UNIVERSITAT POLITÈCNICA DE CATALUNYA
BARCELONATECH
Escola Superior d'Enginyeries Industrial, Aeroespacial i Audiovisual de Terrassa

Study of molecular aggregation in l-tyrosine through superficial tension

Bachelor's Degree in Chemical Engineering

Final Degree Thesis

Document: Report

UNIVERSITAT POLITÈCNICA DE CATALUNYA

Escola Superior d'Enginyeries Industrial, Aeroespacial i Audiovisual de Terrassa

Director: Dr. Manuel José Lis Arias

Author: Berta Mas Campabadal

Delivery date: 27th of April of 2020

Aknowledgments

First and foremost, I would like to express my gratitude to my thesis director, Dr. Manuel José Lis Arias, for his guidance not only during my thesis, but throughout the entire duration of my bachelor's degree. I am truly deeply grateful for having had the privilege of having him not only as a teacher and tutor, but as a mentor.

I would also like to sincerely thank the team at the INTEXTER-UPC Laboratories, for allowing me to use their facilities and thus granting me this immense learning opportunity. Especially Mercè Simó, who was always helpful when any questions arised and provided a very welcoming work environment with her warm and supportive presence.

Abstract

L-tyrosine is a proteinogenic amino acid that presents potential as a therapeutic agent, given that is a precursor of catecholamines, and thus would be of interest in psychiatric diseases associated with low catecholamine synthesis. One of the limits of its free oral administration is its permeation through the blood-brain barrier, which is regulated through specific transporter proteins, as well as the fluctuating concentrations in plasma levels this type of dosage produces. This study presents a possible solution to overcome these challenges through the proposal of a drug delivery system able to penetrate said barrier: microencapsulating l-tyrosine. The development of this design requires a previous study of the physicochemical characteristics of all the components involved in the system, which would enable to find the optimal parameters for encapsulation thus also providing the most efficient drug delivery. The study of l-tyrosine's physicochemical properties is carried out through an extensive review of the literature pertaining to it, along with a study of its surface tension to analyze its self-assembly and aggregation. The same methodology is implemented for the biocompatible surfactant in which the compound will later be entrapped, so as to avoid the formation of large aggregates before the execution of its microencapsulation. After the determination of said parameters, a few other analytical techniques are suggested for further characterization of the complex, alongside an analysis of the best biopolymers to microencapsulate it for future lines of work.

Contents

List of Figures

List of Tables

1. Introduction

1.1 Aim

The aim of this study is to propose an optimized drug delivery mechanism for the amino acid l-tyrosine as an active compound. It will be executed through the interpretation of the surface chemistry and self-assembly of said amino acid in aqueous solution as well as entrapped in a biocompatible surfactant. This research will facilitate the next step in the drug delivery design process: microencapsulation of the active compound, given that it would allow to find the optimal concentration of both the active agent and surfactant to be encapsulated. Its application is meant for the clinical and biomedical field, specifically for the treatment of clinical symptoms associated with low levels of the neurotransmitters dopamine and norepinephrine.

1.2 Scope

The study was executed through an in-depth investigation into the physicochemical properties of l-tyrosine and its current state-of-the-art, the effect of surfactants in formulations, as well as a systematic review of the literature relating to surface chemistry of colloidal systems. Specifically, research regarding protein-surfactant systems was focused on. Surface tension was measured through a method of force tensiometry: Wilhelmy Plate.

1.3 Requirements

A background knowledge on surface chemistry and the assembly of colloidal systems is required. As well as an understanding of the properties, structure, metabolism and biosynthesis of the amino acid is necessary to later interpret the evolution of its surface tension. The same principles apply in order to study the effect the surfactant of choice has on the assembly of the amino acid. Access to a Wilhelmy plate tensiometer is also required in also to execute the experimental design, as well as a comprehension of the equations that govern said method.

1.4 Justification of the need

L-tyrosine is a precursor to neurotransmitters and has been proven to be effective in improving cognitive function under conditions of adverse stimuli. It has also been proposed for the treatment of clinical disorders, specifically those pertaining suboptimal levels of catecholamines, such as: depression, Parkinson's or ADHD. It could even be considered of interest in other disorders such as Schizophrenia. The study of its surface tension and self-aggregation would allow to determine the optimal concentrations for it to be later microencapsulated, in order to administer a controlled release and sustained dosage of the drug so as not to saturate the enzymes responsible for catecholamine synthesis.

2. Active compound: L-Tyrosine

The active compound studied throughout this project will be the aromatic amino acid tyrosine, as a possible drug for therapeutic treatment. Specifically, its levorotatory stereoisomer L-Tyrosine, which only appears in mammalian protein [1]. Therefore, only its application and biosynthesis in mammals will be considered.

2.1. Chemical structure and properties

Tyrosine is one of the 20 standard proteinogenic amino acids, meaning that it is one of the standard genetic code amino acids that are incorporated biosynthetically into proteins during translation [2]. Its chemical structure can be seen in Figure ***2.1***.

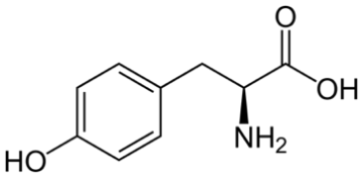


Figure 2.1. Chemical structure of the amino acid L-Tyrosine. Retrieved from[2]

Tyrosine (Y) can be considered an uncharged polar and neutral amino acid, as defined through the international immunogenetics information system. The standardized criteria used to classify the physicochemical properties of the 20 standard amino acids can be seen in Table ***2.1***[3].

Table 2.1 The IMGT 'Physicochemical classes of the 20 standard amino acids. Retrieved from [3]

'Volume' classes		'Hydropathy' classes											
	in Å^3	Hydrophobic				Neutral				Hydrophilic			
Very large	189-228	F		W			Y						
Large	162-174	I	L	M						K	R		
Medium	138-154	V							H			E	Q
Small	108-117			C		P	T					D	N
Very small	60-90	A				G	S						
		Aliphatic		Sulfur			Hydroxyl			Basic		Acidic	Amide
							Uncharged			Charged			Uncharged
		Non polar					Polar						

Therefore, tyrosine can be considered a partially hydrophobic amino acid. Even though it has an aromatic side chain, its hydrophobic character is tempered somewhat by the presence of an uncharged hydroxyl, also granting the amino acid polarity [4].

Hence, it is also the only aromatic amino acid with an ionizable side chain. Its solubility in water between the range of pH of 3.2-7.5 and at 25ºC is that of 0,45 g/L [5].

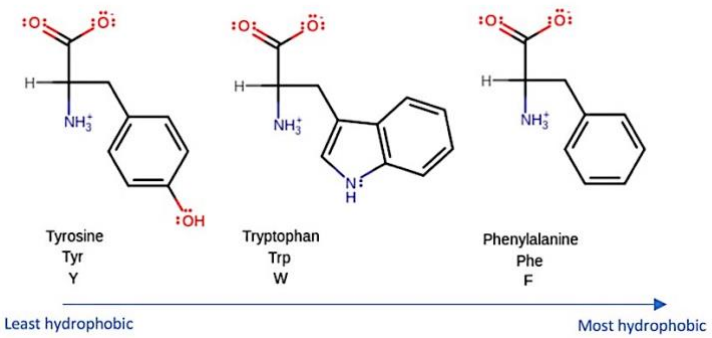


Figure 2.2. Aromatic amino acids classified in increasing order of hydrophobicity.[6]

The acid dissociation constants (pK_a) of the amino acid are key factors in determining its isoelectric point (pI), it being the pH value at which the amino acid has no net charge. Since tyrosine has three functional groups, α-amino($-NH_3^+$), α-carboxyl(-COOH) and α-phenol($-C_6H_5OH$), it has three dissociation constants.

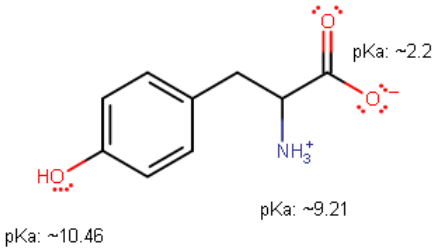


Figure 2.3. Acid dissociation constants of L-Tyrosine side chains of tyrosine at physiological pH (7,4). Retrieved from [7]

The dissociation constants, the values of which can be seen in Figure ***2.3***, are the pH values at which the functional group in question donate or accept a proton. The isoelectric point is therefore calculated as the mean of the pK_a values relevant to the form with no net charge, indicated in red in Figure ***2.4***. In the case of tyrosine its value is pI=5,66 [8].

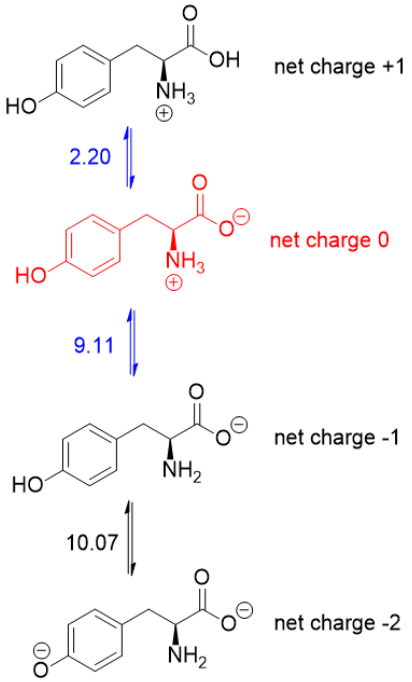


Figure 2.4. Acid-base equilibria for tyrosine. Retrieved from [9]

Amino acids with aromatic residues often participate in *stacking* interactions, which are attractive, noncovalent interactions between aromatic rings. They are also referred to as π stacking, given they take place when aromatic rings are stacked on top of each other in order to align their π electron clouds [4].

There are different stacking geometric configurations, the most energetically favorable being the T-shaped and parallel. Face-to-face stacking is not energetically favorable due to the repulsion forces between the π electron clouds, as can be seen in Figure ***2.5*** [10].

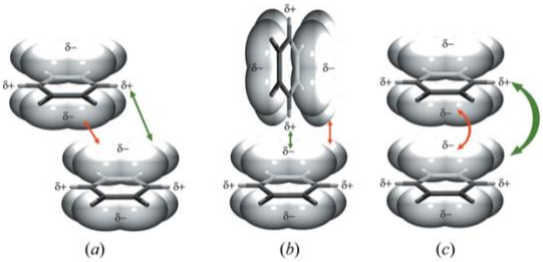


Figure 2.5. Quadrupolar model of stacking of aromatic rings proposed by Hunter & Sanders (1990). (a) Parallel (b) T-shaped and (c) face-to-face configurations. Retrieved from [10]

The relatively weak individual interactions combined with the overall stabilizing effects they have on the system, allows these types of interactions to provide a flexible framework for different biological processes. In fact, this is often taken advantage of during the drug development process, specifically in the drug design of pharmaceuticals that exhibit protein-drug binding [11].

These interactions between amino acids are also determining factors in protein-folding processes. It is because of this that aromatic amino acids play a decisive role in the structure of a protein. The chemical structure of the amino acids interacting also plays a part in the site of the protein it is located in.

For instance, Tyr-Tyr stacking interactions mainly take place on the surface of the protein given that tyrosine is less hydrophobic than phenylalanine (Phe). However, Phe-Tyr interactions more frequently occur at the hydrophobic protein core [12].

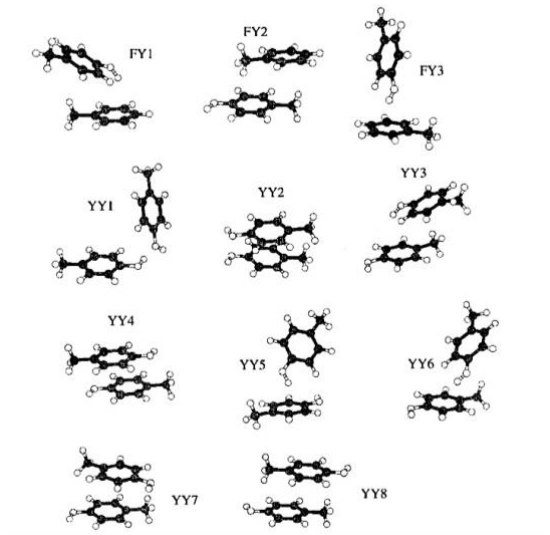


Figure 2.6. Minimum energy structures of Phe-Tyr (FY1, FY2, FY3), and Tyr-Tyr (YY1, YY2, YY3, YY4, YY5, YY6, YY7, YY8) complexes. Retrieved from [12]

2.2. Biosynthesis and metabolism

L-tyrosine is considered a non-essential amino acid given that it is naturally occurring and synthesized in vivo through the hydroxylation of the amino acid phenylalanine (phe), in its para position [6]. There are two other structural isomers of L-tyrosine, which are synthesized through the non-enzymatic free-radical hydroxylation of phenylalanine. These regioisomers are known as *meta*-tyrosine and *para*-tyrosine [2]. The former has been shown application in the treatment of arthritis, as well as neurological disorders such as Parkinson's and Alzheimer's [13].

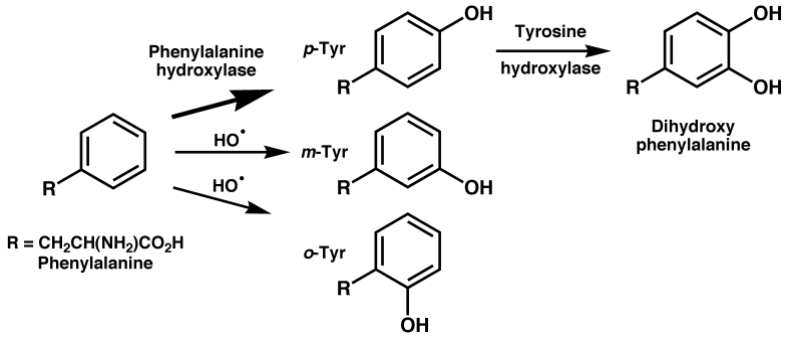


Figure 2.7. The three structural isomers of L-Tyrosine. Retrieved from [2]

Tyrosine, therefore, can be synthesized in the liver through the hydroxylation of phenylalanine. In fact, as much as half of the ingested phenylalanine can be converted to tyrosine in cases where a diet may be low on the latter amino acid [14].

