Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Scienze Chimiche

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE MOLECOLARI

INDIRIZZO: SCIENZE CHIMICHE

CICLO XXVIII

SYNTHESIS AND EVALUATION OF NEW PRODRUG SYSTEMS OF THE NATURAL POLYPHENOL CURCUMIN

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“Libertas, quae non in eo est ut iusto utamur domino, sed ut nullo.”

(Cicerone, De Re Publica, II, 43)

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Abbreviations and acronyms

ACN: Acetonitrile

CMC: Critical micellar concentration

CROP: Cationic Ring Opening Polymerization

Curc: Curcumin

DCM: Dichloromethane

DMAP: 4-dimethylaminopyridine

DLS: Dynamic light scattering

DMF: Dimethylformamide

DMSO: Dimethylsulfoxide

ESI-MS: Electro-spray ionization-Mass Spectrometry

EtOAc: Ethyl acetate

HPLC: High Performance Liquid Chromatography

HSA: Human Serum Albumine

Ile: Isoleucine

Leu: Leucine

MeOH: Methanol

MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium)

NMR: Nuclear Magnetic Resonance

PBS: Phosphate Buffered Saline

PEG: Polyethyleneglycol

PLA: Polylactic acid

PMOXA: Poly-2-methyl-2-oxazoline

POXA: Poly-2-alkyl-2-oxazoline

PVP: Polyvinylpirrolidone

TEM: Transmission Electron Microscopy

TFA: Trifluoroacetic acid

TIPS: Tri-isopropylsilane

UPLC: Ultra Performance Liquid Chromatography

UV: Ultraviolet

Val: Valine

Riassunto

La tesi descrive la sintesi e la caratterizzazione di nuovi prodrugs carbamoilici della Curcumina ((1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), un importante polifenolo naturale cui vengono accreditate molteplici proprietà benefiche ed un ampio spettro d'azione. L'utilizzo in campo farmaceutico del composto naturale tal quale è però limitato dalla sua bassa biodisponibilità dovuta a scarsa solubilità nei liquidi fisiologici ed a un rapido metabolismo epatico. Difatti, dopo somministrazione orale di Curcumina, solo tracce del composto naturale vengono ritrovate in circolo. L'approccio prodrug, rappresenta quindi un'utile opzione per aggirare questi problemi. Questo approccio è basato sulla modificazione del composto naturale mediante protezione bioreversibile e coniugazione a specifici sostituenti (promoieties) per ottenere le proprietà chimico-fisiche desiderate (solubilità, stabilità ecc). Sulla base di risultati ottenuti precedentemente con altri fenoli naturali, ho scelto ed utilizzato il legame carbamoilico per coniugare la Curcumina a due diversi tipi di promoieties, amino acidi naturali e polimeri sintetici poliossazolinici.

Nello specifico, tre amminoacidi, leucina, isoleucina e valina, sono stati selezionati come promoieties più promettenti da coniugare alla Curcumina. Gli amminoacidi sono stati scelti per la loro affinità verso specifici trasportatori presenti a livello intestinale. Questo, dovrebbe favorire l'assorbimento intestinale della Curcumina a seguito della somministrazione orale dei prodrugs. Poiché la Curcumina possiede due gruppi idrossilici, ho scelto di funzionalizzarne rispettivamente uno ed entrambi mediante coniugazione con gli amminoacidi. Sono stati sintetizzati così sei prodrugs della Curcumina classificabili in mono- e di-sostituiti.

Successivamente è stata testata la stabilità della Curcumina e dei prodrugs in soluzione acquosa a diversi valori di pH (1 e 6.8) al fine di mimare le condizioni di stomaco e intestino. Sono state effettuate analisi UPLC-UV, HPLC-MS, spettroscopia UV-Vis, e ^{1}H-NMR. Nel condurre questi esperimenti, ho incontrato però numerosi problemi dovuti in particolar modo alla bassa solubilità dei composti in acqua ed alla loro tendenza a precipitare.

I prodrugs hanno mostrato una maggiore stabilità in soluzioni acide, confermando le aspettative relative ai carbammati *N*-mono-sostituiti, la cui reattività è nota. In soluzioni a pH 6.8 (pH intestinale) sono invece state osservate alcune tracce di degradazione.

L'idrolisi dei derivati è stata studiata anche tramite spettroscopia ^{1}H-NMR usando soluzioni di D_2O/DMSO-d_6 a diverse percentuali di D_2O. Nessuna traccia di degradazione dei prodrugs è stata osservata in queste condizioni.

Gli esperimenti preliminari di farmacocinetica che sono stati condotti su topi modello, hanno inoltre provato che il derivato carbamoilico di-sostituito della Leucina è in grado di aumentare la biodisponibilità della Curcumina dopo somministrazione orale.

Infine, ho verificato l'affinità della Curcumina e dei prodrugs nei confronti delle proteine plasmatiche. In particolare, ho effettuato saggi fluorimetrici di binding utilizzando l'albumina umana (HSA), importante per le sue capacità di complessamento e distribuzione di sostanze farmaceutiche nel sangue. Tutti i sei nuovi prodrugs hanno mostrato un'affinità elevata per l'albumina, superiore a quella della Curcumina stessa, ad eccezione del derivato mono-sostituito della leucina.

Dall'insieme dei risultati ottenuti dallo studio dei prodrugs descritti in questa tesi è emersa la loro buona distribuzione nel circolo sanguigno e un'alta stabilità nei confronti degli enzimi epatici, evidente soprattutto per i composti mono-sostituiti, incoraggiandone così un potenziale utilizzo in campo terapeutico.

L'ultimo capitolo di questa tesi descrive la sintesi di nuovi sistemi di rilascio farmaceutico ottenuti mediante un processo di auto assemblaggio di prodrugs polimerici della Curcumina con caratteristiche anfifiliche. Anche in questo caso è stato scelto il legame carbamoilico per garantire la coniugazione tra i polimeri e la Curcumina. Tra i vari polimeri, la scelta è ricaduta sulle poli-2-metil-2-ossazoline (PMOXAs) la cui somiglianza ai poli-etilenglicoli (PEGs), molto spesso utilizzati in questo campo, sommata ad una minore risposta immunitaria e ad una maggiore idrofilia rende promettente la scelta di questi composti a ruolo di promoieties. In particolare, cinque poli-2-metil-2-ossazoline, differenti tra loro per lunghezza della catena polimerica, sono state coniugate ad un solo gruppo idrossilico della Curcumina.

A causa dell'elevata idrofilicità dei polimeri e della alta idrofobicità della Curcumina, questi nuovi cinque prodrugs possono essere classificati come anfifilici. Essi hanno, infatti, mostrato la capacità di auto assemblare in strutture di tipo micellare in soluzioni acquose garantendo così un'elevata solubilità della Curcumina in condizioni simili a quelle fisiologiche.

Il lavoro si è concentrato in particolare sulla studio della correlazione tra la lunghezza della catena polimerica e la capacità di auto assemblare.

Per tutti i derivati è stata determinata la concentrazione micellare critica (CMC), che si aggira in un intervallo di 10^{-6}-10^{-4} M. Dai risultati emersi, il coniugato Curcumina-PMOXA30, caratterizzato da una CMC intorno a 10^{-6} M, si è dimostrato il più incline ad auto assemblare. Al contrario, sono state identificate CMC più alte per i coniugati Curcumina-PMOXA50 e Curcumina-PMOXA100 dovute probabilmente ad un bilancio sfavorevole tra le parti idrofobiche e quelle idrofiliche che, essendo più lunghe, ostacolano il processo di micellizzazione. Dall'altra parte, il derivato Curcumina-PMOXA10 ha mostrato alta tendenza ad aggregare e precipitare a causa della corta catena polimerica che in questo caso non riesce a bilanciare l'idrofobicità della Curcumina.

La stabilità e le dimensioni delle micelle in soluzione acquosa sono state stimate utilizzando il light scattering dinamico (DLS) e la microscopia a trasmissione elettronica (TEM). La lunghezza della catena polimerica e la dimensione delle particelle sono risultate essere in un rapporto di inversa proporzionalità. Infatti, i coniugati costituiti da PMOXA20 e PMOXA30 presentano un diametro intorno a 100-130 nm, mentre i coniugati con PMOXA50 e PMOXA100 mostrano diametri inferiori intorno a 50-30 nm. Inoltre, tutti i coniugati, ad eccezione di Curcumina-PMOXA10, hanno dimostrato una buona stabilità in soluzione acquosa.

Successivamente, ho testato il rilascio di Curcumina da ogni tipologia di micella mediante analisi UV-Vis in soluzione di buffer salino (PBS) a pH 7.4 ed a 37°C per mimare il pH sanguigno. Il coniugato Curcumina-PMOXA30 è risultato il più promettente mostrando il migliore profilo cinetico e favorendo il rilascio di circa il 90 % della Curcumina che costituisce la micella durante le prime cinque ore. Gli altri coniugati hanno invece mostrato profili diversi rilasciando, nello stesso intervallo di tempo, percentuali di Curcumina intorno al 10-30%.

Abstract

The thesis reports the synthesis and characterization of new carbamoylic prodrugs of the natural bioactive polyphenol Curcumin, found in many foodstuff. Curcumin is a promising drug candidate because of its credited beneficial health effects and wide spectrum of action. However, the low bioavailability due to its poor solubility in aqueous media (i.e. physiological fluids) and to its very fast hepatic metabolism, greatly limits the use of Curcumin in the pharmaceutical field.

The "prodrug approach" represents a valid solution to overcome these issues. This is based on the conjugation of the active compound with a specific promoiety through a bioreversible linkage. This modification is expected to improve the pharmacokinetic profile of the compound (prevent fast metabolism and enhance bioavailability). The choice of the bioreversible linker is crucial for the pharmacokinetic performance of the prodrug. I chose and used a urethane linkage, which has been recently demonstrated by our group to be the best option to protect and enhance the bioavailability of natural polyphenols *in vivo*.

Curcumin was conjugated with two different types of promoieties: natural amino acids and poly-2-alkyl-oxazolines, a class of hydrophilic synthetic polymers.

In particular, leucine, isoleucine and valine were selected as promoieties for the conjugation with Curcumin, since these amino acids show enhanced intestinal absorption due to active transport mechanism. The conjugation of these amino acids with Curcumin could thus improve the absorption of Curcumin following oral administration of the prodrug.

Curcumin has two phenolic hydroxyl groups. I functionalized Curcumin on one and both hydroxy groups by conjugation with amino acids through a urethane linkage, obtaining six new prodrugs (mono- and di-substituted, respectively).

The stability of the new prodrugs and of Curcumin itself in aqueous media was studied using different experimental approaches: UPLC-UV, HPLC/MS, UV-Vis spectroscopy and ^{1}H-NMR. The study of these processes and systems was severely hindered by the solubility of the new Curcumin prodrugs in aqueous media which turned out to be rather poor, either under acidic or near neutral pH conditions, as are found in the stomach and intestine. The prodrugs proved to be more stable in acidic solutions as expected based on the known reactivity of the *N*-monosubstituted

carbamoylic linkage. In solutions at pH 6.8 (as found in the intestine), some degradation was observed. The stability of the new derivatives against hydrolysis was also studied by ^{1}H-NMR spectroscopy in D_2O-DMSO-d_6 solutions with different D_2O contents. No degradation was observed in these aqueous media over three hours.

Preliminary pharmacokinetic experiments were carried out and proved that the di-substituted leucine carbamoyl derivative of Curcumin is capable of improving the bioavailability of the parent phenol in mice model.

Finally, the affinity of these prodrugs for plasma proteins was assed, using fluorescence binding assays with human serum albumin (HSA), which is ubiquitous in the blood stream. All of the new derivatives, except for the mono-leucine prodrug, have higher affinity for HSA than Curcumin itself.

From these combined data a good distribution in the blood stream and high stability against hepatic enzymes can be expected for the new derivatives developed with this thesis, in particular for the mono-functionalized compounds, which make these compounds potential candidates as Curcumin prodrugs.

The last chapter of this thesis is focused on the synthesis of new drug-delivery systems obtained from the self-assembly of amphiphilic polymeric prodrugs of Curcumin. Also in this case a carbamoylic bond was chosen as the linkage between Curcumin and the polymer chain. Poly-2-methyl-2-oxazolines (PMOXAs) were chosen as promoieties for conjugation with Curcumin because of their similarity to the most commonly used poly-ethylene glycols (PEGs), showing the same stealth effects, together with a reduced immune response, and a higher hydrophilicity. In particular, five poly-2-methyl-2-oxazolines of different chain length were conjugated to one hydroxy group of Curcumin. Because of the high hydrophilic character of the PMOXAs, these five new prodrugs can be classified as amphiphiles. Indeed they possess the ability to self-assemble in micelle-like structures when put in aqueous solution, providing good-to-excellent solubility of Curcumin in physiological-like media.

The correlation between the chain length and the self-assembly ability of these prodrugs was investigated. All derivatives have a critical micellar concentration (CMC) in the 10^{-6}-10^{-4} M range. Curcumin-PMOXA30 showed the highest ability to self assemble into micelles at concentrations as low as 10^{-6} M. On the contrary, conjugates with PMOXA50 and PMOXA100 resulted in a higher CMC value, probably due to the unfavourable hydrophilic/hydrophobic ratio, typical of longer polymeric chain, which prevents the self-assembly process. On the other hand the derivative

Curcumin-PMOXA10, showed the tendency to aggregate and precipitate in solution, probably due to a too pronounced hydrophobic character.

The size and the stability in water of all micelles were tested using dynamic light scattering (DLS) and transmission electron microscopy (TEM). An inverse correlation between chain length and size was found. In fact, conjugates with PMOXA20 and PMOXA30 form micelles with a diameter around 100-130 nm, whereas analogues with PMOXA50 and PMOXA100 with diameters of about 50-30 nm. For all conjugates, except Curcumin-PMOXA10, a good stability in aqueous environments was observed.

Furthermore, I tested the release of Curcumin from each type of micelles by means of UV-Vis analysis. The experiments were carried out in phosphate buffered saline (PBS) at pH 7.4 and at 37°C in order to mimic the blood pH value. The conjugate Curcumin-PMOXA30 showed the best kinetic profile with around 90% of released Curcumin during five hours. The other conjugates showed instead a release of Curcumin around 10-30% during the same time.

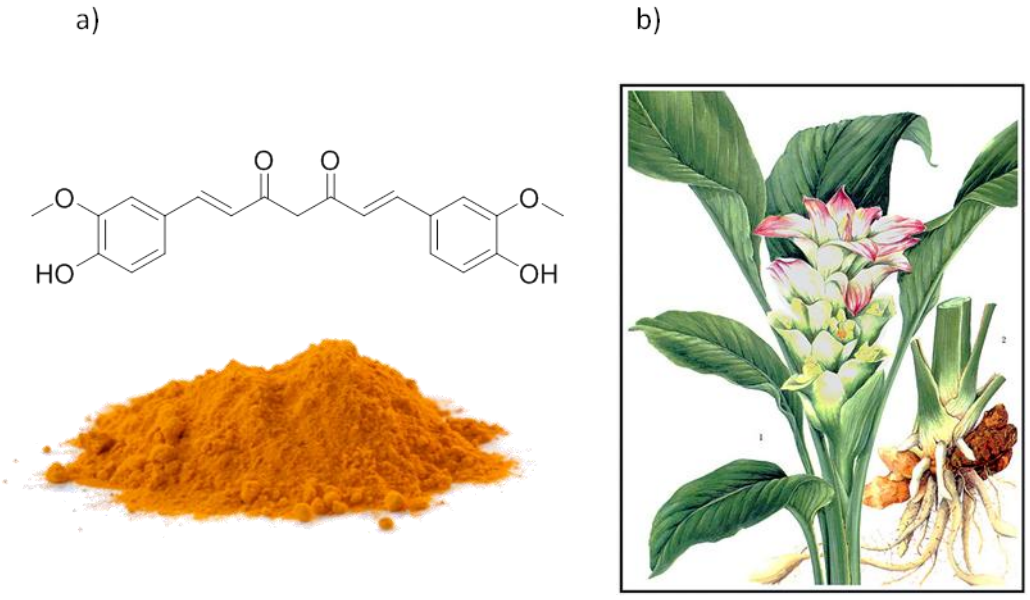
CHAPTER 1

Introduction

1.1 Curcumin, the “golden spice”

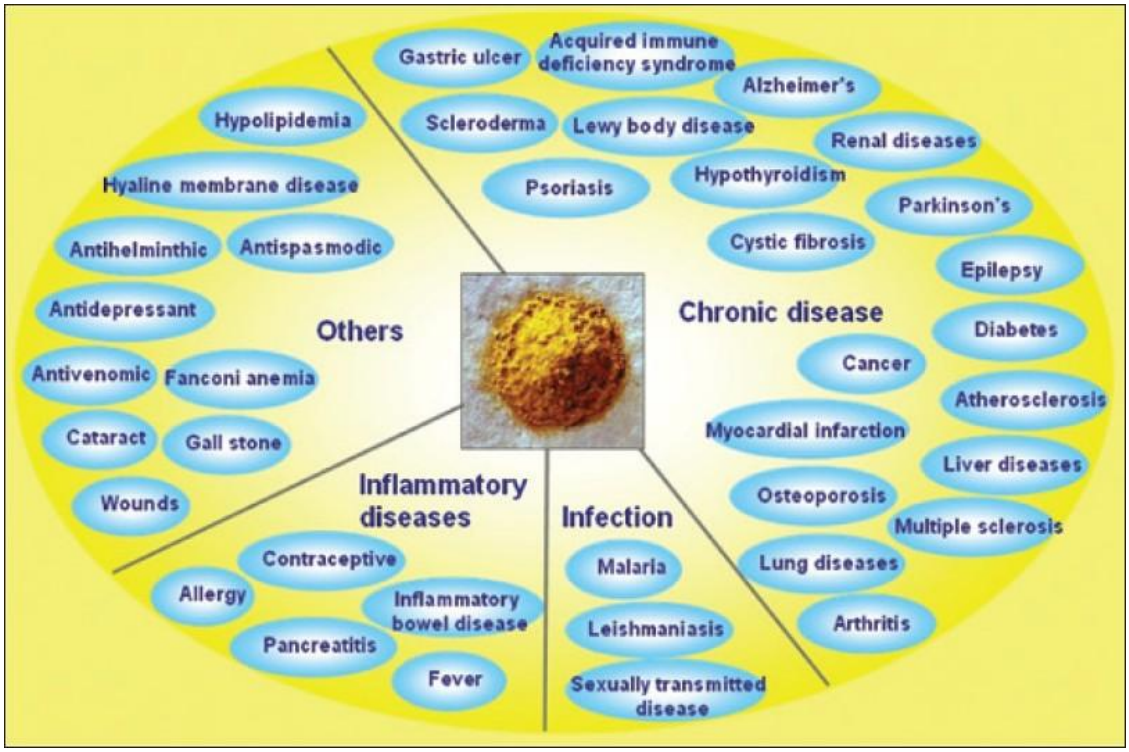
Curcumin, the common name for (1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is one of the active compounds extracted from rhizome of *Curcuma Longa* [Fig.1].
This compounds has different biological activities, and has therefore been attracting increasing interest for its potential use as a drug. Its bioactivity range spans from antioxidant to anti-inflammatory effect [Fig.2].

Figure 1. a) Molecular structure of Curcumin and Curcumin powder. **b)** Representation of *Curcuma longa* and its rhizome



Curcumin was successfully tested in several clinical trials (phase I and II) as anticancer therapeutic, and in the treatment of neurodegenerative diseases such as Parkinson and Alzheimer. [1–3] The broad spectrum of action of Curcumin is due to its chemical structure which makes Curcumin able to interact with several molecular targets.[4]

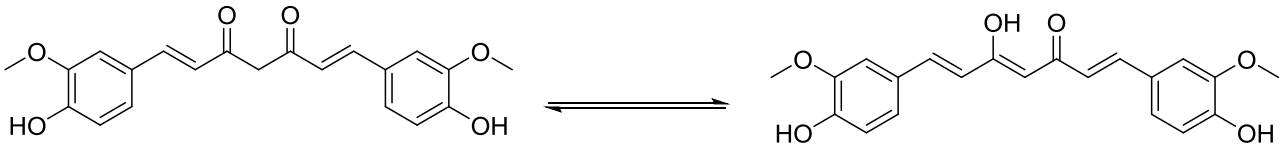
Figure 2. Broad spectrum of action of Curcumin[5]



From a chemical point of view, Curcumin is an amphiphilic molecule characterized by three specific moieties, a β-diketone, two hydroxy groups and two methoxy groups, able to act as hydrogen-bond (H-bond) acceptor and donor. These domains are fundamental for the intermolecular interactions between Curcumin and its molecular targets. Moreover, the β-diketone group which is connected to the aromatic rings via an alkenyl chain of seven carbon atoms, makes Curcumin a good Michael acceptor in conjugate-addition reactions.[4]

One of the peculiarities of this molecule is that it exists in two tautomeric forms [Fig.3] and in different conformations. The enol form is thermodynamically favoured in polar solvents because of the stabilization effect of the intramolecular H-bond.[6] It is known that in polar solvent (DMSO, acetonitrile, alcohols) the tautomeric equilibrium is completely shifted towards the enol form, while in apolar solvents such as toluene, benzene or hexane, a mixture of both tautomers is found.[7–9]

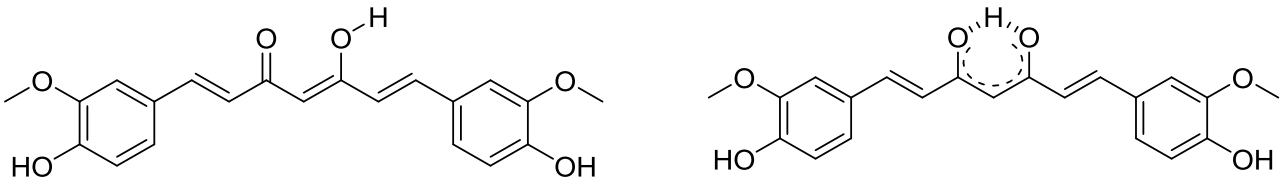
Figure 3. Tautomeric equilibrium of Curcumin



diketo form enol form

Although in polar aprotic solvents Curcumin is found in a closed *cis*-enol conformation, in polar protic solvents the open conformation is adopted as well as in physiological environment where it can interact with proteins directly or via water bridge[9–12] [Fig.4].

Figure 4. Different conformations of enolic tautomer of Curcumin



open *cis*-enol closed *cis*-enol

Nevertheless, its high lipophilicity is envisioned as one of the reasons to explain the high number of interactions between Curcumin and proteins, or to justify its intercalation into cellular membranes.[13–15] Moreover, since Curcumin is able to undergo rotamerization, it can assume different conformations for each molecular target.[16]

Very interesting data were obtained in recent years from studies on the incidence of neurodegenerative disease in developing countries. In particular, India is the first country in which a very low rate of Parkinson and Alzheimer disease was observed.[17,18] A possible explanation to this observation could be related to the high quantity of consumed Curcuma.[2]

For example, a pronounced antiamyloidogenic activity was correlated to Curcumin consumption.[19] The strong interactions between Curcumin and β-amyloid can prevent the aggregation in pathological patches. Furthermore, molecular docking studies on acetylcholinesterase identified three possible sites of interaction in which Curcumin binding might cause the inhibition of the protein and the disaggregation of the patches.[20] This mechanism of action could be responsible of antiAlzheimer activity of Curcumin.

Even for the therapy of Parkinson morb Curcumin and many derivatives were tested.[21]

In particular, Curcumin was modified on one and both hydroxy groups by conjugation with glucose and glucose acetate in order to synthesize mono- and di-glucoside derivatives.[22] The effect of such compounds was investigated *in vitro* on brain tissues affected by Parkinson. Curcumin-glucoside derivatives showed high activity because of their ability to prevent the formation of oligomers of α-synuclein and to avoid their aggregation into fibrils.[22]

Together with the studies and clinical trials on neurodegenerative diseases, Curcumin has also been considered for its antitumoral activity.[23–26] The anti proliferative effect of Curcumin is based on the ability to perturb specific cellular signaling pathways.[27,28] In particular, these latter include:

- β-catenine/TCF4 (T-cell factor) or LEF (lymphoid enhancing factors) system. The formation of β-catenine/TCF complex is responsible of activation in transcription of genes involved in differentiation and cellular proliferation mechanism. The formation of complex is inhibited by Curcumin so that cellular proliferation is stopped.[29]
- Caspase 3 cell signaling and activation of gene p53. Interaction of Curcumin with these targets The apoptosis process is promoted by.[30]
- VEGF (Vascular Endothelial Growth Factor) cellular pathway. The VEGF down-regulation by means of Curcumin reduces the angiogenesis process.[31]

In order to enhance the anti cancer activity and the selectivity toward specific tumors, several analogues of Curcumin were synthesized and tested.[32–36] In particular, an enhancement of anti tumoral activity was showed for that analogues in which phenolic rings were removed and substituted with nitrogen based heterocycles.[37] Similarly, anti proliferative derivatives were obtained by insertion of electron withdrawing substituents in position 2 and 4 of phenol moieties.[38] Selectivity toward specific tumors such as hepatic tumor or breast cancer was instead afforded by insertion of an aromatic domain (e.g. pyrazole, isoxazole) or an alkyl chains on the methylene group in position 5 of Curcumin.[39]

Despite being a promising candidate for the treatment of cancer and neurodegenerative diseases, the use of Curcumin in the pharmacological field is limited by many issues. Unfortunately, Curcumin suffers from poor solubility in water (60 μg/mL) as shown by the high octanol/water partition coefficient (log P) of 2.3-2.6.[40,41]

Moreover, Curcumin is characterized by a low bioavailability and low serum levels were identified after few hours from oral administration in humans even if in high doses (8-12g) of Curcumin.[42] Traces of Curcumin in the intestinal tissue and in liver were detected after oral administration in mice, while no traces were identified upon oral administration of Curcumin in humans.[43–46] Moreover, a fast hepatic metabolism was observed, and many different metabolites were detected. Glucuronide conjugates and sulphates were found after oral and intraperitoneal (i.p.) administration.[47,48] Furthermore products of the reduction of Curcumin, such as dihydrocurcumin, tetrahydrocurcumin, and hexahydrocurcumin were identified.[49,50]

These latter, could be responsible of some biological activities of Curcumin as antioxidant and antidiabetic activity.[50,51]

In order to overcome these bioavailability issues, the prodrug approach and the loading of Curcumin in nanoparticles and liposomes were the most exploited systems in recent years.[52–68]

In terms of prodrugs, several compounds were exploited, and Curcumin was conjugated via different bioreversible linkages to a variety of small molecules, as well as polymers.

