Innovative liquid chromatographic-mass spectometric technologies to purify and identify biologically active antibody-drug conjugates.

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Background

Antibody-Drug Conjugates (ADCs) [Fig.1] are the most powerful tools in cancer treatment [1,2] [Fig. 2] combining targeted therapy and chemotherapy exploiting two fundamental aspects: **SELECTIVITY** and **CYTOTOXICITY.**[3]

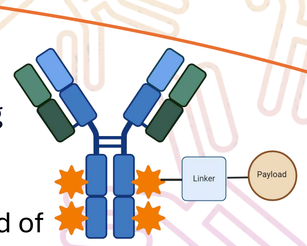


Fig.1: General structure of ADC

ADCs are extremely complex proteins since their *heterogeneity depends* of the monoclonal antibody (mAb) and of the *physico-chemical properties* of the conjugation site, linker chosen and ultimately of the payload.
It is imperative, then, develop an easy, fast and efficient methodology to characterize and purify these molecules.
For this reason, in the past few years the pharmaceutical industry has more and more been interested in exploring the variety of liquid chromatography (LC) techniques by putting more effort and attention in exploring the possibility of combing different LC together a multi dimension liquid chromatography (**mD-LC**). [5]
There are great advantages in performing an mD-LC:

- it is possible to combine different and even complementary separation systems,
- it is possible to achieve a higher resolution,
- it has been proven to be a faster and more convenient system,
- it allows also coupling mass spectrometry (MS) with some chromatography techniques that are not MS-compatible enabling to combine information otherwise not possible.

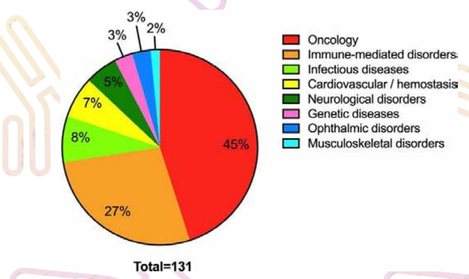


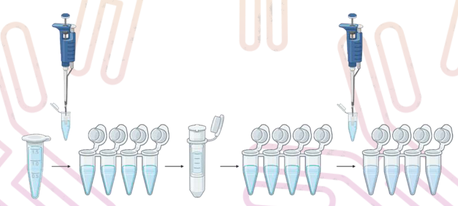
Fig. 2: Therapeutic antibodies approved or in regulatory review in USA and EU. Based on data publicly available on November 15, 2021 [4]

Aim of the work & results

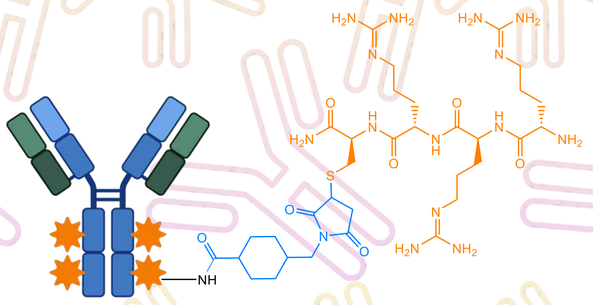
The goal of this work is to develop an innovative 3D LC technology coupled to a high-performance MS system to purify and characterize ADCs.

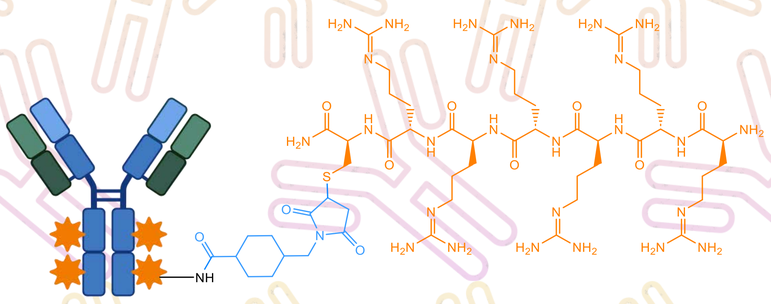
To achieve this goal a model ADC has been produced. We divided the work in four main parts (as shown in the scheme on the right).

Synthesis and purification of Cell Penetrating Peptides, such as short polyarginines sequences composed of three and six units of arginine and bearing a cysteine in C-terminal, necessary for the cross linking, H-R_6C-NH_2 and H-R_3C-NH_2.



Synthesis of the model ADC: by linking through a **non-specific lysine conjugation** a mAb (Rituximab, Matuzumab and Trastuzumab) with units of sSMCC (Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexan-1-carboxylate), a cross-linker commonly used in ADC production and the CPP previously prepared. The conjugation procedure has been optimized to use the less amount of linker (2eq) and payload (1,2, 5, 7 eq) necessary to observe a change in the chromatographic profile.
The success of the the conjugation has been checked by electrophoretic gel by a 12% SDS-PAGE Gel.
(Fig. 3 SDS-Page gel of Rituximab conjugation with 2eq sSMCC and 1, 2, 5, 7 eq of R3C and R6C)


