

Jorge Alexandre Pereira Mota

**Development and optimization of a methodology to
synthetize chitosan/cetuximab conjugates for
encapsulate siRNA and targeting to EGFR
overexpressing cells**

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Development and optimization of a methodology to synthesize chitosan/cetuximab conjugates for encapsulate siRNA and targeting to EGFR overexpressing cells

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group



Author: Jorge Alexandre Pereira Mota

Supervisor: Vitor Seabra, PhD

Co-supervisor: Bruno Sarmiento, PhD

“All cancers are alike but they are alike in a unique way.”

— Siddhartha Mukherjee, *The Emperor of All Maladies*

“The standard treatments for cancer are not meant to heal, but to destroy.”

— Andreas Moritz, *Cancer Is Not a Disease - It's a Survival Mechanism*

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Abstract

The therapeutic use of small interfering RNA (siRNA) has been seen as a new possible potential approach to treat cancer. The key challenge in the clinical field is to deliver the siRNA to the cytosol of target cells within the affected tissues. Therefore, various siRNA delivery systems, such as liposomes, polymers, and inorganic particles, have been developed to improve the therapeutic efficacy of siRNA.

The main goal of this project was to develop new protocols, as well as to optimize already known ones that are used to produce/synthesize chitosan/cetuximab conjugates, so later these could be used to encapsulate siRNA and targeting to EGFR overexpressing cells. Several challenges were presented over this process, thus many were the attempts to produce a viable and in reasonable concentration conjugate, in different molar proportions of cetuximab to chitosan. Not only timings were the concern, but also and mainly, compounds concentrations, and the addition or removals of different steps of the process were taken in account. In the end a new protocol was produced.

After the development and optimization of a new conjugation protocol, the second line of orders was to analyze the conjugates through Fourier transform infrared spectroscopy (FTIR) in order to attest the presence of a true new conjugate in the final product. Protein quantification was, as well, determined in pre and post filtered products, since that would also tell us, by exclusion, that if the pre-ultrafiltered products had some protein that would be conjugated to Low Molecular Weight Chitosan (LMWChi) since that as if it was free it would have pass the filter membrane (150K MWCO membrane).

Finally the last order of works for this project was to test the produced conjugates in cells recurring to 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays, in order to evaluate their effects regarding its cytotoxicity.

A couple of the successful produced conjugates might be used in the future to encapsulate siRNA, produce nanoparticles and improve the therapeutic efficacy of siRNA as a target therapy for non-small cell lung cancer (NSCLC). Still concerning pharmaceutical technology significant efforts have to be done regarding this conjugate in specific, and its siRNA encapsulation efficiency.

Resumo

O uso terapêutico de *Small Interfering RNA* (siRNA) tem sido observado como uma potencial nova abordagem para o tratamento do cancro. O desafio-chave no campo clínico consiste na entrega de siRNA no citosol das células-alvo. Assim, vários sistemas de entrega de siRNA, tais como lipossomas, polímeros e partículas inorgânicas, têm sido desenvolvidos de forma a melhorar a eficácia terapêutica do siRNA.

O nosso principal objectivo neste projeto, que deu origem a esta dissertação, foi desenvolver novos protocolos, bem como otimizar outros já existentes, para o uso na produção/síntese de conjugados de quitosano/cetuximab, de forma a utilizar estes últimos para encapsular siRNA e direccioná-los para as células que sobreexpressam EGFR.

Diversos desafios foram apresentados ao longo deste processo, assim, várias foram as tentativas de produzir um conjugado viável e em concentração razoável, em diferentes proporções molares de cetuximab para quitosano. Não foram apenas os tempos de reacção uma preocupação, mas também e principalmente as concentrações dos compostos e a adição ou remoção de diferentes passos do processo foram tidos em conta. No final, um novo protocolo foi proposto.

Após o desenvolvimento e optimização do novo protocolo de conjugação, a segunda linha de objectivos consistiu na análise de conjugados através de FTIR de forma a confirmar a presença de um verdadeiro novo conjugado no produto final.

A quantificação proteica foi, de igual forma, determinada em pré- e pós- filtração dos produtos, uma vez que nos iria dizer, por exclusão, que se os produtos pré-ultrafiltrados possuísem algum material proteico que pudesse ser, estaria conjugado com o LMWChi, uma vez que se estivesse livre teria passado a membrana de filtração (membrana de 150000 MW)

Finalmente, a última linha de trabalhos deste projeto consistiu no teste em células dos conjugados produzidos, recorrendo aos ensaios com MTT e LDH com o objetivo de avaliar os seus efeitos citotóxicos.

Um par dos conjugados produzidos com sucesso poderão ser utilizados no futuro para encapsular siRNA, produzir nanopartículas e melhorar a eficácia terapêutica do siRNA como terapia direccionada contra o cancro do pulmão das células não

pequenas, ainda assim considerando que muito trabalho haverá a fazer ao nível da eficiência de encapsulação do conjugado.

List of abbreviations

aas	–	aminoacids
Abs	–	absorbance
Chi	–	chitosan
CTX	–	cetuximab
D-MEM	–	Dulbecco's Modified Eagle's Medium
DLS	–	dynamic light scattering
DMSO	–	dimethyl sulfoxide
EDC	–	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
FBS	–	fetal bovine serum
FTIR	–	Fourier transform infrared spectroscopy
HRP	–	horseradish peroxidase (conjugates)
i.e.	–	in example
IEP	–	isoelectric point
LDH	–	lactate dehydrogenase
LMWChi	–	low molecular weight chitosan
MTT	–	3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide
MW	–	molecular weight
NHS	–	N-hydroxysuccinimide
NSCLC	–	non-small cell lung cancer
µg	–	microgram
µL	–	microliters
µM	–	micromolar
mA	–	miliampere
mL	–	millilitres
NCI	–	national cancer institute
nm	–	nanometers
ns	–	non significant
Pdi	–	polydispersity index
RNA	–	ribonucleic acid
rpm	–	rotations per minute
RT	–	room temperature

SD	–	standard deviation
siRNA	–	small interfering RNA
t	–	time
UV	–	ultraviolet

Dissertation organization

Part I – *Introduction*

Part II – *Aims*

Part III – *Materials and methods*

Part IV – *Results and discussion*

Part V – *Conclusions*

Part VI – *Future perspectives*

Part VII – *References*

Part I
Introduction

Introduction

1. Epidemiology of lung cancer

Cancer is a leading cause of morbidity and mortality worldwide (Arnold et al., 2013, WHO, 2013), accounting for 12.7 million new cases and 7.6 million deaths (13% of all deaths) in 2008 (WHO, 2013, IARC, 2013). The latest world cancer statistics provided by the International Agency for Research on Cancer revealed that the number of new cancer cases has risen to 14.1 million in 2012, whereas cancer deaths increased to 8.2 million (IARC, 2013). GLOBOCAN 2012 estimates this trend to increase up to 19.3 million new cancer cases per year by 2025 (IARC, 2013), and cancer deaths are projected to continue rising, with 13.1 million deaths estimated in 2030 (WHO, 2013). In addition, more than a half of all cancers (56.9%) (GLOBOCAN, 2013c) and cancer deaths (64.9%) (GLOBOCAN, 2013j) occurred in less developed countries; these numbers are projected to increase by 2025 (IARC, 2013). Lung, breast, colorectal, liver and stomach cancers are the main responsible for cancer deaths each year (WHO, 2013).

The development of a tumour cell is the result of the interaction between many factors (WHO, 2013). Besides genetic predisposition, population ageing and the increase in life expectancy, environmental factors, behavioural and dietary habits such as obesity, low fruit and vegetable intake, lack of physical activity, viral infections, alcohol and tobacco use, urban air pollution and indoor smoke from household use of solid fuels, constitute some of the leading risk factors for carcinogenesis (WHO, 2013, Jemal et al., 2011). Smoking, dietary habits and reproductive factors have been identified as the main modifiable risk factors for cancer in industrialised populations (Arnold et al., 2013). Modifying or avoiding key risk factors could prevent more than 30% of cancer deaths (WHO, 2013).

In his regard, tobacco deserves special attention, since its use is the most important risk factor for cancer, being responsible for 22% of global cancer deaths and 71% of global lung cancer deaths (WHO, 2013).

Concerning lung cancer, it led the list of the most commonly diagnosed cancers worldwide in 2012, accounting for 1.8 million cases (13.0% of the total), followed by breast (1.7 million, 11.9%) and colorectal cancers (1.4 million, 9.7%) (IARC, 2013).

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Lung cancer was also the first cause of cancer deaths, raising from 1.37 million deaths in 2008 (WHO, 2013) to 1.6 million (19.4% of the total) in 2012, preceding liver (0.8 million, 9.1%) and stomach cancers (0.7 million, 8.8%) (IARC, 2013).

Following the global tendency of all cancers, the number of lung cancer cases was more pronounced in less developed regions (58.4%) than in the more developed ones (41.6%) (GLOBOCAN, 2013o). Likewise, the number of lung cancer-related deaths was also higher in less developed regions (60.6%) than in the more developed ones (GLOBOCAN, 2013p).

Lung cancer has been the most common cancer in the world for several decades, and remains as the most common cancer in men worldwide, with higher incidence rates (the double across all the income groups, and four times higher in upper middle income countries) than those of women (GLOBOCAN, 2013q). It is also the most common cause of cancer death worldwide (responsible for nearly one in five cancer deaths) (GLOBOCAN, 2013q). Furthermore, because of its high mortality rate (the overall ratio of mortality to incidence is 0.87) and the relative lack of variability in survival in different world regions, the geographical patterns in mortality closely follow those of incidence (GLOBOCAN, 2013q).

In Europe, considering both genders, there were more than 3.4 million new cancer cases in 2012 (GLOBOCAN, 2013a). Lung cancer was the 4th cancer with higher incidence, with 410220 new cases (11.9% of the total), preceding breast (464202, 13.5%), colorectal (447136, 13.0%) and prostate cancers (417137, 12.1%) (GLOBOCAN, 2013a). Lung cancer ranked 2nd on the most incident cancers (290904, 15.9% of the total) among men, after prostate cancer (417137, 22.8%) (GLOBOCAN, 2013d), and 3rd on women (119316, 7.4%), after breast (464202, 28.8%) and colorectal cancer (205323, 12.7%) (GLOBOCAN, 2013f).

During the same period, lung cancer has accounted for 353723 cancer deaths (20.1% of the total), preceding colorectal (214814, 12.2%) and breast cancers (131257, 7.5%) (considering both genders) (GLOBOCAN, 2013h). Between men, lung cancer ranked 1st, being responsible for 254610 deaths (26.1% of the total), preceding colorectal (113238, 11.6%) and prostate cancers (92318, 9.5%) (GLOBOCAN, 2013k). Regarding women, lung cancer ranked 3rd on the highest mortality rates (99113, 12.7%), after breast (131257, 16.8%) and colorectal cancers (101576, 13.0%) (GLOBOCAN, 2013m).

In Portugal, there were 49174 new cancer cases in 2012. Considering both genders, lung cancer accounted for 4192 new cases (8.5% of the total), ranking 4th, after colorectal (7129, 14.5%), prostate (6622, 13.5%) and breast cancers (6088, 12.4%) (GLOBOCAN, 2013b). Lung cancer ranked 3rd (3215, 11.3%) on new cases amongst men, after prostate (6622, 23.3%) and colorectal cancers (4209, 14.8%) (GLOBOCAN, 2013e). On women, lung cancer ranked 5th (977, 4.7%), after breast (6088, 29.4%), colorectal (2920, 14.1%), corpus uteri (1485, 7.2%), and stomach cancers (1184, 5.7%) (GLOBOCAN, 2013g).

Considering all the 24112 cancer deaths occurred on both genders, lung cancer ranked 2nd, with 3441 deaths (14.3% of the total), between colorectal (3797, 15.7%) and stomach cancers (2285, 9.5%) (GLOBOCAN, 2013i). Considering men, lung cancer was the first cancer death cause, accounting for 2638 deaths (18.4% of the total), preceding colorectal (2240, 15.7%) and prostate cancers (1582, 11.1%) (GLOBOCAN, 2013l). In women, lung cancer deaths ranked 4th (803, 8.2%), after breast (1570, 16.0%), colorectal (1557, 15.9%) and stomach cancers (GLOBOCAN, 2013n).

All these data highlight the high incidence and mortality rates of lung cancer worldwide. This substantiates the urgent need to search for new treatment approaches and for the improvement of the existing ones in order to overcome their inherent resistance and side effect problems, as well as to enhance their effectiveness and specificity for lung cancer.

The continuous learning about cancer biology, lung cancer histology and its molecular features, as well as the evolution of pharmaceutical technology and the emergence of new targeting formulations, are extremely important to overtake this disease.

2. Lung cancer types

Cancer treatment is a very complex theme that involves multiple considerations. Knowing the histological type/subtype of the cancer, aside of the stage of the disease, is essential to select the adequate treatment approach.

Introduction

In this section, some of the main lung cancer histological types will be addressed, as well as some relevant molecular features of NSCLC (which constitutes the type of cancer that is constitutes the putative target of this work).

2.1. Lung cancer histological types

Among lung cancer cases, two situations must be distinguished: those that started in the lung (primary lung cancer), or those that have spread into the lung (secondary lung cancer) (CRU, 2013). This distinction is important since it may be determinant for the right treatment choice (CRU, 2013).

Primary lung cancer can be categorized in three main groups: small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC) (CRU, 2013, ACS, 2014b) and lung carcinoid tumour (ACS, 2014b). There are many other types of lung tumours, such as adenoid cystic carcinomas, hamartomas, lymphomas and sarcomas, which are rather uncommon (ACS, 2014f), for which they will not be addressed in this work.

SCLC accounts for 10-15 out of every 100 diagnosed lung cancers (ACS, 2014b), and is usually caused by smoking (CRU, 2013). Its name derives from the cells' small size, when observed under the microscope, and often spreads quite early (CRU, 2013). It frequently starts in the bronchi, near the centre of the chest, and tends to grow and spread quickly to other parts of the body earlier than NSCLC (LungCancer.org, 2014c, ACS, 2014f). Since it almost always spreads to distant parts of the body before it is found (ACS, 2014f), chemotherapy may be the first treatment approach, rather than surgery (CRU, 2013). However, it is more responsive to chemotherapy than NSCLC (LungCancer.org, 2014c).

Considering NSCLC, which is the most common lung cancer (about 85 out of 100 lung cancers (ACS, 2014b)), there are three common types grouped together because of their behavioural similarities and different treatment responses from the one of SCLC (CRU, 2013): squamous cell carcinoma (25-30% of lung cancers), adenocarcinoma (40% of lung cancers), and large cell carcinoma (10-15% of lung cancers) (NCI, 2013, ACS, 2014e). The cells on these subtypes differ in size, shape, and chemical make-up when observed under a microscope, but are grouped together because treatment and prognosis approaches are often very similar (ACS, 2014e).

Squamous cell cancer (also called epidermoid carcinoma (NCI, 2014a)) is usually linked more strongly with smoking than other forms of NSCLC (CRU, 2013, NCI, 2013). It develops from the cells that line the airways, most of them are located centrally, in the larger bronchi (CRU, 2013, NCI, 2013). Its incidence has been decreasing in recent years (NCI, 2013).

Adenocarcinoma also develops from the cells that line the airways, but differs from the previous type since it develops from a particular type of cells that produces mucus (phlegm) and is often found in the outer areas of the lungs (CRU, 2013). This is now the most common histologic subtype in many countries, and sub classification is important (NCI, 2013). One of the biggest problems with lung adenocarcinomas is the frequent histologic heterogeneity (NCI, 2013). In fact, mixtures of adenocarcinoma histologic subtypes are more common than tumours consisting purely of a single pattern of acinar, papillary, bronchioalveolar, and solid adenocarcinoma with mucin formation (NCI, 2013).

Large cell carcinoma, is another type of NSCLC. Its name derives from the large and round shape presented by its cells under the microscope (CRU, 2013). It can appear in any part of the lung and tends to grow and spread quickly, which can make it harder to treat (ACS, 2014e).

There are numerous additional subtypes of NSCLC, such as adenosquamous carcinoma and sarcomatoid carcinoma, but they are much less common (ACS, 2014e, NCI, 2013).

Lung carcinoid tumours (also called lung neuroendocrine tumours) represent less than 5% of lung cancers (ACS, 2014b), and are uncommon tumours that tend to grow slower than other types of lung cancers (ACS, 2013b), rarely spreading (ACS, 2014b).

Secondary lung cancers refer to those that have spread from somewhere else in the organism, and cover quite a few different types of cancer, including breast and bowel cancers (CRU, 2013). In these cases, the choice of treatment depends on where the cancer started, since the type of cancer cells corresponds to those that initially metastasized (CRU, 2013). Therefore, the treatment chosen should target them, and not the cells of the tissue they have migrated towards (CRU, 2013).

The process used to find out if cancer has spread within the lungs, to the lymph nodes or to other organs is called staging (NCI, 2014b, LungCancer.org, 2014c). The information gathered from the staging process determines the stage of the disease

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and this is important to know in order to find the treatment approach that best fits the patient (NCI, 2014b).

Since lungs are large, tumours can grow within them for a long period before they are found, even when symptoms (that are often neglected, such as cough) occur, turning early stage lung cancer (stages I and II) difficult to detect (LungCancer.org, 2014c). Most patients with lung cancer are diagnosed at later stages (III and IV) (LungCancer.org, 2014c).

NSCLC may go through four main stages (LungCancer.org, 2014c). In stage I, the cancer is located only in the lungs and has not spread to any lymph nodes (LungCancer.org, 2014c). Stage II occurs when the cancer is in the lung and nearby the lymph nodes (LungCancer.org, 2014c). When the cancer cells are found in the lung and in the lymph nodes in the middle of the chest, stage III has already progressed and has two subtypes: stage IIIa is referred to when the cancer has spread only to lymph nodes on the same side of the chest where the cancer started; and stage IIIb when the cancer has spread to the lymph nodes on the opposite side of the chest, or above the collar bone (LungCancer.org, 2014c). The most advanced stage of lung cancer, stage IV, occurs when the cancer has spread to both lungs, to the fluid in the area around the lungs, or to another part of the organism (LungCancer.org, 2014c).

As far as SCLC is concerned, it has two main stages: the limited stage, when the cancer is found on one side of the chest, involving just one part of the lung and nearby lymph nodes; and the extensive stage, when the cancer is spread to other regions of the chest or other parts of the organism (LungCancer.org, 2014c).

2.2. Molecular features of non-small cell lung cancer

Recent developments in the field of molecular biology have allowed the identification of cancer 'subtypes' according to the molecular profile they present (West et al., 2012, Alamgeer et al., 2013). Specific mutations were found to trigger oncogenic pathways, shedding light into drug sensitivity mechanisms and primary or acquired resistance phenomena (Alamgeer et al., 2013). Their targeting appears as a means to prevent cancer development, setting the ground for personalized

approaches that improve the outcomes for specific subsets of patients with NSCLC, including those with metastatic disease (Alamgeer et al., 2013).