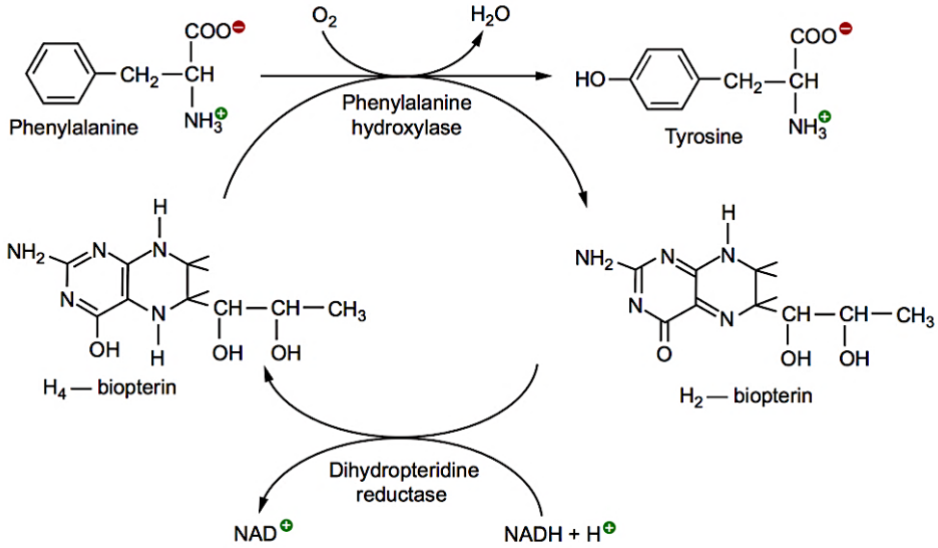


Figure 2.8. Conversion of L-phenylalanine to L-tyrosine catalyzed by phenylalanine hydroxylase. Retrieved from [14]

This reaction is catalyzed through the enzyme phenylalanine hydroxylase (PAH), which is expressed in the liver and kidney. The overall conversion of phenylalanine to tyrosine can be seen in Figure ***2.8***, the enzymatic mechanism simplified by not considering the nonheme atom the coenzyme contains that forms a superoxide. PAH is the rate-limiting factor in the degradation of excess phenylalanine in the body, and mutations in its encoding gene can lead to phenylketonuria, a severe metabolic disease [14]. In patients with phenylketonuria, tyrosine is therefore considered an essential nutrient given that it cannot be converted from phenylalanine [15].

Tyrosine is metabolized into catecholamines, which are physiologically active molecules that can act both as neurotransmitters and hormones [16]. Neurotransmitters transfer signals from neuron to neuron, while hormones regulate physiological functions. Catecholamines contain a catechol group, comprised of a benzene with two adjacent hydroxyl side groups, and an ethylamine side chain. Dopamine, norepinephrine (noradrenaline) and epinephrine (adrenaline) are the predominant catecholamines found in the brain [17].

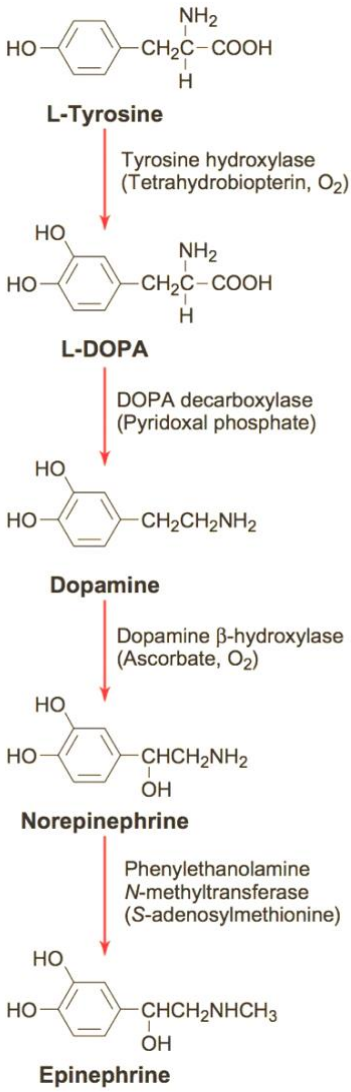


Figure 2.9. Biosynthetic pathway for catecholamines. Retrieved from [17]

The biosynthetic pathway of catecholamine production is regulated through the enzyme tyrosine hydroxylase, which catalyzes the reaction in a mechanism similar to phenylalanine hydroxylase (Figure ***2.8***) [4]. The hydroxylation of l-tyrosine is considered the rate-limiting step in the synthesis of catecholamines.

Once tyrosine has undergone hydroxylation to form DOPA, this substance undergoes decarboxylation to obtain dopamine. This conversion takes place in sympathetic and brain neurons. Norepinephrine and epinephrine are obtained through further metabolization of dopamine, which typically occurs in the adrenal medullary cells and peripheral brain neurons [18].

Catecholamine hormones, norepinephrine and epinephrine, cause physiological changes in the body in order to activate its 'fight-or-flight' response. Increased heart rate and blood pressure due to the response of the sympathetic nervous system are some of the effects they produce when secreted [16]. However, catecholamines only remain on the bloodstream for several minutes, given their short half-life [4].

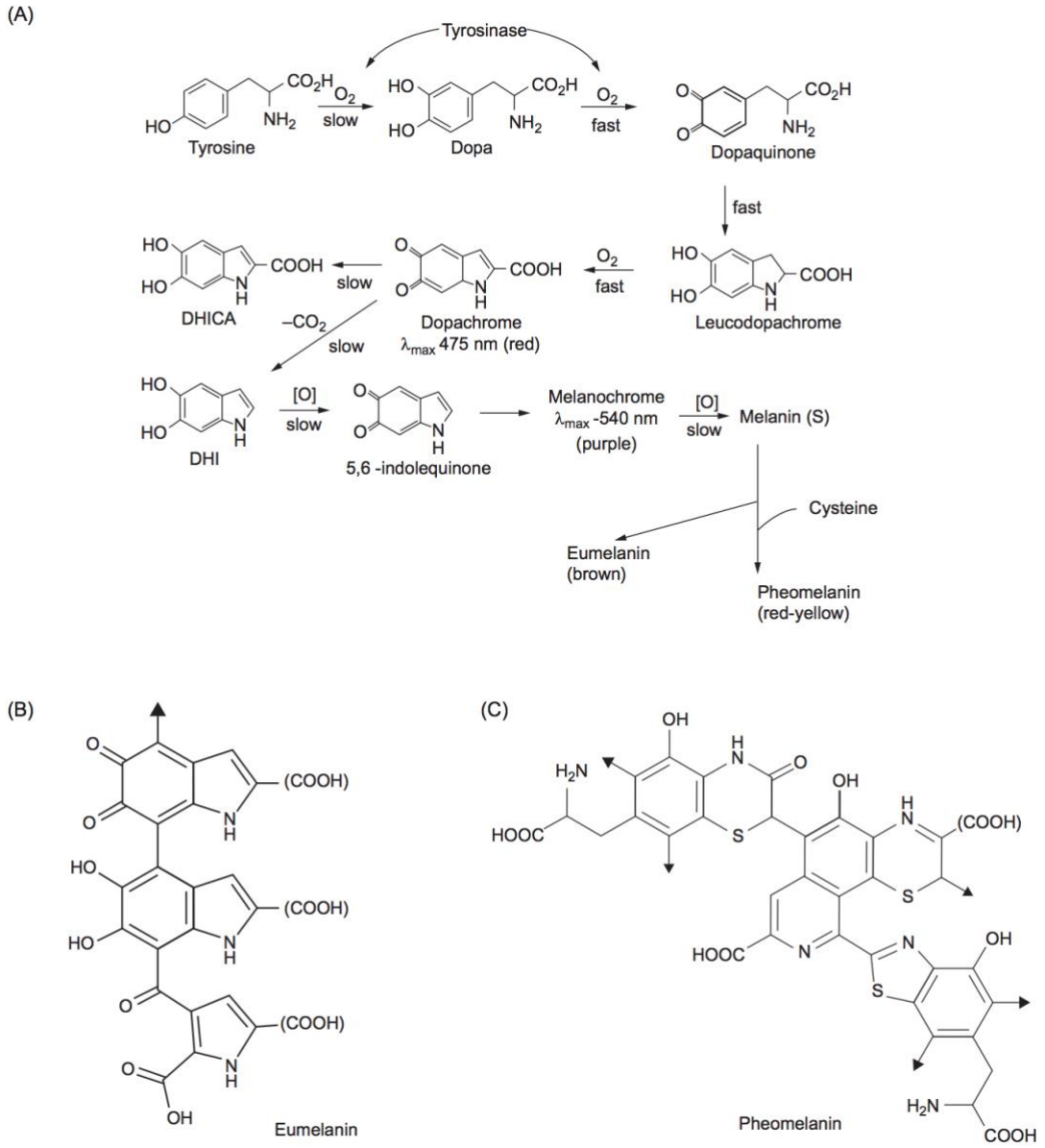
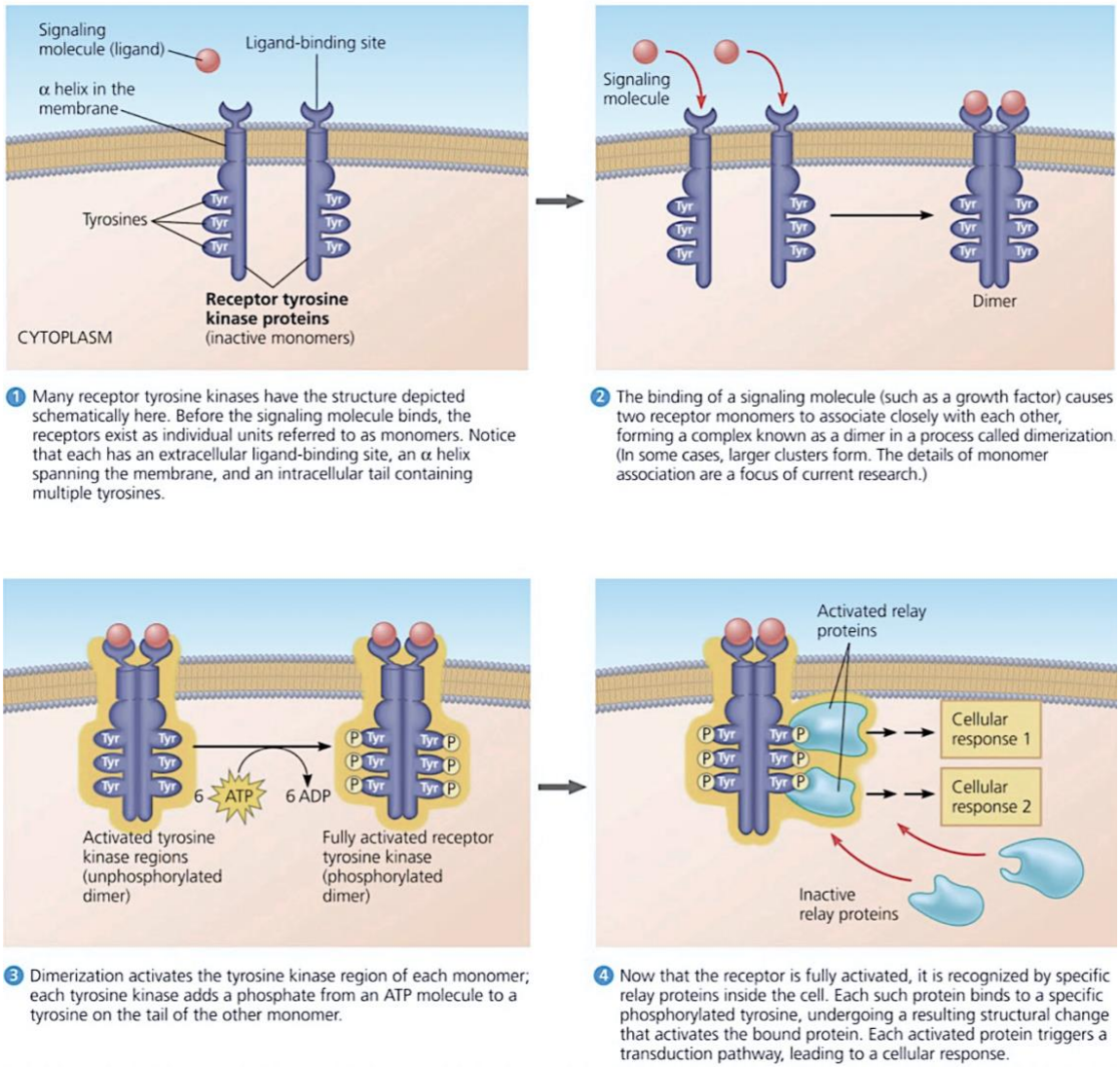


Figure 2.10. Synthesis of melanin through tyrosine. Retrieved from [4]

Tyrosine is also the precursor of thyroid hormones and the tissue pigment melanin. The synthesis of the latter, known as melanogenesis, is shown in Figure ***2.10***.

Due to its phenol functionality, the amino acid tyrosine also plays an important role in intracellular proteins that partake in signal transduction processes. Tyrosine residues are phosphorylated, which activate protein kinases and in turn allow a signal to be propagated through the plasma membrane of the cell [19]. A brief description of this process can be seen in Figure ***2.11***.



1 Many receptor tyrosine kinases have the structure depicted schematically here. Before the signaling molecule binds, the receptors exist as individual units referred to as monomers. Notice that each has an extracellular ligand-binding site, an α helix spanning the membrane, and an intracellular tail containing multiple tyrosines.

2 The binding of a signaling molecule (such as a growth factor) causes two receptor monomers to associate closely with each other, forming a complex known as a dimer in a process called dimerization. (In some cases, larger clusters form. The details of monomer association are a focus of current research.)

3 Dimerization activates the tyrosine kinase region of each monomer; each tyrosine kinase adds a phosphate from an ATP molecule to a tyrosine on the tail of the other monomer.

4 Now that the receptor is fully activated, it is recognized by specific relay proteins inside the cell. Each such protein binds to a specific phosphorylated tyrosine, undergoing a resulting structural change that activates the bound protein. Each activated protein triggers a transduction pathway, leading to a cellular response.

Figure 2.11. Diagram describing the process of receptor tyrosine kinase proteins. Retrieved from [20]

2.3. Medical interest and potential therapeutic treatment

The rates at which neurotransmitters are synthesized have been shown to be influenced by the availability of their precursors in plasma composition [21]. Neutral amino acids, such as tyrosine, enter the brain by an uptake through the blood-brain barrier. Tyrosine administration increases plasma tyrosine levels, which in turn elevates brain tyrosine levels. Given the appropriate conditions, this can therefore stimulate and accelerate the synthesis of catecholamines, particularly dopamine and norepinephrine, in the central nervous system and sympathoadrenal cells [22]. Thus, it has been suggested that precursor administration would allow to produce certain physiological effects by increasing the release of the neurotransmitters [21].

Tyrosine has been shown to enhance cognitive performance most effectively on individuals exposed to stressful situations, that is when there is high cognitive demand. However, it is also suggested that genetic predisposition, for instance the genetically determined dopamine function, influences its effect as a cognitive enhancer [23].

Tyrosine administration is most effective under aversive stimuli due to the fact that biosynthesis of catecholamines is increased under these conditions, resulting in neurotransmitter levels being depleted as well as behavioral depression [18]. The administration of tyrosine would prevent the depletion by replenishing the deficit, as well as reversing the stress-induced degradation of cognitive function and improving working memory [24]. Therefore, tyrosine supplementation has a beneficial effect in situations where neurotransmitter synthesis is stimulated.

Many cognitive and behavioral studies have been executed to investigate the potential of tyrosine as a treatment for clinical symptoms associated with suboptimal catecholamine levels. The characteristics and main outcomes of some of them are presented in Table ***2.2*** [25].

