Final report for NKFIH-K-123917 (Design and synthesis of upconverting nanomaterials enabling targeted, NIR light induced drug release)

Összefoglaló

A kutatás célja olyan upconverting nanorészecske alapú konjugátumok kialakítása volt, melyek lehetővé teszik kemoterápiás szerek célzott bevitelét és közeli infravörös fénnyel történő felszabadítását. A javasolt konjugátumok központi eleme egy upconverting nanorészecske, melynek felületét specifikus célbajuttatást lehetővé tevő célzó elemekkel (pl. folsav) és fotolabilis linkeren keresztül kapcsolódó kemoterápiás szerekkel (pl. doxorubicin, SN38) módosítjuk. Ilyen nanorészecske konjugátumok kialakítása számos fejlesztést igényel, mind a nanorészecskék felületének hatékony módosítása, mind a fotolabilis linkerek terén. A támogatási időszakban, melynek utolsó másfél éve a pandémiás időszakra esett, figyelemreméltó eredményeket értünk el a fotolabilis linkerek fejlesztése terén, valamint hatékony módszert dolgoztunk ki a nanorészecskék megbízható funkcionalizálására is. Bár az általunk tesztelt rendszerek végül nem bizonyultak működőképesnek, a probléma jó eséllyel áthidalható a nanorészecskék méretének növelésével. Ennek ellenére, a fotoloabilis linkerek terén elért eredményeinknek köszönhetően átgondolandó, hogy szükséges-e az upconverting nanorészecskék használata.

Summary

The main objectives of the proposed work were the establishment of an upconverting nanoparticle (UCNP) based construct suitable for targeted delivery and NIR light triggered release of drugs. The proposed systems involve a UCNP core that is functionalized with targeting elements (e.g., folate) and a drug (e.g., doxorubicin, SN38) linked to the nanoparticle surface via a photolabile linker. Such a construct required the development of the means for surface functionalization and bioorthogonally applicable photolabile linkers.

During the grant period (impeded seriously by the pandemic situation) remarkable results were achieved in the development of photolabile linkers (also called photocages), but substantial progress was made towards the final aims as well, yet further fine tuning of the UCNPs is necessary to access functioning systems. However, due to the remarkable progress we made in the development of new photocages the use of UCNPs should be reconsidered.

Development of photolabile linkers / photocages

A set of new linkers sensitive to irradiation with green light were synthesized. Such photolabile linkers (PLs) can be used in combination with green emitting (Eu-doped) UCNPs. We have made three new PLs, based on pi-extended coumarin scaffolds. These PLs were used to cage 3,5-dimethylbenzoic acid as a model compound easily detectable by spectroscopic methods (photorelease was followed by LC-MS). All derivatives had good photochemical quantum yields when using a green LED (one of them even allowed decaging using an orange LED). Furthermore, they all had good aqueous solubility, which makes these PLs superior to existing green sensitive PLs. The new linkers had very good two-photon (2P) cross sections, which allows uncaging of compounds e.g., drugs under irradiation with NIR light (~800 nm). This 2P approach allows consideration of systems without UCNPS in case their efficient surface functionalization or UCNP-mediated uncaging fails. A manuscript summarizing our results with these new linkers was published in *Organic Letters*.[1] We have developed further new, hydrophilic photocages and evaluated their photorelease potential using their drug-conjugates (i.e., SN38). To our

[1] Bojtár, M.; Kormos, A.; Kis-Petik, K.; Kellermayer, M.; Kele, P., **Green-Light Activatable, Water-Soluble Red-Shifted Coumarin Photocages**, *Org. Lett*. **2019**, *21*, 9410-9414.

delight, these constructs showed orders of magnitude less efficiency than the free SN38 (caged drug) with suitable dark stabilities. This is an important feature in terms of therapeutic indices. Upon external light stimulus with green and orange light, efficient release of free SN38 was observed with retained biological effect as indicated by cytotoxicity assays. These results are to be summarized in a manuscript that is *under preparation*. Just very recently, we have also developed new photolabile linkers/photocages that are sensitive to NIR light (up to 700 nm) – *results to be published*.

A remarkable improvement in the field of photolabile linkers / photocages was achieved by the development of conditionally activatable photocages. Based on our extensive knowledge on tetrazine quenched fluorogenic probes, we assumed that that both the fluorescence and light-induced bond dissociation originate from the same excited state, thus, we hypothesized that similarly to fluorescence, the photoresponsivity of photocages can also be modulated by the bioorthogonal and quencher tetrazine moiety. According to our hypothesis we foresaw that light mediated uncaging would be possible only when the tetrazine was previously transformed in a bioorthogonal click reaction. To this end we have made a vinyltetrazine modified coumarin photocage. The proof-of-concept study of this new photochemical concept (conditional photocage or click and uncage) was demonstrated by the release of several small organics. The in vivo applicability of the concept was also demonstrated by the conditional release of fluorogenic dye in live cells. Experimental evidence and theoretical calculations suggested that the presence of the bioorthogonal tetrazine motif efficiently quenches the excited state of the coumarin necessary for photolysis, resulting in disabled photoresponsivity (both in terms of photocaging and fluorescence). Transformation of the tetrazine moiety in a bioorthogonal click reaction fully restores its sensitivity for light. Since bioorthogonal reactions enable highly specific targeting of cells or cellular structures, such conditionally activatable photocages provide an extra level of spatial and temporal control e.g., while photoactivating caged prodrugs. Furthermore, the presented conditionally activatable construct is excitable by visible light and very importantly, inherently fluorogenic, which can be harvested in theranostic applications as well. Thes results were summarized in the *Journal of the American Chemical Society*.[2] These results were also highlighted in the national media (M1, Index).

