Palladium-loaded PLGA-chitosan NPs as efficient catalysts for bioorthogonal chemistry

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Abstract: Bioorthogonal chemistry offers a wide range of reactions, which can be performed in the intracellular platform. Among the wide range of uses, bioorthogonal reactions can be employed for the controlled pro-drug activation *in situ*. Palladium compounds can be exploited as activation agents in the bioorthogonal reactions. However, their properties such as toxicity, poor cellular uptake and inactivation by biogenic thiols hinder their catalytic activity on the cellular level. In our study, we have prepared polymeric palladium-loaded poly(D,L-lactide-*co*-glycolide) (PLGA)-chitosan nanoparticles (NPs). The coating of these two polymers with chitosan has ensured cellular uptake and the gradual release of palladium compounds inside the cell. Their catalytic activity was demonstrated by the conversion of a fluorescent probe made of chemically modified 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt propargyl ether (HPTS-PE).

Key Words: PLGA-chitosan NPs, bioorthogonal chemistry, palladium-mediated catalysis, fluorescent probe activation, nanomedicine

INTRODUCTION

Bioorthogonal chemistry employs specific catalysts to perform bond-forming or cleavage reaction under physiological conditions, thus allowing to interfere with the reactions in biological systems (Li and Chen 2016). In our study, we employed palladium catalyst bis[tri(2-furyl)phosphine] palladium(II) dichloride ($Pd(TFP)_2Cl_2$). Its catalytic activity was confirmed by depropargylation reaction of propargyl-ether modified pyranine fluorescent probe.

Among other, high specificity towards the modified compound, a wide range of catalyzed reaction and the broad spectrum of coordination ligands predestines palladium compounds to be a useful tool in bioorthogonal chemistry (Chankeshwara et al. 2014). The reaction can be mediated by the Pd^0 species (Unciti-Broceta et al. 2012), but also ligand-coordinated Pd^{2+} species (Li et al. 2011, Ma et al. 2014, Martinez-Calvo et al. 2018). The usage of palladium compounds as a promising catalyst for bioorthogonal therapy has been shown previously (Bray et al. 2018, Rubio-Ruiz et al. 2016, Weiss et al. 2014).

However, the intracellular environment provides many obstacles, which are mostly caused by thiol poisoning of the catalyst. Thiols groups, which are irreversibly coordinated to palladium central atoms and therefore inhibit its catalytic activity frequently occur in the biological environment as a part of peptide and protein compounds (Chankeshwara et al. 2014), free amino acids or redox maintaining system such as glutathione. In order to limit access of thiol compounds to the catalyst we have used encapsulation into NPs consisting of PLGA core coated with chitosan polymer. This formulation provides several benefits, such as reduced cytotoxicity, improved cellular uptake and controlled release of the encapsulated agent (Wang et al. 2013).

MATERIAL AND METHODS

PLGA-chitosan NPs preparation

70 µL of organic phase containing PLGA (40 µL of 50 mg/mL dissolved in acetonitrile) and TMP-BODIPY (1 µL of 32 mM dissolved in 30 µL of dimethylformamide) was added into 1000 µL of aqueous phase consisted of low molecular weight chitosan (0.1%), PVA (1%) dissolved in 0.5% acetic acid. The mixture was vortexed at maximum speed to form NPs and incubated for 10 minutes. From this mixture, 400 µL was further processed. The residual cargo and polymers were then separated from NPs using centrifugation (9425 x g, 15 minutes). The obtained pellet was resuspended in cryoprotectant sucrose solution (250 mM) to the final volume of 100 µL. The nanoparticle suspension was then cleared of aggregates using second centrifugation (94 x g, 5 minutes).

In the case of palladium-loaded PLGA-chitosan NPs, the same method for particle preparation was used. The palladium catalyst $Pd(TFP)_2Cl_2$ was added into organic phase (30 µL of 25 mM dissolved in DMF) instead of the fluorescent dye TMP-BODIPY. As a negative control, the PLGA-chitosan NPs without palladium catalyst were synthesized. The harvested NPs pellet was resuspended in 200 µL of sucrose solution.

The raw particle size, morphology and aggregates formed from residual polymers were observed under the microscope (Olympus IX53) on phase-contrast after individual steps in NPs preparation. After vortexing, 10 µL of NPs was resuspended in 100 µL water in a 96-well plate, after second centrifugation, 5 µL of NPs was resuspended in 100 µL water in a 96-well plate. The images of Pd-PLGA-chitosan NPs were obtained using SEM MAIA 3 equipped with a field emission gun from TESCAN Company (TESCAN Ltd. Brno, Czech Republic, EU).

