# Novel nanoparticulated conjugates comprising T-cell epitopes in branched chain arrangement on a lipo-Tuftsin platform

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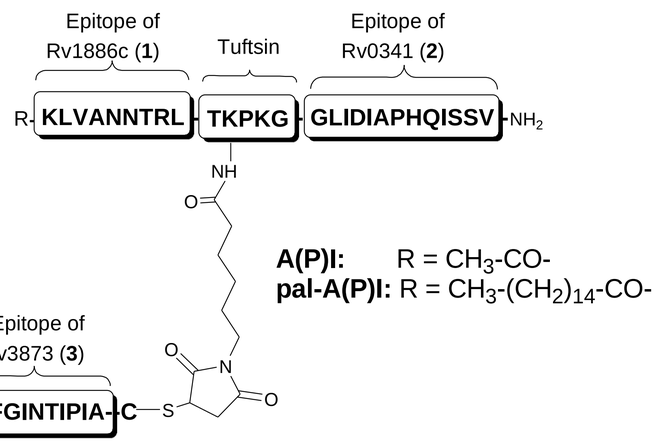
## Introduction

Development of vaccines against a variety of infectious diseases, where the causative agent shows high antigenic diversity due to fast mutation rate or metamorphosis across variation of subtypes, is still challenging. Immunization with a multivalent subunit vaccine, that combines multiple antigens derived from different stages of a pathogen's life cycle, hold promise for overcoming the major obstacles. In this project, promiscuous T-cell epitope peptides derived from immunodominant proteins (Rv1886c **[1]**, Rv0341 **[2]**, Rv3873 **[3]**) expressed during the different stages of *Mycobacterium tuberculosis* (*Mtb*) were conjugated to a Tuftsin carrier in branched chain arrangement. To enhance bioavailability and vaccine efficacy the multi-epitope conjugate was elongated with palmityc acid and encapsulated into poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles. As adjuvant, trehalose-6,6-dibehenate (TDB) was used, which is a synthetic analogue of trehalose-6,6-dimycolate, a cell wall component of *Mtb*. Structure-activity relationship together with *in vivo* immunogenicity and vaccine efficacy study on a murine model of tuberculosis were evaluated and a promising candidate was identified.

## Structure of the conjugates

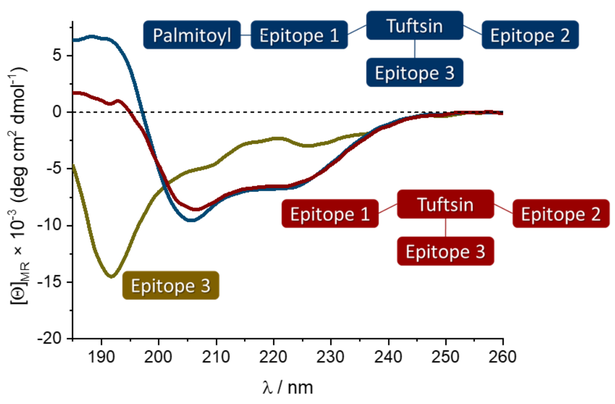
Chemoselective ligation technique (maleimide coupling) was employed in the synthesis of branched chain conjugates. To increase the internalization and bioavailability, the conjugate was elongated with palmytic acid on the *N*-terminus.




| Compound | Sequence | $M_{mo}$ calculated | $M_{mo}$ measured | Rt (min) |
|---|---|---|---|---|
| P/A/I mix | $^{240}$KLVANNTRL$^{247}$ | 1026.6298 | 1026.6295 | 8.4 |
| | $^{124}$FFGINTIPIA $^{133}$ | 1090.6175 | 1090.6174 | 12.7 |
| | $^{33}$GLIDIAPHQISSV$^{49}$ | 1347.7510 | 1347.7508 | 10.7 |
| A(P)I | KLVANNTRL-TK(FFGINTIPIAC)PKG-GLIDIAPHQISSV | 4297.3535 | 4297.3649 | 13.0 |
| pal-A(P)I | Palmitoyl- KLVANNTRL-TK(FFGINTIPIAC)PKG-GLIDIAPHQISSV | 4493.5726 | 4493.5757 | 24.1* |

[a] Exact mass measured on a Thermo Scientific Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer
[b]Analytical RP-HPLC, Agilent Eclipse XDB C8, 5 µm, 80Å, 4.6 x 150 mm, HPLC column, gradient: 5% B, 2 min; 5-100% B, 20 min. *Phenomenex Jupiter C4, 5 µm, 300 Å, 4.6 x 250 mm, HPLC column, gradient: 25% B, 5 min; 5-100% B, 20 min.

