

Revised manuscript

The final version was published in:

Bioconjug Chem. 2018 May 16;29(5):1495-1499.

doi: 10.1021/acs.bioconjchem.8b00156.

Surface Layer Modification of PLGA Nanoparticles with Targeting Peptide: A Convenient Synthetic Route for Pluronic F127 – Tuftsin Conjugate

Kata Horváti†, Gergő Gyulai‡, Antal Csámpai||, János Rohonczy§, Éva Kiss‡ and Szilvia Bősze†*

†MTA-ELTE Research Group of Peptide Chemistry, Hungarian Academy of Sciences Budapest, Eötvös Loránd University, Budapest 112, P.O. Box 32, H-1518 Hungary.

‡Laboratory of Interfaces and Nanostructures, ||Department of Organic Chemistry and

§Department of Inorganic Chemistry, Eötvös Loránd University, Budapest 112, P.O. Box 32, H-1518 Hungary.

* Corresponding author. Dr. Szilvia Bősze, PhD, MTA-ELTE Research Group of Peptide Chemistry, Pázmány P. stny. 1/A, Budapest, Hungary, 1117.

Tel: +36-1-372-2500 ext. 1736, fax: +36-1-372-2620, e-mail: szilvia.bosze@gmail.com

KEYWORDS: PLGA nanoparticles, Pluronic labelling, macrophage targeting, Tuftsin

ABSTRACT: Nanoparticles consist 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 one step reaction. We have also investigated that this change has no effect on the physico-chemical 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*. 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, bactericidal activity and has been reported also as a macrophage-targeting peptide^{3,4,5,6}. During the past decade, a new group of sequential oligopeptide carriers has been developed in our laboratory: oligotuftsin derivatives consisting of a (Thr-Lys-Pro-Lys-Gly) unit⁷. 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 bounds to the Tuftsin receptors more avidly than Tuftsin⁸. It was also reported that the chemical modification of the C-terminus to a carboxamide results in loss of biological function.

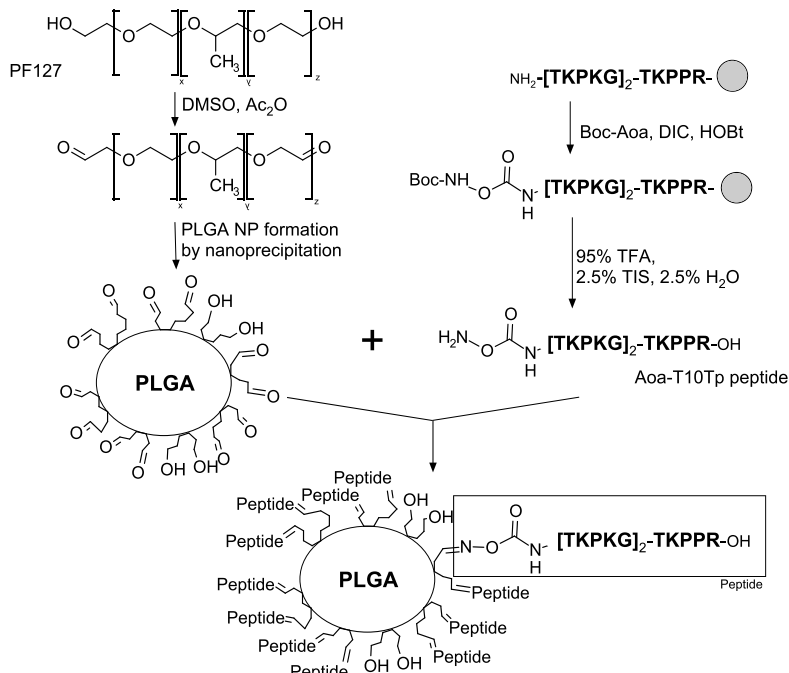
Putting these observations together, a new sequence was utilized in this study: (Thr-Lys-Pro-Lys-Gly)₂-Thr-Lys-Pro-Pro-Arg (T10Tp). The conjugation site of the targeting molecule was designed on the N-terminus of the peptide.