The epidermal growth factor receptor (EGFR) gene has been shown to harbour different activating mutations that cause its overexpression or over-activity (Alamgeer et al., 2013). In particular, this transmembrane receptor is highly expressed in 40-80% of NSCLC patients, with this overexpression being associated

with a poor prognosis (Molina et al., 2008, Pallis, 2012). Upon binding to different growth factors, EGFR activates, through its tyrosine kinase activity, a subset of proteins which, in turn, activate downstream signal transduction cascades, including mitogen-activated protein kinases (MAPK), protein kinase B (Akt) and c-Jun N-terminal kinases (JNK) pathways, which culminate in cell proliferation,

motility, survival and

other cell phenotypes associated with cancer progression (**Fig. 1**) (Pallis, 2012). Monoclonal antibodies such as cetuximab (which will be later addressed) compete with other ligands to bind the extracellular portion of EGFR, thus preventing

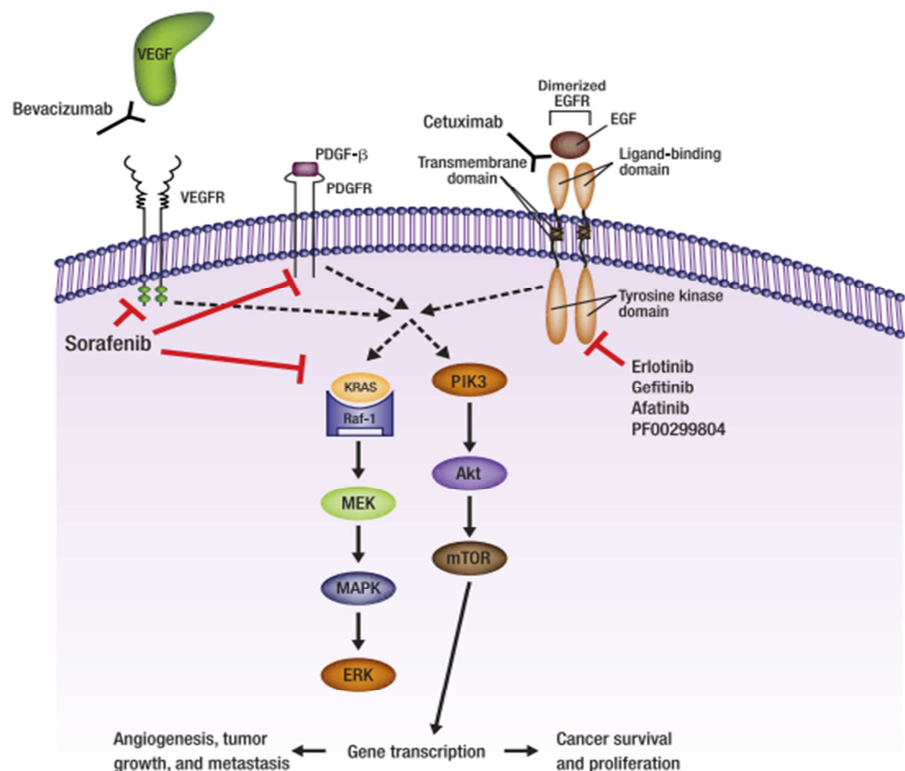


Fig. 1 - Schematic representation of the signalling pathways that are targeted in NSCLC personalized treatment approaches. VEGF inhibitors and EGFR TKIs are depicted. Bevacizumab, a monoclonal antibody, binds VEGF and prevents it from binding VEGFR, thus disrupting angiogenesis, tumor growth and metastasis. Sorafenib, in turn, disrupts angiogenesis by inhibiting multiple receptors, including VEGFR, PDGFR-β, and RAF-1. Erlotinib and gefitinib reversibly bind the tyrosine kinase domain of EGFR, while cetuximab reversibly binds its extracellular portion. Afatinib and PF00299804 irreversibly inhibit EGFR intracellular portion to halt tumor proliferation and survival.

Figure adapted from (Moran, 2011)

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downstream signalling pathways and cell proliferation deriving from uncontrolled EGFR activity (Pallis, 2012).

EGFR mutations represent 17% of the mutations in lung adenocarcinoma (Alamgeer et al., 2013). A deletion at exon 19 and a point mutation in exon 21 (L858R) account for more than 85% of all *EGFR* mutations in NSCLC, being the most common activating mutations and predicting higher response rates to anti-EGFR therapy (Aisner and Marshall, 2012, Sakashita et al., 2014, Alamgeer et al., 2013, Moran, 2011). Gefitinib and erlotinib are two EGFR tyrosine kinase inhibitors (TKIs) currently in use for the treatment of locally advanced or metastatic NSCLC presenting EGFR mutations. By targeting EGFR intracellular domain, they block downstream signalling pathways that regulate proliferation and apoptosis (Sakashita et al., 2014, Alamgeer et al., 2013, Molina et al., 2008, Pallis, 2012). When compared with standard chemotherapy, EGFR TKIs provide less toxicity and an improvement in cancer-related symptoms (Alamgeer et al., 2013). Also, other agents are being developed in order to overcome acquired resistance to EGFR TKIs (Alamgeer et al., 2013). That is the case of afatinib, an orally available irreversible inhibitor of EGFR and human epidermal growth factor receptor 2 (HER2) kinases, dacomitinib, another irreversible inhibitor of EGFR, HER2 and HER4 (Alamgeer et al., 2013), and PF00299804 (Moran, 2011) (Erro! Auto-referência de marcador inválida.)

In turn, bevacizumab is a monoclonal antibody that targets vascular endothelial growth factor (VEGF), which is essential for angiogenesis, a prerequisite for the development of solid tumours and the growth of secondary metastatic lesions (Pallis, 2012, Pillai and Owonikoko, 2014, Molina et al., 2008). Similarly, sorafenib is used as a multitargeted antiangiogenic agent, binding VEGF receptor (VEGFR) and platelet-derived growth factor receptor β (PDGFR- β), for instance (Moran, 2011) (Erro! Auto-referência de marcador inválida.).

Another relevant gene in the context of lung cancer treatment is the anaplastic lymphoma kinase (ALK) gene, which shows rearrangements in about 5-7% of lung adenocarcinomas (Aisner and Marshall, 2012, Alamgeer et al., 2013, Berge and Doebele, 2014). A fusion gene comprising *ALK* and echinoderm microtubule associated protein-like 4 (*EML4*), *EML4-ALK*, is responsible for *ALK* constitutive activation (Alamgeer et al., 2013, Moran, 2011). Crizotinib is an ALK TKI that causes cell cycle arrest in the G1-S phase and induces apoptosis, also presenting activity against tumours with *c-MET* amplification and *c-ROS* kinase mutations (Alamgeer et

al., 2013, Berge and Doebele, 2014, Ulivi et al., 2013). It is expected to be the standard of care for ALK positive NSCLC patients in a near future (Alamgeer et al., 2013, Berge and Doebele, 2014).

Activating mutations in Kirsten rat sarcoma viral oncogene homologue (KRAS) gene account for 30% of all mutations in lung adenocarcinoma and are mutually exclusive with EGFR or ALK mutations (Aisner and Marshall, 2012, Alamgeer et al., 2013, Moran, 2011). They predict resistance to EGFR TKIs and are indicative of a poor prognosis, regardless of the type of therapy (Alamgeer et al., 2013). Unlike *ALK* gene fusions and *EGFR* mutations, *KRAS* mutations are less frequent in never-smokers than in current/light smokers (Berge and Doebele, 2014). Although *KRAS* mutations are frequent in NSCLC, several attempts to develop an efficient inhibitor have been unsuccessful. Nonetheless, the development of inhibitor molecules for downstream targets of *KRAS*, such as mitogen-activated protein kinase kinase (MET), proto-oncogene c-RAF (RAF), extracellular-signal-regulated kinase (ERK) and the phosphatidylinositol 3-kinase (PI3K)/ mammalian target of rapamycin (mTOR) pathway is being pursued (Ulivi et al., 2013, Alamgeer et al., 2013, Berge and Doebele, 2014).

The list of other potential targets with therapies under development is extensive, including: c-ROS oncogene 1, receptor tyrosine kinase (ROS1); human epithelial receptor 2 (HER2); protein kinase B (Akt1); MAPK kinase 1 (MAP2K1 or MEK1); c-MET (encoding for hepatocyte growth factor receptor, HGFR); rearranged in transfection (RET); V-raf murine sarcoma viral oncogene homologue B1 (BRAF); insulin-like growth factor receptor 1 (IGFR1); fibroblast growth factor receptor (FGFR1); discoidin domain receptor 2 (DDR2); neurotrophic tyrosine kinase, receptor, type 1 (NTRK1); phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic protein alpha (PIK3CA); mammalian target of rapamycin (mTOR) (Sakashita et al., 2014, Alamgeer et al., 2013, Berge and Doebele, 2014, Aisner and Marshall, 2012).

In parallel with the approaches discussed so far, a considerable effort is being made in order to identify new predictive biomarkers and molecular targets that characterize lung cancer subtypes, thereby optimizing therapeutics and overcoming acquired resistance issues.

3. Current lung cancer treatments

The treatment approach for lung cancer may be different according to the stage and type/subtype.

In this section, some of the main treatments currently in use for the two most common lung cancers (NSCLC and SCLC), both classic approaches and targeted treatments, will be reviewed.

3.1. Classical approaches for lung cancer

NSCLC treatment may consist of different approaches applied alone or in combination, such as: surgery, radiation, chemotherapy, and targeted treatments (LungCancer.org, 2014b).

Most of the stage I and stage II non-small cell lung cancers are treated with surgery to remove the tumour, which consists of removing the lobe or a section of the lung containing the tumour (LungCancer.org, 2014b). Before surgery, chemotherapy may be applied with the purpose of shrinking the tumour, sometimes along with radiation therapy, which is also known as neoadjuvant therapy (ACS, 2014a).

When non-small cell lung tumours can be surgically removed, chemotherapy after surgery (known as adjuvant chemotherapy) (ACS, 2014a) may help to prevent the cancer from returning, which is particularly true for patients with stage II and IIIA disease (LungCancer.org, 2014b).

In stage III lung cancer cases that cannot be surgically removed, chemotherapy in combination with definitive (high-dose) radiation treatments is recommended (LungCancer.org, 2014b).

For stage IV lung cancer cases, chemotherapy is typically the main treatment, while radiation therapy may be used for palliative purposes (LungCancer.org, 2014b).

Chemotherapy may also be the main treatment for people who are not healthy enough for surgery (ACS, 2014a).

Usually, the treatment for non-small cell lung cancer comprises the combination of two drugs, with the exception of those patients who might not tolerate combination chemotherapy (because of a poor overall health or because they are elderly), to whom a single-drug therapy is applied (ACS, 2014a).

The chemotherapy treatment scheme often consists of a combination of drugs, with the most commonly used being the following: cisplatin (Platinol[®]), carboplatin (Paraplatin[®]), docetaxel (Taxotere[®]), gemcitabine (Gemzar[®]), paclitaxel (Taxol[®]), vinorelbine (Navelbine[®]), pemetrexed (Alimta[®]) (LungCancer.org, 2014b, ACS, 2014a), albumin-bound paclitaxel (nab-paclitaxel; Abraxane[®]), irinotecan (Campto[®]), etoposide (Vepesid[®]), and vinblastine (Velbe[®]) (ACS, 2014a).

Combination chemotherapy often includes either cisplatin or carboplatin plus one other drug (ACS, 2014a). Instead, combinations such as gemcitabine with vinorelbine or paclitaxel may be used (ACS, 2014a).

For advanced lung cancer cases that meet certain criteria, targeted therapies (which will later be addressed) such as cetuximab or bevacizumab may be added to the treatment (ACS, 2014a).

When the initial chemotherapy for advanced lung cancer stops working, a second-line treatment with a single drug such as docetaxel or pemetrexed may be used (ACS, 2014a).

Both the switch to a different drug, prior to cancer progress, and the maintenance of one of the drugs initially used for a longer period have been tested, having shown to have different degrees of success with selected sets of patients (LungCancer.org, 2014b).

As far as small cell lung cancer is concerned, chemotherapy is the main treatment (regardless of the stage) (LungCancer.org, 2014a), given as a combination of two drugs at first (ACS, 2014c). Depending on the stage, radiation treatment also may be used (LungCancer.org, 2014a).

The combinations of drugs that are commonly used are cisplatin and etoposide, carboplatin and etoposide, cisplatin and irinotecan, carboplatin and irinotecan (ACS, 2014c). Other chemotherapeutic drugs may be used if the cancer progresses during treatment or returns after treatment is finished (ACS, 2014c).

Limited-stage small cell lung cancer is usually treated with a combination of a chemotherapy regimen (consisting of etoposide plus cisplatin) plus radiation therapy given at the same time (LungCancer.org, 2014a, NCI, 2014d).

Extensive-stage small cell lung cancer is usually treated with the same chemotherapy regimen alone; however, other regimens comprising carboplatin plus irinotecan (LungCancer.org, 2014a), carboplatin plus etoposide, and cisplatin plus irinotecan (NCI, 2014c) may be used.

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Radiation therapy of the brain may be used before or after chemotherapy for some people whose cancer has spread to this tissue (LungCancer.org, 2014a).

Surgery may only be beneficial in limited-stage small cell lung cancer and no lymph node tumour cases, after which adjuvant chemotherapy may be given (LungCancer.org, 2014a).

3.2. Targeted therapies for lung cancer

Targeted therapies are one of the most promising developments in lung cancer medicine (LungCancer.org, 2014b). Unlike chemotherapy drugs (which are unspecific for cancer cells), targeted therapies are designed to specifically target cancer cells by attaching to or blocking molecular targets on their surfaces (LungCancer.org, 2014b) (which are involved in tumour growth and progression (Widakowich et al., 2007)), and may be addressed alone or in combination with chemotherapy to advanced lung cancer cases with specific molecular biomarkers (LungCancer.org, 2014b, ACS, 2014d). In addition, targeted therapies sometimes work when chemotherapy fails, and often have different and less severe side effects (LungCancer.org, 2014b).

Biomarkers are found on the surface of the cells, or in the genes that program cells, and their presence (such as EGFR, ALK, and KRAS 1) may help to decide which treatment options would work best, with less side effects, and avoid therapeutic strategies that are unlikely to work (LungCancer.org, 2014b).

Currently, there are many different types of targeted therapies being used to treat cancer and new ones are coming out all the time (ACS, 2013a). The two main types of molecules used as targeted therapy drugs consist of antibody drugs (such as cetuximab and bevacizumab), and small-molecule drugs (such as Gefitinib and erlotinib) (ACS, 2013a, Widakowich et al., 2007).

Targeted therapies currently being used against non-small cell lung cancer may be categorized in three main groups: drugs that target EGFR (such as tyrosine kinase inhibitors – erlotinib and gefitinib; and cetuximab – a monoclonal antibody against EGFR); drugs that target tumour blood vessel growth (angiogenesis) (VEGF inhibitors such as bevacizumab); and drugs that target the mutated ALK protein (crizotinib and ceritinib) (ACS, 2014d, LungCancer.org, 2014b).

EGFR is a transmembrane protein that is exposed at cell surface, and has an internal tyrosine kinase domain (Derer et al., 2012, Robinson and Sandler, 2013). The activation of this receptor by ligands (such as EGF or transforming growth factor- α) leads to the activation of multiple pathways that promote cell survival, proliferation, angiogenesis and metastasis (Weihua et al., 2008, Robinson and Sandler, 2013). It is commonly overexpressed or aberrantly active in epithelial cancers, which inhibition has been shown to be a successful strategy in the treatment of NSCLC (D'Arcangelo and Hirsch, 2014). Once it is a widely expressed antigen, it is successfully targeted in tumour patients by monoclonal antibodies or tyrosine kinase inhibitors (Derer et al., 2012).

Cetuximab is a chimeric human-mouse monoclonal antibody that targets EGFR (Carillio et al., 2012, Pirker, 2013), and may be used to treat patients with advanced NSCLC along with standard chemotherapy as part of first-line treatment (Pirker, 2013). It binds to the external domain of the EGFR and competitively blocks the binding of EGF, inhibiting signal transduction (Pirker, 2013). Antibody receptor complexes are then internalized and degraded (Pirker, 2013). This process leads to receptor downregulation on the surface of tumour cells (Pirker, 2013). Cetuximab may also act via antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity (Pirker, 2013). A positive correlation between EGFR expression levels and the therapeutic efficacy of cetuximab in NSCLC patients has already been demonstrated (Derer et al., 2012). It is currently still being evaluated in clinical studies in patients with NSCLC, primarily in combination with first-line chemotherapy in patients with advanced NSCLC (Pirker, 2013). It is not FDA approved for NSCLC; however, since it is approved for use against other cancers (colorectal, head, and neck cancers), it may be used for NSCLC (ACS, 2014d).

Erlotinib (Tarceba[®]) and gefitinib (Iressa[®]) were the first two targeting agents to be approved for clinical use in advanced NSCLC all over the world, with diverse indications (D'Arcangelo and Hirsch, 2014, Sui et al., 2014). They are two small molecules that selectively inhibit the tyrosine kinase activity of EGFR (and thus block cell growth signalling), and both have reversible binding features (D'Arcangelo and Hirsch, 2014). These molecules have shown an impressive activity against lung cancer cells with activating mutations of the *EGFR* gene (D'Arcangelo and Hirsch, 2014). Although EGFR-mutant patients have higher response rates, better quality of life, and longer progression free survival, all patients eventually develop resistance,

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mutations in the tyrosine kinase domain render tumours resistant to erlotinib and gefitinib (Robinson and Sandler, 2013).

A second generation of EGFR inhibitors is currently in an advanced stage of tests, showing promising results regarding their abilities to overcome resistance mechanisms, provide a sustained response through irreversible inhibition and target additional HER receptors (Robinson and Sandler, 2013).

Having initial reversible EGFR TKIs (erlotinib and gefitinib) as models, several irreversible EGFR binders are being developed (Robinson and Sandler, 2013). Afatinib is small molecule that irreversibly inhibits both EGFR and HER2 (Robinson and Sandler, 2013), and was approved to be used, without chemotherapy, as the first treatment for advanced NSCLC that have certain mutations in the *EGFR* gene (ACS, 2014d).

Cells that have a mutation on the EGFR are likely to respond to treatment with erlotinib, gefitinib, and afatinib instead of chemotherapy (LungCancer.org, 2014b, Kohler and Schuler, 2013).

Vascular endothelial growth factor (VEGF) is a protein that stimulates the formation of new blood vessels (angiogenesis), which supply the tumours with nutrients, allowing their growth (Pallis, 2012, Pillai and Owonikoko, 2014, Molina et al., 2008). bevacizumab (Avastin[®]) is a recombinant monoclonal antibody that targets VEGF (Pal et al., 2010) and works by stopping it from stimulating the growth of new vessels (normal tissues are not affected by this drug) (LungCancer.org, 2014b). It was approved by the US FDA as a first-line therapy, in combination with carboplatin and paclitaxel, against metastatic NSCLC (Jardim et al., 2012). The addition of bevacizumab or cetuximab to chemotherapy has shown promising benefits to the patients with NSCLC (Wang et al., 2013).

About 5% of NSCLC have been found to have a rearrangement in a gene called *ALK* (ACS, 2014d). This rearrangement is most often seen in non-smokers (or light smokers) who have the adenocarcinoma subtype of NSCLC (ACS, 2014d). The *ALK* gene rearrangement produces an abnormal ALK protein that causes the cells to grow and spread (ACS, 2014d). Overactivation of the *ALK* gene has been reported in several types of malignancies including NSCLC (Murga-Zamalloa and Lim, 2014). It was shown to be an oncogenic signature that results in tumour dependence on ALK (Murga-Zamalloa and Lim, 2014).

Crizotinib (Xalkori) is a multitargeted small molecule tyrosine kinase inhibitor (Sahu et al., 2013). It was originally developed as an inhibitor of the mesenchymal epithelial transition growth factor (c-MET), and is also a potent inhibitor of *ALK* phosphorylation and signal transduction (Sahu et al., 2013). This inhibition leads to a G1-S phase cell cycle arrest and induction of apoptosis in positive cells (Sahu et al., 2013). Crizotinib also inhibits the related ROS1 receptor tyrosine kinase (Sahu et al., 2013). It is an available treatment that has shown benefits for advanced non-small cell lung cancer patients who have the *ALK* biomarker (LungCancer.org, 2014b), even those who have already had chemotherapy (ACS, 2014d). It is now often the first drug used (instead of chemotherapy) in patients with the *ALK* gene rearrangement (ACS, 2014d).

NSCLC cases that present *ALK* gene rearrangements invariably develop resistance to Crizotinib (Friboulet et al., 2014, Sahu et al., 2013). Ceritinib (Zykadia[®]) is a new FDA-approved *ALK* TKI (FDA, 2014) that can overcome crizotinib resistance (Friboulet et al., 2014). Its use is approved to treat patients with this subtype of metastatic NSCLC, that were previously treated with crizotinib (FDA, 2014)

Nevertheless, targeted therapies have currently no proven efficacy against small cell lung cancer (Jett et al., 2013).

4. Methodologies to synthesize antibody conjugates

4.1. Antibody modification and conjugation

The ability to conjugate an antibody to another molecule is critically important for many applications in life science research, diagnostics, and therapeutics.

