Filipa Grifo Ribeiro Trovão

Licenciatura em Bioquímica

Synthetic Affinity ligands for Human antibodies and its different approaches

Dissertação para obtenção do Grau de Mestre em Bioquímica

Orientador: Professora Doutora Ana Cecília Afonso Roque, Faculdade de Ciências e Tecnologias da Universidade Nova de Lisboa

Co-orientador: Professor Doutor João Manuel Braz Gonçalves, Faculdade de Farmácia da Universidade de Lisboa

Júri:

Presidente: Prof. Doutor Calos Alberto Gomes Salgueiro
Arguente(s): Prof. Doutor(a) Ana Margarida Nunes da Mata Pires de Azevedo
Vogal(ais): Prof. Doutor(a) Ana Cecília Afonso Roque

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Resumo

Os anticorpos são proteínas biofarmacêuticas importantes que podem ser aplicadas numa grande variedade de campos, tais como em investigação, diagnóstico de doenças, terapia e como agentes de purificação.

Este trabalho teve como objetivo o desenvolvimento de ligandos sintéticos de afinidade para a Imunoglobulina G (IgG) humana, explorando estes ligandos em duas abordagens diferentes: na captura de anticorpos, como componente de purificação em cromatografia de afinidade, e como componente de ligação na constituição de Conjugados de Anticorpo-Droga (ADC). Estes ligandos de baixo peso molecular foram baseados numa nova química combinatorial, combinando as reações de Petasis-Ugi. A primeira fase deste trabalho consistiu no estudo e seleção do suporte para a síntese e teste de ligandos sintéticos, tendo-se verificado que a agarose reticulada apresentava resultados mais satisfatórios.

Depois, uma nova biblioteca de ligandos sintéticos baseada na reação de Petasis-Ugi foi desenhada, sintetizada e testada com IgG humana, de onde resultaram dois ligandos principais com capacidades de ligação elevadas: B1Al2A2 e B2Al2A7.

O ligando B1Al2A2 foi explorado para a purificação de IgG proveniente de plasma humano. O tampão de ligação 20 mM HEPES, 500 mM NaCl, pH 7.4 manifestou os melhores resultados de ligação e o 0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10 pareceu ser um tampão de eluição adequado. No entanto, alguns melhoramentos devem ser realizados na recuperação de IgG de plasma humano. As constantes de ligação do sistema estudado (K_a= 3×10^4 M^{-1} and Q_{max}= 6 mg/g) demonstraram resultados promissores no que toca a purificação de proteínas em cromatografia de afinidade.

A segunda abordarem consistiu na exploração dos ligandos de afinidade na constituição de um ADC, utilizando estes ligandos sintéticos como componente de ligação. Ambos os principais ligandos (B1Al2A2 e B2Al2A7) foram testados com um anticorpo monoclonal (mAb) e o ligando B2Al2A7 apresentou melhor capacidade de ligação para com o mAb, sendo um ligando promissor para futuros estudos na área de ADC.

Palavras-Chave: Anticorpos; Ligandos sintéticos de afinidade; Petasis-Ugi; Cromatografia de afinidade; Purificação de proteína; Conjugados Anticorpo-Droga (ADCs)

Abstract

Antibodies are important biopharmaceutical proteins that can be applied in a wide range of fields, such as in research, disease diagnostics, therapy and as purification agents.

This work aimed the development of synthetic affinity ligands towards human Immunoglobulin G (IgG), exploring these ligands in two different approaches: in the capture of antibodies, as a purification component in affinity chromatography, and as the linker component for Antibody Drug Conjugate (ADC) assembly. These low molecular weight ligands were based on a new combinatorial chemistry, the Petasis-Ugi. The first phase of this work consisted in the study and selection of the support for synthesis and screening of synthetic ligands, which turned out to be the traditional cross-linked agarose.

Then, a new library of synthetic ligands based on the Petasis-Ugi reaction was designed, synthesized and screened against human IgG. Two lead affinity ligands with high binding capacities: B1Al2A2 and B2Al2A7 were selected for further studies.

Ligand B1Al2A2 was explored for IgG purification from human plasma. The 20 mM HEPES, 500 mM NaCl, pH 7.4 binding buffer showed the best binding results and the 0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10 seemed to be an adequate elution buffer. However, some improvements should be made in the recovery of IgG from human plasma. The binding constants obtained for the studied system (K_a= 3×10^4 M^{-1} and Q_{max}= 6 mg/g) showed promising results for protein purification through affinity chromatography.

The second approach consisted in exploring the affinity ligands for ADC assembly, using these synthetic ligands as the linker component. Both lead ligands (B1Al2A2 and B2Al2A7) were screened against a monoclonal antibody (mAb) and the B2Al2A7 ligand showed better binding capacity to the mAb, being a promising ligand for further studies in the ADC field.

Key words: Antibodies**;** Synthetic affinity ligands; Petasis-Ugi; Affinity Chromatography; Protein purification; Antibody Drug Conjugates (ADCs)

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Abbreviation List

Abs	Antibodies
ADC(s)	Antibody Drug Conjugate(s)
approx.	Approximately
APS	Ammonium Persulfate
APTES	(3-aminopropyl)triethoxysilane
BCA	Bicinchoninic Acid
BR96-DOX	BR96 antibody-doxorubicin conjugate
BSA	Bovine Serum Albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
Cu	Copper
DCM	Dichloromethane
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
e.g.	*exempli gratia* or for example
Eq.	Equivalent
Fab	Fragment of antigen-binding
Fc	Fragment crystallisable
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
Fmoc	Fluorenylmethyloxycarbonyl
FT	Flow-through
GFP	Green Fluorescent Protein
GFP-RK	Green Fluorescent Protein RKRKRK-tagged
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	Hydrofluoric acid
HI	Hydriodic acid
HIV	Human Immunodeficiency Virus

HSA	Human Serum Albumin
i.e.	*id est* or that is
ICMM-CSIC	*Instituto de Ciencia de Materiales de Madrid – Consejo Superior de Investigaciones*
Ig	Immunoglobulin
IgG	Immunoglobulin G
Ka	Affinity constant
kDa	Kilo Daltons
mAbs	Monoclonal Antibodies
MDR	Multidrug Resistance
MED	Min Effective Dose
MeOH	Methanol
MNP(s)	Magnetic Nanoparticle(s)
MNPs-Si-Si-Dex	Magnetic nanoparticles coated with silica and dextran
MNPs-Si-Si-Dez-NH_2	Magnetic nanoparticles coated with silica and dextran and functionalized with amine groups
MTD	Max Tolerated Dose
MTX	Methotrexate
N_2	Nitrogen
$Na_2S_2O_3$	Sodium thiosulfate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium Hydroxide
NH_2	Amine functional group
NH_4OH	Ammonium hydroxide
NMR	Nuclear Magnetic Resonance
Nü	Nucleophile
PBS	Phosphate buffer saline
PDB	Protein Data Bank
PK	Pharmacokinetics
pKa	Acid dissociation constant logarithm

Q_{max}	Maximum binding capacity
Rink amide MBHA	Rink amide 4-methylbenzhydrylamine
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SiO_3	sodium metasilicate pentahydrate
TEMED	N,N,N,N-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
Tris	Tris(hydroxylmethyl)aminomethane
VEGF	Vascular Endothelial Growth Factor

I. Literature Review

1.1. Antibodies

Antibodies (Abs), also known as Immunoglobulins (Ig), are glycosylated proteins belonging to the globular proteins family. These Y-shaped proteins are mainly produced by plasma cells and used by the immune system to identify and neutralize foreign or non-self molecules [1], [2].

Each immunoglobulin molecule is typically composed by two identical light chains (L), the smaller subunits (25 kDa each), and two identical heavy chains (H), the larger subunits (50 kDa each). By enzymatic or chemical cleavage an antibody can be distinguished in two fragments: The Fab fragment (fragment of antigen-binding) and the Fc fragment (fragment crystallisable). Functionally, an immunoglobulin is composed by two principal regions: the variable region, which confers the versatility and specificity and is responsible for the antigen recognition, and the constant region, that has effectors properties, deciding its biological activity which can lead to complement-mediated lysis, enhanced phagocytosis, or allergy [1], [2].

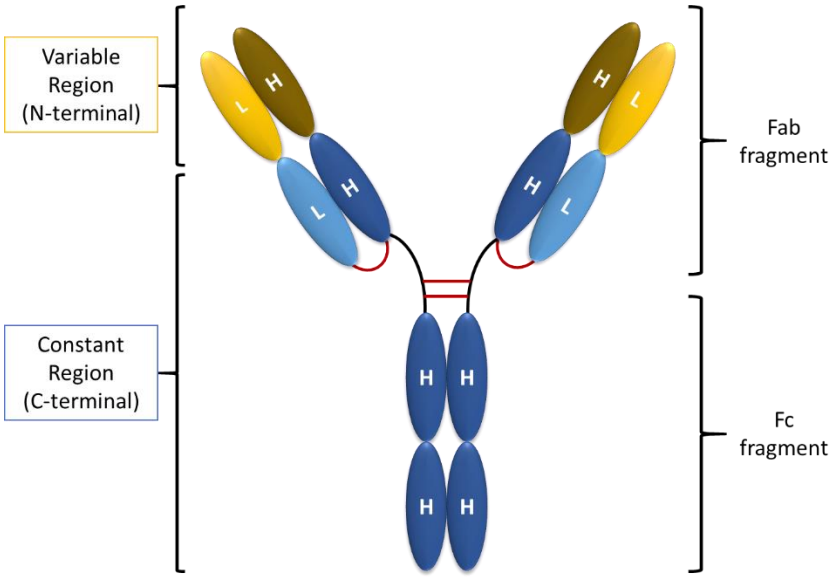


Figure 1.1: Simplified representation of an Antibody structure. **H** - Represents the heavy chains. **L** – Represents the light chains. In red the disulfide bonds.

1.1.1. Monoclonal antibodies

Monoclonal antibodies (mAbs) are monospecific antibodies that are produced by identical immune cells which are all clones of a unique parent cell. They have long been powerful tools in basic scientific research, such as biochemistry, pharmaceutics, medicine and molecular biology, due to their high specificity and affinity for the target antigens [3], [4].

Since the approval of the first therapeutic monoclonal antibody, Orthoclone OKT3 in 1986, mAbs and antibody related products (Fc-fusion proteins, antibody fragments and antibody drug conjugates) became really important and dominant in the biopharmaceutical market [5].

The understanding of diseases at a molecular level has led to a continued interest in the development of antibody products. Monoclonal antibody products show several advantages when compared with other types of therapeutic products, such as long circulating half-life, high target specificity and good tolerance. For these reasons, mAb products are frequently first product candidates to clinical trials, once the risk of unexpected safety issues in human are lower than with other therapeutic products and, if the initial studies are successful, these mAb products can easily move to commercialization [5], [6].

In the biopharmaceutical market, the production of mAb products has been higher than the production of other recombinant protein products. The sales of mAb products have grown 90% from 2008 to 2013, with an increase of approximately $39 billion to almost $75 billion, whilst other recombinant protein therapeutics have only increased 26% [5].

The ability to generate antibodies against tumor-selective antigens and the progression of mAb technology to develop fully human antibodies, to reduce immunogenic risk, has helped drive a steady expansion of the development of monoclonal antibody therapeutics to treat a variety of other diseases and certain cancers. Some researchers predict that the world-wide market of mAb products will increase to $90-94 billion by 2017 and around $125 billion by 2020 [3], [5].

1.1.2. Antibody Drug Conjugates (ADCs)

Through the past decade, significant advances in new cancer treatments were observed. Highly selective small molecules that target specific genetic abnormality responsible for the disease are important advances, despite the fact that they may not sufficiently potent to be therapeutically active on their own. A strategy that combines the powerful cell-killing ability of potent cytotoxic agents with target specificity is the Antibody Drug Conjugates (ADCs) approach [7].

An Antibody Drug Conjugate consist in a tumour-targeting monoclonal antibody linked to a drug compound, usually a cytotoxic molecule. This cytotoxic drug is released specifically into the cancer cell at an appropriate time, ideally without affecting other cells and presenting lower systemic toxicity [8].

The biopharmaceutical industry is investing enormously in the ADC field, as shown by the exponential number of submitted ADCs to FDA authorities [3], [4].

Unlike traditional chemotherapy, ADCs expand the therapeutic window (Figure 1.2). ADC therapeutics can increase efficacy and decrease toxicity, so the administrable dose can be higher, when compared to the traditional chemotherapeutic cancer treatments, due to targeted delivery by the antibody [4].

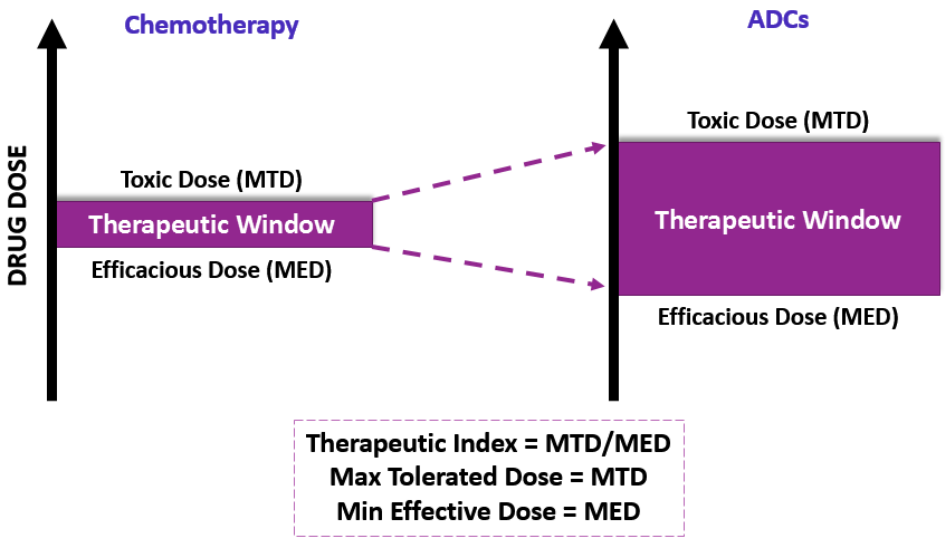


Figure 1.2: Schematic representation of the impact of ADCs in the Therapeutic Window when compared to the Traditional Chemotherapy. Adapted from Panowski et al., 2014.

1.1.2.1. History of ADCs

The beginning of ADCs can be traced back over a century (1913) to the German physician and scientist Paul Ehrlich, who proposed the concept of combining in a single molecule, specific binding to a diseased cell or organism with a toxic activity for that cell or organism. Ehrlich coined the term “magic bullets” to describe this concept. However, at the time the tools that would be needed to make this possible did not exist. About 50 years later, the concept of targeted therapy from Ehrlich was first exemplified when methotrexate (MTX) was linked to an antibody targeting leukemia cells. After that, the researches relied on available targeting agents, such as polyclonal antibodies, to enable preclinical efficacy studies in animal models with both noncovalent-linked ADCs and later covalently linked ADCs [4], [7], [9], [10].

It was only with the development of monoclonal antibodies in 1975 that the concept that antibodies could provide to a cell-killing agent the selective binding became the subject of a large research effort. Kohler and Milstein greatly advanced the ADC’s field developing mouse mAbs by the use of hybridoma technology. Then, followed the first human clinical trial with the antimitotic vinca alkaloid vindesine as the cytotoxic payload, and the production of humanized mAbs with reduced immunogenicity and increased half-life [4], [7], [11].

From the 1980s to 1990s there was several attempts at ADC development, which resulted in the first-generation of ADCs. Although, this generation of ADCs typically used clinically approved drugs with well-established mechanisms of action, they had limited success due to low drug potency, high antigen expression on normal cells and instability of the linker that attached the drug to the mAb. These initial failures led to a new generation of ADCs, several of which entered and later failed human clinical trials, such as the KS1/4 antibody-methotrexate conjugate for non-small cell lung cancer and the BR96 antibody-doxorubicin conjugate (BR96-DOX) for metastatic breast cancer. In 2000 emerged the first ADC approved by Food and Drug Administration (FDA) developed by Wyeth and Celltech, Mylotarg® (gemtuzumab ozogamicin), which a decade later was withdrawn from the market due to a lack of improvement in overall survival [4], [7].

Afterwards, in 2011 Adcetris® (brentuximab vedotin) was approved by FDA for treatment of Hodgkin's and anaplastic large-cell lymphomas, and in 2013 Kadcyla® for treatment of patients with breast cancer, which combines the humanized antibody trastuzumab with a potent antimicrotubule cytotoxic agent using a highly stable linker [7]. Currently, there are at least 25 ADCs undergoing clinical evaluation in oncology [11]. In Figure 1.3, is represented a succinct timeline of ADC's history.

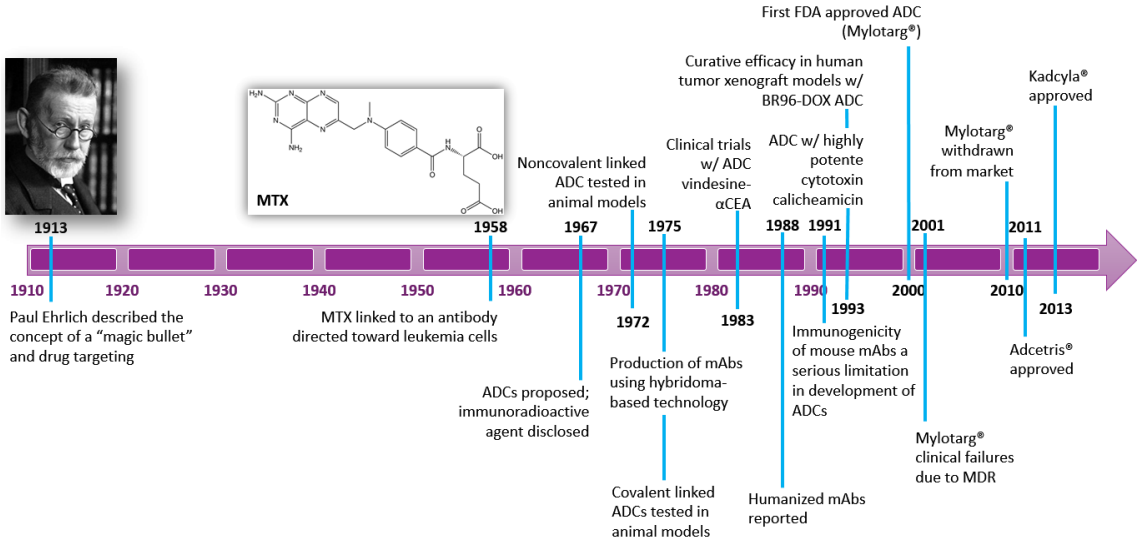


Figure 1.3: Antibody Drug Conjugate (ADC) timeline. Adapted from Perez et al., 2014.

This iterative learning process of Antibody Drug Conjugates development, allowed a better understanding of ADCs function and their clinical performance, which can lead to new improvements in the field.

1.1.2.2. ADCs Structure

An ADC is composed by an antibody, which can bind to a specific tumor antigen, a linker and a cytotoxic drug (Figure 1.4). This four components are critical factors to the success of ADCs [4].

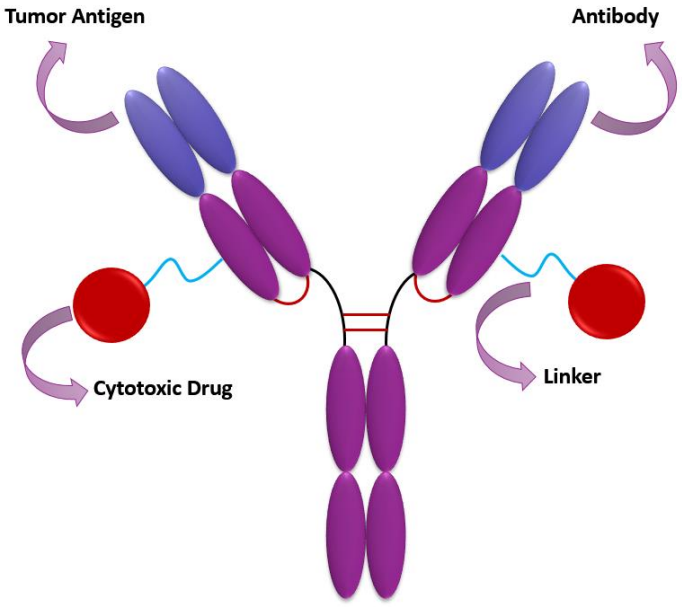


Figure 1.4: Illustrative Antibody Drug Conjugate, with the indication of the critical factors that influence ADC therapeutics. Adapted from Panowski et al., 2014.

Tumor Antigen

The target/antigen is the starting point to build an ADC. Ideally a tumor antigen must be localized at the cell-surface allowing ADC binding, and it is important that the target antigen be highly expressed in the target cells and with low expression in other tissues, in order to limit off-target toxicity and maximize the efficacy of the ADC. Also, the tumor antigen should be highly upregulated in cancer tissue, internalized upon ADC binding, and able to release the cytotoxic agent inside the cell [3], [4], [7], [12].

Antibody

The antibody itself is another critical factor that influences ADC. Some critical attributes that the selected antibody should have are high specificity for the tumor antigen, otherwise it could result in toxicity and removal/elimination of the ADC before it can reach the tumor, then the antibody must bind the target antigen with high affinity, and it is also important to select an antibody with high half-life time and low clearance in plasma [4].

Nowadays, the antibodies used as components of ADCs include chimeric, humanized and fully humanized antibodies [3].

Linker

The linker attaches the antibody to the cytotoxic molecule, so it must be bifunctional. An ideal linker should be stable to the endogenous proteases in circulating blood, but capable of rapid release of active free drug inside tumor cells following antigen-mediated internalization. If the selected linker is not stable in plasma, drug will be lost, which can result in damage of normal tissues, and ADC activity will be decreased. [3], [4], [7], [11].

Current linkers can be classified by their mechanism of drug release, which falls into two groups: cleavable linkers and non-cleavable linkers. Cleavable linkers have sites that are susceptible to chemical (usually by hydrolysis) or enzymatic cleavage, which includes the acid-labile, protease-cleavable, and disulphide linkers. Acid-labile linkers are design to be stable in circulating blood, but become unstable and degrade once lysosomes are reached due to the low pH environment. Protease-cleavable linkers are also stable in blood, but rapidly release free drug inside lysosomes upon cleavage by lysosomal enzymes. Finally, disulfide linkers exploit the high level of intracellular reduced glutathione to release free drug inside the cell [3], [4], [11].

Non-cleavable linkers have no sites for enzymatic or chemical cleavage in biological systems, and release drug via peptide backbone degradation of the antibody in lysosomes of target cells. This linker's category provide high stability in blood, however are dependent on internalization, lysosomal delivery, and degradation of the ADC complex to release active drug and kill cancer cells [3], [4], [11].

Cytotoxic Drug

The drug or payload, as some authors called it, plays a major role in ADC activity and characteristics. Cytotoxic drugs must contain a suitable functional group for conjugation with linker, and need to be stable under physiological conditions. The current generation of drugs falls into two mechanistic classes: microtubule inhibitors and DNA-damaging agents. Microtubule inhibitors are the ones that bind to tubulin, destabilize microtubules polymerization, and cause G2/M phase cell cycle arrest. DNA-damaging agents are those that bind the minor groove of DNA, damaging DNA by stand scission, alkylation or cross-linking [3], [4].

1.2. Affinity Binders to Antibodies

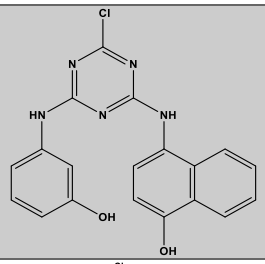
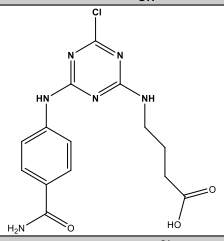
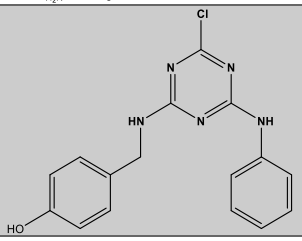
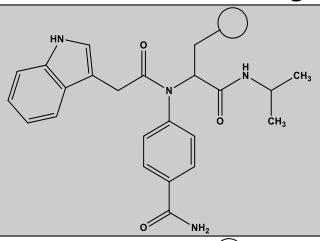
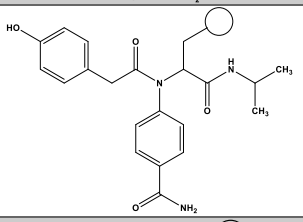
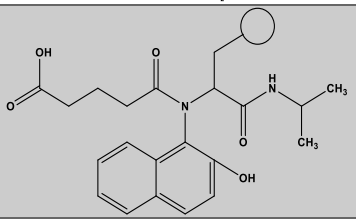
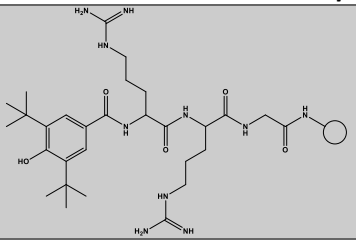
Currently, we can find several molecules that bind antibodies, known as antibody binders or antibody affinity ligands. There are biological affinity ligands that bind naturally to antibodies and synthetic affinity ligands, including *de novo* designed synthetic ligands – such as triazine and Ugi ligands – and peptides designed to bind antibodies. Antibody binders can be used for technological approaches, *i.e.*, affinity chromatography, or analytical methods, as well as to therapeutics and diagnostic fields. Synthetic antibody binders have been essentially developed to capture antibodies in affinity chromatography processes, in order to substitute protein A affinity chromatography. However, these synthetic ligands can be explored for other approaches, such as the linker component in the ADC assembling.

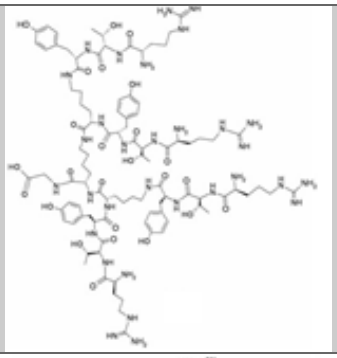
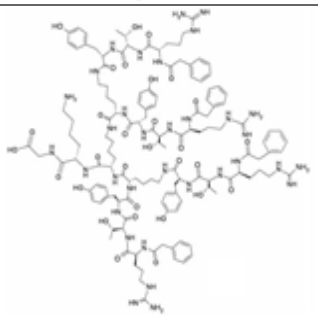
In Tables 1.1 and 1.2 are represented, respectively, all natural/biological and synthetic affinity ligands that bind to antibodies and their respective amino acids interaction, ligands structures and affinity constants.

Table 1.1: Natural/Biological affinity ligands that bind to antibodies.

Protein	Interactions		Affinity constant	Ref.
	Protein	Antibody		
Protein A	Phe124, Gln128, Phe132, His137, Phe149, Ile150, and Leu153.	**Fc region – consensus-binding site (CBS)**: Met252, Ile253, Gln330, His464, Asn465, His466 and Tyr467.	$K_a \approx 10^8\ M^{-1}$	[13]–[15]
Protein G	Glu27, Lys28, Lys31, Gln32, Asn35, Asp40, Glu42 and Trp43.	**Fc region – CBS:** - CH2 domain: Ile253–Ser254 and Gln311; - CH3 domain: Glu380, Glu382 and His433–Gln438. (also shows some affinity to Fab fragments)	$K_a \approx 10^8\ M^{-1}$	[13], [14]
Protein L	Gln35, Thr36, Ala37, Glu38, Phe39, Lys40 and Tyr53.	**Fab region:** kappa light chains – k1, k3 and k4. (PpL domain is sandwiched between two antibodies Fab)	$K_a \approx 10^{10}\ M^{-1}$	[13], [16]

Table 1.2: Synthetic affinity ligands that bind to antibodies.

Ligand	Interactions Struture	Antibody	Affinity constant	Ref.
Triazine Ligands				
22/8 (Artificial protein A)		**Fab region:** Ser7, Gly8, Lys12, Ser17 and Ser21; **Fc region:** CBS.	$K_a \approx 1,4 \times 10^5$ M^{-1}	[15], [17]
8/7 (Artificial protein L)		**Fab region.**	$K_a \approx 10^4$ M^{-1}	[18]
MABsorbent A2P		**Fc region.**	-	[19]–[21]
Ugi Ligands				
Ligand A2C11I1 (Artificial Protein G)		**Fc region:** H-bonds with Asn434**.**	$K_a \approx 2,1 \times 10^5$ M^{-1}	[22]
Ligand A2C7I1 (Artificial Protein G)		**Fab region - CH1 domain:** Pro125 to Val128.	$K_a \approx 1,9 \times 10^4$ M^{-1}	[23]
Ligand A3C1I1 (Artificial protein L)		**Fab region.**	$K_a \approx 3,8 \times 10^5$ M^{-1}	[24]
Peptides				
DAAG		**Fc region:** CBS.	$K_a \approx 10^5$ M^{-1}	[25], [26]

Peptide TG1931/PAM (Mimetic Protein A)	Y-T-R G-K-K-Y-T-R K-Y-T-R Y-T-R	**Fc region.**	$K_a \approx 3,3 \times 10^5$ M^{-1}	[27], [28]
D-PAM (Mimetic Protein A)		**Fc region.**	$K_a \approx 10^4$ M^{-1}	[29], [30]
Phenylacetyl-D-PAM (Mimetic Protein A)	 (More hydrophobic groups than D-PAM)	**Fc region.**	-	[30]
Hexapeptides	**Peptide sequence:** HWRGWV; Acetylated-HWRGWVA (better selectivity).	**Fc region:** Interacts with loop Ser383–Asn389 of CH3 domain.	$K_a \approx 10^5$ M^{-1} $K_a \approx 4,9 \times 10^5$ M^{-1}	[31]–[34]
Octapeptides (Binding capacity: FYWHCLDE > FYCHWALE > FYCHTIDE)	**Peptide sequence:** FYWHCLDE	**Fc region:** CBS - Glu and Asp interact with Lys97 and Lys99 of Fc.	$K_a \approx 6,7 \times 10^5$ M^{-1}	[35], [36]
	Peptide sequence: FYCHWALE	**Fc region:** CBS - His and Glu interact with Glu186 and Lys99 of Fc.	$K_a \approx 1,6 \times 10^5$ M^{-1}	[37]
	Peptide sequence: FYCHTIDE	**Fc region:** CBS - Glu and His interact with Lys99 and Glu186 of Fc; - Phe and Ile with Leu74 and Pro102 of Fc.	$K_a \approx 1,8 \times 10^5$ M^{-1}	[37]
IgGBP	**Peptide sequence:** IgGBP – **DCAWHLGELVWCT** C-terminus mKate - **GGGGS**	**Fc region:** CH2 and CH3 domains.	-	[38]

1.3. Aims of the work

In this work, it was explored a new chemistry, the Petasis-Ugi, to generate a synthetic combinatorial library of affinity ligands designed to bind to antibodies. These affinity reagents were studied for two applications: to capture antibodies, as a purification component in affinity chromatography, and as the linker component for ADC assembly (Figure 1.5). Therefore, the objective of this research project is the development of synthetic affinity ligands towards human IgG.

The backbone thesis and respective aims are:

i) Choice of solid support for library synthesis and screening (Chapter II):

It was explored two supports for the solid-phase synthesis of combinatorial libraries of affinity ligands. The aim here was to study which of the supports are better to do the initial screenings for the selection of the leader ligands, analysing their reproducibility.

ii) Rational design, synthesis and screening of a Petasis-Ugi towards human IgG (Chapter III):

The design of this new library, through the Petasis-Ugi reaction, was based on natural and synthetic molecules that were found to bind antibodies by non-covalent interactions. The aim was to find which of the ligands had the highest binding capacity towards human IgG. From this study, it resulted in two lead affinity ligands that could be used for different purposes: Antibody purification approach and ADC approach.

Exploration of the lead affinity ligands (Chapter IV):

iii) Explore the best ligand for Antibody purification (Part I):

For the purification approach the main goal is to have an affinity ligand that highly binds to IgG and can easily unbind this protein through a “selective” elution buffer, and also characterize the binding capacity of the affinity ligand.

iv) Explore the ligands for ADCs approach (Part II):

For the ADC approach, the main goal is to test the hypothesis that ADCs can be prepared through the action of hybrid affinity pairs which will attach the drug to the mAb by non-covalent interactions and must be very stable under rough conditions. With this strategy we expect to overcome chemical and other conjugation difficulties to mAbs and optimize drug release in order to potentiate specific and bystander effects of cytotoxic drugs.

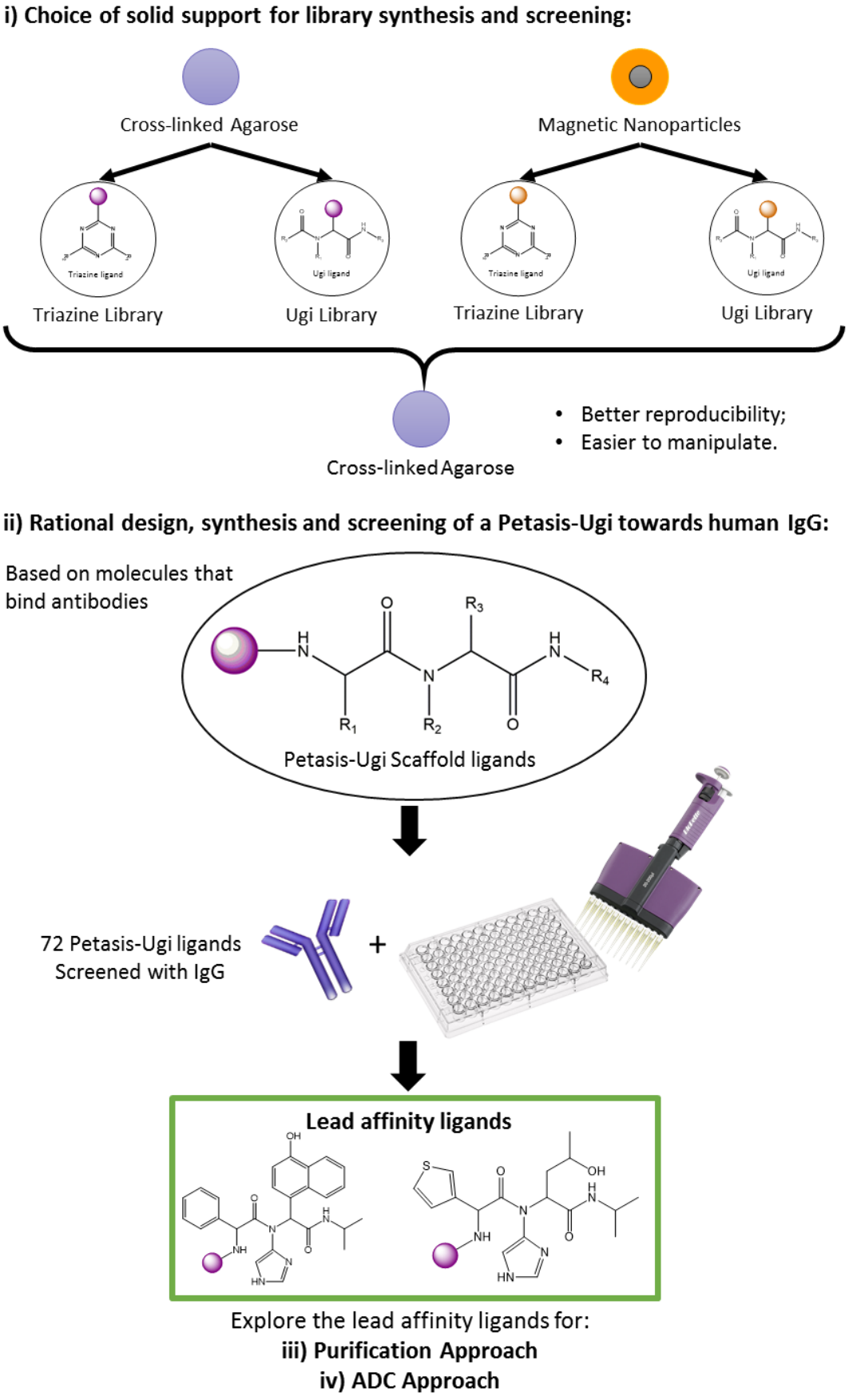


Figure 1.5: Resume of the strategy followed in this project. The purple circles represent the cross-linked agarose and the grey/orange circle represents the MNPs.

