1. PUBLISHABLE SUMMARY

The development of effective drug delivery systems and vaccines for cancer treatment is still nowadays a scientific challenge that requires further exploration and development. The design of such systems involves the consideration of different aspects crucial for their performance such as composition, functionalization, morphology and release mechanism. The present project explores the development of innovative nanocarriers based on degradable (DNA) block copolymers which disassemble under specific stimuli such as reducing environment and light. The use of DNA block copolymers provides the possibility of incorporating multifunctionality by hybridization of complementary DNA sequences linked to specific molecules resulting in systems that combine both a controlled release of the encapsulants and multifunctionality.

Over the past years different delivery systems have been developed with the aim of making the most of their combined features. The use of biodegradable block copolymers which yield to harmless byproducts easy to eliminate from the organism is a fundamental pre-requisite for in vivo applications. Other features such as the morphology, functionality or release mechanism can be tuned as advanced attributes. It has been for example demonstrated that worm-like micelles remain in circulation for longer time than their spherical counterparts due to their ability to avoid uptake by macrophages.[1] On the other hand moieties responsive to specific stimuli have been incorporated into nanoparticle-based systems providing a higher degree of control over the release of encapsulated molecules.[2] The vast majority of the stimuli-sensitive delivery systems developed until the moment have been however limited to spherical aggregates. Thus combining the advantages of shape and controlled disassembly, the creation of novel and advanced delivery systems can be envisioned.

The work performed during the outgoing phase of the project focused in the development of two different stimuli-sensitive worm-like micelles which disassemble under reducing conditions or under irradiation. In the last stage of the project also micellar systems from redox-sensitive DNA block copolymers were developed in order to provide the possibility of incorporating multifunctionality. During this period block copolymers from poly(ethylene glycol) (PEG) and poly(ε-caprolactone) (PCL) containing either a disulfide bond or a photocleavable linker between the two polymer groups were synthesized, purified and characterized. These copolymers were assembled into worm-like micelles and their triggered disassembly was studied *in vitro*. The redox-sensitive micelles containing the anti-cancer drug paclitaxel were also tested *in vivo*. Tumor-bearing mice injected with the formulation showed a systematic reduction in the tumor size during the treatment proving this system as an effective tool in drug delivery for cancer treatment. These results are currently being written in a manuscript for submission to a high impact peer-reviewed journal.

Parallel to this work DNA block copolymers containing a disulfide bond were also synthesized, purified, characterized and assembled into micelles. Their disassembly *in vitro* in a reducing environment was proven.

The return phase of the project focused in applying the acquired knowledge in the development of redox-sensitive DNA block copolymers for the creation of innovative vaccines. The development of nanoparticle-based vaccines involves the delivery of immunostimulatory and co-stimulatory molecules,

antigen and adjuvant respectively, to antigen presenting cells (APCs) to trigger subsequent events that lead to immune responses.

Among the antigens and adjuvants used in the field of vaccines the work was focused on two of them, namely ovalbumin (OVA) or a derived peptide from OVA (SIINFEKL) as the antigen and CpG B as the adjuvant. CpG B is a short single-stranded DNA molecule that acts as immunostimulant. Different CpG B-polymer conjugates containing disulfide bonds were synthesized and purified successfully. CpG B-S-S-PEG conjugates were used in the formation of polyionic complex (PIC) micelles which are formed by the combination of the conjugate with a polycation such as branched polyethyleneimine (B-PEI). Although such kind of micelles has been reported before, their application as vaccines is still missing and that is what is being at the moment explored in our group. PIC micelles from CpG-B in combination with OVA were studied *in vivo* on tumor-bearing mice. Although the results are still inconclusive the micelles showed some effect and resulted in tumor shrinkage. These results represent the first step in the development of PIC micelle-based vaccines and further experimentation is taking place at the moment combining the peptide SIINFEKL.

The results obtained during the three year project represent an advancement in the fields of drug delivery and vaccine development since the combination of different relevant features have been further explored, i.e. morphology, functionality and triggered release, and novel effective systems have been developed. Although their social impact is still far from being patent, these results are a step further in the elaboration of effective systems in both fields and represent a very useful tool for the progress towards that end.

1 Y. Geng, P. Dalhaimer, S. Cai, R. Tsai, M. Tewari, T. Minko, D. E. Discher, *Nature Nanotechnol.* 2007, 2, 249

2 S. Mura, J. Nicolas, P. Couvreur, *Nature Mat.* 2013, 12, 991

2. PROJECT OBJECTIVES FOR THE PERIOD

The objectives set for the return phase in Prof. Hubbell's laboratory at EPFL involved the application of the knowledge acquired during the outgoing phase in stimuli-responsive DNA block copolymers for the development of innovative vaccines. The different steps to be taken involve the synthesis, purification, characterization and assembly of DNA block copolymers and of DNA-antigen conjugates. Using this material the best formulation should be explored and characterized to obtain micelle-based vaccines. In a later stage, *in vivo* studies using the optimal formulation will provide the results for the assessment of the vaccine developed.