Antibody conjugates have become one of the most important classes of biological agents associated with targeted therapy for cancer and other diseases. There literally are dozens of markers that have been identified on tumour cells to which monoclonal antibodies have been developed for targeted therapy (Carter et al., 2004). The preparation of antibody conjugates to find and destroy cancer cells in vivo has become one of the leading strategies of research into investigational new drugs

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(McCarron et al., 2005). In most cases, the site-specific delivery of drugs involves the successful development of defined monoclonal antibody conjugates that can target diseased cells without affecting normal ones (Hermanson, 2008).

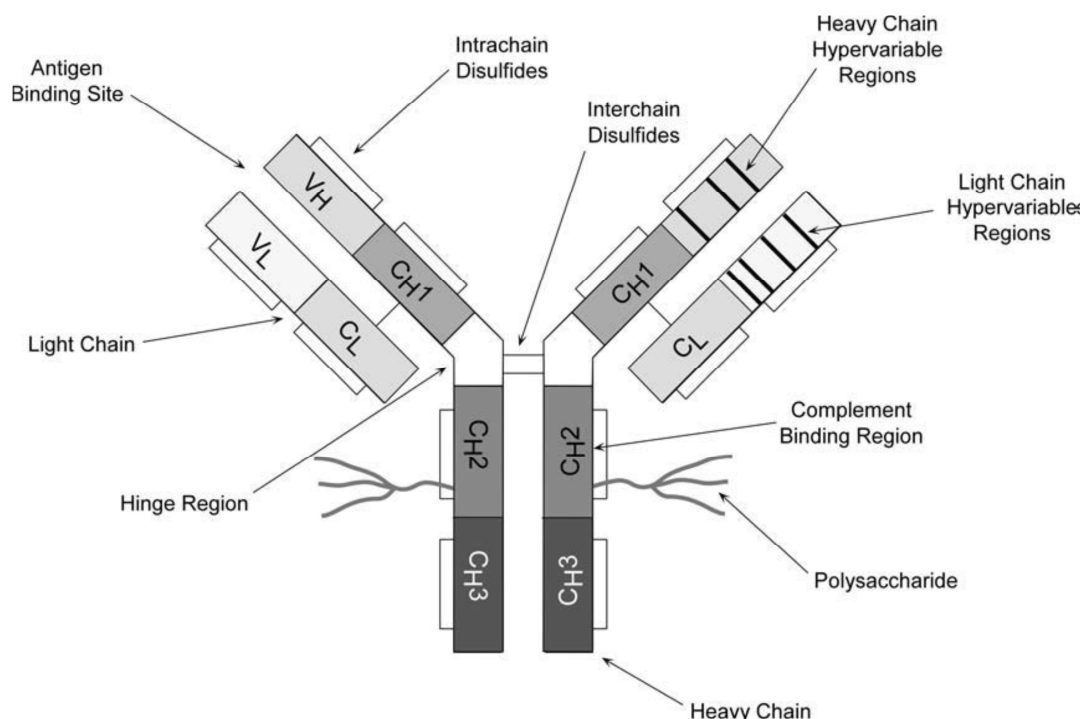


Fig. 2 - Detailed structure of an IgG antibody molecule.

Figure adapted from (Hermanson, 2008)

4.2. Zero-length crosslinkers, their advantages and disadvantages

4.2.1. Carbodiimides

Recurring to zero-length crosslinkers is one of the most used chemical techniques to conjugate antibodies to other molecules. Being our target the conjugation of a relatively small antibody with a large molecule such as LMWChi, it was important not to create much embarrassment with a long connection between molecules. Moreover LMWChi has several amine groups that could interfere with the activated carboxyl groups in the heavy chains of the antibody

Carbodiimides are zero-length crosslinking agents used to mediate the formation of an amide or phosphoramidate linkage between a carboxylate group and an amine or a phosphate and an amine, respectively (Hoare and Koshland, 1966, Chu et al.,

1986, Ghosh et al., 1990). These are called zero-length reagents because in forming these bonds no additional chemical structure is introduced between the conjugating molecules.

4.2.1.1. EDC and NHS

EDC (or EDAC; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) is the most popular carbodiimide used for conjugating biological substances containing carboxylates and amines. In fact, it also may be the most frequently used crosslinking agent of all. Its application in particle and surface conjugation procedures along with NHS (*N*-hydroxysulfosuccinimide) or sulfo-NHS is nearly universal which leads it to being the most common bioconjugation reagent in use today (Hermanson, 2008).

EDC reacts with carboxylic acid groups to form an active *O*-acylisourea intermediate - extremely short-lived - that is easily displaced by nucleophilic attack from primary amino groups in the reaction mixture (**Fig. 3**). The primary amine forms an amide bond with the original carboxyl group, and an EDC by-product is released as a soluble urea derivative (

Fig. 4) (Williams and Ibrahim, 1981).

The *O*-acylisourea intermediate is unstable in aqueous solutions; failure to react with an amine results in hydrolysis of the intermediate, regeneration of the carboxyls, and the release of an *N*-unsubstituted urea.

Other nucleophiles also are reactive. Sulfhydryl groups may attack the active species and form thioester linkages, even though these are not as stable as the bond formed with an amine.

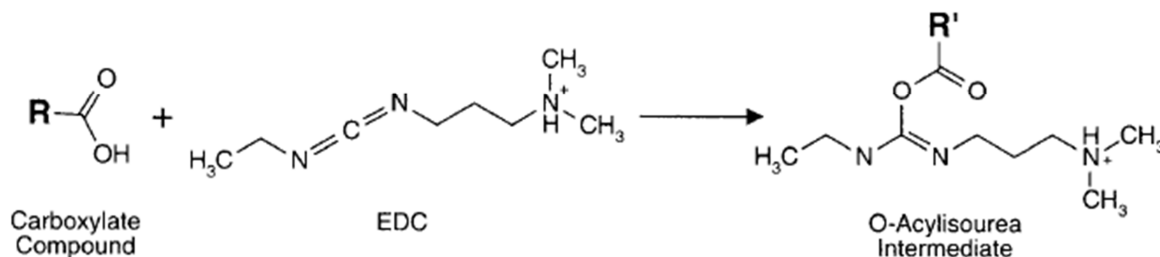


Fig. 3 - Carboxylic acids reacting with activated *N*-substituted carbodiimides (EDC) to form highly reactive, *o*-acylisourea derivatives that are extremely short-lived.

Figure adapted from (Hermanson, 2008)

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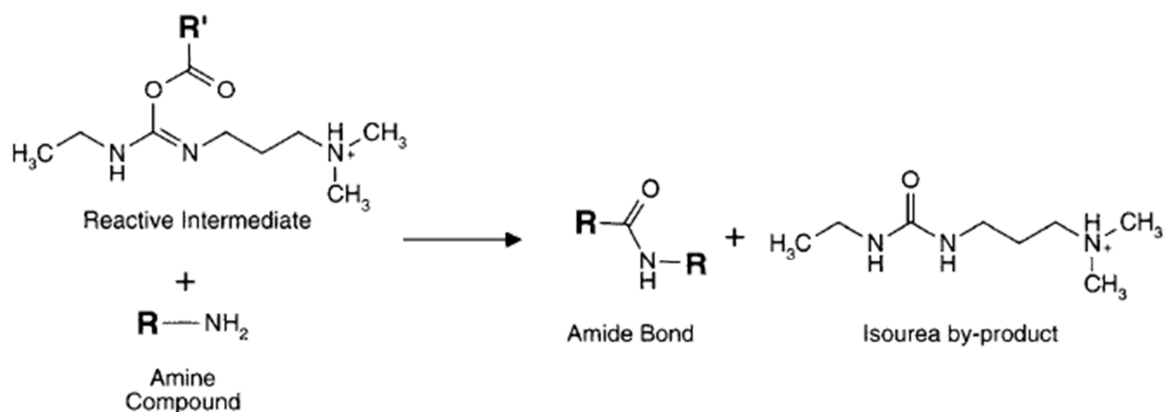


Fig. 4 - The active species reacting with a nucleophile (primary amine) to form an amide bond.

Figure adapted from (Hermanson, 2008)

In addition, oxygen atoms may act as the attacking nucleophile, such as those in water molecules. With this said, in aqueous solution, hydrolysis by water is the major competing reaction and a disadvantage of this linkers, both inactivating EDC itself and cleaving off the activated ester intermediate, forming an isourea, and regenerating the carboxylate group (Gilles et al., 1990).

Results indicate that carboxylate activation occurs most effectively at pH 3.5-4.5, while amide bond formation occurs with highest yield at pH 4-6. However, the maximal rate of hydrolysis of EDC occurs at acidic pH values with increasing stability of the carbodiimide in solution at or above pH 6.5 (Nakajima and Ikada, 1995).

When working with proteins and peptides, fabricants and literature indicate that EDC-mediated amide bond formation effectively occurs approximately between pH 4.5 and 7.5. Buffer systems using MES or phosphate are advisable in order to stabilize the pH during the course of the reaction.

Furthermore molecules containing phosphate groups, such as the 5-phosphate of oligonucleotides, may also be conjugated to amine-containing molecules through a carbodiimide-mediated reaction (**Fig. 5**).

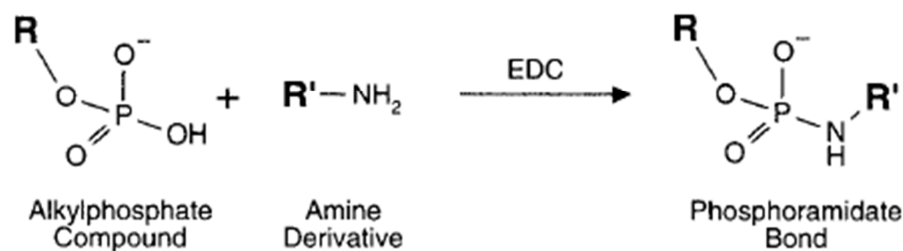


Fig. 5 - The carbodiimide activates the phosphate to an intermediate phosphate ester similar to its reaction with carboxylates. In the presence of an amine, the ester reacts to form a stable phosphoramidate bond.

Figure adapted from (Hermanson, 2008)

EDC has been used in a wide range of applications such as forming amide bonds in peptide synthesis, attaching haptens to carrier proteins to form immunogens, labeling nucleic acids through 5' phosphate groups and creating amine-reactive NHS-esters of biomolecules.

N-hydroxysuccinimide (NHS) or its water-soluble analog (Sulfo-NHS) is often included in EDC coupling protocols to improve efficiency or create dry-stable (amine-reactive) intermediates. EDC couples NHS to carboxyls, forming an NHS ester that is considerably more stable than the *O*-acylisourea intermediate while allowing for efficient conjugation to primary amines at physiologic pH.

4.3. Removal of unconjugated compounds from conjugates

Conjugates of antibodies and enzymes or other compounds are critical components in immunoassay, detection systems and development of targeted therapies. During preparation of the referred conjugates, a molar excess of enzyme, peptide, or polymer typically is cross-linked to a specific antibody to obtain a conjugate of high activity. The result of this ratio is excess left unconjugated after completion of the reaction. The unconjugated reagents confer nothing to the utility of the final product and can be detrimental by contributing to increased backgrounds in assay procedures. With this said the removal of these secondary components it is crucial either as an advantage to improve the resultant signal-to-noise ratio in some assays, or simply because they will interfere with the conjugate detection and characterization.

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Commercial preparations of antibody–enzyme conjugates usually are not purified to remove unconjugated enzyme. Frequently, the major proteinaceous part of these products is not the active conjugate, but leftover peptide or polymer that contributes nothing to the immunochemical activity of what was purchased. Just as an example, unconjugated HRP must be removed from antibody–enzyme/peptide conjugates to obtain optimal staining in immunoassay procedures (Boorsma and Kalsbeek, 1975). In this work specific case, the presence of unconjugated protein (cetuximab) leads to diffuse substrate noise that can obscure FTIR analyses and specially protein quantification methods such as Bradford Method. Several methods may be used to purify an antibody–polymer/protein conjugate and remove unconjugated enzyme. For instances: recurring to HPLC, simple dialysis, gel filtration, or through disposable centrifugal devices for concentration and diafiltration of samples. Being the last method chosen for our project as it easily separated all the unreacted compounds at once, is fast, reliable and less expensive than the others.

5. Polymeric nanoparticles

5.1. Nanocarriers and nanoparticles

Growing interest in nanotechnology-based drug delivery systems within the pharmaceutical technology domain has significantly raised their market potential and diversity (Akhter et al., 2013). Based on their structure and properties, nanocarriers are currently organized into several different classes, comprising liposomes, niosomes, polymeric micelles, polymeric nanoparticles, solid lipid nanoparticles, inorganic nanoparticles, magnetic nanoparticles, dendrimers, virus-based platforms, carbon nanotubes and quantum dots (Akhter et al., 2013, Jin et al., 2014, Tzeng and Green, 2013). Polymeric nanoparticles were the subject under study in the present work, for which they will be addressed in further detail.

Polymeric nanoparticles are those obtained through drug encapsulation, dissolution or entrapment within nanosized particles or drug attachment to a polymeric matrix (Akhter et al., 2013). They may be defined as colloidal systems, which size may

range from 5-10 nm to 1000 nm, although their most common size is about 100-500 nm (Lu et al., 2011).

The term “polymer nanoparticles” comprises any type of polymer nanosized particles, encompassing polymer nanospheres and nanocapsules (Lu et al., 2011). Polymer nanospheres are matrix particles, whose entire mass is solid. They may be used as carriers for other biologically active molecules (e.g., drugs, genes, fluorescent and other functional materials), either adsorbed at the surface or encapsulated within the particle. In turn, polymer nanocapsules are vesicular systems in which the bioactive agents are entrapped in an aqueous core and surrounded by the polymeric shell (Lu et al., 2011). Several approaches have been used to synthesize polymeric nanoparticles, according to their application and type of bioactive molecules or medical drugs encapsulated (Kumari et al., 2010). **Fig. 6** depicts the different types of biodegradable nanoparticles that may be obtained from various methods of encapsulation (Kumari et al., 2010).

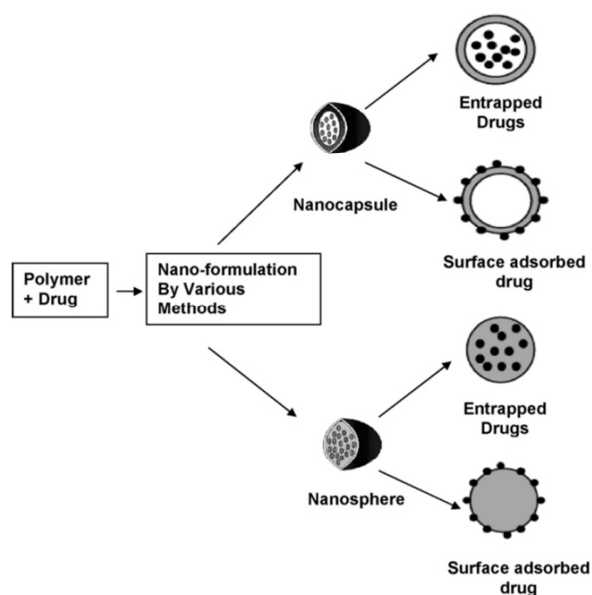


Fig. 6 - Different types of biodegradable nanoparticles according to their structural organization.

Figure adapted from (Kumari et al., 2010)

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5.2. Types of polymers used in polymeric nanoparticles

The encapsulation process with polymeric nanoparticles is in a more advanced condition than that of other nanoparticle systems (Kumari et al., 2010). During the past two decades, many nanomedicines synthesized from biocompatible and biodegradable polymers have arisen and were improved (Kumari et al., 2010). All of the nanomedicine formulation features (including maximum encapsulation efficiency) depend on the choice of a suitable polymeric system (Kumari et al., 2010). Some of the most commonly and extensively used include chitosan, PLGA (poly-d,l-lactide-co-glycolide), PLA (polylactic acid), PCL (poly- ϵ -caprolactone), gelatin, and PAC (poly-alkyl-cyano-acrylates) (Kumari et al., 2010, Akhter et al., 2013). Natural polymers also comprise starch, polypeptides, albumin, sodium alginate, chitin, cellulose, and polyhydroxyalkanoates (PHAs), while polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), polyethylene (PE), polyanhydrides, and poly-orthoesters are other common synthesis polymer materials (Lu et al., 2011). Natural polymers are generally biodegradable, while some of the synthesis polymers are not, which make the former preferable (Lu et al., 2011).

5.3. Advantages of biodegradable polymeric nanoparticles as carriers

Biodegradable nanoparticles have been used in order to improve the therapeutic value of many water soluble/insoluble medicinal drugs and bioactive molecules by improving their bioavailability, solubility and retention time, and by decreasing toxicity risks (Kumari et al., 2010, Akhter et al., 2013). In fact, the nanoencapsulation of drugs (nanomedicine) increases their efficacy, specificity, tolerability and therapeutic index (Kumari et al., 2010). These formulations offer protection from premature degradation and enhance the interaction with the biological environment, absorption into a selected tissue, bioavailability, retention time and intracellular penetration (Kumari et al., 2010). Besides, under *in vivo* enzyme action, biodegradable nanoparticles can produce water and carbon dioxide without adverse effects, and have thus become the focus of increasing research (Wang et al., 2011).

Biodegradable polymeric nanoparticles are therefore preferred because of their potential as drug delivery systems, because of their controlled/sustained release properties, subcellular size and biocompatibility with tissue and cells (Kumari et al., 2010). Moreover, these formulations are stable in blood, non-toxic, non-thrombogenic, non-immunogenic, non-inflammatory, do not activate neutrophils, avoid the reticuloendothelial system, are biodegradable and applicable to various molecules such as drugs, proteins, peptides, or nucleic acids (Kumari et al., 2010).

Many drugs/bioactive molecules (for the treatment of many diseases such as cancer, AIDS, diabetes, malaria, prion disease and tuberculosis) have been successfully encapsulated in order to improve their bioavailability, bioactivity and delivery control (Kumari et al., 2010). In particular, polymeric nanoparticles are the vehicles chosen for various anticancer agents. For instance, a copolymer of *N*-(2-hydroxypropyl) metacrylamide/camptothecin and a NK-105 (PEG-polyaspartate) nanoparticle of cisplatin are in different stages of development (Akhter et al., 2013). Furthermore, paclitaxel, cisplatin and folate have been included in polymeric nanoparticles, with a consequent enhancement of solubility, targeting, cytotoxicity and sustained release profile, when compared with the corresponding free solutions (Akhter et al., 2013).

5.4. Nanoparticle morphological remarks

As before mentioned, when compared with conventional approaches, nanoformulations are advantageous regarding released control, targeted delivery and therapeutic impact, for instance (Kumari et al., 2010). Targeting abilities are modulated by particle size, surface charge, surface modification, and hydrophobicity, with the size and size distributions of nanoparticles being determinant in their interaction with the cell membrane and their penetration across the physiological barriers (Kumari et al., 2010). The release profile may be controlled by the molecular weight of the polymer (Kumari et al., 2010). In fact, the rate of drug release is inversely proportional to the polymer molecular weight (Kumari et al., 2010). The appropriate size for nanoparticles to cross different biological barriers is dependent on the tissue, target site and circulation (Kumari et al., 2010, Yousefi et al., 2013, Tzeng and Green, 2013). In other words, the *in vivo* performance of nanoparticles is

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influenced by parameters such as their morphological features, surface chemistry, and molecular weight (Kumari et al., 2010).

Since electrostatic interaction increases the rate and extent of nanoparticle internalization, and since cell surface is assumed to have a negative charge, cationic nanoparticles are preferable (Kumari et al., 2010). This would also prevent their clustering in the blood flow (Kumari et al., 2010).

The design of these delivery systems, taking into account the nanoparticle features, target and route of administration, may solve the problems associated with new classes of active molecules (Kumari et al., 2010).