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) have been extensively investigated due to its ability to essentially modify the biodistribution of an active compound^{9,10,11,12}. PLGA is synthesized from ring-opening polymerization of cyclic lactide and glycolide monomers and degrades via hydrolysis of its ester bonds in water¹³. 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 delivery¹⁴. During the NP formulation process surfactants are frequently used to stabilize and reduce the surface tension of PLGA¹⁵. 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 PEO-PPO-PEO block copolymer arrangement¹⁶. PF127 has been evaluated for numerous biomedical applications (recently reviewed by Akash and Rehman¹⁷) and due to terminal hydroxyl groups, which are available for conjugation, further functionalities can be added to the pharmaceutical perspectives. Most of the conjugation methods described in the literature, start with the reaction of PF127 with succinic anhydride and the resulted terminal carboxylic groups are then used in a reaction with amino or hydroxyl groups of different ligands such as chitosan, peptides, dendrimers, etc¹⁸. Another synthetic way is to prepare PF127-amine via reductive amination of oxidized polymer and then react terminal amino groups to form amide-conjugates^{19,20}. These methods involve three or more synthetic steps and the coupling agents and other reactants can remain in the polymer product. To overcome the obstacles a new synthetic route was designed in this study based on a

one-step quantitative reaction of aminoxy compounds with aldehydes. This conjugation technique does not require pre-activation and as reaction media water can be used.

Aldehyde terminated PF127 was synthesized according to the method described for PEO by Harris et al.¹⁹. Briefly, PF127 was dissolved in dry DMSO and oxidized with acetic anhydride to result aldehyde end groups. After precipitation and filtration, the aldehyde content of the polymer was measured by Purpald analysis²¹, which revealed that the conversion rate of hydroxyl groups of PF127 to aldehyde groups was 69%. Purpald analysis was adapted to perform a more convenient assay in a 96-well microplate, attaining great sensitivity with sample volume of as little as 80 μ L. Detailed synthesis and Purpald assay together with the calibration curve are provided in the Supporting Information.

To prepare an aminoxy-peptide, a convenient solid-phase synthetic route is available, namely coupling Boc-aminoxyacetic acid (Boc-Aoa) to the peptide resin followed by a standard cleavage procedure (see Scheme 1.). Tuftsin-derivative T10Tp was produced on Wang resin in an automated peptide synthesizer using Fmoc/*t*Bu strategy with DIC/HOBt coupling reagents. The Boc-aminoxy-group was placed at the *N*-terminus of the peptide. After the final cleavage, compound was purified and chemically characterized by RP-HPLC and mass spectrometry (Aminoxyacetyl-TKPKG₂TKPKGTKPPR, M_{av} calcd/found: 1694.0/1694.0, Rt: 7.6 min).



Scheme 1. Outline of the synthesis of PF127-aldehyde and aminoxy-Tuftsin peptide and the oxime bond formation on the surface of PLGA nanoparticles.

Resulted aminoxy-derivative was then dissolved in dist. H₂O and allowed to react with PF127-aldehyde for 30 min. Purpald analysis revealed that the conversion of aldehyde to oxime was up to 90.5%.

To prove the appearance of the oxime bond between PF127-aldehyde polymer and aminoxy-peptide, NMR spectroscopy was evaluated. The conjugate was subjected to NMR measurements carried out in DMSO-d₆ solution at 23 °C (Figure 1 and Figure S2).

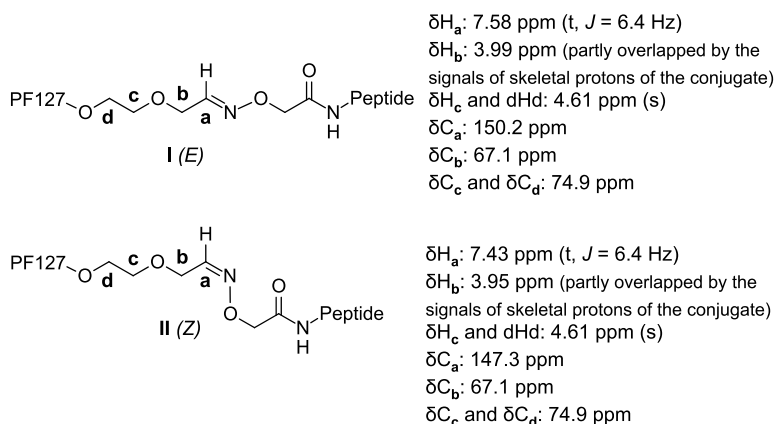


Figure 1. Isomers of the oxime conjugate and the assignment of the NMR signals.