The recommendations from previous reviews involve a better outline of the deviations from the original objectives and their impact on the tasks performed as well as a clarification on the ethical issues. This is taken care in this report under the section of "Project management".

3. WORK PROGRESS AND ACHIEVEMENTS DURING THE PERIOD

During the period reported here the project has been focused in applying the previous knowledge acquired for the development of drug delivery systems during the outgoing phase into the creation and study of innovative vaccines. The new systems under development are based on micelles formed from DNA block copolymers containing a linkage moiety between the DNA and the polymer which is susceptible to breakage under specific conditions (Figure 1).

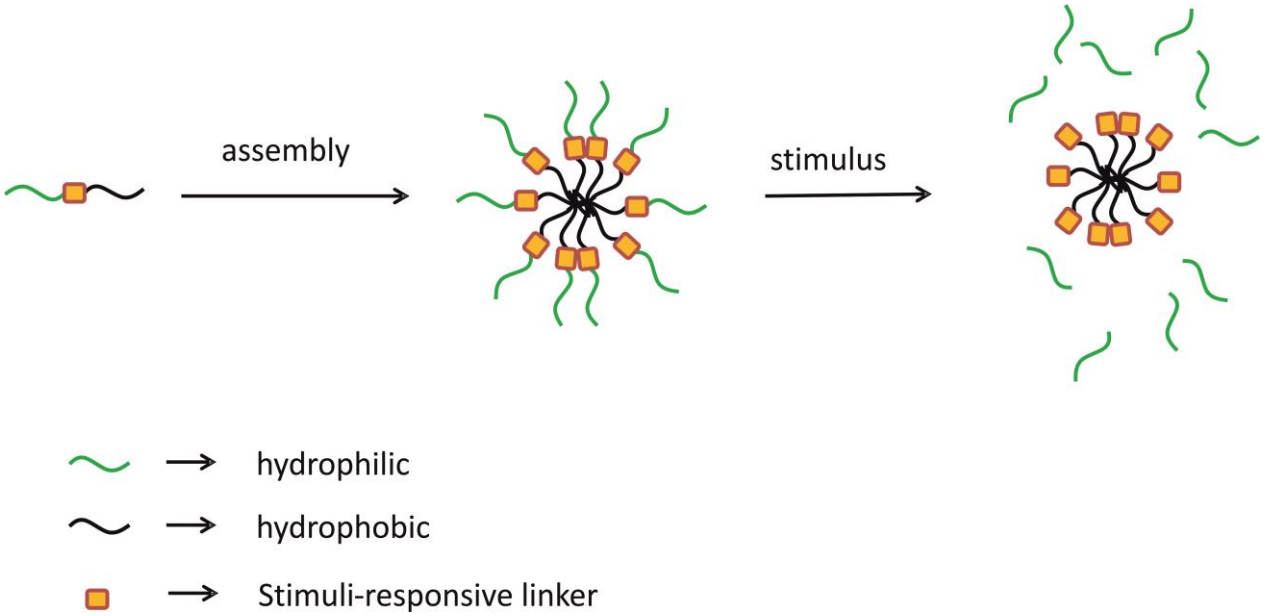

Figure 1. Schematic representation of the assembly of responsive block copolymers in water and the subsequent stimuli-triggered disassembly of the aggregates.

Although the original proposed system involved the use of a photocleavable linker, the research has been mainly focused in the development of systems containing redox-sensitive moieties such as a disulfide bond. This particular chemistry has been used and developed during the outgoing phase of the project and it has been further extended in this later phase to the development of vaccines. During this period the new systems based on polyionic complex (PIC) micelles have been developed and tested *in vivo* for their application as cancer vaccines. The approved protocol for such experiments was submitted to the project coordinator at the time of the experiments and it is also included as an attachment to this document.

In the development of vaccines antigens, which are molecules that induce immune responses, and adjuvants, which are co-stimulatory molecules, are co-delivered to antigen presenting cells (APCs). The APCs display the antigen complexes on their surfaces which can be recognized by T cells. This recognition triggers T-cell stimulation and induces a subsequent immune response.

Ovalbumin (OVA) and CpG are an antigen and an adjuvant used extensively in the group of Prof. Hubbell for vaccine development. CpG is a short oligonucleotide that can be conjugated to polymers in a similar way to the ones used during the outgoing phase of the project. In this case CpG was attached to polypropylene sulfide via a disulfide bond (Figure 2).

