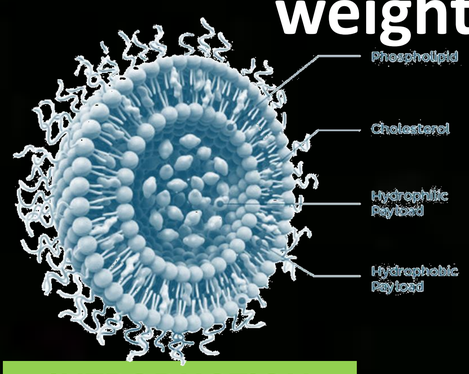
Synthesis of novel reactive MALDI matrices for the analysis of low molecular weight metabolites in hypoxic pancreatic cancer extracellular vesicles



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INTRODUCTION

Applying matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) to low molecular weight compounds has remained challenging due to the spectral interferences of ionizable conventional organic matrices in the low m/z range. To overcome this issue, alternative methodologies based on rationally designed organic matrices have been proposed[1]. The purpose of this study was to synthesize a new functionalized MALDI matrix able to react with small compounds, such as amines or alcohols, with the aim of analyzing these low-mass compounds in a biological sample avoiding interfering matrix background. The study has been performed on nanovesicle (NV) samples obtained from cellular line PANC1 (immortalized PDAC cells), collected and filtered through 100kDa MWCO filters. A total exosome isolation solution was added and left to react overnight and then vesicles were collected through exospin columns. Low molecular weight (LMW) compounds were extracted from NVs by following two rapid protocols on cell pellet and the samples were analysed by MALDI and hydrophilic interaction liquid chromatography (HILIC) coupled with electrospray ionization high-resolution Fourier-transform mass spectrometry (ESI-FTMS) to confirm structural identity.

EXPERIMENTAL

The functionalization was carried out by nucleophilic substitution of two different p-hydroxy compounds, 4-hydroxybenzoic acid (**4-HBA**) and alpha-cyano-4-hydroxycinnamic acid (**CHCA**), with a 2,4,6-trichloro-1,3,5-triazine (**TCT**) in the presence of a base[2]. In our experiments, we first carried out the reaction between CHCA or HBA and TCT exploiting the presence of -OH residue on the matrix. The product was characterized by NMR, ATR_FTIR, LDI-MS and LC-MS/MS

