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EXECUTIVE SUMMARY OF THE THESIS

pH-sensitive tracers for fluorescence-guided glioblastoma surgery

TESI MAGISTRALE IN MATERIALS ENGINEERING AND NANOTECHNOLOGY – INGEGNERIA DEI MATERIALI E DELLE NANOTECNOLOGIE

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1. Introduction

Glioblastoma multiforme (GBM) is the most common primary brain cancer and patients inevitably experience a high recurrence rate [1]. Since ordinary effective therapies to cure GBM are not available yet, surgical resection represents the first crucial step of the treatment to limit cancer relapse [2]. Several measures are used to help the surgeon performing the procedure as precisely as possible and some of the recent advances involve the fluorescent intraoperative navigation. Fluorescein (FL) is one of the most ubiquitous probes and it is already used in clinical practice. However, the main limitation is lack of specificity for cancer cells [3].

The purpose of this project was to target the acidic tumor microenvironment by exploiting the pH-sensitive properties of pH (low) insertion peptides (pHLIPs). Being moderately hydrophobic, pHLIPs have a modest affinity for cellular membranes at physiological pH, but fold and insert across the phospholipid bilayer only at lower pH [4]. In this study, pHLIPs modified at an edge with an additional cysteine were directly conjugated to a Fluorescein-maleimide derivative (FL-pHLIP) in order to develop a specific fluorescent tracer able to more selectively label cancer cells during glioblastoma surgical resection.

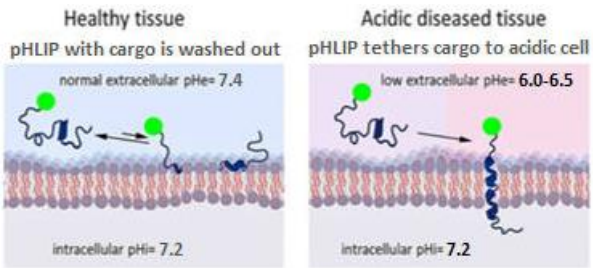

Figure 1: Scheme representing FL-pHLIP insertion into the cellular lipid bilayer in acidic diseased tissues (right), but not in healthy cells at physiological pH (left) [5].

To evaluate the behavior of this novel macromolecule and verify that the linkage with the fluorescent dye did not affect the pH-dependent insertion properties, we characterized both Wild Type (WT) and FL- pHLIP derivatives from a

chemical-physical perspective. The results on peptide stability and self-assembly are reported together with a study of pHLIP interaction with model systems of the cellular membrane, such as liposomes and supported lipid bilayers (SLBs).

Finally, FL-pHLIP tolerance and labelling efficacy were assessed by *in vitro* cellular tests performed on three different patient-derived glioblastoma primary cell lines. Encouraging results were obtained with the effective and specific targeting of proliferative and mesenchymal cells subtypes.

2. Materials and methods

All pHLIP peptides (WT: AEQNPIY WARYADWL FTTPLLLLDLALLVDAD EGT; FL-derivative: AC EQNPIY WARYADWLFTTPLLLLDLALLVDAD EGT, and SC-FL construct: ACEQNPIY WARYAK WLFTTPLLLLKLALLVDAK EGT) were kindly synthesized by Dr. Alessandro Gori from National Research Council (CNR).

pHLIP aqueous solutions. 0.1 M PBS was added to either WT or FL- pHLIP at physiological pH and strongly mixed by the use of a vortex. The resulting solutions (100 μM) were heated at 35 °C for 30 minutes and fully dissolved using an ultrasonic bath (5 minutes at 59 kHz). This procedure turned out suitable for completely dissolving both peptides and all the analyses were then performed on 5 μM pHLIP solutions prepared by diluting the stock 1:20 v/v in either 0.1 M Acetate buffer (pH 5.2) or 0.1 M PBS (pH 6.5 and 7.4).