5.5. Nanoparticle modification, passive and active targeting

The persistence of the nanoparticles in systemic circulation – which is a drawback for the conventional nanoparticles with hydrophobic surface, which are easily opsonized and cleared by the mononuclear phagocytic system – is required for the success of targeted delivery (Kumari et al., 2010, Yousefi et al., 2013, Tzeng and Green, 2013, Jin et al., 2014). In this sense, the surface of conventional nanoparticles can be modified with different molecules in order to increase the circulation time and persistence in the blood (Kumari et al., 2010, Yousefi et al., 2013, Tzeng and Green, 2013). In fact, the coating of hydrophilic polymers creates a cloud of chains around the particle that repels the plasma proteins (Kumari et al., 2010). Surface-modified nanoparticles have anti-adhesive properties because of the extended configuration on the particle surface, which acts as a steric barrier, reducing the extent of clearance by the circulating macrophages, and improving the permeation process (Kumari et al., 2010, Yousefi et al., 2013, Tzeng and Green, 2013).

Modified nanoparticles also have other properties such as improved drug targeting (Wang et al., 2011, Tzeng and Green, 2013). Two distinct approaches may be explored in order to target nanoparticles to their sites of interest: passive and active targeting (Akhter et al., 2013, Jin et al., 2014, Yu et al., 2012, Master and Sen Gupta, 2012).

Passive targeting makes use of the enhanced permeation and retention (EPR) effect that characterizes tumor microenvironment, due to its high vascularization, high

vascular permeability, leakage and defective lymphatic drainage, leading to increased retention of extravasated molecules and nanoparticles (Akhter et al., 2013, Jin et al., 2014, Yu et al., 2012, Master and Sen Gupta, 2012). Given that nanoparticles with 10-100 nm sizes have been shown to accumulate in tumor tissue via EPR effect, both their size and surface may be tailored to avoid phagocytosis and promote accumulation (Akhter et al., 2013, Jin et al., 2014, Yu et al., 2012).

In turn, active targeting is based on the specific interaction between a ligand-presenting nanoparticle and the receptors exposed at the surface of target cells. In fact, the surface of polymer nanoparticles may be functionalized with metal ions, small molecules, peptides, nucleic acids (aptamers), antibodies, vitamins, carbohydrates, surfactants, or polymers, in order to avoid immunological reactions and to obtain better targeting and binding with ligands (Lu et al., 2011, Yu et al., 2012). This interaction not only promotes nanoparticle accumulation in the tumor, but also its uptake by the tumor cell through receptor-mediated endocytosis (Akhter et al., 2013, Jin et al., 2014, Yu et al., 2012, Master and Sen Gupta, 2012). In fact, many tumor cells over-express one or more molecular targets that can be addressed by nanocarriers (Akhter et al., 2013, Master and Sen Gupta, 2012). For instance, approaches using peptides and targeting folate receptors, tumor necrosis factor receptor CD95, transmembrane tyrosine kinase receptors (e.g., HER2, EGFR), luteinizing hormone-releasing hormone receptors, vasoactive intestinal peptide receptors and integrins are currently available (Akhter et al., 2013). Following endocytosis, the drugs carried by the nanoparticle are processed within lysosomes, avoiding multi-drug resistance mechanisms because endocytosed nanoparticle-associated drugs or drug-polymer conjugates cannot be pumped by proteins such as glycoprotein-P (Akhter et al., 2013).

The use of drug-antibody conjugates is a particular form of active targeting that explores the affinity of monoclonal antibodies for specific antigens (Akhter et al., 2013, Jin et al., 2014). For instance, brentuximab vedotin, which binds CD30, a tumor-specific marker of the tumor necrosis factor receptor superfamily, has recently been approved for the treatment of Hodgkin and systemic anaplastic large cell lymphomas. Furthermore, inotuzumab ozogamicin and trastuzumab emtansine (targeting CD22 and HER2, respectively) have shown significant clinical activity in phase 3 clinical trials for non-Hodgkin lymphoma, acute lymphocytic leukemia and metastatic breast cancer (Akhter et al., 2013, Jin et al., 2014).

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As already mentioned, EGFR, a transmembrane receptor that is overexpressed in several cancers, including gliomas, ovarian, head and neck, renal, pancreatic, colon and NSCLC, is also targeted by nanoparticles conjugated with antibodies, including necitumumab, matuzumab, panitumumab, trastuzumab and cetuximab (Master and Sen Gupta, 2012, Maya et al., 2013). It should be further mentioned that the use of other agents, including antibody fragments, peptides, aptamers and the EGF itself, is also being developed as an active targeting methodology (Master and Sen Gupta, 2012).

In particular, cetuximab-conjugated O-carboxymethyl chitosan nanoparticles for targeting EGFR-overexpressing cancer cells have been successfully prepared by the research team, and were shown to be internalized by tumor cells, causing their death (Maya et al., 2013). cetuximab-conjugated chitosan nanoparticles are, in fact, the scope of the present work.

5.6. Chitosan polymeric nanoparticles

Chitosan nanoparticles are drug carriers that may solve many problems related to drug poor stability, water insolubility, low selectivity, high toxicity, side effects, and many others (Wang et al., 2011). As a natural product, chitosan is a renewable pharmaceutical adjuvant, with good biocompatibility (Wang et al., 2011, Singh et al., 2014, Lai et al., 2014, Maya et al., 2013).

These nanoparticles have wide development potential and show the advantage of slow/controlled drug release, which improves drug solubility and stability, enhances efficacy, and reduces toxicity (Wang et al., 2011, Singh et al., 2014, Lai et al., 2014). Because of their small size, they can easily pass through biological barriers *in vivo* (such as the blood–brain barrier), deliver drugs to the lesion site and enhance their efficacy (Wang et al., 2011, Tzeng and Green, 2013). These advantages confer chitosan and its derivatives a strong potential for application as drug carriers, allowing them to carry drugs as diverse as genes, proteins, anticancer agents, antibiotics, antivirals, anti-allergic agents and hormones, and to be suitable for various routes of administration – oral, nasal, intravenous, and ocular (Wang et al., 2011).

Chitosan adhesiveness, biodegradability, safety, and anti-tumor effect particularly stand out for their clinical relevance (Wang et al., 2011, Singh et al., 2014, Maya et al., 2013).

Biodegradability is obviously an important feature for any drug delivery system (Wang et al., 2011, Singh et al., 2014). Chitosan of a suitable molecular weight can be cleared by the kidney *in vivo*, while the excessive molecular weight molecules can be degraded into fragments that are suitable for renal clearance (Wang et al., 2011). Chitosan is degraded mainly by chemical processes and enzyme catalysis (which is the major *in vivo* process) (Wang et al., 2011). The higher the degree of deacetylation, the greater the degradation rate (Wang et al., 2011, Wang et al., 2014). Degradation by enzyme catalysis also depends on the availability of the amino group (Wang et al., 2011). Presently, it is accepted that chitosan is a non-toxic polymer and a safe drug-delivery material, which was certified as wound dressing by the FDA (Wang et al., 2011).

Chitosan also exerts an anti-tumor effect by itself through improving the body's immune function and through the direct action on tumor cells by interfering with cell metabolism, inhibiting cell growth, or inducing cell apoptosis (Wang et al., 2011, Qi et al., 2005). Many studies have already supported chitosan's anti-tumor effects *in vitro* and *in vivo*, as well as the nanoparticle selectivity for tumor cells, leading to good prospects for their application as a supplementary anti-tumor drug and drug carrier (Wang et al., 2011). In fact, chitosan positive charges selectively adhere to and neutralize the tumor cell surface (Wang et al., 2011, Qi et al., 2005). It was also shown to have a targeting function to liver, spleen, lung, and colon (Wang et al., 2011). In particular, ethylene chitosan nanoparticles have higher circulating times in the blood, with greater tumor cell selectivity; doxorubicin-chitosan polymeric micelles were able to target the liver and spleen, while significantly reducing toxicity to the heart and kidney (Wang et al., 2011).

5.7. Characteristics of chitosan nanoparticles

Nanoparticles consist of small-sized colloid particles of 1 to 1000 nm, which have strong mobility, high cell uptake rate, and that can easily enter in the cells and accumulate at the interest site (Wang et al., 2011).

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Chitosan nanoparticles can release drugs/biomolecules through polymer degradation, which leads to a clear sustained-release effect (Wang et al., 2011). Because of the varied degradation rate and time of chitosan with different molecular weights and degrees of deacetylation, as well as other molecular modifications, different types of chitosan may be used to produce different types of nanoparticles with specific drug-release rates and, thus, achieve sustained/controlled release (Wang et al., 2011, Wang et al., 2014).

Also, in order to improve chitosan nanoparticle targeting and bioavailability, several approaches are being pursued, mostly focusing on chitosan modifications. For instance, drug release may be promoted by the changes in carrier properties under a specific acid-base environment. The same purpose is achieved when the carrier is designed to be thermo sensitive and structurally responsive to temperature changes (Wang et al., 2011, Singh et al., 2014). In turn, active targeting may also be accomplished if chitosan nanoparticles are modified in order to allow the identification of the proper drug targets (Wang et al., 2011).

Part II
Aims

Aims

The main goal of this dissertation was to provide new knowledge on the most viable and efficient way to produce cetuximab-LMWChi conjugates in order to later encapsulate siRNA and target to EGFR overexpressing cells.

The work reported in the present thesis aimed at the production of such conjugates through the implementation and optimization of protocols recurring to zero-length crosslinker chemistry, without underestimate the purification, evaluation/characterization of the reaction products through FTIR, protein quantification methods for reaction efficiency, and cell cytotoxicity through MTT and LDH assays.

Furthermore the results of the reported project aimed at being reproduced and applied in future studies so that new reliable target siRNA therapies can be synthesized recurring to cetuximab-LMWChi conjugates.

Part III
Materials
and
methods

Materials and methods

1. Cetuximab

Gently provided by the Pharmaceutical services of Hospital St. António – Porto, clinical leftovers of MerckSerono's 5 mg/dL cetuximab were used for the production of the several conjugates in this project.

This monoclonal antibody blocks ligand binding to EGFR and prevents downstream signaling in EGFR overexpressing cells. It should provide us with the needed driver, to target in the future, by encapsulating siRNA, small cell lung cancer cells without affecting other cells and saving drug.

2. Chitosan

Chitosan (**Fig. 7**) physicochemical properties are greatly dependent on its molecular weight and degree of deacetylation (Wang et al., 2011, Wang et al., 2014, Lai et al., 2014). Since its glycoside bond is hemiacetal, it is unstable under acidic conditions, which thus favor its hydrolysis and solubilization (Wang et al., 2011). Chitosan becomes less viscous, with a lower molecular weight; amino acid protonation occurs, conferring positive charge and triggering gelation and membrane formation (Wang et al., 2011, Lai et al., 2014).

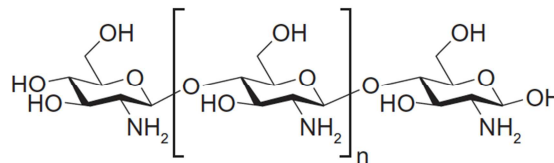


Fig. 7 - Chitosan structure.

Figure adapted from (Wang et al., 2011)

The amino and carboxyl groups in the chitosan molecule can be combined with mucus glycoproteins to form a hydrogen bonds, leading to an adhesive effect that may be strengthened under neutral and acidic conditions (Wang et al., 2011, Lai et al., 2014). The greater the molecular weight and higher the degree of

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deacetylation of chitosan, the stronger its adhesiveness will be (Wang et al., 2011, Wang et al., 2014, Lai et al., 2014).

Chitosans having lower molecular weights and lower degrees of deacetylation exhibit greater solubility however faster degradation than their high-molecular-weight counterparts.(Wang et al., 2000, Zhang and Neau, 2001, Koping-Hoggard et al., 2004, Mao et al., 2004, Ren et al., 2005, Bowman and Leong, 2006).

In this project all the attempts to produce conjugates recurred to Sigma Aldrich's LMWChi, 4,5mg/mL and 5,0mg/mL LMWChi solutions were prepared through the solubilization of the previously referred LMWChi in a 1% glacial acetic acid solution. After dissolution pH was adjusted to 5,0 using a CRISON's pH meter GLP21, while slow magnetic stirring of the solutions.

3. EDC

As previously referred EDC is a zero-length crosslinker and one of the most popular carbodiimide used for conjugating biological substances containing carboxylates and amines. In the present work Sigma-Aldrich's EDC was used.

4. NHS

NHS, as already reported in this dissertation introduction, is often included in EDC coupling protocols to improve efficiency or create dry-stable (amine-reactive) intermediates, improving reaction efficiency. During the several conjugation attempts Sigma-Aldrich's EDC was chosen.

5. Conjugation procedure

The most challenging and crucial procedure, but also the main goal, of this project was the conjugation aiming the formation of a cetuximab-chitosan molecule; several were the unsuccessful attempts and protocol changes until reaching a satisfactory conjugate.

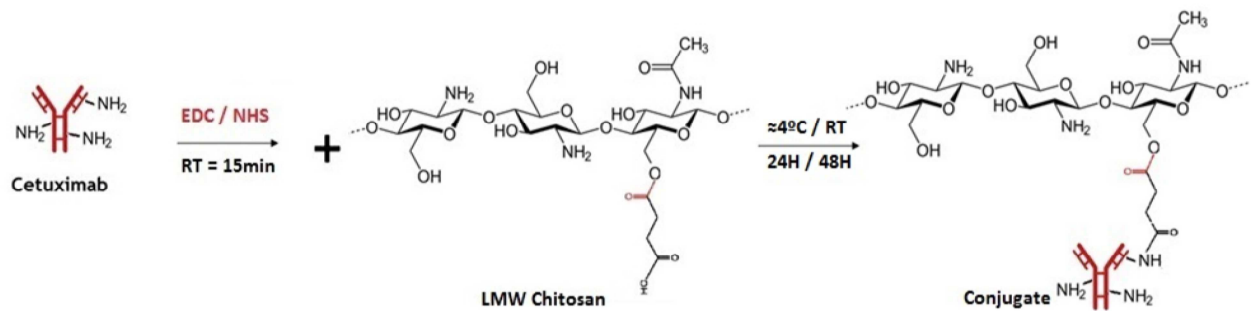


Fig. 8 – Conjugation reaction scheme, representing the activation of carbonyl groups of CTX by EDC/NHS, forwarded by the conjugation with LMWChi through its amine groups.

Low molecular weight chitosan (LMWChi) may help to form small-sized polymer-DNA or small interfering RNA (siRNA) complexes, which made this one of the reasons why LMWChi was chosen. (Fernandes et al., 2012).

Chitosan nanoparticles can be prepared using various formulation methods to release an active ingredient (such as proteins, peptides and DNA vaccines) in a sustained manner over a prolonged period. The ionic crosslinking method has received significant attention in recent years due to the preparation of chitosan nanoparticles containing proteins, peptides and vaccines due to the fact that this is simple and mild for proteins and viruses. It doesn't recur to chemical cross linkers and avoids using organic solvents and high temperatures. (Han et al., 2010, Zhao et al., 2012).

Standard biomolecular conjugation chemistries using homo- or heterobifunctional cross-linkers (e.g., glutaraldehyde, cross-linkers containing amine reactive N-hydroxysuccinimidyl (NHS) ester and thiol reactive maleimide) have inherent drawbacks despite recent progress. These drawbacks include limited stability of the cross-linkers, nonselective conjugation, and self-blocking of active conjugation sites by the cross-linkers. (Hermanson, 2008). Even though EDC/NHS was chosen as the appropriate linker, as it was already available, it's one of the most used zero-length cross-linkers and was compatible with the working pH and reagents to conjugate.

Materials and methods

5.1. Procedure for EDC/NHS crosslinking of carboxylates with primary amines

After trying several alternatives to produce a satisfactory conjugate compound the most satisfactory approach ended up to be the one following the next protocol.

First and foremost the NHS ester of cetuximab had to be activated. As suggested by Thermo Scientific supplier as a strategy, a 10-fold molar excess of EDC were added directly to 1 mL of cetuximab solution of 5mg/dL (Support, 2012-2013). Then ½ molar of NHS relatively to 1 molar of EDC, were added to the reaction to promote the EDC activation. Reaction components were slowly magnetically stirred and reaction was let to proceed for 15 minutes at room temperature, so the carboxylic groups of cetuximab were activated for further conjugation (Support, 2012-2013).

For the second part of this procedure the amine reaction took place. Despite the recommendation from Thermo Scientific technical support to increase the reaction pH above 7,0 (using concentrated PBS or other non-amine buffer such as sodium bicarbonate) - this step was not performed due to the fact that chitosan is only soluble and stable at acidic pH. Thus chitosan solution was introduced to the reaction in the tested ratios of 1:1, 1:25, 1:50 and 1:100mol (cetuximab to chitosan molar ratios). The solution was well mixed and allowed to react while gently magnetic stirred for 24 or 48h hours at room temperature for a batch of ratios, and for the same 24 or 48h for a couple more batch of ratios produced at 4°C (Lee et al., 2012, Gaspar et al., 2013, Support, 2012-2013). As a final step and in order to quench the reaction, the final product was immediately ultra-filtered recurring to disposable concentrators with a 150K MWCO membrane, capable of retaining a predicted conjugated molecule of 195K MWCO. This procedure provided a fast sample processing, high retentate recovery and high concentration factors (> 90 fold) even with low expected concentrations.

After ultra-filtering the produced compound, the retained concentration product was collected and an aliquot freeze-dried for 48h.

During the previously mentioned conjugation procedure the pH was maintained approximately at 5,0, which was measured using a CRISON's pH meter GLP21.

6. Cellulose acetate electrophoresis

On a first approach we recurred to this methodology to identify conjugate formation.

The process consists on the separation of proteins recurring to their different molecular weight and isoelectric point. As the produced conjugates were expected to have a molecular weight (MW) of approximately 195000g/mol - far superior from cetuximab's MW of 145000g/mol and LMWChi's MW of 50000g/mol – samples of the produced conjugates, cetuximab, LMWChi and physical mixture of the last two compounds (without activation by EDC/NHS), should present different migration patterns according to their MW and isoelectric point (IEP) in cellulose acetate stripes after exposition to electrical current.

6.1. Cellulose acetate electrophoresis protocol

First, Cellulose acetate strips were immersed in tampon (tris-hipurate pH=8,8) for 15minutes at room temperature. After this the strips were put to contact with absorbent paper to remove excess tampon, and stretched in the mechanism's support with their extremities immersed in the tampon. With an appropriated micropipette 15µL per sample were applied with low pressure to the strips for 1minute.

A 5mA current per strip was used in the system for 1 hour (Erro! A origem da referência não foi encontrada.). To reveal the migration patterns strips were immersed in Coomassie dye (0,5% m/V Coomassie blue G250, 45% methanol, 10% glacial acetic acid, 45% distilled water) for 5minutes (Erro! A origem da referência não foi encontrada.). Then discoloration was proceeded with bleaching compound I (50% Methanol, 10% Glacial Acetic Acid, 40% distilled H₂O) for 1hour and bleaching compound II (5% Methanol, 7% Glacial Acetic Acid, 88% distilled H₂O) overnight. Cellulose acetate strips were then fixed with pure methanol, and put in a sterilizer at 37°C for 10minutes. After this strips were immersed in a transparency solution (90% methanol, 10% glacial acetic acid) for 3 to 4 minutes. Strips were then drained from excess liquid with absorbent paper and stuck to microscope slides. Finally

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microscope slides, with the cellulose acetate stripes attached, were put in the sterilizer at 80°C for approximately 5 minutes until the strips got transparent.

7. Freeze-drying

After each conjugation a 5 mL sample of each conjugate was freeze at -80°C, after which freeze-drying was executed recurring to a 4K freeze-dryer from Edwards (Crawley, West Sussex, U.K.) for 48H.

8. FTIR analyses

Fourier transform infrared spectroscopy (FTIR) is a technique used to obtain an infrared spectrum of absorption, emission, photoconductivity or Raman scattering of a solid, liquid or gas. An FTIR spectrometer simultaneously collects spectral data in a wide spectral range advantage that, in conjugation with the sensitivity of the this method, and due to the fact that a minimal concentration of conjugate could have been produced, made this the identification method of choice to detect the conjugate presence.