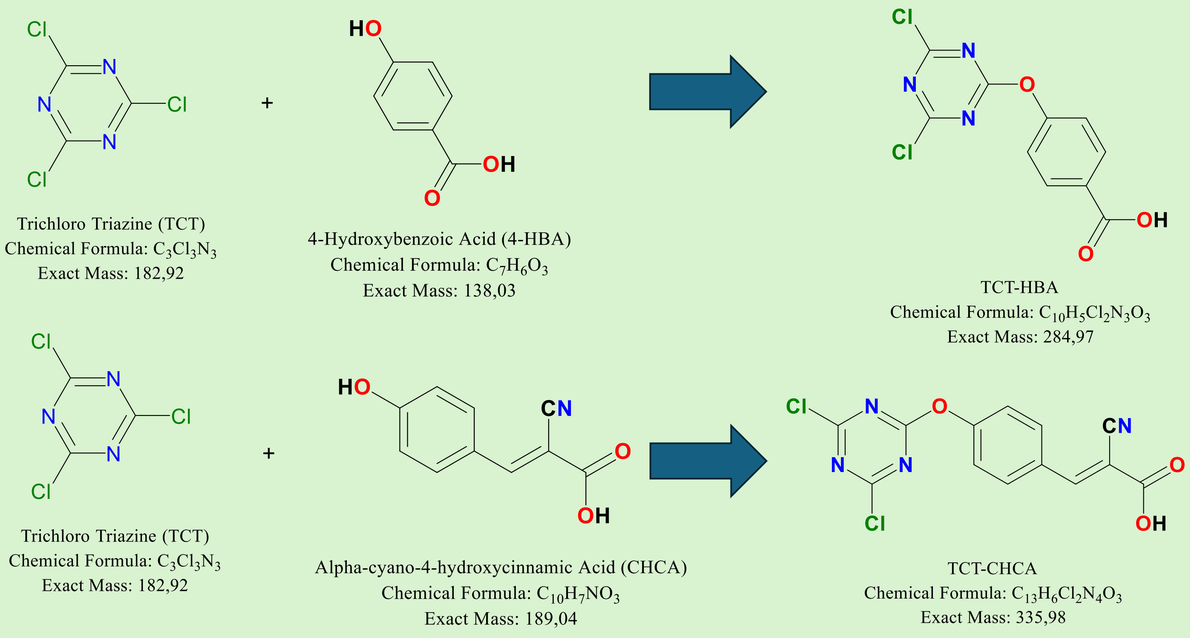


***Figure 1**: Reaction of TCT with 4-HBA and CHCA*

Characterization

MALDI MS

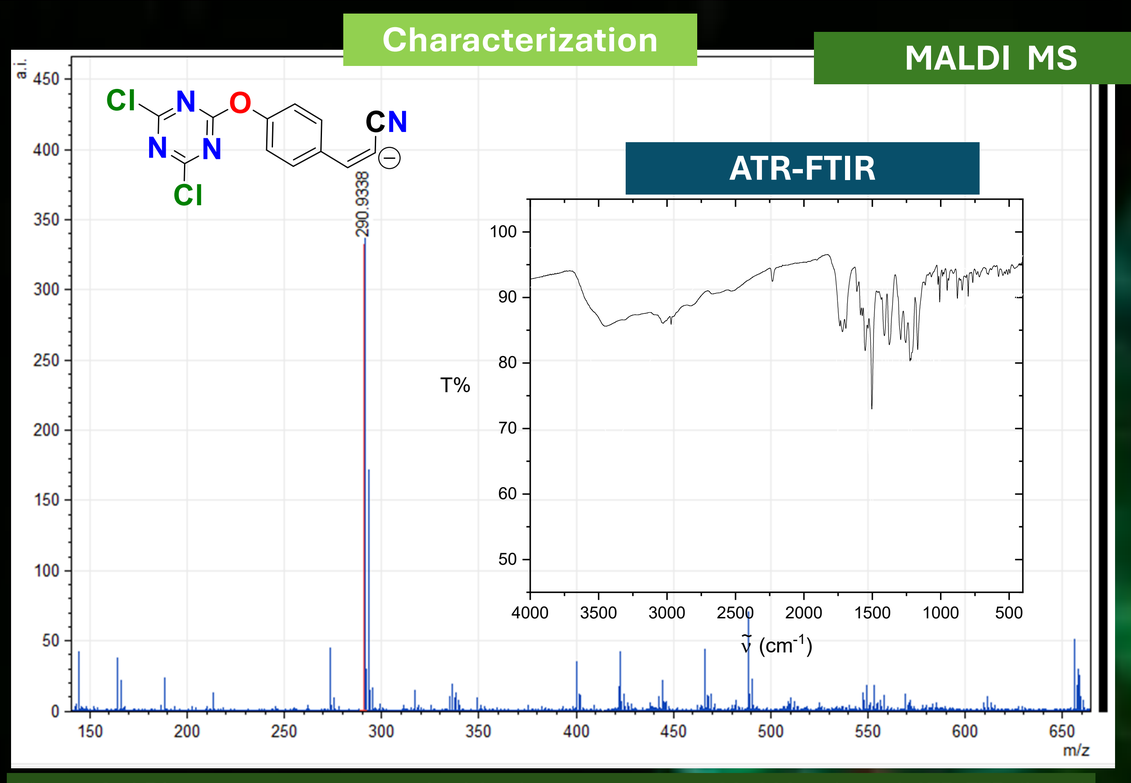


Figure 2: LDI mass spectrum acquired in the mass range 100–800 m/z and ATR-FTR (inset) relevant to TCT-CHCA.