II. Selection of support for the synthesis and screening of synthetic ligands

2.1. Introduction

The use of antibodies has grown exponentially due to their application in different fields, such as research, pharmacology, therapy, diagnostics and as purification agents. Thereby, the need of finding feasible and economical methods to purify these proteins is essential.

Nowadays, affinity chromatography field is a common step on antibody purification and the most used solid support to synthesize and select lead affinity ligands for a specific protein is the cross-linked agarose, due to its properties. Cross-linked agarose is chemically stable, resistant to degradation by enzymes, microbes, elution buffers, regenerating solvents and cleaning agents. It is also known that after the selection of lead affinity ligands, they can be coupled onto different solid supports, such as magnetic nanoparticles, membranes or monoliths [39]. In order to overcome the limitations of usual chromatographic methods, such as high pressure drop, high cost, low flow rates, weak mechanical properties and tendency for fouling, new non-chromatographic techniques have been developed and improved, namely aqueous two-phase system, crystallization/precipitation and magnetic separation [40]–[43].

Magnetic separation is probably one of the most versatile separation processes in biotechnology as it can be used to purify cells, viruses, proteins and nucleic acids directly from crude samples. It presents undisputable advantages, such as low-cost, speed, scalability and compatibility with complex biological suspensions [44]. Since magnetic nanoparticles (MNPs) present low colloidal stability, due to high surface area to volume ratio, which can lead to agglomeration, the coating of MNPs appears to be an essential strategy for stabilization, and different coating layers can be applied, including pullulan, chitosan, gum arabic, alginate, heparin and dextran [45].

Synthetic affinity ligands are interesting alternatives in the purification of high value biopharmaceutical proteins, such as antibodies, since they are less expensive than biospecific affinity ligands (protein A, G and L – isolated from the surface of bacteria), are chemically defined, resistant to biological and chemical degradation, readily immobilized onto various purification supports and can be highly selective [17].

Synthetic ligands so far have been developed based on two distinct combinatorial reactions: The Triazine and Ugi reactions.

2.1.1. Triazine Reaction

The triazine reaction uses cyanuric chloride as the scaffold structure, which is reactive under relatively mild conditions. All three chloride atoms from the triazine ring can be substituted in a sequential and controlled fashion by amines in aqueous solution through

nucleophilic attacks, although there are differences in reactivity. In practice, the synthesis of ligands library occurs directly on the solid matrix functionalized with amine groups, so the first nucleophilic substitution (denoted by Nü) is between the matrix and the cyanuric chloride molecule at approximately 0 °C for 1h. Then two subsequent nucleophilic substitutions will occur: the first substitution (R_1) at 30 °C for 24h, while the second (R_2) occurs at 80 °C for 48h (Figure 2.1). This reaction results in a library of bisubstituted ligands, where R_1 and R_2 can contain a varied functional groups [46]–[48].

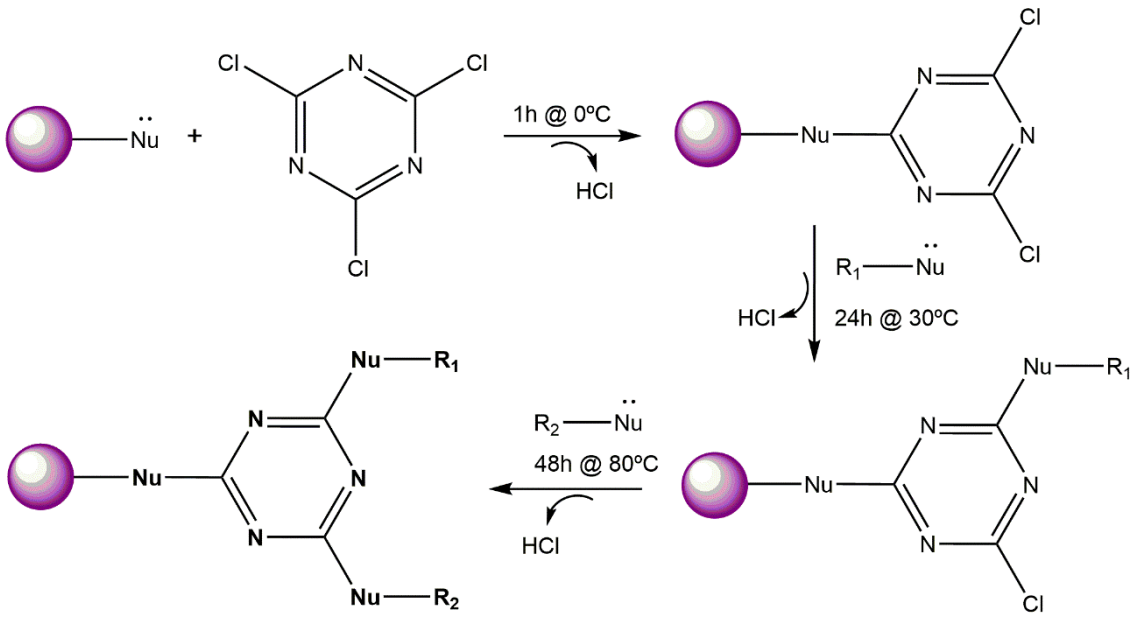


Figure 2.1: Solid-phase synthesis of triazine reaction, where the purple circle represents the solid support. Adapted from Batalha, 2014.

2.1.2. Ugi Reaction

Ivar Ugi and co-workers reported in 1959 a four-component reaction also known as Ugi reaction, in which an oxo-component (aldehyde or ketone), a primary or secondary amine, a carboxylic acid and an isonitrile group are condensed, in a one-pot reaction conducted at a constant temperature (60 °C), to yield a di-amine scaffold product, losing just one molecule of water during the whole process [22], [24], [49].

The Ugi multicomponent reaction shows several advantages in the synthesis of combinatorial libraries of affinity ligands over the triazine reaction, since the procedure it's simpler and saves time. Besides that, it has an increased scaffold diversity, it's able to mimic a native peptide bonds, and can also adopt more structural flexibility by possessing a less planar structure [22], [49].

Ugi reaction on solid-phase is depicted in Figure 2.2. The first step of Ugi reaction mechanism consists in the condensation between the amine component (R_1) and the aldehyde/ ketone in a solid matrix to form an imine. The imine will react in an acid-base reaction with the carboxylic acid (R_2) to form an iminium ion, which reacts with isocyanide component (R_3) to generate the nitrilium ion. Then, the nitrilium ion reacts with carboxylate ion (R_2) produced in the imine activation step, yielding an unstable imino-anhydride. Finally a Mumm rearrangement occurs to generate the final Ugi product [49], [50].

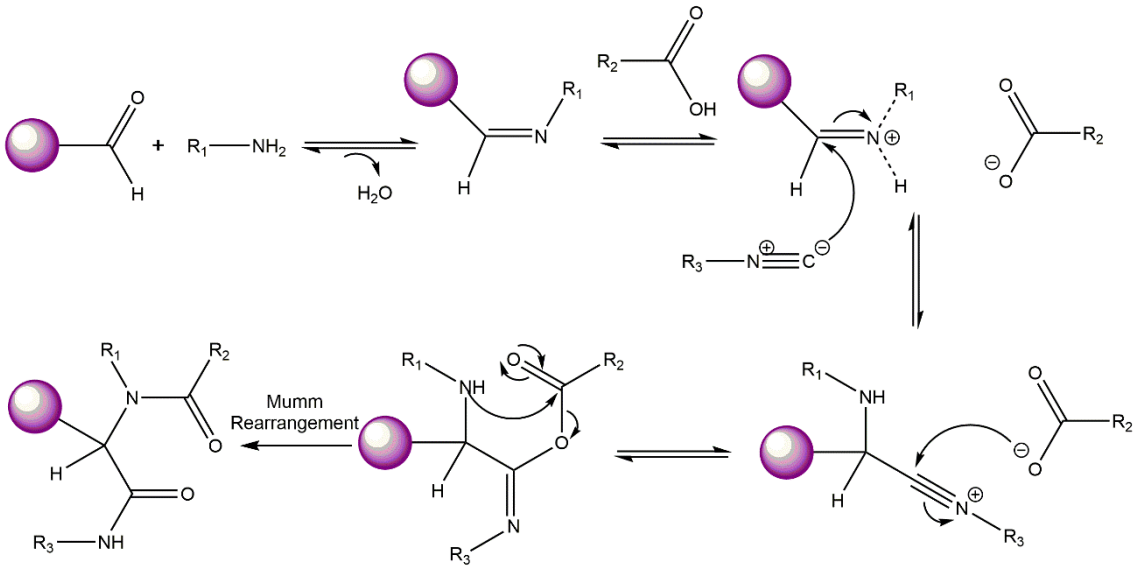


Figure 2.2: Solid-phase Ugi reaction mechanism, where the purple circle represents the solid support. Adapted from Batalha, 2014.

2.1.3. Aims of the chapter

This chapter focuses in exploring the best support to synthesize and screen synthetic affinity ligands for antibody and how these supports influence the binding capacity of the affinity ligands.

As magnetic separation is a simpler and a faster methodology than conventional packed chromatography, and the use of affinity ligands can increase the selectivity of this method, two libraries of affinity ligands for human IgG were design using two different supports: magnetic nanoparticles (MNPs) and cross-linked agarose (Figure 2.3). The libraries were based on the combinatorial reactions Ugi and Triazine, that are the most used and well-studied ones in bioseparation.

Thus, the aim here is to test the reproducibility of the two libraries used, in each support and between supports, and to test if the selection of the lead affinity ligands is possible in a more economic solid support, the magnetic nanoparticles.

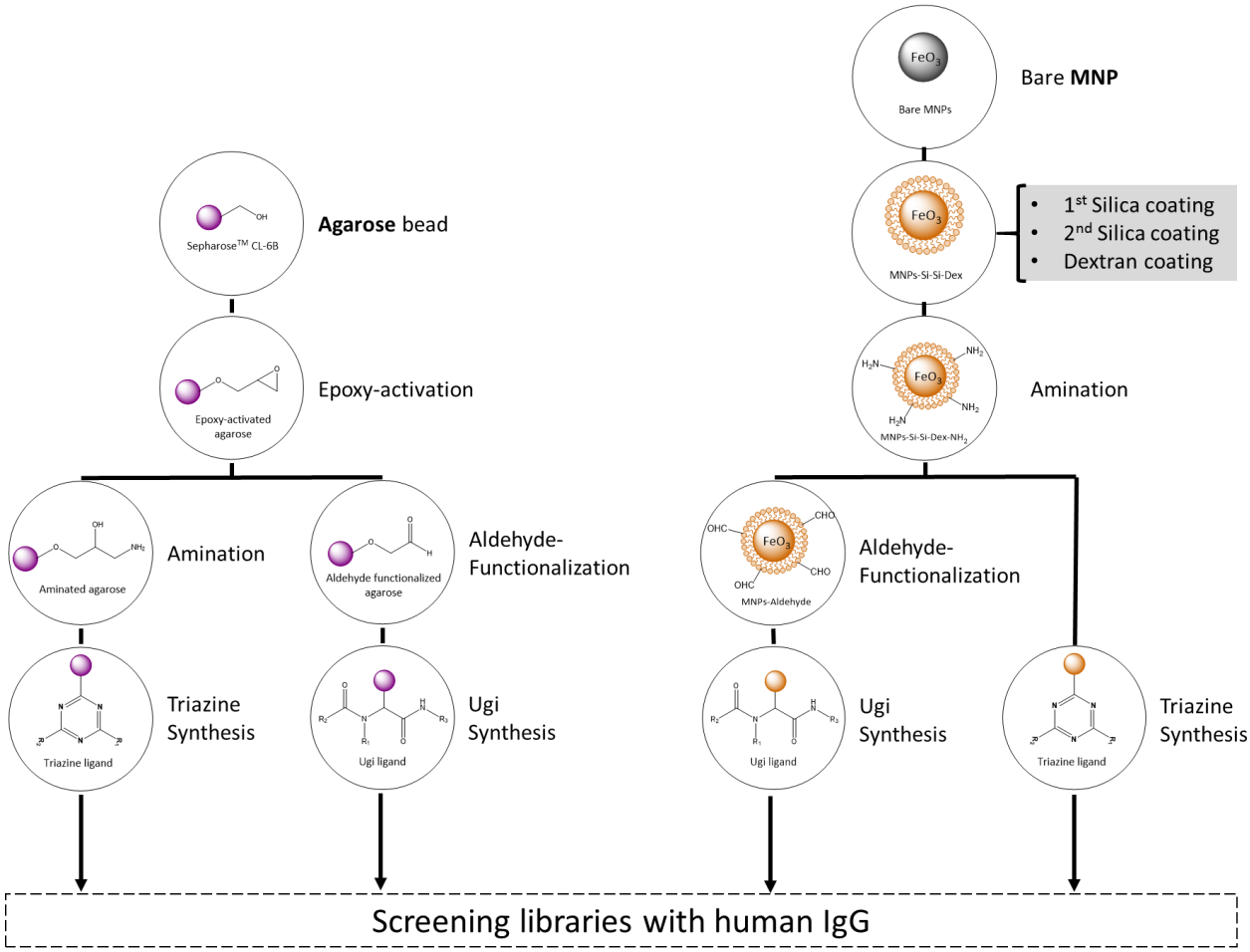


Figure 2.3: Resume of the strategy followed for the synthesis of Triazine and Ugi ligands in the different supports, Cross-linked agarose (left) and Magnetic nanoparticles (right). The purple circles represent the agarose as solid support, and the grey and orange circles represents the MNPs as solid support.

2.2. Materials

2.2.1. Chemicals

All reagents were used with a high purity and the solvents were pro-analysis.

The Iron Oxide Magnetic Nanoparticles used were synthesized by chemical co-precipitation method in an aqueous medium and kindly donated by Dr. Puerto Morales from Spain (ICMM-CSIC). The reagents (3-aminopropyl)triethoxysilane (APTES), 1,4-diaminobutane, 1-amino-2-propanol, 3-aminophenol, 4-amino-1-naphthol hydrochloride, 4-amino-benzamide, 4-aminophenol, 4-hydroxybenzylamine, 4-hydroxyphenylacetic acid, bovine serum albumin, cyanuric chloride acid, dextran sulfate sodium salt from *leuconostoc spp.*, ethanol, glutaric acid, glutaric dialdehyde, isopentylamine, isopropyl isocyanide, phenethylamine, phenylacetic acid, sodium bicarbonate, sodium metasilicate pentahydrate (SiO_3), sodium periodate, sodium phosphate dibasic dehydrate, sodium phosphate monobasic monohydrate, sodium thiosulfate, tetraethyl orthosilicate, tyramine, β-alanine, and γ-aminobutyric acid were acquired from Sigma-Aldrich (Portugal).

Acetone, dimethylformamide (DMF), glycine, methanol, sodium bicarbonate and sodium hydroxide were obtained from VWR. The reagents 2-propanol and ammonium hydroxide were obtained from ROTH, and acetic acid and sodium chloride from Pronalab.

The BCA reagents of BCA kit used in the screenings were purchased from Sigma-Aldrich (Portugal) and the IgG solution Gammanorm (165 mg/ mL normal human immunoglobulin), was produced by Octapharma AB.

2.2.2. Chromatographic Material

Cross linked agarose (Sepharose™ CL-6B) was acquired from GE Healthcare. Deep well plates riplate® sw 2 mL, were purchased from Roth. Half-area UV-Star® 96-well microplates and 96-well transparent microplates were obtained from Greiner Bio-One and Sarstedt, respectively.

2.2.3. Buffers

The following buffers and reagents were used: Phosphate Buffer Saline (PBS) (10mM Sodium phosphate, 150mM Sodium Chloride (NaCl), pH 7.4); Regeneration Buffers (0.1M NaOH in 30% isopropanol and 0.1M HCl).

2.2.4. Equipment

Particle coatings, agarose reactions and ligands synthesis were carried out in Incubator ZKA KS4000i (VWR). Absorbance readings were performed in Microplate reader TECAN Infinite® 200 (Portugal). The oven used in the aldehyde-functionalization of agarose, BCA assays and quantification of particles was a Boekel Big Shot III 230402-2 Hybridization Oven. A BioSan Laboratory Centrifuge LMC-3000 was used for agarose washes after libraries syntheses while Elma Elmasonic S30H Heated Ultrasonic Water Bath was needed to disperse particles.

2.3. Methods

This chapter was accomplished jointly with the chemical engineering MSc. student Ana Lázaro Herrasti from *Universidad Politécnica de Madrid* and with the chemical engineering MSc. student Marina Sayuri Uema from *Escola Politécnica da Universidade de São Paulo*. These students were doing an internship at the Biomolecular Engineering Lab. Both produced a solid-phase library based on Triazine and Ugi reactions and performed the screening of the libraries with human IgG. In my work, I repeated the synthesis of the triazine library, screening of Triazine libraries in MNPs and agarose and performed the data analysis.

2.3.1. Epoxy-activation of agarose

Initially, the Sepharose™ CL-6B was washed with distilled water (10x volume of resin's weight) in a sinter funnel using vacuum suction. Then, the resin was ressuspended in distilled water (1 mL/ g moist agarose) and NaOH 10 M (0.04 mL/ g moist agarose). The suspension was incubated for 30 minutes at 30 °C with orbital shaking at 230 rpm. Posteriorly, Epichlorohydrin was added in a proportion of 0.072 mL/g moist agarose and incubated for 3 hours at 36 °C at 200 rpm. In the end, the agarose was washed with distilled water (10x resin volume).

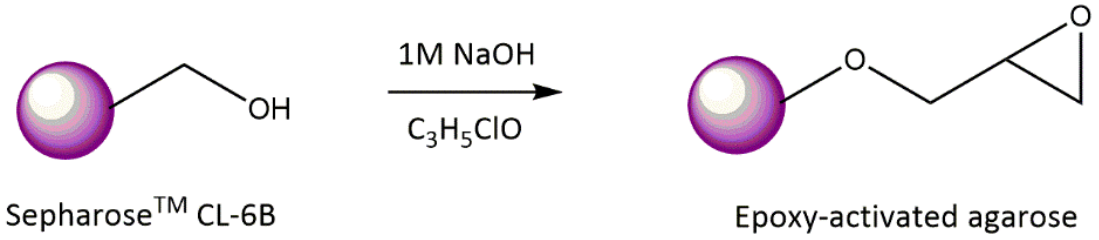


Figure 2.4: Scheme of epoxy-activation reaction of agarose. The purple circle represents the solid support (agarose).

Afterwards, to determine the amount of epoxy groups by the quantification of OH^- groups released from the epoxy ring opening, 1 g of epoxy-activated agarose was incubated with 3 mL of sodium thiosulfate 1.3M for 20 minutes at room temperature. Epoxy groups were then quantified by titration with 0.1M HCl until pH reached 7. The volume added corresponded to the number of OH^- released (10 µmoles per 100 µL added). The epoxy-activation of Sepharose™ CL-6B usually yields 20 µmol epoxy/ g moist agarose [16]. In this work, the epoxy-activation obtained were between 21-23 µmol/ g of agarose.

2.3.2. Functionalization of epoxy-activated agarose with amine groups

Epoxy-activated agarose was ressuspended in 5.0 M Ammonium hydroxide (1.5 ml/g of moist resin) and incubated overnight at 40 °C, with agitation at 200 rpm. The aminated agarose was then washed with distilled water (10x resin volume).

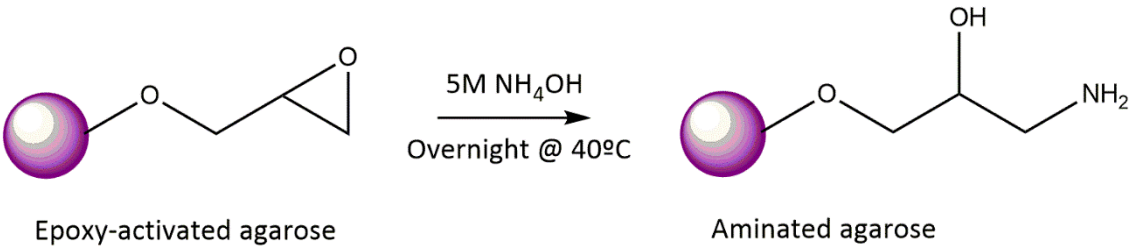


Figure 2.5: Scheme of amine functionalization reaction of agarose. The purple circle represents the solid support (agarose).

Then a Kaiser test was performed in order to confirm the presence of amines in the agarose. This test allows to quantify the free amines (-NH_2) present in a given sample, in this case the agarose, and is based on the reaction of ninhydrin with primary amines, yielding a characteristic dark blue colour. Therefore, 50 µl of 5% ninhydrin in ethanol (w/v), 50 µl of 80% phenol in ethanol (w/v) and 50 µl of 2% 0.001M potassium cyanide in pyridine (v/v) were added to 0.1 g of aminated agarose in 0.9 mL of distilled water. The samples were heated in a water-bath at 100 °C for 5 minutes. A calibration curve with glycine solutions (0-5 µmol/mL) was performed in duplicate, resulting in an equation line ($y = 5.04x - 0.0955$ with a $R^2 = 0.987$) where was determined an average amines concentration of 10 µmol/ g.

2.3.3. Functionalization of agarose with aldehyde groups

Epoxy-activated agarose was ressuspended in 5M NaOH (1 mL/g moist resin) and incubated overnight at 30°C (200 rpm). Then the resin was washed with distilled water (10x resin

volume) using vacuum funnel, ressuspended in sodium periodate 0.1M (1 ml/g moist resin) and incubated in the orbital shaker (200 rpm) at 45 °C for 6 hours.

After the reaction, the functionalized agarose was washed with distilled water (10x resin volume) and stored with 20% (v/v) ethanol/distilled water (1 ml/g of moist resin) at 4°C.

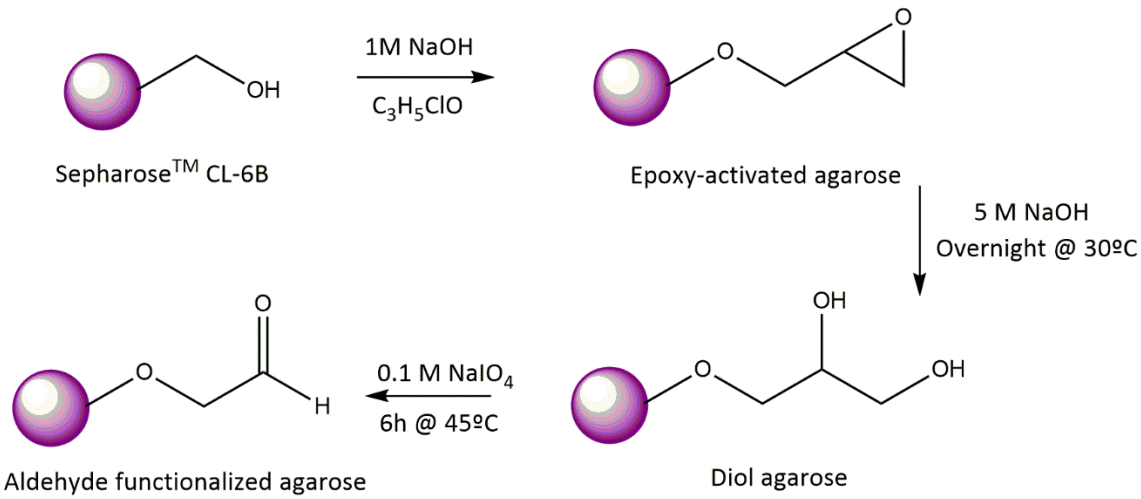


Figure 2.6: Scheme of epoxy-activation and aldehyde functionalization reaction of agarose. The purple circle represents the solid support (agarose).

2.3.4. Synthesis and Preparation of magnetic nanoparticles for libraries synthesis

The MNPs were provided by Ana Lázaro Herrasti, and their synthesis were performed by the co-precipitation method with an average size of 20 nm. The preparation of MNPs for the libraries synthesis, such as the coating and the functionalization steps, were performed according to the procedures present in the Appendix 1 (1.1; 1.2; 1.3 and 1.4), by Ana Lázaro Herrasti and Marina Sayuri Uema.

The synthesis of Triazine and Ugi ligands with MNPs as a solid support were conducted as described in Appendix 2 and 3, also by Ana Lázaro Herrasti.

2.3.5. Synthesis of Triazine ligands in agarose

Aminated agarose was washed with distilled water (10x resin volume). Then two solutions were prepared: a cold solution of 50% (v/v) acetone/distilled water with 1 molar eq. of sodium bicarbonate ($NaHCO_3$) relative to epoxy groups (1 mL/g of moist agarose), and a solution of cyanuric chloride (5 eq. molar excess, relative to epoxy groups) dissolved in acetone

(8.6 mL/ g of cyanuric chloride). These solutions were added to the aminated agarose, followed by 1 hour of incubation in ice (approx. 0 ^{o}C) at 200 rpm.

After reaction, the agarose was washed with acetone (2x resin volume), 50% (v/v) acetone/distilled water (3x resin volume) and distilled water (5x resin volume). The cyanuric-functionalized agarose was ressuspended in distilled water (1 mL/ g of moist agarose), and then distributed by 64 wells of a 96 deep well plate (0.25 g/well), with a 1 mL pipette tip that was cut 4 mm at the end. After that, the plate was centrifuged to take the supernatant out, carefully, with a pipette.

The amines A1 to A8 were added to each column of the deep well plate (2 molar eq. of each, relative to epoxy groups; 0.5 mL/well). A sealing cover was placed and the plate was incubated for 24 hours at 30 ^{o}C (150 rpm), R_1 substitution (Figure 2.7). Thereupon, the ligands were washed with the solvent in which each amine was dissolved (3x 1 mL) and distilled water (3x 1 mL).

Then, the amines A1 to A8 were added to each row of the deep well plate (5 molar eq. of each, relative to epoxy groups; 0.5 mL/well). The plate was then sealed and incubated for 48 hours at 80 ^{o}C (150 rpm), R_2 substitution (Figure 2.7). At the end, the ligands were washed with the solvent in which each amine was dissolved (5x 1 mL) and distilled water (5x 1 mL), ressuspended in distilled water (1 mL/ well) and stored at 4 ^{o}C.

All the amines were dissolved in distilled water, with the exception of amines A2 and A6, which were dissolved in 50% (v/v) DMF/distilled water. Moreover, it was added to each amine 1M of sodium bicarbonate (1 molar eq., relative to epoxy groups).

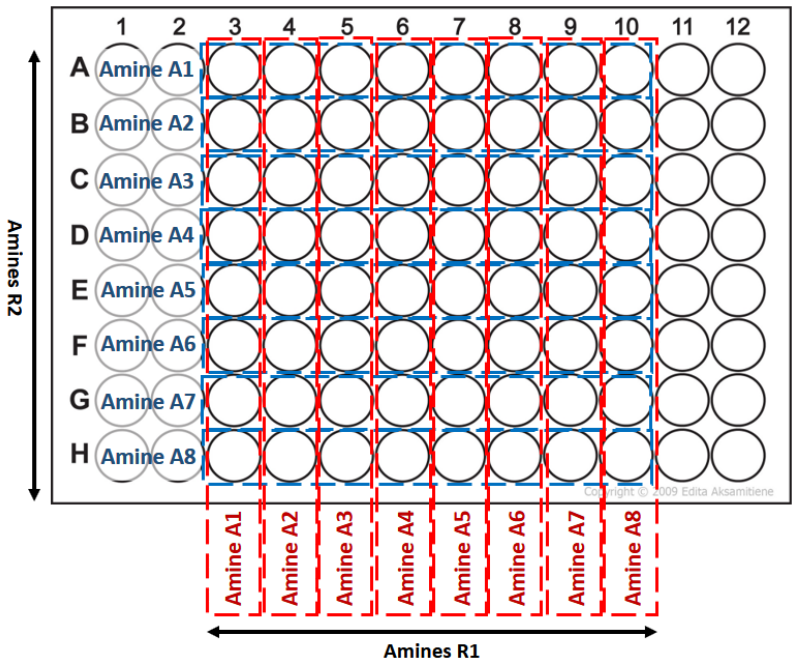


Figure 2.76: Illustrative image of the 96 deep well plate used for the synthesis of the triazine ligands.

2.3.6. Synthesis of Ugi ligands in agarose

The synthesis of Ugi ligands with agarose as a solid support were accomplished by MSc. Marina Sayuri Uema following the protocol described in Appendix 4.

2.3.7. Screenings of solid-phase combinatorial libraries

The libraries were regenerated and equilibrated before starting the screening procedure. The resins and the MNPs were washed for binding with 0.1M HCl followed by distilled water (1x 1 mL/ well), and with 0.1M NaOH in 30% isopropanol followed by distilled water (1x 1 mL/ well). After, the equilibration of the ligands was conducted with the addition of the binding buffer, the 10mM Phosphate Buffer, 150mM NaCl at pH 7.4 (15x 1 mL/ well on average).

To perform the screening procedure, a solution of pure human IgG in Phosphate Buffer at pH 7.4 (1 mg/ mL) was prepared and 0.25mL of this solution and 0.75mL of PBS was loaded to each well. Then the deep well plate was incubated for 1 hour at 25 ^{o}C with agitation (50 rpm). After, the plate with agarose was centrifuged for 30 seconds at 500 rpm, the plate with MNPs was placed in the magnet, and the flow-through of both libraries were taken with a pipette and transferred to a different deep well plate. The same was done with the washes (3x 1 mL of PBS/ well). In the end, the resins and the MNPs were ressuspended in PBS (1mL/ well) and stored at 4 ^{o}C.

Posteriorly, the total protein present in the samples were quantified by the BCA assay according to supplier instructions, where 200µL of BCA reagent was added to 25µL of each sample and incubated at 37 ^{o}C for 30 minutes. The absorbance of the samples was then read at 560 nm. Protein concentration was determined with a calibration line using solutions with known protein concentration, BSA in 10mM Phosphate Buffer 150mM NaCl at pH 7.4 from 0 to 1000 µg/mL with values of $y = 0.001x + 0.0099$ with a $R^2 = 0.99$.

2.4. Results and Discussion

2.4.1. Library Design: Selection of amines, carboxylic acids and isonitrile

The Ugi and Triazine libraries used in this work (Tables: 2.1., 2.2. and 2.3.) were set up based in previous works: [22]–[24], [51], in which Ugi and Triazine affinity ligands successfully developed to bind antibodies in agarose (resin used in chromatography) were described. Some of the reagents described in the original articles were substituted by similar ones.

Table 2.1: Amine compounds (A1 - A8) used in Triazine combinatorial library with structure and name.

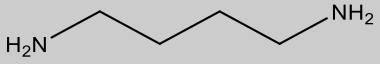
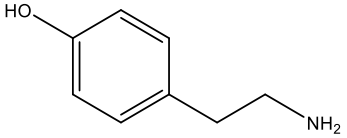
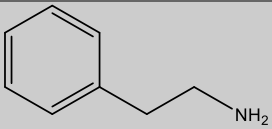
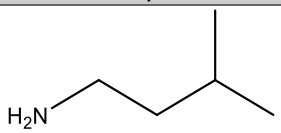
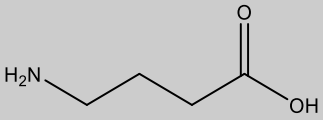
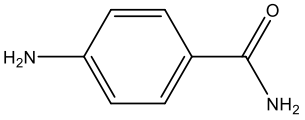
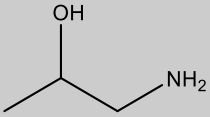
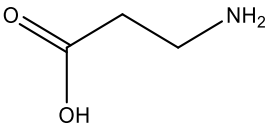
Number	Reagent
A1	 1,4-diaminobutane
A2	 Tyramine
A3	 Phenethylamine
A4	 Isopentylamine
A5	 4-aminobutyric acid
A6	 4-amino-benzamide
A7	 1-amino-2-propanol
A8	 β-alanine

Table 2.2: Amine compounds (A1 – A7) used in Ugi combinatorial library with structure and name.

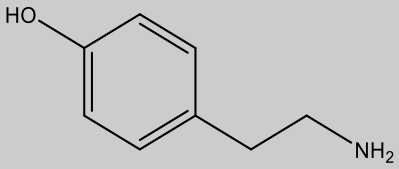
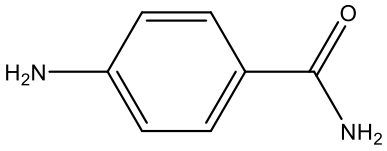
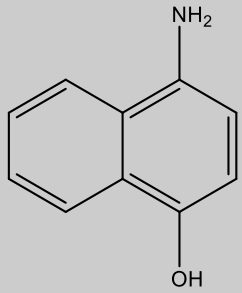
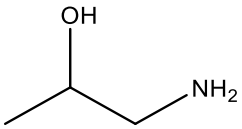
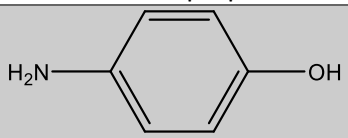
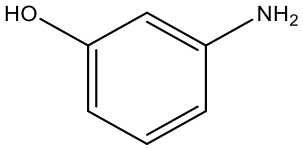
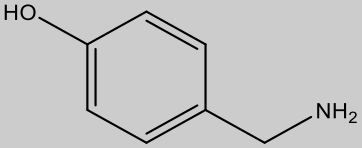
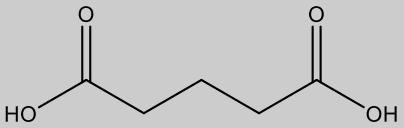
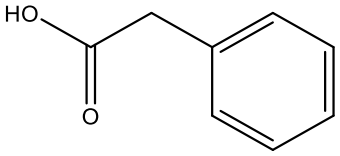
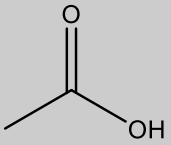
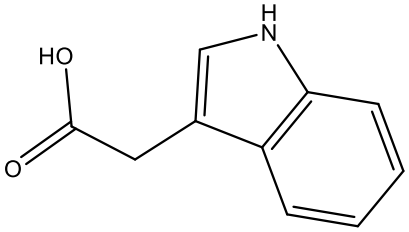
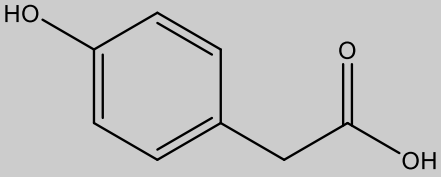
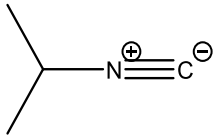
Number	Reagent
A1	 Tyramine
A2	 4-amino-benzamide
A3	 HCl 4-amino-1-naphthol hydrochloride
A4	 1-amino-2-propanol
A5	 4-aminophenol
A6	 3-aminophenol
A7	 4-hydroxylbenzylamine

Table 2.3: Carboxylic acid compounds (C1 – C5) and isonitrile compound (I1) used in Ugi combinatorial library with structure and name.