The two triplets discernible in the ¹H-NMR spectrum at 7.58 ppm and 7.43 ppm (with relative intensity of ca. 2/1 superpositioned on the broadened NH signals of the peptide fragment) were identified as the H_a signals originated from the oxime motifs with configurations *E*- and *Z*-, respectively. This assignment was unambiguously evidenced by the cross-peaks in the ¹H-¹³C-HSQC spectrum allowing indirect detection of C_a signals at 150.2 ppm 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-¹³C-HMBC method between H_a protons and C_b carbons disclosed at 67.1 ppm for both oximes **I**(*E*) and **II**(*Z*). On the other hand, assignment of C_b was based on the cross-peak generated by its three-bond correlation with the H_c signal which appears at 4.61 ppm as a singlet coalesced with the signal of H_d proton situated in a chemical environment practically identical to that of H_c. The ¹H-¹³C-HMBC spectrum to allow the identification of the H_b signals of **I**(*E*) and **II**(*Z*) at 3.99 ppm and 3.95 ppm, respectively, through their cross-peaks with the previously identified C_a signals. It is of diagnostic importance that due to their position in the atomic sequence, exclusively H_b protons can be involved in not more than three-bond correlation with C_a carbon. Finally, note that in the ¹H-¹³C-HMBC spectrum the one-bond correlations are discernible as satellites providing further evidence for the assignments obtained from the cross-peaks in the ¹H-¹³C-HSQC spectrum.

PLGA nanoparticles were prepared by nanoprecipitation method similar to that employed previously²². Instead of acetone, tetrahydrofuran was used during the synthesis because even trace amount of acetone can react with the aminoxy-peptide and consequently decrease the yield of the desired oxime product. Organic solution of PLGA was added to aqueous solution of PF127-aldehyde and stirred till the complete evaporation of THF. The aqueous PLGA sol was further purified by centrifugation and allowed to react with the aminoxy-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 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 growth²⁴. Moreover, the excellent fluorescent property of TB515, which is essential for flow cytometry, emphasises the use of TB515 compound to determine the cellular uptake rate even in its nanoparticulated forms without the need of further fluorescent labelling.

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

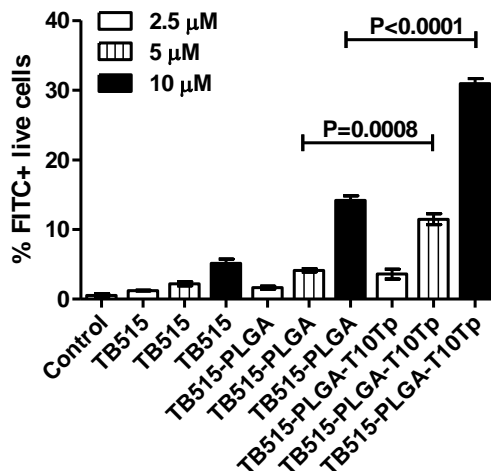


Figure 2. Internalization rate of drug loaded PLGA nanoparticles. MonoMac6 human monocytes were treated with TB515 drug candidate and TB515 containing peptide conjugated and unconjugated PLGA nanoparticles. The intracellular fluorescent intensity was measured by flow cytometry.

Intracellular killing efficacy of the nanoparticles was measured on *Mtb* H₃₇Rv infected MonoMac6 human monocytes as described previously²⁵. 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.