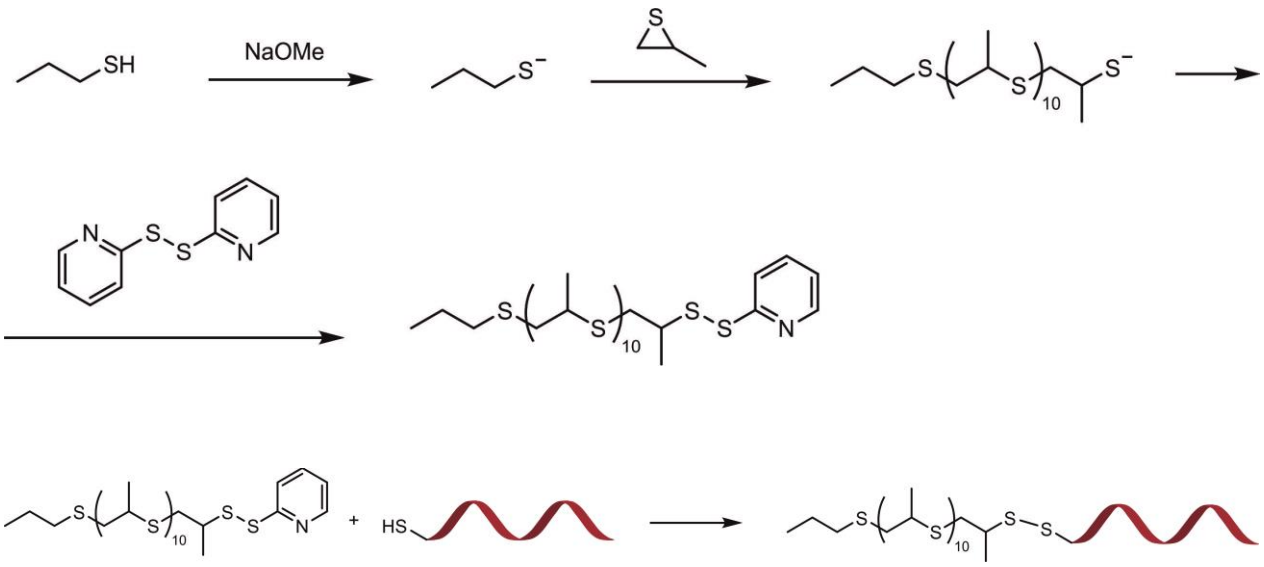

Figure 2. Synthetic route followed for the synthesis of pyridine disulfide-funtionalized polypropylene sulfide (Py-S-S-PPS) and CpG-S-S-PPS.

PPS has been used in the group for the creation of nanoparticles due to the differences in hydrophilicity depending on the redox environment. Under non-oxidative conditions the polymer is hydrophobic thus it can be used as the constituent of the core of nanoparticles. Under oxidative conditions however, the sulfur atoms get oxidized to sulfoxides and sulfones which contribute to the hydrophilicity of the polymer and results in solubilization and therefore disassembly of the particle (Figure 3). Oxidative conditions can be found in sites of inflammation and endolysosomes therefore, this polymer is particularly interesting for molecular delivery *in vivo*.

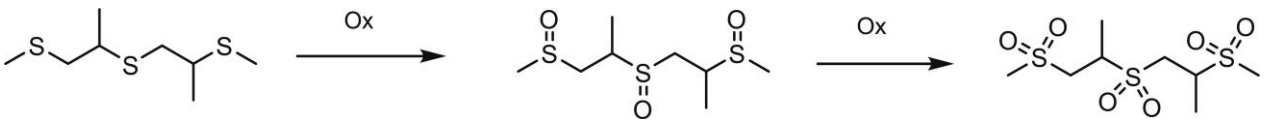

Figure 3. Oxidation of polypropylene sulfide (PPS).

The synthesis and purification of CpG-S-S-PPS was carried out successfully. Unfortunately, only small amounts of pure material were recovered which were insufficient for obtaining aggregates. The low yields obtained were attributed to the non-quantitative coupling reaction and the purification process which resulted in significant product loss.

