**RESEARCH PROJECT**

**TITLE**: "Development of chromatographic and capillary electrophoresis methods for the quality control of therapeutic proteins"

Scholarship within PRIN 2022: Quality by Design approach for the development of validated analytical platforms to be used for recombinant proteins characterization and Quality Control (QubyD4Prot)

In the Laboratory of Pharmaceutical Analysis of the Department of Pharmacy and Biotechnology (FaBiT) of the University of Bologna, the research activity is addressed under the supervision of Prof. R. Gotti, and deals with the development and validation of highly selective and sensitive methods based on chromatography (LC) and capillary electrophoresis (CE) with optical (UV and fluorescence, including LIF laser-induced) and mass spectrometry (MS) detection systems

Recent progress in recombinant DNA technologies has paved the way to producing recombinant proteins that can be used as therapeutics, vaccines, and diagnostic reagents. Therapeutic proteins are complex molecules due to their high molecular weights, numerous possible conformations, post-translational modifications, and micro-heterogeneity. The heterogeneity of biopharmaceuticals is a consequence of their expression from living organisms and by the chemical and enzymatic modifications which can readily occur during production, extraction, purification, formulation, and storage. Chemical and enzymatic modifications (wanted and unwanted) could generate micro-heterogeneity (variability), such as deamidation, isomerization, aggregation, oxidation, C-terminal lysine truncation, glycation, disulfide bond scrambling, alteration of the glycosilation profile, or degradation. Among these modifications, some of them are particularly critical for the efficacy and safety of biopharmaceutical products and are known as critical quality attributes (CQAs) [1].

The CQAs of biopharmaceutical products must be identified, characterized, and monitored routinely using a variety of orthogonal analytical techniques, including LC, CE, MS, and spectroscopy to ensure quality, safety, and efficacy. Ideally, these techniques would be applied in integrated systems (Analytical Platform Technologies, APT) suitable to be used for the quality control (QC) of multiple highly similar products or product sample matrices without modification of the procedure to surpass the QC challenges in biopharmaceutics field [1,2].

In the present research project, the development of APT will be addressed to some biopharmaceutics selected with the collaboration of Istituto Superiore di Sanità (ISS, Centro Nazionale Controllo e Valutazione dei Farmaci) as monoclonal antibodies (mAb) and other therapeutic proteins in order to cover an adequate panel of molecular heterogeneity referring to originators and commercially available biosimilars.

Among the mAbs, Infliximab will be firstly considered; it is constituted of 1328 amino acids (Mw about 145 kDa) with two heavy chains and two light chains. It is administered for the treatment of autoimmune and inflammatory diseases as Crohn's diseases, ulcerative colitis etc...

The goals to be achieved are the identification and definition of the main CQAs for the selected biopharmaceutics, and the development and validation of the analytical methods for their monitoring. The first CQAs to be considered as potentially useful will be: primary structure of light and heavy chains ($CQA_{1,2}$); charge heterogeneity ($CQA_3$) and mass variants ($CQA_4$); glycosylation profile ($CQA_5$). Stress studies will be carried out by high temperature, oxidation by $H_2O_2$ etc., of the considered biopharmaceutics, in order to yield impurities and isoforms.

The CQAs will be simultaneously investigated in collaboration with others research Units of the PRIN2022 project (ISS, University of Pavia and Florence). $CQA_{1,2,3}$ will be mainly studied by means of LC-HRMS in Florence and Pavia Units, whereas the development of the methods addressed to mass and charge variants will be carried out in the laboratory of the Department of Pharmacy and Biotechnology – University of Bologna. In particular, the studies supported by the present funding program/scholarship will be addressed to the application of different CE approaches (e.g., CE-SDS either reduced or non-reduced) to contribute to the development of a suitable APT for QC of biopharmaceutics.