Piperic acid, Glycine and D-alanine were used as promoieties *via* conjugation to the hydroxy groups of Curcumin with an ester bond. Such compounds showed antibiotic activity for infectious diseases, especially to overcome the problem of resistance, as proved by *in vitro* tests.[69]

Other amino acidic ester bioconjugates of Curcumin were synthesized to test the enhancement of solubility, as well as of other biological activities (*i.e.* antioxidant, antimutagenicity and radical scavenging).[70] With respect to Curcumin, enhanced water solubility was observed for derivatives of Curcumin with glycine and proline, and stronger antioxidant effects for Curcumin-leucine and -isoleucine derivatives.[70]

Curcumin-polymer conjugates were synthesized using different linkages (ester, carbamate and thioester) with the particular aim to increase the water solubility of the drug and to promote a higher antitumoral activity taking advantage from enhanced permeability and retention effect.[71–75]

For example, Curcumin was conjugated via a carbamoylic bond to poly ethylenglicols (PEGs) of molecular weight of 750 Da and 3500 Da. A high water solubility and cytotoxycity were observed for both these conjugates. Unfortunately, a fast hydrolysis process in physiological like conditions

(PBS pH: 7.4) was shown.[76] In another work, the synthesis of a Curcumin-oligoethylenglicole (OEG) derivative and *in vitro* studies were reported.[77] A short OEG with chain length of 7 units was conjugated via thioester linkage to both of the hydroxyl groups of Curcumin. For this compound the ability to self assemble into micelles in aqueous environment was investigated, and antitumor activity due to the Curcumin release at physiological pH was observed. Hydrolysis tests were carried out, using the same conditions previously described for Curcumin-PEG derivatives. In this case, only 30% of compound hydrolyzed over 70 hours underlying a high stability of these compounds.[77]

From the above short account on the state of the art for possible therapeutic applications of Curcumin, with the limitations due to its poor bioavailability and consequent attempts to develop effective prodrugs, it is evident that there is presently great interest in new ways to exploit the biomedical potential of this popular active natural compound. My PhD project focussed on this goal and took advantage of the expertise in the development of prodrugs of natural phenolic compounds gained over the years by the research team I associated with. Specifically, I used the carbamoyl linkage, which in previous studies[78–81] proved the best among different tested possibilities, as described in Chapter 2, and developed new Curcumin prodrugs belonging to two families depending on the promoiety used, a natural amino acid or a polyoxazoline polymer. Specifically, I synthesized and tested a set of Curcumin prodrugs using the natural amino acids leucine, isoleucine and valine as promoiety. The reactivity of these conjugates was studied under different experimental conditions in collaboration with the group of Dr. Mario Zoratti of the CNR Institute of Neuroscience and pharmacokinetic experiments were carried out by Dr. Stefano Dall'Acqua at the Department of Pharmaceutical Sciences of the University of Padova. The synthesis and behaviour of these amino acid derivatives of Curcumin are described and discussed in Chapter 2.

I then synthesized and studied a set of five conjugates of Curcumin with polyoxazolines in collaboration with Dr. Edmondo Benetti during a eight-month stage at ETH in Zurich. The behaviour of such polymeric prodrugs was investigated, and all micelles were completely characterized in terms of size, stability and kinetic of Curcumin release.

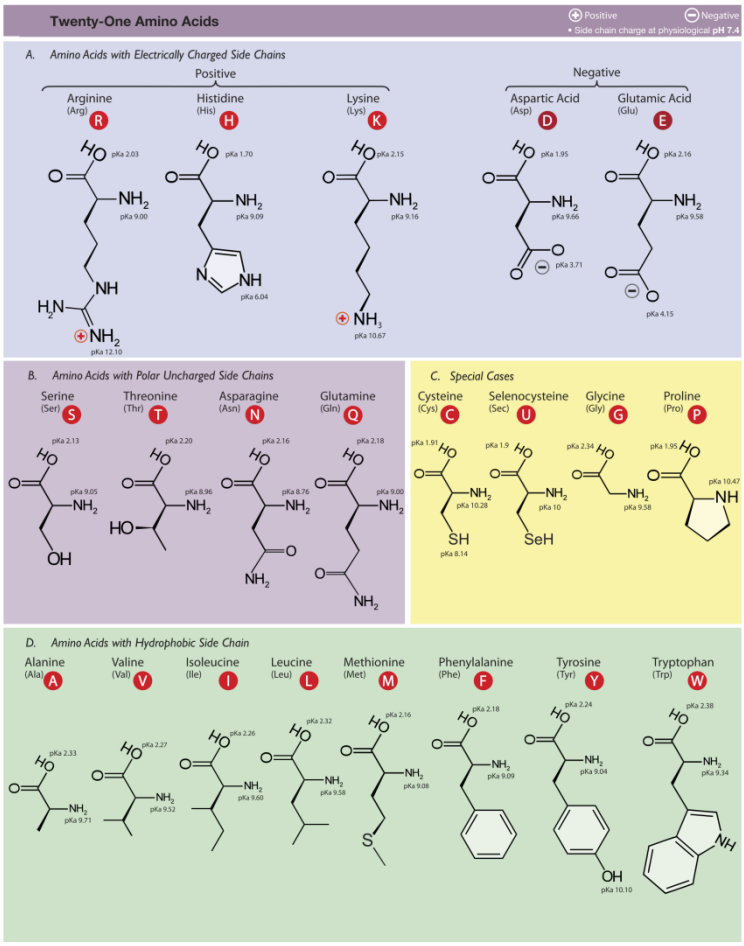
CHAPTER 2

Amino acidic prodrugs of Curcumin

2.1 Amino acids as promoieties

α-Amino acids are excellent compounds often used as promoieties in prodrug synthesis. Classification of the 20 known natural amino acids is based on the polarity/apolarity of these side chains. They are divided in non-polar, uncharged polar and charged polar a.a. [Fig.5]. Their use as promoiety in prodrugs may lead to different advantages: the improvement of drug solubility, that enhances physicochemical and pharmacokinetic drug profile and the possibility to resort to oral administration.

Figure 5. Natural amino acids



Moreover, amino acids can promote drug adsorption in the gastro intestinal environment by active transport processes by exploiting the affinity of transporters to amino acids with specific characteristics (charged or uncharged side chains). Indeed, specific transporters of amino acids and oligopeptides were found in brush border membranes of intestinal epithelium cells.[82] Many examples of amino acidic prodrugs are known in the literature and other are also in commerce as medicament. One of the most known prodrugs is Valtrex®, in which the antiviral drug acyclovir is conjugated to the amino acid valine resulting in a prodrug with enhanced bioavailability due to increase in intestinal uptake by action of the peptide transporter PEPT1.[83,84]

PEPT1, is the most known amino acids and oligopeptide carrier, it belongs to the proton-coupled transporter's family and is specific for apolar side chains.[85] An increase of its expression rate in human tissues, in particular of colon, was noticed in the case of inflammatory bowel disease and cancer.[86] Drugs with poor bioavailability, like *e.g.* methyl-dopa, when conjugated with phenylalanine showed a ~20 fold enhancement of their intestinal permeability due to active transport by PEPT1.[87,88]

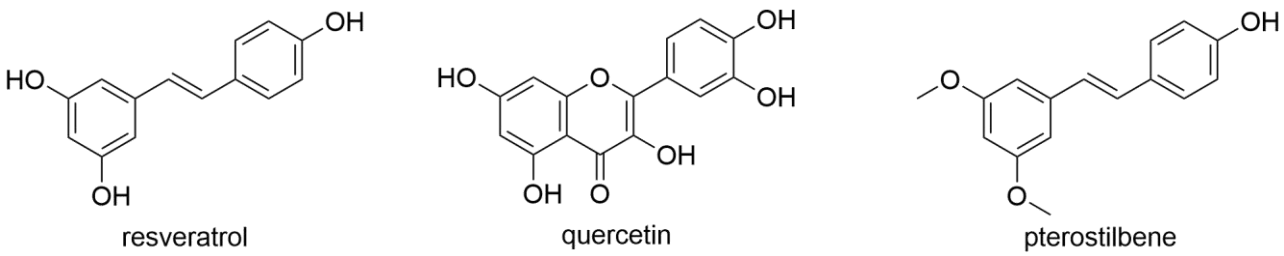
Amino acids characterized by uncharged lateral chains can exploit two other transporters: ATB^0, Na^+ dependent neutral amino acids transporter and LAT1, neutral amino acid transporter, mainly found in the central nervous system.[89,90] For example, amino acidic prodrugs of ketoprofen or valproic acid were targeted to carrier LAT1 in order to improve their permeability of blood brain barrier (BBB).[91,92]

In addition to the enhanced permeation of the intestinal mucosa, amino acids can improve the stability of prodrugs toward hydrolysis or enzymatic degradation .[93,94] Drugs conjugated to amino acids bearing apolar long side chains e.g. leucine, isoleucine and valine, showed higher stability toward hydrolysis and enzymatic metabolism.[95–97] This is probably due to the steric clash between the enzymatic pocket and the amino acid's side chain.[94] It has been demonstrated that the nature of the linkage between the amino acid and the drug plays a crucial role in these mechanisms.[98,99]

2.2 Carbamoylic prodrugs of polyphenols

Different linkages are usually exploited in the synthesis of prodrugs and in particular of prodrugs of natural polyphenols. Several prodrugs of resveratrol, quercetine and pterostilbene [Fig.6] were developed and studied by my research group in order to increase the bioavailability of the natural active compound after oral administration.

Figure 6. Molecular structure of specific polyphenols

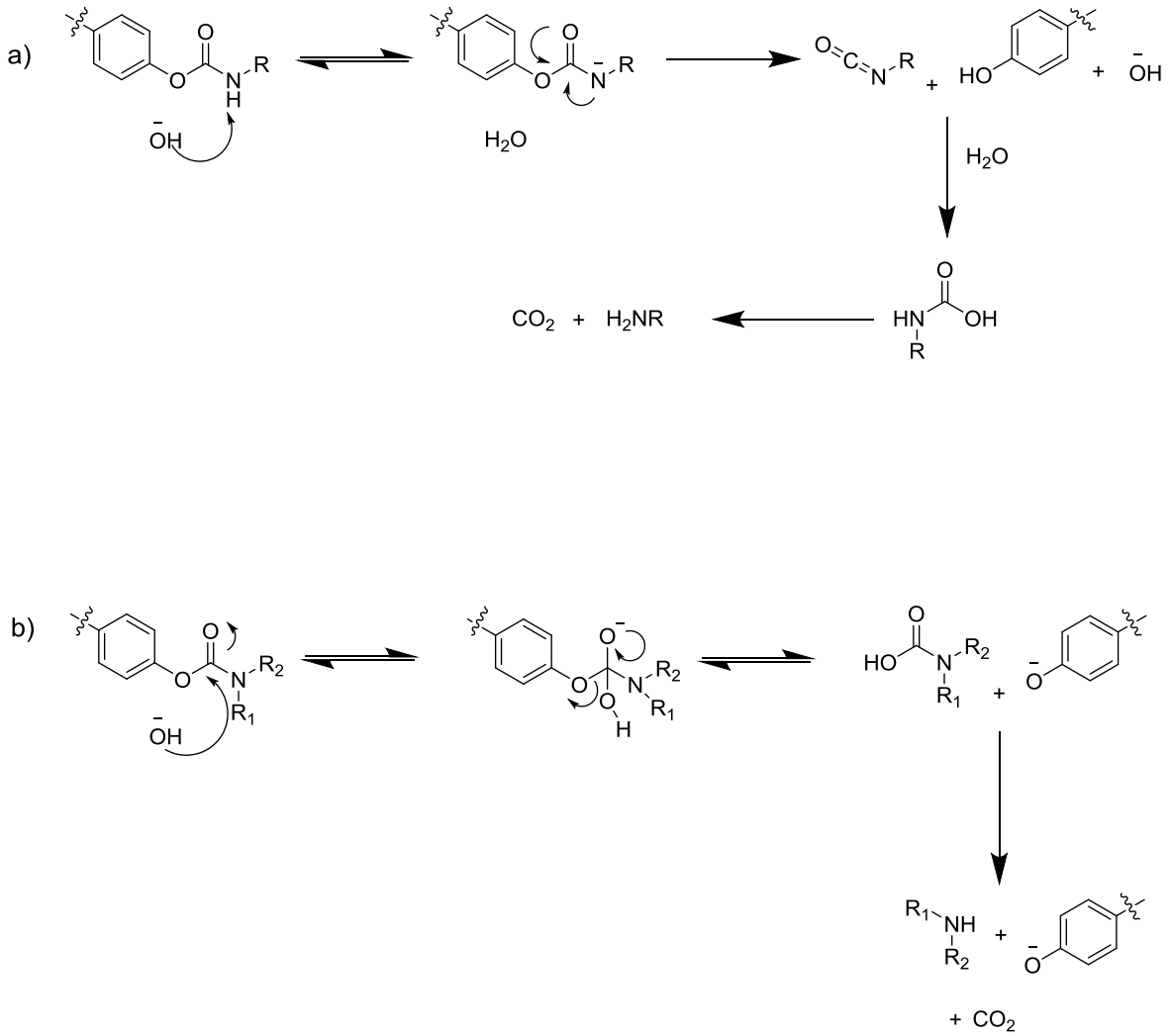


In particular, acetal, sulfonate, carbonate, and carboxyester were tested, among others, as bioreversible linkage between drug and promoiety.[100–105] Results of *in vitro* and *in vivo* experiments of these prodrugs showed a high rate of hydrolysis and promoted the investigation on other types of linkage. Eventually the carbamoylic bond was chosen as more hydrolytically stable linkage than ester or carbonate and tested using resveratrol as model. *N*-mono-substituted and *N,N*-di-substituted carbamoylic derivatives were synthesized. Resveratrol was mono-, di- and tri-substitued using dihydroxipropyl, 6-deoxigalactosyl, butyl-glucosyl and m-PEG350 as promoieties.[80,81] All derivatives showed high water solubility, high hydrophilicity and good response to carbamoyl esterase activity of plasma albumins.[80,81]. In particular, the rate of hydrolysis for *N'*-monosubstituted compounds resulted much faster than *N,N*-disubstituted derivatives.[106,107] The reasons of these differences are related to the different hydrolysis mechanism in primary and secondary carbamates. The first undergoes hydrolysis passing through

the nitrogen deprotonation and the formation of an anionic intermediate [Fig.7a]. In the case of secondary carbamate, in which an acidic proton is absent, carboxylic group is directly attached by the base with consequent formation of tetrahedral intermediate[108] [Fig.7b].

So, since their good rate of hydrolysis, *N*'-monosubstituted carbamates were identified as best compounds for their use in pharmaceutical field.[80,81,109]

Figure 7. Mechanisms of base-catalyzed hydrolysis of **a)** *N*'-monosubstituted carbamate **b)***N,N*-di-substituted carbamate.



In another work[79] resveratrol was functionalized with specific amino acids by carbamoylic bond. Leucine, isoleucine, threonine and phenylalanine were selected as promoieties in order to

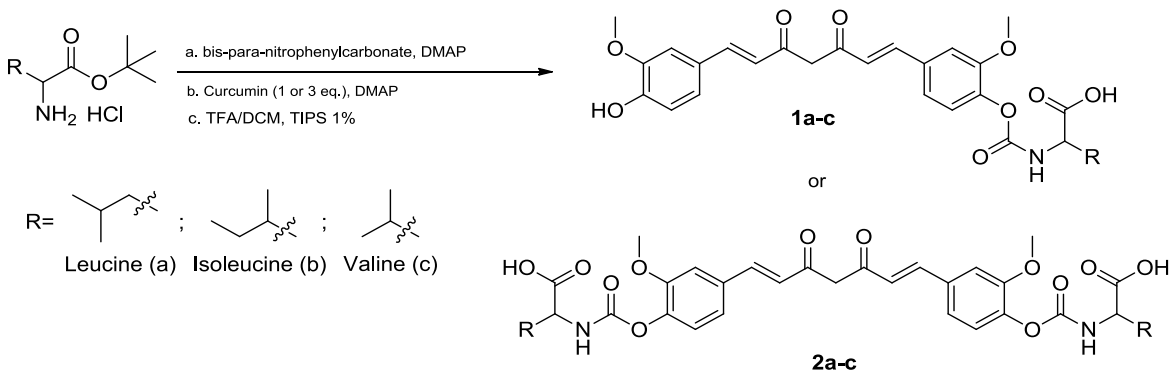
enhance the water solubility of resveratrol and to investigate the intestinal uptake of these compounds by peptide transporter LAT1. All derivatives showed high solubility in water, however the enhanced polarity prevented the penetration of cellular membranes and the conjugation to LAT1.[79]

2.3 Synthesis of amino acidic prodrugs of Curcumin

A large part of my PhD project focussed on the synthesis of new carbamoylic prodrugs of natural polyphenols using amino acids as promoieties. Curcumin was chosen as polyphenol for its remarkable biomedical properties, as described in the Introduction, and for its well known problems of poor bioavailability and solubility. Among the amino acids, valine, leucine and isoleucine were chosen since they are known as best ligands for amino acids transporters. One and both the hydroxyl groups of Curcumin were functionalized in order to protect the polyphenol by the phase 2 conjugative metabolism during absorption after oral administration and to investigate the possible improvement of the ADME (absorption, distribution, metabolism and excretion) profile in pharmacokinetics experiments using the synthesized prodrugs.

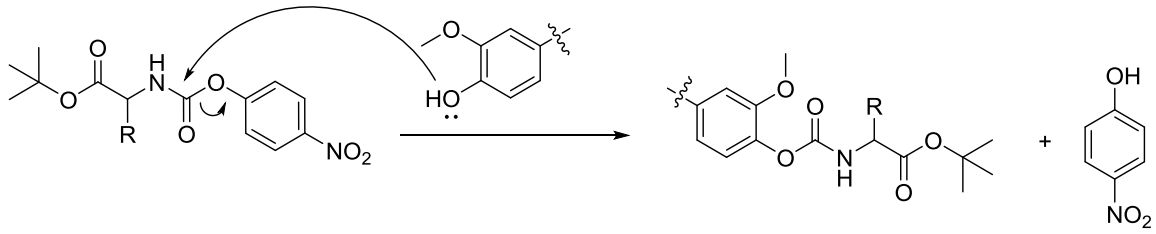
For the synthesis I used a procedure, reported in Scheme 1, previously established and optimized in my research group.[79,81]

Scheme 1. General procedure for the synthesis of Curcumin prodrugs



In the first step, the primary amino group of the carboxy-protected amino acid was activated by reaction with bis(4-nitrophenyl)carbonate in order to obtain a reactive 4-nitrophenyl urethane intermediate. In the second step, which was done in a one-pot procedure without isolation of the active urethane, transesterification with Curcumin was achieved via base promoted addition and elimination of 4-nitrophenol as shown in Figure 8. Finally, cleavage of the tert-butyl ester protecting group at the carboxyl moiety was carried out using TFA in DCM and tri-isopropylsilane (TIPS) as cation scavenger.

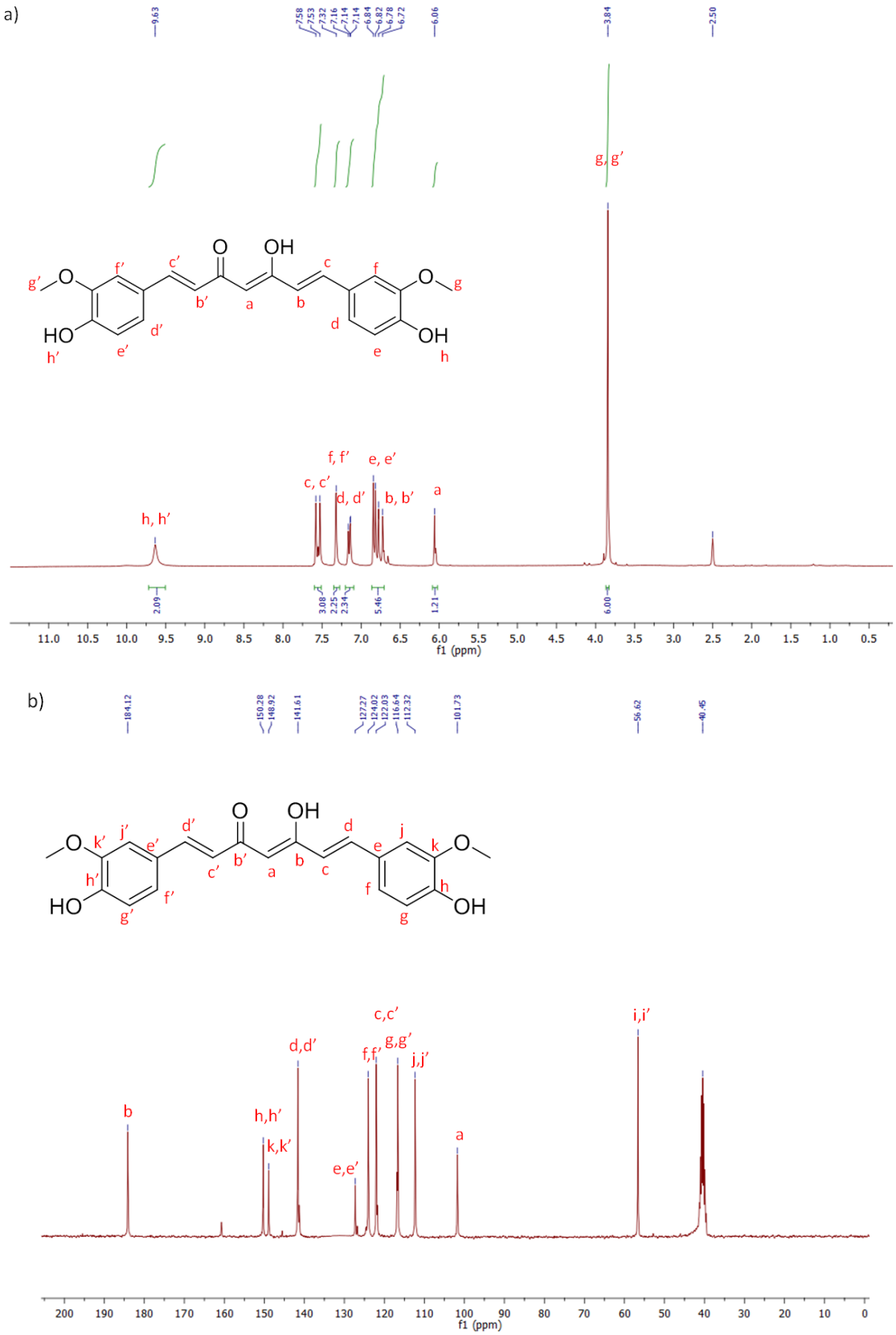
Figure 8. Nucleophilic substitution by Curcumin hydroxyl group on the active urethane intermediate with elimination of p-nitrophenol. The reaction is thought to proceed via and addition/elimination sequence.



To obtain the mono- or di-substituted products, either one or 0.5 equivalents of Curcumin, respectively, were added to the reaction mixture during the second step.

The obtained products were purified by flash chromatography in order to isolate mono- or di-substituted derivatives. Each derivative was then characterized by proton and carbon NMR and by ESI/MS spectrometry. NMR experiments were carried out using DMSO-d_6 as solvent in order to shift largely the tautomeric equilibrium towards a single tautomer, the enol form, thus simplifying the spectrum and signal assignments as in the Curcumin spectrum showed in Figure 9 below. The signal of the enolic proton O-H under these conditions is not observed because of its tendency to be involved in hydrogen bonds which promotes the shift of this signal around 12-16 ppm.[7,109] The existence of Curcumin as enol tautomer is confirmed by the signal of the methylenic proton at 6.2 ppm whose integration results in a single proton as expected for this tautomer. Furthermore, as additional evidence of the existence of the enol tautomer, in the ^{13}C-NMR spectrum of Curcumin it is possible to observe the signal of carbon linked to the enol group around 180 ppm [Fig. 9b].