DLS. For both WT and FL- pHLIPs stability assay, 90° Dynamic Light Scattering (DLS) analyses were performed on 0,8 mL of peptide solution (5 μM) at different pH values (7.4, 6.5 and 5.2).

Liposomes preparation. DOPC (20 mg) was dissolved in 1 mL of chloroform and the solvent was removed by rotary evaporation at 30°C. The hydration of the obtained dry lipid film was accomplished by adding 1 mL of either 0.1 M PBS or 0.1 M Acetate buffer. Freeze-thaw cycles were repeated for 5 times. The resulting liposomes solution was extruded through 200 nm and then 100 nm polycarbonate filters (11 times/each filter) and finally stored at + 4°C.

CD and intrinsic fluorescence. pHLIPs (5 μM) with DOPC liposomes (750 μM) solutions were measured at different pH values (7.4, 6.5 and 5.2). Following the procedure described by T. Crawford *et al*. [6], Circular Dichroism (CD) spectra were recorded in a wavelength range between 200 and 260 nm, with steps of 1 nm and a scanning speed of 100 nm/min. Fluorescence spectra were recorded from 310 to 400 nm using an excitation wavelength of 280 nm.

Sephadex G-25 column. 1 mL of DOPC liposomes (750 μM) solutions, incubated with FL-pHLIP (5 μM) for different times (30 min, 2 h and 24 h) at different pH values (7.4, 6.5 and 5.2), were purified from the non-interacting peptides by following the gravity protocol of a PD-10 Desalting Column containing Sephadex G-25 resin (exclusion limit Mr 50000). The eluate collected was analyzed by DLS and the fractions containing liposomes were pooled and concentrated in centrifuge for 10-15 minutes at 4000 rpm and 25°C by using Amicon® Ultra filters (50 kDa cutoff). UV-Vis analyses were performed in a wavelength range between 200 and 800 nm before and after liposomes purification.

QCM-D. Quartz crystal microbalance with dissipation (QCM-D) analyses were performed on both WT and FL- pHLIPs solutions (5 or 0.5 μM) flowing onto supported lipid bilayers (SLBs) and then left in static incubation (30 or 15 minutes each step). The SLBs were formed on silicon dioxide functionalized QCM-D chips by adding 3 mL of calcium chloride to an equal volume of DOPC liposome solution (750 μM) and insufflating for 8 minutes. 5-minutes flows of alternated buffer and MilliQ water were performed to stabilize the bilayer and prepare the proper peptide environment. In particular, 0.1 M Acetate, 0.1 M TRIS buffer and 0.1 M PBS were used for analyses at pH 5.2, 6.5, and 7.4, respectively.

***In vitro* cellular tests.** FL-pHLIP tolerance and labelling efficacy were evaluated by 7-Aminoactinomycin D (7-AAD) staining and Flow cytometry assay (FACS). Proliferative (BT 592), mesenchymal (BT 1007) and proneural/ mesenchymal derived from cancer recurrence (GBMR16-NS) glioblastoma primary cell lines were kindly provided by Dr. Pellegatta from Carlo Besta Neurological Institute. Cells were seeded at a density of 100k cells/well in a 12-well plate and incubated with the peptide for 2 hours at different concentrations (0.5, 5 and 10 μM). Both acid (pH 6.4 – 6.7) and physiological conditions (pH 7.2 – 7.4) were investigated. Labelled cells were then collected and washed in abundant culture medium (3 × 1000 rpm for 5 min).

3. Results and discussion

3.1. pHLIP colloidal stability

Data obtained from DLS analyses at 90° showed that both WT and FL- peptides formed smaller assemblies at physiological pH that did not evolve over time. Furthermore, at slightly acidic pH (6.5) the FL derivative was more stable and did not change its dimensions at longer timings. Anyway, both pHLIPs tended to aggregate in highly acidic environment (pH 5.2). In fact, the decay of the autocorrelation function shifted towards higher times and the DLS profile moved to larger sizes (Figure 2). Representative DLS graphs are reported only for the FL derivative, however those of WT pHLIP are similar (see details in the main text).