Number	Reagent
C1	 Glutaric acid
C2	 Phenylacetic acid
C3	 Acetic acid
C4	 Indole-3-acetic acid
C5	 4-hydroxyphenylacetic acid
I1	 Isopropyl isocyanide

2.4.2. Screening results and data processing

In order to test and analyse the binding capacity of the synthetic ligands in both supports (agarose and MNPs), the libraries were synthesized in duplicate. All libraries were screened with pure human IgG (250 μg/well) in Phosphate buffer saline (PBS) at pH 7.4.

The unbound IgG was quantified by BCA assay, which gives a linear response from 200 to 1000 μg/L of protein and is based on the formation of a Cu^{2+}-protein complex under alkaline conditions. Peptide bonds and the amino acids cysteine, tryptophan and tyrosine are able to

reduce Cu^{2+} to Cu^{1+}, producing a purple-blue complex which absorbs at 562 nm. The amount of reduction is proportional to the protein.

The quantity of protein binding was determined by Equation 2.1 and Equation 2.2. The binding capacity of the ligands were determined by Equation 2.3.

Equation 2.1:

$$Amount\ of\ protein\ bound\ (\mu g) = Amount\ of\ protein\ loaded\ (\mu g) - \sum amount\ of\ protein\ washed\ (\mu g)$$

Equation 2.2:

$$\%\ Protein\ Bound = \frac{Amount\ of\ protein\ bound\ (\mu g)}{Amount\ of\ protein\ loaded\ (\mu g)} \times 100$$

Equation 2.3:

$$Binding\ capacity\ (mg/g) = \frac{mg\ of\ protein\ bound}{g\ of\ support}$$

Triazine library

The screening of the triazine library in agarose (Figure 2.8 A and C) resulted in a mass bound range from 0.25 mg of protein/ g of agarose to 0.70 mg of protein/ g of agarose. Ligands A3A3 and A3A6 were the synthetic ligands with higher binding capacity, with 0.70 mg of protein/ g of agarose and 0.64 mg of protein/ g of agarose, respectively.

Furthermore, the binding capacity of the triazine library in MNPs is higher than the values obtained for the agarose support (Figure 2.8 B and D). This may be due to the distinct physical properties of MNPs, namely the high surface area to volume ratio. All ligands showed the ability to bind IgG resulting in a mass bound range from 4.14 mg of protein/ g of MNPs to 5.86 mg of protein/ g of MNPs. Overall, 42 out of 64 ligands showed more than 80% of protein binding, where the ligands containing amines A4, A5, A6 and A7 (isopentylamine, 4-aminobutyric acid, 4-amino-benzamide and 1-amino-2-propanol, respectively) in R_2 position are the ones with the highest IgG binding.

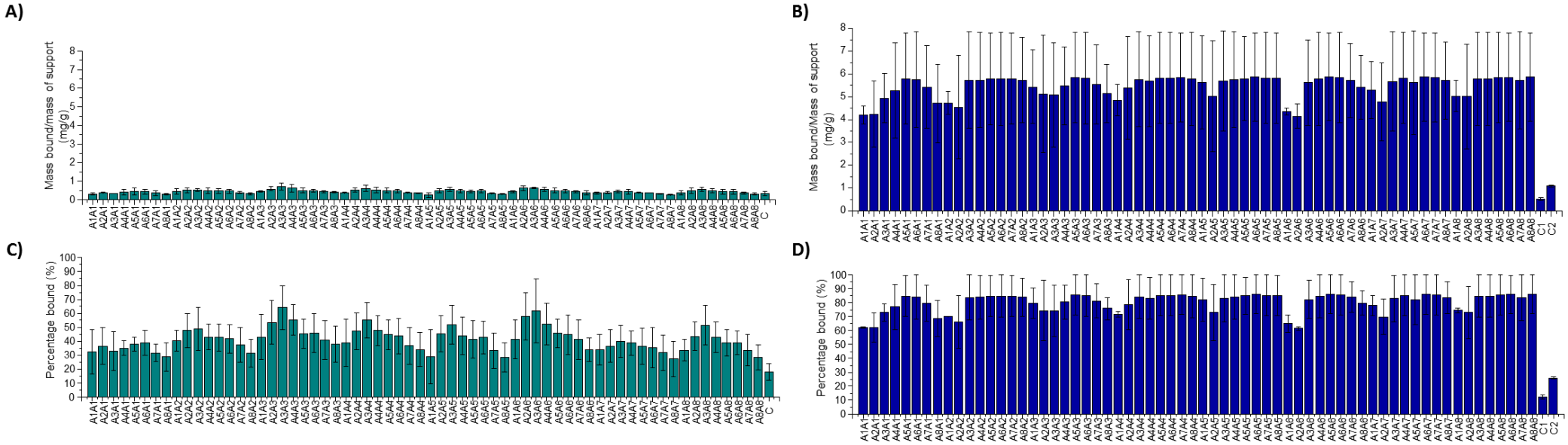


Figure 2.8: Triazine results. Screenings against pure human IgG were performed in duplicate. **A)** Average of screening results of Triazine library in agarose. **B)** Average of screening results of Triazine library in MNPs. **C)** Average of the percentage bound of Triazine ligands in agarose. **D)** Average of the percentage bound of Triazine ligands in MNPs (**C –** Aminated agarose; **C1** – MNPs coated with Dextran (MNPs-Si-Si-Dex); **C2** – Aminated MNPs (MNPs-Si-Si-Dex-NH_2)).

Ugi library

Regarding the screening results of Ugi library in agarose (Figure 2.9 A and C), the mass bound range goes from 0.17 mg of protein/ g of agarose to 1.05 mg of protein/ g of agarose. Ligands A6C4, A7C4 and A6C5 showed the highest amount of protein bound, 1.05 mg of protein/ g of agarose, 0.79 mg of protein/ g of agarose and 0.78 mg of protein/ g of agarose, respectively.

Considering the Ugi library in MNPs (Figure 2.9 B and D), all ligands were able to bind IgG ranging from 1.54 mg of protein/ g of MNPs to 4.65 mg of protein/ g of MNPs. It is possible to observe that 12 out of 35 ligands showed a binding capacity over 50%, where A3C1, A3C2 and A1C3 are the leader ligands with 4.36 mg of protein/ g of MNPs, 4.53 mg of protein/ g of MNPs and 4.65 mg of protein/ g of MNPs, respectively.

As previously observed for the Triazine library, ligands immobilized on magnetic supports presented higher binding capacities when compared with the data observed for cross-linked agarose.

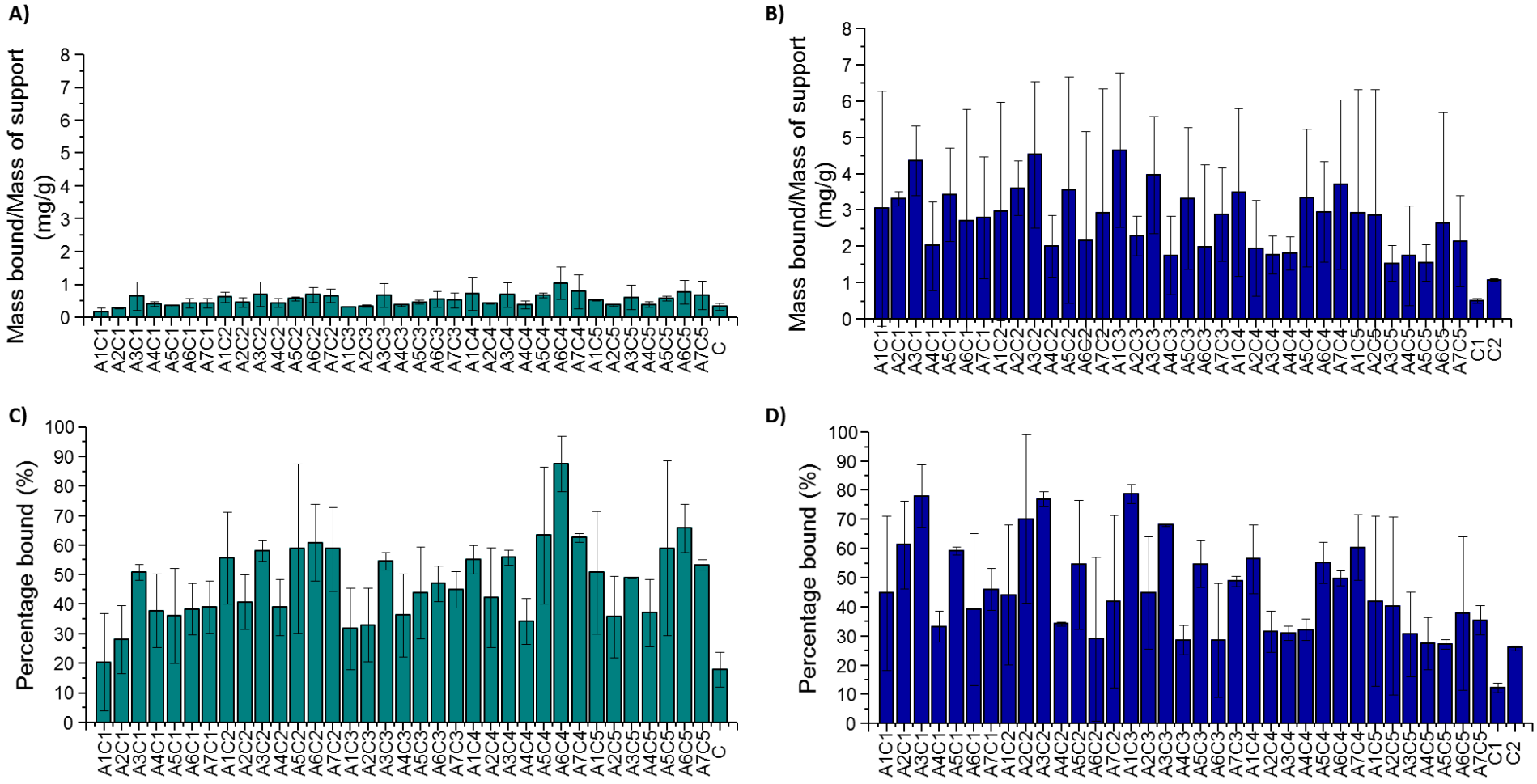


Figure 2.9: Ugi results. Screenings against pure human IgG were performed in duplicate. **A)** Average of screening results of Ugi library in agarose. **B)** Average of screening results of Ugi library in MNPs. **C)** Average of the percentage bound of Ugi ligands in agarose. **D)** Average of the percentage bound of Ugi ligands in MNPs. (**C –** Aminated agarose; **C1** – MNPs coated with Dextran (MNPs-Si-Si-Dex); **C2** – Aminated MNPs (MNPs-Si-Si-Dex-NH_2)).

2.4.3. Reproducibility of libraries

During the experimental work it was observed some differences in the results between the first and the second experiments, for that reason it was necessary to analyse the reproducibility of libraries.

Triazine libraries

For the Triazine libraries (Figure 2.10 A and C) there is a linear trend between the two screening experiments in agarose and we were able to choose the lead ligands with no difficulties, once they coincide in both experiments. Thus, it was possible to have some reproducibility with triazine libraries in agarose. Also, in figure 2.10 C, despite that only two ligands are in the 10% error, it looks like the results from the first to the second experiment increased around 25%.

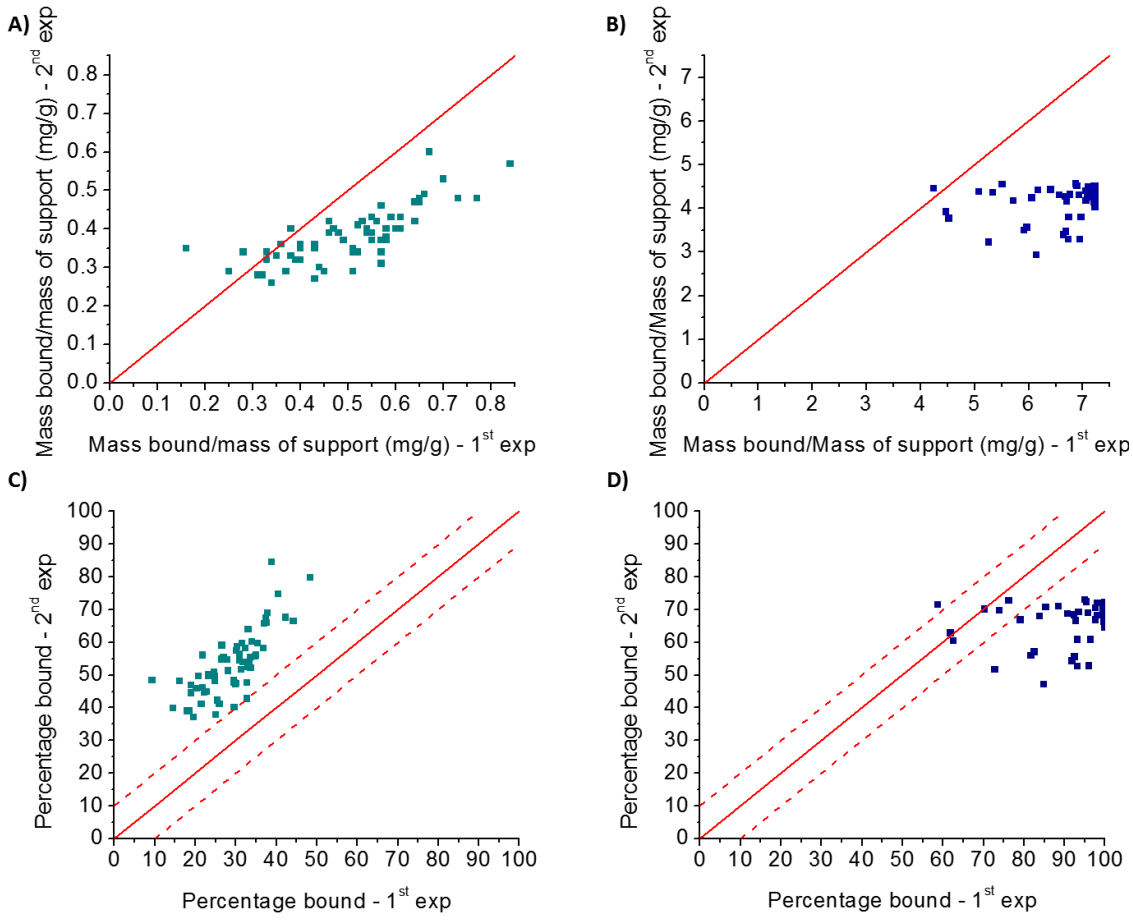


Figure 2.10: Dispersion of Triazine library between the two screenings in agarose and in MNPs. **A)** Comparison in mass of IgG bound per mass of agarose. **B)** Comparison in mass of IgG bound per mass of MNPs. **C)** Comparison in percentage of IgG bound in agarose. **D)** Comparison in percentage of IgG bound in MNPs.

When using MNPs as support (Figure 2.10 B and D), this linear trend is not so evident. The dispersion that is visible can be a consequence of experimental error through the ligands synthesis and/or during the screening procedures. However, it was also possible to choose the lead ligands and is visible 5 ligands in the 10% error (Figure 2.10 D).

Ugi libraries

The reproducibility of the screening of Ugi libraries, the linear trend that was supposed to happen is somehow observable in agarose, but in general the results are scattered in both supports (Figure 2.11), being worst for MNPs libraries. However, it is observed 8 Ugi ligands with agarose as solid-support and 12 Ugi ligands with MNPs as solid-support in the 10% error (Figure 2.11 C and D), which is more when compared with the Triazine libraries. With this dispersion, the selection of the best ligands was a difficult task, once they were not exactly the same in a both experiments.

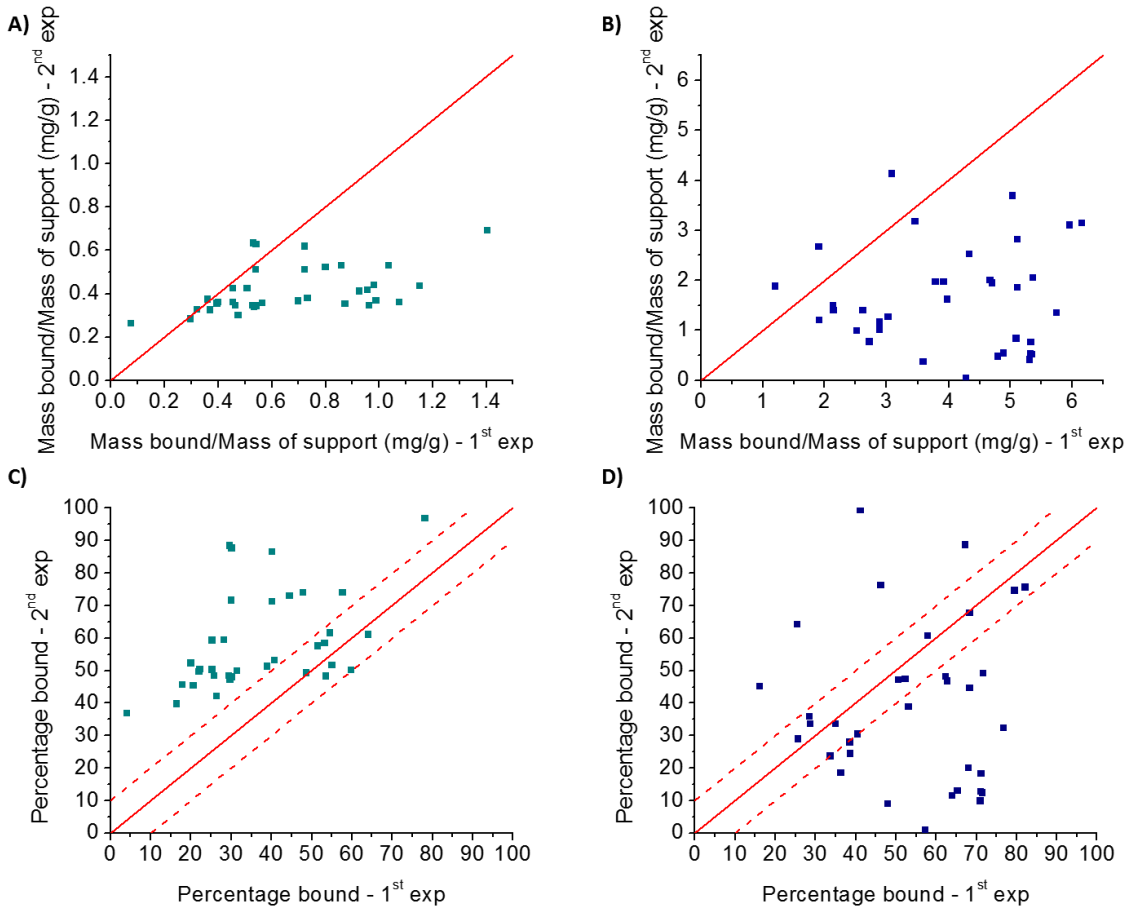


Figure 2.11: Dispersion of Ugi library between the two screenings in agarose and in MNPs. **A)** Comparison in mass of IgG bound per mass of agarose. **B)** Comparison in mass of IgG bound per mass of MNPs. **C)** Comparison in percentage of IgG bound in agarose. **D)** Comparison in percentage of IgG bound in MNPs.

In summary, the reproducibility of both Triazine and Ugi libraries in both supports was low, being more evident when MNPs were used as solid support. To improve the reproducibility of Triazine and Ugi libraries, it is important to control some parameters during the synthesis of the ligands, such as temperature, volumes of reagents, constantly check the solvents due to evaporation and wash carefully the MNPs to minimize their loss. Additionally, once we work with low volumes and quantities, it easily happens some experimental error, which can somehow affect the final results.

2.4.4. Reproducibility between supports

Once this chapter studies the influence of different supports in synthetic ligands, it is relevant to evaluate the capacity of reproducibility between the supports (agarose and MNPs).

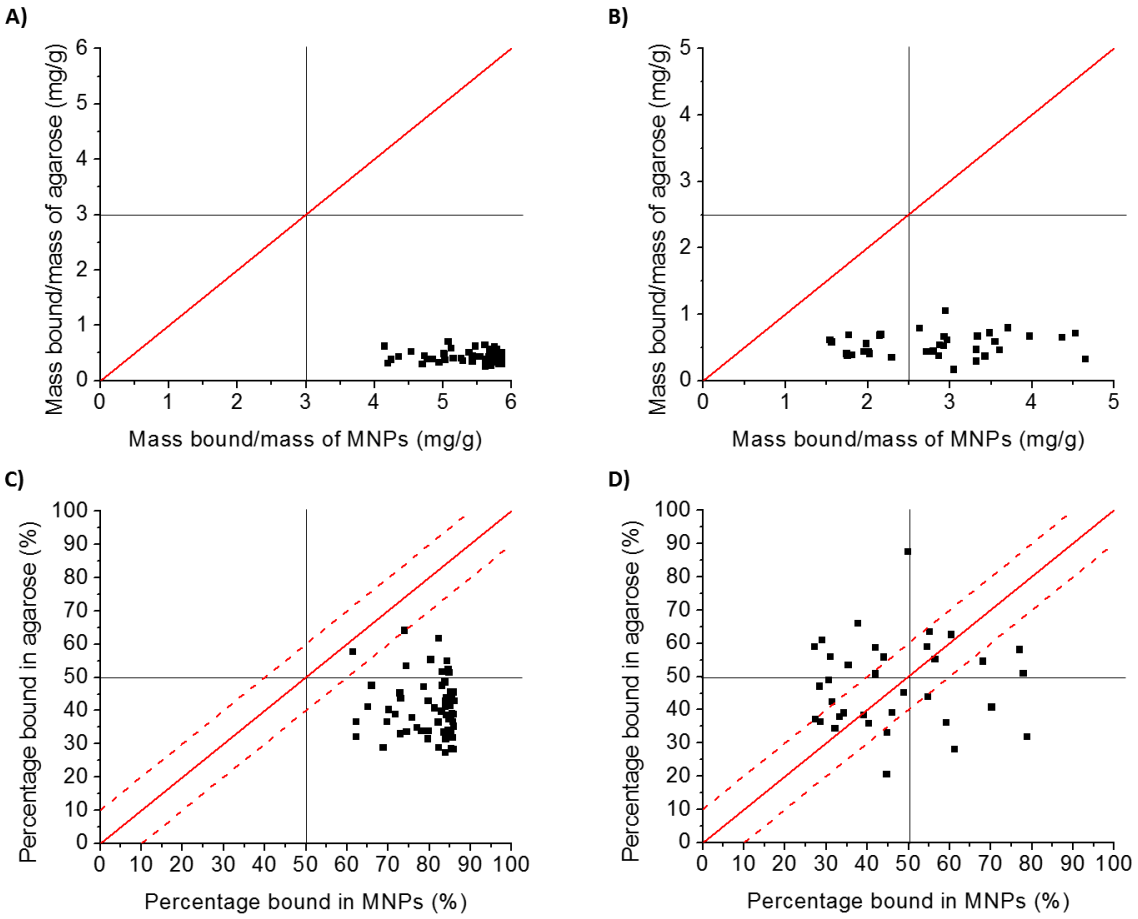


Figure 2.12: Dispersion of the libraries between the two supports (agarose and MNPs). **A)** Comparison of Triazine results in mass of IgG bound per mass of support. **B)** Comparison of Ugi results in mass of IgG bound per mass of support. **C)** Comparison of Triazine results in percentage of IgG bound. **D)** Comparison of Ugi results in percentage of IgG bound.

Analysing the graphs (Figure 2.12), it is clearly observable that none of the libraries follows a trend line, where only 2 Triazine ligands are in the 10% error and in Ugi libraries, despite being the more disperse results, there are 14 ligands in the 10% error. In general, none of them have reproducible results between the supports. However, a common observation is that the amount of protein bound onto MNPs is always higher.

2.4.5. Selection of lead ligands

In Triazine results (Table 2.4), all the best five ligands are different in both supports, so there are no common lead affinity ligands, where we conclude that there is influence of the support with the binding capacity of the affinity ligands.

Table 2.4: Best 5 ligands in Triazine library of both supports and their respective values of binding capacity and protein binding.

Support	Ligand	Binding Capacity (mg Protein bound/g of support)	Protein Binding (%)
Agarose	A3A3	0.70	64%
	A3A6	0.64	62%
	A4A3	0.62	55%
	A2A6	0.62	58%
	A3A4	0.61	55%
MNPs	A6A5	5.86	86%
	A8A8	5.86	86%
	A6A7	5.86	86%
	A6A8	5.85	86%
	A7A4	5.85	86%

However, when we observe the values of the best Triazine ligand in MNPs with the correspondent ones in agarose, A6A5 (=A5A6) has 43-46% of protein binding in agarose, which can be considered a relatively good binding.

Regarding the Ugi results (Table 2.5), it is possible to find two common lead affinity ligands in both supports, the A7C4 and A3C2. In agarose A7C4 is the second best ligand while in MNPs is the fifth best ligand, on other hand A3C2 is the fifth best affinity ligand in agarose and the second in MNPs as support.

Table 2.5: Best 5 ligands in Ugi library of both supports and their respective values of binding capacity and protein binding.

Support	Ligand	Binding Capacity (mg Protein bound/g of support)	Protein Binding (%)
Agarose	A6C4	1.05	87%
	A7C4	0.79	63%
	A6C5	0.58	66%
	A1C4	0.72	55%
	A3C2	0.71	58%
MNPs	A1C3	4.65	79%
	A3C2	4.53	77%
	A3C1	4.36	78%
	A3C3	3.97	68%
	A7C4	3.71	60%

Nevertheless, besides A7C4 and A3C3, the other best ligands found in both supports are different, which implies that the substitution of the support for ligand immobilization strongly influences the choice of the best ligands.

2.5. Conclusion and future perspectives

In this chapter, two combinatorial libraries of affinity ligands based on the triazine and Ugi reaction were designed, synthesized in two different supports (cross-linked agarose and magnetic nanoparticles) and screened against IgG. It should be noticed that the use of magnetic nanoparticles as a purification technique is under development.

In order to create the two libraries, the amines and carboxylic acids chosen were based on Ugi and Triazine affinity ligands that were developed/used to bind antibodies and are already described on literature. In total, 64 Triazine ligands and 35 Ugi ligands were synthesized in agarose and in MNPs, and tested against pure IgG, where it was possible to find synthetic ligands with a high binding capacity for IgG.

The reproducibility of the libraries in each support were evaluated, it was more evident in Triazine with agarose and was really low with MNPs as support in both libraries. To overcome this problem, the synthesis and screenings of the affinity ligands should be repeated and some parameters should be more controlled to minimize the experimental error, such as solvent evaporation and washing steps.

The reproducibility between the supports was in general low, and higher binding capacities were found for the libraries immobilized in magnetic nanoparticles. Therefore, we conclude that the use of different supports can influence the binding capacity of the affinity ligands and consequently the selection of the best synthetic ligands. This can be due to the characteristics of the supports: the crossed-linked agarose has high hydrophilicity and can interact with proteins; the MNPs used were functionalized with amines (NH_4^+), which were not blocked, for that reason the nanoparticle could have an excess of positive charge and present non-specific interactions through electrostatic interactions. In fact, the aminated agarose presented a binding capacity of 0.32 ± 0.12 mg of protein bound/g of support, the MNPs coated with Dextran 0.51 ± 0.06 mg of protein bound/g of support and the aminated MNPs 1.08 ± 0.04 mg of protein bound/g of support.

In conclusion, the cross-linked agarose is still the best support to synthesize affinity ligands and do the initial screenings, in order to choose the lead ligands for further studies, since it's easier to manipulate and gives more reproducible results. For these reasons, cross-linked agarose was the solid support to proceed the work.

III. Design, Synthesis and Screening of a Petasis-Ugi ligands library towards IgG

3.1. Introduction

The low molecular weight ligands, that can mimic biological binders, are of great interest. Small ligands show several advantages and can be used in different areas, such as drug discovery, enzyme inhibition, protein purification applications and, hopefully, to assemble a novel generation of ADCs.

Affinity ligands are classified in two major classes: biospecific and pseudospecific. Biospecific ligands are molecules derived from natural sources with affinity for a target protein-binding site. Pseudospecific ligands may be biological or synthetic molecules that interact with a target protein but do not occur naturally in biological systems. Biomimetic ligands are the youngest class between the synthetic ligands, which mimic the natural biological recognition between a target protein and a natural ligand, by mimicking the amino acids that are involved in the binding site [13], [52].

Biomimetic ligands have been developed and improved through rational design, combinatorial chemistry, and high-throughput screening techniques, in order to overcome drawbacks of their naturally-occurring templates. Biomimetic ligands are chemically defined, easy to produce at large-scale, show high chemical and biochemical resistance, less immunogenicity and reduced production costs [13], [17], [52].

Lowe and co-workers were pioneers in the design and synthesis of biomimetic ligands. Four main design strategies have been followed to the development of biomimetic ligands: (i) use as a template a natural molecule involved in binding to the target protein; (ii) design a molecule that mimics the binding between the complementary molecule and the target site of the protein; (iii) direct mimic of the interactions in the natural biological recognition; and (iv) study of the target protein and selection of suitable binding sites [53].

Biomimetic ligands have been successfully synthetized by different combinatorial strategies, which include a small chemical library synthesis that is screened against a target protein. The library synthesis occurs on a solid support, which eliminates laborious intermediate purification steps, allows ease removal of excess reagents, by-products and solvents by a separation process, such as filtration [50].

3.1.1. Petasis-Ugi Reaction

A new approach has been developed in synthesis of combinatorial libraries of affinity ligands. The Petasis-Ugi reaction is based in Petasis borono-Mannich reaction and in Ugi reaction together and was adapted to cross-linked agarose as a solid support by Irís Batalha in 2014 [54].

The Petasis borono-Mannich reaction was discovered by Nicos A. Petasis in 1993, this reaction consists in adding an aldehyde, an amine, and a boronic acid. Secondary amines are the most reactive, followed by bulky primary amines, and the employment of tertiary amines has been reported as well. Petasis reaction has been used for the synthesis of α-amino acids, amino alcohols, 2-hydroxymorpholines, 2H-chromenes, etc [50], [54].

In this circumstance, the first step of Petasis-Ugi reaction is the Petasis borono-Mannich reaction (Figure 3.1), which consists in a condensation between the amine on the solid matrix and the aldehyde component (glyoxylic acid) obtaining a zwitterion specie. Then, the carboxylate of the zwitterion reacts by nucleophilic addition with the boron from the boronic acid, forming a negatively charged tetra-coordinated boronate intermediate – the "ate complex". The boronic acid substituent (R_1) then migrates to the iminium's electrophilic carbon, which is the irreversible step of this reaction. Both the product and boric acid are then generated by a hydrolysis reaction. This reaction mechanism is currently considered more energetically favourable, however some mechanistic differences may be observed depending on the nature of each of the components [50], [54].

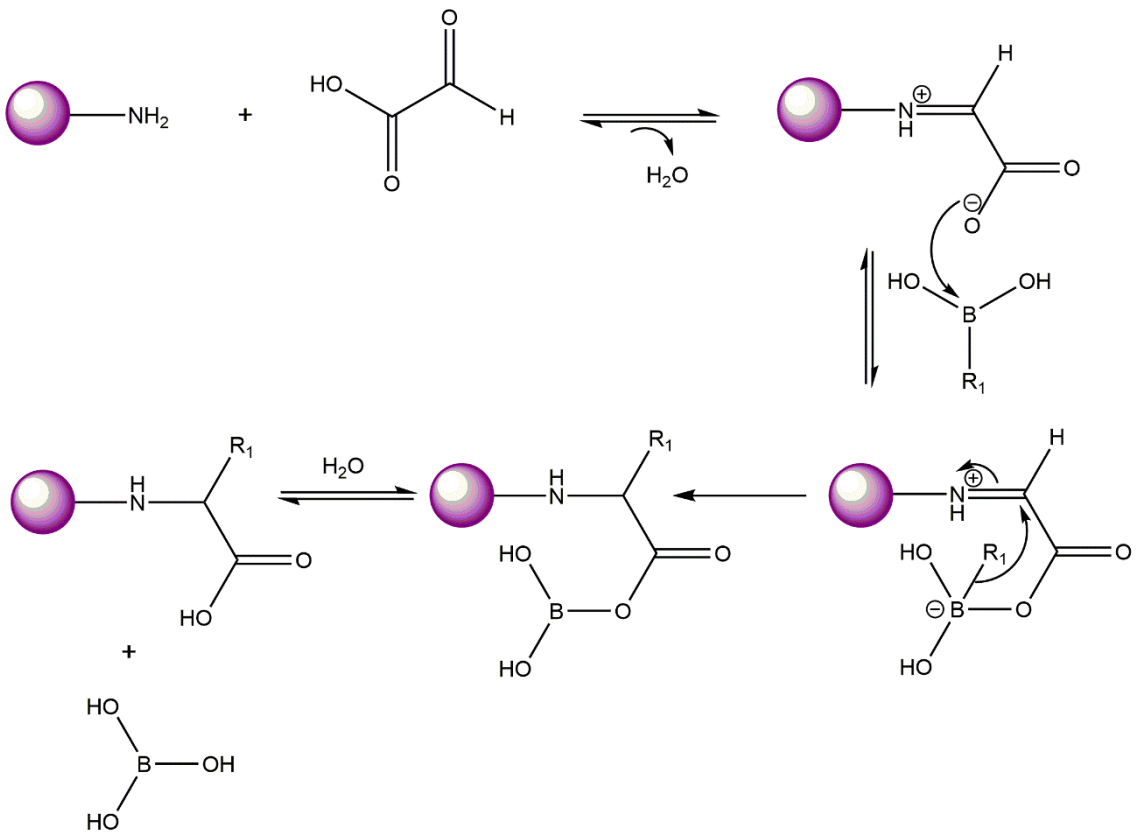


Figure 3.1: Proposed mechanism of Petasis borono-Mannich reaction on solid-phase, where the purple sphere represents the solid support. Adapted from Batalha, 2014.

In the end, Petasis reaction results in a product with a free carboxylic acid, which outcomes from the glyoxylic acid component, and can be further employed as a component in other reactions, such as the Ugi reaction [50], [54].

The next step is the employment of Ugi reaction, but now instead of using an aldehyde as a start point, a free carboxylic acid is the handle in the solid phase. Like in Ugi reaction the first step is the condensation between the amine component (R_2) and the aldehyde component (R_3) in order to form an imine. Then the imine reacts with isonitrile component (R_4) to generate the nitrilium ion. The nitrilium ion reacts with carboxylate ion (R_1) in the solid matrix, generating an unstable imino-anhydride. Finally, a Mumm rearrangement occurs to generate the final Petasis-Ugi product (Figure 3.2).

Thus, the condensation between the carboxylic acid and an amine (R_2), an aldehyde (R_3) and an isonitrile component (R_4) generates the Petasis-Ugi scaffold. The tandem Petasis-Ugi reaction tremendously increases molecular diversity, by allowing the incorporation of a higher number of functional groups [50], [54].