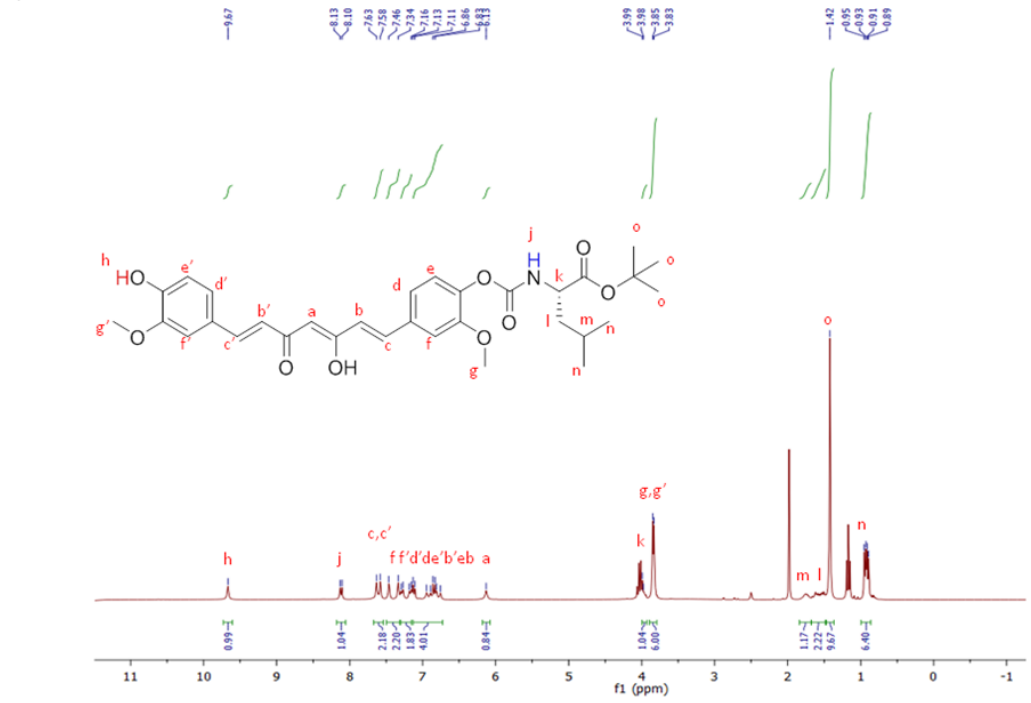
Figure 9. a) ^{1}H-NMR (300 MHz, DMSO-d_6) and **b)**^{13}C-NMR (300 MHz, DMSO-d_6) of Curcumin



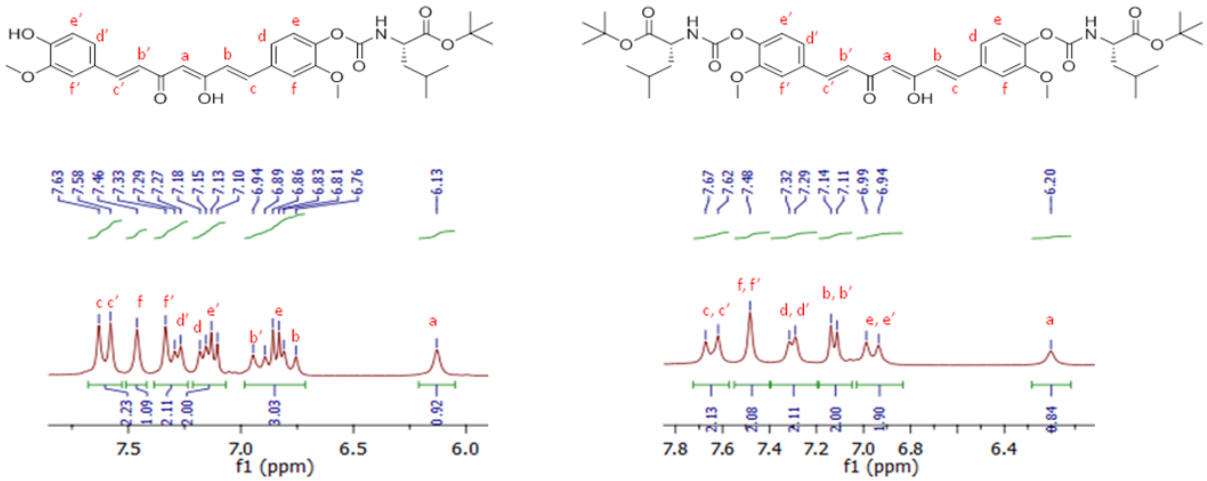
Proton NMR spectra confirmed the achievement of the desired product by identification, among others, of the signal of the carbamoylic proton –N*H* in a range between 8.0 and 8.2 ppm which appears as a doublet [Fig.10a]. Moreover, mono-substituted products showed a characteristic signal at 9.67 ppm, which can be ascribed to the phenolic proton –OH and an increased complexity in the pattern of the aromatic protons in the range 6.13 - 7.63 ppm, consequence of the loss of symmetry in the molecule if compared to the starting material (Curcumin) or to the corresponding di-substituted product as shown in Figure 10b.

Figure 10. a) ^{1}H-NMR (300 MHz, DMSO-d_6) of Curc-*m*-LeutBuO **b)** Comparison of aromatic signals in ^{1}H-NMR spectra between Curc-*m*-LeutBuO (left) and Curc-*d*-LeutBuO (right)

a)



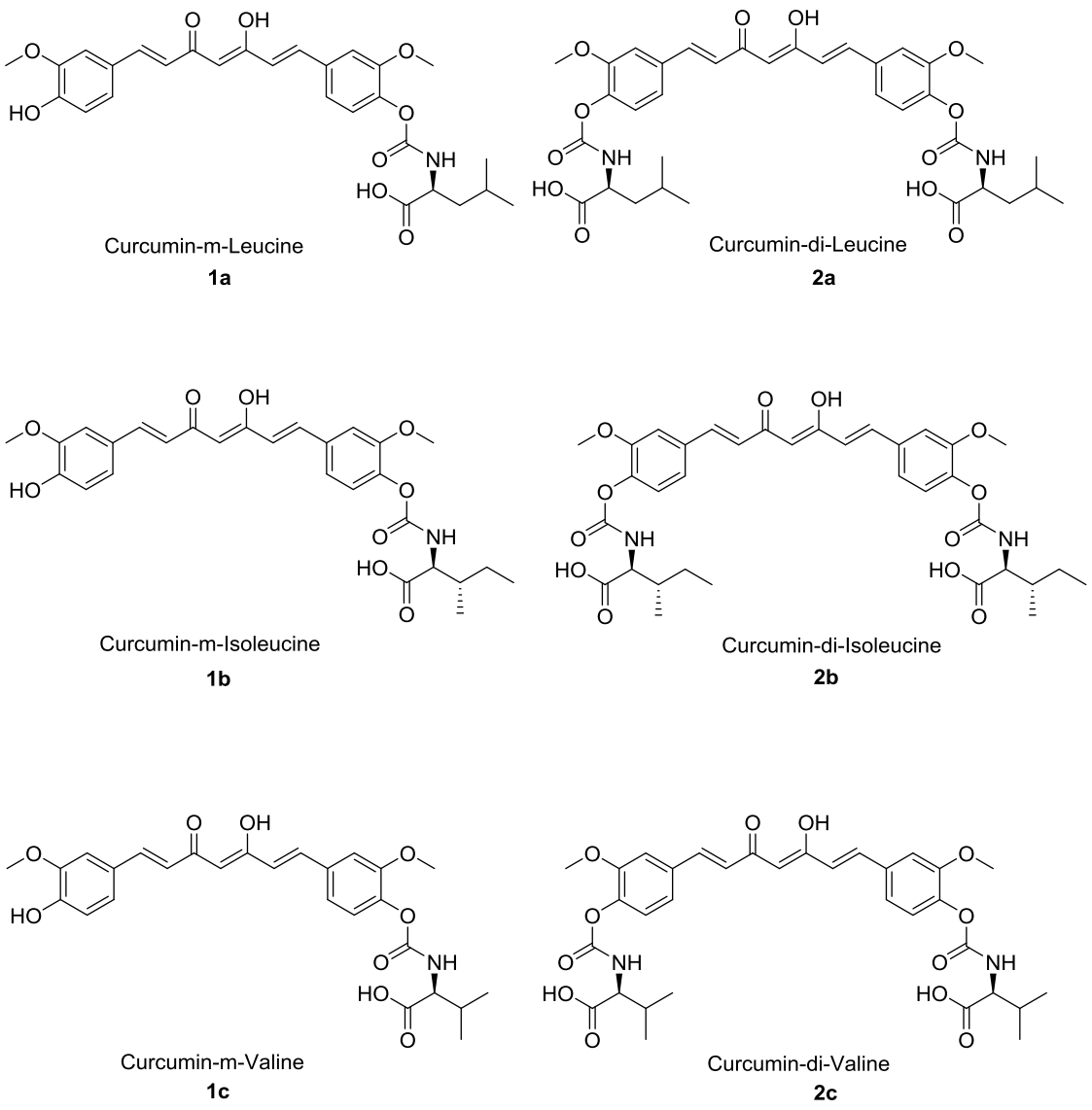
b)



After deprotection with TFA and tri-isopropylsilane, the final products **1a** – **1c** and **2a** – **2c** were obtained. Each was isolated by flash chromatography and purified by preparative HPLC using acetonitrile/water with 0.05% of trifluoroacetic acid and a gradient of eluent composition starting from 20% and reaching 100% acetonitrile. All compounds were then characterized by proton and carbon NMR and by ESI/MS spectrometry.

Following this procedure, six amino acidic derivatives of Curcumin were synthesized [Fig.11].

Figure 11. Molecular structures of the new six amino acidic prodrugs of Curcumin



2.4 Hydrolytic reactivity of Curcumin and derivatives 1a-c and 2a-c

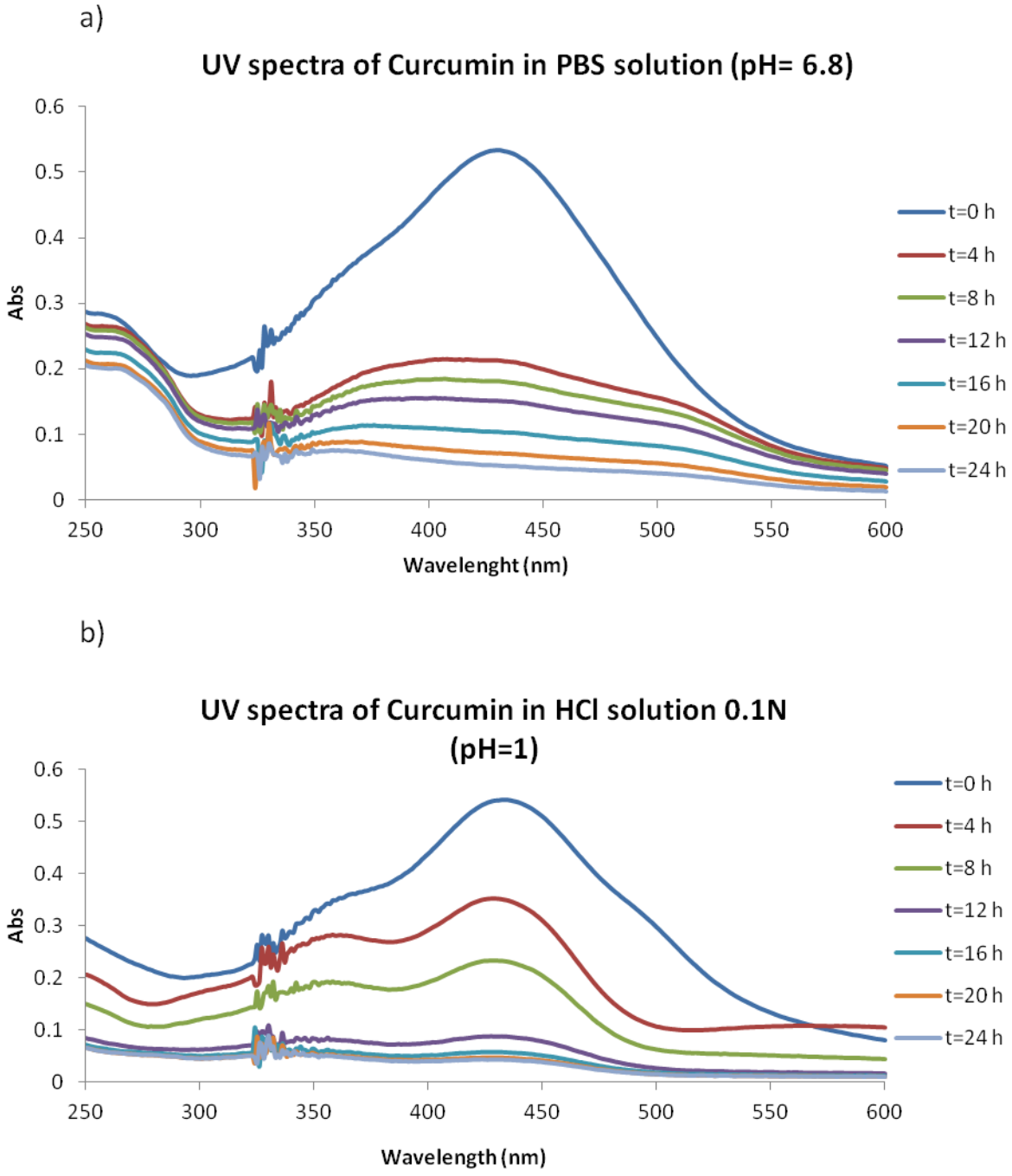
The hydrolytic reactivity and degradation processes of Curcumin have been extensively investigated but not yet fully characterized and understood as testified by a vast, yet conflicting literature.[2,6,109-111] I therefore decided to test the stability of Curcumin itself besides that of its amino acid prodrugs under specific conditions relevant for their possible use via oral administration.

In particular, the stability of the synthesized new derivatives and of Curcumin itself was tested in aqueous solutions at pH 1 and 6.8 at 37°C, to match conditions found in the physiological environment of stomach and gut, respectively. The hydrolytic reactivity of Curcumin was assessed by UV-Vis spectroscopy and HPLC/MS analyses.

2.4.1 UV-Vis stability assay

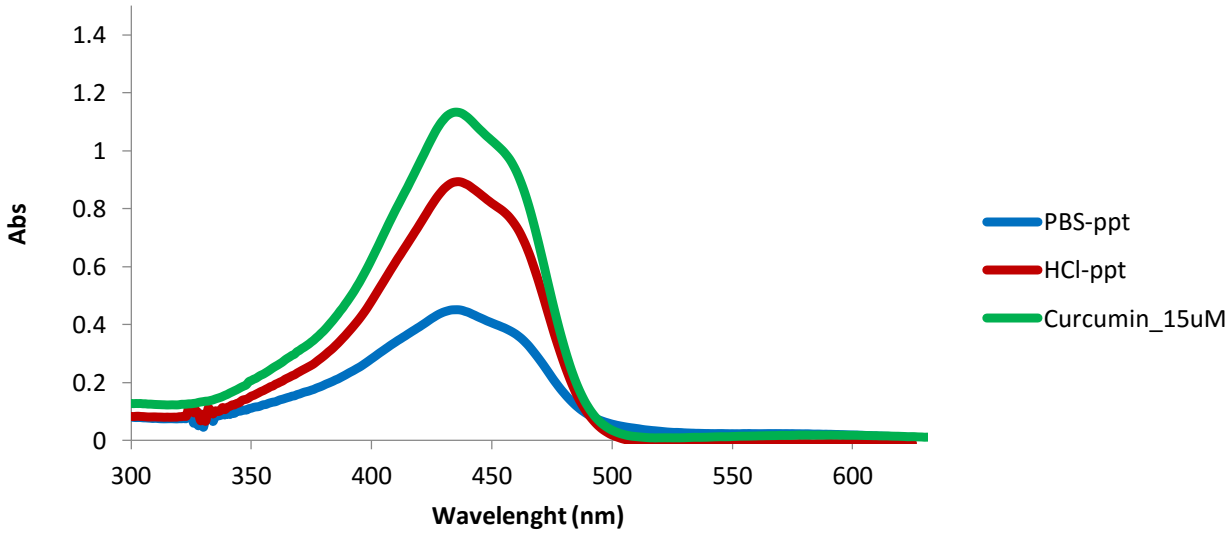
The absorption maximum of Curcumin (425 nm), was determined by analysis of a standard 15 μM solution of Curcumin in DMSO. Absorption spectra of solutions (nominally 15 μM) of Curcumin at pH 1 and 6.8 (PBS buffer) at 37 °C were recorded every 4 hours for 24 hours. From the UV spectra, in acidic and in basic conditions, a decay in the Curcumin absorption at 425 nm was detected already after 4 hours [Fig.12a-b]. In addition, after 24 hours, Curcumin absorption was barely detectable and the presence of a yellow precipitate was observed in both cases. This precipitate was identified as Curcumin by redissolving it in DMSO and acquiring a UV-Vis spectrum. As showed by UV-Vis spectrum in acidic solution, two different peaks can be identified at 363 nm and 425 nm that can be ascribed to the diketo and the enol form of Curcumin, respectively.[112-114] The diketo form, which, according to the literature, is the most favourable in aqueous acidic solutions, is more hydrophobic if compared to the enol form promoting the precipitation within the aqueous environment.[115] The reason for this decay in absorbance could be ascribed to different factors. Namely, the low water solubility, especially at low pH, of Curcumin that results in its precipitation as a yellow solid and the possible decomposition of Curcumin.

Figure 12. **a)** UV-Vis spectra of Curcumin in PBS pH: 6.8 **b)** UV-Vis spectra of Curcumin in HCl pH:1



The spectrum of each precipitate was compared to the spectrum of a Curcumin standard solution [Fig. 13]. An absorption peak at 425 nm was identified for both precipitates, thus confirming the hypothesis of precipitation. This latter is ascribed to the Curcumin keto-enolic equilibrium that, in aqueous solution, promotes the formation of the keto tautomer, more hydrophobic than enol tautomer.[8]

Figure 13. UV-Vis spectra of precipitate found at pH 1 (orange), precipitate found at pH 6.8 (blue) and Curcumin standard solution (grey) in DMSO



These results indicate a good stability of Curcumin in acidic environment while a partial degradation and precipitation occurs under slightly alkaline conditions.

2.4.2 HPLC-MS assessment of the stability of Curcumin

UV-Vis data were also compared with the data acquired by HPLC-MS analysis which allows for an identification of the hydrolysis products. According to the literature, the major degradation

products of Curcumin in aqueous solution (PBS 0.1 M, pH 7.2, at 37°C) are those shown in Figure 14.[109-111] The parameters and the sample concentration used in my experiments were the same as used for the UV tests. A C_{18} column and as eluent a gradient of Acetronitrile/H2O from 10% to 100% in 30 minutes were used.

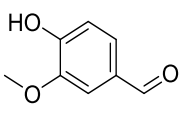
At pH 6.8, the appearance of five new peaks with relatively short retention times indicates a partial degradation although also in this case a major precipitation of the compound was observed as shown by the decrease in the signal intensity after 24 hours [Fig. 15]. The most part of these compounds was poorly ionizable under ESI conditions, preventing their identification. In the chromatograms the peaks with retention times of 16 and 20.5 minutes have m/z 369 which corresponds to protonated Curcumin, as confirmed by their UV-Vis spectra. The presence of two peaks with the same mass value is attributed to the existence of a tautomeric equilibrium of Curcumin.

According to the literature, the first peak, with a retention time of 16 minutes, should be related to the diketo tautomer.[113]

The second peak is indeed representative of the enol form. This latter is considered the thermodynamically favoured tautomer because of intramolecular hydrogen bond and the presence of a conjugated π system.[114,116] Moreover, The presence of the two tautomers has been previously confirmed by the UV-Vis spectrum of Curcumin in HCl 0.1 N solution. The peak with m/z 401 and retention time 10.2 minutes was attributed to compound **6** [Fig.15].

Other known products of the degradation of Curcumin, such as **3**, **4** and **5** were not observed.

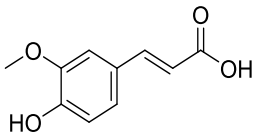
Figure 14. Principal degradation products of curcumin in 0.1 M phosphate buffer (PBS, pH 7.2 , T: 37°C) reported in the literature.[117]



vanilline

M.W. 152.15

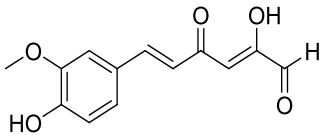
3



ferulic acid

M.W. 194.19

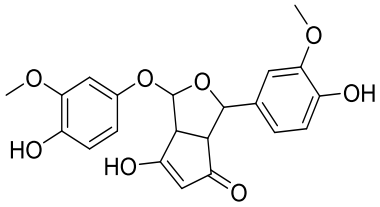
4



(2*Z*,5*E*)-2-hydroxy-6-(4-hydroxy-3-methoxyphenyl)-4-oxohexa-2,5-dienal

M.W. 248.23

5

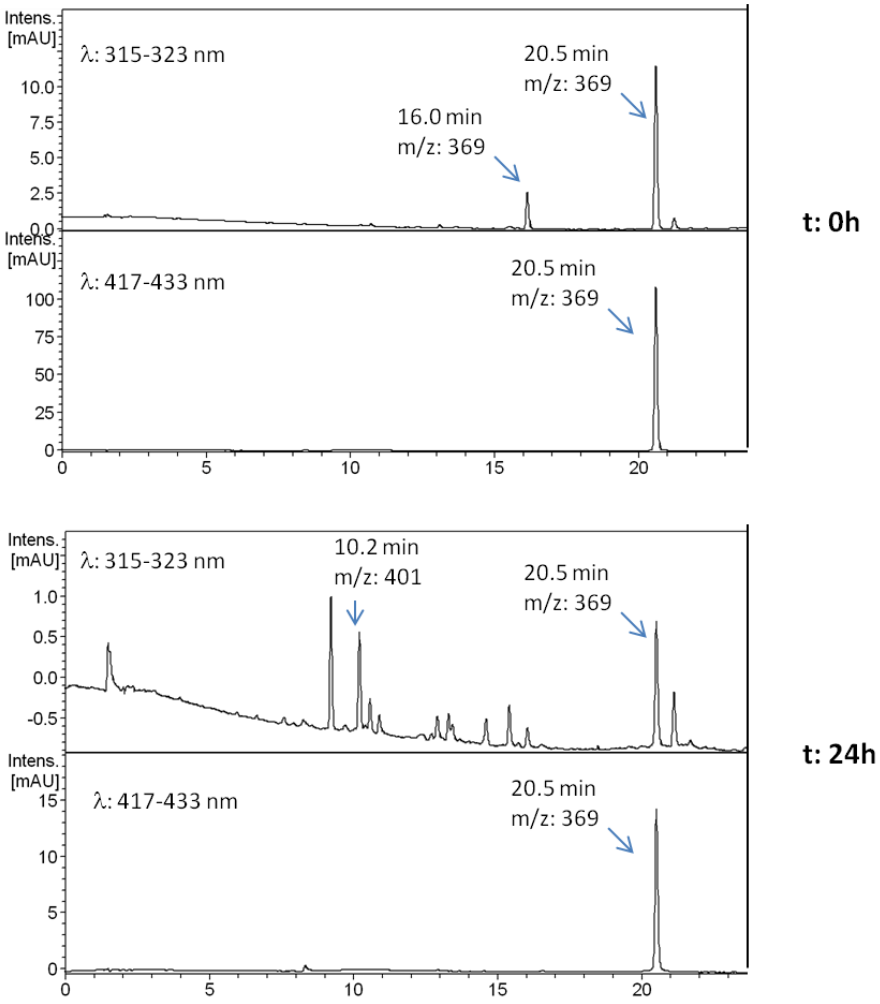


6-hydroxy-1-(4-hydroxy-3-methoxyphenoxy)-3-(4-hydroxy-3-methoxyphenyl)-1,3,3a,6a-tetrahydro-4*H*-cyclopenta[*c*]furan-4-one

M.W. 400.38

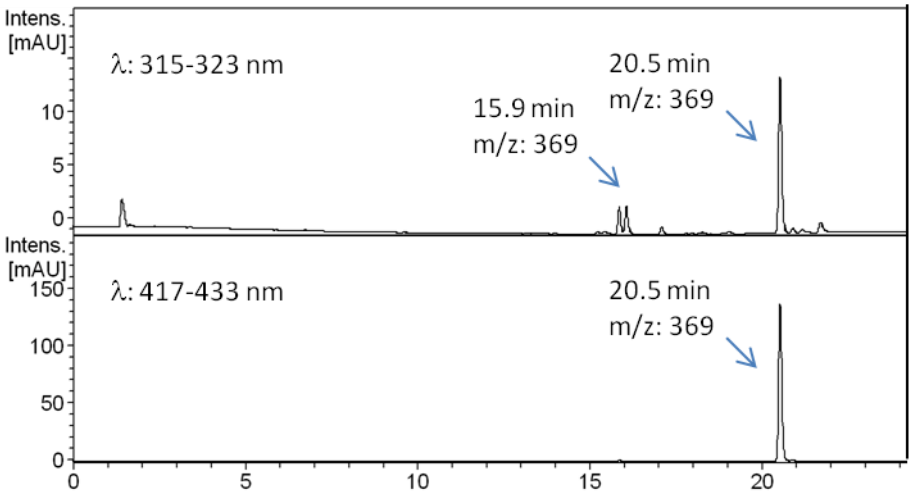
6

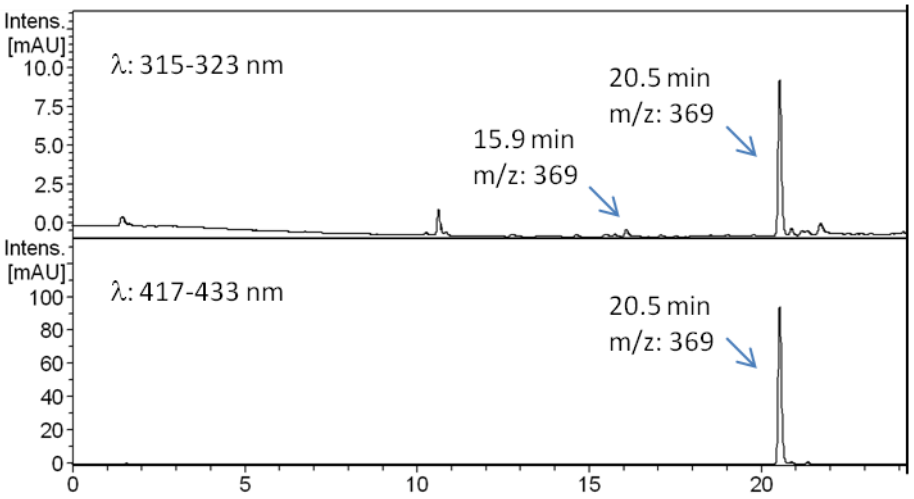
Figure 15. Chromatograms obtained by HPLC/MS analysis of Curcumin in PBS (pH = 6.8) maintained at 37°C for 24 hours. Ranges of wavelength selected for detection: 315-323nm and 417-433 nm



In contrast, Curcumin proved to be stable over a 24 h period in acidic solution (HCl 0.1 N, [Fig. 16]) where only low amount of the keto-form was detected.