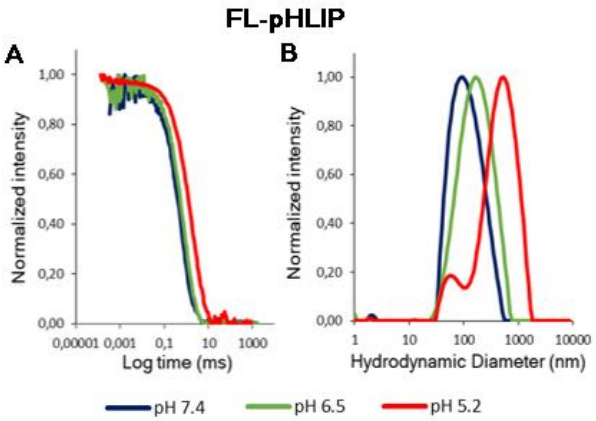

Figure 2: DLS experiments performed at 90°: auto-correlation functions (A) and unweighted size distributions (B) of FL-pHLIP measured at different pH values in aqueous buffers at 22°C.

These results confirmed the increment of hydrophobicity when pHLIPs were exposed to acidic conditions as the net charge was minimized, and the reduced electrostatic repulsion between peptide molecules enhanced their aggregation. However, pHLIP WT aggregated more than FL-pHLIP suggesting that the steric effects related to the presence of Fluorescein could have moderately increased the peptide stability in solution.

3.2. Membrane insertion properties

The interaction between pHLIPs and DOPC liposomes was first evaluated by intrinsic tryptophan (Trp) fluorescence and CD analyses. For both WT and FL- pHLIPs, the wavelength of maximum emission for Trp shifted towards lower values reducing the pH, indicating a progressively more hydrophobic environment around the Trp residues due to their increasing insertion into the lipid bilayer (Figure 3). In case of pHLIP WT, this behavior was also confirmed by the less effective quenching of Trp fluorescence. In fact, when Trp is in the excited state, it appears to be highly sensitive to collisional quenching thanks to the tendency of indole rings to donate electrons [7]. In this process, the excited fluorophore reacts with external molecules (quenchers) to form an excited product that decays by a nonradiative pathway and therefore diminishes the fluorescence quantum yield. For this reason, Trp emission intensity is strongly decreased in presence of charged water-soluble molecules, while the fluorescence of the membrane-inserted residue is less affected by the quenchers [7]. On the contrary, the fluorescence intensity of FL-pHLIP was reduced at acidic pH as a consequence of FL ionization equilibrium (pKa = 6.4) [8].

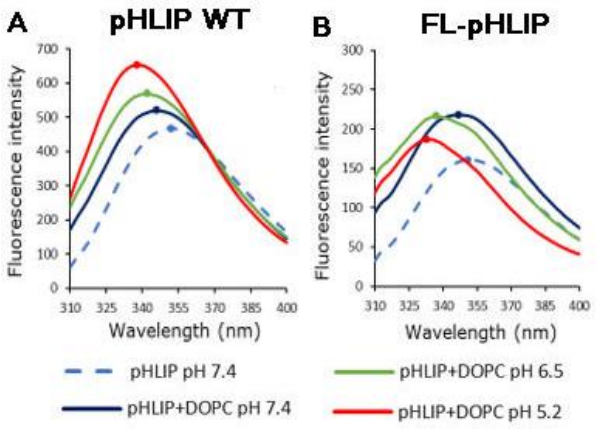

Figure 3: Fluorescence spectra of pHLIP WT (A) and FL-pHLIP (B) measured at different pH values and in presence of DOPC liposomes.

To further prove the pHLIP insertion into liposome-membranes and the formation of the α-helix, the peptide three-dimensional conformation was characterized through CD analysis. Both WT and FL-pHLIPs exhibited a random coil conformation in physiological conditions that progressively changed into an α-helix secondary structure reducing the pH (Figure 4). In fact, a double minimum at 208 and 222 nm could be clearly observed at pH 5.2. This means that the

conjugation with FL does not influence the membrane insertion properties of the peptide.