Table 2.2. Studies reviewed in article [25], along with their characteristics, type and size of samples, potential stressors and main outcomes of tyrosine administration. Retrieved from [25]

Authors	Sample	Dose of TYR	Findings
Banderet and Lieberman (1989)	Healthy, cold exposure ($N = 23$)	100 mg/kg	Reduced symptoms, improved mood, reaction times and vigilance
Chinevere et al. (2002)	Healthy, physically exerted ($N = 9$)	150 mg/kg	No effect of TYR
Colzato et al. (2013)	Healthy, cognition challenged ($N = 22$)	2.0 g	Improved working memory
Colzato et al. (2014a)	Healthy, cognition challenged ($N = 22$)	2.0 g	Improved inhibitory control
Colzato et al. (2014b)	Healthy, cognition challenged ($N = 32$)	2.0 g	Improved convergent thinking
Deutsch et al. (1994)	Schizophrenia patients ($N = 11$)	10.0 g	Increased saccadic intrusions, no effect on behavior o
Deijen and Orbleke (1994)	Healthy, auditory stress ($N = 16$)	100 mg/kg	Improved working memory and Stroop performance
Deijen et al. (1999)	Healthy, intensive combat training ($N = 21$)	2.0 g	Improved memory and tracking performance
Eisenberg et al. (1988)	ADHD patients ($N = 7$)	100 mg/kg	No effect of TYR
Gelenberg et al. (1980)	Depressive patients ($N = 1$)	100 mg/kg	Self-rated improvement of depression
Gelenberg et al. (1990)	Depressive patients ($N = 65$)	100 mg/kg	No effect of TYR
Goldberg et al. (1980)	Depressive patients ($N = 2$)	100 mg/kg	Improvement of symptoms
Growdon et al. (1982)	Parkinson's patients ($N = 23$)	100 mg/kg	Increased levels of TYR and homovanillic acid.
Kishore et al. (2013)	Healthy, heat exposure ($N = 10$)	6.5 g	Reduced delay in event related potentials
Leathwood and Pollet (1983)	Healthy, no manipulation ($N = 60$)	500 mg	No effect of TYR on mood
Lemoine et al. (1989)	Parkinson's patients ($N = 10$)	1.6–4.0 g	Improvement of symptoms
Lieberman et al. (1983)	Healthy, no manipulation ($N = 16$)	100 mg/kg	No effect of TYR on mood
Magill et al. (2003)	Healthy, sleep deprivation ($N = 76$)	150 mg/kg	Improved working memory, reasoning and vigilance
Mahoney et al. (2007)	Healthy, cold exposure ($N = 19$)	150 mg/kg	Improved working memory
Nemzer et al. (1986)	ADHD patients ($N = 14$)	140 mg/kg	No effect of TYR
O'Brien et al. (2007)	Healthy, cold exposure ($N = 15$)	300 mg/kg	Improved working memory
Palinkas et al. (2007)	Healthy, in Antarctica ($N = 43, 42$)	12 g	Improved mood during winter
Pietz et al. (1995)	Phenylketonuria patients ($N = 24$)	100 mg/kg	No effect of TYR
Pollin et al. (1961)	Schizophrenia patients ($N = 12$)	285 mg/kg	No effect of TYR
Posner et al. (2009)	ADHD patients ($N = 1$)	100 mg/kg	Improvement of symptoms
Reimherr et al. (1987)	ADHD patients ($N = 12$)	50–150 mg/kg	Short term, unsustained clinical response
Shurtleff et al. (1994)	Healthy, cold exposure ($N = 8$)	150 mg/kg	Improved working memory
Smith et al. (1998)	Phenylketonuria patients ($N = 21$)	100 mg/kg	No effect of TYR
Steenbergen et al. (2015)	Healthy, cognition challenged ($N = 22$)	2.0 g	Improved cognitive flexibility
Sutton et al. (2005)	Healthy, physically exerted ($N = 20$)	150 mg/kg	No effect of TYR
Thomas et al. (1999)	Healthy, cognition challenged ($N = 20$)	150 mg/kg	Improved working memory
Tumilty et al. (2011)	Healthy, heat exposure ($N = 8$)	150 mg/kg	Increased endurance capacity
Tumilty et al. (2014)	Healthy, heat exposure ($N = 7$)	150 mg/kg	No effect of TYR
Watson et al. (2012)	Healthy, heat exposure ($N = 8$)	150 mg/kg	No effect of TYR
Wood et al. (1985)	ADHD patients ($N = 12$)	150 mg/kg	Short term, unsustained clinical response

Tyrosine has been proposed for the treatment of depression, as the catecholamine hypothesis of affective disorders suggests that this illness may be associated with a decrease in particularly norepinephrine and dopamine in the nervous system [26][27][28].

However, the potential of tyrosine administration as a treatment for psychiatric disorders is limited by genetic predisposition, as mentioned previously, as well as the subject's ability to synthesize neurotransmitters.

In many disorders, catecholamine concentrations are lower than in normal subjects due to an impairment in neurotransmitter metabolism, particularly in dopamine and norepinephrine synthesis. Therefore, the origin of the disorder is not found in a lack in the necessary precursors but rather on the ability to metabolize them. In Parkinson's disease, for instance, tyrosine hydroxylase is less active and therefore tyrosine cannot be converted into l-dopamine at a normal rate [25].

2.3.1. Effect of dosage and routes of administration

There is not yet an agreed upon optimal dosage for tyrosine supplementation. However, doses exceeding the daily intake recommended form the World Health Organization, 14mg/kg, would presumably be unable to grant additional benefits due to the saturation of the enzyme tyrosine hydroxylase [25].

In the vast majority of human studies, tyrosine supplementation was administered orally. The evolution of the plasma levels of tyrosine in time can be seen in the figure below:

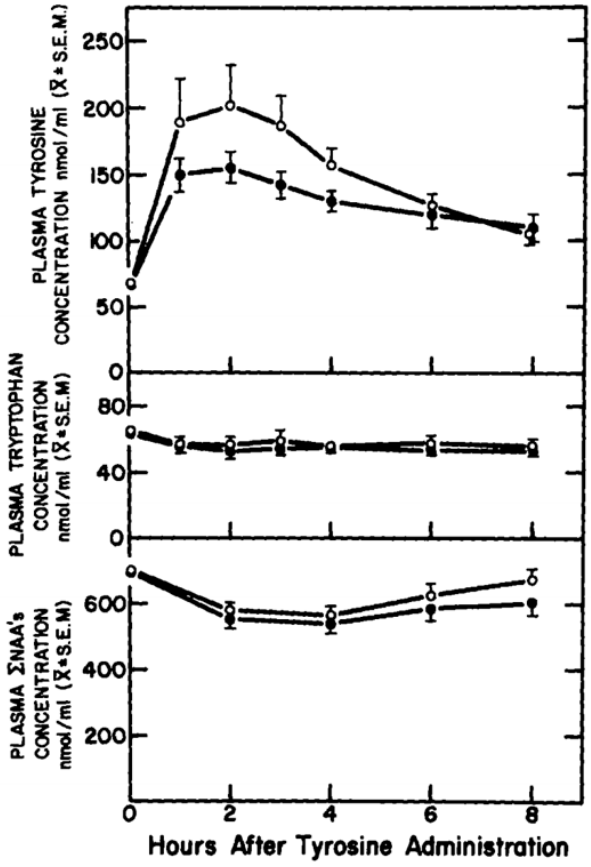


Figure 2.12. From top to bottom: plasma tyrosine, tryptophan and neutral amino acids concentrations after a l-tyrosine oral administration (100mg/kg in black and 150mg/kg in white). Retrieved from [29].

Orally administered tyrosine produces a peak in plasma concentrations approximately two hours after consumption, as can be seen in Figure ***2.12***, and the effects can persist for six to eight hours. It also produced a slight decrease of neutral amino acids in plasma concentration, which could be attributed to competition between amino acids to cross the blood-brain barrier [29].

As a proposal for the next stage of the present study, microencapsulation of the drug is discussed as an alternative drug delivery system in contrast to the direct administration of the free active compound. The entrapment of the active compound in microcapsules would allow for it to be released in a controlled manner, in order to avoid peaks of drug concentration as well as possibly avoid saturation of the enzymes regulating catecholamine synthesis. It would also provide protection and prevent the drug's degradation, as well as improve its efficacy given that factors that difficult its administration could be altered through the materials forming the microcapsule [30].

Further justification on the decision to select this method as the optimal strategy of drug delivery, along with the preferred route of administration is discussed in the following chapter: Drug design.

3. Drug design

The goals of the present study encompass the proposal of an alternative strategy to deliver l-tyrosine in order to maximize its therapeutic impact. Therefore, different routes of administration from the most commonly used to this day are examined, as well as different approaches to enhance the amino acid's physicochemical properties.

3.1. Drug delivery and administration

The ultimate effect of a compound depends on its availability at the site of action once it is administered through the appropriate route in an suitable form, therefore its potential therapeutic effect is determined through its formulation [31]. Accordingly, formulating a drug delivery system able to penetrate the blood-brain barrier, and therefore transport l-tyrosine directly through it, could potentially minimize the effect oral administration of tyrosine has on the competition between neutral amino acids to cross the blood-brain barrier – further explanation of this phenomenon is found in section 3.2.

The direct administration of l-tyrosine could potentially reduce amino acid competition given that l-tyrosine transport into the brain would not be regulated through transport proteins, as can be seen in Figure ***3.1***, as its delivery across the blood-brain barrier would be regulated through drug delivery mechanisms.

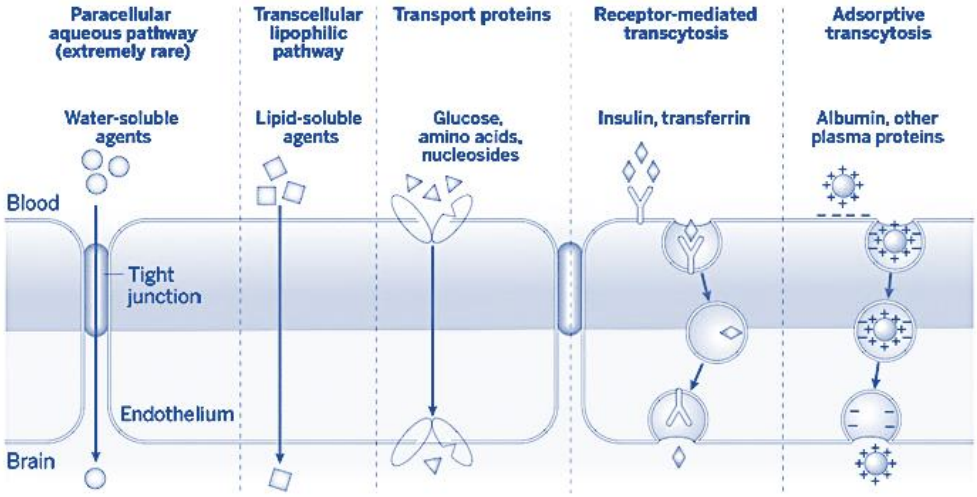


Figure 3.1. Schematic view of various pathways of crossing the blood brain barrier. Retrieved from [32]

As mentioned before, the most common routes of amino acid administration are oral and intravenous, in which the amino acid is distributed as a free compound. Through these routes, the free amino acid is absorbed from the small intestine, in l-tyrosine specifically by a sodium-dependent active transport process [33]. Thus, administration of free amino acids is able to successfully bypass the digestive system. In fact, the later absorption profile of free amino acids is higher than that of the ingestion of whole proteins.

However, plasma amino acid levels of the former administration both peak and decrease more rapidly than the latter, which is why administering an amino acid formulation imitating physiological absorption kinetics could improve the rate of assimilation into proteins, as well as preventing fluctuations of plasma amino acid levels [34].

The difference in plasma amino acid concentrations between ingesting a whole protein and administering free amino acids can be seen in Figure ***3.2***.

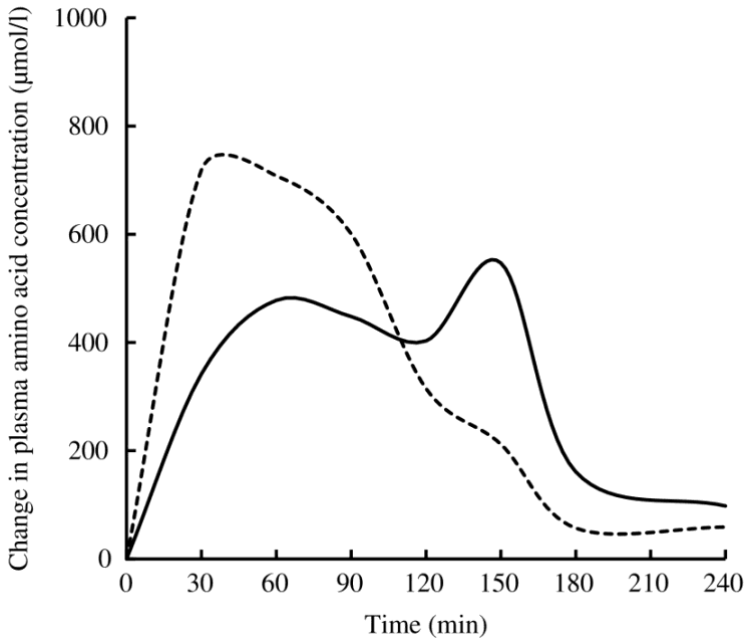


Figure 3.2. Mean change from baseline in total plasma amino acids after feeding whole protein (cottage cheese, —) or an equivalent amount of a free amino acid mixture with identical amino acid composition (---) in fasting healthy volunteers (n=10). Retrieved from [34]

After absorption, amino acids are later metabolized into a variety of different compounds or implemented in protein synthesis. In fact, the latter is stimulated both by plasma amino acid levels and insulin. When the amino acids are artificially maintained at post-absorptive levels, therefore saturating the transporters responsible for their absorption, administration of insulin further increases net protein synthesis and decreases protein catabolism [35].

In contrast, by permeating the blood-brain barrier, namely delivering tyrosine directly to the brain could maximize its therapeutic effect given that the amino acid would already be located at the major site in which it is employed for catecholamine synthesis, and therefore it could potentially minimize the metabolization of the amino acid into other compounds.

However, the specifics regarding the pharmacokinetics and pharmacodynamics involved in the transport of pharmaceutical compounds across the blood-brain barrier should be further examined, in order asses the optimal parameters of drug design and consequently obtain the greatest results after drug administration.

Thus, as the administration of the free amino acid is not able to accomplish such a specific targeted delivery directly, the best manner in which to achieve this would be to develop a specialized drug delivery system to enable the release of the drug only once it had permeated the blood-brain barrier. Said drug delivery system could also enable a controlled drug release over a prolonged period of time, and thus avoid the fluctuations in amino acid concentration as seen through the administration of the free amino acid has.

As can be seen in Figure ***3.3***, there are many strategies available to accomplish this goal.

