

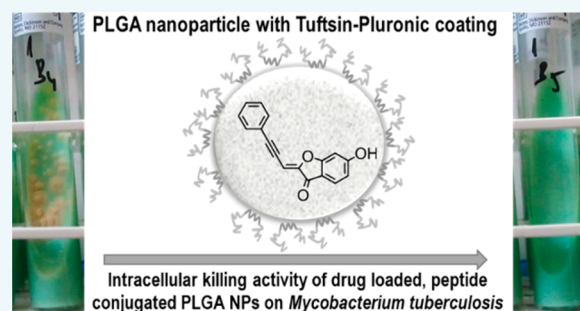
Surface Layer Modification of Poly(D,L-lactic-co-glycolic acid) Nanoparticles with Targeting Peptide: A Convenient Synthetic Route for Pluronic F127–Tuftsin Conjugate

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S Supporting Information

ABSTRACT: Nanoparticles consisting of biodegradable poly(D,L-lactic-co-glycolic acid) (PLGA) are promising carriers for drug molecules to improve the treatment of tuberculosis. Surface modifiers, such as Pluronic F127, are essential for biocompatibility and for the protection against particle aggregation. This study demonstrates a successful approach to conjugate Pluronic F127 coated PLGA nanoparticles with Tuftsin, which has been reported as a macrophage-targeting peptide. Transformation of Pluronic F127 hydroxyl groups—which have limited reactivity—into aldehyde groups provide a convenient way to bind aminoxy-peptide derivatives in a one-step reaction. We have also investigated that this change has no effect on the physicochemical properties of the nanoparticles. Our data showed that coating nanoparticles with Pluronic-Tuftsin conjugate markedly increased the internalization rate and the intracellular activity of the encapsulated drug candidate against *Mycobacterium tuberculosis*. By employing this approach, a large variety of peptide targeted PLGA nanoparticles can be designed for drug delivery.



Facultative and obligate intracellular pathogens are major cause of global health threats such as tuberculosis, HIV/AIDS, and malaria. A key target of many intracellular pathogens are macrophages; therefore, targeted delivery to those immune cells and diseased sites has crucial pharmaceutical benefits. Host recognition of surface modified nano- and microparticles and sufficient phagocytosis of the drug- or vaccine-loaded particulate systems provide attractive therapeutic approaches for prevention and elimination of persistent infections.^{1,2}

Macrophages express a broad range of receptors such as Fc and scavenger receptors, that mediate their interactions as well as cellular uptake. Conjugation of such receptor-binding ligands onto the surface of polymeric nanoparticles can promote macrophage uptake and improve specific delivery through receptor mediated endocytosis. Tuftsin, a natural immunostimulatory tetrapeptide (Thr-Lys-Pro-Arg), has been found to increase macrophage phagocytosis, splenocyte proliferation, and bactericidal activity and has been reported also as a macrophage-targeting peptide.^{3–6} During the past decade, a new group of sequential oligopeptide carriers has been developed in our laboratory: oligotuftsins consisting of a (Thr-Lys-Pro-Lys-Gly) unit.⁷ Based on further structure–activity relationship studies, another pentapeptide derivative of Tuftsin (Thr-Lys-Pro-Pro-Arg) has been described that represents a higher affinity antagonist and binds to the Tuftsin receptors more avidly than Tuftsin.⁸ It was also

reported that the chemical modification of the C-terminus to a carboxamide results in loss of biological function.

With these observations put together, a new sequence was utilized in this study: (Thr-Lys-Pro-Lys-Gly)₂-Thr-Lys-Pro-Pro-Arg (T10Tp). The conjugation site of the targeting molecule was designed on the N-terminus of the peptide.