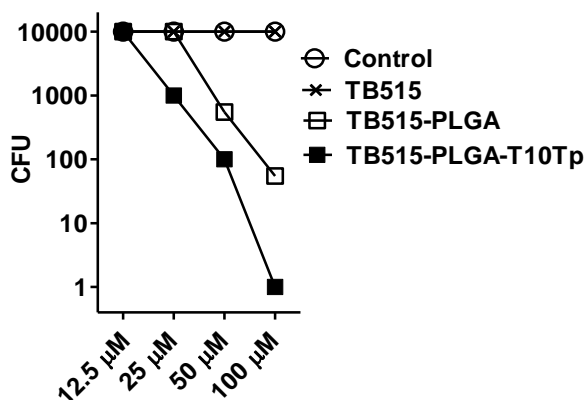


Figure 3. Inhibition of intracellular *Mycobacterium tuberculosis* by peptide conjugated and unconjugated NPs. Cultured MonoMac6 cells were infected with *Mtb* H₃₇Rv bacteria and treated with the compounds. As control, untreated cells were used. TB515 containing peptide conjugated PLGA NPs were more 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 to kill intracellular bacteria.

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 nevertheless,

intensify the antibacterial effect of an entrapped drug molecule against intracellular *Mycobacterium tuberculosis*.

Supporting Information: detailed synthesis and characterization of the compounds, NMR spectra, NP preparation and characterization, detailed description of the biological assays. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Corresponding Author

* Szilvia Bősze, MTA-ELTE Research Group of Peptide Chemistry, Eötvös Loránd University, H-1117 Budapest, Pázmány P. stny. 1/A.
e-mail: bosze@elte.hu

Funding Sources

This work was funded by the Hungarian Scientific Research Fund (115431, 124077, 104275).

ACKNOWLEDGMENT

The authors thank to Dr. Hedvig Medzihradszky-Schweiger for the amino acid analysis and to Mr. Sándor Dávid for the antimycobacterial testing. Kata Horváti was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences and Gergő Gyulai was funded by the Hungarian Academy of Sciences Postdoctoral Research Program.

Conflict of Interest

The authors declare that they have no conflict of interest.

REFERENCES

1. Gordon, S. (2016) Phagocytosis: An Immunobiologic Process. *Immunity* 44, 463-475.
2. Griffiths, G.; Nystrom, B.; Sable, S. B.; Khuller, G. K. (2010) Nanobead-based interventions for the treatment and prevention of tuberculosis. *Nat. Rev. Microbiol.* 8, 827-834.
3. Dutta, T.; Garg, M.; Jain, N. K. (2008) Targeting of efavirenz loaded tuftsin conjugated poly(propyleneimine) dendrimers to HIV infected macrophages in vitro. *Eur J. Pharm. Sci.* 34, 181-189.
4. Amoscato, A. A.; Davies, P. J.; Babcock, G. F.; Nishioka, K. (1983) Receptor-mediated internalization of tuftsin. *Ann. Ny. Acad. Sci.* 419, 114-134.
5. Gottlieb, P.; Stabinsky, Y.; Hiller, Y.; Beretz, A.; Hazum, E.; Tzehoval, E.; Feldman, M.; Segal, S.; Zakuth, V.; Spierer, Z.; Fridkin, M. (1983) Tuftsin Receptors. *Ann. Ny. Acad. Sci.* 419, 93-106.
6. Siebert, A.; Gensicka-Kowalewska, M.; Cholewinski, G.; Dzierzbicka, K. (2017) Tuftsin - Properties and Analogs. *Curr. Med. Chem.* 24, 3711-3727.
7. Mezo, G.; Kalaszi, A.; Remenyi, J.; Majer, Z.; Hilbert, A.; Lang, O.; Kohidai, L.; Barna, K.; Gaal, D.; Hudecz, F. (2004) Synthesis, conformation, and immunoreactivity of new carrier molecules based on repeated tuftsin-like sequence. *Biopolymers* 73, 645-656.
8. Zhang, Y.; Xiao, L.; Chordia, M. D.; Locke, L. W.; Williams, M. B.; Berr, S. S.; Pan, D. (2010) Neutrophil targeting heterobivalent SPECT imaging probe: cFLFLF-PEG-TKPPR-99mTc. *Bioconjug. Chem.* 21, 1788-1793.
9. Bala, I.; Hariharan, S.; Kumar, M. N. (2004) PLGA nanoparticles in drug delivery: the state of the art. *Crit. Rev. Ther. Drug Carrier Syst.* 21, 387-422.