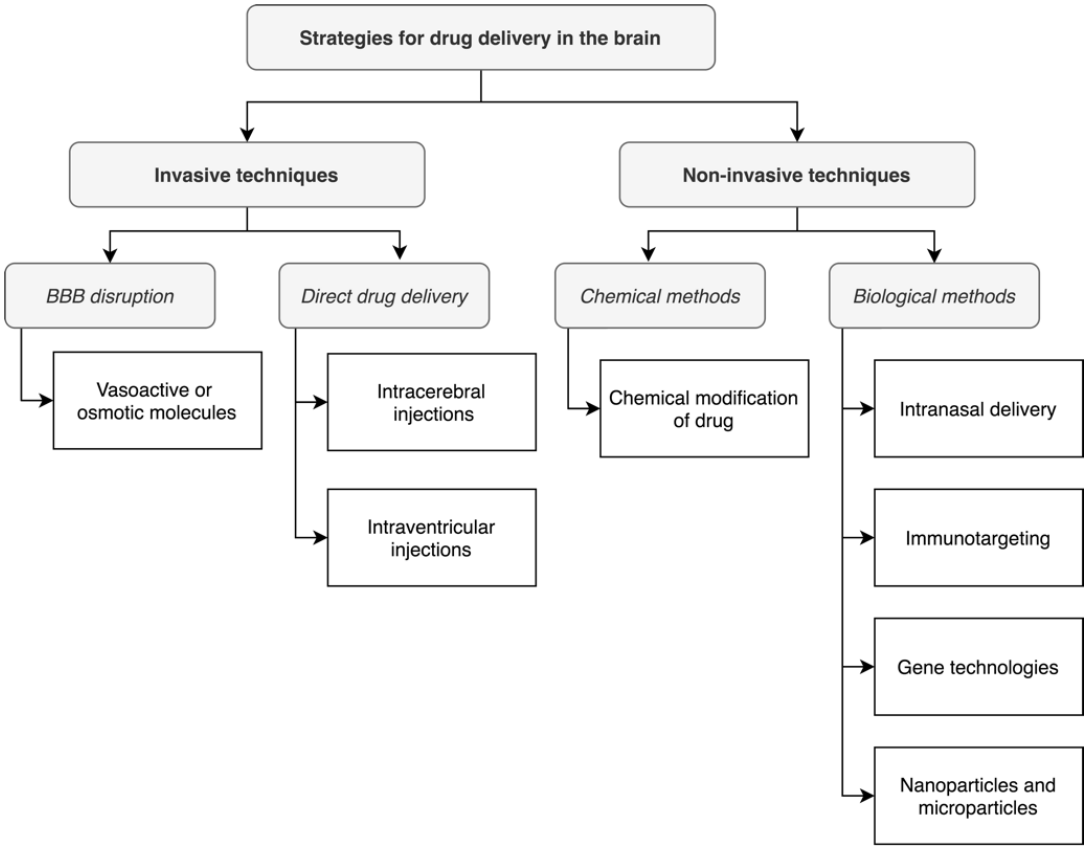


Figure 3.3. Schematic representation of strategies for drug delivery in the brain. Retrieved from [36]

The strategy considered throughout this study is the non-invasive technique of microencapsulating the amino acid in a biocompatible structure, which would confer multiple benefits to the active compound, the main ones represented in Figure ***3.4***.

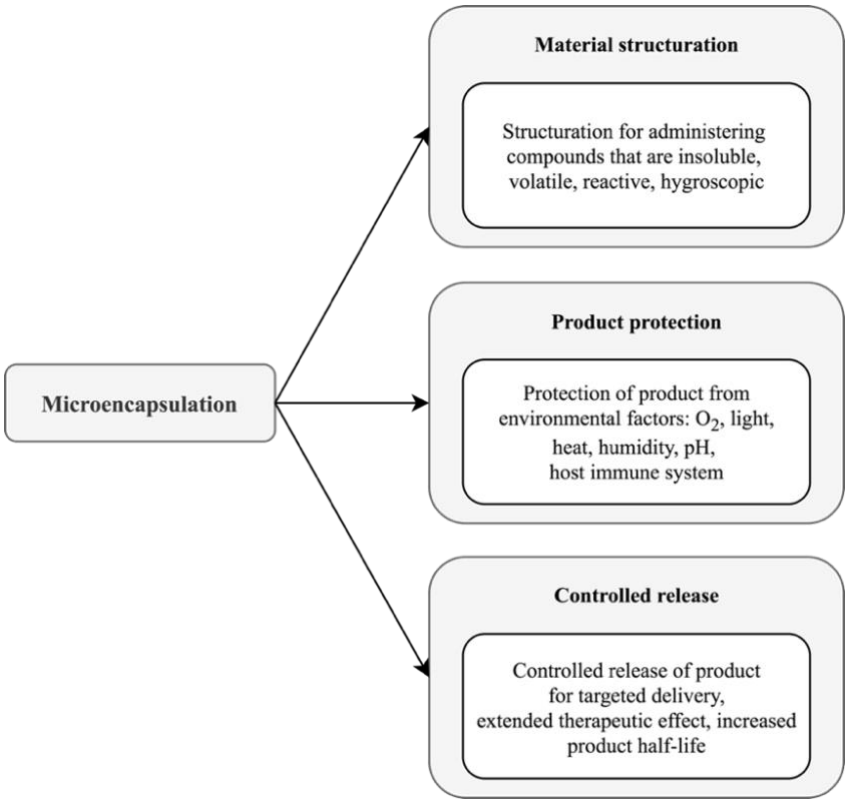


Figure 3.4. The main biopharmaceutical goals of microencapsulation: material structuration, therapeutic product protection and targeted delivery and/or controlled release of the encapsulated biotherapeutics. Retrieved from [30].

Micro and nanoencapsulation offer many advantages in drug delivery, since they are not only able to offer a targeted drug delivery at the site of interest, but to also confer many structural benefits and protection to the active compound in question. Through microencapsulating tyrosine, enzymatic degradation could be prevented, along with an improvement in physical stability and increased membrane permeability [30].

Several studies on the nanoencapsulation of tyrosine have already been conducted [37], as well as the microencapsulation of compounds involved in l-tyrosine's metabolic pathway [38] or the use of l-tyrosine for the surface functionalization drug carriers [39][40][41][42]. A study has also been conducted specifically assessing the antidepressant effect of l-tyrosine loaded nanoparticles [26].

However, in this last particular study [26] the influence of the surface chemistry of tyrosine, its self-assembly and other physicochemical factors were not considered during its nanoencapsulation process. These factors can affect the in vivo performance of a nanoparticle formula, as well influence toxic or negative effects of the treatment [43], which incidentally highlights the importance of the focus of the present study in the previous assessment of the compound's properties.

The administration of l-tyrosine following this approach could also prove helpful to patients with phenylketonuria, which – as mentioned in section 2.2 – is an autosomal recessive disorder caused by deficiency of hepatic phenylalanine hydroxylase, making l-tyrosine an essential amino acid given it cannot be converted from phenylalanine.

Due to this fact, patients suffering from phenylketonuria have been shown to have reduced levels of dopamine. Contributing to these low levels is the fact that high blood phenylalanine levels prevent exogenous tyrosine from crossing the blood-brain barrier (BBB). Moreover, supplementation with the free amino acid may cause blood tyrosine levels to fluctuate greatly during the day in treated patients [13]. The alternative route of drug delivery proposed in this study would be able to reduce the fluctuations produced by the administration of the free amino acid, given that microencapsulation ensures a controlled release of the compound, making the concentrations of the amino acid remain at constant levels, and it would also avoid the competition between amino acids to cross the BBB.

This drug delivery system could also be of interest for the treatment of Schizophrenia, given that the most common hypothesis for the development of this disease is that tyrosine is less available to the brain also due to competition with other amino acids [44].

Lower and constant doses could also lower l-tyrosine's potential toxicity. Even though the amino acid exhibits very low toxicity, and the studies reporting such toxicity are few [8], abnormally high concentrations of tyrosine in the blood have been linked to corneal diseases in rats [45], as well as hepatic and neurological lesions in rats [46].

Another factor to consider would be the route of administration, which are numerous, as can be seen in Figure ***3.5***. However, not all of them exhibit the same level of bioavailability, which is defined as the fraction of drug reaching the systemic circulation [47] and can be expressed as in equation (*3.1*).

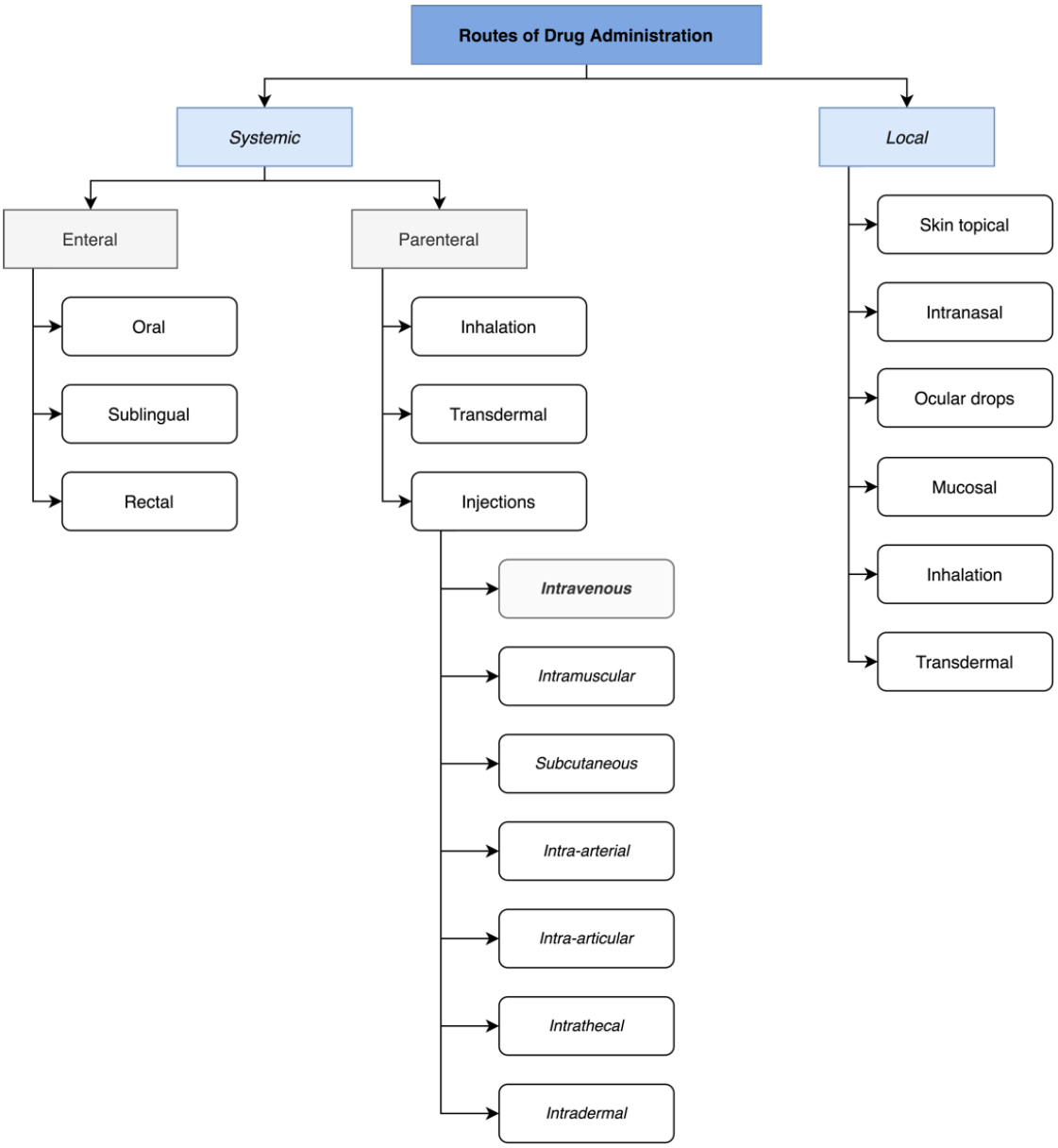


Figure 3.5. Different routes of drug administration.

The route of administration of a formulation heavily influences its bioavailability, and therefore its effectiveness. The bioavailability of a certain compound can also be heavily dependent on various factors, such as: physiological barriers, transporters and the own metabolism of the drug before it reaches the targeted site [47].

$$Bioavailability\ (F) = \frac{Amount\ of\ drug\ in\ circulation}{Amount\ of\ drug\ administered} = \frac{Drug\ absorption}{Drug\ administered} \tag{3.1}$$

In order to ensure the bioavailability of the selected drug delivery mechanism, at least during the first stages of the development of the formulation, the preferred route of administration would be intravenous administration. The basis of this decision is rooted on the high bioavailability that this route presents, as can be seen in Table ***3.1***., compared to the other alternatives, given that through it it's possible to bypass the major biological barriers that could compromise bioavailability.

Table 3.1. Some characteristics of common routes of drug administration. Retrieved from [48]

ROUTE AND BIOAVAILABILTY (*F*)	ABSORPTION PATTERN	SPECIAL UTILITY	LIMITATIONS AND PRECAUTIONS
Intravenous $F = 1$ by definition	Absorption circumvented	Valuable for emergency use	Increased risk of adverse effects
	Potentially immediate effects	Permits titration of dosage	Must inject solutions *slowly* as a rule
	Suitable for large volumes and for irritating substances, or complex mixtures, when diluted	Usually required for high-molecular-weight protein and peptide drugs	Not suitable for oily solutions or poorly soluble substances
Subcutaneous $0.75 < F < 1$	Prompt from aqueous solution	Suitable for some poorly soluble suspensions and for instillation of slow-release implants	Not suitable for large volumes
	Slow and sustained from repository preparations		Possible pain or necrosis from irritating substances
Intramuscular $0.75 < F < 1$	Prompt from aqueous solution	Suitable for moderate volumes, oily vehicles, and some irritating substances	Precluded during anticoagulant therapy
	Slow and sustained from repository preparations	Appropriate for self-administration (e.g., insulin)	May interfere with interpretation of certain diagnostic tests (e.g., creatine kinase)
Oral ingestion $.05 < F < 1$	Variable, depends on many factors (see text)	Most convenient and economical; usually safer	Requires patient compliance
			Bioavailability potentially erratic and incomplete

After ensuring the success of the developed drug delivery system, other less invasive, more practical and cost-effective routes of administration could be considered, such as: the olfactory pathway. The formulation characteristics, such as volatility, should therefore be re-examined and modified in order to guarantee that bioavailability of the drug-delivery system is not hindered by external factors.

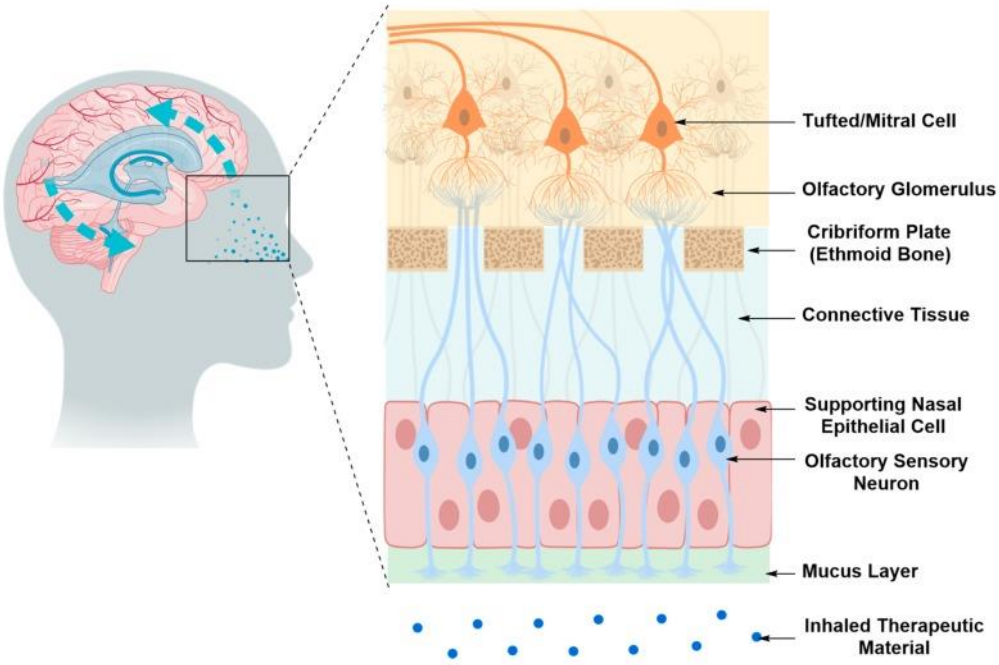


Figure 3.6. Direct nose-to-brain Access via the olfactory pathway. The therapeutic material injected into the nasal cavity diffuses through the olfactory sensory nerves and olfactory glomeruli via passive transport mechanisms. It then reaches the brain tissues via projection neurons, tufted of mitral cells. Retrieved from [32]

3.2. Microencapsulation as a technique for drug enhancement

Provided that the selected route of administration is an intravenous injection, and this technique is classified under the parenteral route of administration, the formulation must be delivered through an injectable solution.