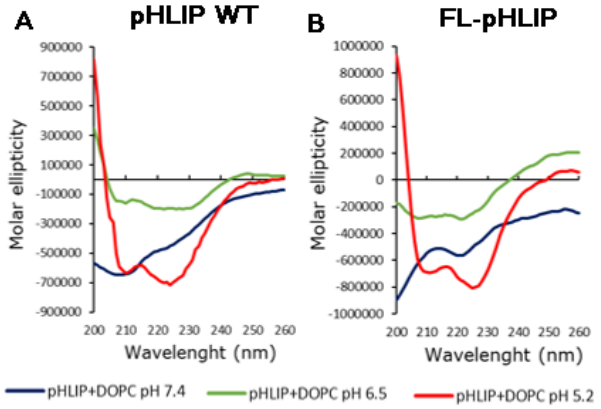

Figure 4: CD profiles of pHLIP WT (A) and FL-pHLIP (B) in presence of DOPC liposomes measured at different pH values in aqueous buffers at RT.

3.3. Quantification of inserted pHLIP

With the purpose of quantifying the amount of inserted FL-pHLIP, DOPC liposomes were incubated with the peptide at different pH values (7.4, 6.5, and 5.2) and purified from the excess of unbound peptide by following the gravity protocol of a Sephadex G-25 column. UV-Vis analyses were performed before and after sample purification to evaluate the decrease in intensity of the peak related to the dye (between 450 and 500 nm).

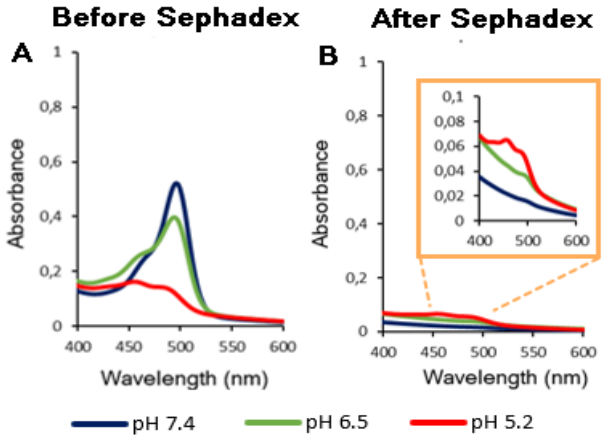

Figure 5: UV-Vis absorption spectra of DOPC liposomes incubated with FL-pHLIP before (A) and after purification (B) at different pH values in aqueous buffer at RT.

Although Fluorescein absorbance generally decreases in acidic environment (Figure 5 A), after purification a higher intensity was observed for lower pHs indicating a clear insertion into liposomes, with respect to physiological conditions where no peak was detected (Figure 5 B).

Comparing FL-pHLIP concentrations before and after Sephadex purification, we calculated the percentage of inserted peptide, which was higher at lower pHs (Figure 6). In particular, at pH 6.5 the amount of FL-pHLIP progressively increased from 14.3% to 17.2% by performing a longer incubation time. Instead, in highly acidic environment (5.2) a considerable content of FL-pHLIP (40%) entered the membrane immediately after 30 minutes and the value remained constant for hours until reaching a maximum of 57% after one day.

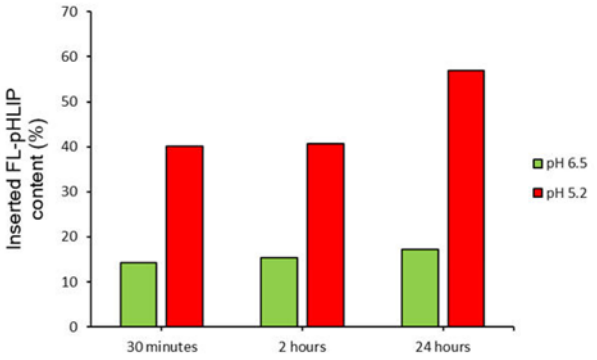

Figure 6: UV-Vis quantification of inserted FL-pHLIP into DOPC liposomes after purification at pH 6.5 (green) and 5.2 (red) at different incubation times.