While exploring new ways for therapeutic applications i.e., in photodynamic therapy using chromogenic compounds as reactive oxygen species (ROS) sensitizers, we ran into an interesting phenomenon. Originally we were prompted by finding alternatives for drug release, and tested rhodaphenothiazines as sensitizers for light triggered $^1O_2$ production. We assumed that the upconverted emission of UCNPs upon NIR light illumination would excite the sensitizer either via radiative or non-radiative processes and induce sensitized $^1O_2$ production. We have designed and synthesized bioorthogonally applicable, tetrazine functionalized rhodaphenothiazines and elaborated their light-assisted sensitizer characteristics. Experimental results suggest that the probes efficiently sensitize $^1O_2$ generation upon illumination with green or orange light. Interestingly, the produced singlet oxygen is consumed by the sensitizer, which undergoes a self-oxidation process leading to intensely fluorescent sulfoxide products. Further studies revealed a marked difference between the photooxidizability of the free tetrazine and the bioconjugated pyridazine forms, which implies a two-orders of magnitude fluorescence increase due to this new fluorogenic mechanism. Although these probes are not suitable for PDT applications, this unique fluorogenic behavior allows selective photoactivation of specifically conjugated probes. We have demonstrated this in the labeling schemes of actin filaments with low background fluorescence even under no-wash conditions. A remarkable feature of the developed “ClickOx” probes is that the same commercial green excitation laser (552 nm)

[2] Bojtár, M.; Németh, K.; Domahidy, F.; Knorr, G.; Verkman, A.; Kállay, M.; Kele, P., **Conditionally activatable visible-light photocages**, *J. Am. Chem. Soc*. **2020**, *142*, 15164–15171.

is suitable for carrying out photooxidation of the probes and subsequent excitation of the product. An added value of the present probe is that it is also suitable for STED super-resolution microscopy using a 660 nm depletion laser. These results were published in *Chemical Communications*.[3]

UCNP surface modification

Following several attempts towards the synthesis of 'clickable' nanoparticles (e.g., via direct surface modification of ligand-free NPs or using phospholipid bilayers) the capping of the oleic-acid-covered NPs was realized through a bilayer method. In order to obtain stable and water-soluble particles enabling further conjugation, the oleic acid-capped particles were further modified with a copolymer previously conjugated to a commercially available bicyclononyne (BCN, Figure 1). The bilayer-modified particles (~30 nm) were soluble in water and cyclooctyne incorporation was confirmed by Raman spectroscopy using the characteristic peak of the triple bond. Modification of the as-prepared UCNPs with model cargo-loaded photolabile linkers with subsequent NIR-light-assisted uncaging of a model cargo was tested using our established conditionally activatable tetrazine-containing photocage bearing a fluorogenic (i.e., fluorescent only when uncaged) cargo. This model construct has the salient