All of the factors that alter a drug's ability to cross biologic membranes, its interaction with pumping mechanisms, or its metabolism will also affect drug bioavailability, drug effect, and drug toxicity [47]. Accordingly, in order to minimize their effect, they need to be inspected and taken into consideration.

One of the problems presented when developing a pharmaceutical formulation using tyrosine as the active agent is its low solubility, which represents a serious limitation. Hence, its bioavailability could be compromised by its efficiency of absorption and transportation to the previously defined target tissues. Therefore, the improvement of its properties in formulation is essential for its therapeutic potential.

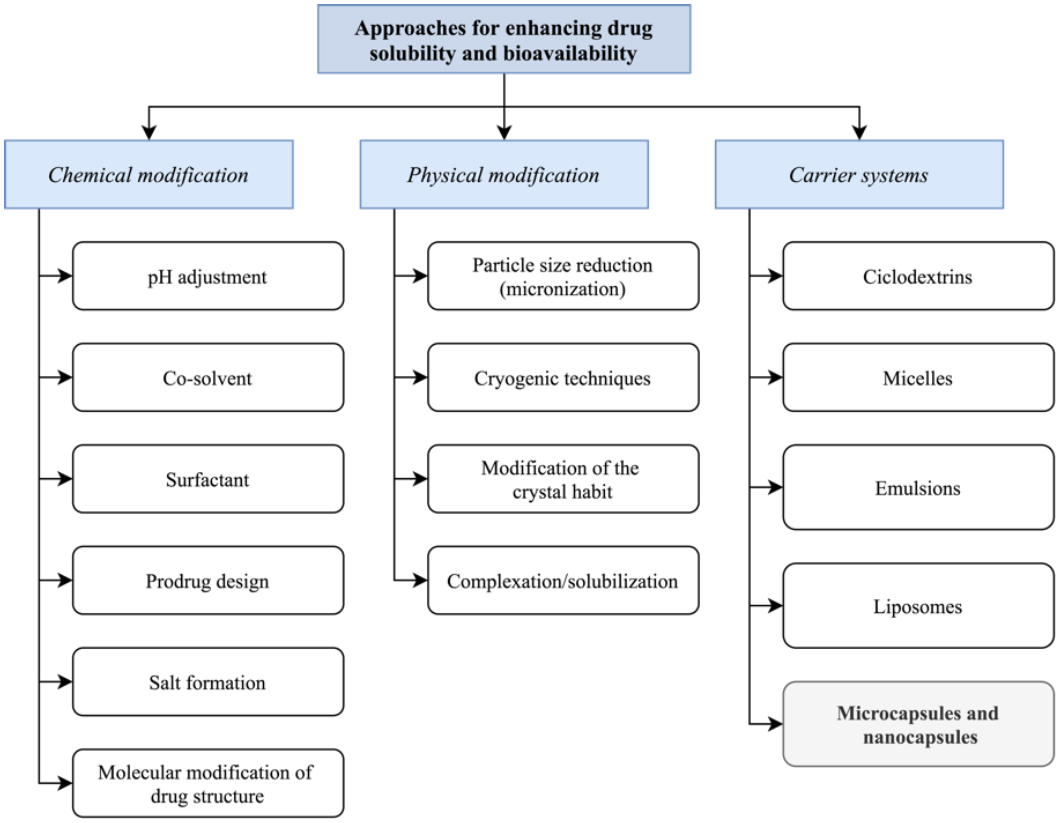


Figure 3.7. Approaches for improving drug solubility and bioavailability.

There are currently various techniques and approaches which are employed to tackle the problem of low solubility, as seen in Figure ***3.7***., in order to prepare an effective and marketable drug. The physicochemical properties of the drug and the carrier, as well as their application, are the decisive factors when selecting the most appropriate method [49]. The decision to microencapsulate l-tyrosine in itself would therefore improve not only its solubility but its bioavailability.

Microencapsulation would also pose a particularly large advantage compared to other drug delivery mechanisms, given that the most commonly involved drug release mechanism is diffusion, which is classified as a form of passive transport [50]. The different mechanisms in which drugs can be transported through the cellular membrane can be appreciated in Figure ***3.8***.

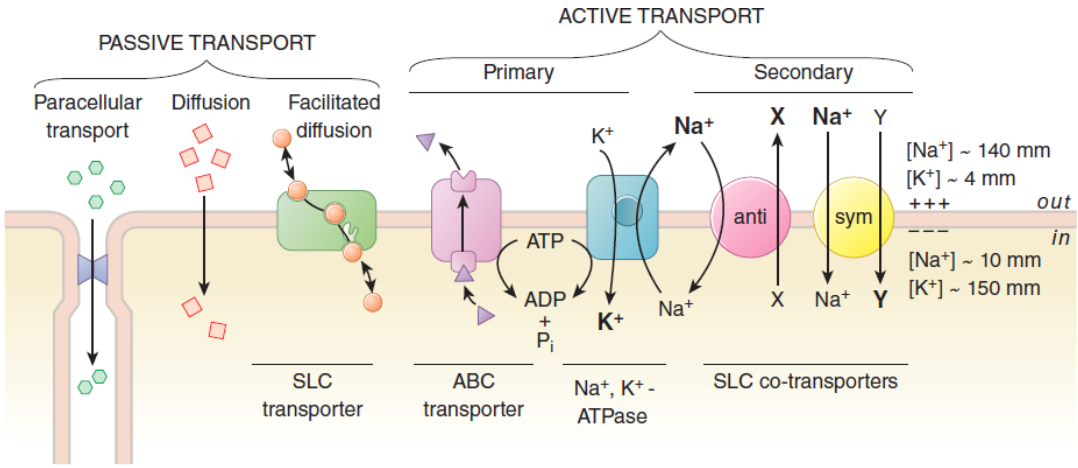


Figure 3.8. Mechanisms of drug transport through cellular membrane. Retrieved from[48]

As previously mentioned in section 3.1, amino acid absorption is mediated by active transport, specifically through a carrier system, involving the use of transporters in the GI track. The transport mechanisms involved in amino acid absorption can be seen in Figure ***3.9***. In contrast to passive diffusion, these carriers often exhibit a concentration beyond which no further increase in transport occurs. This is due to the fact that this type of transport can become saturated at high substrate concentrations, and therefore presents a disadvantage compared to diffusion mechanisms.

These transporters present structural selectivity. Hence, drugs that are structurally similar to the transporter's natural substrates and involve the use of a transporter in its drug delivery mechanism, often have to compete with said substrates to bind to the transporters, resulting in lower absorption [51].

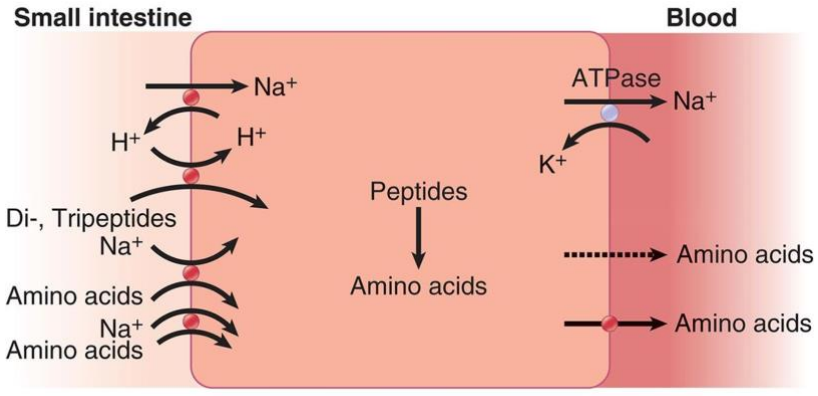


Figure 3.9. Absorption of amino acid and peptides in the small intestine. Retrieved from [52]

As previously established and shown in Figure ***3.8***, drug molecules are be absorbed and transported across membranes through several mechanisms, the main ones being: passive diffusion (transcellular), passage through pores and junctions (paracellular) and transporter-mediated (active transport) [48].

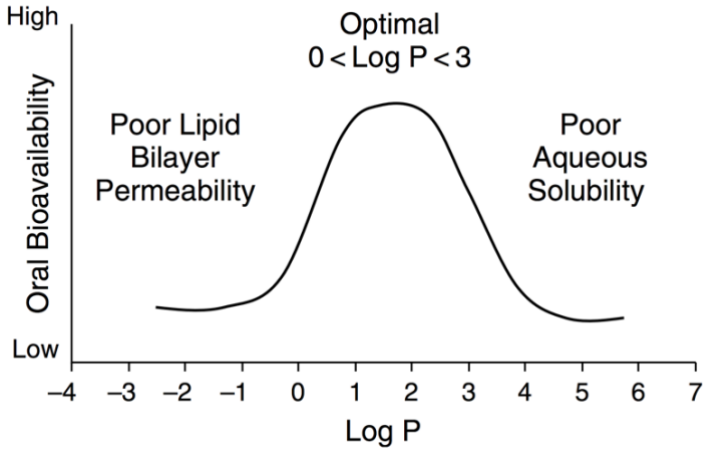


Figure 3.10. Hypothetical example of how Log P can affect oral bioavailability for a compound series. Absorption by passive diffusion permeation after oral dosing is generally considered optimal for compounds having a moderate LogP and decreases for compounds having higher and lower Log P values. Retrieved from [53]

The only means for drugs that do not exhibit binding interactions with transporters to be absorbed through biological membranes is by passive diffusion. The partition coefficient is the factor that directly assesses the ability of a molecule to permeate through membranes. It directly measures the ratio of the concentrations of a solute in two immiscible liquids, usually octanol and water, when it is in equilibrium across the interface between them [48].

As can be observed through Figure ***3.10***, the values at which permeability and solubility present optimal bioavailability is very limited. Values out of this short range would present problems with one of the two parameters.

L-tyrosine presents a particularly low partition coefficient, with a value of log P= -2.26 [8]. The value of this partition coefficient would classify the amino acid's character as partially hydrophilic. Hydrophilicity is defined as the capacity of a molecular entity or substituent to interact with polar solvents, in particular with water, or with other polar groups on a molecular level [54]. However, hydrophilic substances are not necessarily water soluble, the partition coefficient simply determines that a substance interacts more strongly with the polar solvent. Even though l-tyrosine presents a partially hydrophilic character, its solubility in water is relatively low, as determined in section 2.1.

Compounds with low solubility have a higher difficulty diffusing through membranes, hence their permeability is negligible and they consequently present very low bioavailability. In order for a drug to diffuse through membranes, it firstly has to be dissolved in the physiological medium. The bioavailability of poorly soluble drugs is therefore limited by their solvation rate [55].

Table ***3.2*** shows the impact that the value of the distribution coefficient, similar to the permeation coefficient but considering the pH, can have both on the properties of a drug *in vitro* and *in vivo*.

Table 3.2. Impact of Log D at physiological pH on Drug-like properties. Retrieved from [53]

Log $D_{7.4}$	Common Impact on Drug-like Properties	Common Impact *In Vivo*
<1	Solubility high Permeability low by passive transcellular diffusion Permeability possible via paracellular if MW < 200 Metabolism low	Volume of distribution low Oral absorption and BBB penetration unfavorable Renal clearance may be high
1 to 3	Solubility moderate Permeability moderate Metabolism low	Balanced volume of distribution Oral absorption and BBB penetration favorable
3 to 5	Solubility low Permeability high Metabolism moderate to high	Oral bioavailability moderate to low Oral absorption variable
>5	Solubility low Permeability high Metabolism high	High volume of distribution (especially amines) Oral absorption unfavorable and variable

The low value of tyrosine's partition coefficient therefore reflects permeability problems across the intestinal track, as well as difficulties in penetrating the blood-brain barrier, as can be interpreted through Table ***3.2***. These problems would only be present if the amino acid were to be absorbed through the BBB by a mechanism of diffusion and if administered orally

However, since the absorption of free l-tyrosine both into the brain and small intestine is regulated through transporters, it is unable to penetrate the blood-brain barrier without the aid of its structure specific carrier proteins.

In order to administer l-tyrosine into the brain through a mechanism of passive diffusion, its chemical structure and in turn its partition coefficient would need to be modified to fit the optimal values at which permeation of the blood-brain barrier is favorable. Therefore, the value of the new partition coefficient at which optimal activity is observed is Log P =2,00 [56], which will be achieved in its microencapsulation through the use of excipients.

L-tyrosine's tendency to form aggregates in solution is also a challenge that must be addressed during its development into microcapsules. The tendency of proteins to self-assemble and to form aggregations in solution will be extensively analyzed in the following chapters.

In order to confer l-tyrosine the desirable properties to effectively deliver the drug molecules, it would be helpful to resort to the use of a biocompatible surfactant, in order to avoid its aggregation during the process of microencapsulation. The use of surfactants has long been a primary drug delivery strategy to increase the solubility of lipophilic drugs [57], as well as an additive to prevent protein aggregation by isolating the protein form interfacial tension [58]. Thus, the role of surfactants in drug delivery is essential for the determination of the final microcapsule's characteristics.

Therefore, a preliminary study on the surface tension both l-tyrosine's and the combination of the amino acid with the surfactant must be executed, polysorbate 20 being the biocompatible surfactant of choice. By studying the properties of tyrosine in solution and its entrapment in a biocompatible surfactant, it would be possible to determine the ideal conditions of its microencapsulation.

Hence, in order to develop a more sophisticated and effective approach to deliver tyrosine into the brain, an analysis on its surface chemistry must be conducted.

It must be noted, that as much as the direct administration of tyrosine to the brain is presented as the optimal solution for a number of diseases, the fact that the systems in place for amino-acid transport across the BBB have not been fully characterized at a molecular or functional level represents a serious limitation for the long-term application of this study. Through the combination of molecular research, fibroblast techniques, and brain imaging a new basis for clinical research on the role of amino-acid membrane transport could be developed [44]. As previously mentioned, a study on the pharmacokinetics and pharmacodynamics involved in the transport of pharmaceutical compounds across the blood-brain would also enable to assess the optimal parameters of its drug design, as well as obtain the best results after drug administration.

3.3. Outline and current outreach

The present research constitutes the first stage of l-tyrosine's drug delivery design process, which is classified under the preformulation stage of pharmaceutical drug design and comprises the first stage in drug development.