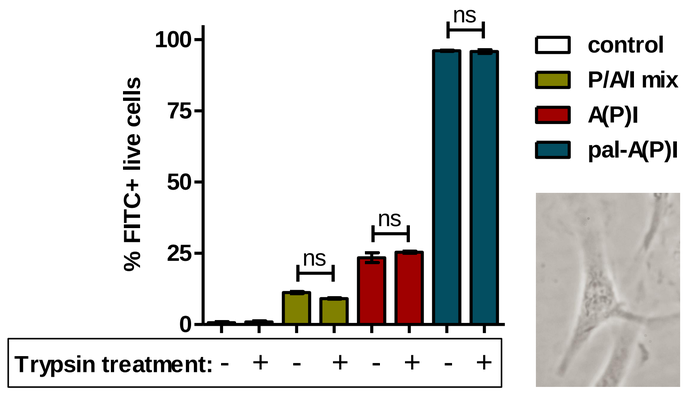
## Conformation studies - ECD




The short, linear epitope peptide has highly dynamic conformation in TFE. The branched conjugates showed a more intense spectrum in the range of 185-210 nm indicating more partially folded states. The palmitoylated analogue has a higher tendency to fold in the membrane inducing environment.

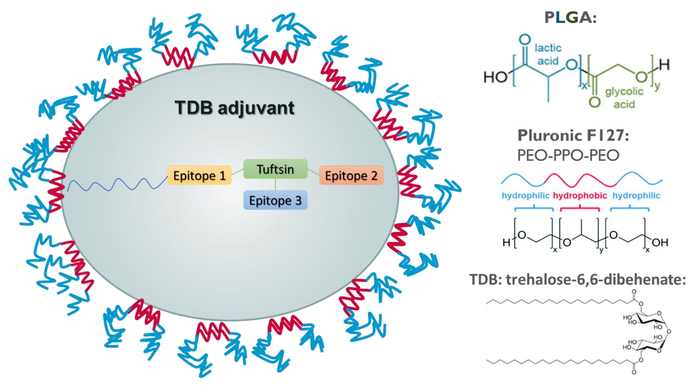
## Improved uptake by murine BMDM

Cellular uptake experiments in trypsin treated and not-treated murine BMDM were conducted in order to determine the internalization rate and to distinguished between internalization and surface binding.

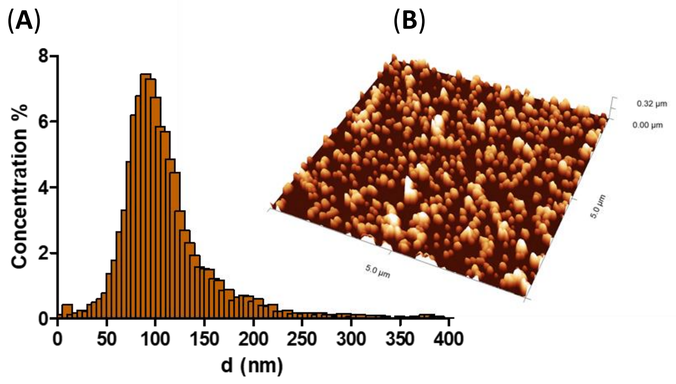



Here we show that palmitoylation of the conjugate dramatically enhanced the cellular uptake rate to antigen presenting cells. Peptides are internalized rather than bind to the surface of the cells

## Formulation: TDB adjuvanted PLGA

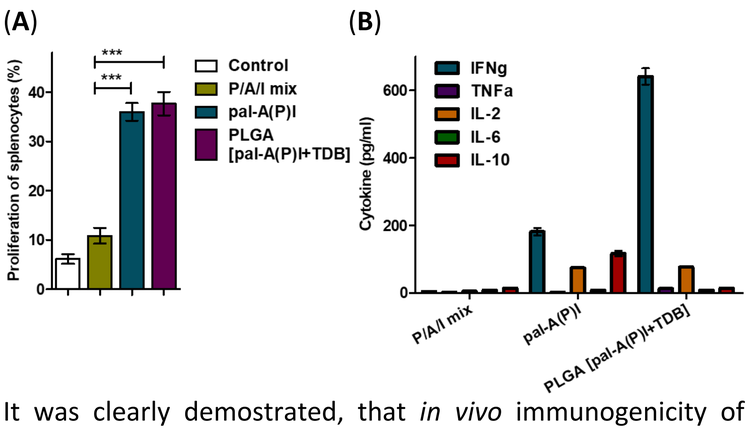



PLGA particles were prepared by nanoprecipitation method **[4]**, analysed by DLS (**A**), AFM (**B**) and aminoacid analysis. The encapsulation efficacy of the pal-A(P)I conjugate was up to 80%.