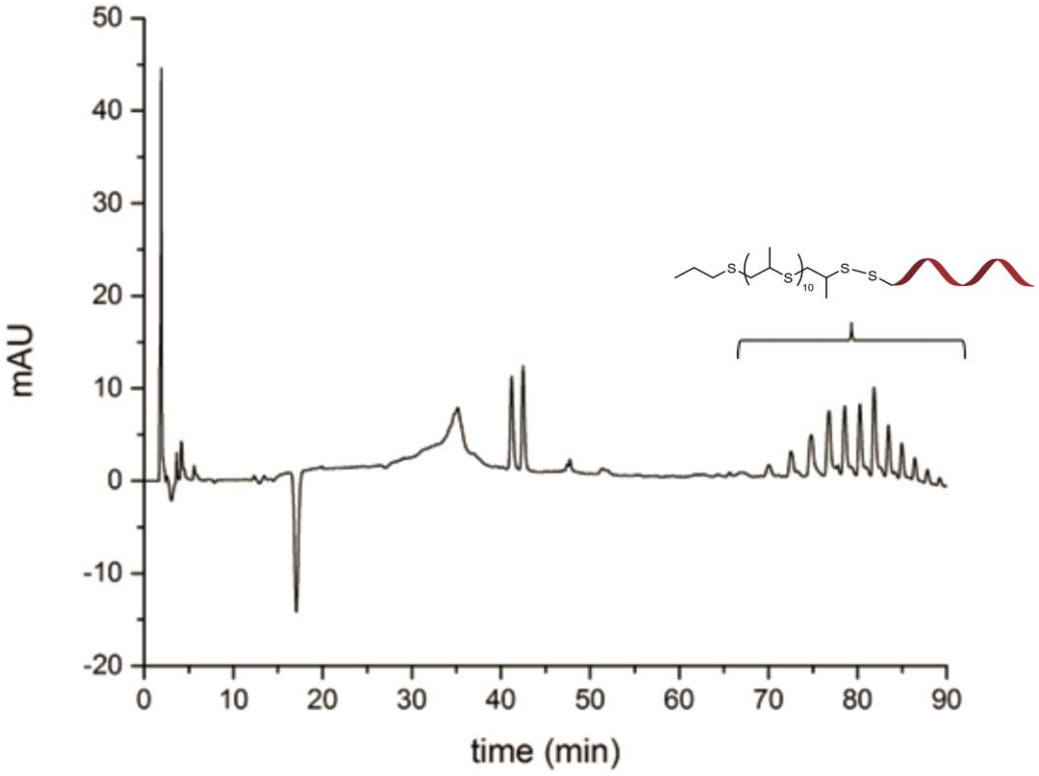

Figure 4. HPLC chromatogram of the coupling reaction for the synthesis of CpG-S-S-PPS. The elution of the product takes place between 70 and 90 minutes.

Alternatively, CpG was conjugated to PEG using the same kind of reaction (Figure 5).

Figure 5. Synthesis of CpG-S-S-PEG

This kind of conjugates can be used in combination with a polycation like branched polyethylenimine (B-PEI) for the formation of polyion complex (PIC) micelles as previously reported (Figure 6).[1]

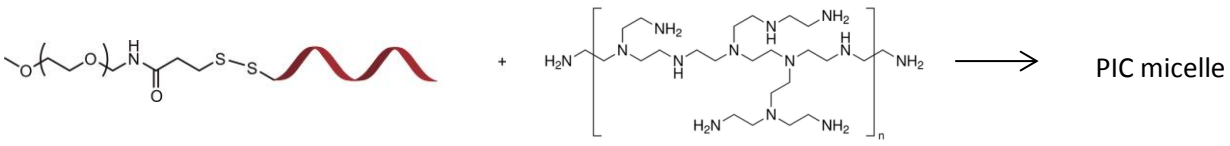

Figure 6. PIC micelle formation by combination of CpG-S-S-PEG with B-PEI

Using this approach the negative charges of the DNA sequence interact with the positive charges of the polycation resulting in the formation of a hydrophobic complex that constitutes the core of the micelle. The PEG chains remain in the outside of the micelle facing the aqueous solution.

The conjugation and purification to PEG-S-S-CpG was found to be easier than PPS-S-S-CpG. After purification of the conjugates by size exclusion chromatography quantitative yields were obtained.

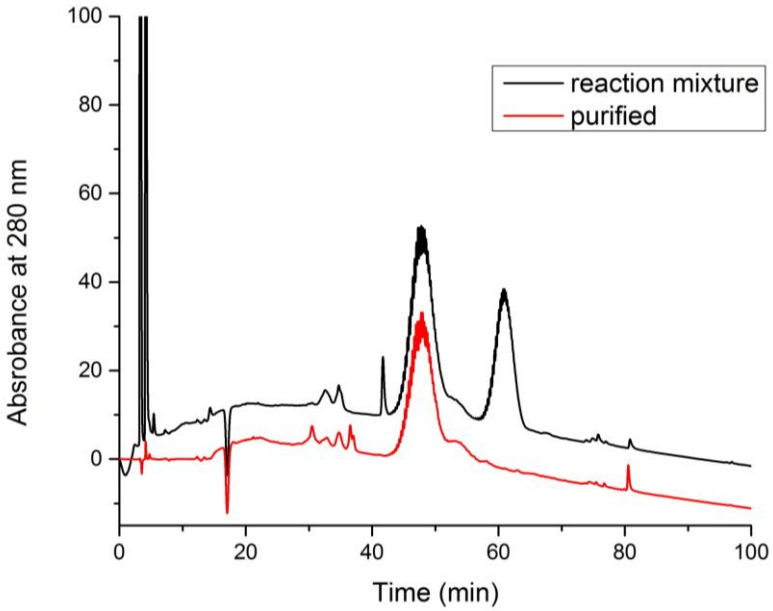

Figure 7. HPLC chromatograms of the coupling between CpG-SH and PEG-S-S-Py (in black) and of the purified product CpG-S-S-PEG (in red). CpG-S-S-PEG elutes between 45 and 52 min.