The aim of preformulation is to provide knowledge of the physicochemical parameters of the drug, establish its physical characteristics and stability, as well as to select the right excipients to optimize the drug's performance while studying their compatibility. Hence, this stage is crucial in the early stages of drug development [59], as it is executed as an aid for the later design of an optimum drug delivery system [60]. This phase of development requires a multidisciplinary approach, as can be seen Figure ***3.11***, along with its basic outlines [31].

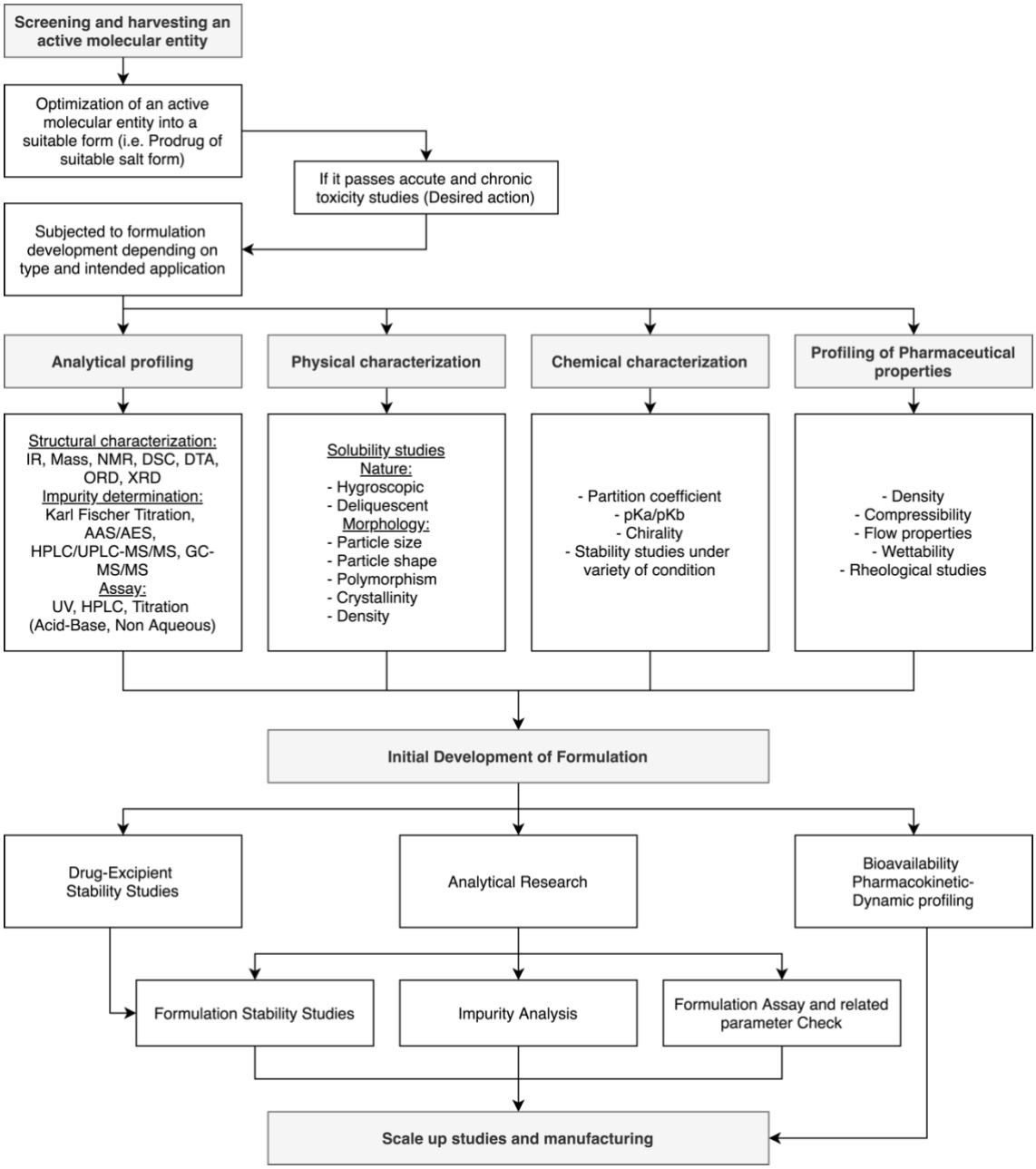


Figure 3.11. Outline of preformulation studies. Retrieved from [31]

As has been established, l-tyrosine's main physicochemical properties have been extensively characterized and analyzed in previous studies, the literature pertaining to which has already been reviewed throughout the previous chapters.

Consequently, from hereon forward, the research focuses on the study of the interactions and the stability between the compound and the first excipient: the surfactant. Specifically, the analysis on drug-excipient stability will be achieved through the study of its surface chemistry.

Once complete, this study will enable the launch of the second phase: microencapsulation. The strategy of the overall drug design process for l-tyrosine proposed in this study can be seen in Figure ***3.12***.

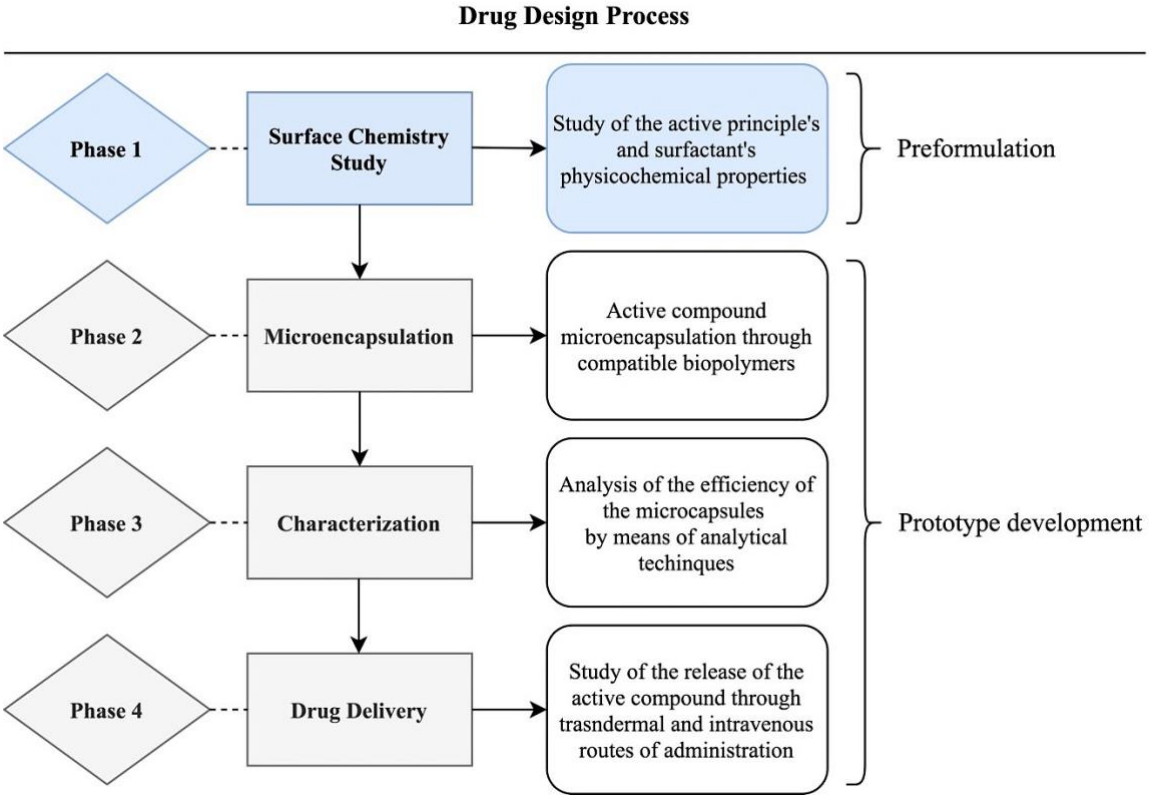


Figure 3.12. Phases involved in drug design in chronological order.

Therefore, the objective of this study is to develop a foundation to transform l-tyrosine into a pharmaceutical formulation that administered in the correct way and in the precise amount, would affect the specified target. Whereas during the following phases, the objective will be to provide longer stability to the formulation by designing and protecting the compound against environmental agents, as well as finally evaluating the formulation's performance.

4. Surface and colloid chemistry

Drug carrier shape and size are important factors in drug delivery [61]. The shape of a drug often affects the affinity of a drug for carrier molecules or its binding site. Drugs that have a similar structure may display competition for their binding sites, as happens between neutral amino acids to cross the BBB, and in turn can affect the pharmacokinetics of a drug [47]. Most synthetically formulated particles tend to have a spherical geometry, in order to minimize its surface energy. Particle size is also a determining factor to consider to when designing particulate systems [61], given that smaller molecules tend to be absorbed more easily [47].

Consequently, in order to optimize the drug delivery of tyrosine for its afterward microencapsulation, a study of its surface tension was carried out to examine the effect of its increasing concentration on its morphology and self-aggregation. Subsequently, another study on its superficial tension, alongside the addition of the surfactant polysorbate 20 was also conducted to determine the ideal concentration at which it could later be microencapsulated.

In order to interpret the aforementioned phenomenon, a further explanation of the principles of surface chemistry, along with a review of a literature pertaining to amino acid self-assembly is required.

4.1. Surface tension

Surface tension is the phenomenon resulting from the cohesive forces between liquid molecules. On the interior of a homogenous liquid, as can be seen in Figure ***4.1***, molecules experience attracting forces equally in all directions, resulting in a net force value of zero.

However, a molecule located in the surface of the liquid experiences an imbalance of forces due to the lack of neighboring molecules. In liquid and air interfaces, molecules on the surface can also develop adhesive forces of attraction to those at the other side of the interface. Nonetheless, they are far weaker than cohesive forces, which causes an unbalance. As a result, a net inward force is developed and pulls the molecules into the interior of the liquid [62].

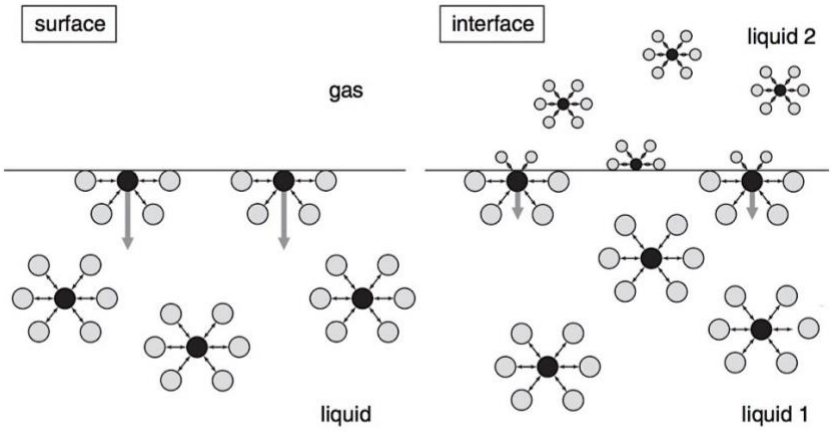


Figure 4.1. The molecular interactions of molecules in adjacent phases can be used to visualize the concept of surface or interfacial tension. Retrieved from [63]

If the surface tension of a liquid is high, it means it is harder to extend the area of said surface and more work must be done to distort it, as can be seen in (*4.1*)[63].

$$W = \gamma \cdot \Delta A \tag{4.1}$$

This work is often referred to as the free energy of the liquid. Hence, the higher the superficial tension, the larger is the free energy required to overcome. Excess free energy per unit surface area is therefore defined as the surface tension of a liquid, expressed in Nm^{-1} [62]. It can also be measured in dyn/cm, referring to the force in dynes required to deform a surface of 1cm.

In equilibrium, the surface free energy of a system must be at a minimum. Since the geometry of a sphere is that of the smallest surface are per unit volume, liquid droplets tend to acquire said shape [64]. A higher surface tension also suggests stronger intermolecular forces, therefore substances that can form hydrogen or those of higher molecular weight could have greater superficial tension [65].

The distinction between dynamic surface tension and static surface tension also needs to be addressed. Static superficial or interfacial tension is the value of the surface tension of a liquid at thermodynamic equilibrium. Therefore, this value is independent of time. In contrast, the dynamic surface tension is the value of the surface tension at a particular place in time.

In non-homogenous systems, or systems with surface-active agents, the value of this parameter is therefore dependent of time and accordingly, can differ from the value at equilibrium. The time it takes for a solution with surface-active agents to reach equilibrium is highly dependent on the diffusion and adsorption rate of the surfactant. The aforementioned properties are discussed more extensively in the following section.

Dynamic surface tension is also of particular interest in processes which involve the rapid production of interfaces, given that the kinetics of interface formation are decisive influences in said processes [66].

4.2. Surfactants and their effect on superficial tension

Surfactants – surface-active agents – are often added in biopharmaceutical applications to offer stability and prevent the physical degradation of the protein structure. They are amphiphilic, meaning they contain a polar hydrophilic head group and a non-polar hydrophobic tail [67].

There are several different types of surfactants, their most usual chemical classification seen in Table ***4.1*** is done according to the nature of their polar head group. Nonionic surfactants are commonly used as a stabilizing agents in protein formulations in order to prevent degradation. In contrast, ionic surfactants are usually used for purposes of pre-treatment of proteins, since that they are effective protein denaturants [67]. This is the reason why throughout this study there will be a specific focus on the former.

Table 4.1. Classification and characteristics of surfactants. Retrieved from [68]

Classification	Characteristic	Example
Anionic	The hydrophilic group carrying a negative charge such as carboxyl ($RCOO^-$), sulfonate (RSO_3^-), or sulfate ($ROSO_3^-$)	$CH_3(CH_2)_{11}SO_4^-Na^+$ Sodium dodecil sulfate (SDS)
Cationic	The hydrophilic group carrying a positive charge as, for example, the quaternary ammonium halides ($R_4N^+Cl^-$)	$CH_3(CH_2)_{15}N^+(CH_3)_3Br^-$ Cetyl trimetyl ammonium bromide (CTAB)
Non-ionic	The hydrophilic group has no charge but derives its water solubility from highly polar groups such as polyoxyethylene ($-OCH_2CH_2O-$) or polyol groups	$CH_3(CH_2)_{11}(OCH_2CH_2)_{23}OH$ Polyoxyethylene (23) dodecanol (Brij 35)
Amphoteric or zwitterionic	Its molecules present both the anionic and cationic groups and, depending of pH, its prevalence the anionic, cationic, or neutral species	$CH_3(CH_2)_{11}N^+(CH_3)_2(CH_3)COO^-$ 4-(Dodecyldimetyl ammonium) butirate (DAB)

In aqueous solution, provided their amphipathic nature, surfactants tend to orient themselves so that the exposure of their hydrophobic portions are minimized. Thus, they accumulate at the interfaces of aqueous and air systems in a way in which only their hydrophilic ends come in contact with the water, forming a surface layer. The adsorption process of the surfactant molecules to the surface of water causes a reduction surface tension and a decrease of the free energy of the system. This is caused by the replacement of high energy water molecules for surfactant molecules, given that the interaction between the former molecules is weaker than between two water molecules [62].

The hydrophobic force driving surfactants to be adsorbed in the interface can be described by the Gibbs energy free equation [69]:

$$\Delta G_t = \Delta H_t - T\Delta S_t \tag{4.2}$$

where ΔH_t and ΔS_t are the enthalpy and entropy of transfer, respectively.