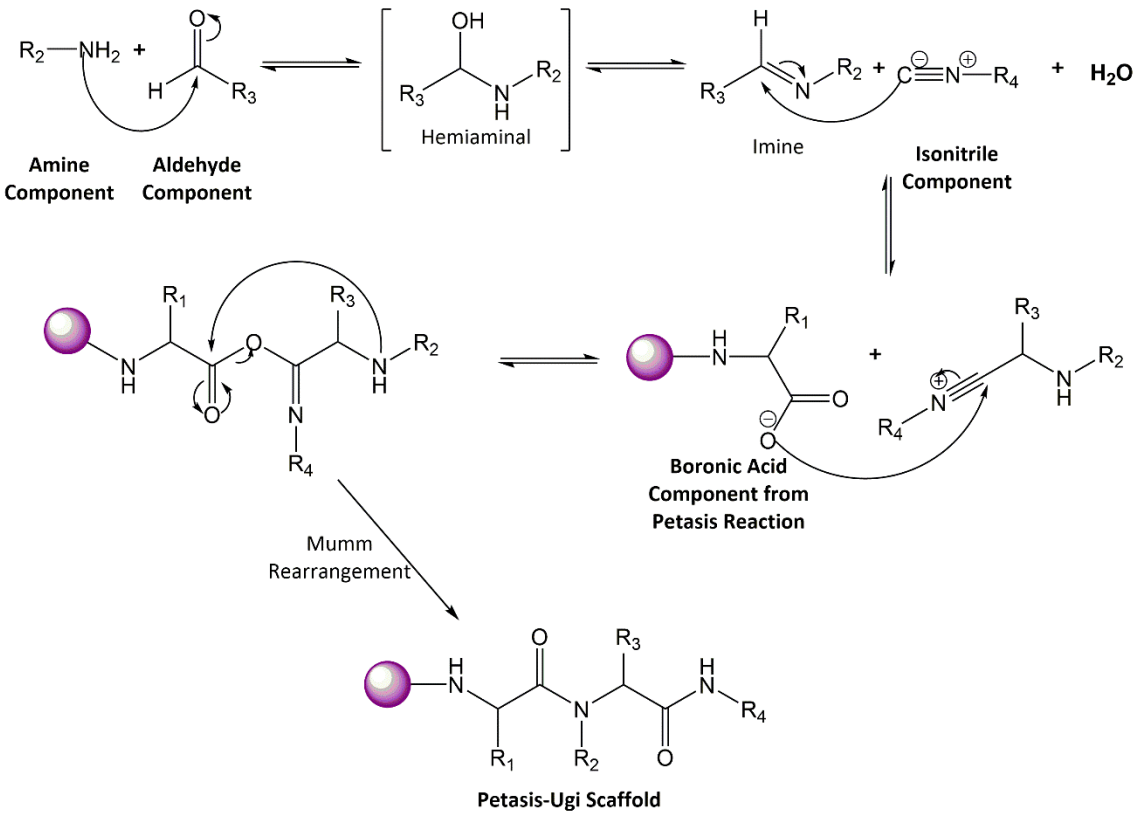


Figure 3.2: Solid-phase Petasis-Ugi Reaction mechanism, where the purple sphere represents the solid support.

The aim of this chapter is to develop a new library of affinity ligands for antibodies, using a new combinatorial chemistry, the Petasis-Ugi reaction. From this library, the main goal is to find affinity ligands with high binding capacity towards IgG, and apply it in two fields: Antibody purification and creation of ADC complexes.

3.2. Materials

3.2.1. Chemicals

All reagents were used with a high purity and the solvents were pro-analysis.

The reagents 3-aminophenol (A1), 4-amino-1-naphthol hydrochloride (A2), 4-aminobenzamide (A3), Tyramine (A4), y-aminobutyric acid (A5), Agmatine sulfate salt (A6), 1-amino-2-propanol (A7), Isopentylamine (A8), Indole-3-carboxaldehyde (Al1), 4-Imidazolecarboxaldehyde (Al2), 3,5-di-*tert*-butyl-2-hydroxybenzaldehyde (Al3), Phenylboronic acid (B1), 3-thienylboronic acid (B2), 4-aminocarbonylphenylboronic acid (B3) were purchased from Sigma-Aldrich (Portugal).

Epichlorohydrin, sodium thiosulfate ($Na_2S_2O_3$), glyoxylic acid monohydrate, isopropyl isocyanide, sodium chloride (NaCl), Tris (hydroxymethyl)aminomethane hydrochloride and glycine were acquired from Sigma-Aldrich (Portugal). Ammonium hydroxide solution (NH_4OH) and Methanol (MeOH) were obtained from Roth (Portugal). Dimethylformamide (DMF) and di-sodium-hydrogen phosphate 2-hydrate were purchased from VWR. Hydrochloric acid 37% (HCl), ethanol absolute PA, acetic anhydride, sodium-di-hydrogen phosphate 1-hydrate and sodium hydroxide (NaOH) were acquired from Panreac (Spain).

The BCA reagents of BCA kit used in the screenings were purchased from Sigma-Aldrich (Portugal) and the IgG solution Gammanorm (165 mg/ mL normal human immunoglobulin), was produced by Octapharma AB.

3.2.2. Chromatographic Material

Cross linked agarose (Sepharose™ CL-6B) was acquired from GE Healthcare. Captiva 96-well filtration block, Agilent bond Elut 3ml and Frits was purchased from Agilent Technologies (USA). Deep well plates riplate® sw 2 mL, were acquired from Roth (Portugal). Half-area UV-Star® 96-well microplates and 96-well transparent microplates were obtained from Greiner Bio-One (Germany) and Sarstedt (Portugal), respectively.

3.2.3. Buffers

The following buffers and reagents were used: Phosphate Buffer Saline (PBS) (10mM Sodium phosphate, 150mM Sodium Chloride (NaCl), pH 7.4); Regeneration Buffers: 0.1M NaOH in 30% isopropanol and 0.1M HCl; Regeneration Buffers for a more extensive regeneration: 0.1M

NaOH in 50% isopropanol; 0.1M glycine-NaOH at pH 9.0 in 50% ethylene glycol; 0.1M Glycine-NaOH at pH 11; 0.1M HCl; 0.1M Glycine at pH 2.5; 0.2M NaOH in 50% isopropanol; and 1M NaCl in Phosphate buffer saline.

The buffers used in the elution steps were 0.1M Glycine-HCl at pH 2 and Tris-NaOH at pH 9.0 for neutralization of eluted samples; 0.1M Glycine-NaOH at pH 11 and PBS at pH 6 (10mM Sodium phosphate, 150mM Sodium Chloride).

3.2.4. Equipment

The synthesis of the synthetic ligands on solid-phase were carried out in Incubator ZKA KS4000i (VWR). Absorbance readings were performed in Microplate reader TECAN Infinite® 200 (Portugal). The oven used for ligand synthesis and BCA assay was a Big Shot III 230402-2 Hybridization Oven from Boekel Scientific. A Laboratory Centrifuge LMC-3000 from BioSan was used for agarose washes after libraries synthesis and for screenings in filter plates or in deep well plates.

3.3. Methods

3.3.1. Epoxy-activation of agarose and functionalization with amine groups

The epoxy-activation and functionalization of agarose with amine groups was performed according to the procedures 2.3.1. and 2.3.2. from chapter 2.

3.3.2. Synthesis of the solid phase combinatorial library based on Petasis-Ugi reaction

3.3.2.1. Petasis reaction on aminated agarose

Aminated agarose was washed with 25% (v/v) ethanol/distilled water (5x resin volume) and 50% (v/v) ethanol/distilled water (5x resin volume). Then, 3 batches were prepared with 5 molar eq. of each compound in excess relative to the epoxy, where only the boronic acid compound differ from each batch. In batch 1), glyoxylic acid monohydrate and phenylboronic acid (B1) in 50% (v/v) ethanol/distilled water (0.5 mL of each/g moist agarose) were added; In batch 2), glyoxylic acid monohydrate and 3-Thienylboronic acid (B2) in 50% (v/v) ethanol/distilled water (0.5 mL of each/g moist agarose) were added; Finally, in batch 3),

glyoxylic acid monohydrate and 4-aminocarbonylphenylboronic (B3) acid in 50% (v/v) ethanol/50% (v/v) distilled water and DMF (0.5 mL of each/g moist agarose) were added.

The reactions occurred for 48 hours at 60 °C with agitation at 200 rpm. Afterwards, the Petasis-scaffolded agarose was washed with 50% ethanol/distilled water (v/v) (5x resin volume) and distilled water (10x resin volume) by vacuum suction.

3.3.2.2. Blocking of unreacted amines on Petasis-functionalized agarose

To block the unreacted amines, Petasis agarose was washed in a sinter funnel with 20% to 100% DMF (in 20% increments). Then, the agarose was ressuspended in 10% (v/v) acetic anhydride in DMF and incubated for 24 hours at room temperature with orbital shaking (200 rpm). Afterwards, the resin was washed with 100% to 20% DMF (in 20% decrements) and distilled water (10x resin volume).

3.3.2.3. Ugi reaction on Petasis-functionalized agarose

In order to perform the final step of Petasis-Ugi reaction for each batch, Petasis-functionalized agarose was washed with 20% to 100% Methanol (in 20% increments) and ressuspended in Methanol (1 mL/g moist agarose). Then with a 1mL pipette tip that was end cut in 4 mm, each Petasis-functionalized agarose was distributed for 24 wells (0.5 mL/well which corresponds to approximately 0.25 g of resin) of a 96-well filter plate. This step is critical to guarantee a uniform distribution of resin in the filter plate. The end cap of the block was removed in order to let the solvent drain by gravity. Afterwards, the reaction block was end capped again.

In the meantime, aldehyde and amine components (5 molar eq. of each, relative to epoxy groups) were dissolved in Methanol (0.25 mL/component/well) and the reaction occurred for 2h at 60 °C in the orbital shaker (200 rpm), in order to facilitate the imine formation required for the Ugi reaction. After incubation, each mixture of amine and aldehyde (0.5 mL/well) was added to the Petasis resin (Figure 3.3), along with isopropyl isocyanide (5 molar eq. relative to epoxy). The filter plate was covered with a plate cover and the reaction was left for 48h at 60 °C in the orbital shaker (150 rpm). Finally, the Petasis-Ugi ligands were washed with 0.75 mL of 100% to 20% Methanol (in 20% decrements) and then with distilled water (10x 0.75 mL).

The amines used were A1, A2, A3, A4, A5, A6, A7, and A8. The aldehydes were Al1, Al2, and Al3. A5, and A6 were dissolved in 50% (v/v) DMF/Methanol and heated. Al1 was dissolved in 25% (v/v) DMF/Methanol. NaOH 1.0 M was added to A2 (5 molar eq.) and A6 (2 molar eq.).

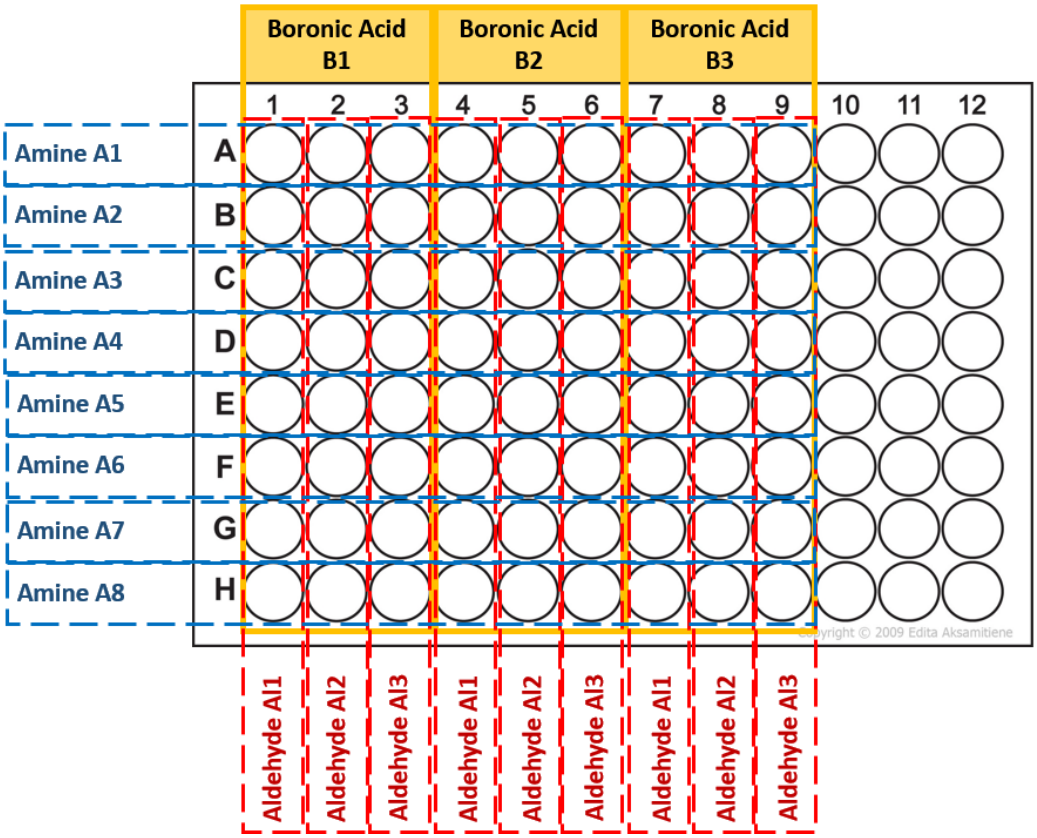


Figure 3.3: Illustrative image of the 96-well filtration block used for the first synthesis of the ligands based on Petasis-Ugi reaction.

3.3.3. Re-synthesis of Petasis-Ugi ligands

Posterior synthesis of the Petasis-Ugi ligands were made following the procedure described in section 3.3.2., but instead of using the filter plate the synthesis were done in deep well plates or in batch.

3.3.4. Regeneration and equilibration of the combinatorial library

To do the screenings it was necessary to regenerate and equilibrate the Petasis-Ugi libraries. The affinity ligands were washed for binding with regeneration buffers, first with 0.1M NaOH in 30% isopropanol followed by distilled water (2 cycles of washes, 0.75mL/well) and then with 0.1M HCl followed by distilled water (2 cycles of washes, 0.75mL/well). After, the equilibration of the ligands was conducted with the addition of the binding buffer, the 10mM Phosphate Buffer, 150mM NaCl at pH 7.4 (15x 0.75mL/well on average). In the last wash, 100µl were collected in a half-area UV-Star 96-Well microplate, and read at 280 nm. The last wash was

performed when the absorbance values were lower than 0.01. Then, binding buffer (0.75ml/well) was added to the libraries and stored at 4 ^{o}C.

3.3.5. Screenings of the synthetic ligands with pure IgG

To perform the library screening, a solution of pure IgG in Phosphate Buffer at pH 7.4 (0.5mg/mL) was prepared and 0.25mL of this solution was loaded to each well of the Petasis-Ugi combinatorial library. Then the block was incubated for 1 hour at 25 ^{o}C with agitation (50 rpm). After, the flow-through and the washes were collected with binding buffer (4x 0.25mL). Both were collected by centrifugation at 500 rpm during 30 seconds in 96-well transparent microplates.

The total protein present in the samples was quantified by the BCA assay according to supplier instructions (Sigma-Aldrich), where 200µL of BCA reagent was added to 25µL of each sample and incubated at 37 ^{o}C for 30 minutes. The absorbance of the samples was then read at 560 nm and determined with a calibration line using solutions with known protein concentration, BSA in 10 mM Phosphate Buffer 150mM NaCl at pH 7.4, from 0 to 1000 µg/mL with values of $y = 0.0019x + 0.0684$ with a $R^2 = 0.99$.

After the first screening, the library was washed with 1mL of the following solutions: 0.1M glycine in NaOH with 50% ethylene glycol (v/v) at pH 9 (1x); 0.1M glycine in NaOH at pH 11 (1x); distilled water (1x); 0.1M HCl (2x); distilled water (1x); 0.1M glycine at pH 2.5 (1x); 0.2M NaOH in 50% isopropanol (v/v) (2x); distilled water (1x); Phosphate buffer saline in 1M NaCl (1x); 0.1M NaOH in 30% isopropanol (2x) and distilled water (1x). Then, the equilibration was made as described in 3.3.4. and the screening was repeated.

The total protein was also quantified by measuring the absorbance of the samples at 280 nm, where 100 µL of each sample was transferred to Half-area UV-Star® 96-well microplates. The protein concentration was determined with a calibration line using protein solutions with known concentration, IgG in 10 mM Phosphate Buffer 150mM NaCl at pH 7.4, from 0 to 1000 µg/mL with values of $y = 0.4341x$ - 0.0003 with a $R^2 = 0.99$.

3.3.6. Elution test of the synthetic ligands (96-well microplate format)

Three elution conditions were tested: 0.1 M Glycine-HCl at pH 2, 10 mM Phosphate Buffer 150mM NaCl at pH 6 and 0.1 M Glycine-NaOH at pH 11. The elution fractions were collected by centrifugation at 500 rpm during 30 seconds to 96-well transparent microplates (3x0.25 mL). To avoid denaturation of the eluted protein at pH 2, 1 M Tris-base pH 9 was added to adjust the pH value to 7. Then, the total protein present in the samples was quantified by

reading the absorbance at 280 nm in the Half-area UV-Star® 96-well microplates, determined with a calibration line using solutions with known protein concentration, IgG in 10 mM Phosphate Buffer 150mM NaCl at pH 7.4, from 0 to 1000 µg/mL with values of $y = 0.4341x$ - 0.0003 with a $R^2 = 0.99$.

3.3.7. Screening and Elution tests of the lead ligands with pure IgG (on-column format)

The ligands B1Al2A2, B2Al2A7, B3Al1A1 and B3Al2A4 packed in a column were tested against IgG. 0.5 g of each ligand-functionalized agarose was packed in a small column, three columns were made for each ligand to test their stability at different pH conditions (pH 2, 6 and 11). Then, the columns were regenerated and equilibrated as described in 3.3.4. section. The last wash was performed when the absorbance at 280 nm reached values lower than 0.005.

To perform the screenings, 0.5 mL of pure IgG (0.5 mg/mL) in 10 mM Phosphate Buffer 150 mM NaCl at pH 7.4 was added in each column and five more washes with Phosphate Buffer Saline at pH 7.4 were performed. After the screening, bound protein was eluted with 0.5 mL of 0.1M Glycine-HCl at pH 2, with 10mM Phosphate Buffer 150mM NaCl at pH 6 or with 0.1M Glycine-NaOH at pH 11. Five elution fractions were collected for each pH condition to each ligand. Again, to avoid denaturation of the eluted proteins at pH 2, 50µL of 1M Tris-base pH 9.0 was added to collect the elution fractions, to adjust the pH value to 7. All samples were collected in 1.5 mL microcentrifuge tubes.

The total protein of each sample was quantified measuring the absorbance at 280 nm, as described in section 3.3.5.

Note: For every screening of the Petasis-Ugi ligands presented in this chapter, the quantity of protein binding was determined by Equation 2.1 and Equation 2.2, and the binding capacity of the ligands were determined by Equation 2.3, as described in section 2.4.2. from chapter 2.

3.4. Results and Discussion

3.4.1. Rational design of Petasis-Ugi library

In this work the solid-phase Petasis-Ugi combinatorial library was designed based on amino acids of biological ligands that bind naturally to antibodies, on *de novo* designed synthetic ligands – such as triazine and Ugi ligands – and on peptides that were also designed to bind antibodies. All these binders are represented and described in tables 1.1 and 1.2 from the Literature Review chapter. The rational design of Petasis-Ugi library was essentially based on these ligands, in order to select the amine, aldehyde and boronic acid compounds.

The Petasis-Ugi reaction is a multicomponent reaction, where it can be added four reagents, allowing a wide variety in the final structure of the ligand (Figure 3.4). In this study, three components were varied – boronic acids (R_1), amines (R_2) and aldehydes (R_3) – which resulted in a library with 72 different affinity ligands. The isopropyl isocyanide (R_4, isonitrile component) was kept constant.

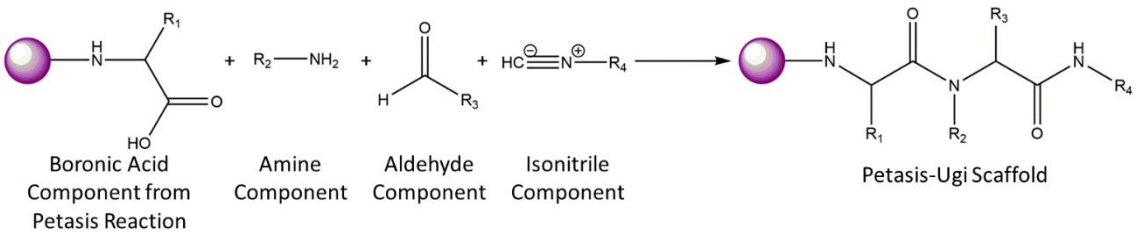


Figure 3.4: Simplified scheme of Petasis-Ugi reaction.

After some analysis of the interactions that occurred between the amino acids of natural ligands and from the groups of synthetic ligands with the antibodies, different boronic acids (Table 3.1), amines (Table 3.2) and aldehydes (Table 3.3) were selected for the synthesis of the Petasis-Ugi library.

Table 3.1: Boronic acid compounds (B1 - B3) used in Petasis-Ugi library with structure and name. The reagent on the right of the compound represents the amino acid which it mimics.

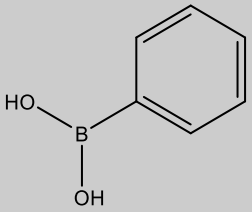
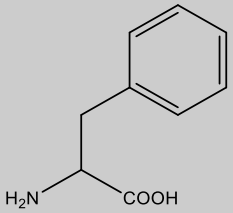
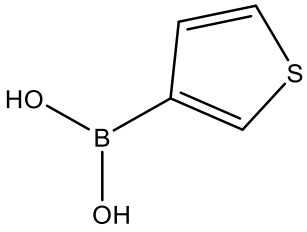
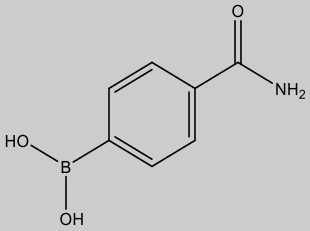
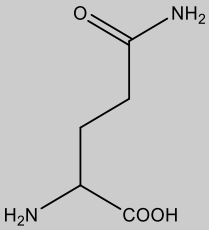
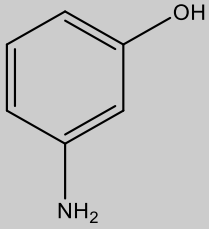
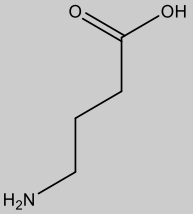
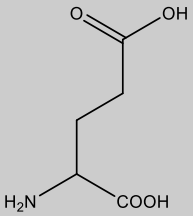
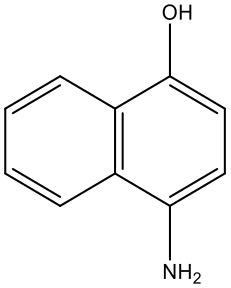
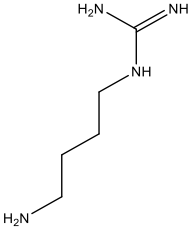
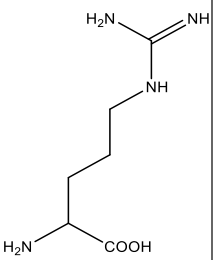
	Reagent	Amino Acid
B1	 Phenylboronic acid	 Phenylalanine
B2	 3-Thienylboronic acid	
B3	 4-aminocarbonylphenylboronic acid	 Glutamine

Table 3.2: Amine compounds (A1 - A8) used in Petasis-Ugi library with structure and name. The reagent on the right of the compound represents the amino acid which it mimics.

	Reagent	Amino Acid		Reagent	Amino Acid
A1	 3-aminophenol		**A5**	 γ-Aminobutyric acid	 Glutamic Acid
A2	 4-Amino-1-naphthol		**A6**	 Agmatine sulfate salt [1-(4-aminobutyl) guanidine]	 Arginine

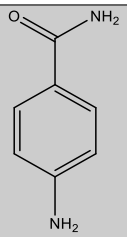
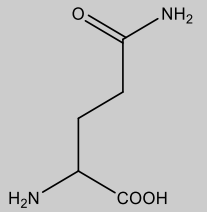
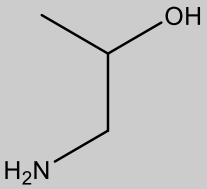
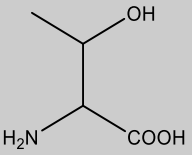
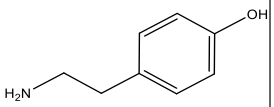
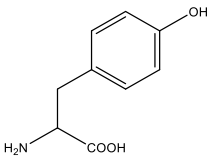
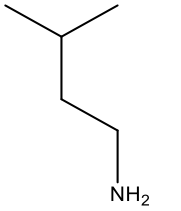
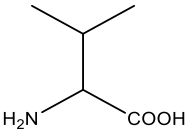
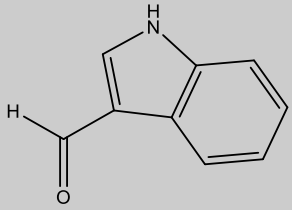
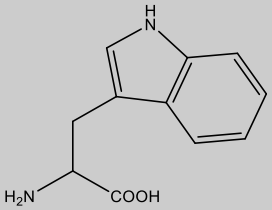
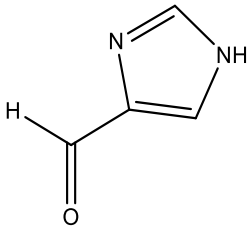
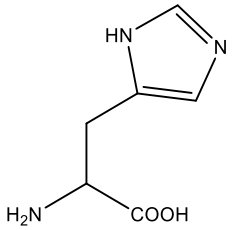
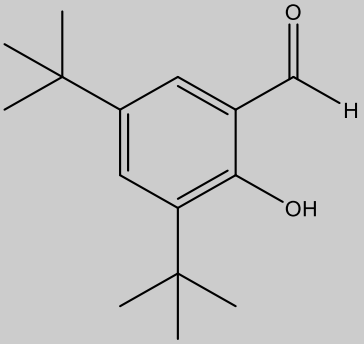
A3	 4-Aminobenzamide	 Glutamine	**A7**	 1-Amino-2-propanol	 Threonine
A4	 Tyramine	 Tyrosine	**A8**	 Isopentylamine	 Valine

Table 3.3: Aldehyde compounds (Al1 - Al3) used in Petasis-Ugi library with structure and name. The reagent on the right of the compound represents the amino acid which it mimics.

	Reagent	Amino Acid
Al1	 Indole-3-carboxaldehyde	 Tryptophan
Al2	 4-Imidazolecarboxaldehyde	 Histidine
Al3	 3,5-Di-tert-butyl-2-hydroxybenzaldehyde	

3.4.2. Initial screenings of the Petasis-Ugi combinatorial library with pure IgG

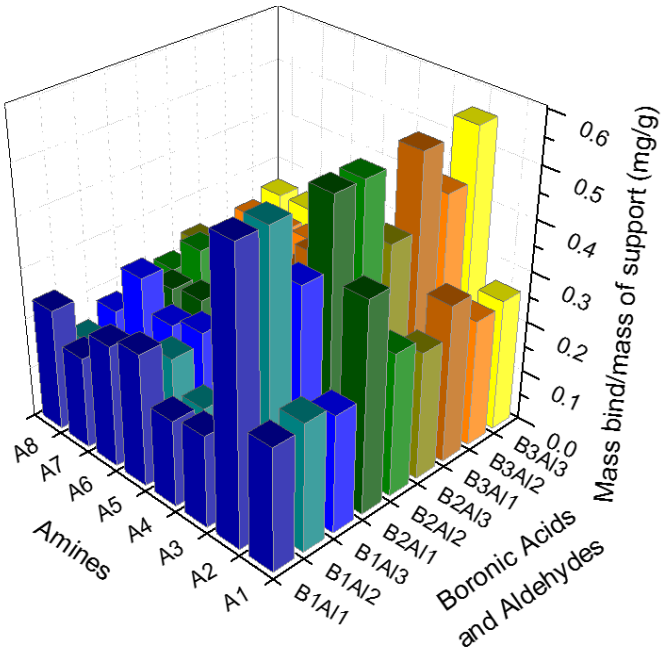
The Petasis-Ugi library was synthesized in a 96-well filtration block and screened with pure IgG (0.5 mg/mL) in PBS at pH 7.4, mimicking physiological conditions. The screenings were also performed in a 96-well filtration block with 0.25g of ligand-functionalized agarose in each well. The second screening was done after a more effective regeneration of the ligands used in the first screening. The unbound protein (flow-through and washes) was collected in 96-well transparent microplates and quantified by the BCA method.

The results obtained from the first screening (Figure 3.5 A and C) resulted in a mass bound range from 0.14 mg of protein/g of support to 0.56 mg of protein/g of support, which corresponds to a % protein binding range from 25% to 100%, respectively. Regarding the second screening (Figure 3.5 B and D), which was performed after a more effective regeneration, it resulted in a mass bound range from 0.05 mg of protein/g of support to 0.58 mg of protein/g of support, which corresponds to a % protein binding range from 8% to 85%, respectively. Most of the ligands decreased their binding capacity in the second screening, with a few exceptions, which may indicate ligand loss during regeneration or resin fouling. From this second screening it was concluded that posterior screenings should be made with freshly prepared ligands.

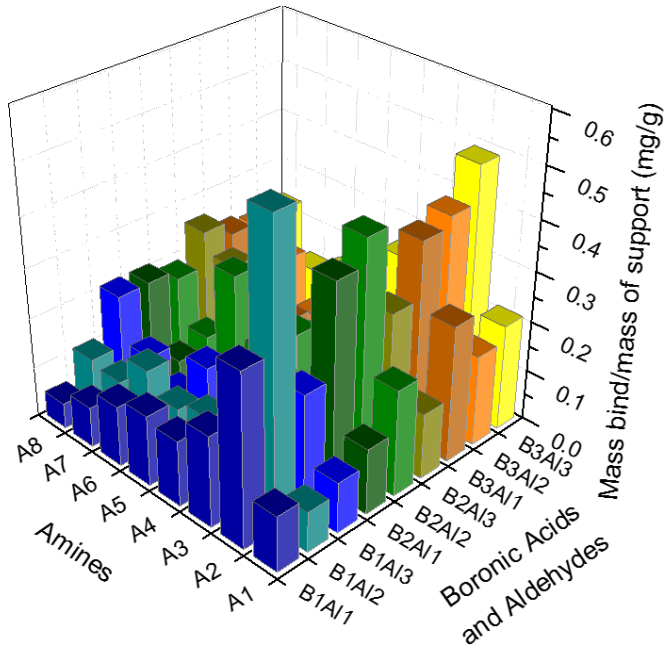
From the first screenings it's evident that the ligands with the A2 component are the ones with a better binding capacity, this amine is the 4-Amino-1-naphthol, which is found in the structure of the triazine ligand 22/8, that was designed to bind antibodies with high affinity.

In order to discover the lead ligands from this library, and since we want ligands with high binding capacity, the ligands that had a percentage of IgG bound higher than 48% (Figure 3.5 C) were chosen to forward screenings, which resulted in a total of 21 ligands. The ligands chosen were: B1Al1A2, B1Al2A2, B1Al3A2, B1Al3A7, B2Al1A1, B2Al1A2, B2Al1A4, B2Al1A5, B2Al2A1, B2Al2A2, B2Al2A5, B2Al2A7, B2Al3A2, B2Al3A6, B3Al1A1, B3Al1A2, B3Al1A3, B3Al1A5, B3Al2A2, B3Al2A4 and B3Al3A2.

A)



B)



C)

	B1			B2			B3		
%	Al1	Al2	Al3	Al1	Al2	Al3	Al1	Al2	Al3
A1	43	44	41	72	49	45	55	45	47
A2	100	100	76	100	100	74	100	81	100
A3	32	37	37	43	43	44	50	47	46
A4	30	28	36	50	48	46	46	49	45
A5	46	39	42	48	51	43	50	43	41
A6	42	30	38	43	37	52	39	45	42
A7	32	25	49	41	50	43	34	42	47
A8	43	27	32	37	38	43	34	42	47

100
50
0

D)

	B1			B2			B3		
%	Al1	Al2	Al3	Al1	Al2	Al3	Al1	Al2	Al3
A1	17	11	15	19	31	20	39	27	31
A2	50	85	34	61	68	43	58	62	72
A3	26	17	22	17	16	28	27	25	20
A4	19	21	13	14	31	17	15	27	37
A5	20	18	25	12	21	19	16	20	16
A6	18	23	12	12	38	23	17	13	21
A7	12	14	17	11	15	32	13	27	20
A8	8	16	31	31	28	38	34	32	33

100
50
0

Figure 3.5: Initial screening results of Petasis-Ugi library in filtration block. **A)** First screening result of Petasis-Ugi library. **B)** Second screening result of Petasis-Ugi library, after ligands regeneration. **C)** Schematic diagram representing the percentage of binding protein of the first screening. **C)** Schematic diagram representing the percentage of binding protein of the second screening, after ligands regeneration.

3.4.3. Re-test of the best 21 affinity ligands

The selected 21 Petasis-Ugi ligands were re-synthesized in a deep well plate and re-screened with pure IgG (0.5 mg/mL) in PBS at pH 7.4. The screenings were performed in triplicate, in the deep well plate, with 0.25g of ligand-functionalized agarose in each well. The unbound protein (flow-through and washes) was collected in 96-well transparent microplates and quantified by the BCA method.

The results from the best 21 ligands are represented in figure 3.6 A and B, the mass of IgG bound range from 0.28 mg of protein/g of support to 0.41 mg of protein/g of support, as it was expected, the ligands have all a good binding capacity. However, the percentage of IgG bound decrease in all of them and the difference of values between them are not so significant, when compared to the previous screening. This may be due to the deep well plate that was used, since it's trickier to handle, or the synthesis wasn't so effective once Petasis-Ugi reaction involves a lot of steps and washes.

To proceed the work, it was made an average of the 4 screenings performed (Figure 3.6 C), the first screening and the three analysed in this section. From the ligands with the A2 component were chosen the best 3: B1Al2A2, B3Al2A2 and B3Al3A2 with 62%, 64% and 72% of percentage of IgG bound, respectively. Then, five more were chosen to basically have more variability to the next screening tests. The ligands chosen were B2Al2A7, B2Al3A6, B3Al1A1, B3Al1A3 and B3Al2A4. These ligands showed a good binding capacity, with 55% to 58% of percentage of IgG bound, and low error.

The 8 ligands resulted from these screenings proceeded work to the stability test with different pH conditions, to see which of them were more stable and which were not.