Figure 16. Chromatograms obtained by HPLC/MS analysis of Curcumin in HCl 0.1N (pH = 1) maintained at 37°C for 24 hours. Ranges of wavelength selected for detection: 315-323nm and 417-433 nm





2.4.3 UPLC and HPLC-MS assessment of the stability of derivatives 1a-c and 2a-c

The stability of the new amino acid prodrugs of Curcumin in aqueous solution at different pH values was also tested. Aqueous solutions (15 μM) were prepared by dilution in the appropriate media (HCl 0.1 N or PBS at pH = 6.8) of a stock solution of the derivative in DMSO in order to have a maximum of 15% of DMSO in the final solution. The hydrolytic stability over 24 hours of derivatives **1a-c** and **2a-c** was assessed by UPLC-UV analysis. During these experiments several issues emerged due to the low solubility and tendency for aggregation of these compounds and after one hour traces of a yellow precipitate were found in the solution. In order to better understand the behaviour of these prodrugs in physiological like conditions, HPLC/MS analysis were performed under the same conditions discussed before. Derivatives **1c** and **2c** were chosen as model compounds to study the stability.

Aliquots of freshly prepared aqueous solutions were analyzed at time 0 and after 24 hours of incubation at 37°C.

The monosubstituted analogue of Curcumin (**1c**) in PBS solution at pH 6.8 showed at time zero two peaks both with *m/z* 512 attributed to the keto and enol tautomers. After 24 hours at 37°C, about 50 % of **1c** remained unmodified as confirmed by the presence of the peak at 21.0 min, and a few unknown peaks appeared with low retention times [Fig. 17] indicating a certain degree of degradation. A trace of yellow precipitate was also observed. Mass spectrometric analysis allowed to identify the precipitate as curcumin confirming a slow hydrolysis of the carbamoyl linkage under these conditions.

Under acidic conditions **1c** showed a relatively slow degradation rate [Fig. 18] leading to a decrease of 25% in the derivative's concentration after 24 hours. As in the case of the PBS solution at pH 6.8, two peaks with same *m/z* values were identified, confirming the presence of a diketo-enol tautomerism but not curcumin was found confirming the stability of the carbamate linkage under acidic conditions.

The same behaviour was also observed for the di-substituted derivative **2c** confirming slow hydrolysis of the carbamate linker at pH 6.8 and higher stability at acidic pH. The analysis for this compound was made difficult by the poor water solubility compared to the mono-substituted analogue.

Figure 17. Chromatograms obtained by HPLC/MS analysis of **1c** in PBS (pH = 6.8) maintained at 37°C for 24 hours. Ranges of wavelength selected for detection: 315-323nm and 417-433 nm

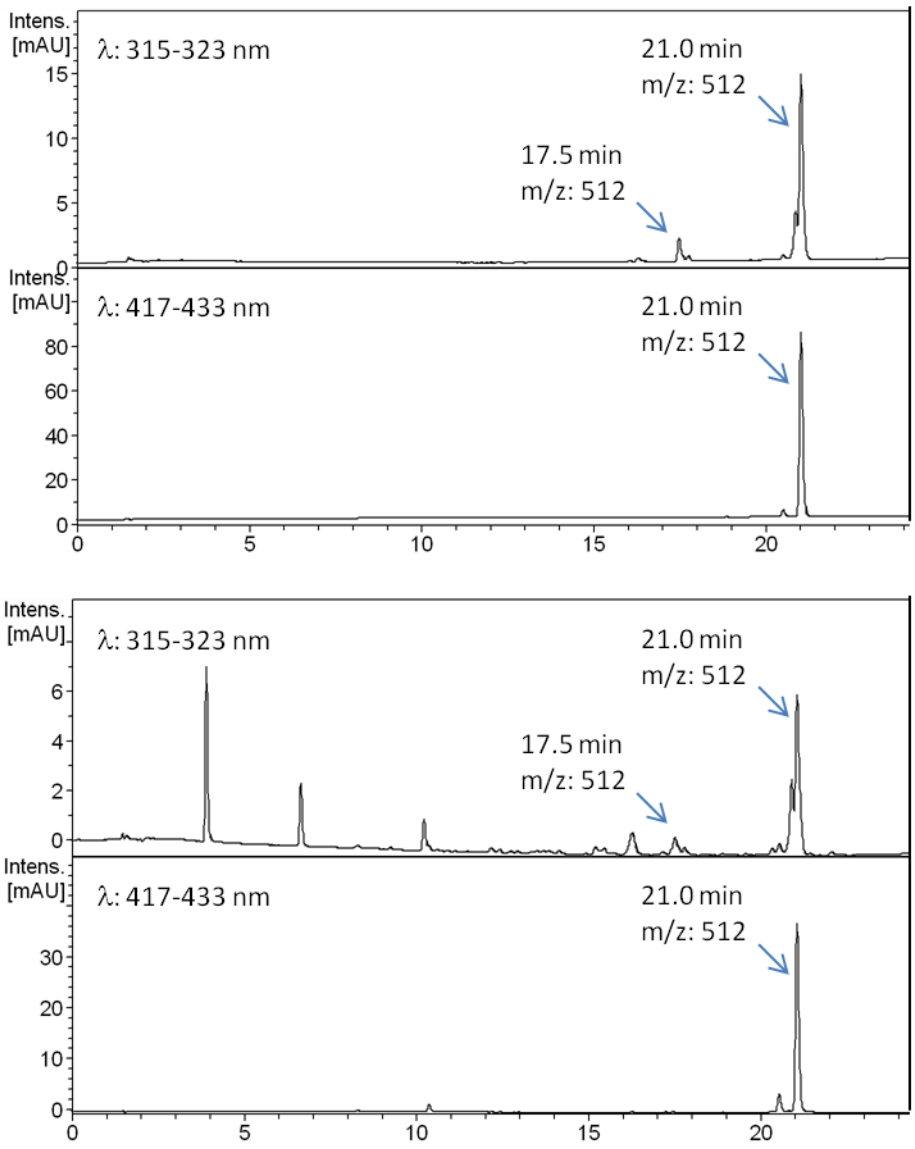
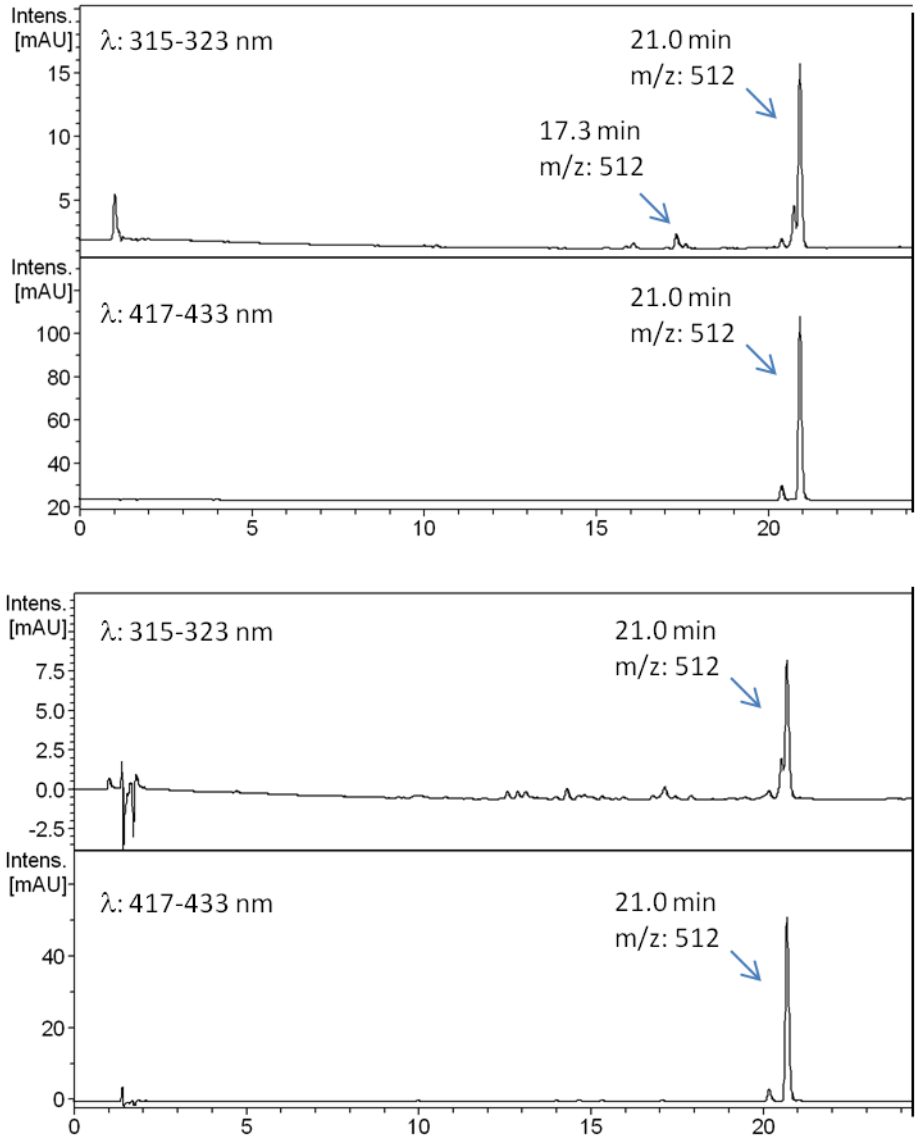


Figure 18. H Chromatograms obtained by HPLC/MS analysis of **1c** in HCl 0.1N (pH = 1) maintained at 37°C for 24 hours. Ranges of wavelength selected for detection: 315-323nm and 417-433 nm



These analyses and analogous trials with the other synthesized compounds show that all derivatives, as well as Curcumin, are characterized by a poor solubility in water, especially at low pH values and in particular for di-substituted derivative. They are stable in acidic solution and can

therefore survive the gastric stage. In near-neutral solution (pH 6.8) the rate of hydrolysis of the carbamoyl linkage is conveniently slow ensuring the protection of the phenolic function during absorption.

2.4.4 NMR assessment of the stability of Curcumin derivatives

The stability of the synthesized compounds in aqueous media was also studied by means of NMR analysis. Larger amounts of DMSO were used compared to those of the previously described stability assays in order to avoid precipitation. Compound **2b** was taken as example. The compound was dissolved in DMSO-d_6 and different amounts of D_2O were added to achieve a final concentration of 1 mM and water percentages of 10%, 30% and 50%, respectively. It turned out, however, that **2b** was not completely solubilized in 50% D_2O-50% DMSO-d_6 at this concentration. Therefore the data from experiments performed in this medium are not included in the present analysis. From NMR analysis of solutions of **2b** in 10% and 30% D_2O in DMSO-d_6 no hydrolysis was observed over 3 hours at room temperature [Fig.s 19a and 20a].

In particular, the signals of the aromatic protons remain unchanged during the 3 hours of duration of the experiment [Fig.s 19b and 20b]. It is also noticed that after 1 hour in both solutions, with 10% and 30% of D_2O, respectively, complete exchange of the carbamoylic N-*H* proton with deuterium has occurred as shown by the disappearance of the characteristic signal around 7.8 ppm [Fig.s 19b and 20b]. The signal appears as a doublet at time = 0 due to the slow exchange rate of carbamoylic proton which allows coupling with the proton on the α carbon of the amino acidic promojety.[118-120] The deuterium exchange of the N-H group is confirmed by the corresponding changes observed in the signal of the proton on the α carbon of the amino acidic promojety around 3.8-3.9 ppm. At time zero, this signal appears as a multiplet centered at 3.9 ppm, due to spin-spin coupling of such proton with both the carbamoylic proton and the proton of the amino acidic side chain respectively. Spectra acquired after 1h, 2h and 3h show that the multiplicity of the signal changes from multiplet to doublet. This change in multiplicity is related to the N-*H* deuterium exchange which results in the disappearance of coupling with the carbamoylic N-*H* proton.

Figure 19. **a)** ^{1}H-NMR spectra (300 MHz) of **2b** in a solution of 90% DMSO-d_6 and 10% D2O. Spectra were acquired every hour for 3 hours. **b)** Expansion of aromatic region between 8 and 6.5 ppm **c)** expansion of amino acidic α-proton

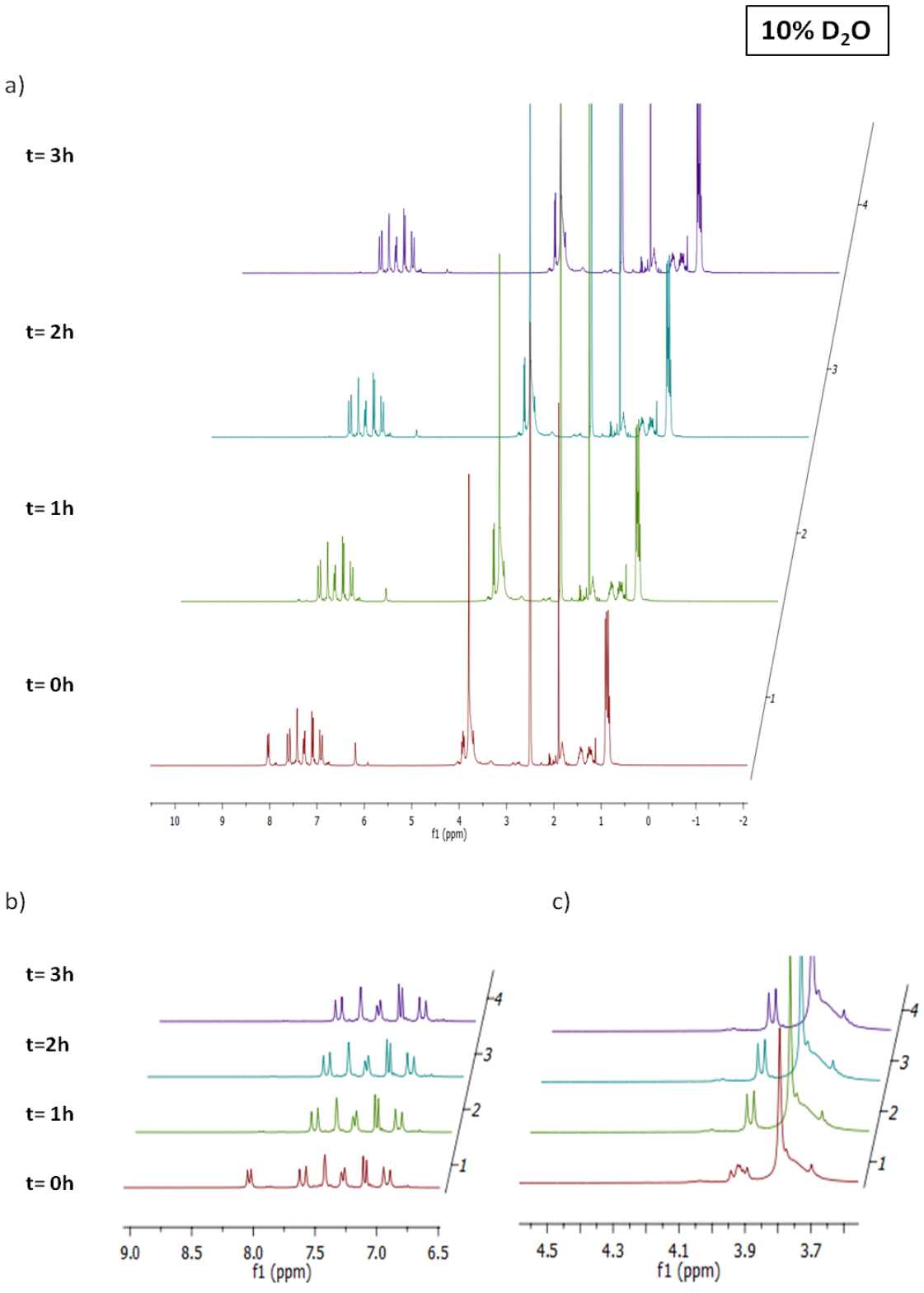
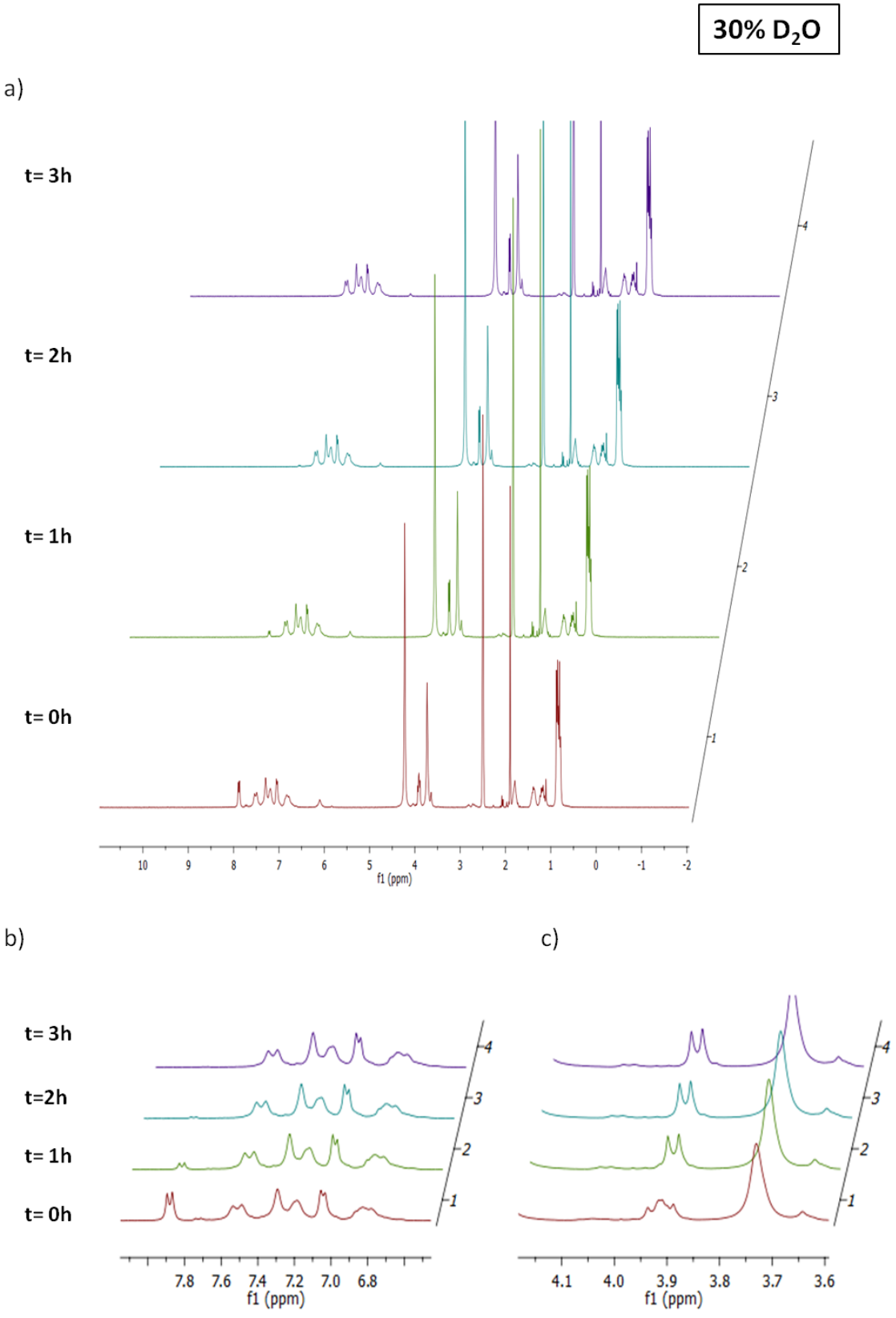


Figure 20. **a)** ^{1}H-NMR spectra (300 MHz) of **2b** in a solution of solution of 60% DMSO-d_6 and 30% D_2O. Spectra were acquired every hour for 3 hours. **b)** Expansion of aromatic region between 8 and 6.5 ppm **c)** expansion of signal of amino acidic α-proton



These data indicate a high hydrolytic stability of the synthesized derivatives in DMSO/water solutions where no precipitation occurred. Extrapolation of these results to interpret those acquired by HPLC analysis of Curcumin and derivatives in aqueous solutions suggests that precipitation processes have probably contributed to loss of signal during the UV-Vis and HPLC analyses more than hydrolysis and degradation processes.

2.5 Pharmacokinetic profile of Curcumin and amino acid prodrugs

In vitro stability studies thus indicated that the rate of hydrolysis of the carbamoyl bond in our new Curcumin derivatives is suitable for application as prodrugs. We therefore proceeded to investigate the behavior of our compounds in pharmacokinetics studies in vivo. These assays are carried out in collaboration with researchers at the Department of Pharmaceutical Sciences and are still ongoing. I report here preliminary results obtained for the mono-leucine and di-leucine derivatives (**1a** and **2a**) in comparison with the natural polyphenol Curcumin.

Following administration of a single intragastric bolus of **1a**, **2a** or Curcumin (50 mg/Kg suspended in H_2O) to overnight-fasted male mice (weight between 20-25 g) aliquots (200 μL) of whole blood were taken at specific intervals (5, 10, 30 minutes and 1, 2, 4, 6 hours) and diluted with methanol (200 μL) to precipitate proteins. Benzanilide (100 μL of 220.8 μg/mL solution) was added as internal standard. Each sample was vortexed, centrifuged (10 minutes, 13000 rpm) and the supernatant was collected, concentrated under vacuum and dissolved in methanol (50 μL). Then the resulting mixture was analyzed by UPLC-UV-MS (Agilent 1260 with Agilent/Varian 320 Triple Quadrupole –TQD) injecting 10 μL in a C18 Phenomenex 2.6 μm 2.1 x 50 mm column and using as eluent a gradient of Acetronitrile/H_2O +2% of HCOOH from 10% to 95% ACN in 9 minutes.