The contribution from the entropic term greatly outweighs the enthalpic. However, the former experiences a decrease with the addition of surfactant molecules. This phenomenon is thought to be the result of the hydrophobic species promoting an ordering of water molecules differently from their normal hydrogen-bonded structure of water, organizing themselves around the hydrocarbon chain. In order to minimize said effect, the surfactant molecules tend to cluster together to reduce the involvement of water molecules, which is enthalpically favored ($\Delta H<0$), but entropically unfavorable. The clustering of hydrophobic molecules is known as the hydrophobic interaction [70].

Another important property that must be taken into account when examining surfactants in solution is interfacial tension. It is of high importance due to the fact that when suitably altered, it can provide a stabilizing influence in various types of dispersions [70].

The Gibbs adsorption equation shows one of the most fundamental interfacial phenomena caused by the addition of surfactants in solution: the decrease of interfacial tension, which is caused through the expanding force that surfactant molecules absorbed in the interface provide against normal interfacial tension [65]. Through this equation, the relationship between interfacial tension and interfacial coverage of the molecules can be derived [63]. It can be expressed through the following equation:

$$d\gamma = -\sum_i \Gamma_i d\mu_i \tag{4.3}$$

Where $d\gamma$ is the change in surface or interfacial tension of the solvent, Γ_i the surface excess concentration of any component of the system, and μ_i the change in chemical potential of any component of the system [65].

The surface excess concentration (Γ) is defined as the area-related concentration of a surfactant at an interface or surface. It is the resulting concentration of the difference between the interfacial (Γ_i) concentration and the concentration at a virtual interface at the interior of the volume phase (Γ_v).

$$\Gamma = \Gamma_i - \Gamma_v \tag{4.4}$$

However, as justified through the hydrophobic effect, surfactants tend to accumulate to in the surfaces or interfaces of liquids, thus making the volume excess concentration practically negligible. Which is why it is most commonly directly equated with interfacial concentration [71].

The practical application of equation (*4.3*) is contingent on the theory that the relative adsorption of a material at an interface can be determined through experimental data of surface tension as a function of bulk solute concentration. In ideal systems, hence isothermal binary systems, where the chemical potential is equal to $\mu_i \approx R \cdot T \cdot \ln C_i$, the Gibbs adsorption isotherm can be expressed as [72]:

$$\Gamma_i = -\frac{1}{RT}\left(\frac{d\gamma}{d\ln C_i}\right)_{T,p} \tag{4.5}$$

Where Γ_i is the surface excess of surfactant (mol/cm$_2$), C_i is the solution concentration of the surfactant (M), and g may be either surface or interfacial tension (mN/m) [70].

Additionally, the Gibbs absorption isotherm for dilute solutions of a nonionic surfactant can be calculated through the following equation [65]:

$$\Gamma_1 = -\frac{1}{2{,}303 \cdot RT}\left(\frac{d\gamma}{dlnC_1}\right)_T \tag{4.6}$$

and the surface concentration can be obtained from the slope of a plot of γ versus log C_1 at constant temperature (when γ is in dyn/cm (ergs/cm^2); R = 8,31·10^7 ergs/mol /K; Γ_1 is in mol/cm^2).

Another determining factor of the interfacial characteristics of a solution is the area per molecule at the interface. It is, as its name suggests: a measure of the surface area per molecule. Hence, it relates to the orientation of the absorbed surfactant molecules, as well as their degree of packing. It is expressed as:

$$a_1{}^s = \frac{10^{16}}{\mathrm{N} \cdot \Gamma_1} \tag{4.7}$$

where N is Avogadro's number and Γ_1 is in mol/cm^2 [65].

The comparison between the surface tension and surface excess concentration as a function of surfactant concentration can be seen below:

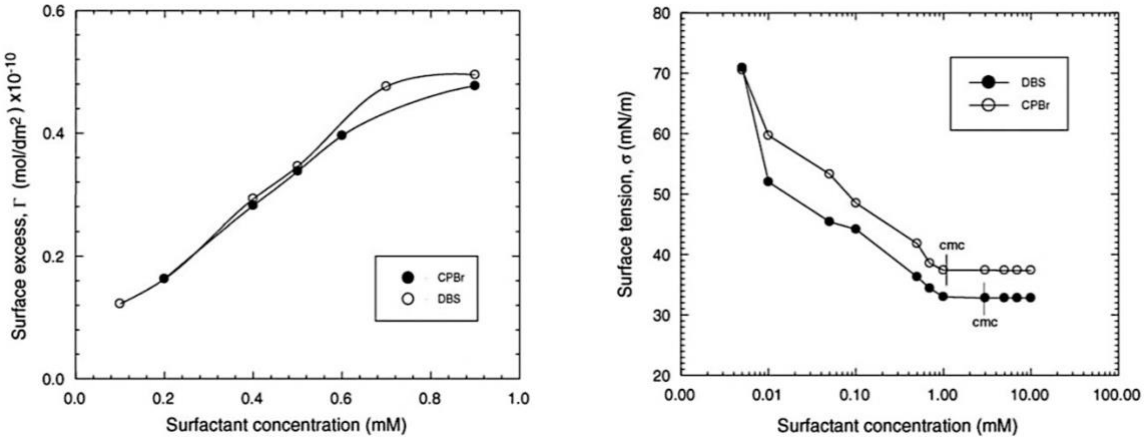


Figure 4.2. Variation of surface excess concentration and surface tension as function of surfactant concentration. Retrieved from [73]

Once the surface excess concentration reaches its maximum value at the critical micellar concentration, this parameter remains constant through the increase of surfactant. It is with the maximum value of excess surface tension, therefore, that the minimum area per molecule of surfactant that lies at the liquid/air interface [73].

Furthermore, the performance of a surfactant is also a crucial factor to be evaluated. In order to be able to interpret it, its two critical parameters must be observed and evaluated individually, as they are not dependent on each other:

- *Efficiency*. It is the amount of surfactant required to reduce surface tension by a significant amount, and can be measured by the negative log of the bulk phase concentration necessary to lower surface tension 20 dyn/cm.

- *Effectiveness.* It is the maximum reduction in tension that can be obtained, regardless of the concentration of the surfactant in order to do so. It can be measured by the amount of reduction in surface tension has been attained at the critical micelle concentration [65]– discussed in the following section.

4.2.1. Critical micelle concentration

Surfactants have the property to form micelles in solution, which is particularly important given it affects a multiple of important interfacial phenomena, as well as the surfactant's physicochemical properties [65].

When the surfactant molecules adsorbed as a monolayer in the interface become so saturated that additional molecules cannot be easily distributed, the excess surfactant will begin to agglomerate in the bulk of the solution forming micelles. The critical micelle concentration (CMC) is the concentration at which this phenomenon occurs. Hence, it is an important parameter for the characterization of aggregation. As can be seen in the figure below, once the CMC concentration is achieved the surface tension of the surfactant does reduce further and rather remains constant [74].

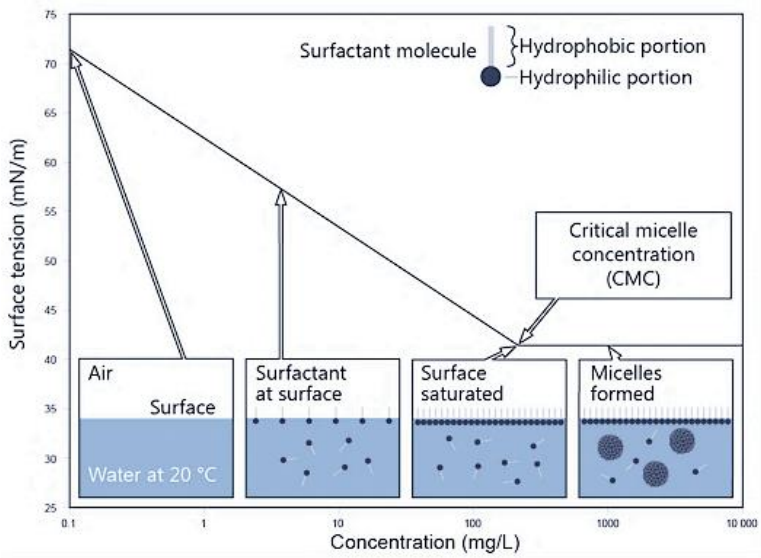


Figure 4.3. Surface tension of a surfactant solution with increasing concentration, formation of micelles. Retrieved from [74]

The physicochemical properties of surfactants, as previously mentioned, are altered once the concentration of surfactant in solution reaches the critical micelle concentration, as can be seen in Figure **4.4** [70].

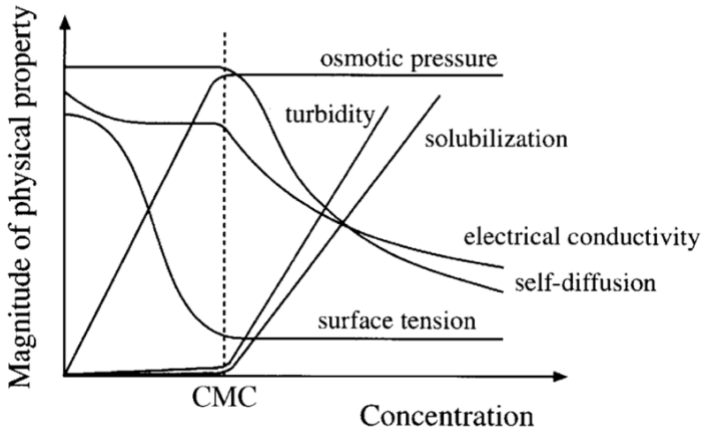


Figure 4.4. Many physical properties exhibit a discontinuity near to the critical micelle concentration (CMC). The CMC is not a thermodynamic quantity but is defined by sharp changes in measurable quantities, which occur in a concentration range close to the CMC. Retrieved from [75]

Among the properties that exhibit a sudden change in slope is osmotic pressure. Similarly to surface tension, after reaching CMC this property has a practical constant trajectory, given that it depends on the number of dissolved particles and the interfaces and surfaces are practically saturated with surfactant molecules. Electrical conductivity, in the case of ionic surfactants, exhibits a sharp decrease since only the counterions of surfactant molecules that are not aggregated can carry current.

Overall, the vast majority of physicochemical properties of a given surfactant and solvent system will show an abrupt change in slope in a narrow concentration range, which is characterized as its critical micelle concentration. Therefore, this suggests that a highly cooperative association process between surfactant molecules takes place above this concentration [70].

Resembling the trend surface tension has with increasing concentrations of surfactant past its CMC, interfacial tension is also somewhat stabilized –although not to the same degree as surface tension – after reaching said point. The stability of both tensions is due to the crowding of surfactant molecules at surfaces and interfaces. The comparison between the two tensions can be seen in the figure below:

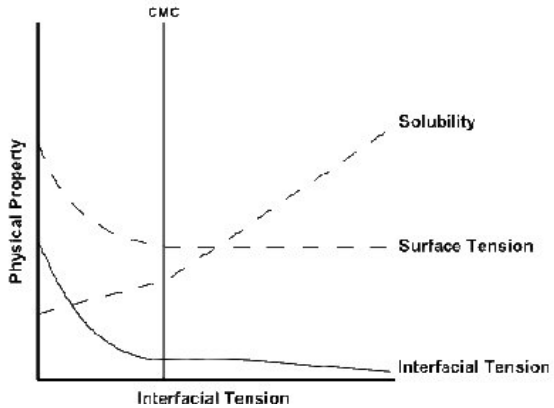


Figure 4.5. Relationship of Surface tension, interfacial tension, solubilization and CMC with biosurfactant concentration. Retrieved from [76]

As previously mentioned in section 4.2, critical micelle formation is a parameter that is commonly used to measure both the effectiveness and efficiency of a surfactant. Hence, efficient biosurfactants exhibit lower CMC values, meaning less concentration of surfactant is necessary to decrease surface tension [76].

The property of surfactants to self-assemble forming micelles has also been taken advantage of for the creation of drug delivery systems. Niosomes are vesicle structures derived from the spontaneous micellation of nonionic surfactants [77]. They have been researched as a drug delivery system able to offer better permeability to otherwise hydrophilic aqueous solutes, by entrapping them in the interior of their aqueous compartment, as seen in Figure ***4.6*** [78].

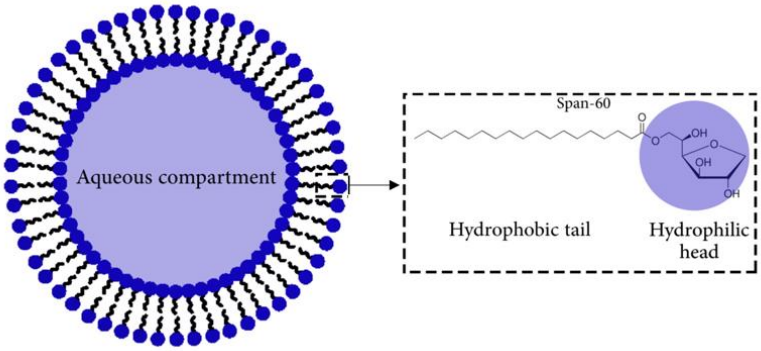


Figure 4.6. Schematic representation of a niosome. Retrieved from [79]

4.2.2. Surfactant of choice: Polysorbate 20

Polysorbate 20 (Tween 20) is commonly used in biopharmaceutical formulations as an excipient, in order to obtain a long-term stabilized formulation containing the active compound, enhance its solubility and also facilitate drug absorption acting as a vehicle for the active compound [80].

The purpose of this surfactant throughout this study is to optimize the drug delivery of tyrosine, by entrapping a determined concentration of the amino acid in surfactant-based micelles, thus preventing amino acid aggregates from forming.

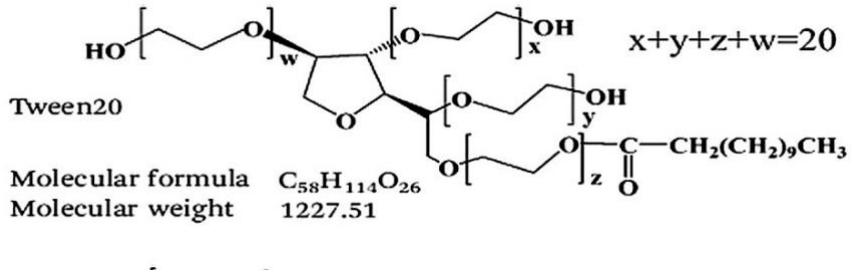


Figure 4.7. Chemical structure of polysorbate 20. w+x+y+z refers to the total number of oxyethylene subunits on each surfactant molecule and may not exceed 20. Retrieved from [81]

Polysorbates are nonionic, amphipathic surfactants derived from the esterification of ethoxylated sorbitan with fatty acids. Polysorbate 20 in particular is composed of the fatty acid esters of polyoxyethylene sorbitan monolaurate, its chemical structure shown in Figure ***4.7***.

Its hydrophobic nature is provided by the carbohydrate chain, while its hydrophilic character is given through the ethylene oxide subunits. The structure in the previous figure represents a chemically homogenous polysorbate [69].

However, most polysorbate solutions sold by manufacturers are generally a heterogeneous mixture of the fatty acids seen in Table **4.2**. These compositions are defined through the European Pharmacopoeia [69].