Cellular uptake of TMP-BODIPY-PLGA-chitosan NPs

For all experiments, non-malignant mammalian cell line HEK 293T was used. Cells were incubated in complete medium (DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin) in a humidified incubator with 5% CO_2. Particle aggregates and cells images were taken using fluorescent microscope Olympus IX53. Cells are imaged using phase-contrast microscopy and fluorescent microscopy with green filter (excitation wavelength = 460–495 nm, emission wavelength = 510 nm, fluorescent lamp intensity = 12, exposition time = 800 ms).

Cells were seeded in a 24-well plate at 50% confluence and allowed to attach for 4 hours. Then, the medium was replaced with 500 µL of medium containing 5 µL of TMP-BODIPY-PLGA-chitosan NPs. Before taking the pictures after 17 h incubation, the media were replaced with 500 µL of fresh complete media.

Cell-free fluorescent probe precursor activation using Pd-PLGA-chitosan NPs

A mixture of fluorescent sensor 8-hydroxypyrene-1,3,6-trisulfonic acid propargyl ether (HPTS-PE) (50 µM) in 200 µL of complete cell media was added into 3 µL of palladium-loaded PLGA-chitosan NPs or $Pd(TFP)_2Cl_2$ catalyst (50 µM). The suspension was placed in a 96-well plate and the fluorescent probe depropargylation was measured at the beginning of the reaction, and after 24, 72 hours and 6 days as fluorescence on TECAN reader (excitation wavelength = 450 nm, emission wavelength = 515 nm).

Pd-PLGA-chitosan NPs intracellular catalytic activity evaluation

Cells were seeded in a 6-well plate at 50% confluence and allowed to attach for 4 hours. Then, the old medium was replaced with 2 mL of complete cultivation medium containing 50 µL of Pd-PLGA-chitosan NPs. As a negative control, 50 µL PLGA-chitosan NPs without catalyst was used. After overnight incubation, cells were split into a 24-well plate to 50% confluence and allowed to attach for 6 hours. Afterwards, cells were treated with 500 µL of media containing HPTS-PE (50 µM) and Hoechst 33258 (4 µg/mL). After overnight incubation, images of fluorescence were taken. Cells were imaged using a phase contrast microscopy and fluorescent microscopy with blue light emission filter (Hoechst nuclear staining) (excitation wavelength = 360–370 nm, emission wavelength =

420–460 nm, fluorescent lamp intensity = 25, exposition time = 20 ms) and with green filter (excitation wavelength = 460–495 nm, emission wavelength = 510 nm, fluorescent lamp intensity = 25, exposition time = 500 ms).

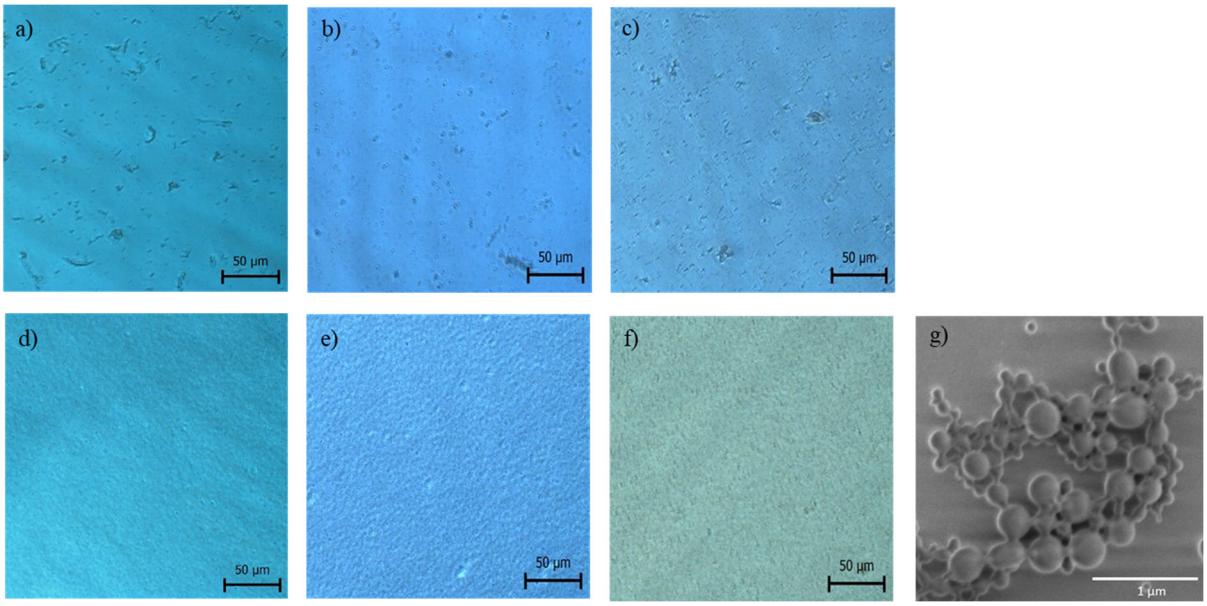
RESULTS AND DISCUSSION

The aim of our study was to prepare the palladium-loaded PLGA-chitosan NPs (Pd-PLGA-chitosan NPs), which would be able to catalyze a deprotection reaction in cells under physiological conditions.

