molecular Pharmaceutics, doi: 10.1021/acs.molpharmaceut.7b00889, **2018.** [#]***Equal contribution.*** Impact factor: 4.44

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b. Publications, which are not part of the thesis

Lukas Balek, Marcela Buchtova, Michaela Kunova Bosakova, Miroslav Varecha, Silvie Foldynova-Trantirkova, Iva Gudernova, Iva Vesela, Jan Havlik, **Jitka Neburkova**, Stuart Turner, Mateusz Adam Krzyscik, Malgorzata Zakrzewska, Lars Klimaschewski, Peter Claus, Lukas Trantirek, Petr Cigler, Pavel Krejci: Nanodiamonds as “artificial proteins”: Regulation of a cell signalling system using low nanomolar solutions of inorganic nanocrystals, Biomaterials, doi: 10.1016/j.biomaterials.2018.05.030, **2018.** Impact factor: 8.402

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