For the formation of micelles, CpG-S-S-PEG conjugates were mixed with B-PEI. The formation of aggregates is dependent on the number of positive charges added and it is expressed as the N/P molar ratio where N corresponds to the amine groups on the polycation and P to the phosphate groups on the CpG. For this particular conjugate the optimum N/P ratio was found to be 3 as confirmed by dynamic light scattering (DLS) measurements (Figure 8).

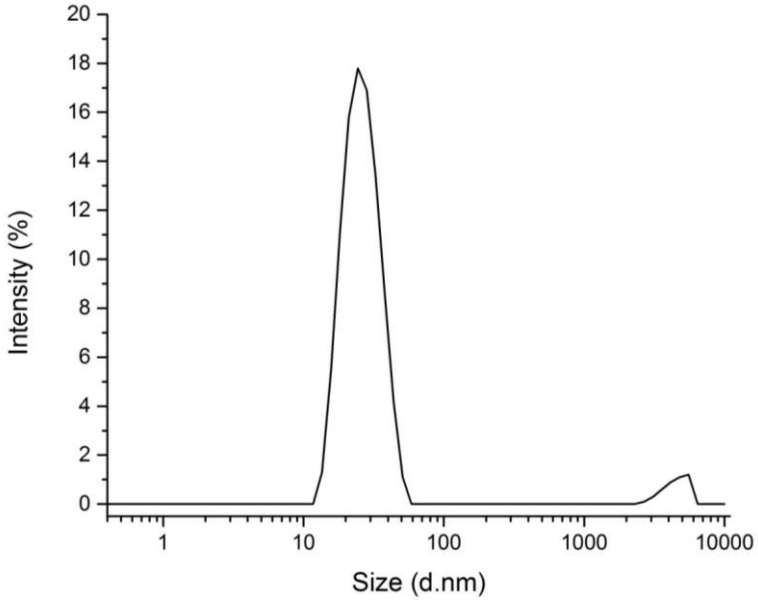

Figure 8. DLS measurement for PIC micelles from CpG-S-S-PEG.

The obtained aggregates possessed an average diameter of ~ 30 nm with a polydispersity index of 0.2. This size of aggregates is perfectly suitable for our intended application since it is known that micelles of a diameter around 50 nm or lower can effectively target resident dendritic cells in the lymph nodes which are antigen presenting cells. The formation of the CpG micelles (MC-CpG) was also confirmed by cryo-TEM (Figure 9).

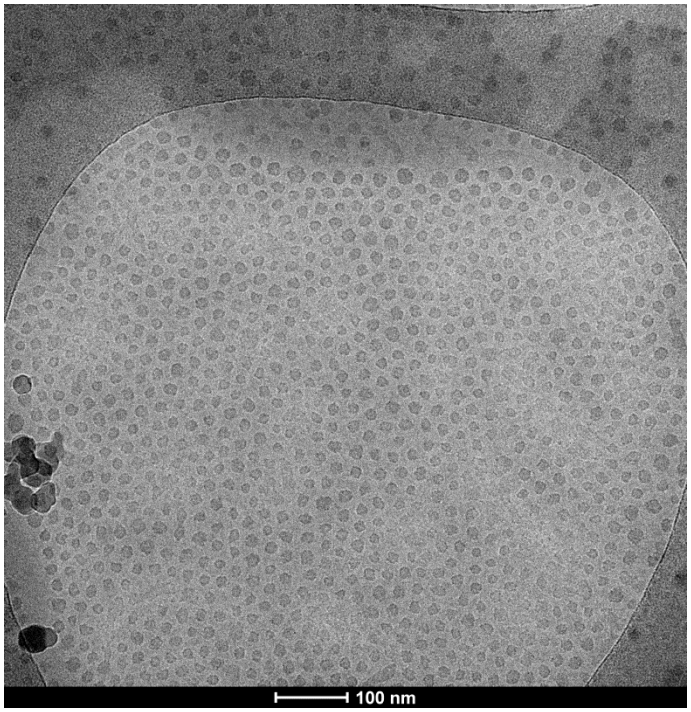

Figure 9. cryo-TEM image of PIC micelles from CpG-S-S-PEG.