Model ADC bearing R3C as CPP


Model ADC bearing R6C as CPP

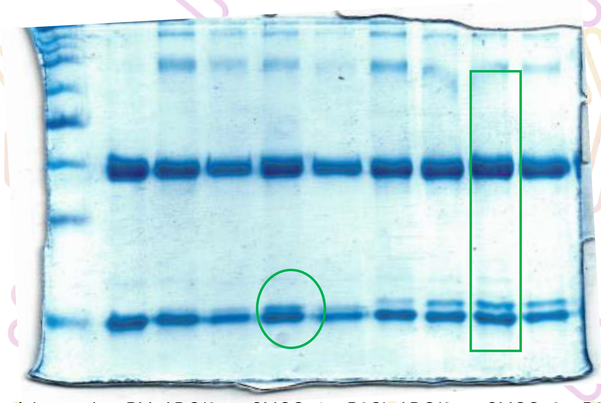
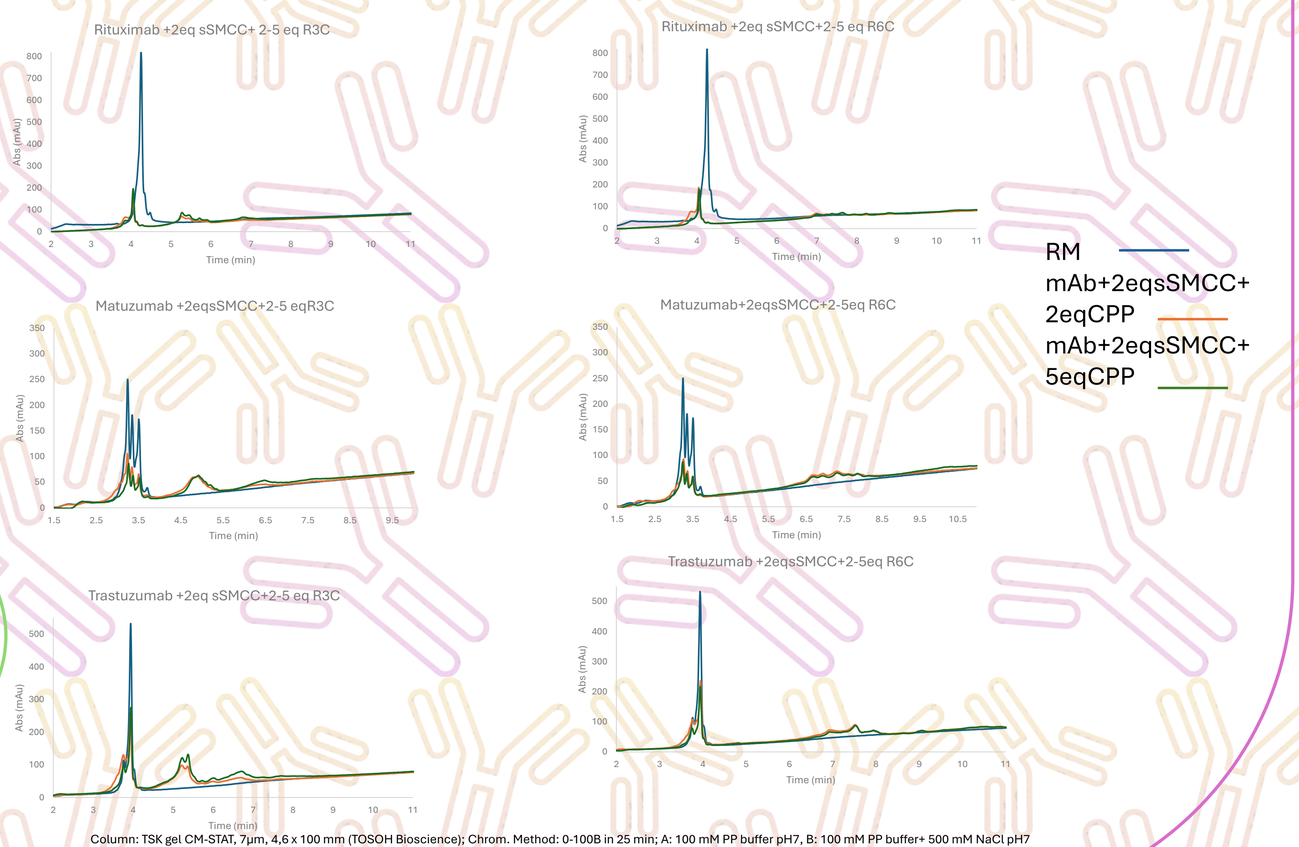


Fig. 3: From left to right: marker, RM, ADC(2eq sSMCC+1eqR3C), ADC(2eq sSMCC+2eqR3C), ADC(2eq sSMCC+5eqR3C), ADC(2eq sSMCC+7eq R3C), ADC(2eq sSMCC+1eqR6C), ADC(2eq sSMCC+2eqR6C), ADC(2eq sSMCC+5eqR6C), ADC(2eq sSMCC+7eqR6C),

Purification, NON DENATURATING CHROMATOGRAPHY TECHNIQUES such as Size Exclusion Chromatography (SEC), Weak Cation Exchange (WCX) and Hydrophobic Interaction Chromatography (HIC) and DENATURATING CHROMATOGRAPHY as Reverse Phase Liquid Chromatography (RP-LC) have been tested by using different columns and changing the chromatographic parametrers.
The best separation has been observed by using WCX
The chromatograms showed the presence of new peaks with higher retention time («ADCs' peaks») but since the couplings happen on random mAb's lysine residues the new peaks could not be sharper and separated.



Column: TSK gel CM-STAT, 7µm, 4,6 x 100 mm (TOSOH Bioscience); Chrom. Method: 0-100B in 25 min; A: 100 mM PP buffer pH7, B: 100 mM PP buffer+ 500 mM NaCl pH7

Conclusions

Model ADCs have been designed and synthesized by using sSMCC as cross-linker and two different length polyarginines as CPPs with a new optimised method and monitored by SDS-PAGE gels.
Both non-denaturating and denaturing chromatography have been used to analyse the complex matrix obtained by the coupling steps and among them WCX showed the best separation and resolution.

Future studies will be aimed to improve the chromatographic separation and coupling LC techniques to build a 3D LC-MS purification system.

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