In the first part of our research, we have focused on the tuning of the PLGA-chitosan NPs formulation. For the optimization process, the fluorescent dye TMP-BODIPY was encapsulated to facilitate the visualization of NPs and cellular uptake. The process of optimization of the PLGA-chitosan NPs consisted of the testing of the correct ratio of the particles-forming polymers PLGA, chitosan, and polyvinylalcohol (PVA) stabilizer. Hydrophobic PLGA polymer provides a gradual release of the encapsulated compound inside the cells and interacts with the hydrophobic fluorescent dye or hydrophobic organometallic palladium catalyst. Poly-cationic polymer chitosan serves as a particle coating and its positive charge also improves the cellular uptake. The PVA stabilizer prevents particles from aggregation.

During the synthesis of PLGA-chitosan NPs, different amounts of each component, as well as different emulsification technologies (sonication and vortexing) were tested. The main problem during the NPs preparation turned out to be the formation of aggregates induced by the presence of chitosan polymer in reaction mixture. To control the production of aggregates in NPs suspension, we made microscopic observation (phase contrast) of macro-aggregate formation. The key steps leading to aggregates-free suspension were the acidic pH of aqueous phase that ensured a more soluble form of chitosan polymer, the emulsification method using vortexing and the centrifugation of the NPs suspension at low speed to remove the largest aggregates. The comparison between the suspensions after vortexing (A) and after the second centrifugation, when the final product was reached (D) is shown in Figure 1. The formation of NPs in range below 500 nm has been confirmed by SEM (Figure 1g).

Figure 1 Microscopic observation of PLGA-chitosan NPs

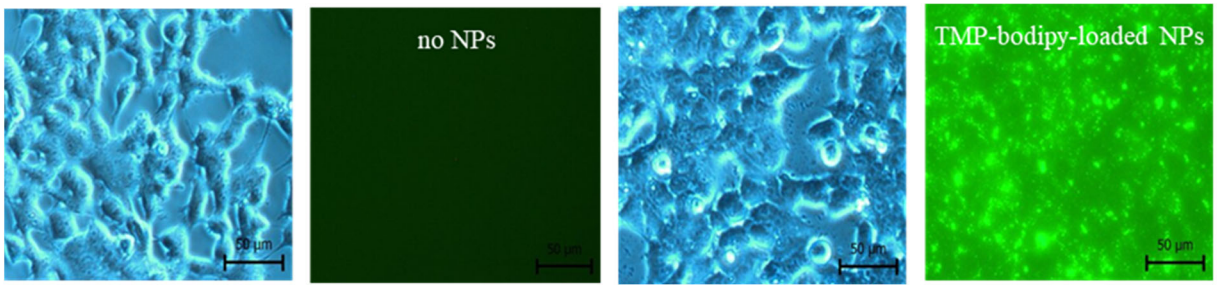


Legend: Phase contrast microscopy of PLGA-chitosan NPs (a-f). Samples a), b) and c) show the NPs after vortexing. Samples d), e) and f) show the final NPs after the second centrifugation leading to removal of chitosan aggregates. Samples a) and d) contained TMP-BODIPY-PLGA-chitosan NPs, samples b) and e) contained PLGA-chitosan NPs without any encapsulated compound and c) and f) contained Pd-PLGA-chitosan NPs. Scanning electron microscopy analysis of Pd-PLGA-chitosan NPs (g).

After the NPs preparation, their cellular uptake into non-malignant HEK cells has been tested. NPs with the encapsulated fluorescent dye were incubated with cells overnight. After incubation,

the cell media were changed to remove non-internalized NPs. Figure 2 shows that TMP-BODIPY-PLGA-chitosan NPs have been effectively internalized by HEK cells. Since TMP-BODIPY is completely insoluble in water, the green fluorescence indicates inclusion of the dye in the NPs.