Rather than shining a monochromatic beam of light at the sample, with this technique a beam containing many frequencies of light at once is produced (Multiplex or Fellgett advantage), after which how much of that beam is absorbed by the sample is measured. This beam is then modified to contain a different combination of frequencies, giving a second data point. The process is repeated continuously and afterwards, a computer program uses all these data and works backwards to infer the absorption at each wavelength.

The previously described beam is generated by starting with a broadband light source—one containing the full spectrum of wavelengths to be measured. The light shines into a Michelson interferometer (a certain configuration of mirrors), one of which is moved by a motor. As this mirror moves, each wavelength of light in the beam is periodically blocked, transmitted, blocked, transmitted, by the interferometer,

due to wave interference. Different wavelengths are modulated at different rates, so that at each moment, the beam coming out of the interferometer has a different spectrum.

Computer processing is crucial to turn the raw data (light absorption for each mirror position) into the desired result (light absorption for each wavelength). The processing required turns out to be a common algorithm called the Fourier transform, and so the name FTIR.(Griffiths, 2007)

In this project an ABB MB3000 FTIR Laboratory Spectrometer equipped with an ATR was used throughout the analysis. For process and analyses of the data collected, the Horizon MBTM FTIR software was elected.(ABB.pt, 2014).

9. Protein quantification – Bradford method

As a protein quantification method to help determine the amount of conjugated and unconjugated cetuximab after filtration with Pierce's Protein Concentrators, 150K MWCO, the Bradford method was elected as it's simple, rapid, readily automated, relatively reliable, available and didn't interfere with any of the compounds in solution (CTB, 2005).

This method is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to the amine groups of proteins (particularly in the lysine aminoacids (aas) occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change (Bradford, 1976, CTB, 2005).

The main disadvantage and thus limitation of this method relies on the it's high dependency on protein's quality and composition, since it's more sensible to amine groups in lysine aas (Sapan et al., 1999, Okutucu et al., 2007).

Protein measurements in the final products were made using a Bradford assay kit (Bio Rad Protein Assay; Bio Rad Laboratories).

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9.1. Bradford method protocol

The Bradford reagent is prepared dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, added 100 ml 85% (w/v) phosphoric acid. Diluted to 1 liter when the dye is completely dissolved, then it is filtered through Whatman#1 paper before use. In the present work, the Bio-Rad[®] concentrate (Bradford reagent) was used as it was available and it's more sensible than the lab made reagent.

First a standard curve of absorbance was determined. Briefly, standards of 0,01mg/mL to 2mg/mL were prepared from a concentrated 5,0mg/dL cetuximab solution diluting volumes of this solution in LMWChi filtrate so that the noise due to the later could be eliminated when running the samples. Then to individual falcons containing 5mL of Bradford reagent, 100 μ L of each of the referred previous standards were added, and vortex. These were left to incubate at room temperature for 5min, and immediately, after new vortex, 100 μ L of the solutions were transferred to a 96 well plate as displayed below.

Plate A – CTX standards

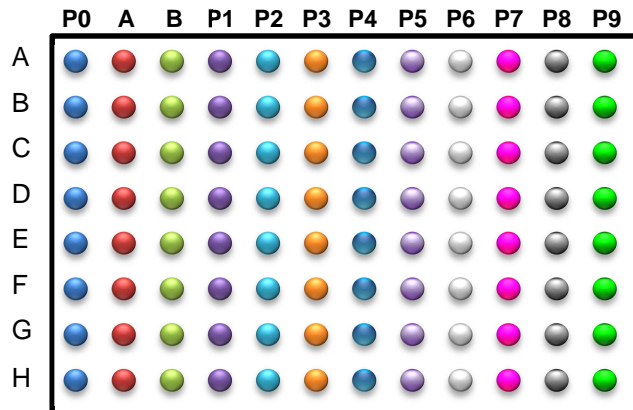


Figure 1 - Schematic representation of the experimental design of a 96-well plate for absorbance reading of CTX standards, through the Bradford method.

P0	Distilled water	P4	CTX(0,1mg/mL) + Coomassie reagent
A	Distilled water + Coomassie reagent	P5	CTX(0,2mg/mL) + Coomassie reagent
B	CTX(5mg/mL) + Coomassie reagent	P6	CTX(0,5mg/mL) + Coomassie reagent
P1	CTX(0,01mg/mL) + Coomassie reagent	P7	CTX(1mg/mL) + Coomassie reagent
P2	CTX(0,02mg/mL) + Coomassie reagent	P8	CTX(1,5mg/mL) + Coomassie reagent
P3	CTX(0,05mg/mL) + Coomassie reagent	P9	CTX(2mg/mL) + Coomassie reagent

Table 1 – Bradford method: Plate A, disposition of cetuximab standards

Standards for LMWChi were created and read since that free LMWChi was presented in the “filtered” samples (post-ultra-filtered products that passes the 150K MWCO membrane), but also LMWChi could be found conjugated to CTX. Thus LMWChi would present itself as noise in the detection and quantification of the successfully produced conjugate. The same procedure previously described was applied to this plate.

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A schematic representation of the 96 well plate with the LMWChi standards is presented below.

Plate B – LMWChi standards

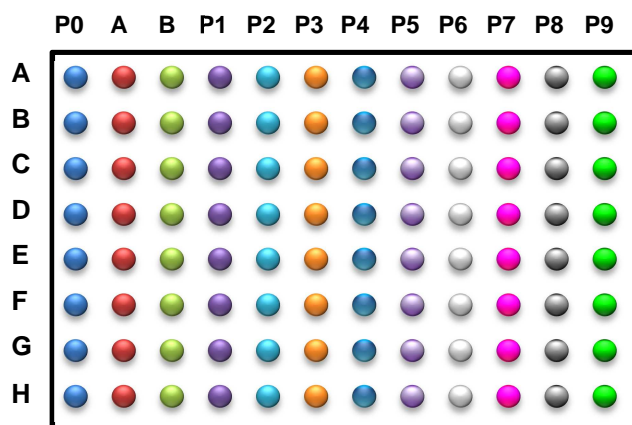


Figure 2 - Schematic representation of the experimental design of a 96-well plate for absorbance reading of LMWChi standards, through the Bradford method.

P0	LMWChi (5mg/mL) + Coomassie reagent	P4	LMWChi (0,1mg/mL) + Coomassie reagent
A	Distilled water + Coomassie reagent	P5	LMWChi (0,2mg/mL) + Coomassie reagent
B	LMWChi (5mg/mL) + Coomassie reagent	P6	LMWChi (0,5mg/mL) + Coomassie reagent
P1	LMWChi (0,01mg/mL) + Coomassie reagent	P7	LMWChi (1mg/mL) + Coomassie reagent
P2	LMWChi (0,02mg/mL) + Coomassie reagent	P8	LMWChi (1,5mg/mL) + Coomassie reagent
P3	LMWChi (0,05mg/mL) + Coomassie reagent	P9	LMWChi (2mg/mL) + Coomassie reagent

Table 2 - Bradford method: Plate B, disposition of LMWChi Standards

For the samples in which was needed protein (cetuximab) quantification, the same procedure was taken, and a schematic representation of the plates and samples disposition can also be seen below.

Plate C – Samples

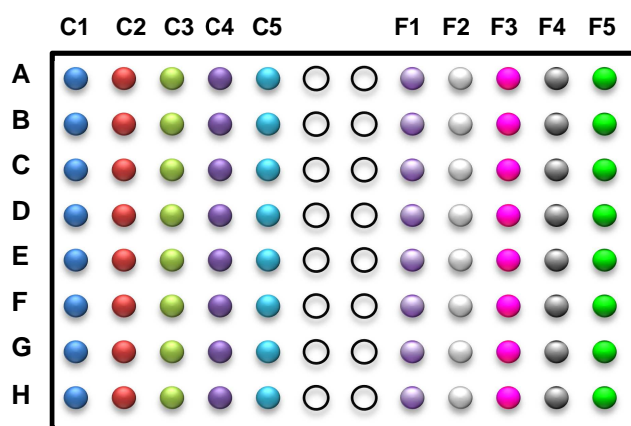


Figure 3 - Schematic representation of the experimental design of a 96-well plate for protein quantification of samples, through the Bradford method.

C1	Sample: Concentrate 1:1 + Coomassie reagent	F1	Sample: Filtrate 1:1 + Coomassie reagent
C2	Sample: Concentrate 1:25 + Coomassie reagent	F2	Sample: Filtrate 1:25 + Coomassie reagent
C3	Sample: Concentrate 1:50 + Coomassie reagent	F3	Sample Filtrate: 1:50 + Coomassie reagent
C4	Sample: Concentrate 1:100 + Coomassie reagent	F4	Sample Filtrate: 1:100 + Coomassie reagent
C5	Sample: Concentrate 1:200 + Coomassie reagent	F5	Sample Filtrate: 1:200 + Coomassie reagent

Table 3 - Bradford method: Plate C, disposition of «concentrate» and «filtrate» samples in test

Finally, after transferring 100µL of each of the compounds to their respective and previously represented wells, the reading took place in a Biotek Synergy 2 plate reader. Briefly: the device was previously warmed up before use to room temperature, plates were programmed to be gently shaken for 1min., and absorbance was read at 595 nm.

Data was analyzed using GraphPad Prism[®] version 6.0c.

10. Size characterization - Dynamic light scattering

Dynamic light scattering (DLS), sometimes referred to as Quasi-Elastic Light Scattering (QELS), is nowadays used on a routine basis for the analysis of particle sizes in the sub-micrometer range. As a non-invasive, well-established technique for measuring the size and size distribution of molecules and particles typically in the submicron region, and with the latest technology lower than 1nm, It provides an estimation of the average size and its distribution within a measuring time of a few minutes.

Sub-micron particles suspended in a liquid are in constant motion as a result of the impacts from the molecules of the suspending liquid. This movement is known as Brownian Molecular Movement and was correctly suggested by W. Ramsay in 1876 and confirmed by A. Einstein and M. Smoluchowski in 1905/06.

In the Stokes-Einstein theory of Brownian motion, the particle motion at very low concentrations is depending on the viscosity of the suspending liquid, the temperature, and the size of the particle (Russel, 1981). If viscosity and temperature are known, the particle size can be evaluated from a measurement of the particle motion. At low concentrations, this is the hydrodynamic diameter.

DLS probes this motion optically. The particles are illuminated by a coherent light source, typically a laser, creating a diffraction pattern, a fine structure from the diffraction between the particles, i.e. its near-order. As the particles are moving from impacts of the thermal movement of the molecules of the medium, the particle positions change with the time, (t). The change of the position of the particles affects the phases and thus the fine structure of the diffraction pattern. So the intensity in a certain point of the diffraction pattern fluctuates with time. The fluctuations can be analyzed in the time domain by a correlation function analysis or in the frequency domain by frequency analysis. Both methods are linked by Fourier transformation.

The measured decays rate G are related to the translational diffusion coefficients D of spherical particles by

$$\Gamma = Dq^2 \quad \text{with} \quad q = \frac{4\pi}{\lambda_0} \sin \frac{\theta}{2} \quad \text{and} \quad D = \frac{k_B T}{2\pi\eta x}$$

were q is the modulus of the scattering vector, k_B is the Boltzmann constant, T the absolute temperature, and η the hydrodynamic viscosity of the dispersing liquid. The

particle size x is then calculated by the Stokes-Einstein equation from D at fixed temperature T and h known.

DLS covers a broad range of diluted and concentrated suspension. As the theory is only valid for light being scattered once, any contribution of multiple scattered light leads to erroneous PCS results and misinterpretations. With this said different measures have been taken to minimize the influence of multiple scattering.

DLS measurements were performed using a Malvern zetasizer Nanoseries nano-ZS. Conjugates were characterized in terms of average size, polydispersity index (PDI), and zeta potential.

11. Cell culture

11.1. Cell lines

This project used two different immortalized human cell lines, as both were already available as well as the fact that both are adenocarcinomic cell lines from the respiratory tract where it's pretended that a future compound as pharmacologic action.

11.1.1. A549

These adenocarcinomic human alveolar basal epithelial cells were first developed in 1972 by D. J. Giard, et al. through the removal and culturing of cancerous lung tissue in the explanted. Morphologically they are squamous and physiologically are responsible for the diffusion of some substances, such as water and electrolytes across the alveoli of lungs. If cultured in vitro, A549 cells grow as monolayer cells, adherent or attaching to the culture flask. A549 cell line are widely used as an in vitro model for a type II pulmonary epithelial cell model for drug metabolism and as a transfection host.(ATCC.org, 2014a, a549.com)

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11.1.2. Calu3

Lung adenocarcinoma; derived from metastatic site by pleural effusion epithelial cells, Calu3 is a non-small-cell lung cancer cell line that grows in adherent culture and displays epithelial morphology. This cell line was established in 1975 from a 25-year-old Caucasian male with adenocarcinoma of the lung (ATCC.org, 2014b).

These cells have constitutively active ErbB2/Her2 due to amplification of the ERBB2 gene. They express wildtype EGFR and mutant K-Ras (G13D). In addition, they harbor mutations in TP53 and CDKN2A genes. The Calu3 cells are sensitive to erlotinib (EGFR tyrosine kinase inhibitor) and cetuximab. These cells are also capable of forming tumors in immunocompromised mice (ATCC.org, 2014b).

Being this cell line a suitable model not only to examine the transport of low molecular weight substances and xenobiotics, as for studying the contributions of bronchial epithelial cells to mechanisms of drug delivery at the respiratory epithelium, it was a natural choice to test the produced conjugates on (Foster et al., 2000).

12. Cell culture conditions

Both cell lines were cultured in complete growth medium consisting of Dulbecco's Modified Eagle's Medium (D-MEM, PAA – The Cell Culture Company), supplemented with 5% (v/v) Foetal Bovine Serum (FBS, Gibco). All cell lines were grown in monolayer cultures in adequate flasks and kept permanently in exponential growth in a humidified incubator (ESCO Celculture CO₂ Incubator – CCL-170B-8 170L), at 37°C and a 5% CO₂ atmosphere. Cells were trypsinized and subcultured mostly every three to four days, in order to avoid confluence and ensure their healthy exponential growth.

All procedures involved in cell culture were performed in a ESCO Class II BSC laminar flux biological safety cabinet, biosafety level II, with aseptic techniques being always regarded.

13. Cell subculture

Cells were mainly maintained in 25cm³, in some occasions also in 75cm³ flasks, and regularly observed under microscopy (Zeiss Primo Vert) to check for the confluence and to assure they looked healthy and free of contamination to use in the experiments. At approximately 70-80% confluence, they were subcultured to new flasks, according to the following procedure.

For 25cm³ flasks, growth medium was discarded and the cells were washed with 2ml of sterile Phosphate-Buffered Saline (PBS, pH≈ 7.4: 137mM NaCl; 2.7mM KCl; 6.4mM K₂HPO₄; 1mM NaHPO₄) in order to remove trypsin inhibitors. To promote cell detachment, 500μL of Trypsin EDTA 1× (ScienCell) were added and allowed to act for 5 to 10 minutes at 37.0 °C. Cell detachment was then confirmed through microscopic observation. 4.5ml of complete growth medium were added to stop the reaction. At this point, two different approaches were performed, in accordance to the final purpose of the cell suspension (experimental assays or cell culture maintenance).

When cells were intended to be seeded at a determined density for experimental assays, they were counted through the trypan blue exclusion method. In such cases, a 30μL aliquot of the cell suspension was collected. 30 μl of trypan blue were then added to this aliquot and the suspension was homogenized. Aliquots of 15 μl were then transferred to a Neubauer counting chamber. After this cells were counted using the trypan blue exclusion method and cell density (number of cells per ml) was determined. Finally, an equivalent volume to the intended density of cells (according to each experiment) was transferred to an adequate final volume of fresh growth medium.

Whenever the purpose was to maintain cell culture stocks, cells were split and diluted. Cell splits were based on the percentage of cells taken from the trypsinized flask and put into a new flask. In general, according to the amount of cells recovered from the trypsinized flask, 10% splits were performed. To perform a 10% split (1:10) to a new T25 flask, 500 μL of cell suspension were transferred into a new flask containing 4.5 ml of growth medium.

14. Cell defrosting

Cryovials containing cells were removed from the freezer and left to warm to room temperature. When already in the liquid state, cells were then transferred to pre-warmed growth medium and centrifuged at 1,000 rpm for 5 minutes so the supernatant could be discarded due to DMSO toxicity. After which cells were transferred to growth medium supplemented with 10% (v/v) FBS for both cell lines.

15. Cytotoxicity assays

15.1. MTT assays

This toxicity testing, for cell viability and metabolism, is done in some ways which are by measuring the number of cells and their development after being exposed with the tested materials, by examining cell status after the changing of membrane permeability, and by measuring toxic response based on enzymatic activities. Nevertheless, toxicity testing based on enzymatic cell activities is often done since this method can monitor specific function of cell metabolism, it needs only short duration (4 hours), gives quantitative results, has high sensitivity towards toxic materials, and has potentials for standardization of testing method.

MTT is a yellow soluble molecule, which can be used to analyze cellular enzymatic activities. If the cell can reduce MTT, the formazan produced will be blue-purple, insoluble, and will precipitate in cell. The amount of formazan formed is proportional to enzymatic activities. This testing, furthermore, is measuring cellular dehydrogenizing activities, and changing the chemical material, MTT, through the number of cellular reductive materials into blue and insoluble formazan compound. MTT assay actually is based on the capability of living cells in reducing MTT salt. The principals of this assay are to break tetrazolium MTT ring (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the existence of dehydrogenase in active mitochondria, and then to produce insoluble blue-purple formazan product. The

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mechanism is that the yellow tetrazolium salt will be reduced in cell which has metabolic activities. Mitochondria of living cell has important role in producing dehydrogenase. If the dehydrogenase is not active because of cytotoxic effects, formazan will not be produced. Formazan production can be measured by diluting it and measuring the optic density of the solution produced. There are actually many protocols in using MTT assay, but the concentration of MTT used must be the same as to dilute 5mg/ml yellow MTT powder in PBS. The reaction of blue-purple color is used as the measurement of the number of living cells. The number of living cells can be measured as the product result of MTT by using spectrophotometer with 570–690 nm wave length. (Maretaningtias Dwi Ariani, 2009)

This metabolic/toxicity assay was used to quantitate cell proliferation and cytotoxicity after exposure to different produced conjugates,

Cells were trypsinized and counted according to the procedure described previously in the “Cell culture” section. After plating cells in ideal densities (previously determined by plotting calibration curves), in 96-well plates, and allowed to stabilize and adhere for 24 hours. Cell suspensions with a final density of 9×10^4 cells/ml were prepared, for both cell lines, in appropriate growth medium containing 5% FBS and without antibiotic/antimitotic mixture. A volume of 100 μ l cells were plated per well. Following dilution, the compounds under analysis were then added, according to the schematic representations below.

Following 24 or 48 hours of exposure to the compound at 37°C and 5% CO₂ (in a ESCO Celculture CO₂ incubator CCL-170B-8 170L) 20 μ l of a 5 mg/ml MTT ((3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) solution in PBS were added to each well and incubated for 4 additional hours in the same conditions. Formazan crystals were then solubilized by adding 100 μ l of solubilization solution (89% (v/v) isopropanol, 10% (v/v) triton X-100, 0,37% (w/v) HCl). Upon homogenization, absorbance values were read at 550 nm in a plate reader (Biotek Synergy 2) and retrieved using Gene5 software (Biotek). Data was analyzed using GraphPad Prism[®] version 6.0c.

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Below are schematic representations of the MTT plates.

Plates A, A1, C and C1:

A – A549 Cell line – 24H exposure to compounds

A1 – A549 Cell line – 48H exposure to compounds

C – Calu3 Cell line – 24H exposure to compounds

C1 – Calu3 Cell line – 48H exposure to compounds

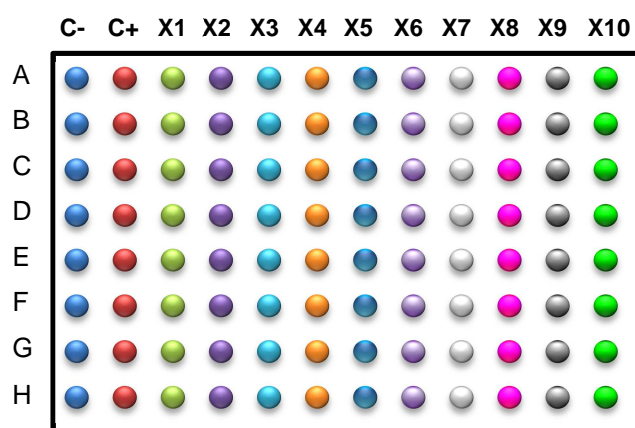


Figure 4 - Schematic representation of the experimental design of a 96-well plate for absorbance reading of MTT exposed cells after contact with compounds in test for 24H.