3.4. Eukaryotic cells membrane in-flow model

To deeply investigate pHLIP sensitivity in a context closer to the cellular environment, we proposed an in-flow mimic model system of the lipid membrane based on supported lipid bilayers (SLBs). In particular, pHLIP-SLB interaction was observed by quartz crystal microbalance with dissipation monitoring (QCM-D) experiments. After the introduction of both WT or FL- pHLIPs in the SLB-based system at physiological pH, just a slight increase in the dissipation occurred indicating a mild interaction between peptides and lipid bilayer. However, during the final washing in

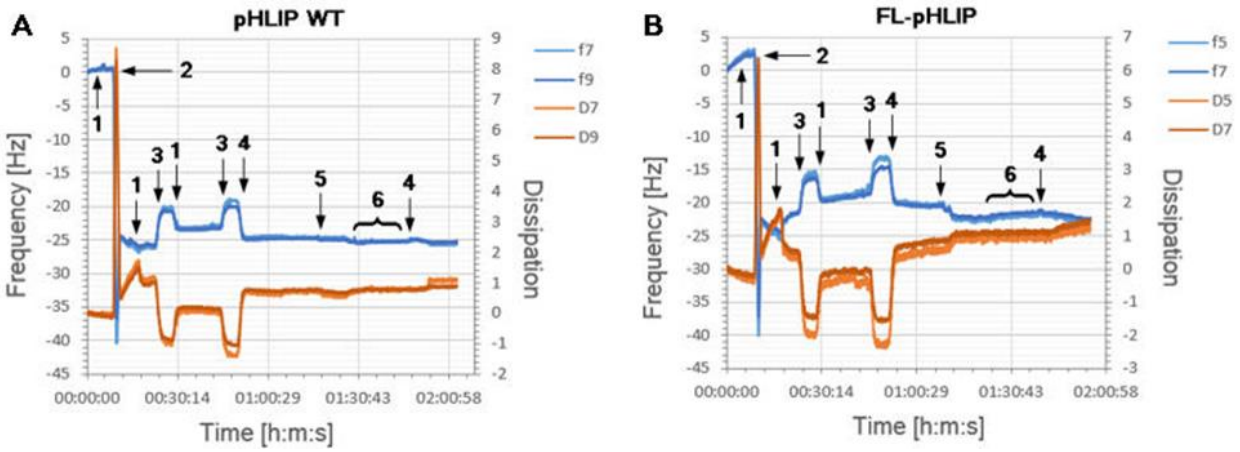

Figure 7: Representative QCM-D measurements of DOPC SLBs incubated with the WT (A) and FL- (B) pHLIP at pH 6.5: dissipation (red line) and frequency (blue line). The numbers indicate the flow of the following solutions: 1 = PBS 0.1 M pH 7.4, 2 = Liposomes solution + CaCl2, 3 = MilliQ water, 4 = TRIS 0.1 M pH 6.5, 5 = pHLIP solution (0.5 μM), 6 = Static incubation.

PBS, the original value was restored meaning that the pHLIP was only weakly adsorbed onto the SLB and easily removed by washing.

Analogous experiments were also performed at very low pH (5.2). Here, the increase in dissipation and decrease in frequency upon either WT or FL-pHLIP insufflation were significant, indicating a strong interaction with the SLB. Moreover, the peptides were not removed during the washings suggesting a stable insertion into the lipid bilayer. Interestingly, if the final washing was performed using a buffer at physiological pH, the deposited pHLIP was completely lost, demonstrating a reversibility of the insertion process.