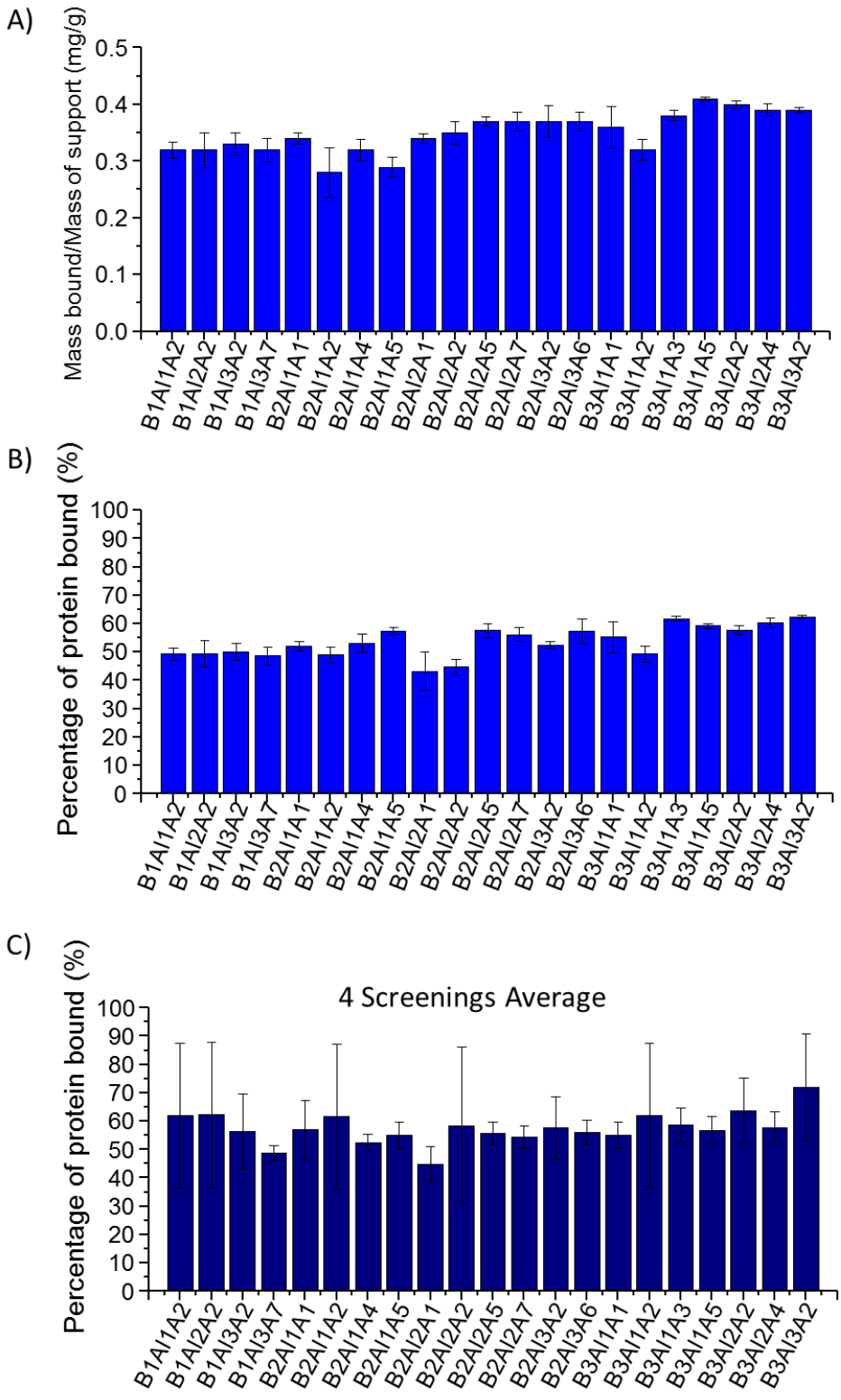


Figure 3.6: Screening results of the best 21 Petasis-Ugi ligands. The screenings were performed in triplicate for each ligand in a deep well plate. **A)** Average of screening results of the 21 Petasis-Ugi ligands in mg of protein bound/g of support. **B)** Average of the percentage bound of the 21 Petasis-Ugi ligands. **C)** Average of the percentage bound from the 4 screening results of the 21 Petasis-Ugi ligands (the first one in filtration block and the triplicates represented here in deep well plate).

3.4.4. Stability test of the lead ligands

To perform the stability test of the lead ligands, it was chosen three different pH conditions: pH 2 with 0.1M Glycine-HCl buffer, pH 6 with PBS (10mM Sodium phosphate, 150 NaCl) and pH 11 with 0.1M Glycine-NaOH buffer. These three buffers were used to elute the previously bound IgG, and the binding stability of the ligands was evaluated. Since we want to apply these ligands in two different approaches – Purification Approach and ADC Approach – the choices were made according to the requirements of each approach. For the purification approach, a ligand that is capable to bind and then unbind is needed. For the ADC approach, it is required a very stable binding.

The quantity of protein eluted was determined by Equation 3.1, and the percentage of protein eluted were determined by Equation 3.2.

Equation 3.1:

$$Amount\ of\ protein\ eluted\ (mg/g) = \frac{\Sigma\ mg\ of\ protein\ washed}{g\ of\ support}$$

Equation 3.2:

$$\%\ Protein\ Eluted = \frac{Amount\ of\ protein\ eluted\ (\mu g)}{Amount\ of\ protein\ bound\ (\mu g)} \times 100$$

3.4.4.1. Confirmation of IgG Binding for 8 lead ligands

The 8 Petasis-Ugi ligands were re-synthesized and re-screened with pure IgG (0.5 mg/mL) in PBS at pH 7.4 and then eluted with three different pH conditions. The ligands B1Al2A2, B3Al1A1, B3Al2Al2, B3Al2A4 and B3Al3A2 were synthesized directly in the deep well plate, while the other ones, B2Al2A7, B2Al3A6 and B3Al1A3, were synthesized in batch. The screenings were performed in triplicate for each pH condition (pH=2, pH=6 and pH=11) in a deep well plate with 0.25g of ligand-functionalized agarose in each well. The unbound protein (flow-through, washes and elution) was collected in 96-well transparent microplates and quantified by measuring the absorbance at 280 nm. The quantifications were made by this method instead of BCA assay, because glycine can interfere with BCA reagents and give false results.

About the binding results (Figure 3.7 A and B) there is some divergences between the ligands that were synthesized in the deep well plate and the ones in batch. In batch the reagents are mixed in a more efficient way with agarose, than in the deep well plate, where the space is

reduced to a 2 mL well. Which is concluded that the synthesis in batch is more effective, resulting in higher values of binding capacity, than in wells. In figure 3.7 B the differences are even more evident, since the ligands synthesized in batch were not tested in the same day as the ones synthesized in the deep well plate.

In the elution results (Figure 3.7 C e D), almost all the ligands are stable at different pH condition, with the exception of B1Al2A2, and in overall they elute more at pH 11. These ligands seem all to be great candidates for ADC assembly as the linker component, once they showed good stability. However, the ligands that showed less mass of IgG eluted per mass of support (Figure 3.7 C) were B2Al2A7, B3Al1A1 and B3Al2A4. The B1Al2A2 was the affinity ligand that showed higher percentage of eluted protein (Figure 3.7 D) and for this reason is the best ligand to explore for the purification approach.

These 4 ligands proceeded to the on-column format in the same conditions as performed in this section, to a more accurate results.

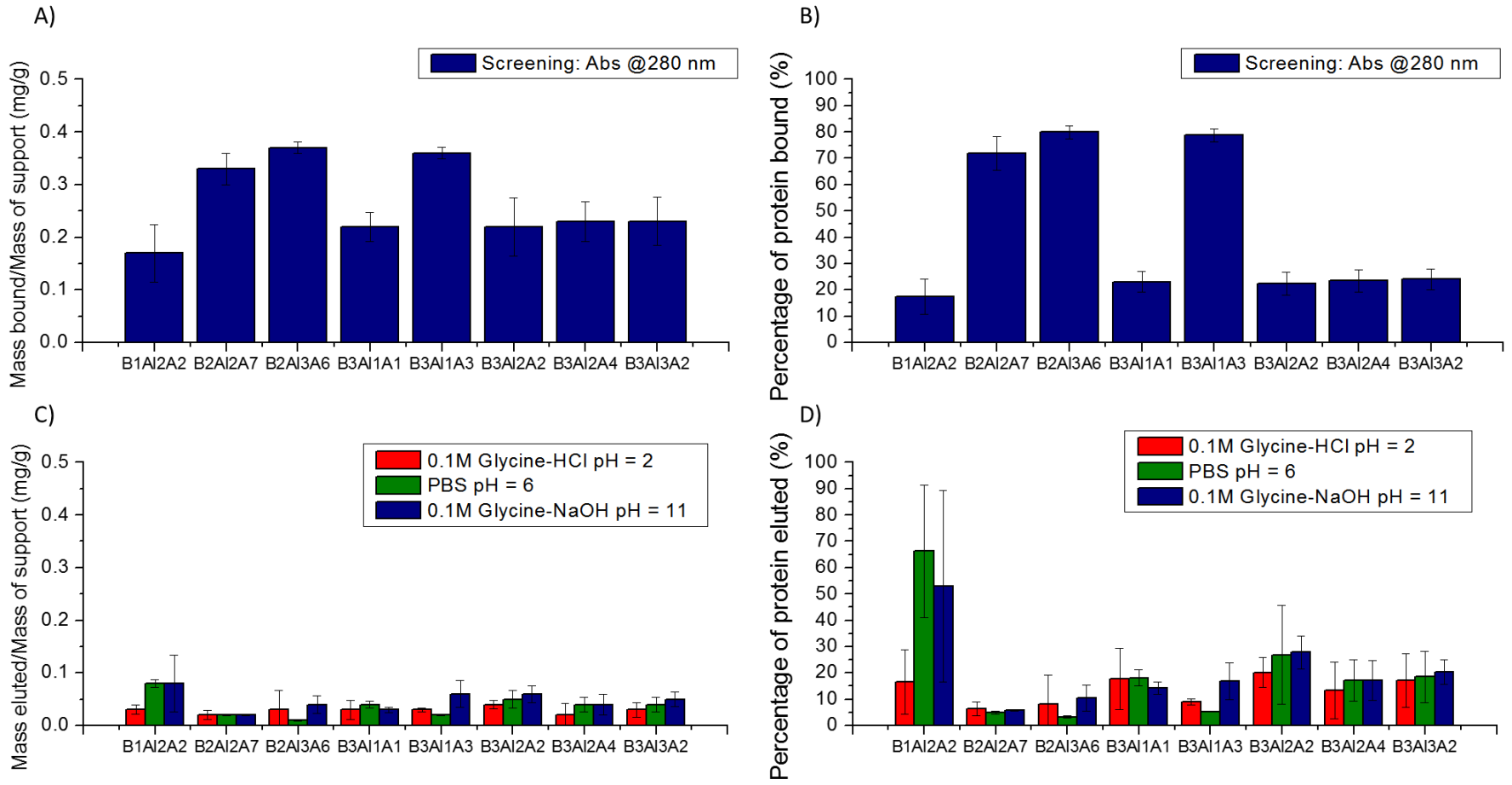


Figure 3.7: Screening results of the best 8 Petasis-Ugi ligands. The screenings were performed in triplicate for each pH condition in a deep well plate. **A)** Average of binding results of the 8 Petasis-Ugi ligands and the previous binding results in mg of protein bound/g of support. **B)** Average of the percentage bound of the 8 Petasis-Ugi ligands and the previous percentage bound results. **C)** Average of elution results of the 8 Petasis-Ugi ligands in mg of protein bound/g of support for the different pH condition. **D)** Average of the percentage of protein eluted of the 8 Petasis-Ugi ligands for the different pH condition.

3.4.4.2. On-column studies of 4 lead ligands

The 4 lead Petasis-Ugi ligands were re-synthesized in batch and re-screened with pure polyclonal human IgG (0.5 mg/mL) in PBS at pH 7.4 and then eluted. A total of 3 columns per ligand were packed (0.5 g of ligand-functionalized agarose), one column for each pH condition. The samples (flow-through, washes and elution) were collected in 1.5 mL microcentrifuge tubes and total protein was quantified by measuring the absorbance at 280 nm.

From the binding results (Figure 3.8 A and B), the binding capacity decreased in general, with exception of B1Al2A2 ligand, this may be due to screening method used (Table 3.4). In the deep well plate method, the screenings were performed by incubation followed by centrifugation to collect the supernatant from each well (0.8 cm diameter and 3 cm well height). On-column format (0.8 cm of diameter and 6.5 cm of height), the screenings were performed under gravitational force with a constant flow rate (around 0.75 mL/min). The higher values of IgG bound in the screenings with deep well plate can be explained by the higher incubation times used, enhancing non-specific interactions [55].

Table 3.4: Comparison of the binding capacities from lead ligands between the deep well plate and the on-column method.

Petasis-Ugi lead ligands	Deep well plate (mg protein bound/g support)	On-column (mg protein bound/g support)
B1Al2A2	0.17 ± 0.05	0.26 ± 0.05
B2Al2A7	0.33 ± 0.03	0.13 ± 0.02
B3Al1A1	0.22 ± 0.03	0.11 ± 0.04
B3Al2A4	0.23 ± 0.04	0.14 ± 0.01

Regarding the elution results (Figure 3.8 C e D), they seem to be in agreement with the previous ones, with the exception of pH 6 condition. In a deep well plate, the risk to add some error is higher, while on-column the risk of error is lower and the results are more accurate.

Therefore, analysing the results obtained from the elution of the on-column format screenings, B2Al2A7 is the ligand that showed more stability at different pH conditions and B1Al2A2 is the affinity ligand that, once again, showed more amount of protein eluted. These two ligands will proceed the work and be explored for the ADC approach and for the purification approach, respectively.

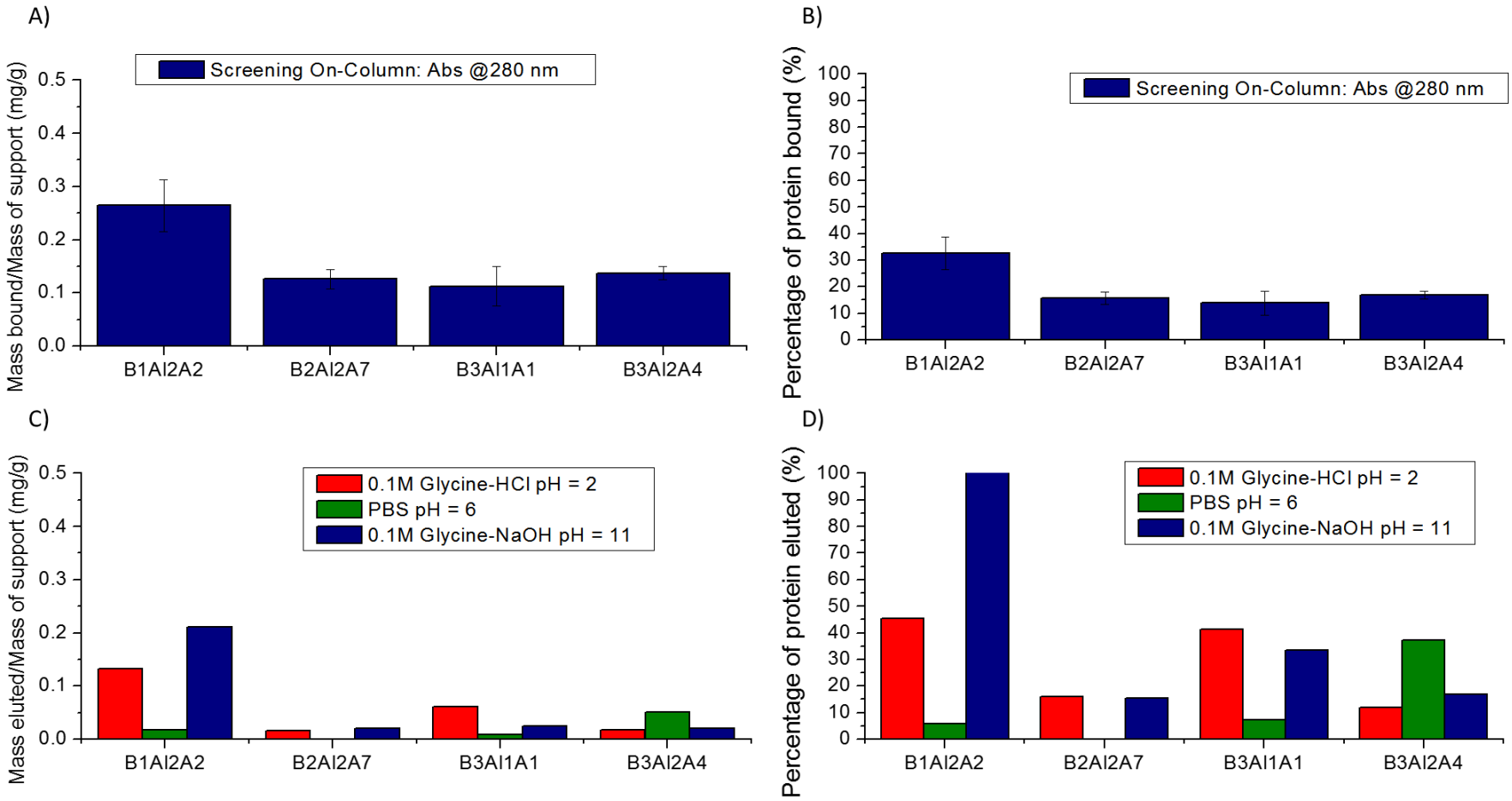


Figure 3.8: Screening results of the best 4 Petasis-Ugi ligands. The screenings were performed in on-column format. **A)** Average of binding results of the 4 Petasis-Ugi ligands and the previous binding results in mg of protein bound/g of support. **B)** Average of the percentage bound of the 4 Petasis-Ugi ligands and the previous percentage bound results. **C)** Elution results for the 4 Petasis-Ugi ligands in mg of protein bound/g of support for each pH condition. **D)** Percentage of protein eluted for the 4 Petasis-Ugi ligands for each pH condition.

3.5. Conclusions and Future Approaches

A combinatorial library of affinity ligands based on the Petasis-Ugi reaction was designed, synthesized and screened towards polyclonal human IgG. In order to create this multicomponent library, the boronic acid, amine and aldehyde components were chosen based on interactions between antibodies and natural binders (Protein A, G and L), on synthetic ligands with triazine and Ugi scaffold and on peptides, both designed to bind antibodies.

This library resulted in 72 synthetic ligands, which were tested against IgG at pH 7.4. From this, 21 ligands were found to bind to human IgG, between 48% and 100%. After re-screening the 21 ligands, 8 ligands were selected, which consequently were tested at three different pH conditions (pH 2, pH 6 and pH 11) to analyse the stability of binding between ligand and protein. Finally, 4 lead ligands were chosen to test again their stability in the same conditions, but in on-column format.

Through the screening tests some features were understood. The deep well plate method increases the introduction of error whereas the on-column format seems to give the most accurate results.

Ligands B1Al2A2 and B2Al2A7 (Figure 3.9) were selected as the lead ligands, both with good binding capacity for IgG. The B1Al2A2 showed to elute more protein, which is desirable for a purification approach. The interaction between B2Al2A7 and IgG was the most stable under different pH conditions and, therefore, it is a great candidate for the ADC approach as the linker component.

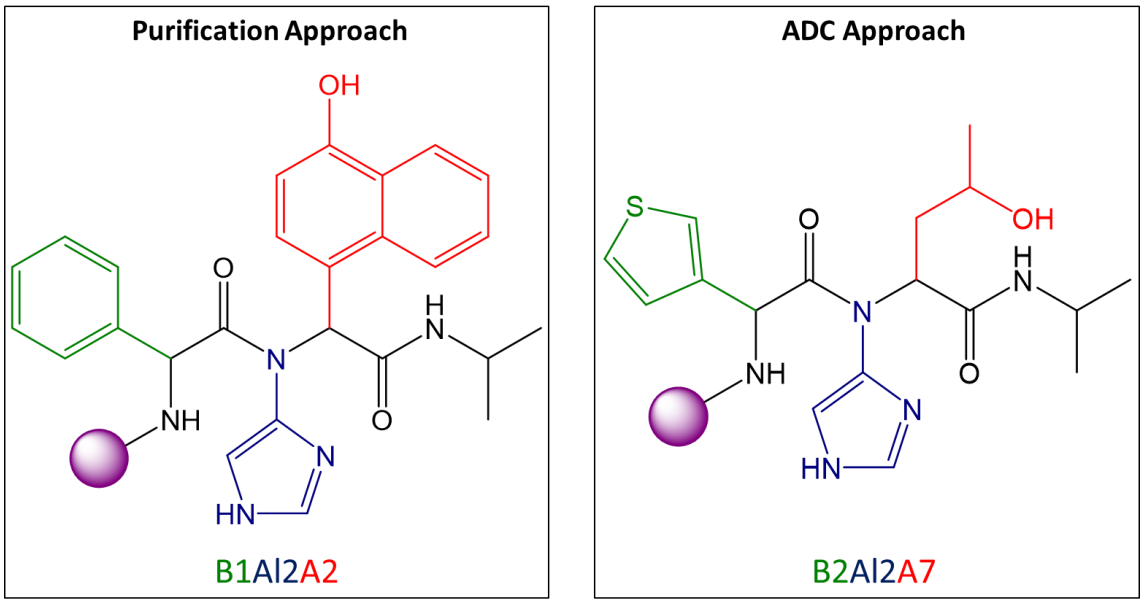


Figure 3.9: Structures of the two Petasis-Ugi lead ligands immobilized on agarose. The purple sphere is a schematic representation of an agarose bead.

IV. Exploration of the lead ligands in different approaches

Part I – Purification Approach

I.4.1. Affinity chromatography

The term of affinity chromatography for protein purification was first described in 1968 by Pedro Cuatecasas, Chris Anfinsen and Meir Wilchek in an article where is briefly described the purification of an enzyme via immobilized substrates and inhibitors. Since then, this method is commonly used to purify biomolecules. Basically, the affinity chromatography consists on an affinity ligand coupled to a solid matrix – usually agarose – to allow specific capture of the protein from a complex mixture. This capture is based on reversible interactions between the protein and the affinity ligand. The affinity ligand is designed to a specific protein target and can be natural or synthetic [39], [56] .

Purification by affinity chromatography involves three main steps (Figure I.4.1.): 1) Incubation of the complex mixture samples with the affinity support, this will permit the binding of the target protein to the immobilized affinity ligand; 2) Wash away the non-bound molecules and other impurities from the support; and 3) Elute the target protein from the immobilized ligand, changing the buffer conditions so it can dissociate from the affinity ligand [39].

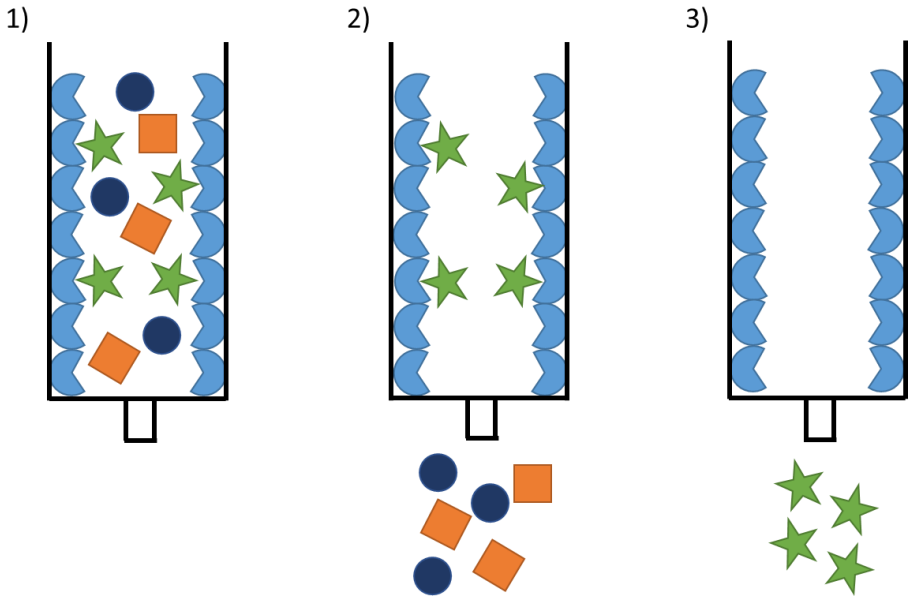


Figure I.4.1: Schematic representation of protein purification using affinity chromatography.

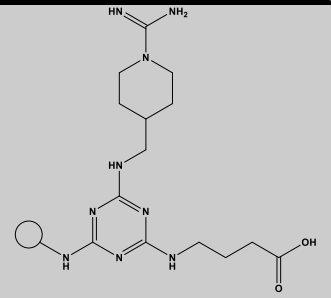
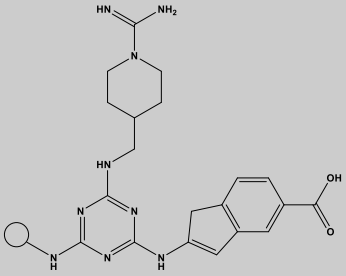
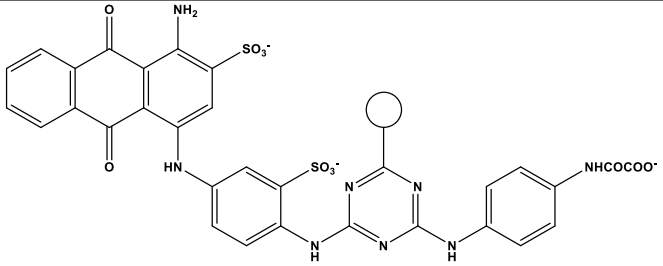
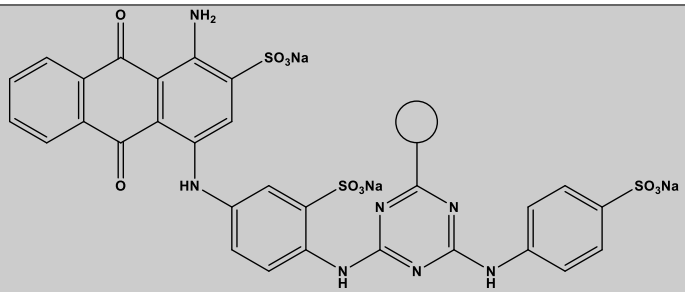
The advantages of using affinity chromatography for protein purification relies on its excellent specificity, easy operation, yield and throughput. Also, in purification of biomolecules for clinical applications, affinity chromatography allows the removal of pathogens [39].

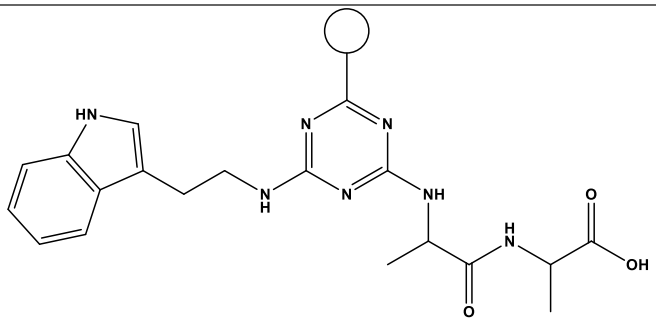
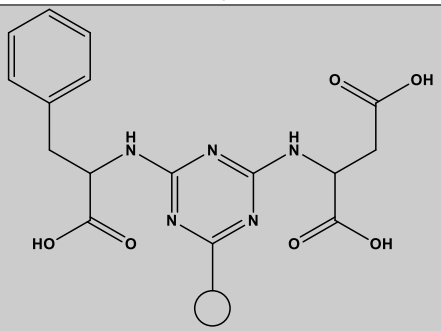
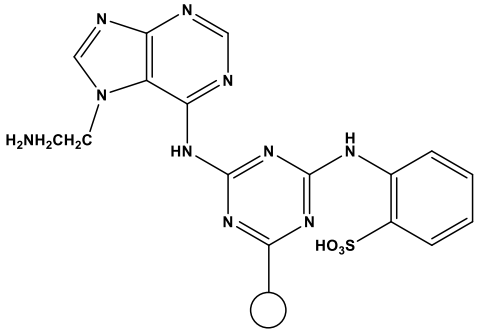
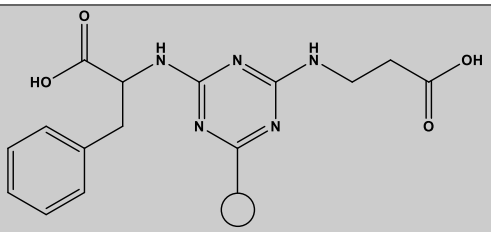
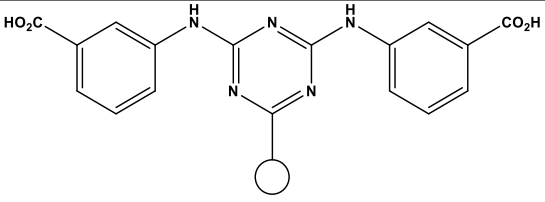
The specificity and the binding constant of the affinity ligand controls the purity and recovery of target protein, and in general the association constants of these ligands range between 10^3 to 10^8 M^{-1} in the purification field [39].

I.4.1.1. Synthetic Ligands in Affinity chromatography

The use of synthetic affinity ligands is important in the affinity chromatography of high value biopharmaceutical proteins, such as antibodies, since they are not so expensive as the biospecific affinity ligands (protein A, G and L – isolated from the surface of bacteria), are chemically defined, resistant to biological and chemical degradation, readily immobilized and can be highly selective [17]. For these reasons, synthetic affinity ligands have been developed and studied for purification of several proteins. The most abundant synthetic affinity ligands in affinity chromatography are based on the Triazine and Ugi reactions, which are two of the most well studied and well characterized combinatorial reactions in bioseparation. In tables I.4.1 and I.4.2 are present some examples of succeeded synthetic affinity ligands in the purification field.

Table I.4.1: Examples of Triazine-based used in protein purification field. Sphere represents the solid support.

Ligand	Protein target	Ref.
 **A5A10**  **A10A11**	Amphotropic murine leukemia virus envelope (AMPHO4070A)	[57]
 **BM1**	Glutathione-recognizing enzymes	[58]–[60]
 **Cibacron Blue 3G-A**	BSA and HSA	[58]

 **C4/6**	Elastase	[47]
 **LAK-mimetic ligand**	Anti-HIV mAb 2F5	[61]
 **oABSAd**	DNA polymerase	[62]
 **4E10lig**	Anti-HIV mAb 4E10	[63]
 **5/5**	Human recombinant clotting factor VIIa	[64]

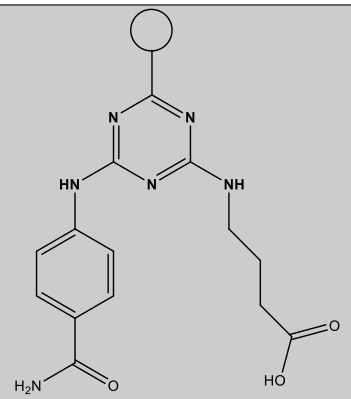
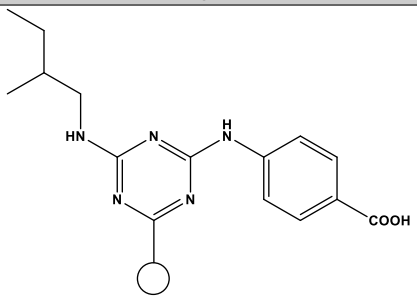
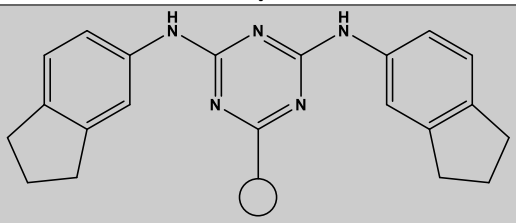
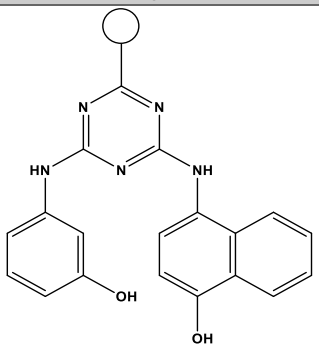
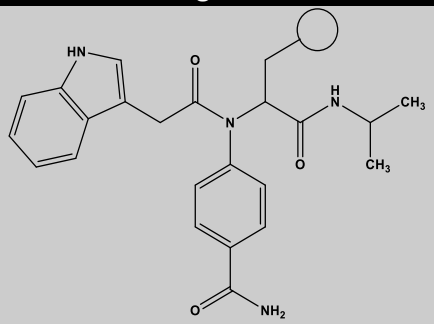
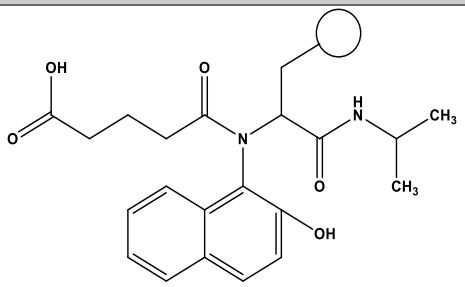
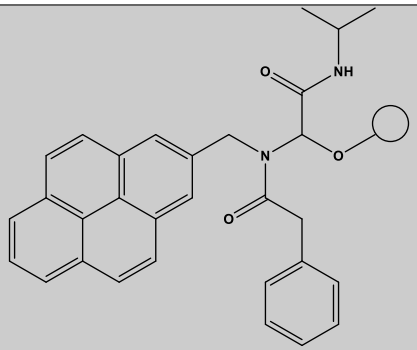
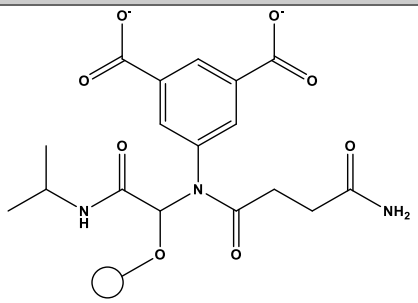
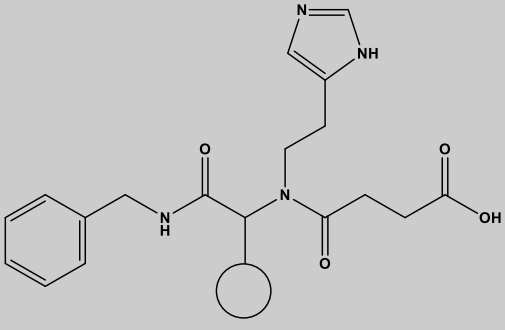
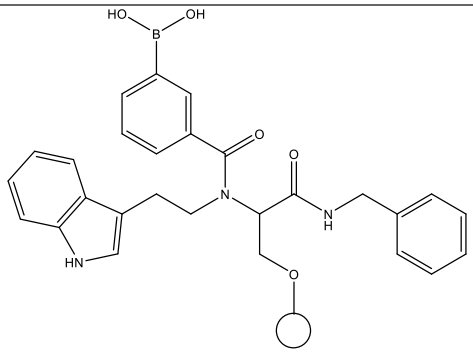
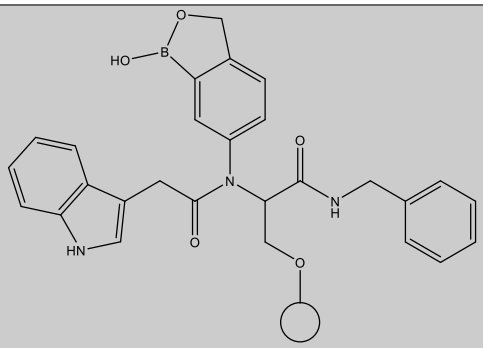
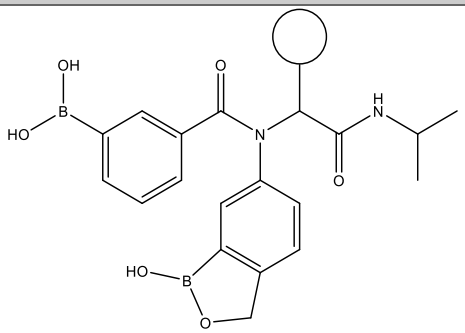
 **8/7**	Immunoglobulins	[18]
 **11/3**	Cutinase	[65]
 **18/18**	Glycoproteins	[66]
 **22/8**	Immunoglobulins	[15], [17]

Table I.4.2: Examples of Ugi-based ligands used in protein purification field. Sphere represents the solid support.