C-	Negative control	X5	Conjugate 1:25 (75µg/mL)
C+	Positive control	X6	Conjugate 1:25 (25µg/mL)
X1	Conjugate 1:1 (200µg/mL)	X7	Conjugate 1:50 (200µg/mL)
X2	Conjugate 1:1 (75µg/mL)	X8	Conjugate 1:50 (75µg/mL)
X3	Conjugate 1:1 (25µg/mL)	X9	Conjugate 1:50 (25µg/mL)
X4	Conjugate 1:25 (200µg/mL)	X10	---

Table 4 - MTT: Plate A, A1, C and C1 disposition of controls and compounds in test

Plates B, B1, D and D1:

B – A549 Cell line – 24H exposure to compounds

B1 – A549 Cell line – 48H exposure to compounds

D – Calu3 Cell line – 24H exposure to compounds

D1 – Calu3 Cell line – 48H exposure to compounds

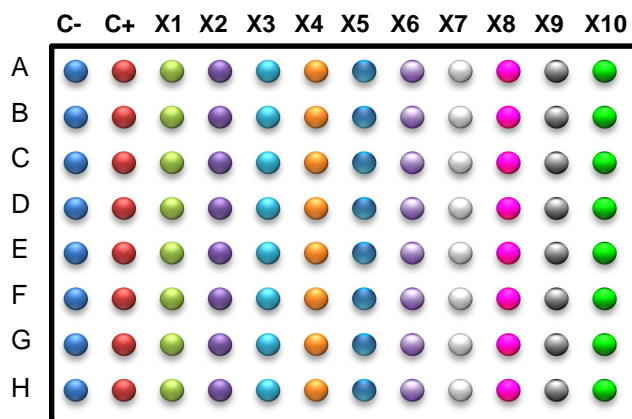


Figure 5 - Schematic representation of the experimental design of a 96-well plate for absorbance reading of MTT exposed cells after contact with compounds in test for 24H.

C-	Negative control	X5	Conjugate 1:200 (75µg/mL)
C+	Positive control	X6	Conjugate 1:200 (25µg/mL)
X1	Conjugate 1:100 (200µg/mL)	X7	Physical mixture: CTX+LMWChi (200µg/mL)
X2	Conjugate 1:100 (75µg/mL)	X8	Physical mixture: CTX+LMWChi (75µg/mL)
X3	Conjugate 1:100 (25µg/mL)	X9	Physical mixture: CTX+LMWChi (25µg/mL)
X4	Conjugate 1:200 (200µg/mL)	X10	---

Table 5 - MTT: Plate B, B1, D and D1 disposition of controls and compounds in test

15.2 LDH assays

LDH assays can be performed by assessing LDH released into the media as a marker of dead cells or performing lysis LDH as a marker of remaining live cells. Unlike many other cytoplasmic enzymes which exist in many cells either in low amount (e.g., alkaline and acid phosphatase) or unstable, LDH is a stable

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cytoplasmic enzyme present in all cells and rapidly released into the cell culture supernatant upon damage of the plasma membrane.

Lactate dehydrogenase (LDH) is an oxidoreductase present in a wide variety of organisms. LDH catalyzes the interconversion of pyruvate and lactate, with the concomitant interconversion of NADH and NAD. When disease, injury or toxins damage tissues, cells release LDH into the bloodstream. Being a fairly stable enzyme, LDH has been widely used to evaluate the presence of damage and toxicity of tissue and cells. Quantification of LDH has broad range of applications. In this colorimetric LDH quantification assay, LDH reduces NAD to NADH, which then interacts with a specific probe to produce a color ($\lambda_{max} = 450 \text{ nm}$). This assay is quick, convenient, and sensitive (Sigma-Aldrich, 2014b, Halprin and Ohkawara, 1966).

To save time and reagents the LDH assays took place at the same time as the MTT. Since the LDH is performed taking in consideration the release of LDH to the extracellular medium after cell exposure to the compounds in test, after the previously described procedure in the «MTT assay» section until the 24 or 48H of exposure step, 100 μL of each well was transferred to new 96-well plates. In the first plates (where MTT assays took place) 100 μL of cell culture medium was added so the MTT could proceed, while in the new plates the LDH assay proceeded as described next. With this said the LDH 96-well plates took the same disposition as the MTT plates.

Briefly at time of use the lactate dehydrogenase assay mixture was prepared by mixing equal volumes of LDH assay substrate solution, LDH assay dye solution, and LDH assay cofactor preparation, as the manufacturer protocol (Sigma-Aldrich, 2014a). As the LDH plates already had 100 μL of extracellular medium exposed to the compounds, 100 μL of the lactate dehydrogenase assay mixture were added to each well in a volume equal to twice

the volume of medium removed for testing (200 μL). Plates were covered with opaque aluminum foil to protect from light, and left to incubate at room temperature for 30 minutes. At this point the reaction was terminated by the addition of 20 μL of 1N HCl to each well. Finally spectrophotometrically measure of absorbance at a wavelength of 490nm took place in a multiwell plate reader. Background absorbance of the multiwell plates was read at 690nm since this value had to be subtracted from

the primary wavelength measurement (490 nm) (Sigma-Aldrich, 2014a). Data was analyzed using GraphPad Prism[®] version 6.0c.

16. Statistical analysis

Results for MTT, LDH and DLS (physical characterization of the conjugates) were expressed as the mean \pm SD and statistical significance was tested by one-way-ANOVA with a Tukey's multiple comparison *post hoc* test comparing formulations results to control, where P-values below 0.05 were considered statistically significant, in GraphPad Prism[®] version 6.0c.

Bradford method assays results were expressed by means \pm SD of at least three experiments and statistical significance was tested by paired t-test, where P-values below 0.05 were considered statistically significant, in GraphPad Prism[®] version 6.0c.

Part IV
Results
and
discussion

Results and discussion

Synthesis of cetuximab modified chitosan is outlined in **Fig. 8**. Here, the carboxylic group of modified cetuximab is reacted with the NH₂ group of chitosan in the presence of the chosen linker carbodiimide linker EDC and its coadjutant NHS.

Regarding the temperature and time of main reaction at which conjugates synthesis was conducted, as previously mentioned, a combination of two different approaches were taken in consideration: ≈4°C and room temperature (RT) for 24 and 48H (Lee et al., 2012, Gaspar et al., 2013, Support, 2012-2013).

1. Cellulose acetate electrophoresis

It was expected a clear difference in microscope slides containing cellulose acetate strips with the results after the electrophoresis protocol, showing a much heavier product than cetuximab, LMWChi or the physical mixture of both of them without the linker action.

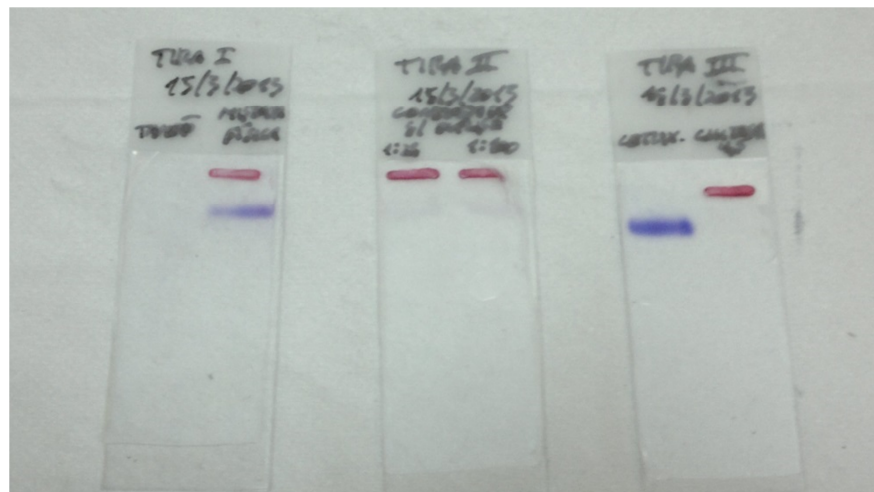


Fig. 9 Figure depicting one of the obtained products from a cellulose acetate electrophoresis assay, showing three strips where samples of: buffer, physical mixture (CTX+LMWChi), conjugate 1:25, conjugate 1:100, CTX and LMWChi, run being separated by molecular weight.

Results and discussion

Despite the expected, the obtained results, assay after assay, show no difference between physical mixture of cetuximab with chitosan (2nd product from left), and the several produced conjugates using the same molecular ratios (in the above figure can be seen conjugates 1:25 and 1:100, although all the other conjugates presented similar results). Thus not indicating that a conjugate wasn't produced but not confirming it either.

As results show cellulose acetate electrophoresis is not a reliable conjugate confirmation method in this case. Probably due to conjugates, LMWChi and other compounds sensibility to pH, or even their inadequate separation by the cellulose acetate membrane due to cetuximab as well as conjugates high molecular weight.

2. Chemical analysis interaction

2.1. FTIR analysis

FTIR spectral analysis was used to study the chemical modifications occurring in the conjugation procedure between cetuximab (CTX) and low molecular weight chitosan (LMWChi). In the below FTIR spectra, typical peaks were identified around 1633 cm⁻¹ which corresponds to the N-H bending vibration band of CTX, through the presence of amide and amide bands (arrow 1500-1690cm⁻¹ range) (Amoozgar et al., 2012). It was also identified a rudimentary peak at around 1734 cm⁻¹ due to carbonyl stretching of C-N (typical C-N aliphatic stretch: 1025-1250 cm⁻¹).

Specially for the 1:1, 1:25 and 1:50 conjugate's spectrums, a peak was observed near 1633 cm⁻¹ confirming the formation of amide bond between LMWChi and CTX (Anitha et al., 2009, Anitha et al., 2011, Deepagan et al., 2012).

Despite these results do not provide the ultimate evidence for the formation of the conjugate they add prove to the presence of Cetuximab in the final product, and so indirectly pointed out for a successful conjugation. As the Thermo Scientific Pierce Concentrators, with a specifically membrane pore of 150K MWCO, have been used as disposable ultrafiltration centrifugal devices for concentration and diafiltration of our conjugation residues: free CTX≈145K MW, free LMWChi≈50K to 37500 MW ,

free EDC \approx 191,70 MW and free NHS \approx 115,09 MW – these are non-reacted compounds with a lower MW than that of the membrane pore, - it can almost be assured that the non diafiltered compound as having a MW of 150K+, so a conjugate of the initial compounds. Even though, the presence of cetuximab and conjugation efficiency of CTX, should be confirmed via BCA assay or other protein analysis techniques like the Bradford method.(Maya et al., 2013)

Concerning synthesis temperature, it doesn't appear to have that much of an impact in the final product. With the same protocol except for synthesis temperature, a first synthesis was carried at 4°C (Lee et al., 2012) (**Fig. 10** and **Fig. 11**) to make sure the stability of cetuximab was maintained and there weren't significant changes to FTIR signal compared to the conjugates produced at room temperature (RT) (**Fig. 12** and **Fig. 13**).

Furthermore, a deeper analysis of the FTIR spectrum shows that ratios of 1:200 mol and 1:100 mol (CTX:LMWChi) seem to be unfavorable to conjugation procedure (**Fig. 10** and **Fig. 11**), probably due to the presence of multiple free amine groups in LMWChi, which might be conditioning the reaction, or probably masking the FTIR analysis.

Results and discussion

2.1.1. Synthesis of conjugates at 4°C

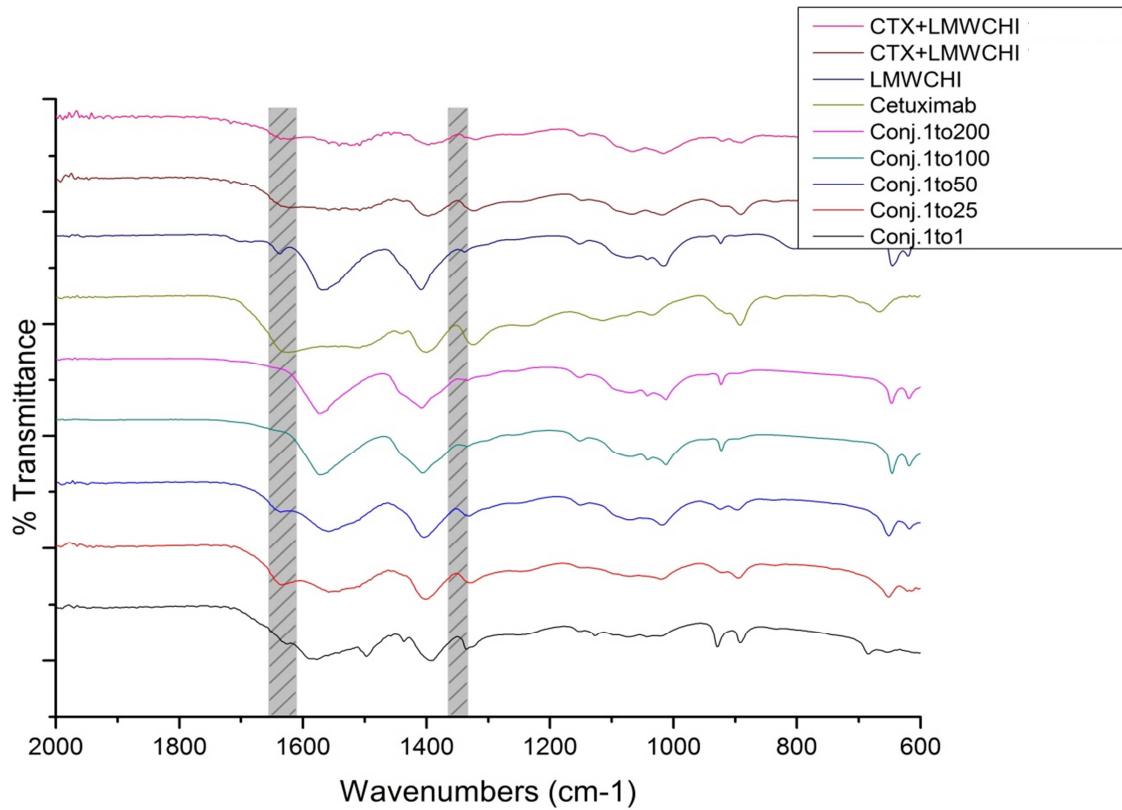


Fig. 10 – Conjugates 1:1, 1:25, 1:50, 1:100, 1:200, synthesized at 4°C FTIR spectrum, compared to the ones of mixtures of CTX+LMWChi without reaction.

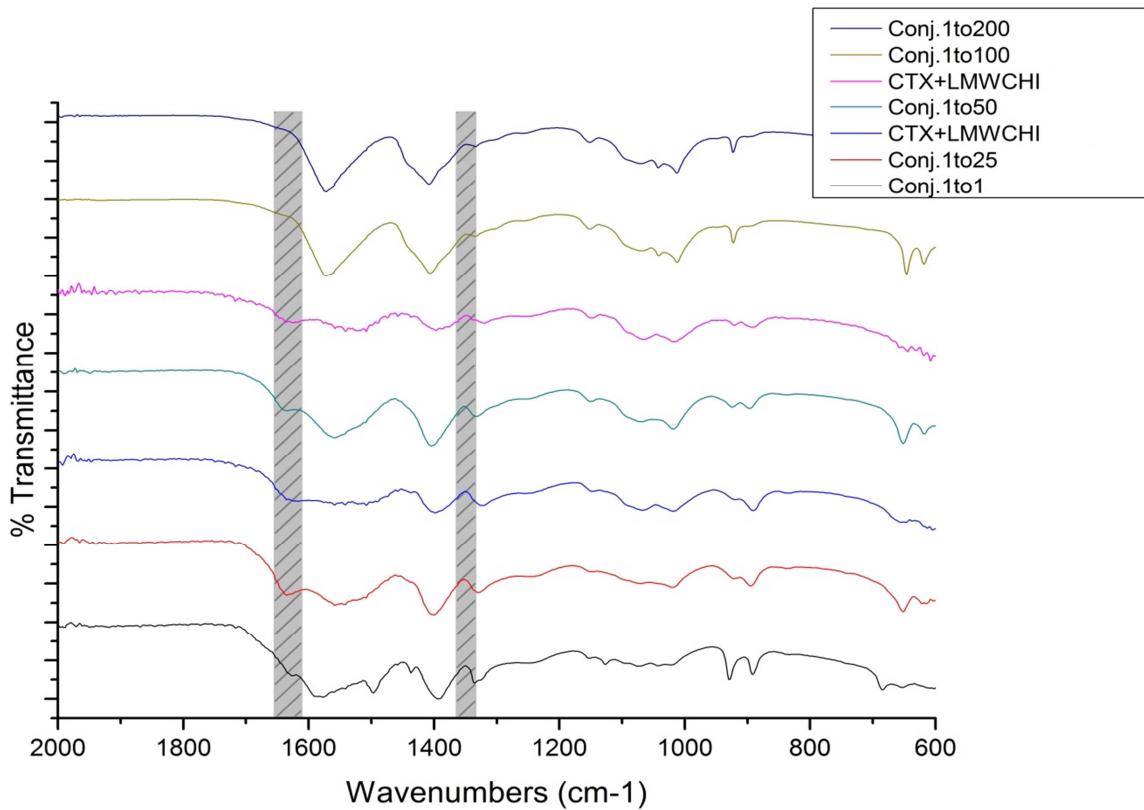


Fig. 11 - Conjugates 1:1, 1:25, 1:50, 1:100, and 1:200 synthesized at 4°C FTIR spectrum, compared to the ones of mixtures of CTX+LMWChi without reaction.

2.1.2. Synthesis of conjugates at RT

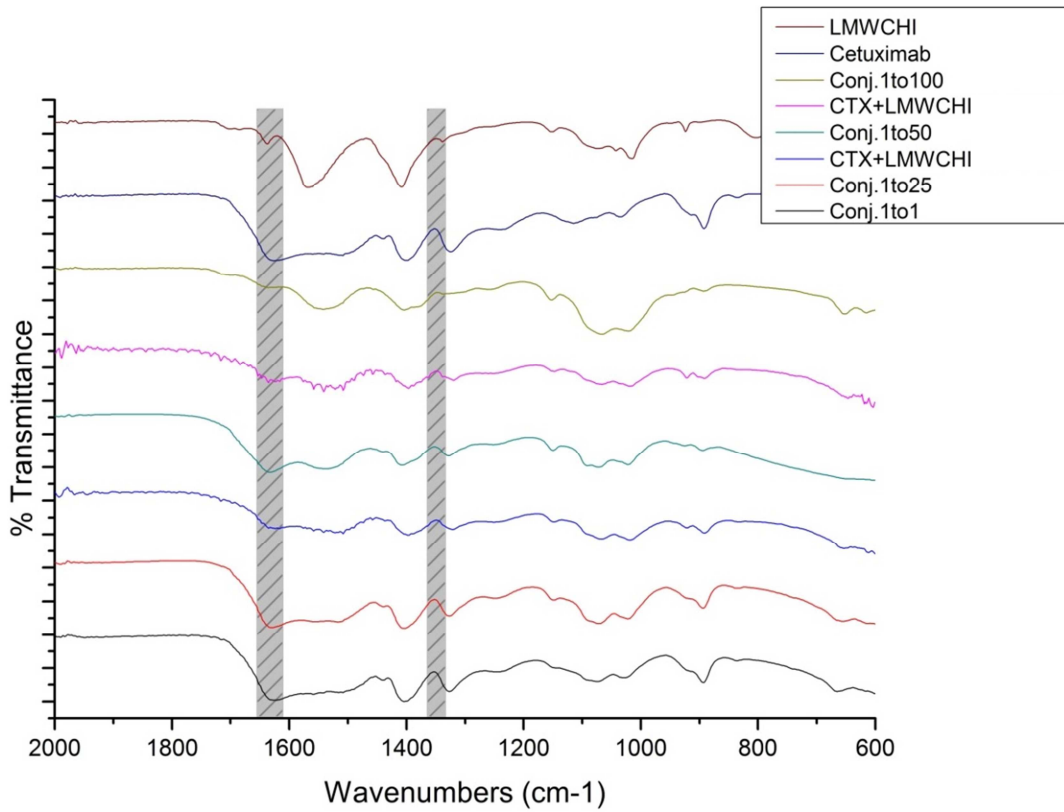


Fig. 12 - Conjugates 1:1, 1:25, 1:50, and 1:100, synthesized at $\approx 22^\circ\text{C}$ (RT) FTIR spectrum, compared to the ones of CTX, LMWChi and mixtures of CTX+LMWChi without reaction.