One of the major complexity of biopharmaceuticals is the glycosylation heterogeneity which deeply affects product solubility, stability, pharmacokinetics, and pharmacodynamics (PK/PD), bioactivity, and safety (e.g., immunogenicity). Thus, in the QC of biopharmaceuticals, monitoring the glycan profiles ($CQA_5$) is of utmost importance [3]; it can be favorably obtained in CE, by the removal of glycans from the protein, followed by separation of the individual glycans upon insertion by derivatization of suitable chromophore/fluorophore, together with the necessary charge to support proper electromigration. In most cases enzymatic release of N-linked glycans is done by peptide-N4-(N-acetyl-β-glucosaminyl)-asparagine amidase (PNGase F) which maintains the free amino group from the side chain of the parent asparagine and the resulting glycosylamine can be derivatized with amine reactive dyes under basic conditions. Implementation of derivatization strategies of therapeutic proteins will involve conjugation with high quantum yield dyes which in turn undergo to large spectral shift thus allowing detection without removing the reagent excess; this approach is expected to facilitate the overall handling of the analytical procedures limiting the general bias and improving robustness. Even though structural information cannot be achieved by LIF detection, the easiness of operation and the versatility of the technique allow a fast method optimization for the development of a ready-to-be used approach in QC of therapeutic proteins [4].

The project will be organized in the following Work Packages and according to the Gantt chart below.

WP1: Bibliographic research

WP2: Training on CE, CE-LIF and derivatization techniques in CE

WP3: Glycosilation profile ($CQA_5$)

WP4: Mass variants ($CQA_4$)

Gantt chart

| Time/months | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WP 1. Bibliorapic research | | | | | | | | | | | | |
| 1.1 CQ of biopharmaceutics | | | | | | | | | | | | |
| 1.2 CE-SDS e CE-LIF | | | | | | | | | | | | |
| WP 2. CE training | | | | | | | | | | | | |
| WP 3. CQA5 | | | | | | | | | | | | |
| Glycosilation profile; CE-LIF | | | | | | | | | | | | |
| WP4. CQA4 | | | | | | | | | | | | |
| Mass variants; CE-SDS | | | | | | | | | | | | |

## References


[1] N. Nupur et al., Analytical similarity assessment of biosimilars: global regulatory landscape, recent studies and major advancements in orthogonal platforms, Front. Bioeng. Biotechnol. 10 (2022) 832059.

[2] H. Kaur et al., Capillary electrophoresis and the biopharmaceutical industry: Therapeutic protein analysis and characterization, Trends Analyt. Chem. 144 (2021) 116407.

[3] B.L. Duivelshof et al., Glycosylation of biosimilars: Recent advances in analytical characterization and clinical implications, Anal. Chim. Acta 1089 (2019) 1–18.

[4] R. Gotti et al., Recent applications of the derivatization techniques in capillary electrophoresis, J. Pharm. Biomed. Anal Open, 1 (2023) 100003.

**ACTIVITY PLAN**

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**Needs for the scholarship request**

AIFA has recently estimates that there are more than 200 biotechnological drugs on the market and research has expanded to include more than 900 biotechnological products in clinical trials.

Thus, there is a need for researchers possessing specific skills in the field of the development and validation of analytical separation methods addressed to the quality control of biotechnological drugs.

**Objective**

Training a researcher with the suitable scientific skills to address emerging issues related to quality control of biotechnology drugs using analytical methods (HPLC-UV, -FL, -MS and CE-UV, CE-LIF), and able to interact with the other Research Units (ISS- Rome, Florence and Pavia) in the pursuing the funded PRIN2022 project.

**Applicant profile**

The applicant has a Master's degree in Pharmaceutical Chemistry and Technology, Chemistry, Pharmaceutical Biotechnology; should preferably possess technical and scientific skills in analytical chemistry areas including those gained during thesis internship.

**Scholarship and project terms, supervision, training verification**

The scholarship is for 12 months, and the supervision and training verification is by Prof. Roberto Gotti at the Department of Pharmacy and Biotechnology – University of Bologna.

Signed,

Prof. Roberto Gotti

(Electronic signature)