Ligand	Protein target	Ref.
 **A2C11I1**	Immunoglobulins	[22]
 **A3C1I1**	Immunoglobulins	[24]
 **A4C7**	GFP (Green Fluorescent Protein)	[55]
 **A7C1**	GFP-RK (Green Fluorescent Protein RKRKRK-tagged)	[67]
 **A9C10I8**	Recombinant Human Erythropoietin	[68]

 **A13C24I8**	Glycoproteins	[69]
 **A21C11I8**	Glycoprotein: Glucose oxidase	[70]
 **Ligand B4**	Glycoprotein: Glucose oxidase	[71]

The aim of this chapter is to test if it is possible to use a synthetic affinity ligand, based on a new combinatorial chemistry, the Petasis-Ugi reaction, for the purification of human IgG from human plasma and additionally, optimize the binding and elution conditions and determine the binding constants.

I.4.2. Materials

I.4.2.1. Chemicals

All reagents were used with a high purity and the solvents were pro-analysis.

The reagents 4-amino-1-naphthol hydrochloride (A2), 4-Imidazolecarboxaldehyde (Al2) and Phenylboronic acid (B1) were purchased from Sigma-Aldrich (Portugal).

Glyoxylic acid monohydrate, isopropyl isocyanide, sodium chloride (NaCl), Tris (hydroxymethyl)aminomethane hydrochloride, glycine, HEPES, imidazole, sodium bicarbonate ($NaHCO_3$), bovine serum albumin and Human plasma were acquired from Sigma-Aldrich (Portugal). CHAPS and Methanol (MeOH) were obtained from Roth (Portugal). Dimethylformamide (DMF) and di-sodium-hydrogen phosphate 2-hydrate were purchased from VWR. Hydrochloric acid 37% (HCl), ethanol absolute PA, acetic anhydride, sodium-di-hydrogen phosphate 1-hydrate, sodium hydroxide (NaOH) and ethylene glycol were acquired from Panreac (Spain).

The BCA reagents of BCA kit used in the screenings were purchased from Sigma-Aldrich (Portugal) and the IgG solution Gammanorm (165 mg/ mL normal human immunoglobulin), was produced by Octapharma AB.

For SDS-PAGE, β-mercaptoethanol, bromophenol blue and glycerol were obtained from Sigma-Aldrich (Portugal). Tris-Base from Nzytech (Portugal). Low weight protein marker, 30% acrylamide, bis-acrylamide solution 19:1, sodium dodecyl sulphate (SDS) and silver stain plus were acquired from Bio-Rad (Portugal). Ammonia persulfate (APS), tetramethylethylenediamine (TEMED), Coomassie Blue R-250 from Roth (Portugal). The glacial acetic acid was obtained from Pronalab.

I.4.2.2. Chromatographic Material

Cross linked agarose (Sepharose™ CL-6B) was acquired from GE Healthcare. Half-area UV-Star® 96-well microplates and 96-well transparent microplates were obtained from Greiner Bio-One (Germany) and Sarstedt (Portugal), respectively. Agilent bond Elut 3ml and Frits from Agilent Technologies (USA).

I.4.2.3. Buffers

The following binding buffers were used: 10mM Sodium phosphate, 150mM Sodium Chloride (NaCl) (PBS), pH 7.4; 10mM Sodium phosphate, 500mM NaCl (PBS), pH 7.4; 20 mM HEPES, 150 mM NaCl, pH 7.4 and 20 mM HEPES, 500 mM NaCl, pH 7.4.

The buffers used in the elution steps were 0.1M Glycine-HCl at pH 2.5 and Tris-NaOH at pH 9.0 for neutralization of eluted samples; 0.1M Glycine-NaOH at pH 11; 10mM Sodium phosphate, pH 5.7 and 8; 20mM HEPES, pH 6.8, 7.4 and 8.2; 20 mM HEPES, 50 mM Imidazole, pH=7.4; 0.1 M NaHCO3 in 30% ethylene glycol, pH=10; 0.1 M NaHCO3, 0.1% (w/v) CHAPS, pH=10 and 0.1 M NaHCO3, 1% (w/v) CHAPS, pH=10.

Regeneration Buffers: 0.1M NaOH in 30% isopropanol and 0.1M HCl.

I.4.2.4. Equipment

The synthesis of the synthetic ligands on solid-phase were carried out in Incubator ZKA KS4000i (VWR). Absorbance readings were performed in Microplate reader TECAN Infinite® 200 (Portugal); The oven used for ligand synthesis and BCA assays was a Big Shot III 230402-2 Hybridization Oven from Boekel Scientific. Mini-Protean Tetra System from BIO-RAD was utilized for the electrophoresis SDS-PAGE gels.

I.4.3. Methods

I.4.3.1. Re-Synthesis of the Petasis-Ugi lead ligand: B1Al2A2

The aminated agarose was prepared according to the procedures 2.3.1. and 2.3.2. from chapter 2, and washed with 25% (v/v) ethanol/ distilled water (5x resin volume) and 50% (v/v) ethanol/ distilled water (5x resin volume). Then, a batch was prepared with 5 molar eq. of each compound in excess relative to the epoxy, glyoxylic acid monohydrate and phenylboronic acid (B1) in 50% (v/v) ethanol/distilled water (0.5 mL of each/g moist agarose). The reaction occurred for 48 hours at 60 °C with agitation at 200 rpm. Afterwards, the Petasis-scaffolded agarose was washed with 50% ethanol/ distilled water (v/v) (5x resin volume) and distilled water (10x resin volume) by vacuum suction.

Petasis-functionalized agarose was washed with 20% to 100% DMF (in 20% increments), ressuspended in 10% (v/v) acetic anhydride in DMF and incubated for 24 hours at room temperature with orbital shaking (200 rpm) to block the unreacted amines. Afterwards, the resin

was washed with 100% to 20% DMF (in 20% decrements), distilled water (10x resin volume) and with 20% to 100% Methanol (in 20% increments).

In meantime, aldehyde (Al2) and amine (A2) component (5 molar eq. of each, relative to epoxy groups) were dissolved in Methanol (0.5 mL/g moist agarose) and the reaction occurred for 2h at 60 °C in the orbital shaker (200 rpm). 1.0 M NaOH was added to A2 (5 molar eq.). After incubation, the mixture of amine and aldehyde was added to the Petasis resin, along with isopropyl isocyanide (5 molar eq. relative to epoxy). The reaction was left for 48h at 60 °C in the orbital shaker (200 rpm). Finally, the Petasis-Ugi ligand was washed with 100% to 20% Methanol (in 20% decrements) and then with distilled water (10x 0.75 mL). The ligand was stored with 20% (v/v) ethanol/distilled water (1 mL/g moist agarose) at 4^{o}C.

I.4.3.2. Packing, Regeneration and Equilibration of the lead ligand

For each test, 0.5 g of B1Al2A2 agarose was packed in small columns and, before screenings, the ligand was washed with regeneration buffers, first with 0.1M NaOH in 30% isopropanol followed by distilled water (2 cycles of washes, 2x resin volume) and then with 0.1M HCl followed by distilled water (2 cycles of washes, 2x resin volume). After, the equilibration of the ligand was conducted with the addition of the respective binding buffer (10x resin volume). In the last wash, 100µl were collected in a half-area UV-Star 96-Well microplate, and read at 280 nm. The last wash was performed when the absorbance values were lower than 0.005 and the columns were stored in binding buffer or in 20% (v/v) ethanol/distilled water at 4^{o}C.

I.4.3.3. Screenings and Elution tests of the lead ligand with pure IgG and pure BSA in on-column format

Pure IgG and pure BSA were diluted, separately, in respective binding buffer (0.5 mg/mL) and 0.5 mL of each was added to their respective column. Then, five more washes were performed with respective binding buffer. After the screenings, to each elution test, it was added 0.5 mL of elution buffer in its respective column. Again, to avoid denaturation of the eluted proteins at pH 2.5 condition, 1M Tris-base pH 9.0 was added to collect the elution fractions, to adjust the pH value to 7. All samples (flow-through, 5 washes and 5 elution fractions) were collected in 1.5 mL microcentrifuge tubes.

The total protein of each sample was quantified by BCA assay or measuring the absorbance at 280 nm, as described in section 3.3.5, from chapter 3, but the calibration lines were done with the respective buffer of each test.

I.4.3.4. Test B1Al2A2 ligand with Human Plasma

To test the ligand B1Al2A2 for Human IgG purification, a screening with Human Plasma was performed on-column format, in duplicate. The Human plasma was diluted in 20mM HEPES with 500mM NaCl, pH 7.4 (0.5 mg/mL) and 0.5 mL was added to each column. The flow-through was collected and five washes were done with the binding buffer (20mM HEPES with 500mM NaCl, pH 7.4). Then, five elution fractions were collected with 0.5 mL of 0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10. All samples were collected in 1.5 mL microcentrifuge tubes and the total protein was quantified by BCA assay as described in section 3.3.5, from chapter 3.

I.4.3.5. SDS-PAGE preparation, staining and analysis

In this work, 12.5% acrylamide/bisacrylamide SDS-PAGE gels were used. The running gel was prepared first according to table I.4.3. and the solution was transferred to the glass plates of the casting frame, then 1mL of isopropanol was added on the top of the gel solution, to prevent bubbles. The gel was left to polymerize for 30 minutes. Afterwards, the isopropanol was removed and the 5% acrylamide stacking gel was prepared according to table I.4.3 and the mixture was added on top of the running gel. The comb of 10 wells was inserted and the gel was polymerized for 30 minutes.

Table I.4.3: Volumes necessary to prepare a 12.5% acrylamide gel for SDS-PAGE.

Stock solutions	**Running gel 12.5% Acrylamide**	**Stacking gel 5% Acrylamide**
Solution I (3M Tris-Base, pH 8.8-9.0)	750 µl	-
Solution II (0.5M Tris-Base, pH 6.6-6.8)	-	450 µl
Solution III (Acrylamide:Bisacrylamide 30:08)	2080 µl	0.3 µl
10% SDS	50 µl	18 µl
Distilled water	2100 µl	940 µl
10% APS	38 µl	13.5 µl
TEMED	2.5 µl	2.0 µl

Then, the samples were prepared, their concentrations were normalized accordingly to the BCA results and boiled at 100^{o}C for 5 minutes. The electrophoresis buffer (0.25M Tris-Base, 1.92M glycine, 0.1% SDS pH 8.3) was added to the tank and the polymerized gel was introduced in the running module. 15 µL of previously prepared samples were applied in each well and the running conditions were set as 90V for 2h.

Silver staining

To perform the silver staining, a Bio-Rad kit was used. First, the gels were transferred into the fixative solution (100 mL methanol, 20 mL acetic acid, 20 mL fixative enhancer concentrate and 60 mL distilled water), and incubated for 20min with agitation. Then, the gels were washed twice in 400ml of distilled water for 10min. Secondly, the gels were transferred into the staining solution (35 mL distilled water, 5ml silver complex solution, 5ml reduction solution, 5ml image solution and 50ml accelerator solution) and incubated for 20 minutes. Finally, the gels were incubated in 5% acetic acid solution in order to stop the previous reaction. The gels were washed in deionized water.

Then the results obtained were analysed and the purity of the elution fractions was calculated by the ImageJ software.

I.4.3.6. Static partition equilibrium studies

The B1Al2A2 functionalized agarose was regenerated with 0.1 M NaOH in 30% isopropanol followed by distilled water and with 0.1 M HCl followed by distilled water, then it was equilibrated with 20 mM HEPES, 500 mM NaCl pH 7.4. Different concentrations of pure IgG (0 to 3.5 mg/mL) in binding buffer were incubated, in duplicate, with 0.25 g of B1Al2A2 functionalized agarose in a total volume of 250 μL. To achieve the chemical equilibrium, the ligand was incubated with the respective IgG solution overnight, at 25^{o}C. The supernatants were collected and a BCA assay was performed to quantify the total protein. The adsorption phenomenon followed a Langmuir isotherm and the experimental data was fitted accordingly with OriginPro (v8.5.1). It was also made an analysis through Hill plot.

Note: For every screening of the B1Al2A2 Petasis-Ugi ligand presented in this chapter, the quantity of protein binding was determined by Equation 2.1 and Equation 2.2, and the binding capacity of the ligands were determined by Equation 2.3, as described in section 2.4.2. from chapter 2. The quantity of protein eluted and the percentage of protein eluted were, respectively, determined by Equation 3.1 and 3.2., as described in section 3.4.4. from chapter 3.

I.4.4. Results and Discussion

I.4.4.1. Re-screening of B1Al2A2 ligand with pure IgG on-column

The lead Petasis-Ugi ligand, B1Al2A2, was synthesized in batch and re-screened with pure IgG (0.5 mg/mL) in PBS at pH 7.4 and then eluted, in on-column format. Three columns were packed, one column for each pH condition, and the screenings were performed with 0.5g of B1Al2A2-functionalized agarose. The unbound protein (flow-through, washes and elution) were collected in 1.5 mL microcentrifuge tubes and quantified by measuring the absorbance at 280 nm.

In table I.4.4 is represented the two screening results of B1Al2A2 with pure IgG that have been done in on-column format. Comparing the results from the present screening with the previous one, discussed in chapter 3, at the same conditions, the binding results as well as the elution results are in general at the same range of values. This consistence of results confirms the capacity and the reproducibility of the B1Al2A2 affinity ligand.

Table I.4.4: Screening results of B1Al2A2 affinity ligand, performed on-column with pure IgG and comparison with the previous one discussed in chapter 3. Both screenings were performed on-column at the same conditions and the quantifications were made by measuring the absorbance at 280 nm.

	Condition	Previous Screening	Present Screening
Mass of protein bound/Mass of support (mg/g)	PBS pH 7.4	0.26 ± 0.05	0.24 ± 0.01
Percentage of protein bound (%)		32.61 ± 6.09	40.92 ± 1.38
Mass of protein eluted/Mass of support (mg/g)	pH 2	0.13	0.13
	pH 6	0.02	0.00
	pH 11	0.21	0.25
Percentage of protein eluted (%)	pH 2	45.39	54.59
	pH 6	5.84	0.00
	pH 11	100.00	100.00

I.4.4.2. Explore best binding condition with pure IgG and pure BSA

In order to increase the binding capacity of the B1Al2A2 ligand to IgG, 4 binding buffers at physiologic pH were studied: B1: 10mM Sodium phosphate, 150mM NaCl, pH 7.4; B2: 10mM Sodium phosphate, 500mM NaCl, pH 7.4; B3: 20 mM HEPES, 150 mM NaCl, pH 7.4 and B4: 20 mM HEPES, 500 mM NaCl, pH 7.4.

The lead ligand, B1Al2A2, was re-synthesized in batch and screened with pure IgG and pure BSA (0.5 mg/mL each) in respective binding buffer. The screenings were performed with 0.5g of B1Al2A2-functionalized agarose, in duplicate for each binding buffer condition on-column format. The unbound protein (flow-through and washes) was collected in 1.5 mL microcentrifuge tubes and quantified by BCA assay. BSA was added to the tests once it's the most abundant plasma protein and B1Al2A2 ligand is being developed for IgG purification.

Two kind of buffers were tested, the phosphate buffer saline and the 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), with two different salt concentrations each. The PBS is the buffer solution with the osmolarity and ion concentrations similar from those of the human body. The HEPES is an amine-based buffer with zwitterionic properties. The purpose of increasing the salt concentration in PBS and HEPES was to promote the hydrophobic interactions between IgG and the B1Al2A2 affinity ligand [72], [73].

The results of B1Al2A2 with pure IgG and pure BSA in different binding conditions are represented in figure I.4.2. From the binding results with PBS (B1 and B2), the increase of sodium chloride did not improve the binding capacity of the affinity ligand towards IgG and the binding towards BSA kept the same. When the HEPES buffer with 150mM NaCl (B3) was tested, it was observed a better binding capacity towards IgG (Figure I.4.2 A) than with the PBS as binding buffer. However, the difference of percentage of protein bound between IgG and BSA (Figure I.4.2 B) was not very significant, so the salt concentration of HEPES buffer was increased to 500mM (B4). With B4 (20 mM HEPES, 500 mM NaCl, pH 7.4) IgG bound almost 100% to the affinity ligand and BSA bound 48%, it is observed a difference of almost 50% in the percentage of protein bound result between IgG and BSA. Thus, these results suggest that B3 (20 mM HEPES, 150 mM NaCl, pH 7.4) and B4 (20 mM HEPES, 500 mM NaCl, pH 7.4) are the most suitable binding buffers, due to its higher selectivity for IgG.

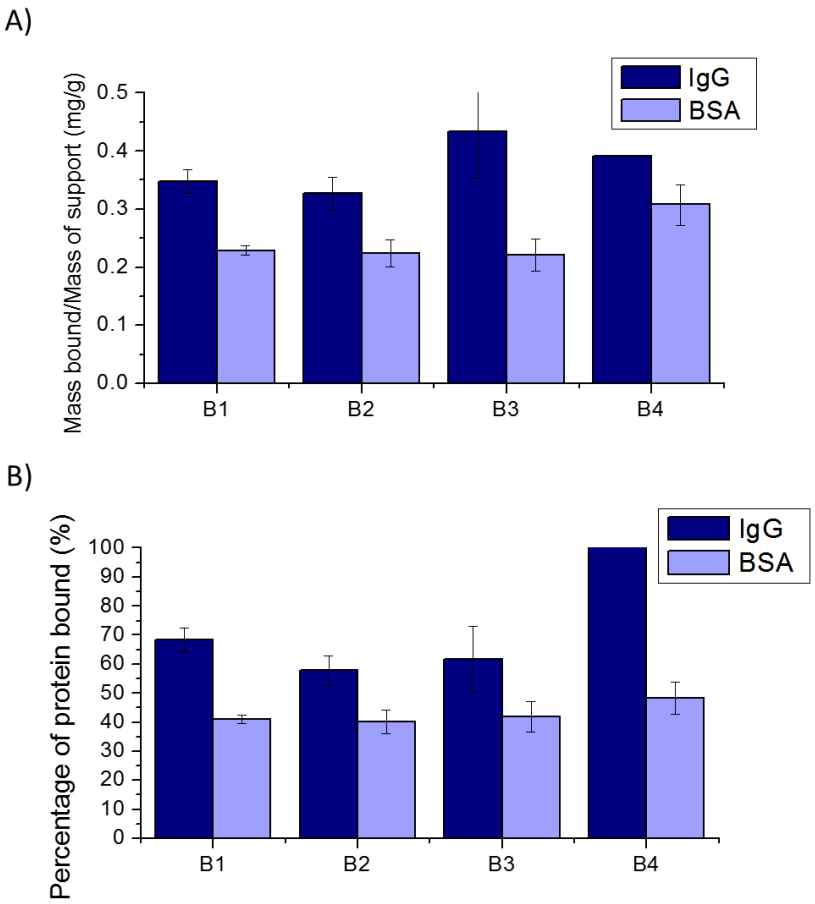


Figure I.4.2: Screening results of the B1Al2A2 Petasis-Ugi ligand with different binding conditions. **B1:** 10mM Sodium phosphate, 150mM NaCl, pH 7.4; **B2:** 10mM Sodium phosphate, 500mM NaCl, pH 7.4; **B3:** 20 mM HEPES, 150 mM NaCl, pH 7.4; **B4:** 20 mM HEPES, 500 mM NaCl, pH 7.4. The screenings were performed in duplicate for each binding condition to each protein in on-column format. **A)** Average of binding results of pure IgG and pure BSA in mg of protein bound/g of support. **B)** Average of the percentage bound of pure IgG and pure BSA.

B1Al2A2 can bind to IgG mainly through hydrophobic interactions due to the presence of aromatic groups in its structure. Depending on the solution pH and the charge of the NH and OH groups, the ligand can also make some electrostatic interactions (Figure I.4.3). Although IgG is mostly hydrophilic, it is also observed some hydrophobic regions (Figure I.4.4).

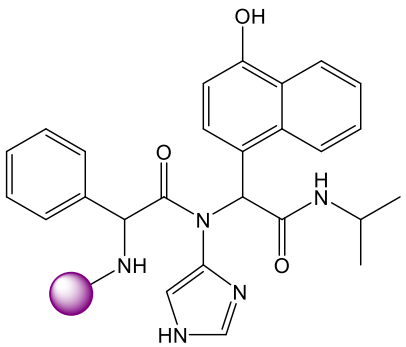


Figure I.4.3: Structure of B1Al2A2 Petasis-Ugi ligand immobilized on agarose. The purple sphere is a schematic representation of an agarose bead.

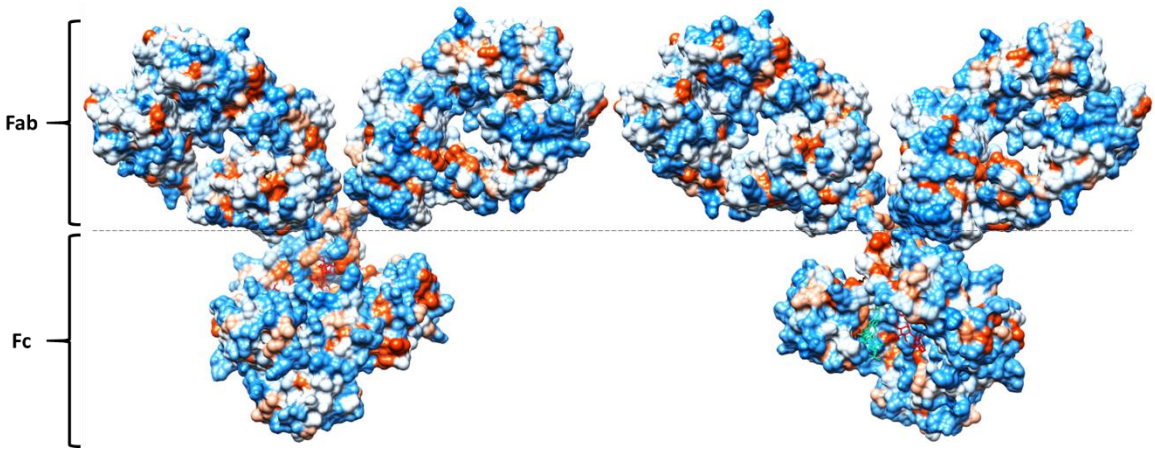


Figure I.4.4: Hydrophobicity Surface analysis from a crystallographic structure of an intact IgG_1 monoclonal antibody from *Mus musculus* (House Mouse). Blue represents the most hydrophilic regions and red the most hydrophobic regions. Images obtained from *Chimera 1.10.1* software. PDB code 1IGY.

Since HEPES is a zwitterionic buffer, without salt prevails the electrostatic interactions, but the addition of sodium chloride enhances the hydrophobic interactions present in the protein. The ions interact with some charged parts of the protein making the hydrophobic residues more available to bind the affinity ligand [72], [73]. The binding condition B3 appears to be a good binding buffer, but in order to reduce unspecific interactions B4 was chosen to continue the work. 20 mM HEPES, 500 mM NaCl, pH 7.4 buffer (B4) makes a balance between these two kind of interactions, which is apparently suitable to this approach.

I.4.4.3. Optimization of Elution conditions with pure IgG and pure BSA

After choosing a suitable binding condition, it was time to find an elution buffer capable to dissociate IgG, selectively, from the affinity ligand. For that, in a total of 11 elution buffer conditions were tested: E1: 20 mM HEPES, pH=7.4; E2: 20 mM HEPES, pH=6.8; E3: 20 mM HEPES, pH=8.2; E4: 10mM Sodium phosphate, pH=5.7; E5: 10mM Sodium phosphate, pH=8; E6: 0.1 M Glycine, pH=2.5; E7: 0.1 M Glycine, pH=11; E8: 20 mM HEPES, 50 mM Imidazole, pH=7.4; E9: 0.1 M $NaHCO_3$, 30% ethylene glycol, pH=10; E10: 0.1 M $NaHCO_3$, 0.1% (w/v) CHAPS, pH=10 and E11: 0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10.

B1Al2A2 affinity ligand, previously synthesized in batch, was screened with pure IgG and pure BSA (0.5 mg/mL each) in 20 mM HEPES, 500 mM NaCl, pH 7.4. The screenings were performed with 0.5g of B1Al2A2-functionalized agarose, in duplicate for each elution condition on-column format. The unbound protein (flow-through, washes and elution) were collected in 1.5 mL microcentrifuge tubes and quantified by BCA method and/or measuring the absorbance

at 280 nm. The bindings were quantified by both methods, however some of the elution conditions were made by BCA and others with the absorbance at 280 nm, due to the interference of the elution buffers. Samples from E1, E2, E3, E4, E5, E8, E10 and E11 were quantified by BCA method, while E6, E7 and E9 were quantified by measuring the absorbance at 280 nm.

From the binding results (Table I.4.5), it is observed a little decrease in the percentage of IgG bound, but in general the results are consistent and in the same range of values, when compared with previous screening.

Table I.4.5: Average of the binding results from the elution tests of the B1Al2A2 affinity ligand with pure IgG and pure BSA, performed on-column with the binding buffer B4 (20mM HEPES, 500 mM NaCl, pH 7.4).

	Quantification method	IgG	BSA
Mass of protein bound/Mass of support (mg/g)	BCA	0.25 ± 0.02	0.18 ± 0.01
	280 nm	0.46 ± 0.01	0.21 ± 0.02
Percentage of protein bound (%)	BCA	82.27 ± 8.50	40.71 ± 3.06
	280 nm	93.05 ± 0.20	48.21 ± 3.52

Regarding the elution buffers chosen to test with pure IgG and pure BSA, the conditions E1 to E7 were essentially to study the pH and salt influence in the binding between the affinity ligand and both proteins. It was decided to remove the sodium chloride from the elution buffers, to minimize the hydrophobic interactions and, since the useful pH range of PBS and HEPES buffer are, respectively, 5.6 to 8 and 6.8 to 8.2, five elution conditions were made (E1 to E5) [74]. E6 and E7 are more extreme conditions, with a very acidic pH and a very basic pH, respectively.

B1Al2A2 has a histidine-like component in its structure, so a buffer with imidazole reagent (E8) was tested to compete with this histidine structure and see if it could dissociate IgG from the ligand.

Ethylene glycol is an alcohol with two hydroxyl groups, which disrupts hydrophobic interactions and was used in elution buffer of A2C11I1 Ugi ligand, which was also designed to bind IgG. Elution condition E9 was basically inspired in its elution buffer [22]. CHAPS is a zwitterionic surfactant used in the elution buffer of A3C1I1, this ligand was developed to bind IgG and has some similar groups with B1Al2A2 structure, E10 was inspired on the elution buffer used in A3C1I1 [24]. Finally, condition E11 was just an improve of E10.

Although with buffer at pH 11 (table I.4.4, section I.4.4.1.) IgG was eluted almost 100%, in conditions E9 to E11, instead of pH 11, pH 10 was used. Buffers at pH 11 are too alkaline and could denature the protein eluted.

In elution results, from E1 to E5 condition nothing eluted, removing salt and simply change of pH was not enough, once the pH range was not close to the pKa values of the functional groups from B1Al2A2 (Figure I.4.5), so the protonation of these groups remains the same. With the elution conditions E6 and E7 there are groups that are protonated and deprotonated, respectively, however it was also not enough to elute the protein. The pKa values of each functional group of the B2Al2A2 ligand was calculated by the software MarvinSketch (Figure I.4.5). Also with condition E8, the imidazole wasn't critical in the bond between the proteins and the ligand, and nothing eluted.

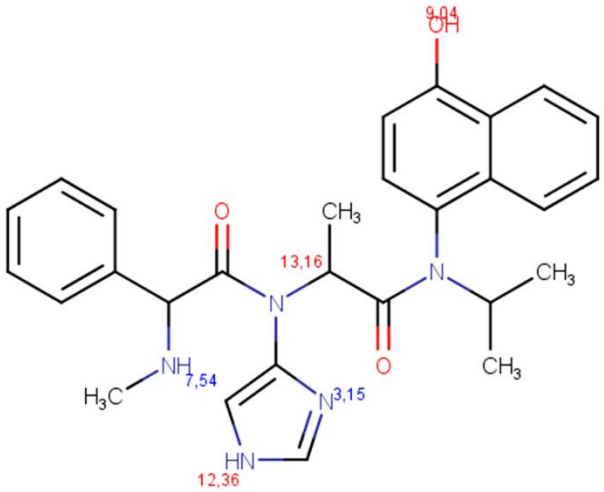


Figure I.4.5: pKa values of B1Al2A2 Petasis-Ugi ligand. Values were determined using the pKa Plugin from MarvinSketch (ChemAxon). A methyl group was included in the structure of the ligand in place of the agarose bead.

The only conditions that showed results were E9, E10 and E11, which are represented in figure I.4.6. The 0.1 M $NaHCO_3$ in 30% ethylene glycol, pH=10 buffer (E9) eluted more BSA than IgG and consequently this condition was discarded for IgG elution. Fortunately, with 0.1 M $NaHCO_3$, 0.1% (w/v) CHAPS, pH=10 (E10) it was observed that only IgG eluted, which is what was pretended.

CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) is usually used for solubilisation of membranes, as well as membrane proteins. This nondenaturing detergent is very effective at breaking protein-protein interactions [75]. Since the structure of B1Al2A2 ligand is based on amino acid interactions with IgG, it is possible to say that the interactions between IgG and the affinity ligand mimics a protein-protein interaction. So, with 0.1 M $NaHCO_3$, 0.1% (w/v) CHAPS, pH=10, the hydroxyl group of B1Al2A2 is deprotonated and CHAPS helps to disrupt the binding between the affinity ligand and IgG.

Therefore, to make the elution more effective it was decided to increase the amount of CHAPS from 0.1% to 1%. With 0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10 (E11) the percentage of

protein eluted kept in the same range, but when we look to the mass of eluted protein/mass of support results (Figure I.4.6 A) it is observed an increase of IgG eluted. Due to these results, elution condition E11 was chosen for IgG purification from human plasma.

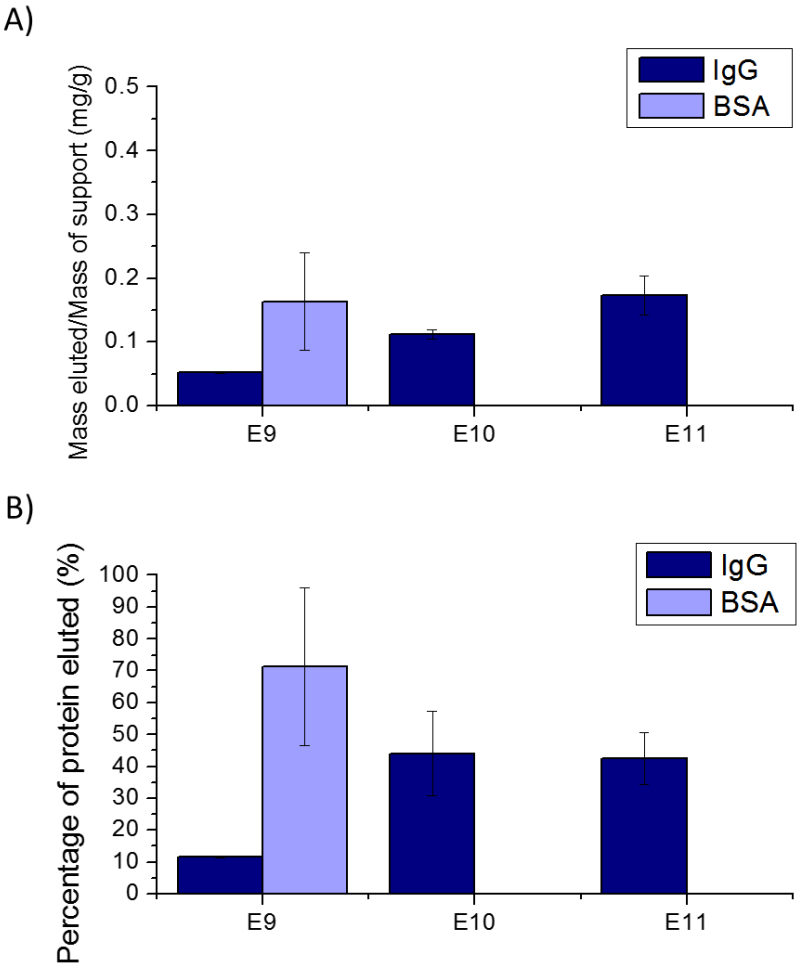


Figure I.4.6: Screening results of the B1Al2A2 Petasis-Ugi ligand with three different elution conditions. **E9:** 0.1 M $NaHCO_3$ in 30% ethylene glycol, pH=10; **E10:** 0.1 M $NaHCO_3$, 0.1% (w/v) CHAPS, pH=10; **E11:** 0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10.The screenings were performed in duplicate for each elution condition to each protein in on-column format. **A)** Average of elution results of pure IgG and pure BSA in mg of protein eluted/g of support. **B)** Average of the percentage of protein eluted for pure IgG and pure BSA.

In conclusion, 20 mM HEPES, 500 mM NaCl, pH 7.4 is the binding buffer and 0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10 is the elution buffer for IgG purification. Once the binding and the elution conditions are chosen, a test with Human plasma, a more complex mixture, is needed to confirm the previous results.

I.4.4.4. Test of B1Al2A2 ligand for IgG Purification with Human Plasma

The plasma is the liquid part from blood and represents 55% from total volume, which 7% of the plasma content are proteins. There are over 300 proteins present in human plasma with a wide range of functions. Albumin is the most abundant plasma protein and represents around 58% of the plasma protein content. Albumin is a single polypeptide chain protein with 66 kDa of molecular weight and is responsible for the transport of hormones, free fatty acids, drugs, bilirubin, metal ions and amino acids. It also maintains oncotic pressure and buffers pH, among other functions [76].

Globulins represent 38% of human plasma proteins, and is where immunoglobulins are found. IgG is the major circulating form of immunoglobulin, about 75% of plasma immunoglobulin content, has a half-life of 22 days and its total molecular weight is 150 kDa. The light chains have 25 kDa each and the heavy chains have 50 kDa each. The remaining 4% of the human plasma protein content is fibrinogen and others [76].

To test the B1Al2A2 affinity ligand for human IgG purification, a screening on-column format with Human plasma was made. B1Al2A2, previously synthesized in batch, was screened with Human plasma (0.5 mg/mL) diluted in 20 mM HEPES, 500 mM NaCl, pH 7.4. The screenings were performed with 0.5g of B1Al2A2-functionalized agarose, in duplicate, on-column format and the elution step was performed with 0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10. The total unbound proteins (flow-through, washes and elution) were collected in 1.5 mL microcentrifuge tubes and quantified by BCA method. The results are represented in table I.4.6 and figure I.4.7, the calculations were made considering plasma total protein present in samples.

Table I.4.6: Screening results of the B1Al2A2 affinity ligand with Human Plasma in the best binding and elution conditions. The screenings were performed on-column, in duplicate.

	Condition	Human Plasma Screening
Mass of protein bound/Mass of support (mg/g)	B4: 20mM HEPES, 500 mM NaCl, pH 7.4	0.28 ± 0.01
Percentage of protein bound (%)		49.07 ± 2.24
Mass of protein eluted/Mass of support (mg/g)	E11: 0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10	0.12 ± 0.01
Percentage of protein eluted (%)		48.81 ± 1.98

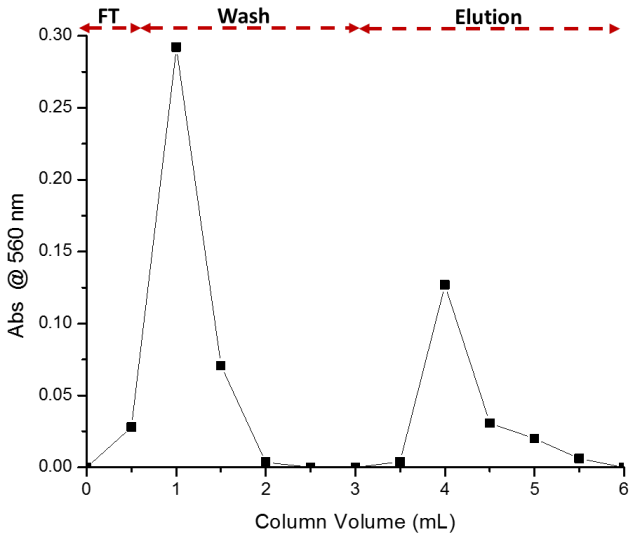


Figure I.4.7: Chromatogram obtained for the screening results of the B1Al2A2 affinity ligand with Human Plasma in the best binding and elution conditions. The screenings were performed on-column, in duplicate.