In order to study their application as cancer vaccines, *in vivo* studies were performed on mice containing EG7-OVA tumors. These tumor cells express ovalbumin (OVA) epitope as a unique antigent thus, delivering OVA antigen and CpG adjuvant to antigen presenting cells, the production of cytotoxic T lymphocytes can be induced resulting in the destruction of the cancer cells.

Tumor-bearing mice were immunized on days 4 and 11 on the footpad draining the tumor with free OVA + free CpG, free OVA + free C6-CpG, free OVA + MC-CpG or MC-CpG. C6-CpG corresponds to the modified CpG used to synthesize the CpG-S-S-PEG conjugate (Figure 10)

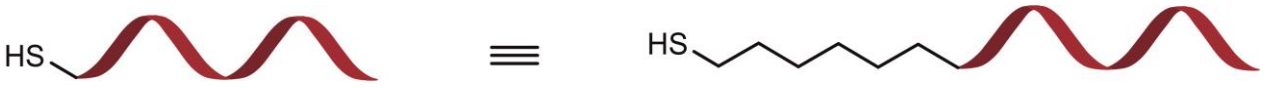

Figure 10. Schematic structure of the thiol-modified CpG (C6-CpG) used in the conjugation and *in vivo* studies.

The amounts of OVA and CpG per injection were 10 µg and 1 µg respectively. The tumor growth was monitored over the period of 21 days and blood samples were taken on days 11 and 14.

Tumor growth curves show a clear effect on the tumor size on mice injected with CpG and OVA when compared to the control group (non-injected mice) (Figure 11).

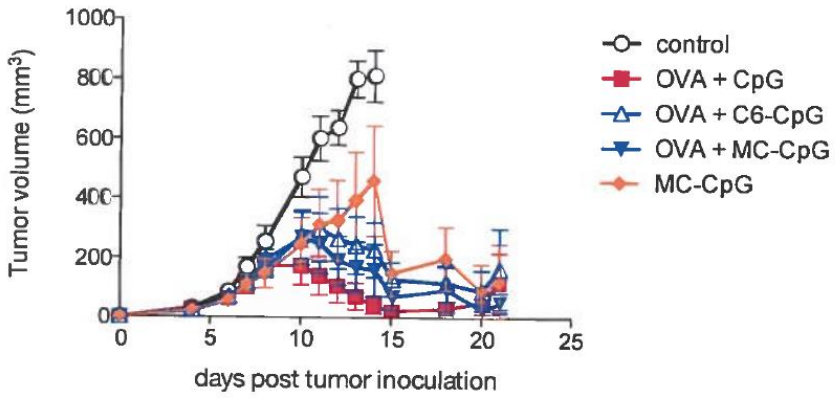

Figure 11. EG7OVA tumor growth curves for the study groups OVA + CpG, OVA + C6-CpG, OVA + MC-CpG and MC-CpG.

Although the tumor shrinkage is clear in all cases, the most effective treatment corresponds to free OVA + free CpG. The results show however that there is an effect in the tumor growth when injecting MC-CpG and that is an encouraging result for further development of the system.

The blood samples were analyzed for the production of antigen-specific T-cells (Figure 12). In all cases the production of T-cells takes place with lower amounts when OVA was not present (sample MC-CpG). These results are also not conclusive because the amount of T-cells produced for samples OVA + CpG and OVA + MC-CpG fall in the same range.

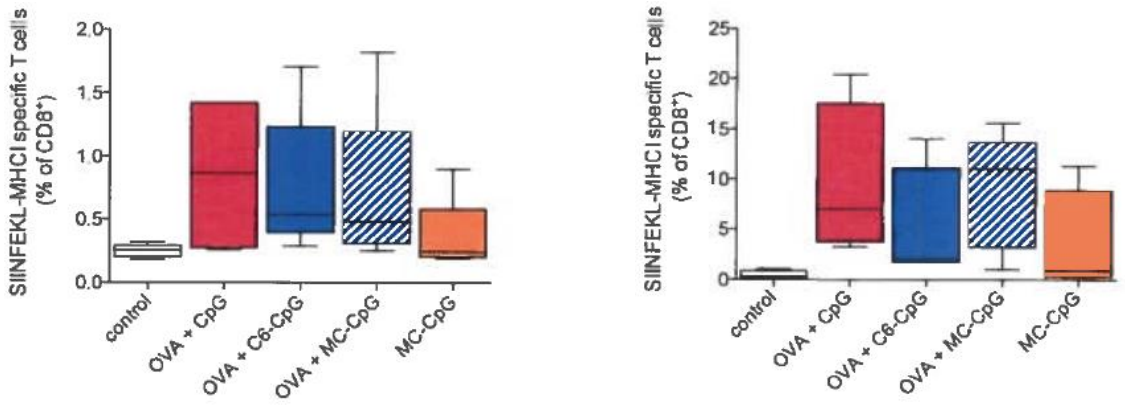

Figure 12. T-cell production on day 11 (left) and day 14 (right) of the treatment.