Over the years, a variety of synthetic polymer based nanoparticles (NPs) have been tested as delivery platforms, of which poly(D,L-lactic-co-glycolic acid) (PLGA) has been extensively investigated due to its ability to essentially modify the biodistribution of an active compound.^{9–12} PLGA is synthesized from ring-opening polymerization of cyclic lactide and glycolide monomers and degrades via hydrolysis of its ester bonds in water.¹³ Drug delivery and release can be efficiently controlled by the size and composition of the NP, and with surface modification they can be designed for host cell directed delivery.¹⁴ During the NP formulation process surfactants are frequently used to stabilize and reduce the surface tension of PLGA.¹⁵ Pluronic F127 (PF127) is a non-ionic poloxamer type surfactant consisting of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) blocks in the PEO–PPO–PEO block copolymer arrangement.¹⁶ PF127 has been evaluated for numerous biomedical applica-

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unambiguously evidenced by the cross-peaks in the ^1H – ^{13}C -HSQC spectrum allowing indirect detection of C_α signals at 150.2 and 147.3 ppm, respectively, in the well-documented region characteristic for oximes. This view gains further support from the cross-peaks generated by two-bond interactions detected by ^1H – ^{13}C HMBC method between H_α protons and C_β carbons disclosed at 67.1 ppm for both oximes I(E) and II(Z). On the other hand, assignment of C_β was based on the cross-peak generated by its three-bond correlation with the H_ϵ signal which appears at 4.61 ppm as a singlet coalesced with the signal of H_δ proton situated in a chemical environment practically identical to that of H_ϵ . The ^1H – ^{13}C HMBC spectrum to allow the identification of the H_β signals of I(E) and II(Z) at 3.99 and 3.95 ppm, respectively, through their cross-peaks with the previously identified C_α signals. It is of diagnostic importance that, due to their position in the atomic sequence, exclusively H_β protons can be involved in not more than three-bond correlation with C_α carbon. Finally, note that in the ^1H – ^{13}C HMBC spectrum the one-bond correlations are discernible as satellites providing further evidence for the assignments obtained from the cross-peaks in the ^1H – ^{13}C HSQC spectrum.

PLGA nanoparticles were prepared by a nanoprecipitation method similar to that employed previously.²² Instead of acetone, tetrahydrofuran was used during the synthesis because even a trace amount of acetone can react with the aminooxy-peptide and consequently decrease the yield of the desired oxime product. An organic solution of PLGA was added to an aqueous solution of PF127-aldehyde and stirred until complete evaporation of THF. The aqueous PLGA sol was further purified by centrifugation and allowed to react with the aminooxy-peptide (Scheme 1). Purpald analysis of PF127-aldehyde coated NPs revealed that 1 mg NP lyophilizate contained 35 nmol aldehyde, while the conversion rate of aldehyde to oxime on the surface of the NPs was up to 98%. The formed peptide coated NPs were then characterized by dynamic light scattering (DLS) and atomic force microscopy (AFM) which showed spherical particle shape (Figure S3) with an average hydrodynamic diameter of 145 nm and a polydispersity of 0.11. The loading amount of peptide on the surface of NPs as measured by amino acid analysis was in correlation with the data of Purpald analysis. Namely, 1 mg NP lyophilizate contained 38 nmol peptide. (Detailed NP preparation and characterization can be found in the Supporting Information.)

To provide the proof of principle, an antitubercular agent was encapsulated into PLGA NPs and into peptide-Pluronic coated PLGA NPs, and the internalization rate together with the intracellular killing of *Mycobacterium tuberculosis* H₃₇Rv (*Mtb*) was compared (both measurements are detailed in the Supporting Information). As a drug candidate, a previously described coumaron derivative (6-hydroxy-2-(3-phenylprop-2-yn-1-ylidene)-2,3-dihydro-1-benzofuran-3-one, shown as TB515) was employed, which was found to be active against virulent *Mtb* H₃₇Rv bacteria at a relatively low minimal inhibitory concentration (19 μM).²³ The possible target of TB515 drug candidate is the species-specific loop of *M. tuberculosis* dUTPase enzyme (EC 3.6.1.23; Rv2697), which plays an important role in preventive DNA repair mechanism and thymidilate biosynthesis, and essentially required for mycobacterial growth.²⁴ Moreover, the excellent fluorescent property of TB515, which is essential for flow cytometry, emphasizes the use of TB515 compound to determine the

cellular uptake rate even in its nanoparticulated forms without the need of further fluorescent labeling.