To study the efficiency of IgG purification with B1Al2A2 affinity ligand, SDS-PAGE gels were performed in order to analyse the proteins present in each fraction and to calculate the purity in the elution fractions (Figure I.4.8). SDS-PAGE gels allows a qualitative analysis of the resulted IgG purification by B1Al2A2 affinity ligand with 0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10 elution condition.

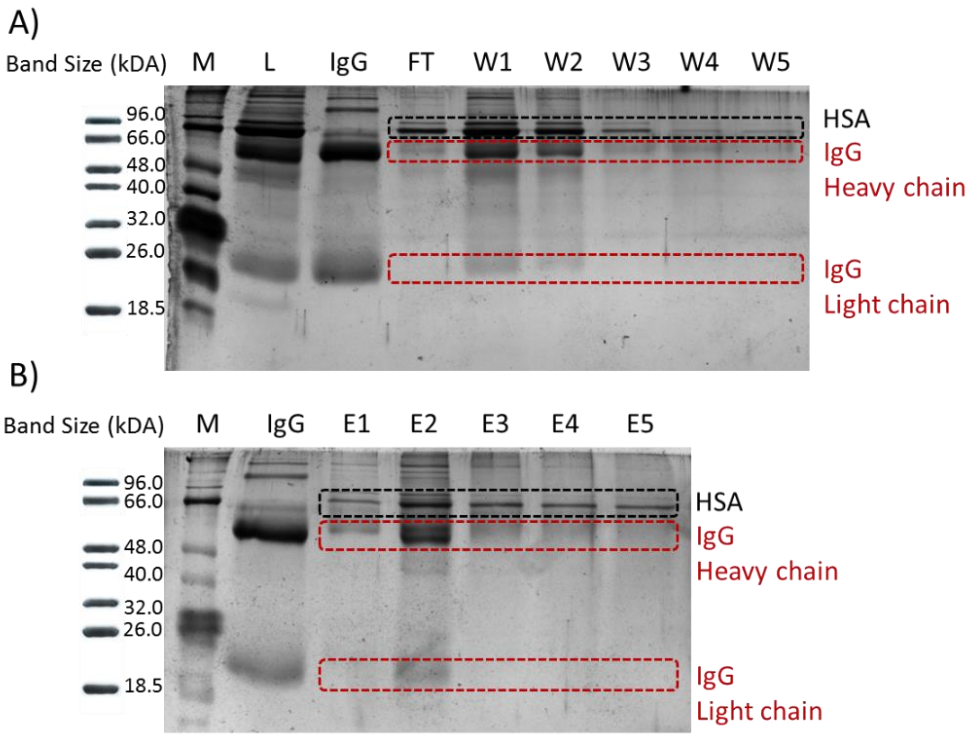


Figure I.4.8: SDS-PAGE gel obtained for Human IgG purification from human plasma with B1Al2A2 affinity ligand. The molecular marker, loading, pure IgG, flow-through, washes and elution fractions correspond to M, L, IgG, FT, W and E, respectively. The present gels are 12.5% acrylamide and stained by silver staining.

Observing the figure I.4.8 A, HSA along with some other plasma proteins are being washed away as desired, however some IgG bands are observed in the washes, this means that some percentage of human IgG did not bind to the B1Al2A2 adsorbent.

From the elution fractions (Figure I.4.8 B), it was observed the IgG bands but also the HSA band. For an ideal purification it was supposed to only elute IgG, which did not happen. Proceeding to the calculation of the purity from the elution fractions with ImageJ software, the IgG purity was about 41%, which is low.

With these results, it is concluded that the purification was not successful in a single step. Since HSA is the most abundant protein from human plasma, there is some competition with IgG in the binding for B1Al2A2. To improve IgG purification in order to have a IgG sample with high purity, some alternatives should be tested, such as a previous HSA depletion, an upgrade of the elution buffer, increasing to pH 11, or use condition E9 (0.1 M $NaHCO_3$, 30% ethylene glycol, pH=10) to recover HSA and then elute IgG with condition E11 (0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10).

I.4.4.5. Static partition equilibrium studies for the determination of binding constants

In an affinity purification method, the affinity binding constant (K_a) and the maximum binding capacity of the support (Q_{max}) are the binding constants that characterize the reversible interaction between an affinity ligand and the target protein. Herein, the binding constants were determined from partition equilibrium studies by applying a Langmuir model to data. The procedure consisted in incubating, at temperature constant, a known amount of partitioning solute (pure IgG) with a fixed amount of affinity matrix (B1Al2A2), until the mixture reach a chemical equilibrium. In the end, the total protein was quantified by a BCA assay and the results obtained followed the Langmuir isotherm model and were also analysed through the Hill plot using a linear fitting (Figure I.4.9) [77].

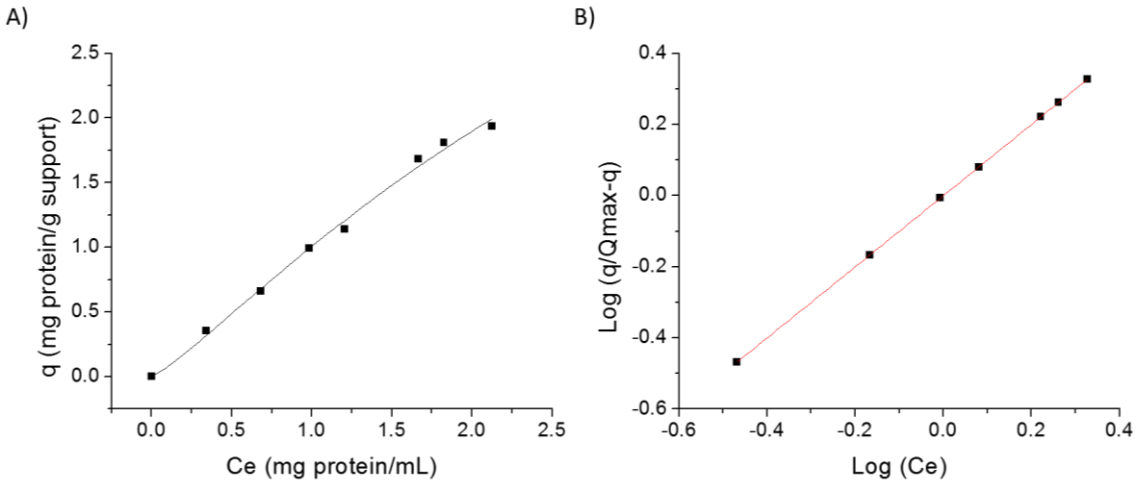


Figure I.4.9: Static partition equilibrium studies plots of IgG for B1Al2A2 ligand. **A)** Adsorption isotherm. The obtained results were fitted to the Langmuir model using OriginPro (v8.5.1). **B)** Hill plot analysis, obtained through a linear fitting using OriginPro (v8.5.1).

The Langmuir isotherm assumes that all binding sites are identical, each one retains one target molecule and have uniform and independent adsorption energies. This model is the most popular one in affinity chromatography and is described by Equation 4.1 [77]:

Equation 4.1:

$$q = \frac{Qmax\ Ka\ Ce}{1 + Ka\ Ce}$$

The Hill equation from Hill plot analysis is represented by Equation 4.2:

Equation 4.2:

$$\log \frac{q}{Qmax - q} = \log Ka + nH \log Ce$$

Where q is the bound protein per mass of support (mg protein bound/g support), Ce is the concentration of unbound protein in equilibrium (mg/mL), Q_{max} is the maximum binding capacity (mg protein bound/g support), K_a is the association equilibrium constant (mL/mg) and n_H represents the type of cooperative binding [77]. The K_a, Q_{max} and n_H parameters of B1Al2A2 affinity ligand were determined and are summarized in table I.4.7.

Table I.4.7: Binding constants: K_a (M^{-1}) and Q_{max} (mg protein bound/g of support), and n_H after fitting with Langmuir model and analysis with Hill plot. R^2 is the correlation factor.

Model	K_a	Q_{max}	n_H	R^2
Langmuir isotherm	2.88 x 10^4 M^{-1}	6.24 ± 5.28 (mg/g)	-	0.98
Hill Plot	2.92 x 10^4 M^{-1}	-	1.13	0.99

The K_a values obtained for B1Al2A2 affinity ligand are similar and within the affinity ligands range used in affinity purification, which is 10^3 to 10^8 M^{-1} [39]. Some other biomimetic ligands designed to bind IgG, such as triazine ligand 22/8, triazine ligand 8/7, Ugi ligand A2C11I1 and others have K_a values in a range of 10^4 and 10^5 M^{-1}, which is the same range as the B1Al2A2 ligand [17], [18], [22]. Comparing this K_a values to the ones found in the literature, it is possible to affirm that B1Al2A2 has a good association constant for a protein purification system, particularly for human IgG. About the obtained value of Q_{max}, when compared to other ligands immobilized to agarose [55], it is superior to what was expected, which means 6.24 mg/g of support is a good maximum binding capacity. However, once the isotherm displays a linear profile (Figure I.4.9 A), this means that the concentrations used weren't enough to saturate the resin, so the Q_{max} obtained could be a non-realistic value. Regarding the n_H value, it is superior to 1, which indicates a positively cooperative binding.

I.4.5. Conclusions and Future Directions

The goal of this chapter was to explore the B1Al2A2 ligand as an affinity adsorbent for human IgG purification. After the confirmation of the results from the previous chapter, ligand B1Al2A2 was tested against four different binding buffers in order to increase the binding capacity towards IgG. From these screenings, it was found that 20 mM HEPES, 500 mM NaCl, pH 7.4 (B4) was the most suitable binding condition, once it bound almost 100% of IgG. The B1Al1A2 binds to IgG mostly through hydrophobic interactions and some electrostatic interactions.

At a second stage, different elution conditions were tested to selectively recover IgG. The 0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10 seemed to be an adequate elution buffer, since the screening results with pure IgG and pure BSA showed that only IgG was being eluted.

However, when B1Al2A2 was tested against human plasma, at equal conditions, the same was not verified. Perhaps the BSA eluted, in the screening with pure BSA, was below the quantification limit of the BCA method and wasn't detected. With human plasma, the purification of IgG with B1Al2A2 adsorbent still needs improvements, a lot of HSA was eluting and the obtained IgG purity was low, about 41%. To overcome this, some alternatives are needed and should be tested, such as a previous HSA depletion, an upgrade of the elution buffer, testing at pH 11, or use condition E9 (0.1 M $NaHCO_3$, 30% ethylene glycol, pH=10) to recover HSA and then elute IgG with condition E11 (0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10) or with a more suitable elution buffer.

Regarding the partition equilibrium studies, the experimental data was fitted according to Langmuir isotherm and also analysed by Hill plot. The K_a and Q_{max} achieved are very promising values within the same range or superior as the ones found in literature for affinity systems used in the purification field. However, the obtained isotherm displays a linear profile, this means that the concentrations used didn't saturate the B1Al2A2 adsorbent and the Q_{max} value could not be real.

Part II – Antibody Drug Conjugate (ADC) Approach

II.4.1. Introduction

Antibody Drug Conjugates are a promising approach in cancer therapeutics field, since they fuse the ability of mAb specificity with the delivery of a cytotoxic agent to the target tumor, which seems to overcome the major problems related to cytotoxic drugs and the mAbs on their own.

II.4.1.1. ADCs: Function and Mechanism of Action

Mechanistically, ADCs are designed to be stable in circulation and only kill cancer cells in a target-dependent manner [4], [11]. The first step in this process is binding of the antibody on the cell-surface antigen (Figure II.4.1). Following binding, the antigen-ADC complex is internalized through receptor-mediated endocytosis. For this process to occur a cascade of events must be initiated, including recruitment of adaptins and clathrin, inward budding of the plasma membrane, formation of early endosomes, and lastly trafficking to late endosomes and lysosomes. The efficiency of this step depends at least in part on the identity of the target antigen [3], [4].

Once inside lysosomes, ADCs are degraded due to the acidic and proteolytic enzyme-rich environment, which results in intracellular release of the cytotoxic drug in order to cause cell death. Depending on the class of cytotoxic drug used, the mechanism of action can vary, it can disrupt cytokinesis by tubulin polymerization inhibitors or damage DNA by DNA interacting agents. Neighbouring cancer cells may also be killed by a process known as the bystander effect, which is caused by the released free drug into the tumor environment by the dying cell [3], [4].

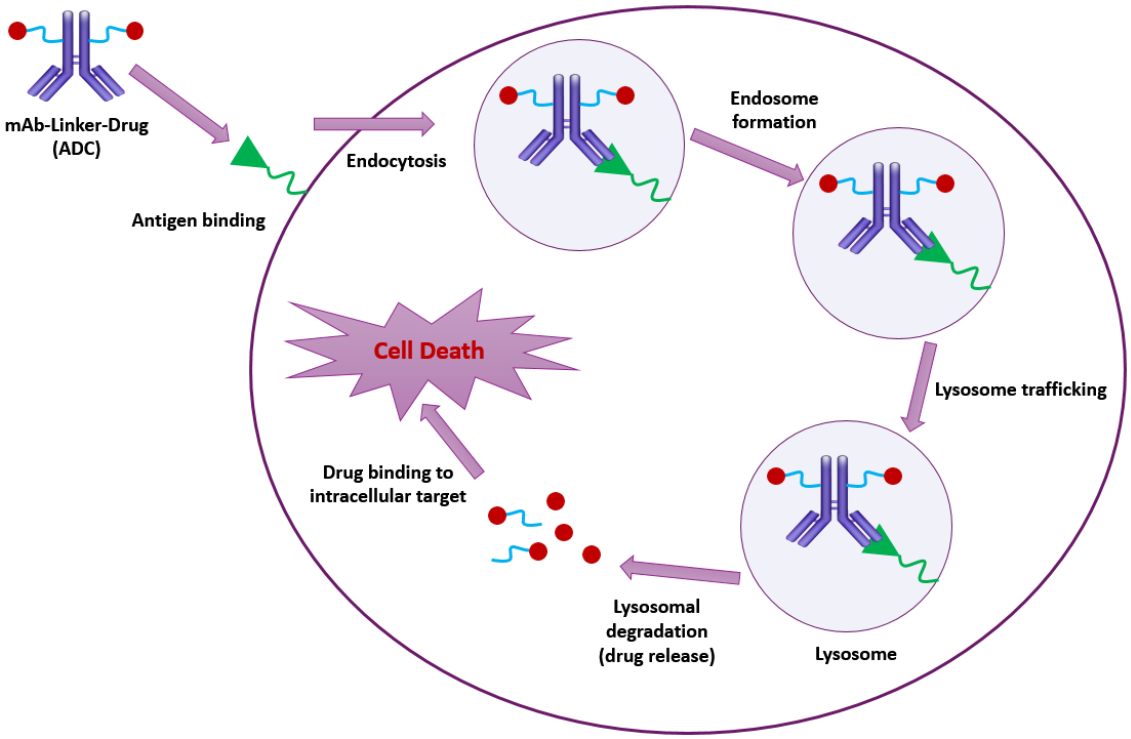


Figure II.4.1: Schematic representation of the processes associated to the mechanism of action and biological activity of an Antibody Drug Conjugates. Adapted from Bouchard, Viskov, & Garcia-Echeverria, 2014.

II.4.1.2. ADCs' conjugation strategies

Classical ADC platforms often rely on the random chemical modification of the mAb at solvent accessible reactive amino acids such as lysines or cysteines derived from the reduction of inter-chain disulfide bonds in the antibody. However, this conventional ADC conjugation strategy generates heterogeneous products [4], [78].

For that reason, several strategies were developed in order to optimize the homogeneity of ADCs' product, and consequently with improved pharmacologic properties. The second strategy of conjugation focuses on the site-specific conjugation, which can be through antibody engineering-based methods or by the use of enzymes, which will catalyse bond formation between the mAb and the linker-drug complex. Beyond that, there already are other strategies under investigation [4], [78].

In Table II.4.1 are described all ADCs conjugation strategies that have been already performed, such as some characteristics and their respective advantages and disadvantages.

Table II.4.1: Relevant features about ADCs' conjugation strategies.

Conjugation Strategy	Characteristics	Advantages	Disadvantages	Ref.
Conventional ADC Conjugation				
Lysine conjugation	- Direct conjugation with the ε-amino terminus of lysine residues; - Results in 0 to 8 drug molecules per antibody;	- Use of native amino acid; - FDA approved ADC: Adcetris®.	- Generates heterogeneity of ADC species; - Each species may have distinct properties, which may result in a wide range of *in vivo* solubility, stability and pharmacokinetics (PK) properties; - Require diligent manufacturing capabilities.	[4], [7], [10], [78], [79]
Cysteine conjugation	- Cysteine conjugation occurs after reduction of four inter-chain disulfide bonds; - Depends on sulfhydryl portion of a reduced cysteine residue.	- Use of native amino acid; - FDA approved ADC: Kadcyla®.		
Site-specific Conjugation – Antibody engineering-based methods				
Inter-chain disulphide bonds	- Reduction of all inter-chain disulphide bonds; - This approach showed that stoichiometry of drug attachment is more critical to ADC properties than the drug site attachment and conjugate homogeneity.	- Optimal *in vivo* performance was observed with the four drug-loaded form.	- Limited to conjugates generated with the use of inter-chain disulfide bonds of IgG1, which are all located in the highly solvent accessible hinge region.	[78]
Engineered cysteine residues	- Insertion of cysteine residues in the antibody sequence by mutation or insertion; - Called THIOMABs.	- Minimal heterogeneity; - Preserves inter-chain disulfide bridges intact; - Similar *in vivo* activity; - Improved PK; - Superior therapeutic index.	- If cysteine residues are not inserted in proper sites, can alter protein structure or function; - Reversible conjugation and unstable; - Requires production of recombinant antibodies.	[4], [7], [78], [80]
Unnatural amino acids	- Insertion of unnatural amino acids with bio-orthogonal reactive handle; - p-acetylphenylalanine contains a keto group that can be selectively conjugated to a drug with an alkoxy-amine through an oxime ligation.	- Conjugates active *in vivo*; - Homogeneous ADCs; - Improved PK; - Good efficacy, specificity and stability in blood serum.	- Requires production of genetic engineered antibodies.	[4], [7], [78]–[80]
Selenocysteine	- Very similar to cysteine, but contains a selenium atom in place of the sulfur atom.	- Selenolate group is a more reactive nucleophile than the thiolate.	- Requires production of genetic engineered antibodies; - *In vivo* experiments not reported.	[4], [78]

Site-specific Conjugation – Enzymatic Conjugation				
Glycotransferases	- Uses a mutant glycotransferase to attach a chemically active sugar moiety to a glycosylation site on an antibody (Asn297 of the Fc fragment).	- Highly homogeneous conjugate; - Exhibited *in vivo* cell-killing activity.	- Lower drug load; - Requires glycoengineering.	[4], [78]
Transglutaminases	- Form a bond between an amine group from the linker/drug and an engineered glutamine side chain from a **glutamine tag** (LLQG) on the antibody.	- Stable isopeptide bond; - High degree of conjugation; - *In vitro* and *in vivo* efficacy, with double potency; - Improved stability;	- Pharmacokinetics not well studied; - Requires engineered antibodies with a "glutamine tag";	[4], [7], [78], [79]
Formylglycine-generating enzyme (FGE)	- Recognizes a CxPxR sequence (where X is any amino acid) and converts a cysteine residue to formylglycine generating antibodies with an **aldehyde tag**; - Chemistry: Hydrazino-iso-Pictet-Spengler (HIPS) ligation.	- Aminooxy- or hydrazide-functionalized molecules were successfully attached to the model proteins.	- Requires production of genetic engineered FGE and antibodies; - *In vitro* and *in vivo* experiments not reported.	[78], [80]
Other Approaches – Under studying				
Other Chemical approaches	- Bis-sulfone reagents that undergo bis-alkylation to conjugate both thiols of the two cysteine residues that were obtained through the reduction of native disulphide bonds.	- Conjugates showed antigen binding and stability; - Exhibited *in vitro* and *in vivo* antitumor activity.	- Under optimization.	[78]
	- Chemical modification of maleimides: facilitates a reaction with two nucleophilic thiol groups derived from a reduced disulfide bridge.	- Highly stable and homogeneous ADCs.	- *In vitro* and *in vivo* experiments not reported.	
Photoactive Protein Z	- Conjugation of a photoactive protein Z (Z domain), which derived from the IgG-binding B domain of protein A, upon exposure to long wavelength UV.	- Binds most of IgG isotypes specifically to the Fc fragment with high affinity.	- Requires production of engineered protein Z with benzoylphenylalanine (BPA): enables to covalently couple protein Z to the antibody; - Premature study; - Needs optimization.	[78]

Some of the strategies mentioned before showed improvements from the first generation of ADCs, however these approaches can activate undesired amino acids and are difficult to implement, which results in an unpredictable stability, solubility, and product quality. In addition, it is desirable that the attachment of drugs should not alter the ability of a mAb to recognize its specific antigen [81].

Drug conjugation to the mAb must be performed under mild conditions to avoid protein denaturation and be well exposed to facilitate cleavage in cancer cells. Linkage of a larger number of cytotoxic drugs can affect pharmacokinetics of mAb, since these drugs are usually hydrophobic and poorly soluble in the predominantly aqueous environment of the antibody in solution, so the ideal number of drug molecules per antibody for most current ADCs appears to be about four. The linkers must be stable in circulation but, upon cell internalization, they should facilitate efficient drug release, due to a pH lowering in the lysosomes (pH 4.5-5.0) [81].

To accomplish these considerations and to overcome the obstacles mentioned before, the use of affinity ligands as the linker component seems to be a promising strategy to assemble an ADC and specifically deliver non-linked cytotoxic drugs to tumor cancer cells.

II.4.2. Materials

II.4.2.1. Chemicals

All reagents were used with a high purity and the solvents were pro-analysis.

The reagents 3-aminophenol (component 8), 4-amino-1-naphthol hydrochloride (A2 and component 22), 1-amino-2-propanol (A7), 4-Imidazolecarboxaldehyde (Al2), Phenylboronic acid (B1), 3-thienylboronic acid (B2), glyoxylic acid monohydrate, isopropyl isocyanide and sodium bicarbonate ($NaHCO_3$) were purchased from Sigma-Aldrich (Portugal).

Methanol (MeOH) was obtained from Roth. Dimethylformamide (DMF), Dichloromethane (DCM) and di-sodium-hydrogen phosphate 2-hydrate were purchased from VWR. Hydrochloric acid 37% (HCl), ethanol absolute PA, acetic anhydride, sodium-di-hydrogen phosphate 1-hydrate and sodium hydroxide (NaOH) were acquired from Panreac.

The monoclonal antibody, Bevacizumab IgG_1, was kindly given by prof. João Gonçalves Lab. The BCA reagents of BCA kit used in the screenings were acquired from Sigma-Aldrich (Portugal).

For the synthesis of the lead ligand in a hydrolysable resin, the Rink Amide MBHA resin (100-200 mesh) was bought from Merck (Portugal). The piperidine, trifluoroacetic acid (TFA) and diethyl ether were obtained from Sigma-Aldrich (Portugal) and dimethyl sulfoxide from Fisher Scientific (Portugal).

II.4.2.2. Chromatographic Material

Cross linked agarose (Sepharose™ CL-6B) was acquired from GE Healthcare. Deep well plates riplate® sw 2 mL, were acquired from Roth. Half-area UV-Star® 96-well microplates and 96-well transparent microplates were obtained from Greiner Bio-One and Sarstedt, respectively. A Laboratory Centrifuge LMC-3000 from BioSan was used for the screenings.

II.4.2.3. Buffers

The following buffers were used: Phosphate Buffer Saline (PBS) (10mM Sodium phosphate, 150mM Sodium Chloride (NaCl), pH 7.4) for binding. Regeneration Buffers: 0.1M NaOH in 30% isopropanol and 0.1M HCl. Elution buffer: Phosphate Buffer Saline (PBS) (10mM Sodium phosphate, 100mM Sodium Chloride (NaCl), pH 6).

II.4.2.4. Equipment

The synthesis of the synthetic ligands on solid-phase were carried out in Incubator ZKA KS4000i (VWR). Absorbance readings were performed in Microplate reader TECAN Infinite® 200 (Portugal); The oven used for ligand synthesis and BCA assay was a Big Shot III 230402-2 Hybridization Oven from Boekel Scientific. A Laboratory Centrifuge LMC-3000 from BioSan was used for agarose washes after libraries syntheses and for the screenings.

II.4.3. Methods

II.4.3.1. Re-Synthesis of the Petasis-Ugi lead ligands

The aminated agarose was prepared according to the procedures 2.3.1. and 2.3.2. from chapter 2, and washed with 25% (v/v) ethanol/ distilled water (5x resin volume) and 50% (v/v) ethanol/ distilled water (5x resin volume). Then, two batches were prepared with 5 molar eq. of each compound in excess relative to the epoxy: 1) glyoxylic acid monohydrate and phenylboronic acid (B1) in 50% (v/v) ethanol/distilled water (0.5 mL of each/g moist agarose); 2) glyoxylic acid monohydrate and 3-Thienylboronic acid (B2) in 50% (v/v) ethanol/distilled water (0.5 mL of each/g moist agarose). The reactions occurred for 48 hours at 60 °C with agitation at 200 rpm. Afterwards, the Petasis-scaffolded agarose were washed separately with 50% ethanol/ distilled water (v/v) (5x resin volume) and distilled water (10x resin volume) by vacuum suction.

The two Petasis-functionalized agarose was washed with 20% to 100% DMF (in 20% increments), ressuspended in 10% (v/v) acetic anhydride in DMF and incubated for 24 hours at room temperature with orbital shaking (200 rpm) to block the unreacted amines. Afterwards, the resins were washed with 100% to 20% DMF (in 20% decrements), distilled water (10x resin volume) and with 20% to 100% Methanol (in 20% increments).

In meantime, aldehyde (Al2) and amines (A2, A7) component (5 molar eq. of each, relative to epoxy groups) were dissolved in Methanol (0.5 mL/g moist agarose) and the reaction (aldehyde + amine component) occurred for 2h at 60 °C in the orbital shaker (200 rpm). 1M NaOH was added to A2 (5 molar eq.). After incubation, the mixture of amine and aldehyde was added to the respective Petasis resin, along with isopropyl isocyanide (5 molar eq. relative to epoxy). The reactions were left for 48h at 60 °C in the orbital shaker (200 rpm). Finally, the Petasis-Ugi ligands were washed with 100% to 20% Methanol (in 20% decrements) and then with distilled water (10x 0.75 mL). The ligands were stored with 20% (v/v) ethanol/distilled water (1 mL/g moist agarose) at 4^{o}C.

II.4.3.2. Synthesis of Triazine ligand: 22/8

Aminated agarose was washed with distilled water (10x resin volume). Then two solutions were prepared: a cold solution of 50% (v/v) acetone/distilled water with 1 molar eq. of sodium bicarbonate ($NaHCO_3$) relative to epoxy groups (1 mL/ g of moist agarose), and a solution of cyanuric chloride (5 eq. molar excess, relative to epoxy groups) dissolved in acetone (8.6 mL/ g of cyanuric chloride). These solutions were added to the aminated agarose, followed by 1 hour of incubation in ice (approx. 0 ^{o}C) at 200 rpm.

After reaction, the agarose was washed with acetone (2x resin volume), 50% (v/v) acetone/distilled water (3x resin volume) and distilled water (5x resin volume). The amine 4-amino-1-naphthol hydrochloride (22 component) was dissolved in 50% DMF/H_2O (2 molar eq. of each relative to epoxy) and the solution was added to the cyanuric-functionalized agarose. The reaction occurred for 24 hours at 30 ^{o}C (200 rpm), R_1 substitution. Thereupon, the ligand was washed with 50% DMF/H_2O (3x resin volume) and distilled water (3x resin volume).

Posteriorly, the amine 3-aminophenol (8 component) was dissolved in 50% DMF/H_2O (5 molar eq. of each relative to epoxy), added to the previous resin and incubated for 48 hours at 80 ^{o}C (200 rpm), R_2 substitution. At the end, the ligand was washed with 50% DMF/H_2O (5x resin volume) and distilled water (5x resin volume), ressuspended in 20% (v/v) ethanol/distilled water (1 mL/g moist agarose) and stored at 4 ^{o}C.

It was added to each amine 1M of sodium bicarbonate (1 molar eq., relative to epoxy groups) and 1M NaOH to 4-amino-1-naphthol hydrochloride (2 molar eq.).

II.4.3.3. Screenings and elution tests of the lead ligands with a Monoclonal Antibody

Before screenings, the affinity ligands, B1Al2A2, B2Al2A7 and 22/8 were regenerated with 0.1M NaOH in 30% isopropanol followed by distilled water and with 0.1M HCl followed by distilled water, and then equilibrated with 10mM Sodium phosphate, 150mM NaCl pH 7.4.

Then, 0.25g of each ligand, in duplicate, was transferred to a deep well plate, and the monoclonal antibody, Bevacizumab, was reconstituted in 10mM Sodium phosphate, 150mM NaCl pH 7.4. to a concentration of 0.5mg/mL. 0.25mL of the mAb was loaded to each ligand and the plate was incubated for 1 hour at 25 ^{o}C with agitation (50 rpm). After, flow-through and washes (5x 0.25mL with binding buffer) were collected. Then, the ligands were incubated for another 1 hour at 25 ^{o}C with agitation (50 rpm) with 0.25mL of elution buffer (10mM Sodium phosphate, 100mM NaCl pH 6) and the elution fractions (5x 0.25 mL with elution buffer) were collected. All samples were collected by centrifugation at 500 rpm during 30 seconds to 96-well transparent microplates from Sarstedt. The triazine ligand 22/8 was used as a positive control.

Posteriorly, the total protein present in the samples was quantified by using the BCA assay as described in section 3.3.5, from chapter 3.

The quantity of protein binding was determined by Equation 2.1 and Equation 2.2, and the binding capacity of the ligands were determined by Equation 2.3, as described in section 2.4.2. from chapter 2. The quantity of protein eluted and the percentage of protein eluted were, respectively, determined by Equation 3.1 and 3.2., as described in section 3.4.4. from chapter 3.

II.4.3.4. Solid-phase synthesis of the lead ligand on a hydrolysable resin

For the synthesis of the B2Al2A7 ligand, on a hydrolysable resin, was used the Rink amide MBHA. This resin is usually used for peptide synthesis and since this ligand is somehow similar to a peptide the synthesis was adapted. Note that this approach was merely exploratory, so the protocol is not optimized.

Rink amide resin was swell on dichloromethane (DCM) for 1h at room temperature, on stir and then the DCM was removed with vacuum filter. Rink amide is protected with Fmoc (Fluorenylmethyloxycarbonyl) group, deprotection is needed to continue the synthesis. The deprotection was performed with a solution of 20% (v/v) piperidine in DMF (figure II.4.2) for 5 minutes and, then, 15 more minutes, to ensure the deprotection. A Kaiser test was performed to confirm the presence of free amines.

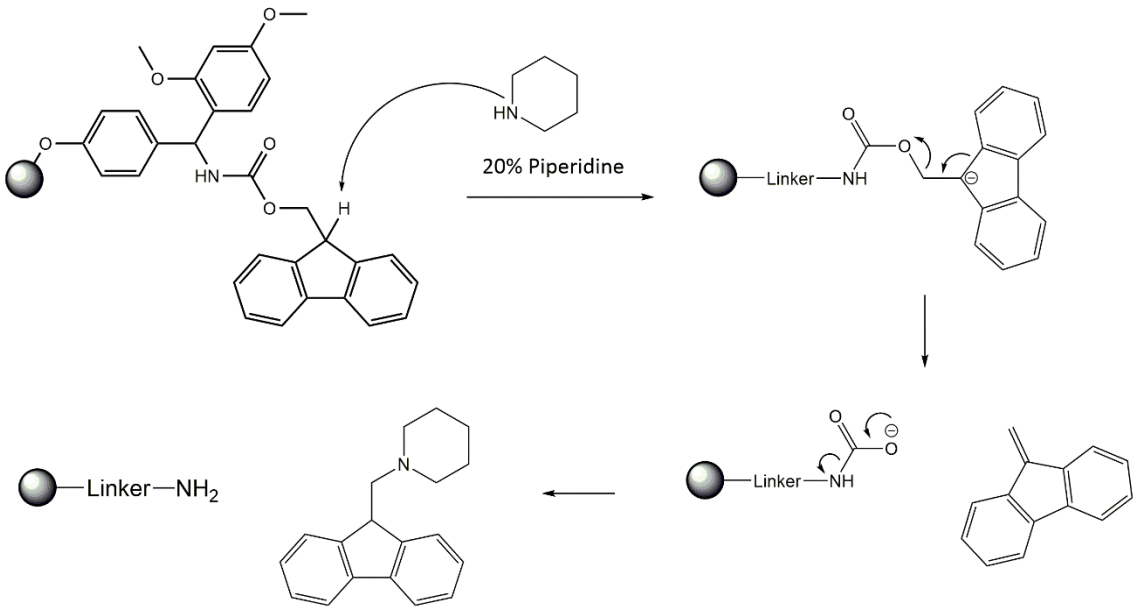


Figure II.4.2: Mechanism of Fmoc deprotection with piperidine. The grey sphere represents the solid support. Adapted from Stephan Steinmann, 2007.

A batch of glyoxylic acid monohydrate and 3-Thienylboronic acid (B2) in DMF was prepared with 5 molar eq. of each compound in excess relative to the resin load (0.78mmol/g) and was added to the resin. The reaction occurred for 48 hours at 60 °C with agitation at 200 rpm. Afterwards, the rink amide resin was washed with DMF (5x resin volume) and DCM (5x resin volume) by vacuum suction.

The Petasis-functionalized resin was ressuspended in 10% (v/v) acetic anhydride in DMF and incubated for 24 hours at room temperature with orbital shaking (200 rpm) to block the unreacted amines. Afterwards, the resin was washed with DMF (5x resin volume) and DCM (5x resin volume) by vacuum suction.

In meantime, aldehyde (Al2) and amine (A7) component (5 molar eq. of each, relative to the resin load) were dissolved in DMF and the reaction (aldehyde + amine component) occurred for 2h at 60 °C in the orbital shaker (200 rpm). After incubation, the mixture of amine and aldehyde was added to the resin, along with isopropyl isocyanide (5 molar eq. relative to the resin load) and incubated for 48h at 60 °C in the orbital shaker (200 rpm). Then, the resin was washed with DMF (5x resin volume) and DCM (5x resin volume) by vacuum suction and proceeded to TFA cleavage.

To cleave the synthetic ligand from the Rink amide resin, it was followed the procedure that is used to cleave the peptides. The resin was incubated with 100% TFA (figure II.4.3.), on stir and with N_2 (nitrogen) flow, for 2h. Then the resin was filtrated by vacuum suction and the TFA was evaporated with N_2 flow. The previous filtrate was added to a cold solution of diethyl ether in order to form a precipitate, which is the product.