An often debated issue in the development of nanoparticle-based vaccines is if the co-delivery of antigen and adjuvant in the same delivery system is advantageous for the trigger of the immune responses. Bearing this possibility in mind another delivery system containing both CpG and OVA-derived peptide SIINFEKL was designed. Since the formation of the micelles is a result from the electrostatic interaction between the negative charges on CpG and the positive charges of B-PEI, SIINFEKL peptide was conjugated to CpG to provide the source of charges and therefore be able to incorporate it in the formulation by electrostatic interactions. The conjugation was done following the synthetic route shown in Figure 13.

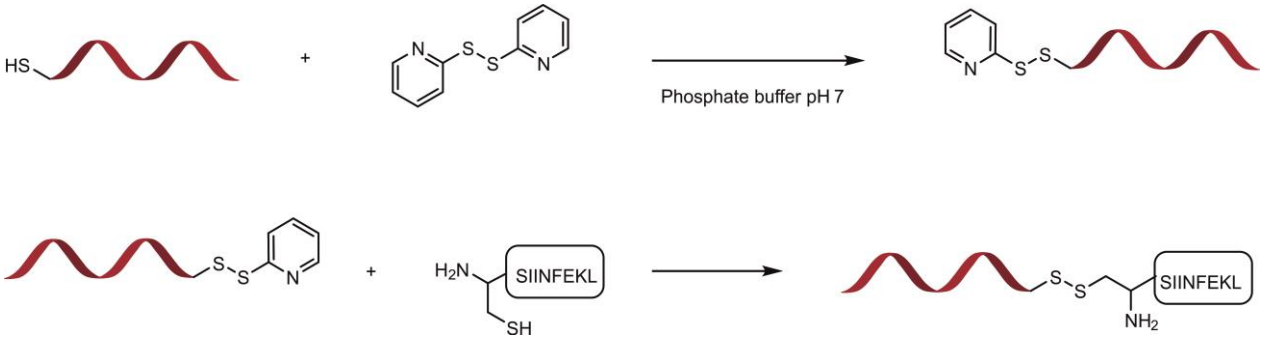

Figure 13. Synthesis of CpG-S-S-SIINFEKL

Micelles were formed by mixing CpG-S-S-PEG and SIINFEKL-S-S-CpG and adding B-PEI in N/P = 2 and were checked by DLS and cryo-TEM (Figure 14). The average size of the micelles was ~ 70 nm.

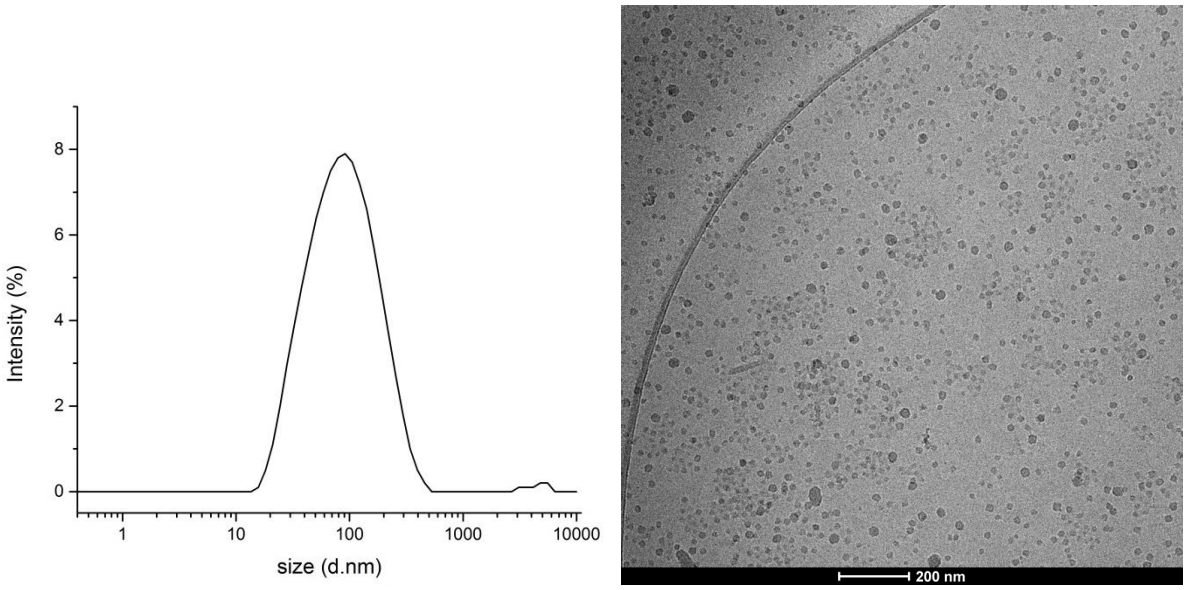

Figure 14. DLS measurement (left) and cryo-TEM image (right) of PIC micelles from CpG-S-S-PEG and CpG-S-S-SIINFEKL

This formulation is under study at the moment using tumor-bearing mice and unfortunately the results are not yet obtained.