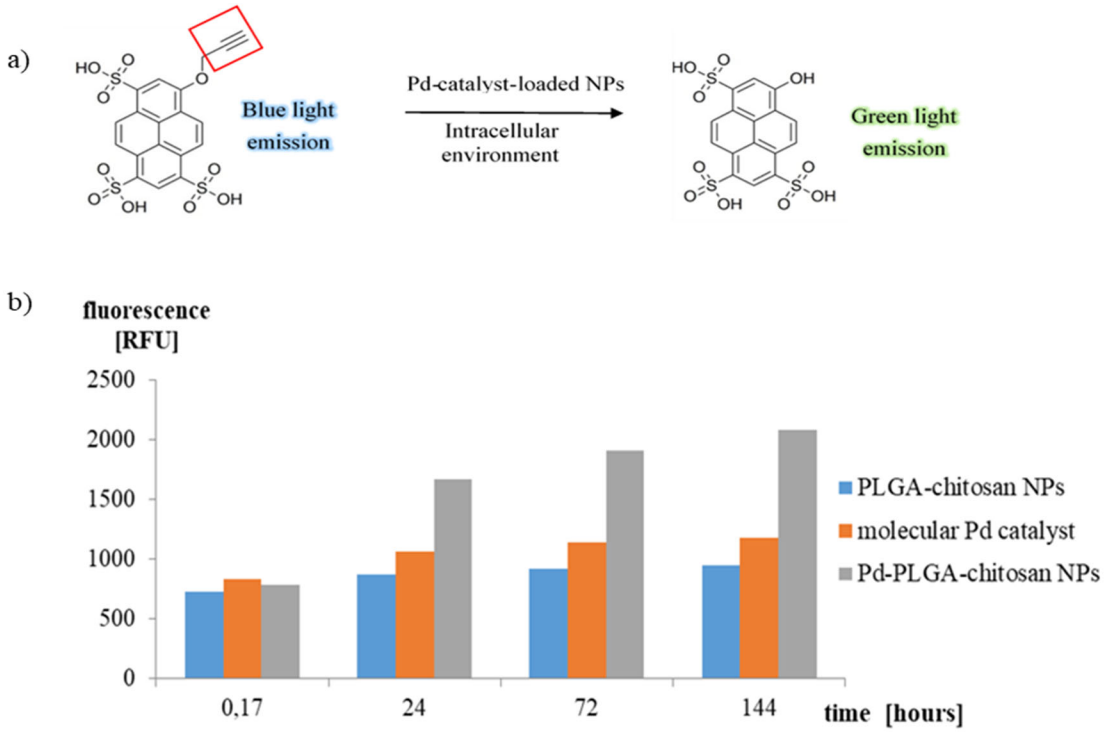
Figure 2 Cellular uptakes of TMP-BODIPY-loaded PLGA-chitosan NPs



Legend: Cellular uptake of TMP-BODIPY-loaded PLGA-Chitosan NPs after 17 h hours of incubation.

After confirmation of model NPs cellular uptake, we prepared NPs with the palladium catalyst $Pd(TFP)_2Cl_2$ using the same method. As a negative control, PLGA-chitosan NPs without a catalyst were prepared (see Figure 1b, c, e, f).

Figure 3 Fluorescence emission intensity in samples evaluating cell-free precursor fluorescent probe activation in complete cell media