10. Hamdy, S.; Haddadi, A.; Hung, R. W.; Lavasanifar, A. (2011) Targeting dendritic cells with nano-particulate PLGA cancer vaccine formulations. *Adv. Drug Deliv. Rev.* 63, 943-955.
11. Silva, A. L.; Rosalia, R. A.; Varypataki, E.; Sibuea, S.; Ossendorp, F.; Jiskoot, W. (2015) Poly-(lactic-co-glycolic-acid)-based particulate vaccines: particle uptake by dendritic cells is a key parameter for immune activation. *Vaccine* 33, 847-854.
12. Xu, Y. H.; Kim, C. S.; Saylor, D. M.; Koo, D. (2017) Polymer degradation and drug delivery in PLGA-based drug-polymer applications: A review of experiments and theories. *J. Biomed. Mater. Res. B* 105, 1692-1716.
13. Astete, C. E.; Sabliov, C. M. (2006) Synthesis and characterization of PLGA nanoparticles. *J. Biomater. Sci. Polym. Ed.* 17, 247-289.
14. Banyal, S.; Malik, P.; Tuli, H. S.; Mukherjee, T. K. (2013) Advances in nanotechnology for diagnosis and treatment of tuberculosis. *Curr. Opin. Pulm. Med.* 19, 289-297.
15. Menon, J. U.; Kona, S.; Wadajkar, A. S.; Desai, F.; Vadla, A.; Nguyen, K. T. (2012) Effects of surfactants on the properties of PLGA nanoparticles. *J. Biomed. Mater. Res. A* 100, 1998-2005.
16. Batrakova, E. V.; Kabanov, A. V. (2008) Pluronic block copolymers: evolution of drug delivery concept from inert nanocarriers to biological response modifiers. *J. Control. Release* 130, 98-106.
17. Akash, M. S.; Rehman, K. (2015) Recent progress in biomedical applications of Pluronic (PF127): Pharmaceutical perspectives. *J. Control. Release* 209, 120-138.
18. Zhang, W.; Gilstrap, K.; Wu, L.; K, C. R.; Moss, M. A.; Wang, Q.; Lu, X.; He, X. (2010) Synthesis and characterization of thermally responsive Pluronic F127-chitosan nanocapsules for controlled release and intracellular delivery of small molecules. *ACS Nano* 4, 6747-6759.
19. Harris, J. M.; Struck, E. C.; Case, M. G.; Paley, M. S.; Vanalstine, J. M.; Brooks, D. E. (1984) Synthesis and Characterization of Poly(Ethylene Glycol) Derivatives. *J. Polym. Sci. Pol. Chem.* 22, 341-352.
20. Gyulai, G.; Magyar, A.; Rohonczy, J.; Orosz, J.; Yamasaki, M.; Bosze, S.; Kiss, E. (2016) Preparation and characterization of cationic Pluronic for surface modification and functionalization of polymeric drug delivery nanoparticles. *Express. Polym. Lett.* 10, 216-226.
21. Quesenberry, M. S.; Lee, Y. C. (1996) A rapid formaldehyde assay using purpald reagent: Application under periodation conditions. *Anal. Biochem.* 234, 50-55.
22. Gyulai, G.; Penzes, C. B.; Mohai, M.; Csempesz, F.; Kiss, E. (2013) Influence of surface properties of polymeric nanoparticles on their membrane affinity. *Eur. Polym. J.* 49, 2495-2503.
23. Kiss, E.; Gyulai, G.; Penzes, C. B.; Idei, M.; Horvati, K.; Bacsa, B.; Bosze, S. (2014) Tuneable surface modification of PLGA nanoparticles carrying new antitubercular drug candidate. *Coll. Surf. A* 458, 178-186.
24. Varga, B.; Barabas, O.; Takacs, E.; Nagy, N.; Nagy, P.; Vertessy, B. G. (2008) Active site of mycobacterial dUTPase: Structural characteristics and a built-in sensor. *Biochem. Biophys. Res. Comm.* 373, 8-13.
25. Horvati, K.; Bacsa, B.; Szabo, N.; David, S.; Mezo, G.; Grolmusz, V.; Vertessy, B.; Hudecz, F.; Bosze, S. (2012) Enhanced cellular uptake of a new, *in silico* identified antitubercular candidate by peptide conjugation. *Bioconjug. Chem.* 23, 900-907.

Table of Contents Graphic