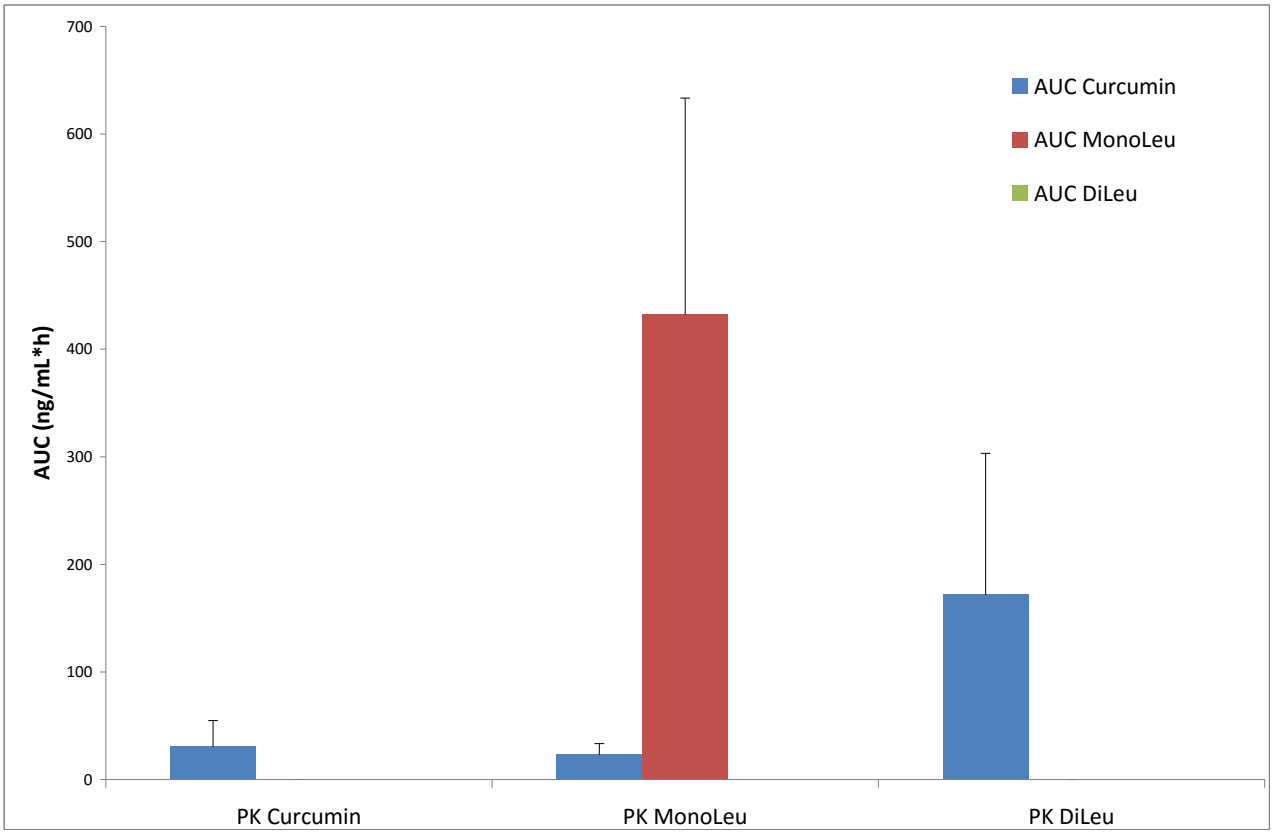
The results obtained [Fig 21] clearly indicate that the mono-substituted Curcumin derivative **1a** is rapidly and extensively absorbed by the intestine after intragastric administration, resulting in high concentration in blood of the unmodified prodrug and also releasing free Curcumin during the 6

hours of the pharmacokinetic experiment in amounts of the same order of magnitude as when Curcumin was administered as such.

Administration of the di-substituted Curcumin derivative **2a** instead resulted in a sustained delivery of the active polyphenol Curcumin during the 6 hours of pharmacokinetics in larger amount (7.3 times more) as compared to the amount of Curcumin administered as such.

These are preliminary results which need to be confirmed and extended. They might be explained by the low absorbtion rate of the di-substituted Curcumin analogue which transits through the gut slowly hydrolyzing to the mono-substituted analogue, which is in turn rapidly absorbed and hydrolyzed in the bloodstream to give Curcumin.

Figure 21. $AUC_{0\text{-}6h}$ of the species circulating in the bloodstream after oral administration of Curcumin, **1a** (mono-leucine derivative) and **2a** (di-leucine derivative). Error bars represent + standard deviation (an error bar of the same size in the negative direction is also implied but not shown for graphic clarity). n = 3



These results suggest that carbamoyl bond-based di-substituted amino acid prodrugs of Curcumin might be quite effective in increasing its bioavailability. The other derivatives synthesized are currently under investigation.

2.6 Studies about binding affinity to Human serum albumin

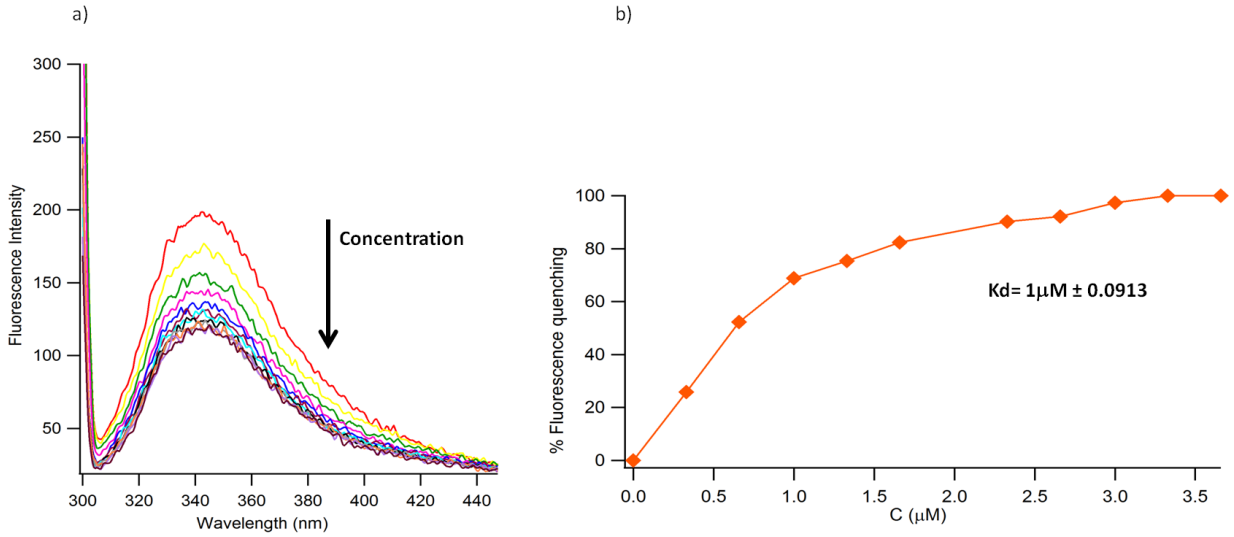
The low solubility of prodrugs and Curcumin itself in aqueous solution, led us to investigate on their affinity for proteins, in particular toward human serum albumin, which is ubiquitous in the blood stream and is known to have function of transport for highly hydrophobic small molecules and peptides. Binding assays for Curcumin and all synthesized prodrugs were thus carried out. Human Serum Albumin (HSA) is characterized by the presence of a tryptophan residue in the proximity of the binding site, thus quenching of fluorescence emission at 340 nm was used as parameter to monitor the binding. As shown in figure 22a, the higher is the concentration of prodrug in HSA solution the higher is the fluorescence quenching of protein.

A 3 μM solution of HSA in PBS at pH 7.4 and 1 mM ethanolic stock solutions of the synthesized new Curcumin prodrugs were prepared. The HSA solutions were maintained at 37°C and were titrated with aliquots of 1 μL of the Curcumin prodrug (**1a-c**, **2a-c**). After each addition an emission spectrum was acquired.

The dissociation constant (K_d) of the prodrug/albumin complex was extrapolated by non-linear regression of the function of prodrug concentration versus percent fluorescence quenching, considering a 1:1 binding stoichiometry.

A control experiment with Curcumin was also performed. The K_d determined in this experiment was 1 μM in agreement with data reported in literature [Fig. 22b].[121]

Figure 22. a) Fluorescence spectra of aqueous solution of HSA at different concentration of Curcumin. Fluorescence quenching band of tryptophan residues at 340 nm was considered. **b)** Binding curve of Curcumin. A binding constant (K_d) of 1 μM was deteremine by extrapolation of the curve.



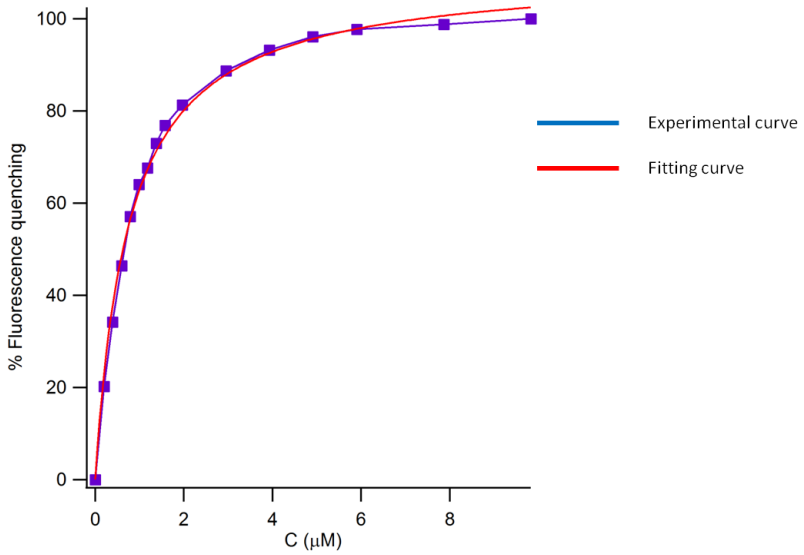
For the major part of the new Curcumin prodrugs developed in this thesis, the measured binding constants underline a strong affinity for albumin, higher than that of Curcumin, probably due to their hydrophobicity. As shown in Table 1 binding constants are in the 640 -730 nM range, with the exception of Curcumin-di-leucine which shows a significantly higher affinity, with a K_d = 225 nM. This means that this derivative is a very strong ligand for albumin.

Table 1. Dissociation constants extrapolated for compounds **1a-c**, **2a-c**

Compound	K_d (nM)
Curcumin-m-Leucine (**1a**)	$3x10^4$
Curcumin-di-Leucine (**2a**)	225
Curcumin-m-Isoleucine (**1b**)	640
Curcumin-di-Isoleucine (**2b**)	709
Curcumin-m-Valine (**1c**)	670
Curcumin-di-Valine (**2c**)	730

For the equilibrium binding constant calculation a one site binding model was used [Fig. 23].

Figure 23. Fitting of binding curve of compound **1b**. The experimental curve (blue) and the fitting curve (red) obtained for this compound are compared.



The equation used for the fitting of the curves was the following:

$$Y = B_{max}\frac{C}{K_d + C}$$

Where:

Y is the experimentally determined quenching in fluorescence emission of HSA

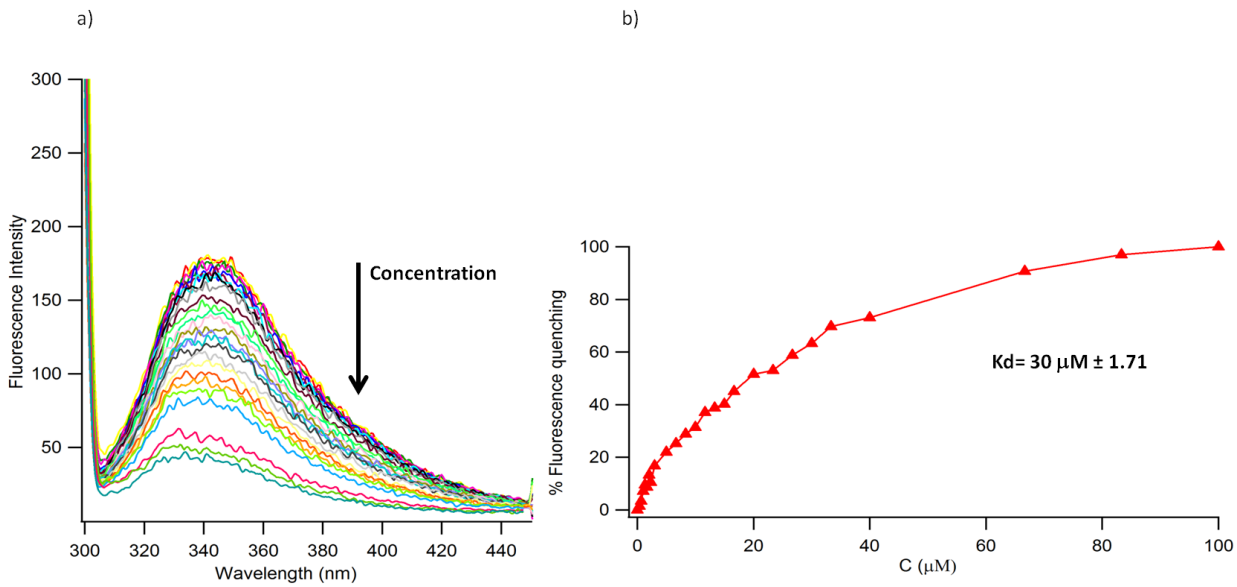
B_{max} is the plateau value of the curve at high concentration of ligand

C is the ligand concentration

Because of the good fitting observed in all cases, which is indicative of the reliability of the extrapolated constants, we could conclude that all the new synthesized Curcumin prodrugs are able to bind to HSA more strongly than Curcumin itself.

Curcumin-*m*-Leucine (**1a**) showed, however, a particular behavior, a high dissociation constant (30 μM) which is about 30 times higher than Curcumin, indicating the poor activity of this derivative as ligand for albumin if compared to the other prodrugs [Fig. 24].

Figure 24. a) Fluorescence spectra of aqueous solution of HSA at different concentration of **1a**. Fluorescence quenching band of tryptophan residues at 340 nm was considered.
b) Binding curve of **1a**. A binding constant of 30 μM was calculated by extrapolation of the curve.



2.6 Conclusions and outlook

In summary, six new prodrugs of Curcumin, based on a carbamoylic linkage with three different amino acids, were synthesized. The carbamoylic linkage was chosen in order to enhance hydrolytic stability of the compounds in particular at low pH values. The optimized synthetic routes proved to be robust and all derivatives were fully characterized by proton and carbon NMR and mass spectrometric analyses.

The stability of the new prodrugs and of Curcumin itself in aqueous media was studied using different experimental approaches: UPLC, HPLC/MS, UV-Vis spectroscopy and ^{1}H-NMR. However, the study of these processes and systems was severely hindered by the solubility of the new Curcumin prodrugs in aqueous media which is rather poor, either under acidic or near neutral pH conditions. Compounds **1a-c** and **2a-c** proved to be more stable in acidic solutions as expected based on the known reactivity of the *N*-monosubstituted carbamoylic linkage. In solutions at pH 6.8 (as found in the intestine), some degradation was observed. The stability of the new derivatives against hydrolysis was also studied by ^{1}H-NMR spectroscopy in water-DMSO-d6 solutions. No degradation was observed in these aqueous media over three hours.

Preliminary pharmacokinetic experiments proved that the disubstituted leucine carbamoyl derivative of Curcumin is capable of improving the bioavailability of the parent phenol in mice model.

Binding affinity toward plasma proteins was also tested for all derivatives. Fluorimetric binding assays to human serum albumin (HSA) showed that all the new derivatives, except for the mono-leucine prodrug **1a**, have higher affinity for HAS than Curcumin itself.

From these combined data a good distribution in the blood stream and high stability against hepatic enzymes can be expected for the new derivatives developed with this thesis, in particular for the mono functionalized compounds, which make these compounds potential candidates as Curcumin prodrugs. Pharmacokinetic studies for all the other synthesized derivatives are currently in progress.

2.7. Experimental section

2.7.1. Materials and methods

Curcumin was purchased from TCI Europe, amino acids were purchased from Iris Biotech. (Marktredwitz, Germany). Other starting materials and reagents were purchased from SigmaAldrich (Milan, Italy), Fluka (Milan, Italy), Merck-Novabiochem (Milan, Italy), Acros Organics (Milan, Italy), Carlo Erba (Milan, Italy), and were used as received. TLC analyses were performed on silica gel supported on plastic (MachereyNagel Polygram®SIL G/UV254, silica thickness 0.2 mm, Duren, Germany) and visualized by UV detection. Flash chromatography was performed on silica gel (Macherey-Nagel 60, 230–400 mesh, 0.063–0.040 mm) under air pressure. Solvents were analytical or synthesis grade and were used without further purification. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AVIII spectrometer operating at 300 MHz or on a Bruker AVII500 spectrometer operating at 500 MHz. Chemical shifts (δ) are given in ppm relative to the signal of the residual undeuterated solvent peak. UPLC-UV analyses were performed with an Agilent 1290 Infinity LC System (Agilent Technologies, Milan, Italy), equipped with, a diode array detector (190–500 nm) and a reverse phase column (Zorbax RRHD Eclipse Plus C18, 1.8 µm, 50 x 2.1 mm i.d.; Agilent Technologies). Solvents A and B were water containing 0.1% formic acid and acetonitrile, respectively. HPLC/ESI-MS analyses were performed on a 1100 Series Agilent Technologies (Milan, Italy) system, equipped with binary pump (G1312A) and reverse phase column (Phenomenex Kinetec C-18 2.6 µm 2.1 x 50 mm) a triple quadrupole mass spectrometer detector (Varian 320-MS). Solvents A and B were water containing 2% formic acid and acetonitrile, respectively. MSD SL Trap mass spectrometer (G2445D SL) with ESI source. ESI-MS positive spectra of reaction intermediates and final purified products were obtained from solutions in acetonitrile, eluting with Acetonitrile containing 0.1% formic acid.

UV Analysis. Samples (500 ul) were analyzed on a Cary 300 UV-Vis spectrophotometer using a quartz cell with optical pathlength of 10 mm. All spectra were collected in a specific range of wavelength from 300 to 700 nm. For each compound different concentrations (5, 10, 15, 20, 25, 30 uM) were analyzed in order to acquire a calibration curve.

Fluorescence analysis. Fluorescence emission spectra were recorded at 37°C on a Perkin-Elmer LS-55 spectrofluorimeter. Each compound was dissolved in Ethanol (1mL) to obtain a solution 1mM. Human Serum Albumin (HSA) was dissolved in PBS (pH:7.4) to obtain a solution 3uM. Aliquots (1uL) of a specific compound were added to the albumin solution into quartz cells and spectra were acquired. All spectra were collected in a specific range of wavelength between 300 and 450 nm.

Quartz cells with an optical pathlenght of 10 mm were used for measurement of both fluorescence emission and UV-Vis absorption spectra.

Hydrolysis Reactions. The chemical stability of all new compounds was tested in aqueous media approximating gastric (0.1 N HCl, NormaFix) and intestinal (0.1 M PBS buffer, pH 6.8) pH values. A 5 μM solution of the compound was prepared from a 5 mM stock solution in DMSO, and incubated at 37°C for 24 hours; aliquots of 1 μL were sampled at regular time intervals and analyzed by UPLC. The gradient for solvent B is: 10% (0.5 min) then from 80 to 100% in 8 min. Flow rate: 0.6 mL/min. The eluate was preferentially monitored at 286, 320 and 425 nm (corresponding to absorbance maxima of the internal standard, and curcumin, respectively). The column compartment was maintained at 20°C.

Pharmacokinetics in blood. Rats of weight between 20 and 25 g were treated via "gavage" with suspension of each compound (50 mg/Kg). Samples were prepared suspending each compound in water and stirring suspension for 20 minutes. After oral administration, blood sample were sampled after 5 minutes, 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 6 hours. All blood samples were collected in heparinised analysis tubes and treated as follows.

Blood Sample Treatment and Analysis. Aliquots (200 μL) of whole blood were taken at specific intervals (5, 10, 30 minutes and 1, 2, 4, 6 hours) and diluted with methanol (200 μL) to precipitate proteins. Benzanilide (100 μL of 220.8 μg/mL solution) was added as internal standard. Each sample was vortexed, centrifuged (10 minutes, 13000 rpm) and the supernatant was collected, concentrated under vacuum and dissolved in methanol (50 μL). Then the resulting mixture was

analyzed by UPLC injecting 10 μL in a C18 Phenomenex 2.6 μm 2.1 x 50 mm column and using as eluent a gradient of Acetronitrile/H_2O +2% of HCOOH from 10% to 95% ACN in 9 minutes.

2.7.2 Synthesis and analytical data of compounds 1a-c

General procedure for the synthesis of protected amino acidic monosubstituted Curcumin [Curc-m-AAtBuO]:

Amino acid tert-butyl ester hydrochloride (1 eq.) and 4-(dimethylamino)pyridine (1.5 eq.) were dissolved in DCM (5mL). The mixture was cooled at 0°C and a solution of bis(4-nitrophenyl) carbonate (0.27 mmol, 1 eq.) in DCM was added dropwise. After stirring for 4 hours at room temperature a fresh solution of Curcumin (0.27 mmol, 1 eq.) and 4-(dimethylamino)pyridine (0.27 mmol, 1 eq.) in THF was added dropwise and mixture was stirred at 50°C overnight. Reaction mixture was then washed with HCl 0.1 N (2x10 mL) and the aqueous layer extracted from EtOAc (3x20 mL). Organic layer was dried over Na_2SO_4 and concentrated under vacuum. The resulting solid was purified by flash chromatography to obtain the desired compound as an orange solid.

tert-butyl((4-((1E,3Z,6E)-3-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-5-oxohepta-1,3,6-trien-1-yl)-2-methoxyphenoxy)carbonyl)-L-leucinate [Curc-*m*-LeutBuO]: Flash chromatography conditions: SiO_2, eluent Petroleum ether/ethyl acetate 6:4. Yield: 82%. ^{1}H-NMR (300 MHz, d_6 -DMSO) δ= 9.67 (s, 1H), 8.12 (d, J=8.0 Hz, 1H) 7.60 (d, *J* = 15.7 Hz, 2H), 7.40 (d, *J* = 38.1 Hz, 2H), 7.22 (dd, *J* = 33.7, 8.0 Hz, 2H), 7.14 – 6.87 (m, 2H), 6.81 (dd, *J* = 18.5, 12.0 Hz, 2H), 6.13 (s, 1H), 3.96 (m, 1H), 3.84 (s, 3H), 3.83 (s, 3H) 1.81 – 1.68 (m, 1H), 1.67 – 1.48 (m, 2H), 0.92 (dd, *J* = 11.3, 6.5 Hz, 6H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ= 184.71, 181.60, 171.60, 153.79, 151.79, 149.57, 148.02, 141.45, 139.15, 133.12, 126.26, 124.26, 123.46, 123.27, 121.14, 115.74, 112.04, 111.46, 101.26, 80.55, 55.84, 55.69, 53.20, 27.60, 24.33, 22.72, 21.26, 14.04 ppm. ESI/MS *m/z* [M+H$^+$]: 582

tert-butyl((4-((1E,3Z,6E)-3-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-5-oxohepta-1,3,6-trien-1-yl)-2-methoxyphenoxy)carbonyl)-L-isoleucinate [Curc-m-IletBuO]: Flash chromatography conditions: SiO_2, eluent Petroleum ether/ethyl acetate 6:4. Yield: 89%. ^{1}H NMR (300 MHz, DMSO-d_6) δ= 9.67 (s, 1H), 8.07 (d, *J* = 8.3 Hz, 1H), 7.60 (d, *J* = 15.7 Hz, 2H), 7.40 (d, *J* = 39.6 Hz, 2H), 7.23 (dd, *J* = 34.2,

8.4 Hz, 2H), 7.17 – 6.86 (m, 2H), 6.88 – 6.72 (m, 2H), 6.13 (s, 1H), 3.89 (m, 1H), 3.84 (s, 3H), 3.83 (s, 3H), 1.90 – 1.73 (m, 1H), 1.43 (s, 9H), 1.28 (m, 2H), 0.89 (m, *J* = 7.1 Hz, 6H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ 184.71, 181.59, 170.47, 153.97, 151.79, 149.54, 148.00, 141.44, 139.16, 133.12, 126.23, 124.26, 123.48, 123.29, 121.14, 115.72, 112.03, 111.46, 101.23, 80.64, 59.35, 55.87, 55.69, 36.40, 27.65, 24.81, 15.34, 11.17 ppm. ESI/MS *m/z* [M+H$^+$]:582

tert-butyl((4-((1E,3Z,6E)-3-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-5-oxohepta-1,3,6-trien-1-yl)-2-methoxyphenoxy)carbonyl)-L-valinate [Curc-m-ValetBuO]: Flash chromatography conditions: SiO_2, eluent Petroleum ether/ethyl acetate 6:4. Yield: 91%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.70 (s, 1H), 8.09 (d, *J* = 8.4 Hz, 1H), 7.60 (dd, *J* = 15.8, 2.1 Hz, 2H), 7.49 – 7.33 (m, 2H), 7.32 – 7.15 (m, 2H), 7.15 – 6.90 (m, 2H), 6.86 – 6.76 (m, 2H), 3.85 (s, 3H), 3.83 (s, 3H), 3.81 (m, 1H), 2.08 (dt, *J* = 12.4, 6.7 Hz, 2H), 1.44 (s, 9H), 0.95 (d, *J* = 6.8 Hz, 6H) ppm. ^{13}C NMR (76 MHz, DMSO-d_6) δ 184.72, 181.59, 154.08, 151.80, 149.53, 148.00, 141.42, 139.17, 133.12, 126.22, 124.26, 123.50, 123.31, 121.23, 121.13, 115.70, 112.03, 111.43, 101.24, 80.64, 60.43, 55.88, 55.69, 29.96, 27.66, 18.89, 18.03 ppm. ESI/MS *m/z* [M+H$^+$]: 568

General procedure for the synthesis of Amino acidic monosubstituted Curcumin [1a-c]:

Curc-m-AAtBuO (0.21 mmol, 1eq.) was dissolved in DCM and cooled to 0°C. TFA (8.64 mmol, 40 eq.) was added dropwise then 1% of TIPS was added. Reaction was stirred for 5 hours at room temperature. DCM was evaporated under vacuum and TFA was diluted with toluene and evaporated under vacuum. The resulting solid was purified by flash chromatography to afford the final product.