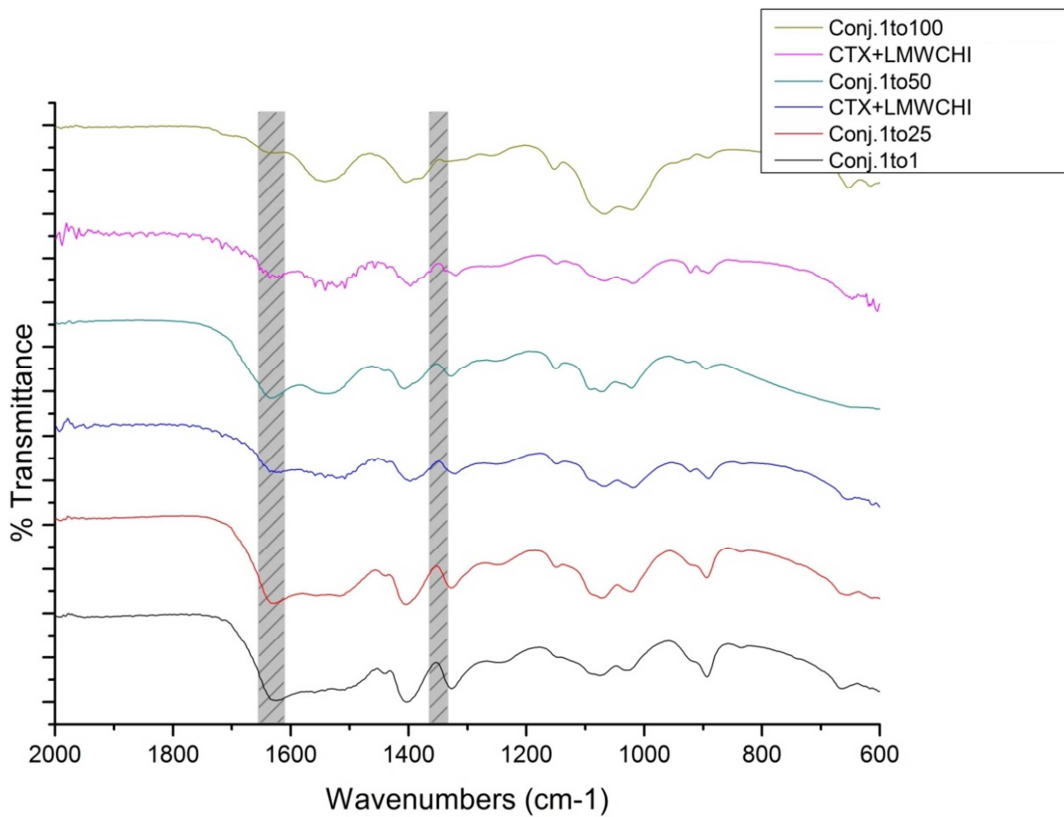


Fig. 13 - Conjugates 1:1, 1:25, 1:50, 1:100, synthesized at RT, FTIR spectrum, compared to the ones of CTX, LMWChi and mixtures of CTX+LMWChi without reaction

Results and discussion

Clearly, and despite the literature (Lee et al., 2012), conjugation reaction taken at 4°C was contra-productive when comparing results with the synthesis at room temperature, since the results obtained through the FTIR analyses show a weaker signal - **Fig. 10** to **Fig. 13**.

3. Protein quantification

3.1. Bradford method

The results obtained on protein quantification through the Bradford method seem to support the FTIR results indicating a higher percentage of protein concentration relatively to the initial cetuximab concentration used to synthesize the conjugate, in the final product in contrast to the filtered compounds remains.

At this point after the ultrafiltration process recurring to the disposable 150K MW filters, we would either have cetuximab in membrane retained «conjugate» or its corresponding, and membrane trespassed, «filtrate». What can be seen in the graphic represented on **Fig. 14** is the final cetuximab concentration (mg/mL) in relation to the initial used concentration for each conjugation process.

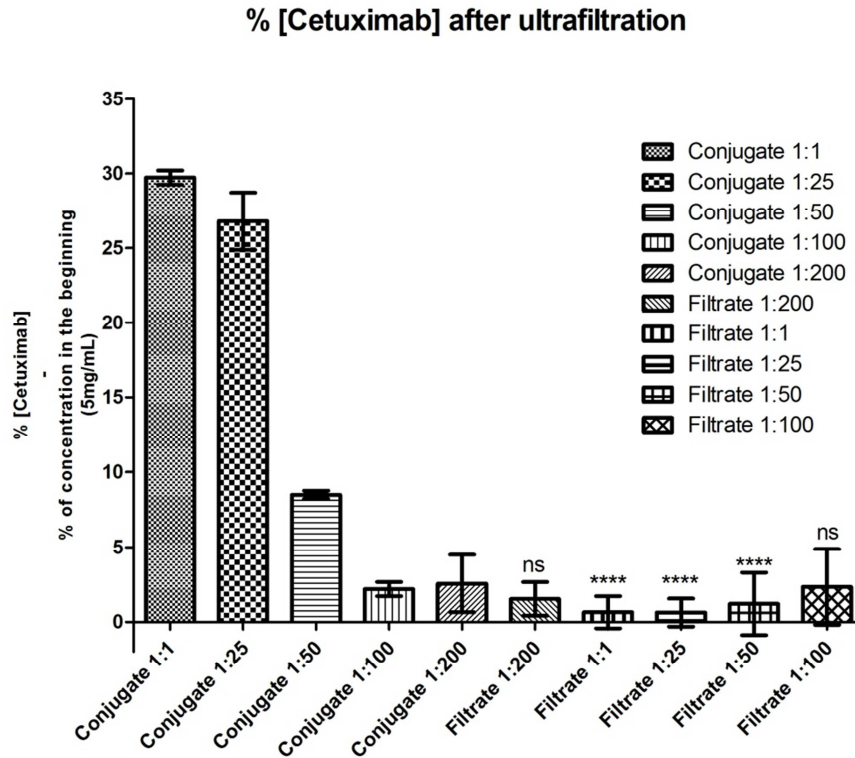


Fig. 14 - Relationship of final [CTX] with the initial concentration, for ultrafiltration products: retained conjugates in the filter's membrane, and filtered products that crossed the membrane.

Results are means \pm SD from at least three independent assays. Statistical significance was accessed by paired t-test. * Indicates $p \leq 0.05$; ** Indicates $p \leq 0.01$; *** indicates $p \leq 0.001$; **** Indicates $p \leq 0.0001$.

Clearly there is a significant difference between pre-membrane (conjugates) products and post-membrane (filtrates) product, conjugates with a cetuximab chitosan molecular ratio of 1:1, 1:25, and 1:50, presented a higher protein concentration, being this protein the used antibody cetuximab. This supports data from FTIR where as previously mentioned conjugates 1:25 and 1:50 seem to be closer to what was expected, a successful cetuximab-chitosan conjugate.

Despite some possible weak interference from LMWChi, presented the same conditions is reasonable to say, at very least, that a molecular proportion of CTX to LMWChi of 1 to 1; 1 to 25 and 1 to 50 seems to produce the most viable conjugates than the rest of tested ratios.

Even though the results obtained, a more sensitive assay for protein quantification should be used to validate and confirm the presented results. Conjugation efficiency of CTX with LMWChi could be confirmed recurring to a BCA assay (Deepagan et al., 2012), though Bradford method for protein quantification was elected due to economic and availability reasons.

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4. Size characterization by Dynamic Light Scattering

Despite the positive results obtained in the other techniques, all the samples from conjugate 1:25 were flocculated when submitted to DLS analyses, meaning that a non-viable compound was formed and unable to be measured.

With this said results obtained are displayed in the table below, where is presented the mean and SD of three samples for each compound.

Compound		Z-average (nm)	Pdi	Zeta Potential (mV)
LMWChi	Mean	539±71	0.66±0.12	77.9±3.6
Conjugate 1:1	Mean	1156±216	0.96±0.07	33.2±2.0
Conjugate 1:50	Mean	270±49	0.76±0.07	82.5±4.6
Conjugate 1:100	Mean	414±82	0.89±0.07	98.8±6.1
Conjugate 1:200	Mean	484±60	0.90±0.07	102.7±4.0

Table 6 - Physical-chemical properties of prepared LMWChi and developed conjugates

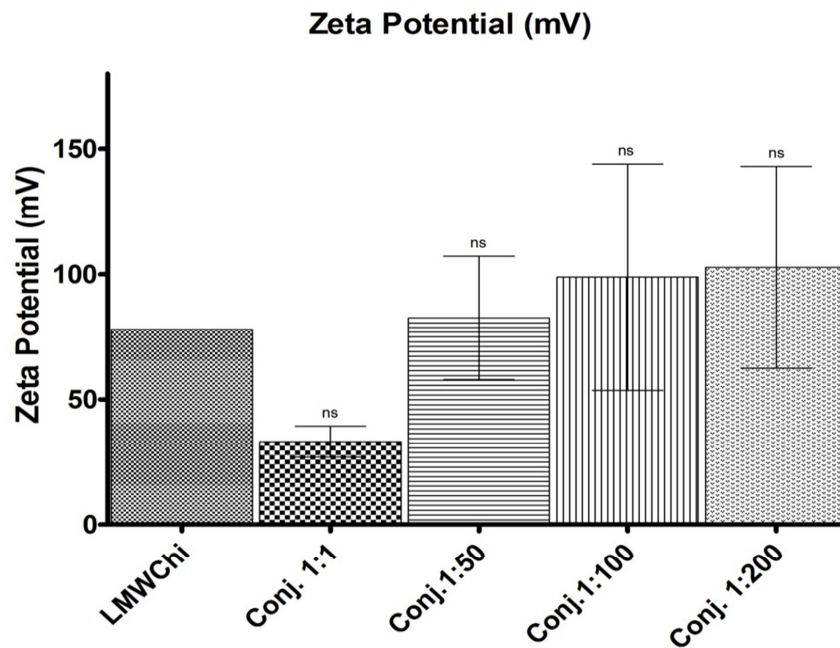


Fig. 15 - Zeta Potential (mV) statistical analysis for Conjugates 1:1, 1:50, 1:100 and 1:200, comparing to LMWChi as control.

Results are means \pm SD from, at least, eight replicates. Statistical significance was accessed by One Way ANOVA with a Tukey's multiple comparison post hoc test comparing conjugates results to LMWChi.

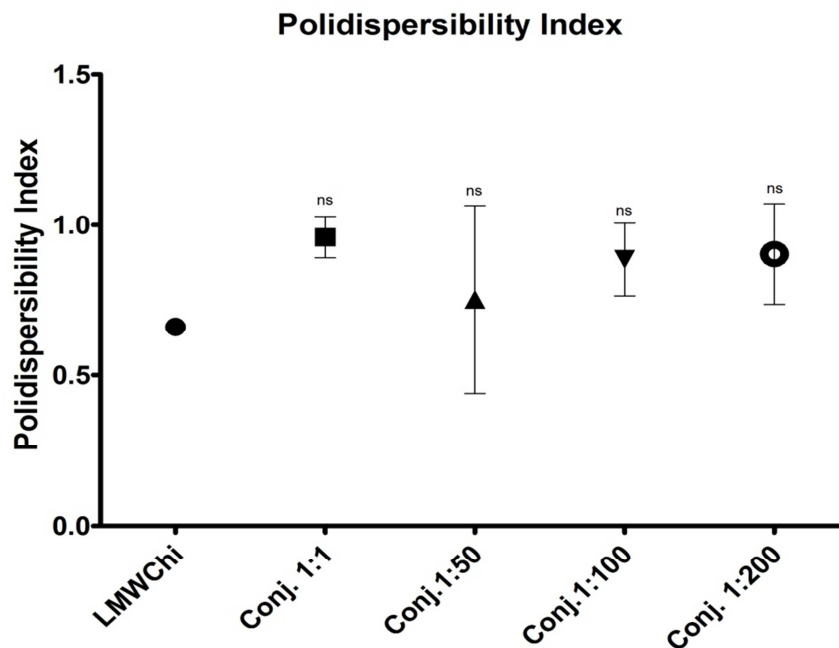


Fig. 16 - Polidispersibility index (Pdi) statistical analysis for Conjugates 1:1, 1:50, 1:100 and 1:200, comparing to LMWChi as control.

Results are means \pm SD from, at least, eight replicates. Statistical significance was accessed by One Way ANOVA with a Tukey's multiple comparison post hoc test comparing conjugates results to LMWChi.

Results and discussion

When it comes to Pdi, values greater than 0.7 indicate that the sample has a very broad size distribution, which indicates us that none of the analyzed conjugates was a homogeneous solution.

The Z-average size increases as the particle size increases, therefore it provides a reliable measure of the average size of a particle size distribution.

Looking to results above, Z-average values seem to be inconsistent, and actually in some samples unexpectedly high. However, these results are not totally consistent with data resulting from the FTIR, this might have something to do with the samples stability over time since some samples were prepared weeks before DLS measurements thus resulting in these values. Also that can be attested by samples Zeta Potential since the same samples with a high Z-average have a low Zeta Potential, which probably has to do with their stability over time. Still, samples from conjugate 1:50 seem to present stability as their average Zeta Potential > 60 (mV).

5. Evaluation of compounds cytotoxicity

5.1. MTT assays

As previously described in «Materials and Methods» cells were exposed to cycles of 24H and 48H to the different compounds/chemicals used in the conjugation reaction, in order to evaluate conjugates cytotoxicity as well as all other compounds, and then MTT and LDH assays were performed.

Metabolic activity/cell viability was expressed in percentual terms, as a fraction of the initial amount of cells (set as 100%).

5.1.1. MTT – 24H Exposure – A549

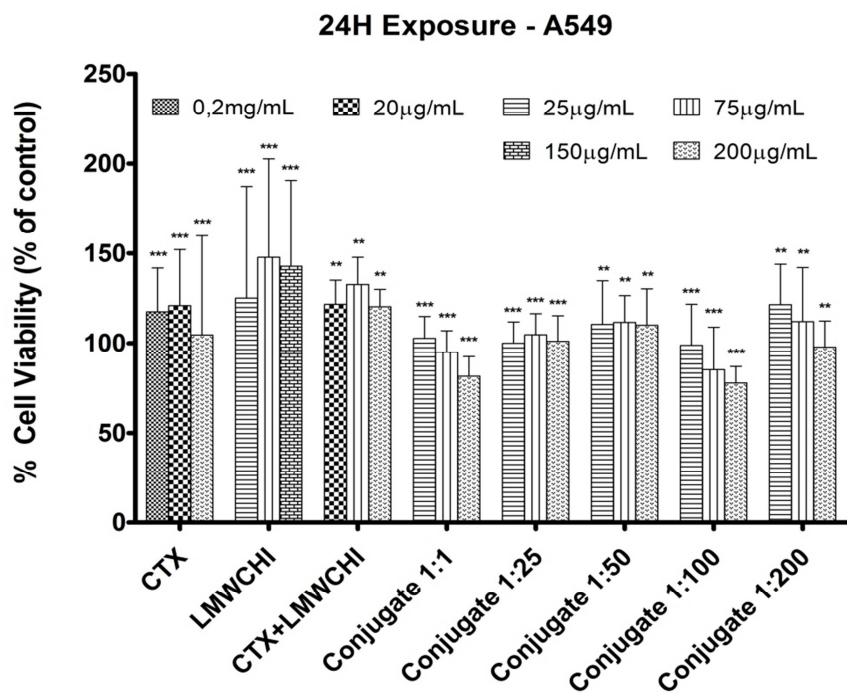


Fig. 17 – MTT assays - % Cell Viability (% of control) for 24H Exposure to several conjugates and their predecessors, on A549 cells

Cell viability was expressed as the ratio between the number of viable cells and the total amount of cells. Results are means \pm SD from, at least, eight replicates. Statistical significance was accessed by One Way ANOVA with a Tukey's multiple comparison post hoc test comparing results to control.

* Indicates $p \leq 0.05$; ** Indicates $p \leq 0.01$; *** Indicates $p \leq 0.001$; **** Indicates $p \leq 0.0001$

Results and discussion

5.1.2. MTT – 24H Exposure – Calu3

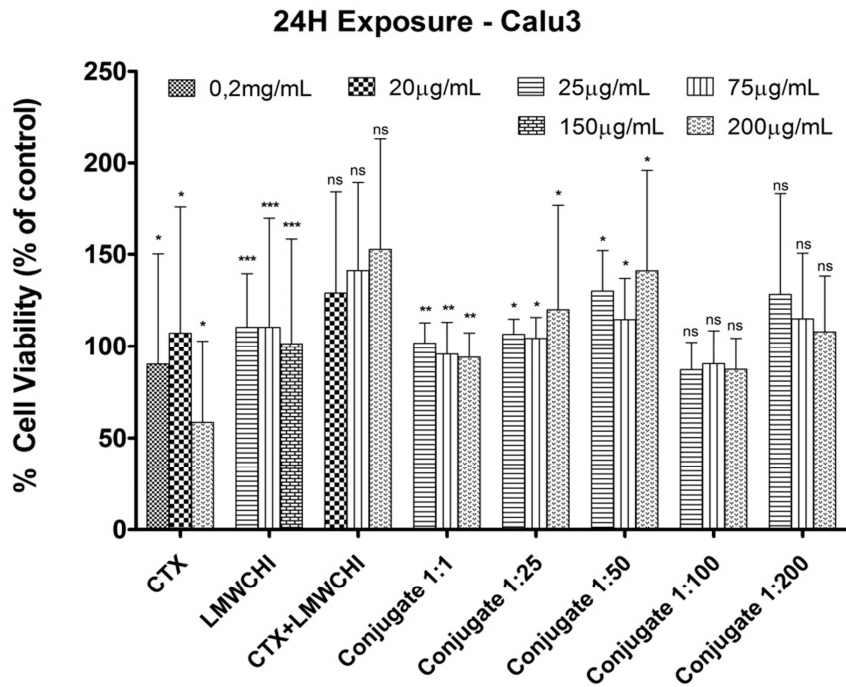


Fig. 18 – MTT assays - % Cell Viability (% of control) for 24H Exposure to several conjugates and their predecessors, on Calu3 cells

Cell viability was expressed as the ratio between the number of viable cells and the total amount of cells. Results are means \pm SD from, at least, eight replicates. Statistical significance was accessed by One Way ANOVA with a Tukey's multiple comparison post hoc test comparing results to control. * Indicates $p \leq 0.05$; ** Indicates $p \leq 0.01$; *** Indicates $p \leq 0.001$; **** Indicates $p \leq 0.0001$.