RESULTS

Fig. 2 shows the LDI mass spectrum relevant to TCT-CHCA acquired in the mass range 100-800 m/z. The main peak, observed at *m/z* 290.93, was assigned to the decarboxylated residue $[M\text{-}CO_2\text{-}H]^-$ of TCT-CHCA. ATR-FTIR spectrum confirmed also the successful reaction between TCT and CHCA since typical vibrations of C-Cl stretching produce strong bands in the region 710-505 cm^{-1} alongside typical C-N, C=N and C=O stretching bands in the range 1700-1400 cm^{-1}. Also, NMR spectroscopy confirmed these findings. Trichloro-triazine, also known as cyanuric chloride, has reported a marked reactivity toward hydroxyl and amine groups[3]. Thus, the other two chlorines on **TCT-CHCA** or **TCT-HBA** can further react with amines or alcohols allowing to increase the molecular weight of these small compounds towards higher *m/z* ratio. The newly obtained compounds were tested with different amino acids (Fig. 3) such as phenylalanine, glycine and isoleucine and to a complex mixture of amino acids-based supplements, which contained mainly valine, leucine and isoleucine. Further the matrix was employed for **NV cells** after applying two protocols, the first (I) based on one phase methanol followed by ACN and the second one (II) based only on a mixture of MeOH/H_2O 95/5 (v/v). Figure 4 shows the MALDI spectra of the two extracts highlighting as the first protocol (upper) was more informative. As evident, MALDI spectra reported the occurrence of many peaks; the typical isotopic pattern of chlorine allowed to establish that derivatization with reactive matrix was likely. Most peaks have been assigned thanks to the high mass accuracy of Orbitrap to amino acids such as leucine, phenylalanine, methionine, and other LMW biomolecules as alcohols, amides, amino acids, carboxylic acids, and sugars.