((4-((1E,3Z,6E)-3-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-5-oxohepta-1,3,6-trien-1-yl)-2-methoxyphenoxy)carbonyl)-L-leucine [1a]:]: Flash chromatography conditions: SiO_2, eluent Dichloromethane/Acetone/Acetic acid 95:5:0.2. Yield: 90%. ^{1}H-NMR (300 MHz, d_6 -DMSO) δ= 9.67 (s, 1H), 8.09 (d, *J* = 8.2 Hz, 1H), 7.59 (d, *J* = 16.0 Hz, 2H), 7.42 (m, 2H), 7.31 – 7.14 (m, 2H), 7.15 – 6.88 (m, 2H), 6.87 – 6.72 (m, 2H), 6.13 (m, 1H), 4.02 (s, 1H), 3.84 (s, 3H), 3.82 (s, 3H), 2.75 (dd, *J* = 21.4, 7.0 Hz, 1H), 1.66 – 1.48 (m, 2H), 0.92 (dd, *J* = 9.8, 6.6 Hz, 6H) ppm.^{13}C NMR (75 MHz, DMSO-d_6) δ 184.72, 181.63, 173.94, 171.98, 153.82, 151.76, 149.54, 148.01, 141.46,

139.21, 133.05, 126.24, 124.25, 123.43, 123.32, 121.16, 115.72, 112.01, 111.46, 101.22, 55.87, 55.71, 52.40, 24.32, 22.88, 21.14, 21.02 ppm. ESI/MS *m/z* [M+H$^+$]: 526

((4-((1E,3Z,6E)-3-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-5-oxohepta-1,3,6-trien-1-yl)-2-methoxyphenoxy)carbonyl)-L-isoleucine [1b]: Flash chromatography conditions: SiO_2, eluent Dichloromethane/Acetone/Acetic acid 95:5:0.2. Yield: 85%. ^{1}H NMR (300 MHz, DMSO-d_6) δ), , 9.66 (s, 1H), 8.04 (d, *J* = 8.6 Hz, 2H),), 7.60 (d, *J* = 15.3 Hz, 2H), 7.40 (d, *J* = 40 Hz, 2H), 7.23 (dd, *J* = 34.2, 8.4 Hz, 2H), 7.17 – 6.86 (m, 2H), 6.88 – 6.72 (m, 2H), 6.13 (s, 1H), 3.94 (m, 1H), 3.84 (s, 3H), 3.83 (s, 3H), 1.55-1.37(m, 1H), 1.36-1.20 (m, 2H), 0.89 (m, *J* = 6.9 Hz, 6H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ 184.70, 181.61, 172.87, 171.97, 154.03, 151.79, 149.53, 148.01, 141.46, 139.19, 133.07, 126.23, 124.25, 123.48, 123.31, 121.15, 115.72, 112.00, 111.45, 101.22, 58.81, 55.90, 55.71, 36.22, 24.59, 21.02, 15.52, 11.20 ppm. ESI/MS *m/z* [M+H$^+$]: 526

((4-((1E,3Z,6E)-3-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-5-oxohepta-1,3,6-trien-1-yl)-2-methoxyphenoxy)carbonyl)-L-valine [1c]: Flash chromatography conditions: SiO_2, eluent Dichloromethane/Acetone/Acetic acid 95:5:0.2. Yield: 95%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.66 (s, 1H), 8.03 (d, *J* = 8.6 Hz, 1H), 7.59 (dd, *J* = 15.8, 3.1 Hz, 2H), 7.40 (d, *J* = 39.6 Hz, 2H), 7.31 – 7.14 (m, 2H), 7.14 – 6.88 (m, 2H), 6.86 – 6.75 (m, 2H), 6.13 (s, 1H), 3.91 (dd, *J* = 8.7, 6.1 Hz, 1H), 3.84 (s, 3H), 3.83 (s, 3H), 2.12 (m, 1H), 0.95 (dd, *J* = 6.8, 2.9 Hz, 6H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ 184.70, 181.59, 170.48, 154.08, 151.80, 149.53, 148.00, 141.44, 139.16, 133.12, 126.22, 124.25, 123.49, 123.29, 121.13, 115.71, 112.04, 111.45, 101.22, 80.63, 60.43, 55.87, 55.69, 29.96, 27.65, 18.87, 18.02 ppm. ESI/MS *m/z* [M+H$^+$]: 512

2.7.3 Synthesis and analytical data of compounds 2a-c

General procedure for the synthesis of protected Amino acidic di-substituted Curcumin [Curc-d-AAtBuO]:

A solution amino acid tert-butyl ester hydrochloride (2 eq.) and 4-(dimethylamino)pyridine (2.5 eq.) in DCM was added dropwise to a cold (0°C) solution of bis(4-nitrophenyl) carbonate (2.5 eq.) in DCM (5mL). After stirring for 4 hours at room temperature a fresh solution of Curcumin (1 eq.) and 4-(dimethylamino)pyridine (2.5 eq.) in DCM was added dropwise and mixture was stirred at 50°C overnight. Solvent was evaporated under vacuum, the residue dissolved in EtOAc and washed with HCl 0.1 N (2x10 mL). The combined aqueous layer was extracted from EtOAc (3x20 mL). Organic layer was dried over Na_2SO_4 and concentrated under vacuum. The resulting solid was purified by flash chromatography to obtain a yellow solid.

di-tert-butyl-2,2'-((((((1E,3Z,6E)-3-hydroxy-5-oxohepta-1,3,6-triene-1,7-diyl)bis(2-methoxy-4,1-phenylene))bis(oxy))bis(carbonyl))bis(azanediyl))(2S,2'S)-bis(4-methylpentanoate) [Curc-di-LeutBuO]:

Flash chromatography conditions: SiO_2, eluent petroleum ether/Ethyl acetate 7:3. Yield: 73%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.13 (d, *J* = 8.0 Hz, 2H), 7.65 (d, *J* = 15.8 Hz, 2H), 7.48 (s, 2H), 7.30 (d, *J* = 7.6 Hz, 2H), 7.13 (d, *J* = 7.9 Hz, 2H), 6.96 (d, *J* = 15.6 Hz, 2H), 6.20 (s, 1H), 4.02 – 3.91 (m, 2H), 3.83 (s, 6H), 1.73 (m, 2H), 1.66 – 1.48 (m, 4H), 1.42 (s, 18H), 0.92 (dd, *J* = 11.8, 6.5 Hz, 12H) ppm. ^{13}C NMR (76 MHz, DMSO) δ 183.20, 170.30, 153.75, 151.78, 141.56, 139.61, 132.99, 124.30, 123.49, 121.38, 112.11, 101.58, 80.57, 55.88, 53.16, 27.61, 24.30, 22.74, 21.26 ppm. ESI/MS *m/z* [M+H$^+$]:795

di-tert-butyl-2,2'-((((((1E,3Z,6E)-3-hydroxy-5-oxohepta-1,3,6-triene-1,7-diyl)bis(2-methoxy-4,1-phenylene))bis(oxy))bis(carbonyl))bis(azanediyl))(2S,2'S,3S,3'S)-bis(3-methylpentanoate) [Curc-di-IletBuO]: Flash chromatography conditions: SiO_2, eluent petroleum ether/Ethyl acetate 7:3. Yield: 71%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.08 (d, *J* = 8.6 Hz, 2H), 7.64 (d, *J* = 16.0 Hz, 2H), 7.48 (s, 2H), 7.30 (d, *J* = 7.9 Hz, 2H), 7.13 (d, *J* = 8.0 Hz, 2H), 6.97 (d, *J* = 15.8 Hz, 2H), 6.20 (s, 1H), 3.88 (m, 2H), 3.83 (s, 6H), 1.82 (m, 2H), 1.46 (m, 4H), 1.43 (s, 18H), 0.89 (dd, *J* = 13.8, 6.9 Hz, 12H) ppm. ^{13}C NMR (76 MHz, DMSO) δ 183.20, 170.48, 153.97, 151.82, 141.59, 139.92, 133.02, 124.30, 123.51,

121.38, 112.11, 101.61, 59.36, 55.87, 36.43, 27.65, 24.83, 20.71, 15.35, 11.18 ppm. ESI/MS *m/z* [M+H$^+$]: 795

di-tert-butyl-2,2'-((((((1E,3Z,6E)-3-hydroxy-5-oxohepta-1,3,6-triene-1,7-diyl)bis(2-methoxy-4,1-phenylene))bis(oxy))bis(carbonyl))bis(azanediyl))(2S,2'S)-bis(3-methylbutanoate) [Curc-di-ValetBuO]: Flash chromatography conditions: SiO_2, eluent petroleum ether/Ethyl acetate 7:3. Yield: 80%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.08 (d, *J* = 7.5 Hz, 2H), 7.65 (d, *J* = 15.8 Hz, 2H), 7.48 (s, 2H), 7.30 (d, *J* = 8.1 Hz, 2H), 7.13 (d, *J* = 8.1 Hz, 2H), 6.95 (d, *J* = 15.9 Hz, 2H), 6.19 (s, 1H), 3.86 (dd, *J* = 6.1, 4.0 Hz, 2H), 3.83 (s, 6H), 2.10 (m, 2H), 1.44 (s, 18H), 0.96 (d, *J* = 6.8 Hz, 12H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ 183.21, 170.52, 154.11, 151.86, 141.62, 139.91, 133.05, 124.32, 123.52, 121.36, 112.14, 101.62, 80.65, 60.46, 55.88, 30.02, 27.66, 18.89, 18.02 ppm. ESI/MS *m/z* [M+H$^+$]: 767

General procedure for the synthesis of Amino acidic disubstituted Curcumin [2a-c]:

Curc-d-AAtBuO, (1eq.) was dissolved in DCM (40 eq.) and cooled to 0°C. TFA (40 eq.) was added dropwise then 1% of TIPS was added. Reaction was stirred for 5 hours at room temperature. DCM was evaporated under vacuum and TFA was diluted with toluene and evaporated under vacuum. The resulting solid was purified by flash chromatography to afford the product.

(2S,2'S)-2,2'-((((((1E,3Z,6E)-3-hydroxy-5-oxohepta-1,3,6-triene-1,7-diyl)bis(2-methoxy-4,1-phenylene))bis(oxy))bis(carbonyl))bis(azanediyl))bis(4-methylpentanoic acid) [2a]: Flash chromatography conditions: SiO_2, eluent Dichloromethane/Acetone/Acetic acid 95:5:0.2. Yield: 90%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.10 (d, *J* = 8.1 Hz, 2H), 7.64 (d, *J* = 16.0 Hz, 2H), 7.48 (s, 2H), 7.30 (d, *J* = 8.3 Hz, 2H), 7.13 (d, *J* = 8.1 Hz, 2H), 6.96 (d, *J* = 16.0 Hz, 2H), 6.20 (s, 1H), 4.04 (m, 2H), 3.83 (s, 6H), 1.71 (d, 2H), 1.66 – 1.44 (m, 4H), 0.92 (dd, *J* = 9.3, 6.7 Hz, 12H) ppm. ESI/MS *m/z* [M+H$^+$]: 685

(2S,2'S,3S,3'S)-2,2'-((((((1E,3Z,6E)-3-hydroxy-5-oxohepta-1,3,6-triene-1,7-diyl)bis(2-methoxy-4,1-phenylene))bis(oxy))bis(carbonyl))bis(azanediyl))bis(3-methylpentanoic acid) [2b]: Flash chromatography conditions: SiO_2, eluent Dichloromethane/Acetone/Acetic acid 95:5:0.2. Yield: 85%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.05 (d, *J* = 8.7 Hz, 2H), 7.64 (d, *J* = 16.0 Hz, 2H), 7.48 (s, 2H), 7.30 (d, *J* = 8.0 Hz, 2H), 7.13 (d, *J* = 8.1 Hz, 2H), 6.96 (d, *J* = 15.9 Hz, 2H), 3.94 (dd, *J* = 8.3, 6.4 Hz, 4H), 3.83 (s, 6H), 1.85 (m, 2H), 1.54 – 1.20 (m, 4H), 0.97 – 0.81 (m, 12H) ppm. ^{13}C NMR (76 MHz, DMSO) δ 183.23, 172.91, 172.01, 154.04, 151.82, 141.66, 139.97, 132.99, 124.30, 123.53, 121.41, 112.10, 101.61, 58.83, 55.92, 36.24, 24.61, 21.05, 15.54, 11.22 ppm. ESI/MS [M+H$^+$]: 685

(2S,2'S)-2,2'-((((((1E,3Z,6E)-3-hydroxy-5-oxohepta-1,3,6-triene-1,7-diyl)bis(2-methoxy-4,1-phenylene))bis(oxy))bis(carbonyl))bis(azanediyl))bis(3-methylbutanoic acid) [2c]: Flash chromatography conditions: SiO_2, eluent Dichloromethane/Acetone/Acetic acid 95:5:0.2. Yield: 95%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.04 (d, *J* = 8.7 Hz, 2H), 7.64 (d, *J* = 15.8 Hz, 2H), 7.48 (s, 2H), 7.30 (d, *J* = 8.1 Hz, 2H), 7.13 (d, *J* = 8.1 Hz, 2H), 6.96 (d, *J* = 16.0 Hz, 2H), 6.20 (s, 1H), 3.91 (dd, *J* = 8.6, 6.0 Hz, 2H), 3.83 (s, 6H), 2.12 (dq, *J* = 13.2, 6.7 Hz, 2H), 0.95 (dd, *J* = 6.8, 2.8 Hz, 12H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ 183.33, 172.81, 154.11, 151.96, 141.45, 139.94, 132.95, 124.12, 123.50, 121.38, 112.09, 101.25, 59.75, 55.90, 29.76, 19.06, 17.93 ppm. ESI/MS *m/z* [M+H$^+$]: 657

CHAPTER 3

New polymeric prodrug systems of Curcumin

3.1 Polymer therapeutics

The use of polymers in pharmaceutical field has attracted major interest in recent years, such derivatives are generally addressed as "polymer therapeutics".[122-125] In particular, new complex polymer-based systems were developed, and new biomaterials were investigated in order to create new drug delivery systems.[126-131] According to Ringsdorf's model the composition of polymeric prodrugs is characterized by three different parts:

- a polymeric backbone;
- a drug, with poor water solubility and instability at physiological pH preferably;
- a spacer, which is able to control the rate of drug release.[132-134]

In more complex systems, targeting groups called "homing devices", that increase the selectivity of drug delivery, and imaging agents, for diagnostic purposes can also be employed.[135]

Polymer therapeutics involves several types of systems: polimer-drug conjugates, polymer-protein conjugates, polymeric micelles, aggregates and nanoparticles.[123,130,136-151]

These latter correspond to the most largely employed systems in drug delivery because of their potential to extend towards more complex systems and tuning their properties. Furthermore, these systems are characterized by high thermodynamic stability, low critical micellar concentration (CMC) values (10^{-8}-10^{-2} mol L^{-1}) that allow to keep their assembled conformations upon dilution.[152] Working with polymers offers several advantages, in particular the straightforward synthesis, the water solubility, and the possibility to tailor specific architectures.[153-154] Furthermore polymers usually show low toxicity and weak protein interactions, and their conjugation with hydrophobic drugs promotes an increase in water solubility, protection by metabolic enzymes and an enhancement of drug bioavailability.[135,155-157]

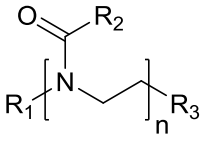
Several random coil polymers were employed in polymer therapeutics, but the most commonly used are poly-glutamic acid (PGA), poly-ethylene glycol (PEG), poly-hydroxypropyl methacrilate (HPMA), poly-vinylpyrrolidone (PVP) and poly-L-lysine (PLL).[158] Other commonly employed macromolecules include chitosan, dextran, and poly-2-alkyl-2-oxazolines (POXA).[159,160] In particular, poly-2-alkyl-2-oxazolines (polyoxazolines) have shown good biocompatibility and

stability over time, thus becoming the object of several studies for the synthesis of new pharmaceutical systems.[159,161-163]

3.2 Polyoxazolines

Polyoxazolines are a family of polymers isomeric to polypeptides or poly-acrylamides bearing a primary amide moiety [Fig.25]. These macromolecules are characterized by a high chemical stability, a lower critical solution temperature (LCST) , and they are generally soluble in water and in common organic solvents.[164,165]

Figure 25. Common structure of poly-oxazolines



R_1: initiating group
R_2: lateral chain
R_3: ending group

The use of POXA is advantageous under many points of view. In particular the straightforward synthesis via living cationic ring opening polymerization (CROP) which offers wide possibilities to tune the architecture of the polymer.[166] As all other polymerization reactions, it is divided into three steps: initiation, prolongation and termination[167] [Sch.2]. Many studies were carried out to investigate the possibility to modulate the structure and the architecture of these polymers by the choice of initiating and terminating compounds.[168-170] Initiation step involves activation of the 2-oxazoline ring by an electrophilic compound generating a 2-oxazolinium cation, alkyl triflate or tosylate proved to be the best for this purpose.[171-175] Halides, silicates and zirconium-based compounds can also be used as initiators. In order to allow for further functionalization of the polymer via click chemistry, alkene and alkyne based initiators were also considered.[168]

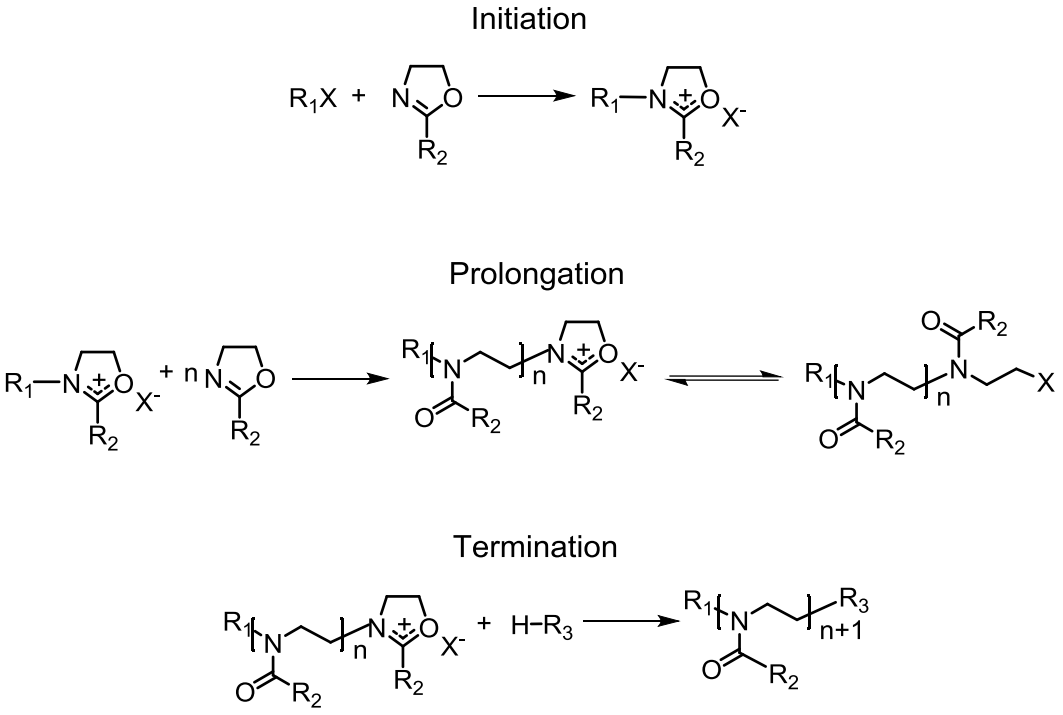
In the propagation step, the activated 2-oxazolonium can react in position *4* with another oxazoline molecule alkylating the nitrogen and transferring the positive charge, which can then

further react with another monomer. The propagation reaction can proceed as long as monomer is present in solution and the cationic head group of the growing chain is not quenched by a nucleophile. For this reason the polymerization is called “living”. This feature allows for a high control over the final molecular weight of the polymer as well as for the synthesis of block-copolymers.[176]

Terminating agents are represented by nucleophiles able to stop the living cationic process by quenching the cation reactive species in 5’ position. Water, alcohols and amines can be identified as the most used terminators. Azides also showed good ability to stop the polymerization.[174] For this reason, nucleophilic groups, such as hydroxyl (OH), mercapto (SH) and amino (NH_2), on the initiator are tolerated but need to be protected in order to reduce potential interferences with the polymerization process.[175]

Furthermore it has been shown that the chain end-group is the main responsible of antimicrobial activity and hemocompatibility of these polymers.[176,177]

Scheme 2. The three steps of POXA synthesis reaction



In particular, poly-2-alkyl-2-oxazolines (POXA) emerged as suitable replacements for PEGs, virtually in all the biomaterials-related applications, thanks to their biocompatibility coupled to their distinctive chemical stability.[178-180]

However, PEGs and their derivatives undergo oxidative and enzymatic degradation in physiological media, originating toxic compounds and limiting their application in drug formulations.[181,182] In addition, PEG therapeutics are known to show accelerated blood clearance (ABC) due to the formation of anti-PEG antibodies by the immune system after the initial treatment.[183] Moreover there has been an increasing evidence of the formation of anti-PEG antibodies also in individuals who have never been exposed to PEGylated drugs, presumably due to its extensive use in cosmetics, household, and hygiene products.[184,185]

Conjugates with proteins as well as with drugs generally showed a similar behavior between POXA and PEG. Hoogemboom, Veronese and coworkers reported that the conjugation of PEtOXA to

Trypsin does not affect the enzymatic activity towards low molecular substrates.[178] Comparison with the corresponding PEG derivative showed similar protein repelling properties. Furthermore conjugation of PEtOXA with cytosine arabinose demonstrated an equivalent behavior towards cytidine deaminase inhibition as for the corresponding PEG derivative.[178] Textor *et al.* pioneered the use of PMOXAs for the preparation of protein repellent surfaces, providing evidence for a similar antifouling activity of surface-grafted PMOXA compared to PEG.[179,180]

In particular poly-2-methyl-2-oxazolines (PMOXA) and poly-2-ethyl-2-oxazolines (PEtOXA) represent the most commonly used PAOXAs for drug formulations due to their high hydrophilicity, the absence of in vivo toxicity, and rapid blood clearance via the kidneys.[163-165,186] In particular Veronese and coworkers showed that the lipophilicity of PMOXA is significantly lower than PEtOXA, and PPrOXA.[165] Moreover Jordan et al. studied the biodistribution and excretion of radiolabeled PMOXA of different molecular weight showing no significant accumulation in tissues and only 2% of the inoculated amount being still present in the blood after 30 min.[186]

Direct conjugation of POXAs with drugs to obtain polymer-therapeutics is not extensively studied and only few examples are known. Among others cytosine arabinose-PEtOXA conjugate by Hoogenboom, and irinotecan and rotigotine conjugates that showed comparable properties with the corresponding PEG conjugates.[158,178-186]

Inspired by the attractive features of PMOXA we present the first example of PMOXA-based carbamoyl prodrugs of curcumin. Such systems are capable to self-assemble in water to form curcumin-PMOXA core-shell nanoparticles.

The influence of PMOXA chain length on the size and the stability of the particles, and the release rate of curcumin was systematically investigated.

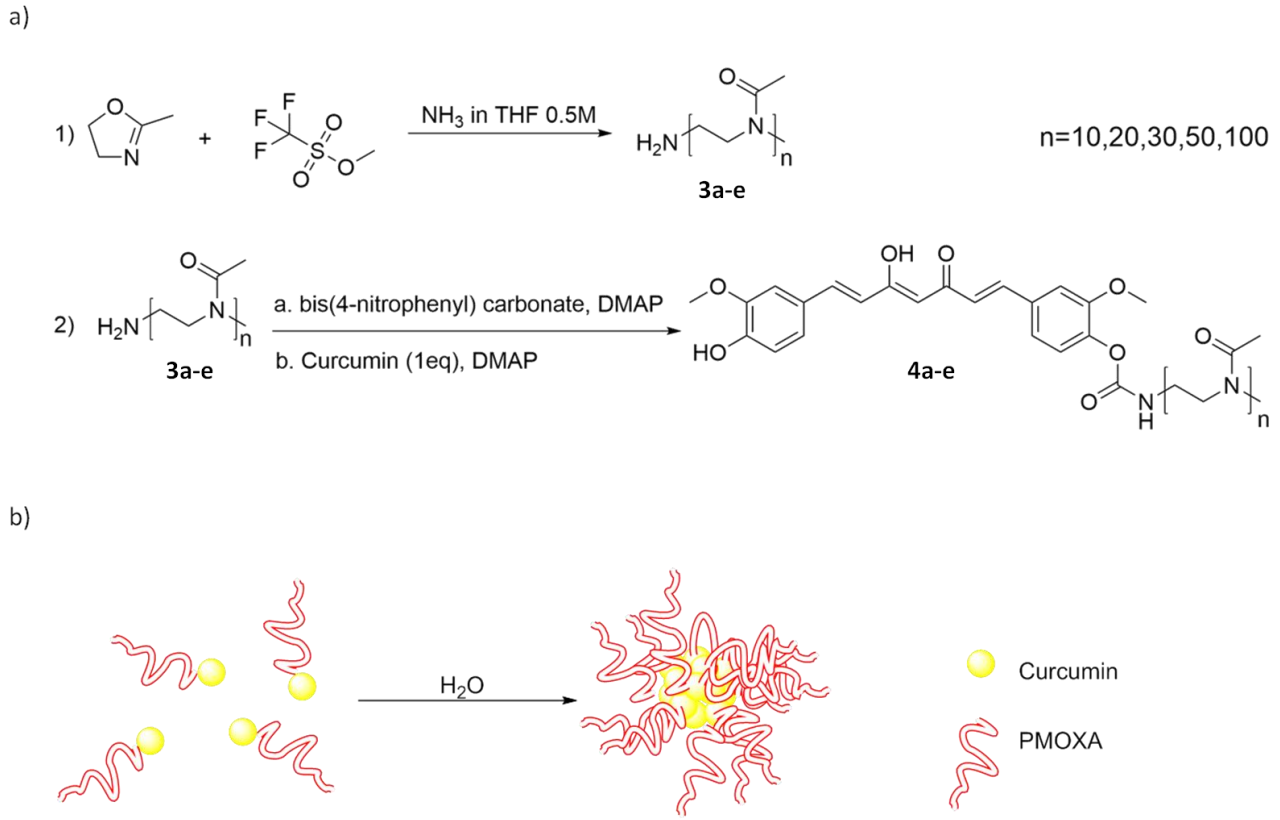
3.3 Synthesis and Self-Assembly of Curcumin-PMOXA Conjugates

Amino-end-functional PMOXAs of different lengths were firstly synthesized by cationic ring opening polymerization (CROP).