To better simulate the extracellular pH value expected in GBM tumor cells, we also examined a slightly less acidic environment (pH 6.5). Both WT and FL- peptides insufflation caused again an increase in dissipation and a decrease in frequency, implying a considerable pHLIP-SLB interaction (Figure 7, arrow 5). Importantly, these trends were not reversed during the final buffer washing meaning that the interacting peptides were permanently inserted into the lipid bilayer. These encouraging results confirmed that the proposed system works well in pH conditions consistent with glioblastoma tumor extracellular environment and that the FL-conjugation does not affect either the affinity of the peptide for the lipid bilayer at pH 7.4 or its insertion properties in acid environment even when a dynamic model is considered.

These results were further confirmed by the mass deposition quantification. Specifically, in the case of pHLIP WT a less deposited mass was detected either reducing the concentration (from 5 to 0.5 μM) or the incubation time (from 30 to 15 minutes) (Figure 8 A). While the amounts of deposited WT peptide mass at low concentration and fast dynamics were comparable both at pH 6.5 and 5.2, for FL-pHLIP the mass at lower pH (5.2) was much higher compared to slightly acidic conditions (pH 6.5) (Figure 8 B).

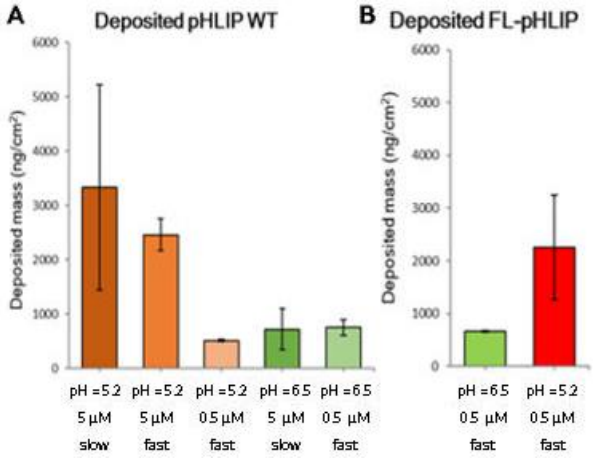

Figure 8: Quantification of deposited WT (A) and FL- (B) pHLIPs mass during QCM-D analysis at pH 5.2 (red) and 6.5 (green) in different experimental conditions.

Anyway, the reduced concentration (0.5 μM) was sufficient to detect the peptide-lipids interaction for both WT and FL- pHLIPs. Of note, the great standard deviation observed at pH 5.2 could be

associated to aggregation phenomena. Therefore, working at non-excessive pHLIP doses and shorter incubation times could help to avoid peptide-lipids non-specific interactions and aggregation.

3.5. *In vitro* cellular tests

FL-pHLIP labelling efficiency and cytotoxicity were evaluated on three different patient-derived glioblastoma primary cells (BT 592, BT 1007, and GBMR16-NS). Cells were incubated for 2 hours with the FL-peptide at different concentrations at both physiological and acidic pHs. A scrambled peptide (SC-FL pHLIP) that was not pH-sensitive was used as a control. Results related to peptides cellular tolerance showed an almost negligible cytotoxicity as the cellular viability after incubation remained in an acceptable range comparable to the untreated cells, especially for the mesenchymal (BT 1007) (Figure 9) and proneural (GBMR16-NS) subtypes.

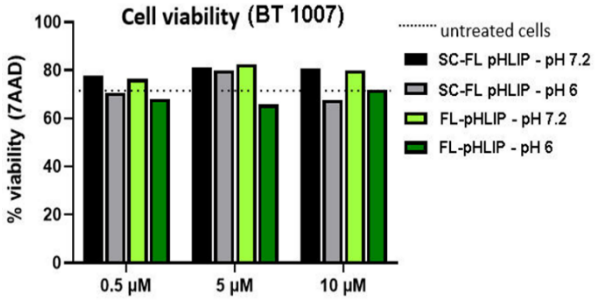

Figure 9: Viability of mesenchymal (BT 1007) subtype of glioblastoma cells incubated for 2 hours with FL- and SC-FL pHLIPs measured at different pHs by FACS.