These results are preliminary and set the basis for the development of improved vaccines. The research is at the moment being taken a step further using formulations of antigen and adjuvant into the same micelle. Although the design of the system has been deviated from the originally proposed it still falls into the frame of stimuli-responsive micelle-based vaccines using DNA block copolymers. This new design represents an easier approach compared to the initially proposed what is an advantage from the production point of view, a very important point to take into account if considered in the future for commercialization. Furthermore this approach also brings the concept of PIC micelles one step further in their application since until now they were mainly limited to gene therapy purposes.

1 M. Oishi, T. Hayama, Y. Akiyama, S. Takae, A. Harada, Y. Yamasaki, F. Nagatsugi, S. Sasaki, Y. Nagasaki, K. Kataoka, Biomacromol. 2005, 6, 2449

4. DISEMINATION ACTIVITIES

The researcher wrote and submitted a review article that got accepted in the journal « Annual Review of Chemical and Biomolecular Engineering » being her the first author of the manuscript:

N. Sancho Oltra, P. Nair, D. E. Discher, From Stealthy Polymersomes and Filomicelles to ‘Self’ Peptide-Nanoparticles for cancer therapy, *Annu. Rev. Chem. Biomol. Eng.* 2014, vol. 5, 281

Furthermore, the work carried out using redox-sensitive worm-like micelles from PEG and PCL has been written into a manuscript by the researcher and it is being revised and edited by the co-authors of the work for submission to a high impact peer-reviewed journal.

The researcher was also selected shortly before the end of the outgoing phase of the project to attend the prestigious Lindau Nobel Laureate Meeting which took place from 29th June until 4th July 2013. During this meeting the work developed by the researcher was presented via de Marie Curie Actions frame. Furthermore, the researcher took active part during the meeting by contributing as a video-blogger discussing what the participants of the event can learn from each other:

http://www.youtube.com/watch?v=8add95BDYAg

Further dissemination activities involved internal presentations within EPFL where the researcher presented her results in a regular basis.

5. PROJECT MANAGEMENT

The work performed during the period reported in this document has been slightly deviated from the original proposal. The most important deviations include the linkage moiety between the DNA and the polymer in the block copolymer synthesized and the nature of the polymer. Originally it was proposed to link both molecules with a photocleavable moiety to trigger the disassembly of the micelles by irradiation with light. Although the synthesis of such molecule has also been (and still is) developed in parallel, the project has focused in the use of a disulfide bond as the linkage. The reason for choosing this linkage involves on one hand the work performed during the outgoing phase of the project where also this linkage was used and the fact that this linkage is used extensively in the group of Prof. Hubbell for the development of nanoparticle-based vaccines. The standing experience of the group and of the researcher using this kind of linkage favored the faster development of a stimulus-triggered system that can have relevance in the field of vaccines. Considering the nature of the polymer used, the proposal suggested the use of hydrophobic polymers for the formation of micelles where the polymer would be the constituent of the core. That approach was followed in the beginning using polypropylene sulfide (PPS) which is the polymer used in the nanoparticle vaccines developed in Prof. Hubbell's lab. As explained in the section « Work progress and achievements during the period » the use of this polymer resulted in the desired DNA block copolymer but a limited yield of pure material was obtained. Due to the better solubility of poly(ethylene glycol) (PEG) in water and therefore easier handling, the strategy was shifted to DNA block copolymers containing PEG. These alterations in the original plan did not affect the overall goal of the project since a new kind of stimulus-responsive vaccine based on DNA block copolymers was developed and the planned steps were followed in the same way as proposed : synthesis, purification, characterization, assembly and testing.

With reference to the ethical issues of the project, the date for the deliverable of the animal protocol for the experiments performed at EPFL was different to the one proposed. The reason for this was that the

material necessary for performing the experiments was obtained in a later stage than expected due to the necessary modifications in the polymer therefore, the *in vivo* studies were also done in a later stage. The approved protocol was sent to Mr. Bode on July 1st and is attached also in this report. The *in vivo* experiments were done by Dr. Laura Jeanbart from Prof. Swartz's group with which Prof. Hubbell's laboratory collaborates closely. The protocol followed was approved for the development of nanoparticle-based vaccines as therapies against cancer and the *in vivo* experimentation of the project reported here was incorporated into that frame.