## Immunogenicity of the compounds

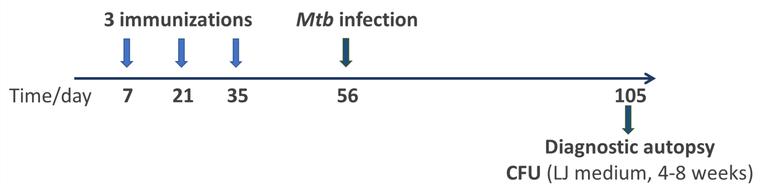
Compounds were injected (*sc*) three times (2 weeks apart) and 6 weeks after the last immunization mice were sacrificed. Single cell suspension was prepared from the spleen and restimulated with the epitopes. Proliferation of the splenocytes (**A**) was examined with CFSE staining **[5]** and cytokine production (**B**) was determined by using Legendplex™ Mouse Th Cytokine Panel (Biolegend).

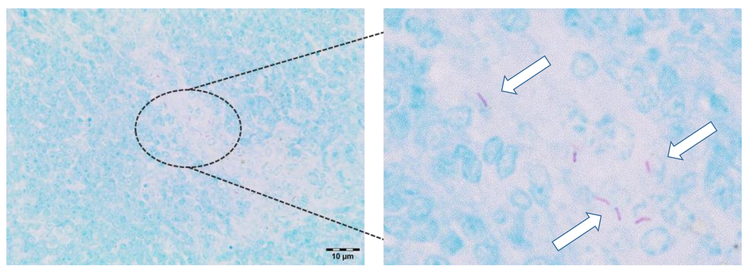



It was clearly demostrated, that *in vivo* immunogenicity of linear epitope peptides was significantly improved by conjugation and adjuvanted PLGA encapsulation.

## Vaccine efficacy study

For vaccine efficacy study, 6-8 weeks old female BALB/c mice were housed at a BSL3 laboratory in ventilated cages. Groups of 5 mice were injected with the compounds (50 µg peptide content / 100 µl PBS, *sc*) 3-times. Mice were infected with *Mtb* $H_{37}$Rv ($10^6$ / ml bacteria in 200 µl PBS, *ip*). Seven weeks after, bacteria were enumerated from tissue homogenates.

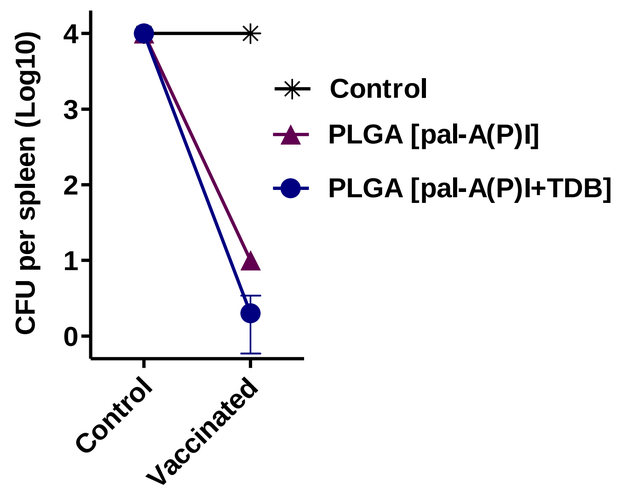



Photomicrographs revealed rod-shaped *Mtb* bacteria in histologic sections from the spleen using acid-fast Ziehl-Neelsen stain.

## Effective vaccine candidate on a murine model of tuberculosis

Significant reduction in the colony forming units (CFU) was observed when mice were vaccinated with PLGA encapsulated epitope-conjugate. The co-administration with TDB adjuvant further improved the vaccine efficacy.




## *In vivo* MRI imaging

In order to follow the compounds by MRI, DTPA was coupled to the conjugate and complexed with gadolinium (Gd).




At the site of injection, PLGA encapsulated conjugate showed a significantly longer half life indicating a delayed clearance compare to the free conjugate.

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