CROPs of 2-methyl-2-oxazoline (MOXA) were initiated by methyl-trifluoromethansulfonate (MeOTf) and terminated by addition of ammonia, yielding PMOXA-NH_2 presenting five different molar masses, as determined by varying the initiator/monomer feed ratio. PMOXA-NH_2 presenting average chain lengths of 10, 20, 30, 50 and 100 monomer units (as estimated by H-NMR) were named PMOXA10, PMOXA20, PMOXA30, PMOXA50 and PMOXA100, respectively (**3a-e**). The conjugation of the polymers to one hydroxyl function of Curcumin was carried out using the same one pot synthetic procedure previously described in chapter 2. In particular, amino terminal groups were activated by reaction with bis(4-nitrophenyl) carbonate. The resulting active urethane was reacted with Curcumin in a 1:1 stoichiometry in order to ensure mono functionalization [Fig. 26]. Five derivatives were synthesized according to this procedure:

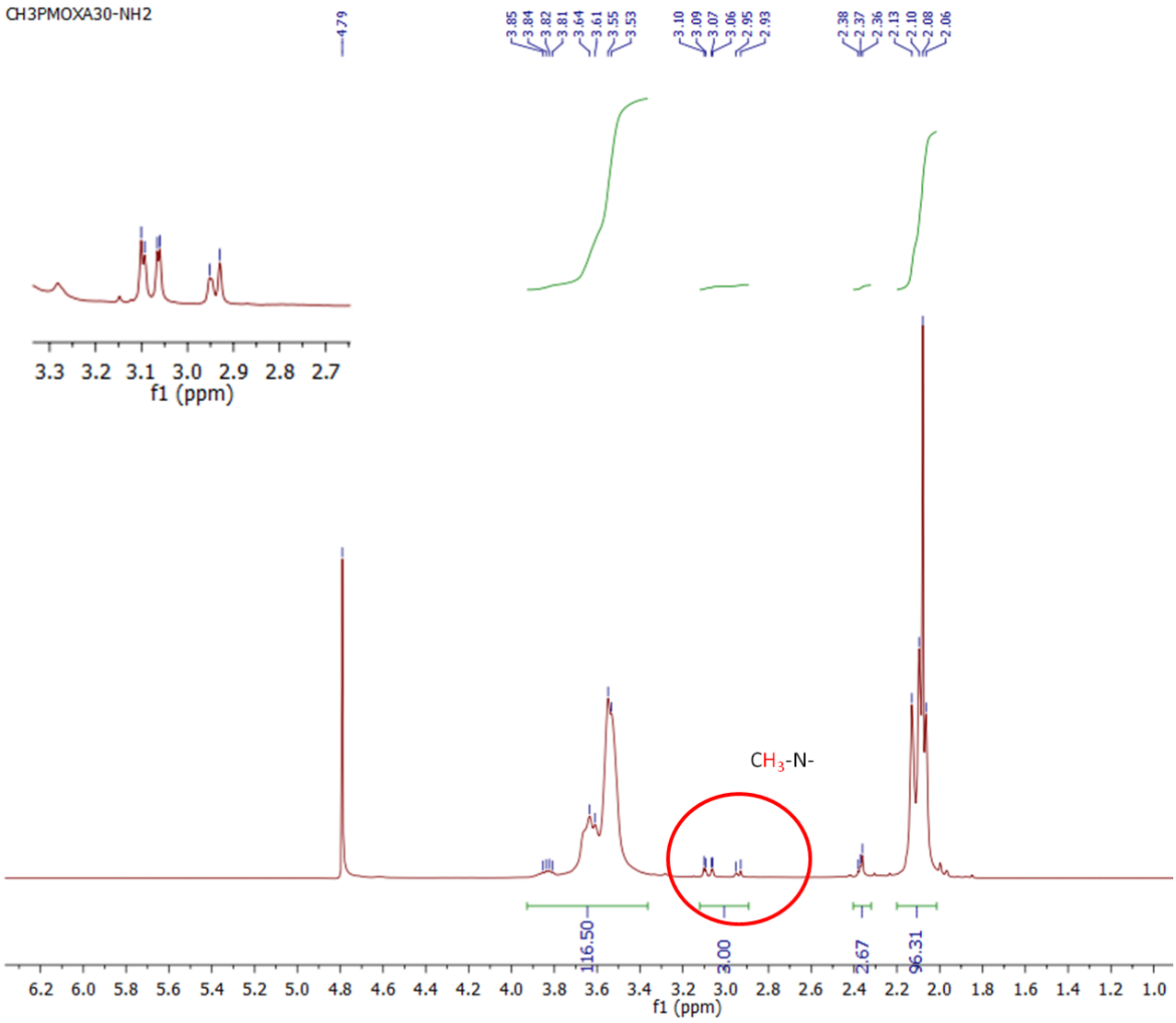
- Curcumin-PMOXA10 (**4a**)
- Curcumin-PMOXA20 (**4b**)
- Curcumin-PMOXA30 (**4c**)
- Curcumin-PMOXA50 (**4d**)
- Curcumin-PMOXA100(**4e**)

Figure 26. a) Synthesis of amino-terminated PMOXA (1) and conjugation with Curcumin via carbamoyl linkage (2). **b)** Nanoparticle formation by self-assembly of the Curcumin-PMOXA conjugates in water.



A common ^{1}H-NMR of a PMOXA is characterized by a specific pattern of signals as shown in figure 27 below. In particular, the signal related to the protons (N-CH_3) of the terminal methyl group between 2.9 and 3.1 ppm appears splitted because of the attachment to an amide backbone than is subjected to cis/ trans isomerism. This specific signal was used to normalize the number of protons in the spectrum.[187]

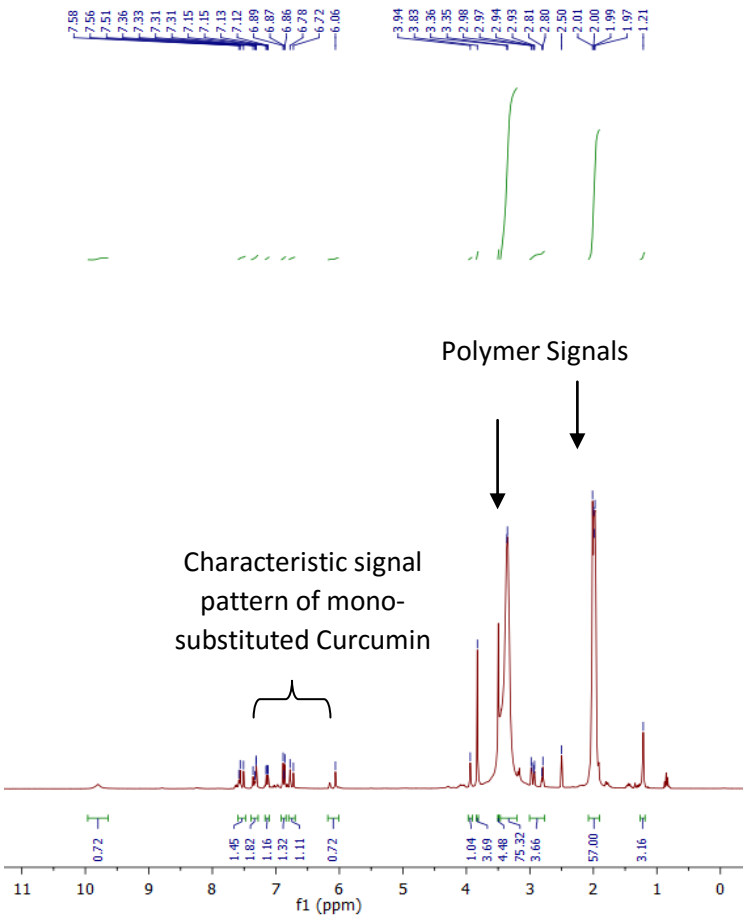
Figure 27. ^{1}H-NMR (300 MHz, D_2O) spectrum of PMOXA30. Signal pattern of terminal methyl group (red) is expanded.



The compounds were purified by dialysis, and characterized by ^{1}H NMR. As an example ^{1}H-NMR spectrum of **4c** were reported in Figure 28.

In the ^{1}H-NMR spectrum, aromatic protons showed a characteristic signal pattern due to the loss of symmetry by molecule. The singlet at 9.8 ppm was identified as phenolic proton of Curcumin as evidence of mono substitution. Between 2 and 4 ppm another pattern of signals related to the polymeric backbone was identified.

Figure 28. ^{1}H-NMR (300 MHz, DMSO-d_6) spectrum of Curcumin-PMOXA30



Curcumin-PMOXA micelles were subsequently prepared by dissolving each polymer conjugate in a mixture of acetonitrile:dimethylsulfoxide (9:1) and dialyzing it against water for 24 hours [Fig. 26b]. The critical micellar concentration (CMC) was later determined according to the literature procedure using pyrene as fluorescence probe sensitive to the hydrophobicity of the environment.[188]

Pyrene's fluorescence is indeed very sensitive to changes in polarity of the chemical environment. Thus when pyrene is trapped inside the micelles its fluorescence is quenched and the relative ratio of intensity of the peaks at 373 and 383 nm decreases. The critical micellar concentration can be calculated by interpolation from the plot of the intensity ratio of the peaks at 373 nm and at 383 nm as a function of concentration [Fig.29b].

As reported in Table 2, Curcumin-PMOXA20 and Curcumin-PMOXA30 derivatives showed the lowest values of CMC indicating the formation of micelles already at μM range of polymer concentrations. On the contrary, Curcumin-PMOXA50 and curcumin-PMOXA100 showed very high CMC values, presumably due to the long hydrophilic polymer segments which stabilized the conjugates in water hindering the self-assembly process into micelles of the molecules. The high CMC value observed for Curcumin-PMOXA10 could be related to the short hydrophilic segment yielding low water-solubility of this conjugate, which resulted in aggregation and precipitation as confirmed by dynamic light scattering (DLS) and transmission electron microscopy (TEM).

Figure 29. a) Fluorescence spectra of solutions of **4c** at different concentrations. λex: 340 nm; [Pyrene]: 1 ppm; **b)** linear extrapolation of the CMC

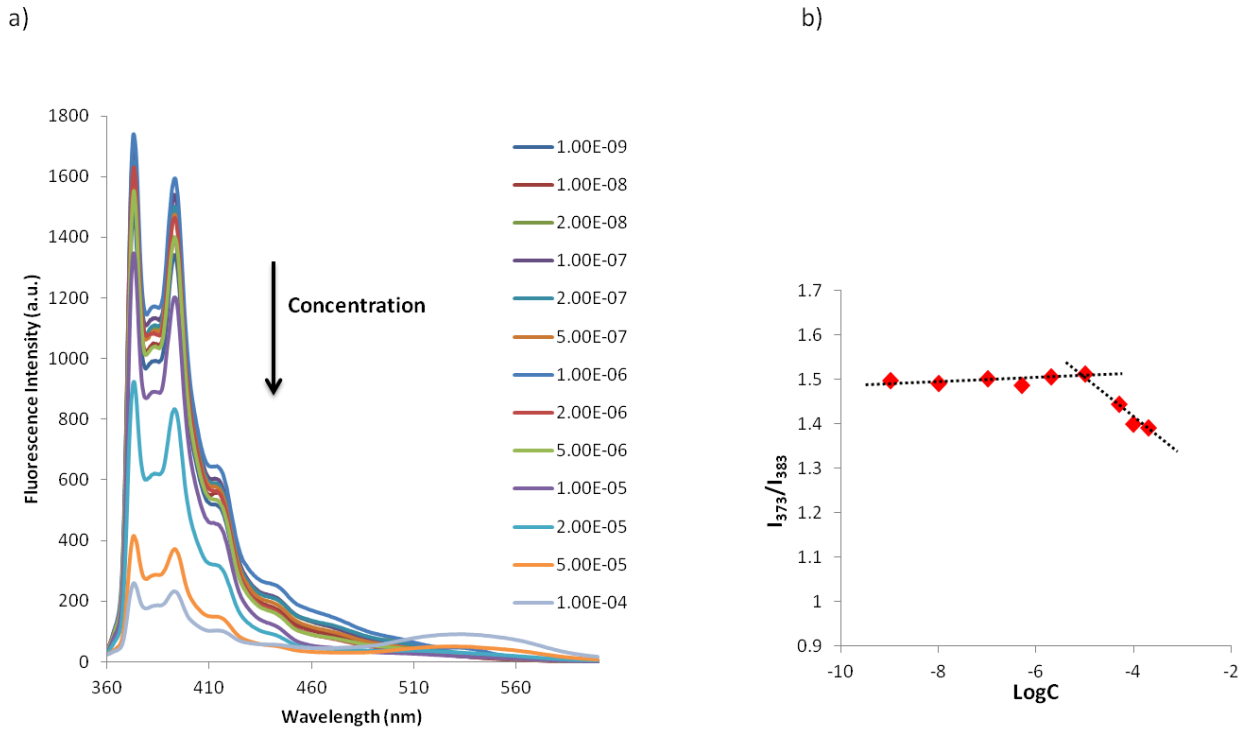


Table 2. Experimental critical micellar concentration (CMC) values for derivatives CuPMOXA10-100.

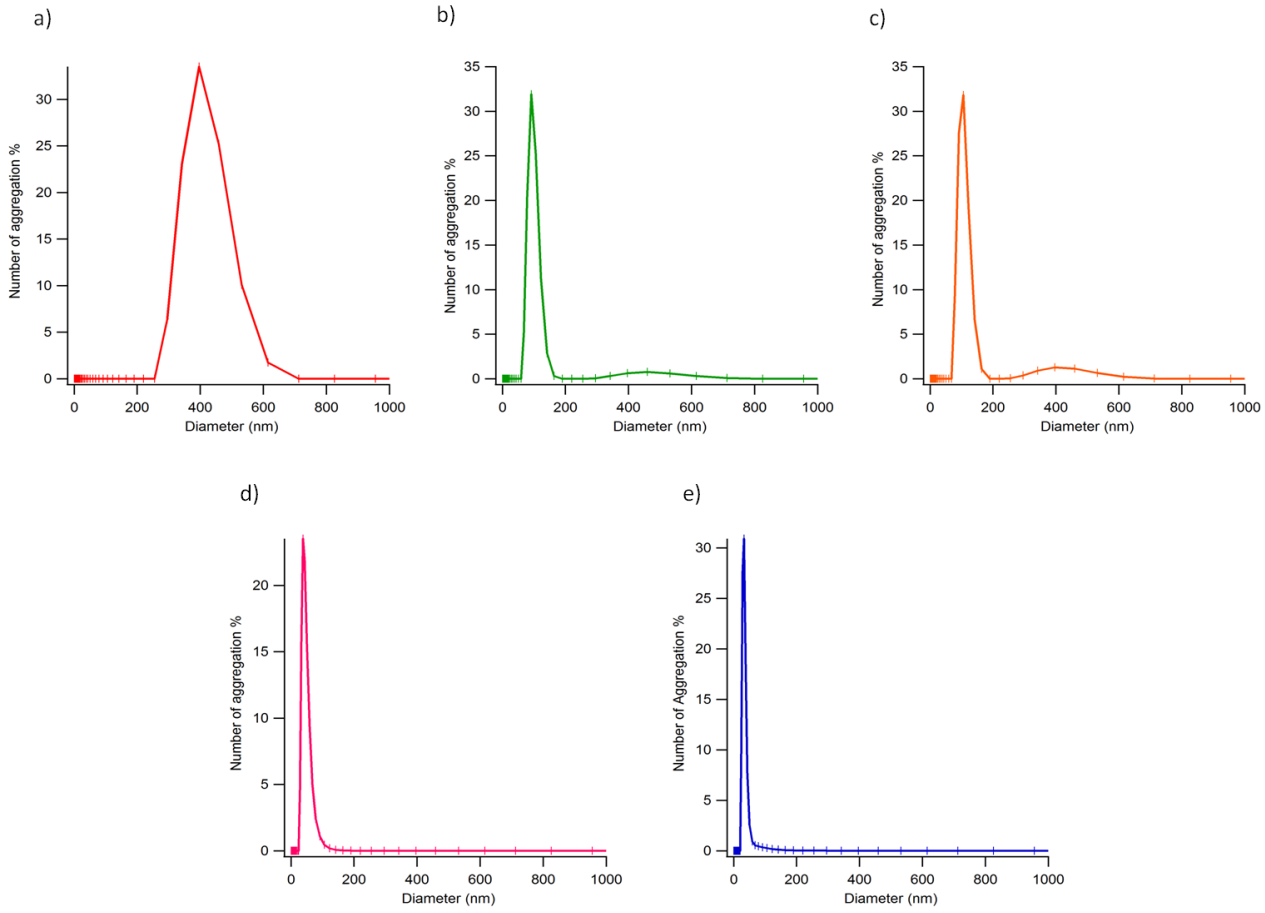
Compound	M.W. (Da)	CMC (mol L^{-1})
CuPMOXA10 (**4a**)	1275	$1.2x10^{-4}$
CuPMOXA20 (**4b**)	2094	$8.6x10^{-6}$
CuPMOXA30 (**4c**)	2975	$8.6x10^{-6}$
CuPMOXA50 (**4d**)	4676	$3.9x10^{-4}$
CuPMOXA100 (**4e**)	8925	$3.5x10^{-4}$

3.4 Size and stability of polymeric micelles

In order to evaluate the size and the morphology of the different Curcumin-PMOXA micelles, DLS TEM were employed.

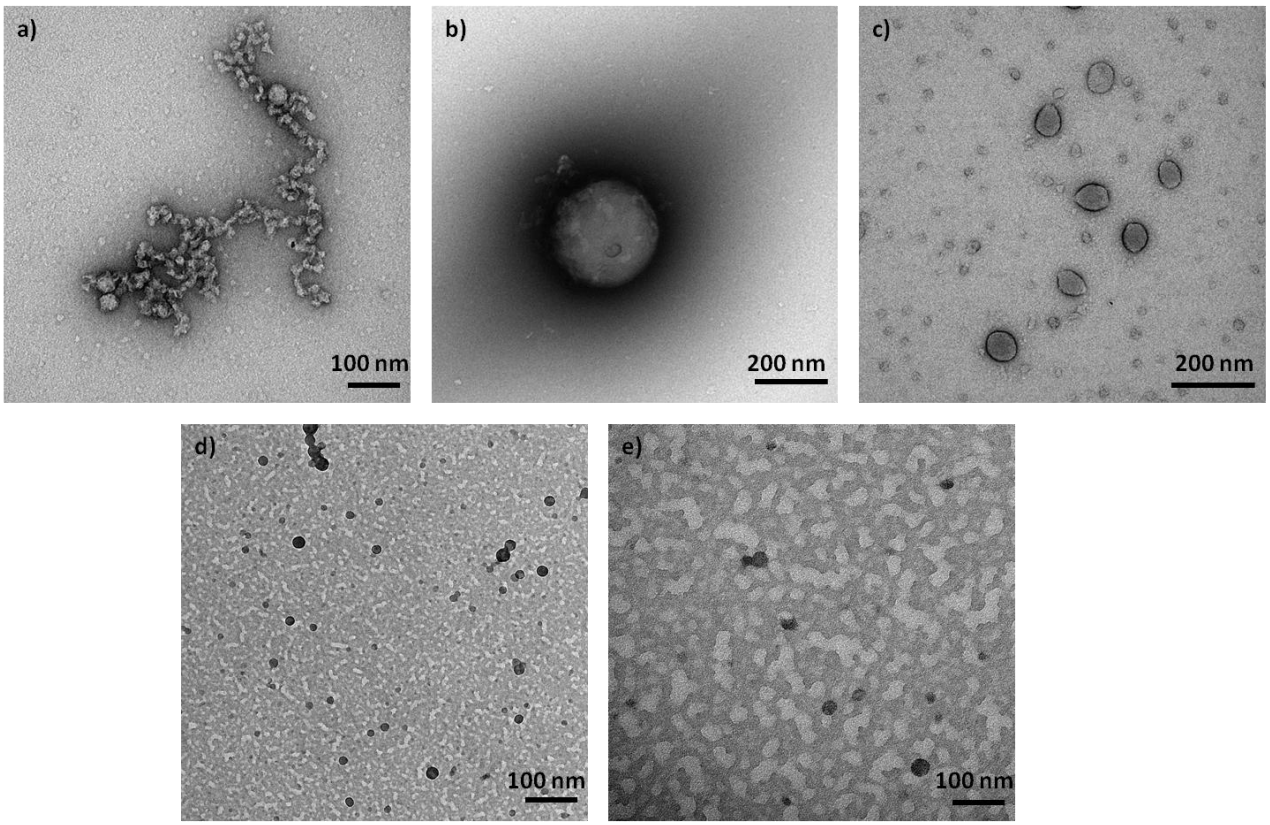
DLS measurements performed in water highlighted the influence of PMOXA chain length on the average size of the formed micelles. As reported in Figure 30 an average diameter of about 411±98 nm was recorded for Curcumin-PMOXA10 (**4a**), and confirmed by TEM micrographs [Fig.31]. The large diameter of the micelles originated from this compound was due to the relatively short PMOXA segment, which was not sufficient to balance the hydrophobic interactions between the Curcumin moieties within the aggregates.

Figure 30. Dynamic light scattering (DLS) analysis of micelles formed by derivatives **a)** CuPMOXA10, **b)** CuPMOXA20, **c)** CuPMOXA30, **d)** CuPMOXA50, **e)** CuPMOXA100.



The DLS profiles of the micellar suspensions prepared from Curcumin-PMOXA20 (**4b**) and Curcumin-PMOXA30 (**4c**) showed similar average diameters for both the samples, with typical values around 100 ± 23 and 104 ± 24 nm, respectively. Interestingly, the TEM micrographs recorded for **4b** showed the presence of larger aggregates than what expected from the DLS analysis, with micelles presenting typical diameters over 200 nm [Fig. 31b]. The discrepancy between DLS and TEM measurements for this particular sample was probably due to the intrinsic instability of the micellar suspensions originated from Curcumin-PMOXA20, as it was subsequently demonstrated by the stability studies (*vide infra*).

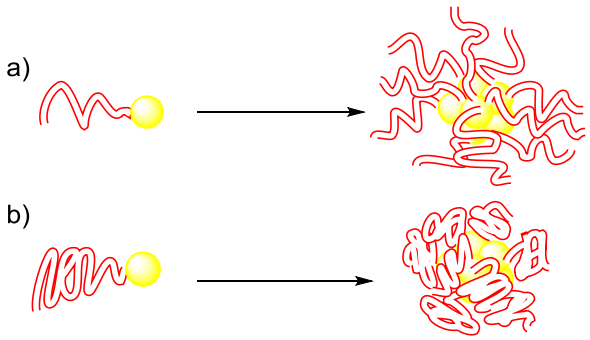
Figure 31. TEM micrographs of nanoparticles formed in water by **a)** CuPMOXA10, **b)** CuPMOXA20, **c)** CuPMOXA30, **d)** CuPMOXA50, **e)** CuPMOXA100. Aggregation is visible for CuPMOXA10.



The micelles formed from **4d** and **4e** showed average diameters of 43 ± 13 and 32 ± 8 nm, respectively, and monodisperse populations, with a good agreement between DLS and TEM analyses [Fig.s 30d, 30e, and 31d, 31e]. The smaller diameters recorded for these micellar aggregates was ascribed to the relatively long PMOXA segments, which favoured the formation of colloidally stable micelles constituted by a limited number of Curcumin-PMOXA conjugates.

Specifically, the polymeric backbone “crumble” is due to the random coil disposition of the polymeric chains. These latter are able to create steric hindrance which influences the density and the thickness of the shell [Fig. 32].