FACS analyses regarding the cellular positivity to Fluorescein showed that FL- and SC-FL pHLIPs have a good affinity for the cellular membrane even at physiological pH for all the cell lines. This result was expected considering the hydrophobic residues that are present in pHLIPs. However, the SC-FL pHLIP exhibited either a similar affinity in both physiological and acidic environments, or an opposite tendency with respect to pHLIP depending on the concentration (Figure 10 A), confirming that the peptide was not pH-sensitive. On the other hand, FL-pHLIP presented the expected trend as cellular positivity to FL increased in acidic conditions, especially in the range of concentrations from 0.5 to 5 µM. In particular, the most relevant result was obtained for BT 1007 cells using a peptide concentration of 0.5 µM (Figure 10 B). Here, the cell labelling at physiological pH was minimal, and thus useful to avoid unspecific targeting. On the contrary, FL-positivity at pH 6 reached a very high value meaning that the contrast between marked and unmarked cells was really evident. Only GBMR16-NS subtype presented an ambiguous behavior that may be attributed to the high response variability of the recurrent cell lines.

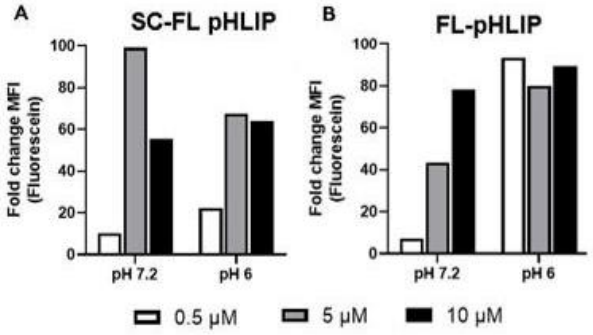

Figure 10: FL-positivity of mesenchymal subtype of primary glioblastoma cells (BT 1007) incubated for 2 hours with SC-FL pHLIP (A) and FL-pHLIP (B) measured at different pH values by FACS.

4. Conclusions

FL-pHLIP was characterized from a chemical-physical perspective as a novel fluorescent tracer able to target the acidic tumor microenvironment. The comparison with the WT derivative successfully demonstrated that the conjugation with the fluorophore did not affect the peptide properties and in particular the pH-dependent insertion into cellular membranes. In detail, we developed a solubilization procedure and verified the enhanced hydrophobicity due to protonation in acidic environment by DLS analysis. Data obtained from the evaluation of the intrinsic tryptophan fluorescence proved the increasing insertion of the residues inside a lipid bilayer by reducing the pH and CD measurements confirmed the formation of the transmembrane α-helix. As a further proof, the percentage of inserted FL-pHLIP into liposomes increased for more acidic conditions. Even when an in-flow model was considered to better mimic a real cellular environment, a quantifiable mass of deposited pHLIP onto SLBs was detected by QCM-D only at

low pH values (6.5 and 5.2). Motivated by these encouraging results, further QCM-D experiments will be performed by flowing the peptides into cell culture media to evaluate possible competition processes. Moreover, fluorescence microscopy experiments on SLB incubated with both FL-pHLIP and SC-pHLIP are planned in order to better elucidate how the peptide insertion affects the lipid bilayer.

Finally, FL-pHLIP compatibility was assessed on three different lines of patient-derived glioblastoma primary cells. Importantly, data obtained from cellular positivity to FL analyzed by FACS suggested that FL-pHLIP could be effectively used as fluorescent tracer for proliferative (BT 592) and mesenchymal (BT 1007) cells subtypes. In particular, the most relevant result was obtained by the high labelling efficacy on BT 1007 at a peptide concentration of 0.5 μM. For this reason, further in-depth analyses will be carried out on this cell line in order to evaluate all the benefits that FL-pHLIP can offer as an imaging agent. Specifically, confocal microscopy studies will be conducted to better understand the peptide folding.

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5. Acknowledgements

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