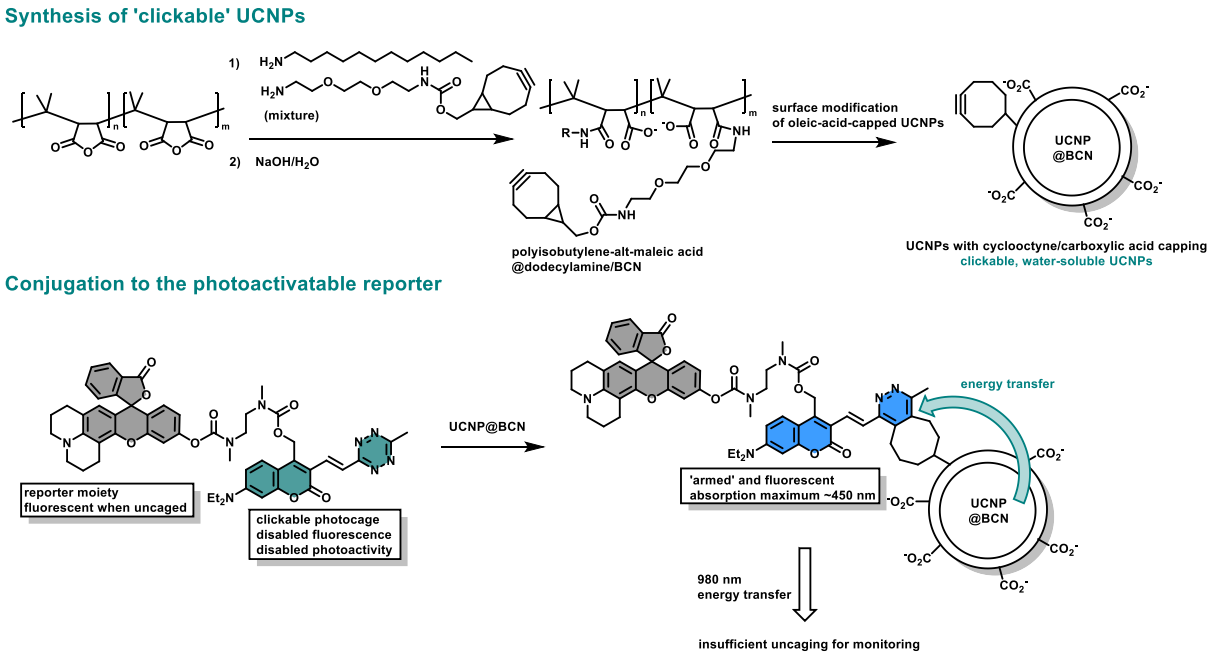

Figure 1. Successful strategy for the cyclooctynylation of UCNPs and subsequent modification with photolabile linker bound fluorogenic cargo.

feature that it is double-fluorogenic i.e., it becomes fluorescent (in the cyan/green range) upon conjugation first (the coumarin-type of proto-photocage becomes fluorescent and photoresponsive upon reacation with the cyclooctyne) and the released cargo becomes fluorescent in the orange range upon external light stimulus (488 nm). The click reaction was fast and reproducible indicating significant loading (around 500-1000 molecule per NP) of the photocage-cargo construct. The covalent conjugation was also confirmed by the NIR-induced emission of the coumarin photocage. The uncaging reaction was also tested in pure water and in buffered media upon NIR (980 nm) irradiation by monitoring the fluorescence of the fluorogenic cargo in the orange range. However, no effect was observed even after prolonged NIR irradiation. When, however, a 488 nm blue light was applied

[3] Kormos, A.; Kern, D.; Egyed, A.; Söveges, B.; Németh, K.; Kele, P., **Microscope laser assisted photooxidative activation of bioorthogonal ClickOx probes**, *Chem. Commun.* **2020**, *56*, 5425-5428.

directly on the UCNP constructs, successful release of the fluorogenic cargo was observed indicating efficient photolysis of the linker-cargo bond.

Conclusions

We believe that the low amount of emitted photons from the 30 nm nanoparticles and the minimal absorbance of the chromophore (photocage) monolayer is insufficient to induce the uncaging reaction. We hypothesize that larger UCNPs could give the expected results, however, our currently developed photocages able to release their cargo upon red/NIR light (up to 700 nm) irradiation (1P absorption) or our above mentioned photocages suitable for 2P absorption could give superior results without the need for UCNP cores.

Oral presentations:

International events:
Péter Kele: Bioorthogonal approaches in fluorescent labeling of biomolecules – *Invited speech* – Chemistry Towards Biology-9, Budapest, Hungary (2019)

Péter Kele: Fighting against auto- and background fluorescence – *Invited speech* – Wold Chemistry Congress and Exhibition, Brusselles, Belgium (2019)

Péter Kele: Multiply Quenched Fluorogenic Probes – *Invited speech* – 3rd International Caparica Conference on Chromogenic and Emissive Materials (IC3EM), 3rd – 6th September 2018| Caparica |

Péter Kele: Fighting against auto- and background fluorescence – *Invited speech* – Radboud University, Nijmegen, The Netherlands (2018)

Péter Kele: Multiply Quenched Fluorogenic Probes – *Invited speech* – Symposioum on Molecular Architectures for Fluorescent Imaging of Cells, Karlsruhe, Germany (October 4-6, 2017)

Hazai rendezvények:
Bojtár Márton, Kormos Attila, Kis-Petik Katalin, Kellermayer Miklós és Kele Péter**:** Látható fénnyel aktiválható fotolabilis vegyületek Szerves Kémiai Előadói napok, Telki (2020)

Péter Kele: Challenges and solutions in the fluorescent labeling of biomolecules Milestone Institute, Budapest, Hungary (2019)

Péter Kele: Bioortogonálisan alkalmazható jelzővegyületek STED alkalmazása, Szegedi Szuperrezolúciós Szimpózium (2019)

Bojtár Márton, Kele Péter: Upconverting nanorészecskékkel aktiválható fotolabilis linkerek *Heterociklusos* és Elemorganikus Kémiai *Munkabizottsági ülés, Balatonszemes (2018)*

Poster presentations:

Márton Bojtár, Thomas Hirsch, Péter Kele: Seeing and Believing: NIR Light Controlled Release and Monitoring with Upconversion Nanoparticles Matrafured 2019: International Meeting on Chemical Sensors, 2019. június 16-21., Visegrád

Márton Bojtár, József Kozma, István Bitter, Péter Kele: Supramolecular analytical applications of pillararenes 3rd International Caparica Conference on Chromogenic and Emissive Materials (IC3EM), 3rd – 6th September 2018| Caparica |Portugal Book of Abstracts, page 206.