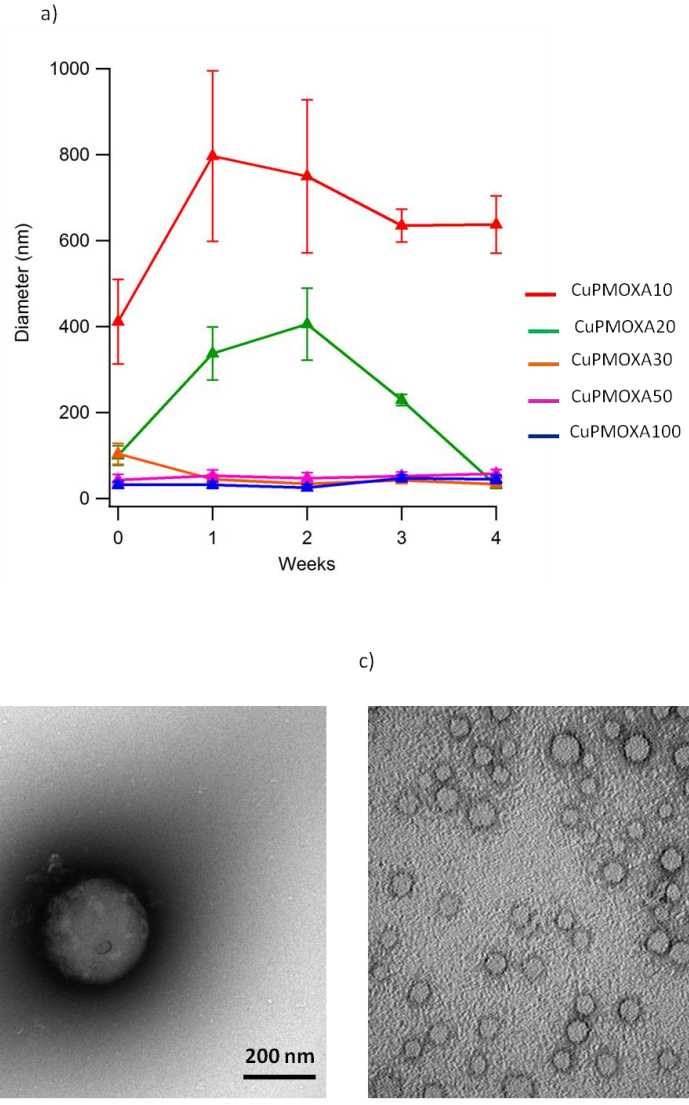
Figure 32. a) Micellization process and disposition of polymeric chains of the shell for lower chain length derivatives **b)** micellization process and disposition of polymeric chains of the shell for higher chain length derivatives



The stability of the different micellar suspensions was subsequently tested by DLS over one month of incubation in water at 4 °C.[145] Suspensions form Curcumin-PMOXA30, 50 and 100 conjugates showed good colloidal stability over the tested time, with diameter values which remain constant over 4 weeks. On the contrary, suspensions of Curcumin-PMOXA10 and Curcumin-PMOXA20 showed a marked variation of average micellar diameter over time. Micelles originated from Curcumin-PMOXA10 underwent a very fast aggregation, which was macroscopically mirrored by the precipitation of aggregates already after 10 minutes of incubation at 4 °C.

Conversely, the average diameter recorded for Curcumin-PMOXA20 showed a gradual decrease during the tested 4 weeks of incubation at 4°C. This phenomenon could be ascribed to digestive ripening, i.e. a progressive variation of the aggregates size in order to reach a thermodynamically more favourable state.[189-192] In this case, the initially large micelles observed for Curcumin-PMOXA20 gradually dissolved into a monodispersed micellar population presenting an average diameter of 31 ± 2 nm. This phenomenon was also confirmed by TEM, which showed the formation of relevantly smaller micelles after 4 weeks of incubation [Fig. 33b-c].

Figure 33. a) Stability over time of the micellar structures assessed by repeated DLS inspection. **b)** TEM micrograph of CuPMOXA20 at time 0 and **c)** after 4 weeks, showing shrinking of the self-assembled structures.

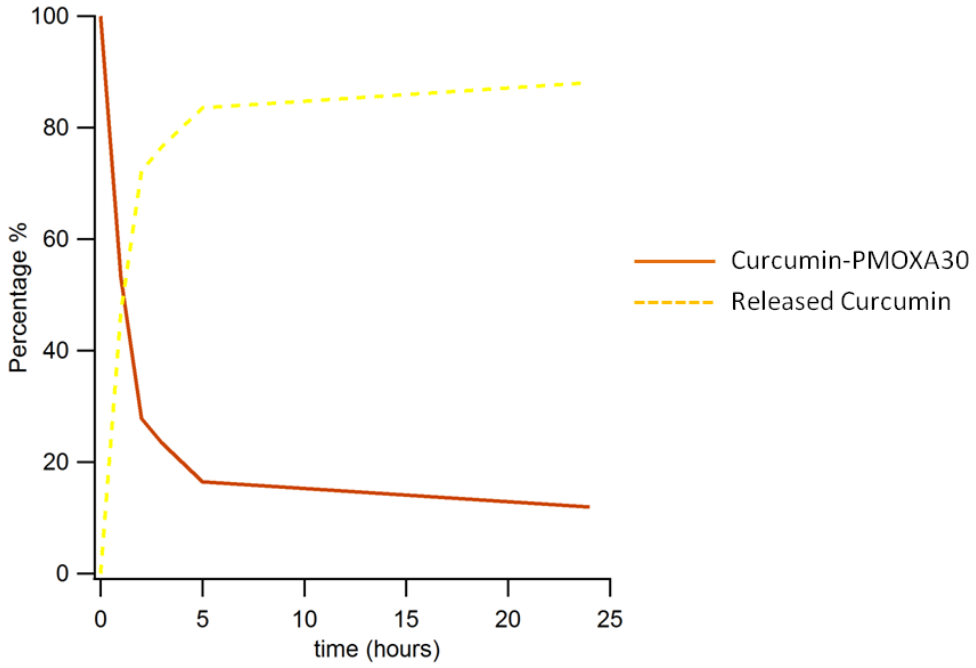


3.5 Curcumin Release from Micelles and Biocompatibility

The release of curcumin from the different micellar suspensions was evaluated under physiological conditions (PBS solution at pH 7.4 and at 37°C) by UV-Vis spectroscopy, monitoring the decrease in the relative absorption of the Curcumin peak at 430 nm during 24 hours, assuming that all the released Curcumin precipitates upon hydrolysis.

As highlighted in Figure 34, the most efficient release of active compound was recorded for Curcumin-PMOXA30. Namely, about 70% of the initial Curcumin content in the micelles was released within the first 2 hours of incubation, while after 24 hours the released fraction reached 88%, following a pseudo-first order kinetic.

Figure 34. Release profile of Curcumin (yellow) from **4c** (orange) at pH:7.4. Temperature 37°C.



All the other conjugates exhibited a slower release kinetics [Fig.36]. About 64% of the Curcumin was released from Curcumin-PMOXA10, and 30% from Curcumin-PMOXA20 after 24 hours. The faster release of Curcumin-PMOXA10 compared to Curcumin-PMOXA20, being partially due to the already observed tendency to precipitate by the former conjugate. For these samples, the release kinetics were characterized by an initial, fast release rate of Curcumin followed by a marked slowing down at longer incubation times. This was presumably due to the occurrence of two different release mechanisms from the micellar suspensions. One could be ascribed to the dissociation of the micelles and another one, presumably taking place after several hours of incubation, which was correlated to the hydrolytic cleavage of carbamoyl bond between curcumin and the polymer.[193,194]

The release of Curcumin further decreased by increasing the length of the PMOXA segments within the conjugates (and thus by decreasing the average diameter of the micelles). In fact, the release of 16% and 8% of Curcumin was recorded for micellar suspensions of Curcumin-PMOXA50 and Curcumin-PMOXA100, respectively. In these two cases, the high stability and relatively small size of the formed micelles hindered the dissociation of Curcumin by hydrolysis and/or the dissolution of the entire micelles [Fig.35].

Figure 35. Illustration of the two supposed mechanism involved in drug release from micelles

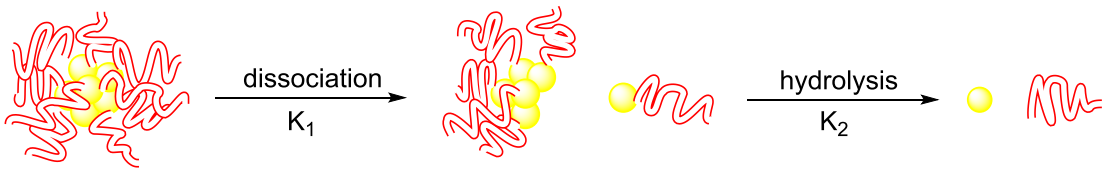
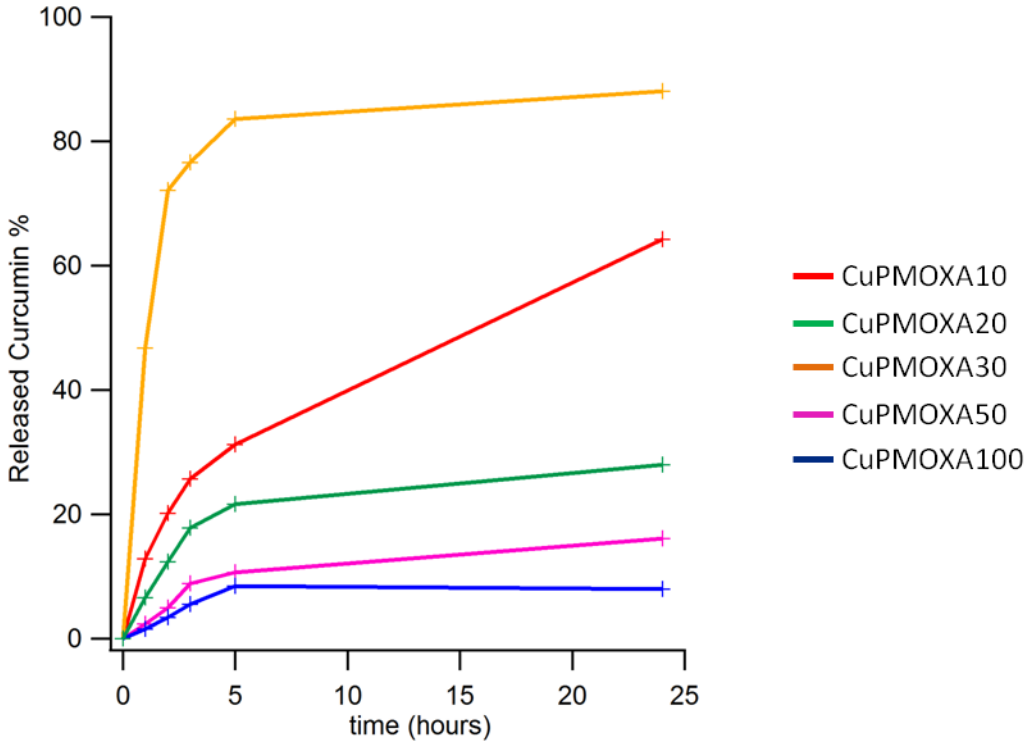


Figure 36. Curcumin release profiles of the CuPMOXA conjugates. Releasing was tested at a concentration of the conjugates of 10^{-3} M, under simulated physiological conditions: PBS buffer pH 7.4 and 37°C.

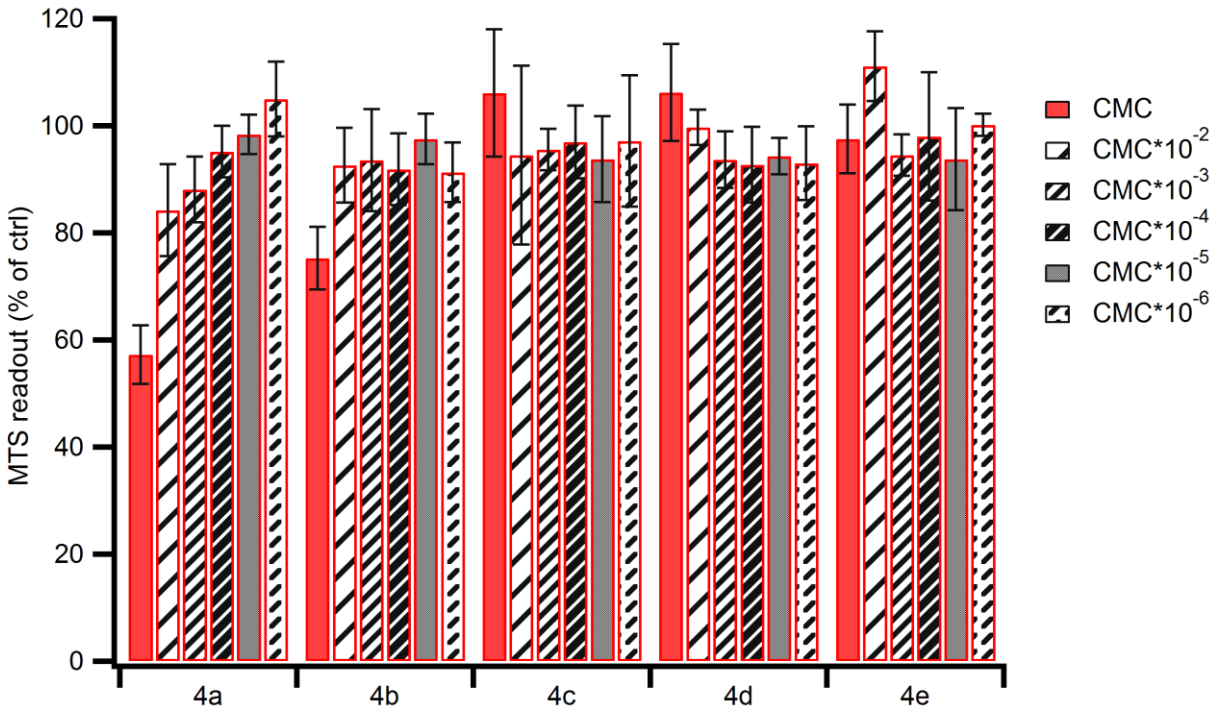


Cell viability tests were performed in order to assess the biocompatibility and cytotoxicity of the different CuPMOXA conjugates [Fig.37]. In particular, macrophages (raw 264.7 cell line) were incubated in cell culture medium containing different concentrations of the curcumin-conjugates for 24h and the MTS assay showed cell viability above 85% for all the systems at the lower concentrations tested. At the highest concentration (corresponding to the CMC) only the conjugates bearing shorter hydrophilic chains (CuPMOXA10 and CuPMOXA20) showed a decrease in cell viability.

In particular as cells, macrophages were used in order to verify whether micelles are able to induce cell toxicity. At different concentrations, none of the micelles showed toxicity against macrophages except for conjugates with PMOXA10 and PMOXA20. These latter, at their CMC are

able to induce cellular death probably because of their tendency to form aggregates dangerous for cells.

Figure 37. MTS cytotoxicity results on macrophages RAW 264.7 cell line treated with different concentrations of the curcumin-conjugates.



3.6 Conclusions and outlooks

New polymeric prodrugs of Curcumin were synthesized. A new class of polymer, poly-2-methyl-2-oxazolines, was used in order to create a valid alternative to the use of common polymers as PEGs. Five amino terminated PMOXA with different chain length of their backbone were synthesized and completely characterized. Coupling with Curcumin was carried out and five new prodrugs systems were created. Such systems are able to self assemble in water as well as in physiological like

conditions because of the hydrophic interactions of Curcumin moieties. Each type of micelles was characterized using DLS and TEM in order to investigate how the length of polymeric backbone could influence the self assembly process. In particular, differences in terms of size were observed and an opposite trend was observed between diameter and chain length. For each type of micelle the CMC was indentified and conjugate Curcumin-PMOXA30 showed the higher ability to self assemble in aqueous solution. Physical and chemical stability over one month was tested. Except for Curcumin-PMOXA10 whose aggregates were identified, for other compound a good stability appeared. Curcumin release in physiological like conditions (PBS, pH 7.4, 37°C) was tested and Curcumin-PMOXA30 showed the best behaviour over 24 hours because of its ability to release around 80-90% of Curcumin. Cytotoxicity assays were carried out, and no evidence of cellular death was identified for nanoparticles of Curcumin-PMOXA30, Curcumin-PMOXA50 and Curcumin-PMOXA100.

3.7 Experimental Section

3.7.1 Materials and methods

Curcumin was purchased from TCI Europe. Other starting materials and reagents were purchased from SigmaAldrich (Milan, Italy), Fluka (Milan, Italy), Merck-Novabiochem (Milan, Italy), Acros Organics (Milan, Italy), and were used as received. 2-methyl-2-oxazoline and methyl trifluoromethanesulfonate were previously distilled over KOH and CaH respectively. The solvents were analytical or synthetic grade and were used without further purification. Dialysis membranes were purchased by Spectra and were used as received. 1H-NMR spectra were recorded with a Bruker spectrometer operating at 300 MHz. Chemical shifts (δ) are given in ppm relative to the signal of the solvent. UPLC-UV analyses were performed with an Agilent 1290 Infinity LC System (Agilent Technologies, Milan, Italy), equipped with binary pump and a diode array detector (190–500 nm). Fluorescence/UV-Vis spectra were recorded at 37°C with a Perkin-Elmer LS-55 spectrofluorimeter cell holder. Quartz cells with an optical pathlenght of 10 mm were used for measurement of both absorption fluorescence and UV-Vis spectra.

Cell culture and mts/fluorescence microscopy experiments materials Cells were cultured in DMEM medium (Gibco 11965-092) supplemented with 10% of FBS and 1% penicillin-streptomycin. For cytotoxicity studies: two Dulbecco's Modified Eagle media were obtained from Invitrogen: a) the DMEM, High Glucose, GlutaMAX™, Pyruvate (Catalog No. 31966021), herein named as “DMEM with phenol red” b) the DMEM, High Glucose, no Glutamine, no Phenol Red (Catalog No. 31053028), herein named as “DMEM without phenol red”. Also Penicillin-Streptomycin Solution, liquid (Catalog No.15140122), herein named as “PenStrep”, Sodium Pyruvate 100 mM Solution (Catalog No. 11360039), herein named as “Sodium Pyruvate”, GlutaMAXTM Supplement (Catalog No. 35050038), herein named as “GlutaMAX”, Foetal Bovine Serum (FBS, Catalog No. 10270106) and Phosphate Buffered Saline (PBS) (1x) (Catalog No. 10010015) were obtained from Invitrogen. The DMEM with phenol red was supplemented with 1 vol% PenStrep and 10 vol% FBS and used for normal cell growth. The DMEM without phenol red was used as “complete” and as “serum-free” medium. The “complete” medium contained 1 vol% PenStrep, 1 vol% Sodium Pyruvate, 2 vol%

GlutaMAX and 10 vol% FBS. The “serum-free” medium had similar composition with the complete medium but with 0 vol% FBS.

Dynamic light scattering (DLS) The size distribution of polymeric micelles was investigated with DLS using a Nano-ZS (Malvern Instruments Inc., U.K.). Each sample was diluted in Milli-Q water in order to obtain a solution at specific micelle’s CMC. The intensity-mean z-averaged particle size (effective diameter) and the polydispersity index (PDI) obtained from cumulant analysis were given to reflect hydrodynamic diameters of Curc-PMOXA micelles.

Transmission Electron microscopy (TEM) micrographs were recorded on a Philips CM12 operating at 100 kV. Colloidal solution of each sample were deposited on 200 mesh carbon-coated copper grids previously submitted to negative glow discharge. Solution was allowed to absorb for 1 minute, then the excess fluid was removed. To enhance contrast, all specimens were negatively stained for 30 seconds using a 2% aqueous solution of uranyl acetate.

Drug release studies To investigate the percentage of released Curcumin from each type of micelle UV (Varian Cary® 50-Uv-vis) was used. Each micelle was dissolved in 1 mL of PBS (pH: 7.4) at its specific CMC. Every sample was heated at 37°C for 24 hours. Every hour a specific aliquot (100 μL) was taken, and was diluted in a quartz cuvette with DMSO (900 μL). A Uv-Vis spectrum between 300 and 700 nm was acquired. The percentage of released Curcumin was calculated following the decrease in the absorption band of Curcumin at 430 nm over time.

Mts cytotoxicity/fluorescence microscopy studies protocol In 96-well plates 12500 cells per well (i.e. 12.5*104 cells/mL in DMEM+10%FBS without phenol red) were used and seed 100 μ for 24 h prior to experiment. During the experiment a mixture of sterilized Milli-Q with serum-free DMEM (without phenol red) in volume ratio 1:3 and incubate for 6 h (100 uL per well) as negative control was used. After this time, 20 μL MTS solution were added. After 3 hours, the absorbance was read using a multi-plate reader set at 490 nm.

3.7.2 Synthesis and analytical data of compounds 3a-e

General procedure for the synthesis of poly-2-methyl-2-oxazolines of different length 2-methyl-2-oxazoline previously distilled (n eq.) was dissolved in dry ACN and cooled to 0°C under Ar atmosphere. Methyl trifluoromethanesulfonate (1 eq.) was added to the mixture and solution was heated to 70°C. Reaction was stirred for 20 hours under Ar atmosphere then was warmed up to room temperature. Ammonia in dry THF solution (3 eq.) was added and stirring was maintained for 48 hours. Solvent was evaporated under vacuum, solid was dissolved in mq water and dialyzed against water for 48 hours. Aqueous solution was freeze dried in order to afford a white solid.

Poly-2-methyl-2-oxazoline850 (3a): Yield: 75%. ^{1}H NMR (300 MHz, D_2O) δ 3.83 (m, 2H), 3.60 (m, 40H), 3.16 – 2.87 (m, 3H), 2.20 – 1.97 (m, 30H) ppm.

Poly-2-methyl-2-oxazoline1700 (3b): Yield: 80%. ^{1}H NMR (300 MHz, D_2O) δ 3.86-3.81 (m, 2H), 3.64-3.54 (m, 80H), 3.16 – 2.87 (m, 3H), 2.20 – 1.97 (m, 60H) ppm.

Poly-2-methyl-2-oxazoline2550 (3c): Yield: 80%. ^{1}H NMR (300 MHz, D_2O) δ 3.85-3.81 (m, 2H), 3.64-3.53 (m, 120H), 3.10-2.93 (m, 3H), 2.13-2.06 (m, 90H) ppm.

Poly-2-methyl-2-oxazoline4250 (3d): Yield: 75%. ^{1}H NMR (300 MHz, D_2O) δ 3.84 – 3.22 (m, 200H), 3.09-2.92 (m, 3H), 2.33 – 1.80 (m, 150H) ppm.

Poly-2-methyl-2-oxazoline8500 (3e): Yield: 78%. ^{1}H NMR (300 MHz, D_2O) δ 3.96 – 3.16 (m, 400H), 3.09-2.92 (m, 3H), 2.23 – 1.76 (m, 300H) ppm.

3.7.3 Synthesis and analytical data of conjugates 4a-e

General procedure for the synthesis of curcumin-poly-2-methyl-2-oxazoline conjugates bis(4-nitrophenyl) carbonate (1.2 eq.) in was dissolved in CAN and the mixture was cooled at 0°C. PMOXAn (1 eq.) and 4-(dimethylamino)pyridine (1.2 eq.) were dissolved in ACN and this fresh

solution was added dropwise to the mixture. After stirring for 12 hours at room temperature a fresh solution of Curcumin (1 eq.) and 4-(dimethylamino)pyridine (1 eq.) in THF was added dropwise and mixture was warmed up to 50°C for 24 hours. Solvent was evaporated under vacuum, solid was dissolved in mq water and dialyzed for 48 hours. Aqueous solution was dried in order to afford a yellow solid.

Curcumin-Poly-2-methyl-2-oxazoline850 (4a): Yield: 60%. ^{1}H NMR (300 MHz, DMSO) δ 8.15 (d, *J* = 7.6 Hz, 2H), 7.55 (d, *J* = 15.9 Hz, 2H), 7.32 (s, 2H), 7.15 (d, *J* = 8.0 Hz, 2H), 6.91 – 6.70 (m, 2H), 6.07 (s, 1H), 3.83 (s, 6H), 3.41 (m, 40H), 3.02 – 2.89 (m, 3H), 2.09 – 1.86 (m, 30H).

Curcumin-Poly-2-methyl-2-oxazoline1700 (4b): Yield: 53%. ^{1}H NMR (300 MHz, DMSO) δ 7.54 (d, *J* = 15.8 Hz, 2H), 7.33 (s, 2H), 7.14 (d, *J* = 8.2, 2H), 6.91 – 6.69 (m, 3H), 6.06 (s, 1H), 3.83 (s, 6H), 3.36 (m, 80H), 2.95 (m, 3H), 1.99 (m, 60H).

Curcumin-Poly-2-methyl-2-oxazoline2550 (4c): Yield: 65%. ^{1}H NMR (300 MHz, DMSO) δ 8.09 (d, *J* = 6.2 Hz, 2H), 7.54 (d, *J* = 15.8 Hz, 2H), 7.31 (s, 2H), 7.14 (d, *J* = 8.0 Hz, 3H), 6.78 (m, 2H), 6.05 (s, 1H), 3.83 (s, 6H), 3.40 (m, 120H), 2.99 – 2.90 (m, 3H), 1.99 (m, 90H).

Curcumin-Poly-2-methyl-2-oxazoline4250 (4d): Yield: 52%. ^{1}H NMR (300 MHz, DMSO) δ 7.59 (dd, *J* = 27.8, 11.0 Hz, 2H), 7.42 – 7.27 (m, 2H), 7.22 – 6.92 (m, 2H), 6.80 (m, 3H), 6.06 (s, 1H), 3.84 (s, 6H), 3.40 (m, 200H), 2.96 (m, 3H), 2.13 – 1.88 (m, 150H).

Curcumin-Poly-2-methyl-2-oxazoline8500 (4e): Yield: 50%. ^{1}H NMR (300 MHz, DMSO) δ 8.15 – 6.64 (m, 9H), 6.09 (s, 1H), 3.42 (m, 400H), 2.96 (m, 1H), 1.93 (m, 300H).

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