5.1.3. MTT – 48H Exposure – A549

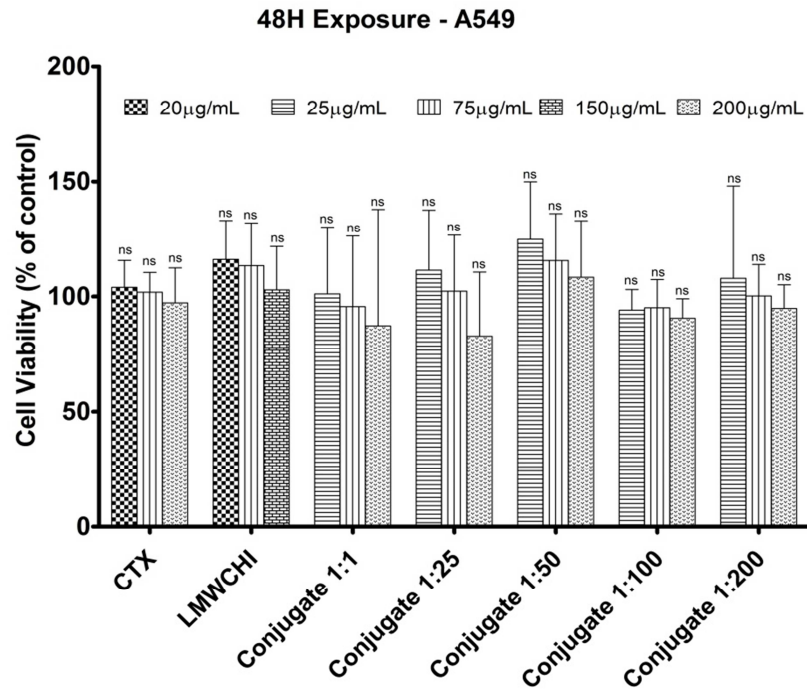


Fig. 19 – MTT assays - % Cell Viability (% of control) for 48H Exposure to several conjugates and their predecessors, on A549 cells

Cell viability was expressed as the ratio between the number of viable cells and the total amount of cells. Results are means \pm SD from, at least, eight replicates. Statistical significance was accessed by One Way ANOVA with a Tukey's multiple comparison post hoc test comparing results to control. ns Indicates $p \geq 0.05$ (non-significant).

Results and discussion

5.1.4. MTT – 48H Exposure – Calu3

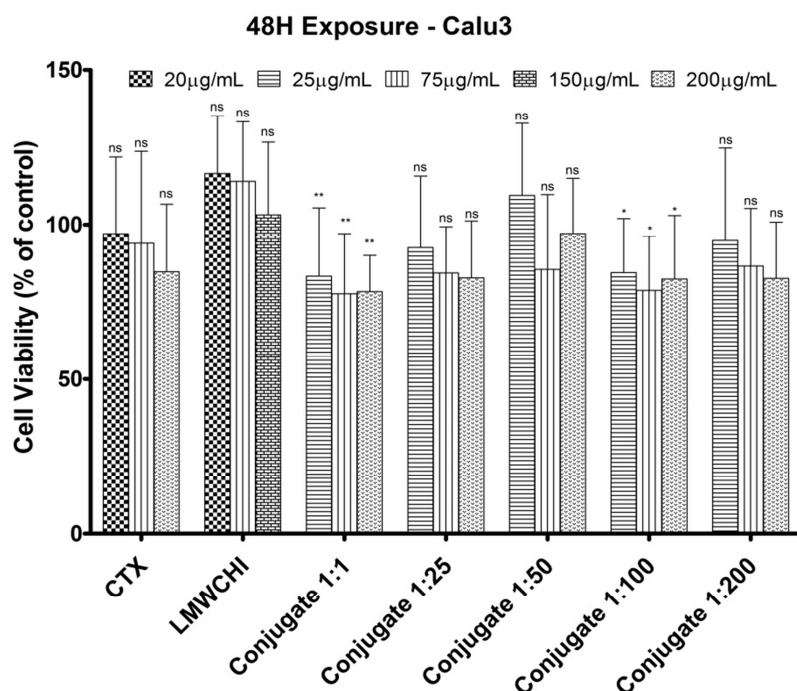


Fig. 20 – MTT assays - % Cell Viability (% of control) for 48H Exposure to several conjugates and their predecessors, on Calu3 cells

Cell viability was expressed as the ratio between the number of viable cells and the total amount of cells. Results are means \pm SD from, at least, eight replicates. Statistical significance was accessed by One Way ANOVA with a Tukey's multiple comparison post hoc test comparing results to control. * Indicates $p \leq 0.05$; ** Indicates $p \leq 0.01$; *** Indicates $p \leq 0.001$; **** Indicates $p \leq 0.0001$.

Upon solubilization, the amount of formazan formed is determined through spectrophotometry. MTT reduction increases with metabolic activity; the amount of formazan is thus an indicator of cell viability (Houghton et al., 2007). In this sense, MTT assays provide additional information, besides that provided by other cytotoxicity assays. If a compound is proven to have cytostatic effect, MTT assays may inform if the cells are still viable, that is, if they are still able to proliferate when in non-adverse conditions. However, it should be mentioned that these MTT assay presents some disadvantages as well. For instance, it shows poor linearity at high cell densities and their end-point requires time-sensitive measurements. Besides, since it's an assay dependent on mitochondrial activity, the results given are more prone to variability between different cell lines, thus requiring cell line-specific optimization (Houghton et al., 2007, Keepers et al., 1991, Pauwels et al., 2003).

Results and discussion

As tested concentrations were different for the compounds to which cells were exposed, in the statistical analysis it was disposed as «Low», «Medium» and «High» concentrations so some comparison could be made.

Evaluation of compounds cytotoxicity through MTT assays reveals no apparent toxicity to A549 and Calu3 cell lines, when exposed to 24 and 48H cycles, despite this further study should be taken to guarantee safety.

Results and discussion

5.2. LDH assays

LDH results turned out to be similar to those of MTT: evaluation of compounds cytotoxicity through LDH assays reveals no apparent toxicity to A549 and Calu3 cell lines, when exposed to 24 and 48H cycles.

Metabolic activity/cell viability was expressed in percentual terms, as a fraction of the initial amount of cells (set as 100%), and the method was followed as described in the manufacturer guidelines (Sigma-Aldrich, 2014b)

5.2.1. LDH – 24H Exposure – A549

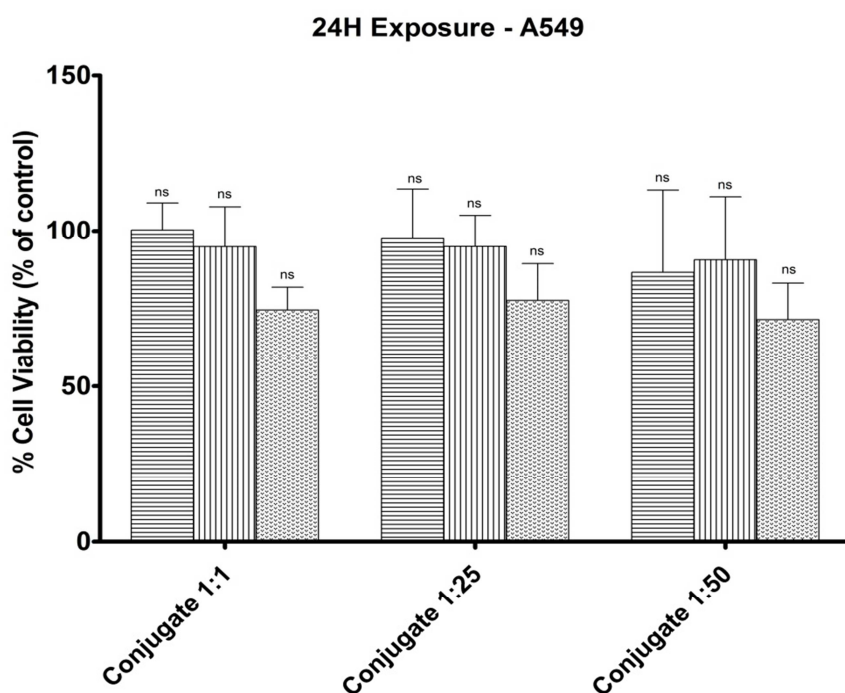


Fig. 21 - LDH assays - % Cell Viability (% of control) for 24H Exposure to several conjugates, on A549 cells

Cell viability was expressed as the ratio between the number of viable cells and the total amount of cells. Results are means \pm SD from, at least, eight replicates. Statistical significance was accessed by One Way ANOVA with a Tukey's multiple comparison post hoc test comparing results to control. ns Indicates $p \geq 0.05$ (non-significant).

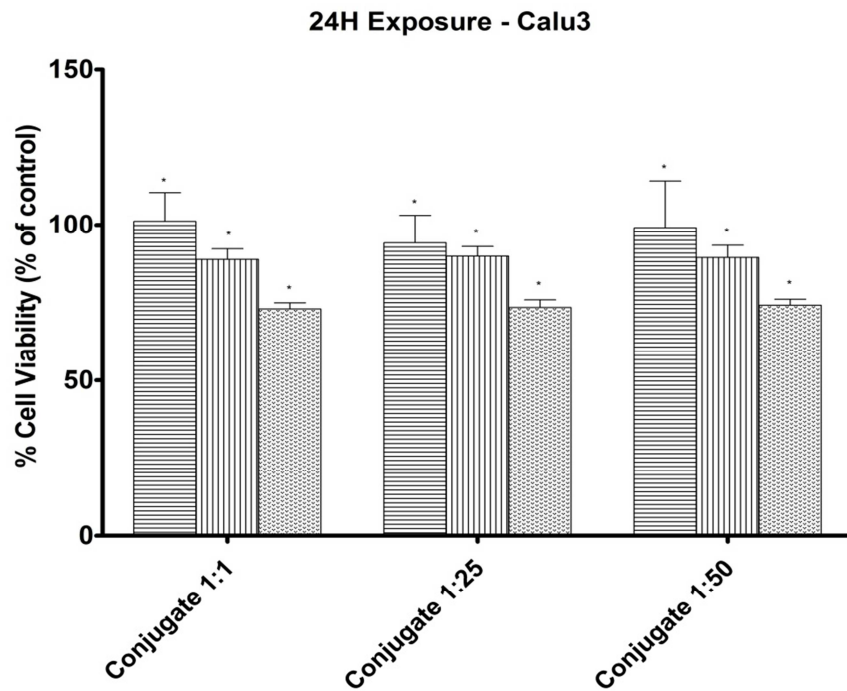
5.2.2. LDH – 24H Exposure – Calu3

Fig. 22 - LDH assays - % Cell Viability (% of control) for 24H Exposure to several conjugates, on Calu3 cells

Cell viability was expressed as the ratio between the number of viable cells and the total amount of cells. Results are means \pm SD from, at least, eight replicates. Statistical significance was accessed by One Way ANOVA with a Tukey's multiple comparison post hoc test comparing results to control. * Indicates $p \leq 0.05$; ** Indicates $p \leq 0.01$; *** Indicates $p \leq 0.001$; **** Indicates $p \leq 0.0001$.

Results and discussion

5.2.3. LDH – 48H Exposure – A549

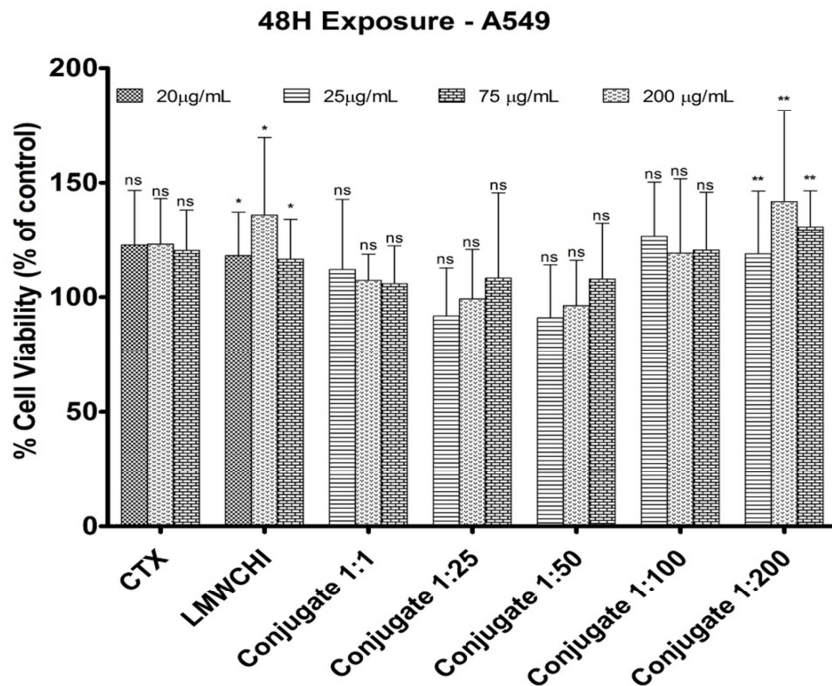


Fig. 23 - LDH assays - % Cell Viability (% of control) for 48H Exposure to several conjugates and their predecessors, on A549 cells

Cell viability was expressed as the ratio between the number of viable cells and the total amount of cells. Results are means \pm SD from, at least, eight replicates. Statistical significance was accessed by One Way ANOVA with a Tukey's multiple comparison *post hoc* test comparing results to control. * Indicates $p \leq 0.05$; ** Indicates $p \leq 0.01$; *** Indicates $p \leq 0.001$; **** Indicates $p \leq 0.0001$.

Statistical analysis reveals no significant difference in the percentage of viable cells after a 48 hours exposure to most of the compounds for the same cell line.

5.2.4. LDH – 48H Exposure – Calu3

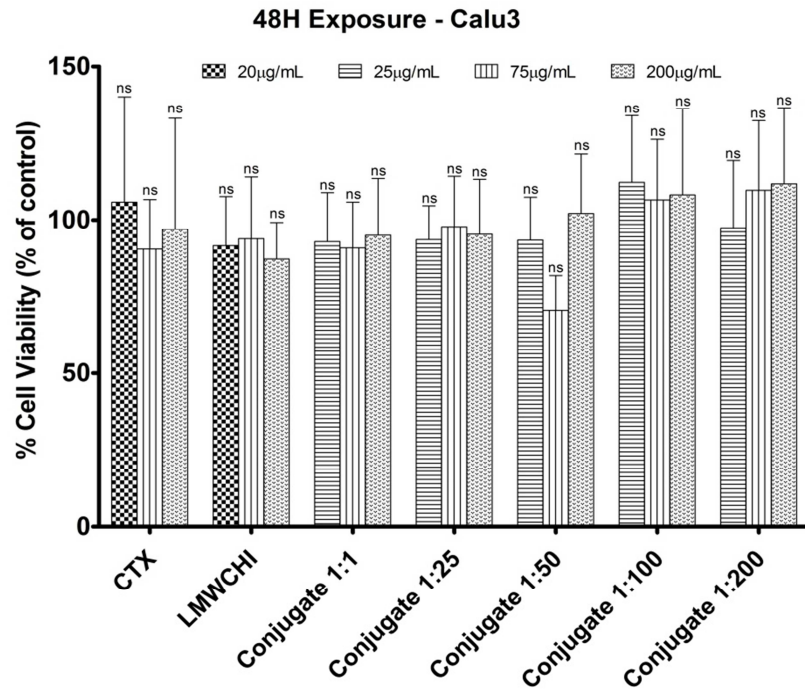


Fig. 24 - LDH assays - % Cell Viability (% of control) for 48H Exposure to several conjugates and their predecessors, on Calu3 cells

Cell viability was expressed as the ratio between the number of viable cells and the total amount of cells. Results are means \pm SD from, at least, eight replicates. Statistical significance was accessed by One Way ANOVA with a Tukey's multiple comparison post hoc test comparing results to control. ns Indicates $p \geq 0.05$ (non-significant).

Some LDH assays couldn't be completed or even done at all seen the unavailability of LDH reagents; this was the case for 24H cycles of exposure to the majority of conjugates, CTX, LMWChi, and the last ones physical mixture. For 24H exposure only conjugates 1:1; 1:25 and 1:50 were tested since these were the ones with better results in the previous evaluated parameters.

Clearly results obtained in the above LDH assays are far from satisfactory as some undisclosed interference occurred leading to some of statistic results, in fact results for 24H exposure to the test compounds shouldn't be taken in consideration as, if there was time and materials, they would have been redone.

Part V
Conclusions

Conclusions

Since Richard Nixon declared War on Cancer in 1971, the US National Cancer Institute has poured some \$90 billion into research and treatments. Yet a cure remains elusive and some claim that Cancer will probably never be completely eradicated. Recent research raises a sobering possibility: that cancer may simply be here to stay. Researchers at Kiel University, the Catholic University of Croatia and other institutions discovered that hydra — tiny, coral-like polyps that emerged hundreds of millions of years ago — form tumors similar to those found in humans. These findings suggest that human cells ability to develop cancer is an intrinsic property that has evolved at least since then (Domazet-Loso et al.). All this means that cancer genes, plus the mechanisms that allow tumor cells to evade death and invade healthy tissue, “have deep evolutionary roots,” the researchers wrote.

While human cells will probably always have the ability to “make mistakes” that trigger cancer, biotechnology will allow humanity to successfully treat and clean a patient completely and forever of cancer cells in a more or less earlier stage. In the future one cancer fighting strategy that’s already gaining expression will be to trigger the immune system potential against these cells. Cancer Immunotherapy have recently cemented its potential in patients and swayed even the skeptics. Another strategy is already in action and recurs to biotechnology to improve the action or transform already used drugs, into more efficient and with less side-effects ones.

From this work we can conclude that the use of cetuximab-LMWChi conjugates as drivers to targeted siRNA therapies seems viable, even though further work must be done and some alternatives should be taken into consideration.

FTIR analyses show us that the ideal cetuximab:LMWChi molecular ratio seems to be 1:25mol and 1:50mol, when using the zero length carbodiimide linker EDC/NHS for the conjugation procedure. These results are confirmed by the Bradford method for protein quantification as conjugates produced with a ratios of 1:1, 1:25 and 1:50mol (CTX:LMWChi), have higher protein (CTX) content.

Despite the identification of a possible link between CTX and LMWChi - thus the formation of a conjugate - in the previously mentioned compounds, DLS analyses

Conclusions

revealed flocculation for conjugates 1:25 samples. Leaving only conjugate 1:50 and 1:1 as the most successful compounds within the expected, this may raise the question if another more stable chitosan shouldn't be studied as an alternative.

Moving forward, conjugate 1:50 presented Z-average values far below the expected which doesn't support the idea of a successful conjugation formation, leaving us with some doubts about this product, nevertheless this compound presented promising results from FTIR.

Furthermore, and as expected from previous work, none of the intervenient compounds seems to be hazardous to A549 or Calu3 cell lines as MTT and LDH results seem to support, despite the fact that a too high cellular density might have been used. As previously mentioned some undisclosed interference occurred in the LDH assays leading to results unable to be interpreted, this probably as to do with the referred high cellular density used or perhaps, undetected contamination of cell cultures, plates or even reagents. With this said it's highly advisable for future studies to be previously determined the Ideal cell densities by plotting calibration curves for each cell line.

Taken together, these findings demonstrate that, when it comes to cetuximab:LMWChi conjugates, ratios of 1:1; and 1:50 are the most promising to be considered in future development of nanoparticles for encapsulate siRNA and targeting to EGFR overexpressing cells

Such characteristics make these promising lead compounds for further studies.

Part VI
Future
perspectives

Future perspectives

The present work here described sets the ground for the prosecution to further studies, in order to test the possible application of the obtained conjugates into target nanoparticles to be developed.

Referring to molecular ratios of cetuximab to LMW Chitosan a reasonable amount of ratios was already tested in this work even though, if another linker is chosen different ratios might be taken into consideration, as well as other Chitosans.

Our best tool to characterize and identify the presence of the expected conjugate was FTIR, despite some purification of the final product recurring to ultrafiltration, HPLC with an appropriate column should be considered in future characterization studies. It would also be a plus to validate the referred HPLC method if this kind of separation method is meant to be considerate.

Concerning protein/conjugate quantification methods it would be interesting to recur to a BCA protein assay kit in future studies, seen its sensibility, reliability and easy reproduction.

Regarding cytotoxicity studies, cellular density should be reviewed prior new MTT and LDH assays, it was probably our main interference.

Furthermore, electron microscopy could be useful to confirm the effect of conjugates/future developed nanoparticles on cells.

Finally, *in vivo* assays should be pursued in future work within the research group, since it will also add valuable information about conjugates efficiency, safety and feasibility as to be used in future siRNA targeting therapies.

Part VII
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“History repeats, but science reverberates.”

— Siddhartha Mukherjee, *The Emperor of All Maladies*