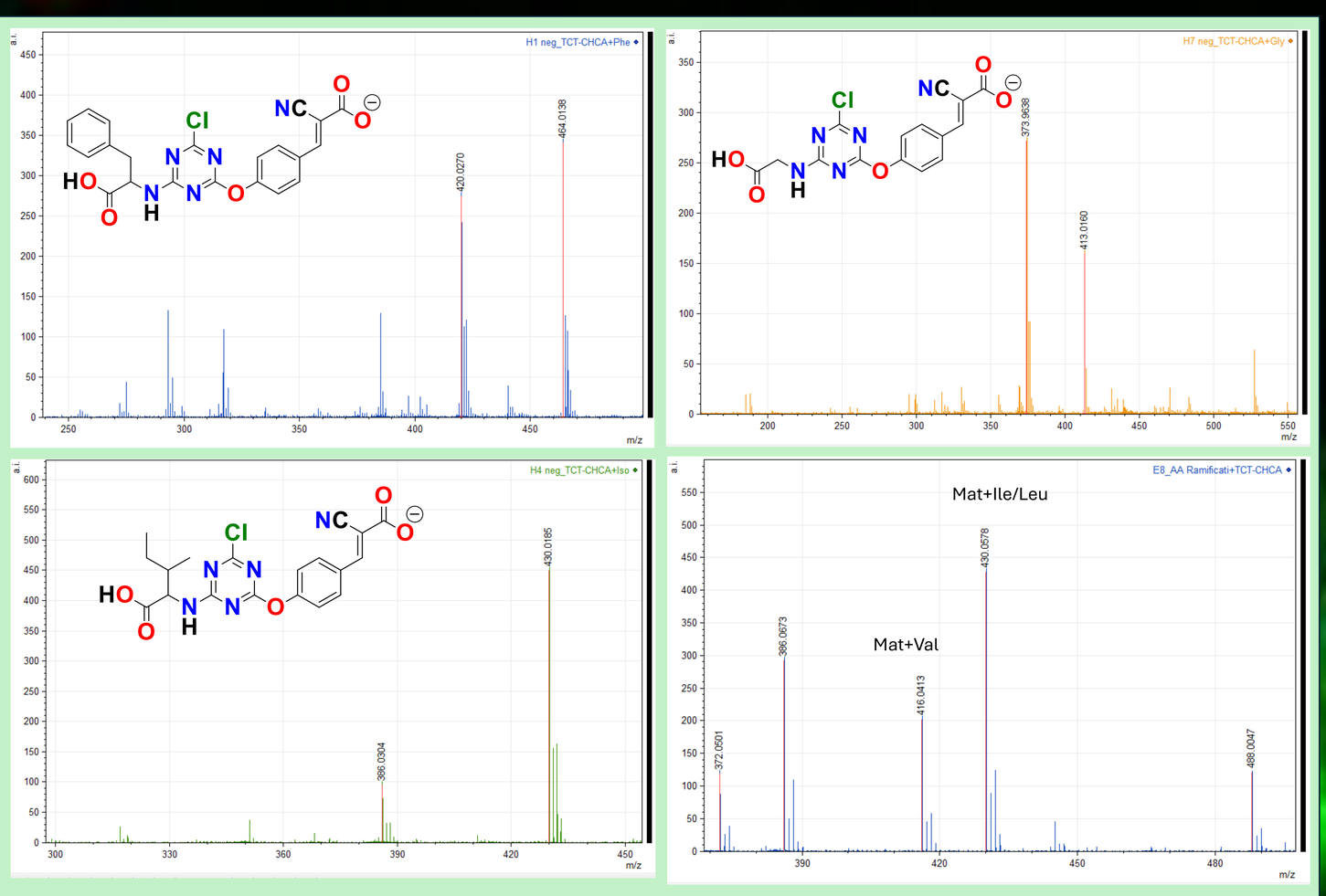


***Figure 3**: MALDI MS spectra of reaction product between matrix and different amino acids (A, phenylalanine, B, glycine, C, isoleucine and D, branched amino acids). The decarboxylated form is mainly observed in the spectra.*

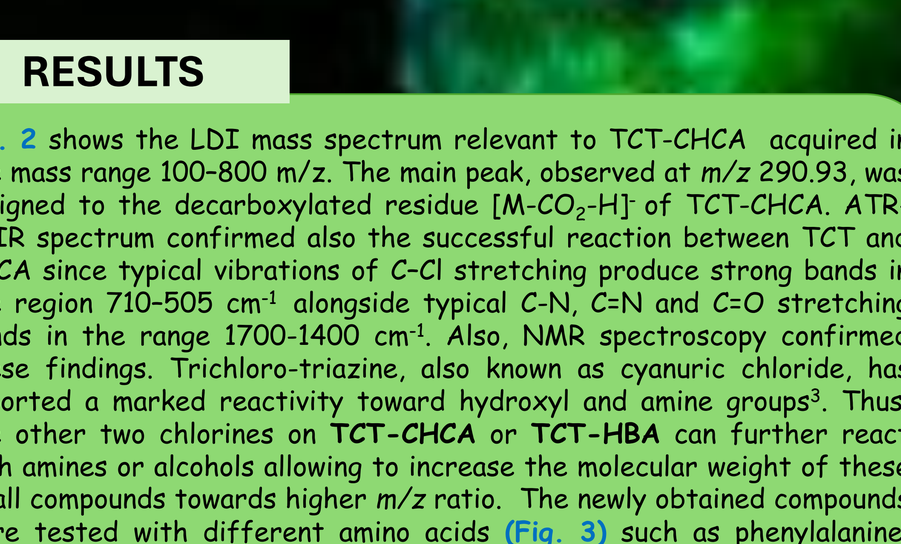


***Figure 4**: MALDI MS spectra of reaction products between matrix and NV extract obtained by protocol I (upper) and II (lower panel).*

CONCLUSIONS

We developed and optimized a comprehensive workflow for the analysis of low molecular weight compounds in extracellular vesicles, suitable for comparative studies across different biological conditions. The whole procedure will be applied to compare control and pancreatic NVs cells grown in hypoxia and normoxia conditions by MALDI for a rapid screening followed by high resolution high accuracy MS analysis employing Orbitrap technology for a detailed structural attribution.

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ACKNOWLEDGEMENTS

This work is supported by project “LIpidome of extracellular VEscicles in hypoxic pancreatic cancer” acronimo “LIVE” CUP - H93C24000620006, Bando a cascata emanato dallo Spoke 6 del Tuscany Health Ecosystem a valere sulle risorse del PNRR, M4C2 - Investimento 1.5 – creazione e rafforzamento di “Ecosistemi dell’innovazione per la sostenibilità”, finanziato dall’Unione Europea – NEXTGENERATIONEU