TB515 drug candidate was dissolved in the PLGA/THF organic solution and was added to the aqueous solution of PF127-aldehyde followed by the conjugation with the aminooxy-peptide. Drug-loaded nanoparticles were purified and characterized as described above. TB515 containing systems were found to have somewhat increased average diameter of 200 nm, along with 0.15 polydispersity. This size increase can be interpreted by the broader size distribution of the particles indicated by the polydispersity causing the shifting of the determined size. Drug content of the NPs was determined spectrophotometrically, which revealed that TB515 content was 3.5 w/w %.

Cellular uptake of MonoMac6 monocytes, that can be considered as a human host cell model, was studied by flow cytometry. Cells were treated with the drug loaded, peptide conjugated nanoparticles (TB515-PLGA-T10Tp) and, for comparison, unconjugated TB515-PLGA NPs and free TB515 compound. After trypsination, intracellular fluorescence intensity of the cells was measured by a BD LSR II flow cytometer. As shown in Figure 2, significantly higher uptake

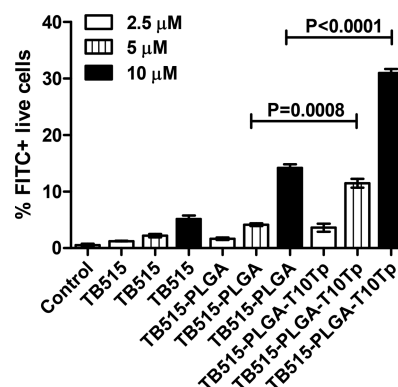


Figure 2. Internalization rate of drug loaded PLGA nanoparticles. MonoMac6 human monocytes were treated with TB515 drug candidate and TB515 containing peptide conjugated and unconjugated PLGA nanoparticles. The intracellular fluorescent intensity was measured by flow cytometry.

rate was measured for the peptide conjugated nanoparticle (TB515-PLGA-T10Tp) than for unconjugated TB515-PLGA, which result indicates that Tuftsin peptide labeling increased the internalization of the nanoparticles.

Intracellular killing efficacy of the nanoparticles was measured on *Mtb* H₃₇Rv infected MonoMac6 human monocytes as described previously.²⁵ TB515 compound alone is not active against the intracellular bacteria. In contrast, PLGA encapsulation is an effective method to improve the intracellular antitubercular effect (Figure 3). Results also showed that Tuftsin peptide conjugation further enhanced the intracellular killing efficacy which can be the consequence of the higher internalization rate observed for the peptide conjugated nanoparticles.

In conclusion, we report here a convenient synthetic route to obtain peptide conjugated PLGA nanoparticles together with diagnostic NMR data providing unambiguous evidence for the presence of oxime bond connecting PF127 polymer with Tuftsin peptide chain. Peptide conjugation significantly increased the internalization rate to human monocytes and

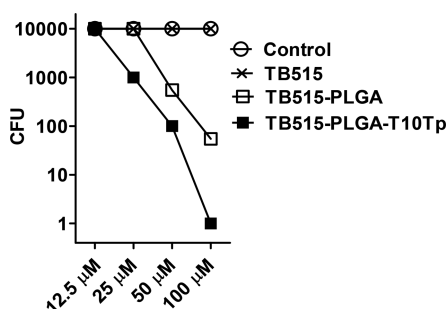


Figure 3. Inhibition of intracellular *Mycobacterium tuberculosis* by peptide conjugated and unconjugated NPs. Cultured MonoMac6 cells were infected with *Mtb* H₃₇Rv bacteria and treated with the compounds. As control, untreated cells were used. TB515 containing peptide conjugated PLGA NPs were more effective than unconjugated NPs against intracellular *Mtb* bacillus and expressively lower the number of detectable mycobacterial colonies, while free TB515 drug candidate was unable to penetrate effectively and kill intracellular bacteria.

nevertheless, intensifying the antibacterial effect of an entrapped drug molecule against intracellular *Mycobacterium tuberculosis*.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.8b00156.

Detailed synthesis and characterization of the compounds, NMR spectra, NP preparation and characterization, detailed description of the biological assays (PDF)

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Notes

The authors declare no competing financial interest.

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