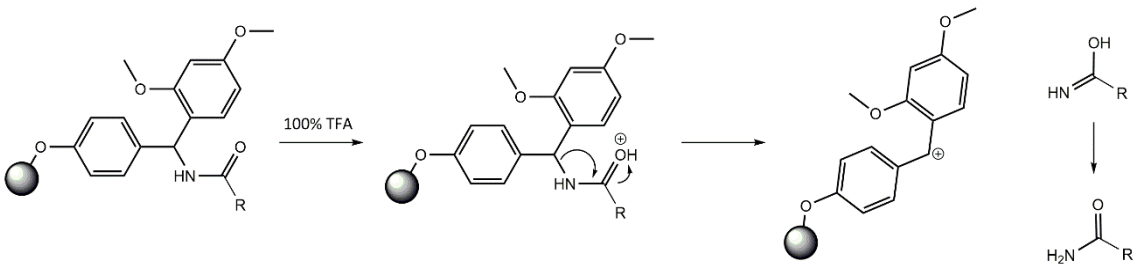


Figure II.4.3: Cleavage from the Rink Amide resin with TFA (trifluoroacetic acid). The grey sphere represents the solid support. Adapted from Stephan Steinmann, 2007.

II.4.4. Results and Discussion

II.4.4.1. Testing lead ligands with a Monoclonal Antibody

The triazine 22/8 ligand, first reported in 1999, is a protein A biomimetic ligand designed to bind with high affinity and selectivity the human immunoglobulin G (IgG). 22/8 is a bifunctional ligand substituted with 3-aminophenol (22) and 4-amino-1-naphtol (8), has an affinity constant (K_a) of $1.4x10^5$ M^{-1} and a theoretical maximum capacity of 151.9 mg IgG/g moist weight gel [17], [48]. Due to its features and since 22/8 is a highly selective affinity adsorbent for antibodies, it was used as a positive control for the monoclonal tests.

Petasis-Ugi B1Al2A2 ligand was included in these tests. Apart from its lower stability at different pH conditions (pH 2 and 11), was the ligand that showed the higher binding capacity to IgG.

The Bevacizumab IgG_1 was the monoclonal antibody used in these tests. Bevacizumab, with commercial name of Avastin, is a recombinant humanized monoclonal antibody used for metastatic colorectal cancer as angiogenesis inhibitor. This mAb binds and inactivates all isoforms of VEGF (vascular endothelial growth factor) resulting in a slow growth of new blood vessels, inhibiting tumor growth and proliferation [82].

The two Petasis-Ugi lead ligands (B1Al2A2 and B2Al2A7) were synthesized in batch as well as the ligand Triazine 22/8 (positive control). The ligands were screened with a monoclonal antibody (0.5 mg/mL) in PBS at pH 7.4 and then eluted with PBS at pH 6 to check their binding stability under the acidic tumor microenvironment. The screenings were performed in duplicate for each ligand, in a deep well plate, with 0.25g of ligand-functionalized agarose in each well. The unbound protein (flow-through, washes and elution) was collected in 96-well transparent microplates and quantified by the BCA method.

The binding results (Figure II.4.4 A and B) showed that the positive control, triazine ligand 22/8, has the higher binding capacity and percentage of protein bound, 0.34 mg of protein bound/g of support and 80% of protein bound, respectively, as it was expected once it was developed to bind antibodies with high affinity. Regarding the Petasis-Ugi ligands, its binding capacity and percentage of mAb bound are in the same rage, however the B2Al2A7 has slightly higher bind to the monoclonal antibody (0.24 mg of protein bound/g of supports and 55% of protein bound).

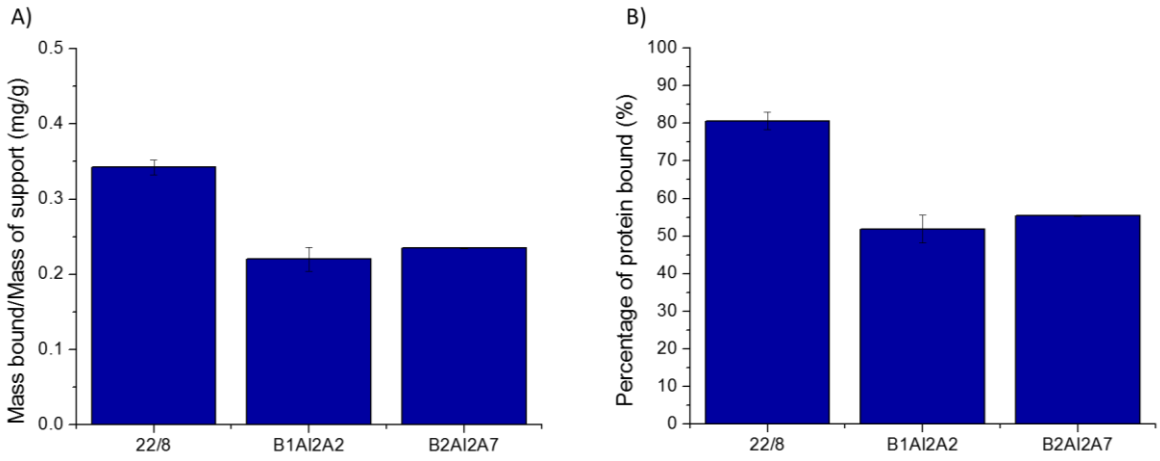


Figure II.4.4: Screening results of the lead Petasis-Ugi ligands. The screenings were performed in duplicate for each ligand in a deep well plate. The triazine ligand 22/8 was used as positive control. **A)** Average of binding results in mg of protein bound/g of support. **B)** Average of the percentage of mAb bound.

The elution test at pH 6 was performed in order to mimic the acidic tumor microenvironment, that is caused by the increase of fermentative metabolism from glucose metabolism, results in H^+ production and excretion. The extracellular pH in malignant tumors is around 6.5 to 6.9 and in some cases can be lower [83]. For this reason, it is useful to see if the ligands were stable at pH 6.

As it was expected from previous tests with the polyclonal IgG and from the analysis of the pKa values from each functional group of each ligand (Figure II.4.5), no protein was eluted at pH 6, which means that these ligands should be stable under the acidic tumor microenvironment and, therefore, good candidates in the ADC assembling.

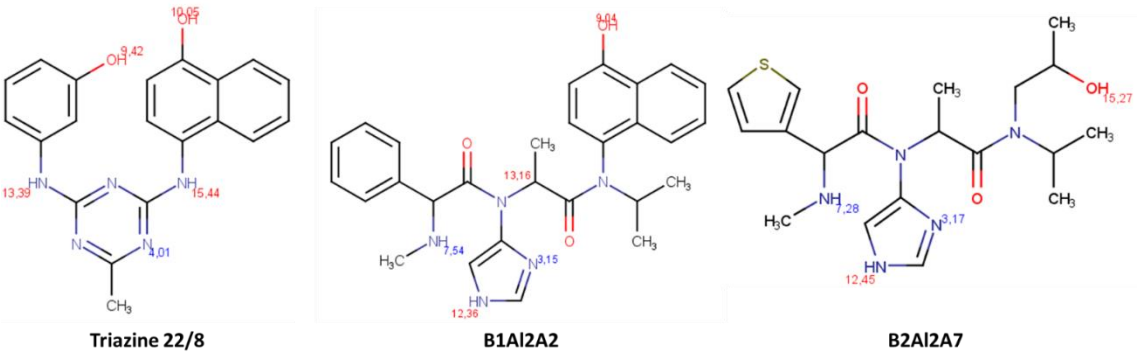


Figure II.4.5: pKa values of 22/8, B1Al2A2 and B2Al2A7 ligands. Values were determined using the pKa Plugin from MarvinSketch (ChemAxon). A methyl group was included in the structure of the ligands in place of the agarose bead.

Once the B2Al2A7 ligand showed more binding capacity to the monoclonal, when compared with B1Al2A2, was the one chosen to proceed to the synthesis in a hydrolysable resin. The objective is to have the ligand in liquid solution and have the possibility to couple with FITC (Fluorescein isothiocyanate) for further studies, such as toxicity evaluation, and posteriorly couple with a drug.

II.4.4.2. Solid-phase synthesis of the B2Al2A7 ligand on Rink amide MBHA resin

The solid-phase synthesis technique was first described by Bruce Merrifield, in 1963, and is a strategy in which molecules are bound on a bead and synthesized step by step. This method is often used to synthesize peptides, or even DNA and RNA, and recently has been used in combinatorial chemistry. Unlike the traditional organic synthesis, the solid-phase synthesis allows to remove impurities or unreacted material without need of chromatographic purification. Also, once the resin does not dissolve in solvent, the organic starting material can be exposed to large excesses of reagent in order to complete the reaction [84].

All these advantages can avoid undesired side reactions or other synthesis complications. Therefore, the solid-phase strategy was chosen to synthesize the B2Al2A7 Petasis-Ugi ligand, so in the end we expect to have a bifunctional ligand (Figure II.4.6) that can bind through non-covalent interactions to monoclonal antibodies and with a free amine in its structure, resulted from the cleavage of the support. To this free amine we can couple FITC or, lately, a drug.

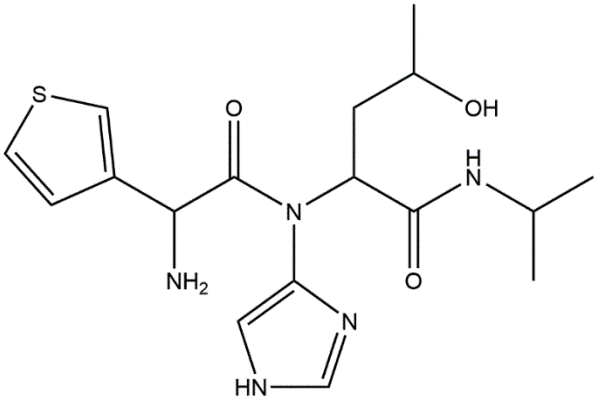


Figure II.4.6: Expected structure of B2Al2A7 Petasis-Ugi ligand.

The resin used for the B2Al2A7 synthesis was the rink amide MBHA resin (Figure II.4.7), this support comprises the modified Rink amide linker, an acid labile linker. The Rink amide resin was designed for chemical synthesis of peptides.

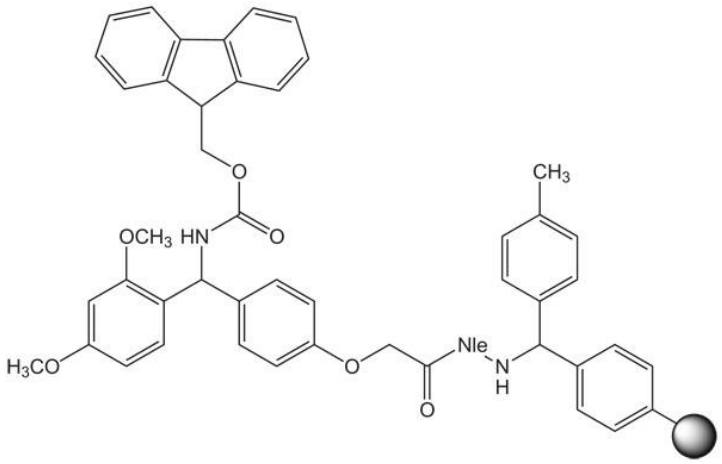


Figure II.4.7: Rink Amide MBHA resin structure.

The protocol followed for the Fmoc deprotection was adapted from the one that is typically used in peptides synthesis. The synthesis of B2Al2A7 itself was adapted from the Petasis-Ugi reaction protocol used in cross-linked agarose.

At end of the reaction, the resin with B2Al2A7 ligand proceeded to the cleavage with 100% of TFA solution. When the cold solution of diethyl ether was added to the solution, where the ligand supposedly was, nothing precipitates. Once this was not a real peptide, the final sample was sent and analysed by NMR in deuterated DMSO, expecting it was present in solution.

The ^{1}H NMR result is in Figure 2 of Appendix 5 and in Figure 3 is the theoretical ^{1}H NMR spectrum of the expected final structure of B2Al2A7 Petasis-Ugi ligand (Figure II.4.6). Analysing the NMR spectrum, it is observed the DMSO characteristic peak around 2.5 ppm and other little peaks are observed, perhaps some impurities from the NMR tube, that were not well washed, or even from the reaction. The ^{1}H NMR spectrum is not at all similar to the theoretical one, confirming the absence of the B2Al2A7 ligand.

The solid-phase synthesis was not monitored, so we are not sure if it occurred as expected, but once the starting point was an amine, like in the cross-linked agarose, the reaction should happen as predicted. Thus, the point where this procedure failed was in the cleavage step. The TFA only acidifies the solution and that is enough when the cleavage point is an amide group (see figure II.4.3 from II.4.3.4 section). However, since this is not a peptide, at the cleavage point of B2Al2A7 was an amine and the TFA was not enough. A good nucleophile must be added and theoretically the cleavage of the B2Al2A7 ligand from the rink amide linker should be succeeded. Some alternative reagents to this cleavage step would be hydrofluoric acid or hydriodic acid, the surround becomes acidic, and fluoride and iodide are both good nucleophile to make the cleavage.

II.4.5. Conclusions and Future Perspectives

Herein the lead Petasis-Ugi ligands were explored for the ADC approach, where the main goal is to apply these ligands in the Antibody Drug Conjugates assembly as the linker component. This element is the one that binds the drug/payload to the monoclonal antibody. Therefore, with these affinity ligands we expect to overcome some difficulties found in the conjugation methods used nowadays, taking advantage of the non-covalent binding, and evolve the cancer therapy field.

From the screening results with the monoclonal antibody, Bevacizumab, both Petasis-Ugi ligands showed a good binding capacity to the mAb, ranging 0.22 to 0.24 mg of protein bound/g of support. Once the B2Al2A7 Petasis-Ugi ligand had more binding capacity and it was the ligand with better stability at different pH conditions, it seems to be the more appropriate affinity ligand to proceed the work.

Nevertheless, a few more tests to B2Al2A7 should be made to make this research more reliable, such as its stability in human plasma for at least 15 days. This test would give an idea of the half life time of the ligand in the presence of other plasma proteins and observe if the affinity of the Petasis-Ugi ligand remains steady, bound to the monoclonal antibody, or if the binding is lost.

Regarding the attempt to synthesize the B2Al2A7 ligand through the solid-phase strategy, the procedure used must be optimized. One alternative would be the use of a good nucleophile at the cleavage step, such as hydrofluoric acid (HF) or hydriodic acid (HI). But, these reagents are really dangerous to handle, and HF is extremely toxic, corrosive and volatile. For these reasons, another linker strategy of solid-phase synthesis should be search, preferably free of not so toxic reagents, suitable to organic synthesis and in the end the ligand shall have a free amine in its structure. Another approach, for the B2Al2A7 Petasis-Ugi ligand synthesis, might be the traditional organic synthesis in liquid state, although more challenging.

V. Concluding Remarks

5. Concluding Remarks

Antibodies are one of the most important and growing sectors in the biopharmaceutical market. In this work, we developed synthetic affinity ligands designed to bind human IgG, based on combinatorial Petasis-Ugi reaction, with the objective to apply it in different fields.

In the first chapter, the best solid support for the synthesis and initial screenings of synthetic ligands was studied, through a comparison between magnetic nanoparticles and the traditional cross-linked agarose. For that, two combinatorial libraries based on the Triazine and Ugi reactions were developed and the reproducibility of results in both supports were evaluated. These libraries were based on previous works, where the ligands were successfully designed to bind antibodies. Despite magnetic nanoparticles are a lower cost procedure, the MNPs support showed lower reproducibility than the crossed-linked agarose. Since the agarose showed more reproducible results, and it is easier to manipulate, it was concluded that the traditional support still is the more suitable support for initial screenings of synthetic affinity ligands.

In the second chapter, a new library of synthetic affinity ligands to bind human IgG was developed. This library was based on Petasis-Ugi reaction, which was adapted to cross-linked agarose as a solid support by Irís Batalha in 2014 [54]. The library was designed based on the interactions between biological ligands, *de novo* synthetic ligands and peptides with antibodies. This library resulted in 72 different affinity ligands, which were screened against pure human IgG. In the end, the two selected lead ligands were B1Al2A2 and B2Al2A7, which showed a good binding capacity. The B1Al2A2 eluted more protein and proceeded to the purification approach. The B2Al2A7 was the most stable under different pH conditions and, therefore, was a promising ligand for the ADC approach. Also, visualising the structure of both ligands, it was concluded that these affinity ligands bind to IgG mostly through hydrophobic interactions and through some electrostatic interactions.

In the third chapter, both lead ligands were explored in different approaches, the Purification approach and the ADC approach.

For the human IgG purification approach, the B1Al2A2 was studied and the binding and elution conditions were optimized, and the binding constants determined. From the screening results with pure IgG and pure BSA, the HEPES buffers with different NaCl concentrations showed better binding capacities. But, in order to reduce unspecific interactions 20 mM HEPES, 500 mM NaCl, pH 7.4 buffer was chosen as the binding buffer, once a higher concentration of salt induces hydrophobic interactions.

Regarding elution optimization, 11 conditions were tested, but only three resulted in protein eluted. Conditions E10 and E11 were the ones that showed promising results, where only IgG was eluted and, therefore, 0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10 (E11) was the elution condition chosen for IgG purification. However, when this condition was tested with human plasma the same was not verified, once the purity obtained for IgG was 41%. Due to these results, improvements are needed and some alternatives should be tested, such as a previous HSA depletion, upgrade the elution buffer, testing at pH 11, or use condition E9 (0.1 M $NaHCO_3$, 30% ethylene glycol, pH=10) to recover HSA and then elute IgG with condition E11 (0.1 M $NaHCO_3$, 1% (w/v) CHAPS, pH=10) or with a more suitable elution buffer.

The results obtained for partition equilibrium studies followed a Langmuir isotherm and were also analysed by Hill plot. The K_a and Q_{max} determined are very promising values and within the same range or superior as the ones found in literature for affinity systems used in the purification field.

In the ADC approach, both lead ligands were explored for the assembling of ADC complexes. The B2Al2A7 was the ligand that showed more binding capacity for the monoclonal antibody, Bevacizumab, and it was the ligand with better stability at different pH conditions. With this it was concluded that B2Al2A7 is a promising affinity ligand to proceed the work in the ADC field. About the solid-phase synthesis strategy that was attempted for B2Al2A7 ligand, the procedure used must be optimized, searching other linker strategies of solid-phase synthesis. As an alternative, traditional organic synthesis of the ligand should be attempted for a full characterization.

VI. Bibliography

6. Bibliography

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VII. Appendix

Appendix 1. Preparation of particles for ligands synthesis

Appendix 1.1. *First silica coating*

Synthetized MNPs were diluted in water to achieve the concentration of 10 mg/mL. Thus, 300 mL of this aqueous solution was placed in an ultrasonic bath for 15 minutes. After it, a solution composed by 3.6 g of sodium silicate in 126.32 mL of 50% (v/v) ethanol/ distilled water was added to the MNPs and reacted for two hours with mechanical stirring in a heated bath (40 °C). In the end, the particles coated with first silica layer (MNP-Si) were washed by magnetic decantation with distilled water (±10 times).

Appendix 1.2. *Second silica coating*

The supernatant was taken from MNP-Si dispersion and 126.32 mL of a solution of 80% (v/v) ethanol/water was added. The suspension was sonicated for 10 min, followed by the addition of 4.47 mL of 5M ammonium hydroxide and 2.37 mL of TEOS while sonicating. The reaction was maintained with mechanical stirring in a heated bath (40 °C) for two hours and then, the particles were washed 10 times with distilled water and ressuspended in 292.1mL of distilled water.

Appendix 1.3. *Dextran coating*

The aqueous solution of particles coated with the second silica layer (MNP-Si-Si), previously prepared, was placed in an ultrasonic bath for 10 minutes, while a polymer solution was prepared by adding 3.34 g of dextran from *Leuconostoc mesenteroides* in 41.73 mL of distilled water. After sonication, the dextran solution was added to the particles, the solution was sonicated for 10 minutes and reacted for two hours at 60°C in an orbital shaker (200rpm). Posteriorly the magnetic nanoparticles with two silica and dextran coatings (MNP-Si-Si-Dex) were washed with distilled water until pH 7 was achieved and ressuspended in 261.43mL 50% (v/v) ethanol/ distilled water.

Appendix 1.4. *Amination of MNPs*

The MNP-Si-Si-Dex dispersion was ultra-sonicated for 5 minutes at 30kHz in an ultrasonic bath, followed by the addition of 29.21 mL APTES (10% v/v). Then, the mixture was incubated for 1 hour at 70 °C in an orbital shaker (200rpm). Lastly, the particles were washed with distilled water by magnetic decantation.

The amine groups of MNPs were quantified by a Kaiser test.

Appendix 2. Synthesis of Triazine libraries in MNPs

The magnetic nanoparticles were washed with cold distilled water (2x volume) and with 50% (v/v) acetone/ distilled water. Then two solutions were prepared: a cold solution of 50% (v/v) acetone/distilled water with 1 molar eq. of sodium bicarbonate ($NaHCO_3$) relative to amine groups and it was added to the nanoparticles until it reached a final concentration of 10 mg/mL, and also a solution of cyanuric chloride (5 eq. molar excess, relative to amine groups) dissolved in acetone (8.6 mL/ g of cyanuric chloride). This solution was also added to the MNPs, followed by 1 hour of incubation in ice (approx. 0 ^{o}C) at 200 rpm.

After reaction, the nanoparticles were washed with acetone (2x volume), 50% (v/v) acetone/distilled water (3x volume) and distilled water (5x volume). The cyanuric-functionalized MNPs were ressuspended in 64 mL of distilled water (concentration approx. 60 mg/ mL), and then distributed by 64 wells of a 96 deep well plate (1 mL/ well). After that, the plate was placed in the magnet to take the supernatant out, carefully, with a pipette.

The amines A1 to A8 were added to each column of the deep well plate (2 molar eq. of each, relative to amine groups; 1 mL/ well). A sealing cover was placed and the plate was incubated for 24 hours at 30 ^{o}C (150 rpm), R_1 substitution. Thereupon, the ligands were washed with the solvent in which each amine was dissolved (5x 1 mL).

Posteriorly, the amines A1 to A8 were added to each row of the deep well plate (5 molar eq. of each, relative to amine groups; 1 mL/ well). The plate was then sealed and incubated for 48 hours at 80 ^{o}C (150 rpm), R_2 substitution. At the end, the ligands were washed with the solvent in which each amine was dissolved (5x 1 mL) and distilled water (5x 1 mL), ressuspended in distilled water (1 mL/ well) and stored at 4 ^{o}C.

All the amines were dissolved in distilled water, with the exception of amines A2 and A6, which were dissolved in 50% (v/v) DMF/distilled water. Moreover, it was added to each amine 1M of sodium bicarbonate (1 molar eq., relative to amine groups).

Appendix 3. Synthesis of Ugi libraries in MNPs

In order to perform the Ugi reaction in magnetic nanoparticles, they had to be functionalized with aldehyde groups. The MNPs were ressuspended in 5% (v/v) of glutaric dialdehyde/ distilled water with 1M NaOH and sonicated for 5 minutes. After the MNPs were incubated for 1 hour at 30 ^{o}C (250 rpm) and then washed with distilled water (5x volume).

The aldehyde-functionalized MNPs were ressuspended in distilled water and distributed for 35 wells of a 96 deep well plate (1.1 mL / well). Then, the supernatant was removed and the amines A1 to A8 (5 molar eq. each relative to amine groups, 1.1 mL/ well) were added to each column of the plate, followed by incubation for 2 hours at 60 ^{o}C (200 rpm) with the plate sealed. Afterwards, the carboxylic acids C1 to C5 (5 molar eq. each relative to amine groups, 0.25 mL/ well) were added to each row of the block along with the isopropyl isocyanide (5 molar eq. relative to amine groups). The cover was placed and incubated for 48 hours at 60 ^{o}C (200 rpm).

In the end of the reaction, the ligands were washed with the solvent in which each compound was dissolved (5x 1 mL) and distilled water (5x 1 mL), ressuspended in distilled water (1 mL/ well) and stored at 4 ^{o}C.

All the amines and carboxylic acids were dissolved in methanol, with the exception of A1, A2, C4 and C5, which were dissolved in 50% (v/v) DMF/ methanol. The A3 had to be neutralized with 1M NaOH (5 molar eq.).

Appendix 4. Synthesis of Ugi libraries in agarose

The aldehyde-functionalized agarose was washed with methanol, from 0% (v/v) methanol/ distilled water to 100% (v/v) methanol (in increments of 20%). Then, the agarose was ressuspended in methanol (1 mL/ g moist agarose) and distributed in 35 wells of a 96 deep well plate (0.25 g/ well), with a pipette tip that was cut 4 mm.

The supernatant was removed and the amines A1 to A8 (5 molar eq. each relative to epoxy groups, 0.5 mL/ well) were added to each column of the plate, followed by incubation for 2 hours at 60 ^{o}C (200 rpm) with the plate sealed, in order to form the imine compound that is required in the Ugi reaction. Afterwards, the carboxylic acids C1 to C5 (5 molar eq. each relative to epoxy groups, 0.5 mL/ well) were added to each row of the block (Figure 1) along with the isopropyl isocyanide (5 molar eq. relative to epoxy groups). The cover was placed and incubated for 48 hours at 60 ^{o}C (200 rpm).

In the end of the reaction, the ligands were washed with methanol, from 100% (v/v) methanol to 0% methanol/ distilled water (in decrements of 20%) and water (10x resin volume). The ligands were ressuspended in water and stored at 4 ^{o}C.

All the amines and carboxylic acids were dissolved in methanol, with the exception of A1, A2, C4 and C5, which were dissolved in 50% (v/v) DMF/ methanol. The A3 had to be neutralized with 1M NaOH (5 molar eq.).

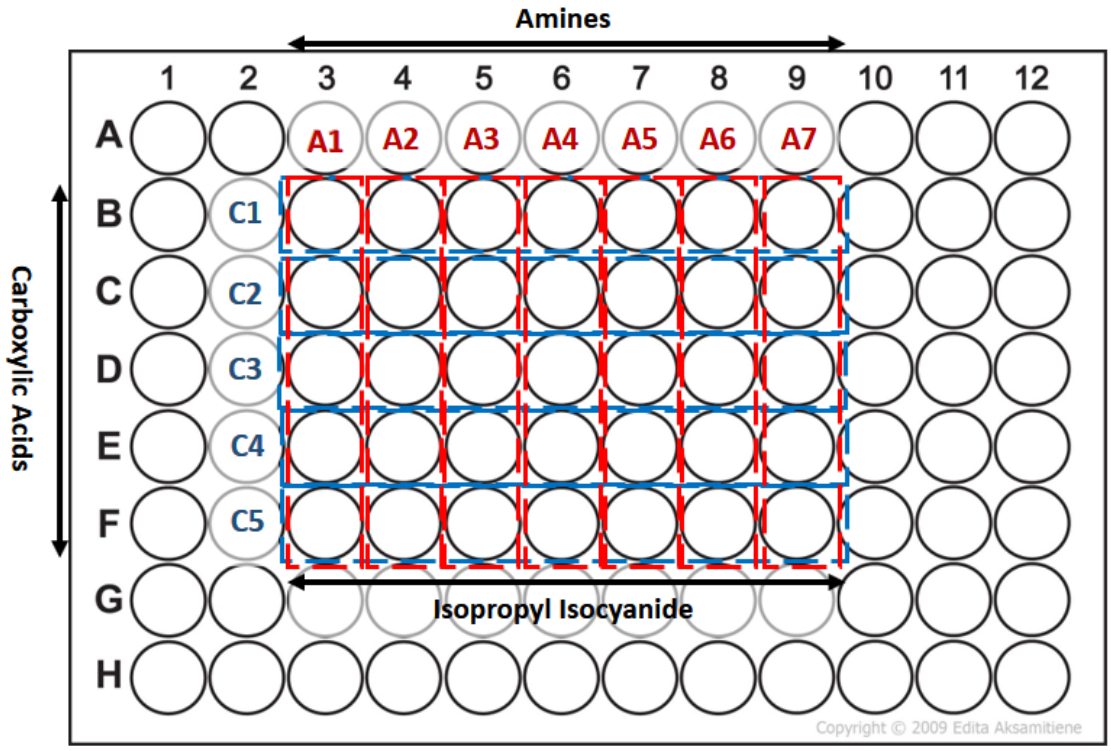


Figure 1: Illustrative image of the 96 deep well plate used for the synthesis of the Ugi ligands.

Appendix 5. ^{1}H NMR Result

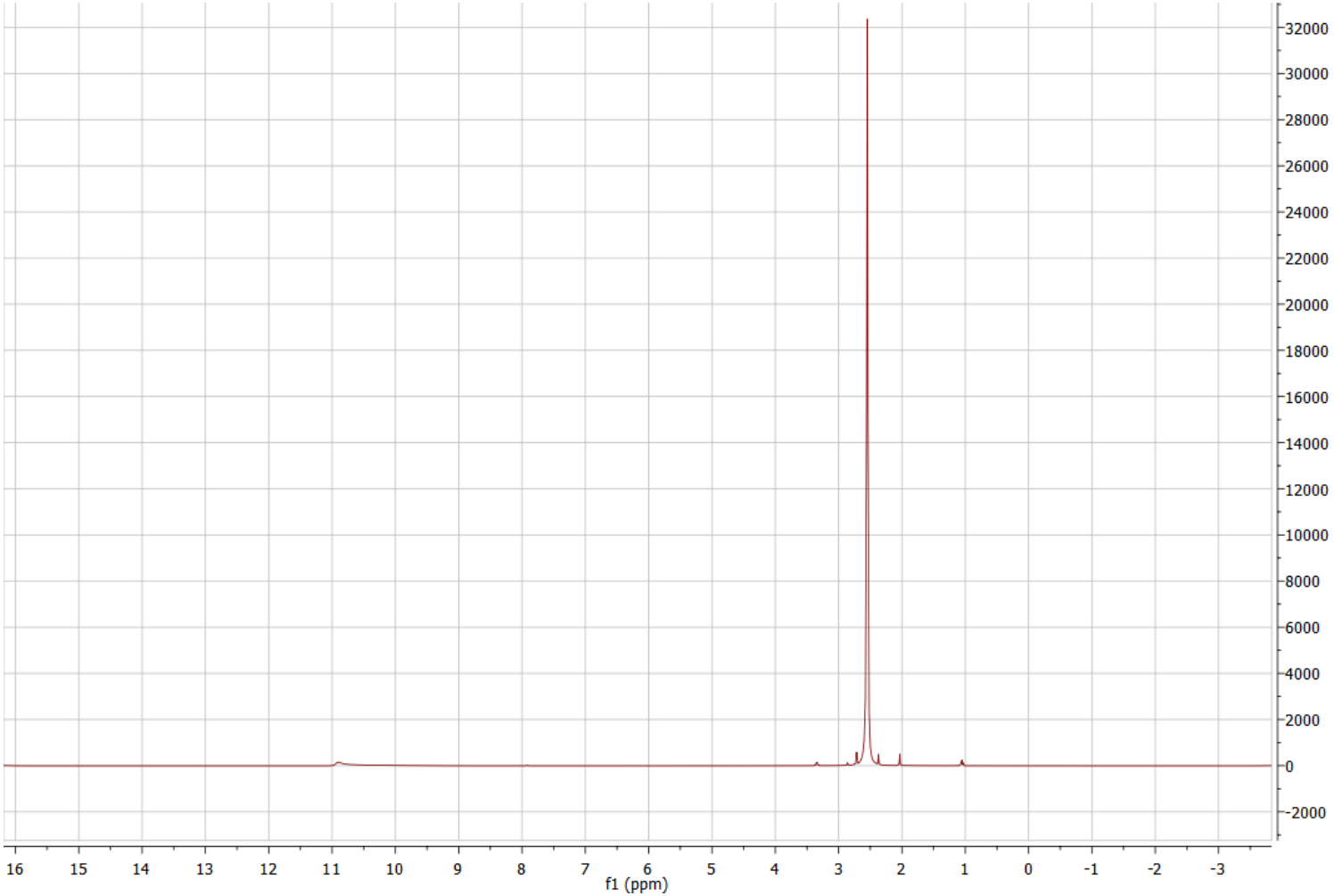
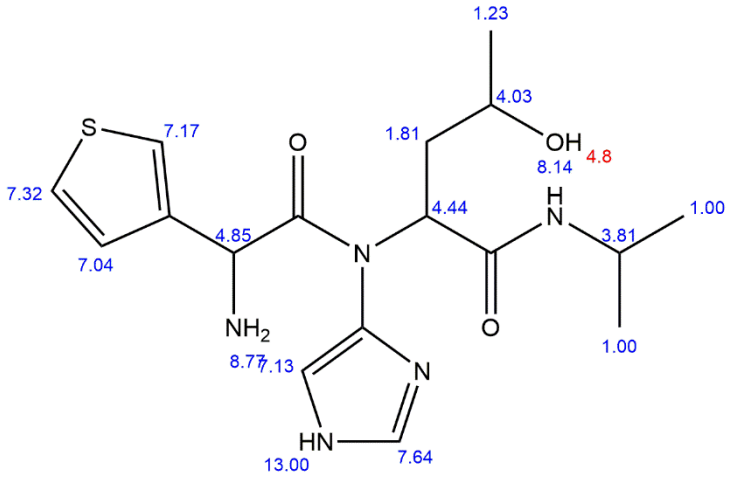


Figure 2: ^{1}H NMR spectrum from the solid-phase synthesis of B2Al2A7 in the Rink Amide MBHA resin in deuterated DMSO.



Estimation quality is indicated by color: good, medium, rough

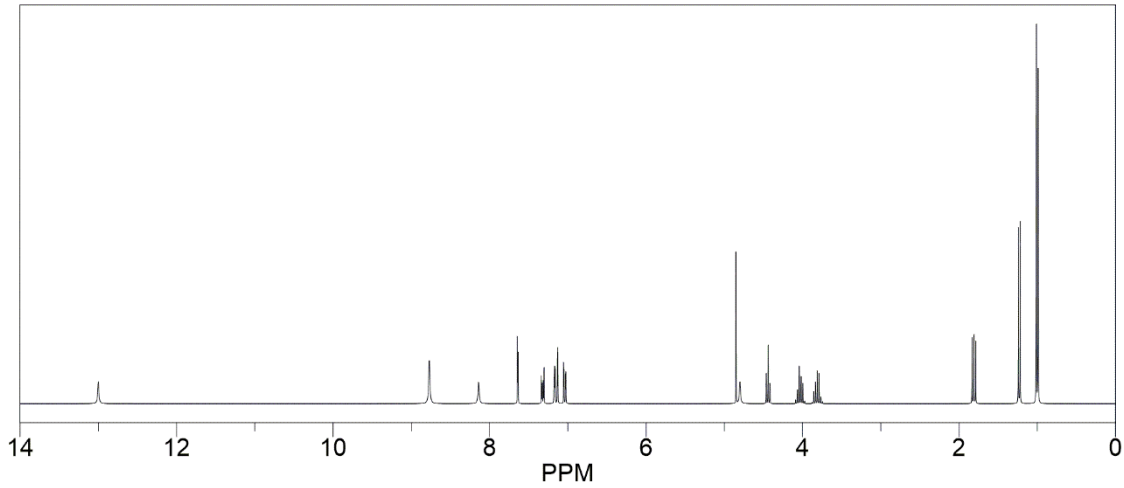


Figure 3: ^{1}H NMR estimation spectrum from the B2Al2A7 Petasis-Ugi ligand expected structure.