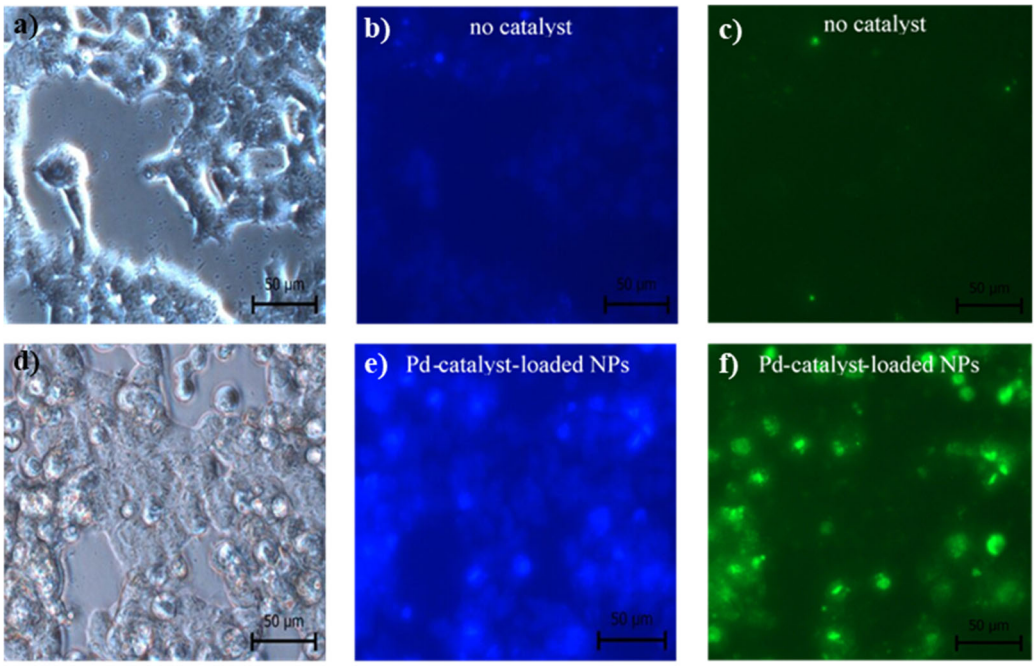


Legend: a) Pd-catalyst-mediated HPTS-PE precursor fluorescent probe activation reaction. b) Probe activation in cell-free conditions. Sample "PLGA-chitosan NPs" did not contain any palladium catalyst, sample "molecular Pd catalyst" contained non-encapsulated $Pd(TFP)_2Cl_2$ catalyst and sample "Pd-PLGA-chitosan NPs" contained $Pd(TFP)_2Cl_2$ catalyst encapsulated in PLGA-chitosan polymer.

Pd-PLGA-chitosan NPs were tested for the ability to deprotect palladium sensitive probe HPTS-PE. The cleavage of the propargylic group is exclusively mediated by palladium catalyst (see Figure 3a). At first, *in vitro* test was conducted. The encapsulated and free palladium catalysts were incubated in complete cell medium supplemented with FBS. The catalytic activity was evaluated as an increase in fluorescence of deprotected HPTS within 6 days. Figure 3b shows that the palladium catalyst gradually activates the HPTS-PE probe, upon release from the NPs. On the contrary,

$Pd(TFP)_2Cl_2$ alone has almost no catalytic activity. In the presence of proteins and biogenic thiols, palladium compounds suffer from "thiol poisoning" which precludes palladium catalytic activity. In our study, the reaction mixture was supplied with fetal bovine serum, to resemble the biological environment rich in thiol-group-containing compounds. The encapsulation protected the catalyst from its immediate inactivation, which encouraged us to evaluate the NPs catalytic activity in cellular system. There are reports using palladium with thiol ligands for catalytic purposes, however, these reactions are executed at high temperatures or in organic solvents where the thiol dissociation from active metal center is facilitated by the conditions. In physiological conditions, the thiol poisoning is a real concern.

Figure 4 Microscopic observation of HPTS-PE fluorescent probe activation inside cells using fluorescent microscope



Legend: Intracellular HPTS-PE fluorescent probe activation. Cells were incubated with negative control PLGA NPs (a, b, c) or Pd-catalyst-loaded NPs overnight (d, e, f). The next day, Hoechst33258 and the probe were added and incubated overnight.

At first, cells were incubated with Pd-PLGA-chitosan NPs. The NPs with Pd catalyst slightly inhibit cells growth. Cells treated with NPs were seeded at the same confluence. Cells were treated with the probe overnight. The activation was detected as green fluorescence emission. The results in Figure 4 show that in cell culture treated with Pd-PLGA-chitosan NPs, the fluorescent probe was activated. To the contrary, the negative control cell culture containing PLGA-chitosan NPs without Pd catalyst did not exhibit any catalytic activity. The blue signal is derived from Hoechst 33258 dye, which was added to all samples as nuclei marker.

In summary, we have developed an efficient method for the PLGA-chitosan NPs preparation, which is more user friendly compared to commonly used solvent-evaporation emulsification methods employing long solvent evaporation (de Castro et al. 2020). Some bioorthogonal chemistry approaches rely on the pre-activation of a protected compound in extracellular space (Martinez-Calvo et al. 2018, Weiss et al. 2014). This approach might not be useful in many applications. The benefit of the encapsulation into PLGA-chitosan polymeric NPs is the ability to catalyze the reactions inside the cells. Owing to the incorporation of the Pd catalyst to the polymer network the catalyst retains its activity during prolonged incubation in the cellular environment. Therefore, these particles are promising nanocarrier of bioactive catalysts for potential bioorthogonal therapies.

CONCLUSIONS

We have prepared non-toxic Pd-PLGA-chitosan NPs. The NPs are effectively internalized into cells and their catalytic activity is preserved during two-day incubation in the cellular environment as documented by activation of fluorescent probe. These are key properties, which are needed from the catalyst employed in bioorthogonal pro-drug activation.

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