Table 4.2. Fatty acid contents of Polysorbate 20 and 80. Retrieved from [69]

	EU Specifications		
Acid	PS-20 (%)[a]	PS-80 (%)[b]	Structure
Caproic	≤1		$CH_3(CH_2)_4COOH$
Caprylic	≤10		$CH_3(CH_2)_6COOH$
Capric	≤10		$CH_3(CH_2)_8COOH$
Lauric	40–60		$CH_3(CH_2)_{10}COOH$
Myristic	14–25	≤5	$CH_3(CH_2)_{12}COOH$
Palmitic	7–15	≤16	$CH_3(CH_2)_{14}COOH$
Palmitoleic		≤8	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
Stearic	≤7	≤6	$CH_3(CH_2)_{16}COOH$
Oleic	≤11	≥58	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
Linoleic	≤3	≤18	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
Linolenic		≤4	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$

[a]European Directorate for the Quality of Medicines and Healthcare. European Pharmacopoeia On-line. 5th Edition 2007, 01/2005:0426.
[b]European Directorate for the Quality of Medicines and Healthcare. European Pharmacopoeia On-line. 5th Edition 2007, 04/2006:0428.

Since throughout this study the superficial tension of polysorbate 20 in an aqueous solution by itself will not be measured, a representation of it retrieved from [82] can be seen in Figure ***4.8***.

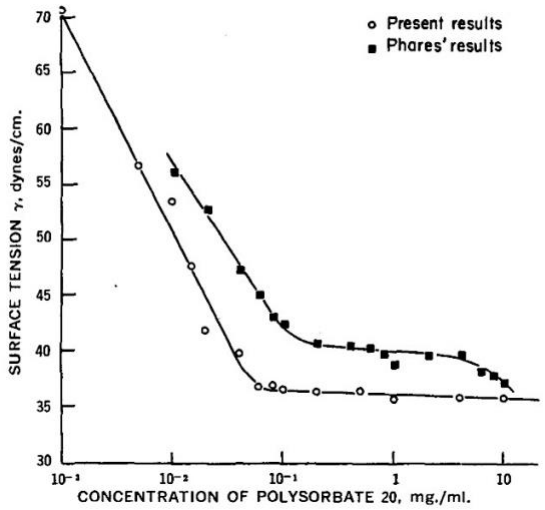


Figure 4.8. Plot of surface tension versus log concentration for polysorbate 20 aqueous solutions. Retrieved from [82]

As can be seen in the figure above, the superficial tension initially decreases linearly with the logarithm of the concentration until it reaches its critical micelle concentration at a value of 0.06 mg/ml. This value can be obtained, as seen in the graph, through the extension of the two lines determining the two distinct linear behaviors of the surfactant at different concentrations.

Therefore, the referenced article is able to determine a definite value of the CMC of polysorbate 20. It can also be observed that extremely low concentrations of surfactant in solution exhibit practically the same behavior as that of pure water. Lastly, the linear behavior exhibited in the graph suggest the applicability of Gibb's adsorption isotherm (*4.6*) to such solutions [82].

The results of this study suggest that this surface tension method is quick, reliable, and accurate for the determination of the CMC of alleged heterodisperse nonionic surfactants [82], thus establishing the perfect conditions for the development of the present study.

Additionally, a table summarizing the properties of said surfactant can seen below:

Table 4.3. Chemical properties of Tween 20. Retrieved from [83][80]

Chemical properties of Polysorbate 20 (Tween 20)	
Chemical formula	$C_{58}H_{114}O_{26}$
Molas mass	1227.51 [g/mol]
Density	1.1 [g/mL]
Boiling point	>100ºC
CMC	60mg/ml
Water solubility	100 [g/L]

4.3. Self-assembly of amino acids

Protein, peptide and amino acid stabilization is one of the most challenging factors in the process of biological drug development, their aggregation and self-assembly being one of the most common problems encountered throughout all phases of said process [84]. Moreover, the fact that that self- assembling systems often play a critical role in physiological functions, has garnered them a lot of attention since they present an untapped potential to be applied in biomedical fields, as well as nanotechnology [85].

As can be seen in Figure ***4.9***, amino acids mostly exist as zwitterions in aqueous solution. This unique hydration behavior that amino acids present, although it may not seem very significant for solutions of free amino acids in water, it is critical for protein functionality [86].

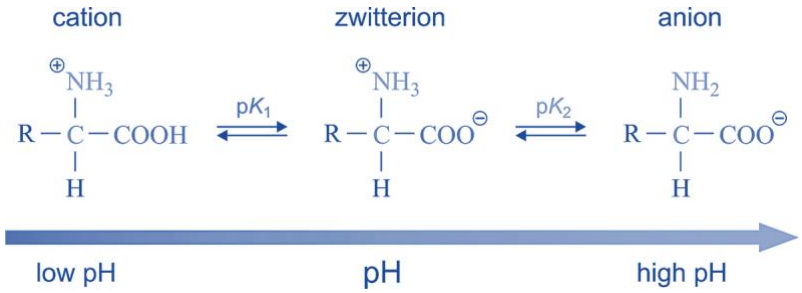


Figure 4.9. Schematic representation of the equilibrium between the zwitterion form of an amino acid and its cationic counterparts. Retrieved from [87]

In fact, by studying thermodynamic and viscometric properties of amino acids in aqueous solution and examining their interactions with the solvent, could in turn improve the understanding of biochemical processes such as protein hydration, denaturation and aggregation [86].

By increasing the concentration of amino acids in aqueous medium, and especially for those that exhibit low solubility or are partially hydrophobic, the formation of colloidal aggregates is favored. This phenomenon is driven by the inability of molecules to form energetically favorable interactions with water [88].

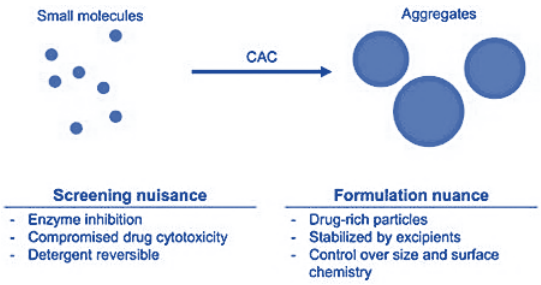


Figure 4.10. Formation of colloidal aggregated from small molecules. Retrieved from [89]

The effect that concentration has on protein aggregation, as can be seen in Figure **4.11**, is also true for other molecules that present surface activity, such as proteins. The aggregation or self-assembly of this molecules, as seen in the figure below, is usually executed at an interface in order to allow said compounds the necessary motion to aggregate between each other [90]. This adsorption of the protein or amino acid molecules to the interface can be related to the superficial tension of a solution through the Gibbs absorption equation (*4.5*), therefore the adsorption of a protein to the interface thus lowers the interfacial tension, while making its unfolding less likely as more protein is adsorbed in the interface.

It must be noted, however, that the application of the Gibbs absorption isotherm is dependent on degree of surface hydrophobicity, as well as the physicochemical properties of the protein or amino acid. If the hydrophobic character is very pronounced, it may lead to additional processes, such as: surface precipitation, the formation of surface sublayers or air-to-liquid surface phase transitions. Hence, the adsorption of the protein to the interface cannot be accurately described by the Gibbs surface equation [67]. Nonetheless, this is not the case for the present study since as previously discussed, l-tyrosine is a partially hydrophobic amino acid.

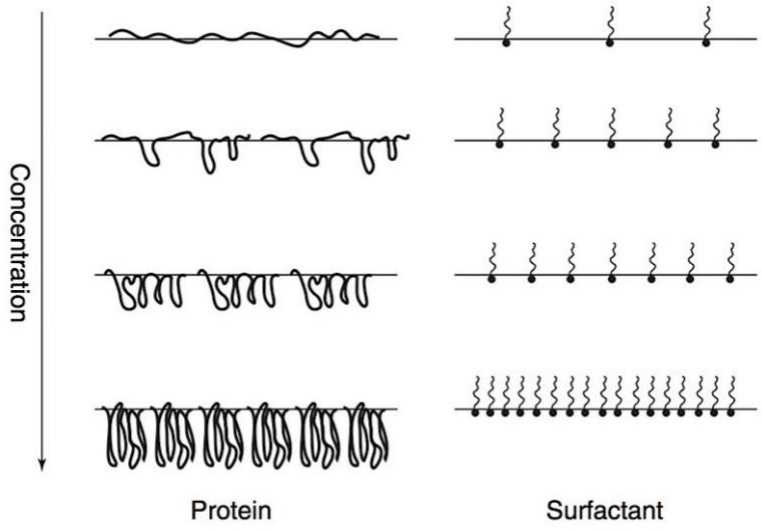


Figure 4.11. Molecular reorientation of surface-active molecules at the interfaces. (Left) Protein molecules unfold less extensively at interfaces as the protein concentration increases. (Right) Orientation of small-molecule surfactants is independent of surfactant concentration in the bulk phase Retrieved from [63]

Colloidal aggregaion is the prime source of false positive readout, which as previously mentioned, presents a large problem in drug development. The formation of said aggregates is largely driven by the chemical structure of the molecule and the medium conditions. It is therefore highly dependent on pH value, buffer composition and concentration, as well as the amount and identity of surfactant in the buffer [91].

Hence, as the formation of these colloidal systems is highly dependent on the chemical structure of the compound, it is also highly dependent on the synergistic effect of various intermolecular non-covalent interactions, such as: hydrogen-bonding, π–π stacking, hydrophobic, and van der Waals interactions. Therefore, the process of self-assembly is mainly driven by thermodynamics; however, another critical factor for structural modulation and integration are the kinetics involved[85].

4.3.1. Critical aggregation concentration

The critical aggregation concentration (CAC) is analogous to the critical micelle concentration for surfactants. Some compounds, similarly to micelles, when found above their critical aggregation concentration nucleation occurs and colloidal aggregates start forming [91], regardless of any definite shape or morphology. Likewise, when diluted below the CAC, said aggregates will spontaneously disassemble and return to a monomeric state [89]. This references the ability of these structures to be reversed, which can also be achieved through the use of a surfactant. Further explanation of the reversibility of said systems is explained in section 4.3.2.

Hence, the formation of aggregates must be either reversable or allow its components mobility once created. The forces which constitute the bonds formed between the molecules of the aggregate must therefore be comparable to the forces that tend to disrupt said aggregate [90].

The particular mechanisms and kinetics on how these aggregates form is still a matter of active research, as is the shape they tend to form and their specific morphology [91]. However, as it has been discussed in the previous section, since this phenomenon is grounded on the physical chemistry of the molecules responsible for aggregation, a prediction on the formation of the aggregation could be made through the study of the physicochemical characteristics.

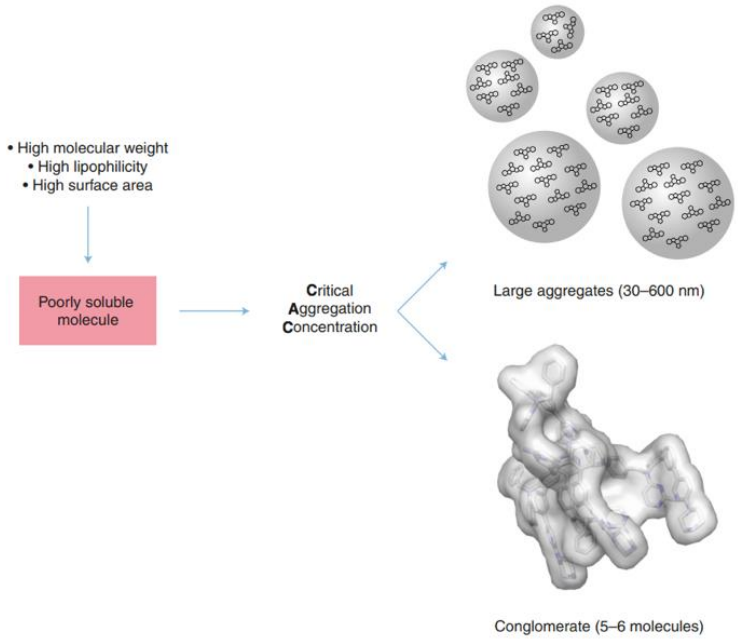


Figure 4.12. Poorly water-soluble molecules aggregate at concentrations above the critical aggregation concentration. Retrieved from [91]

4.3.2. Surfactant-amino acid interactions

Colloidal systems are heavily influenced by the addition of surfactant. In fact, one of their defining properties is their detergent reversibility, which is defined as the disruptive effect detergents have on colloidal systems, at concentrations greater than the aggregator concentration [89].

This could be explained through the effect surfactants have on the increase of solubilization, as seen in Figure **4.4**, therefore adding surfactant into a protein or amino acid solution would also increase their solubility in their aqueous solution. In fact, this phenomenon can be rationalized through the hydrophobic effect. When the hydrophobic portion of nonionic surfactants bind to hydrophobic patches of proteins or to a hydrophobic amino acid, thus only its hydrophilic groups are exposed to the solvent, it results in a "hydrophobicity reversal". This effect signifies that the protein-surfactant or amino acid-surfactant complex is more hydrophilic than any of them individually, which can in turn increase the solubility of the complex [67].

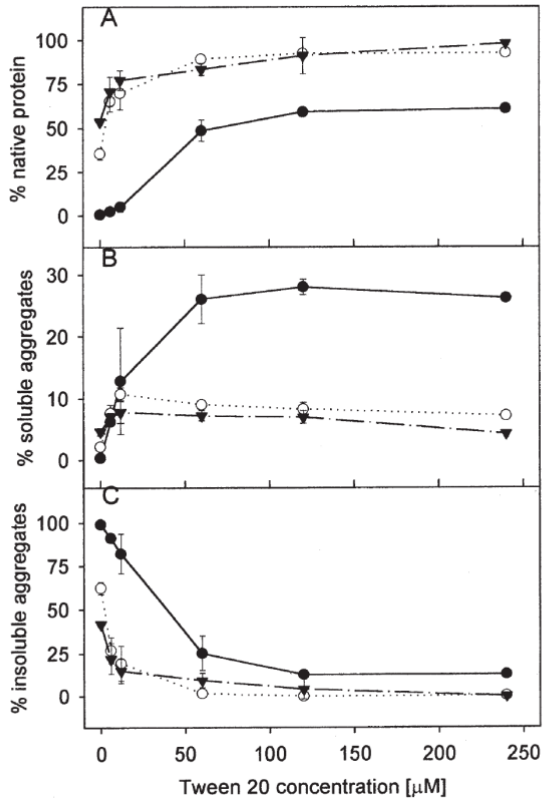


Figure 4.13. Recovery of native rFXIII (A) and formation of soluble (B) and insoluble aggregates (C) as a function of Tween 20. Retrieved from [67]

As can be seen in Figure ***4.13***, the addition of Tween in factor XIII did not completely block protein aggregation, but very effectively prevented the formation of insoluble aggregates. aggregates. The addition of surfactants, thus can reduce the propensity of the amino acids to form higher-order aggregates [67].These interactions can also influence the surfactant's micellization process [92], by potentially reducing the concentration at which CMC is achieved, given that the ability to form micelles would be promoted by the interaction of surfactants with the presence of hydrophobic compounds in solution. The more hydrophobic an amino acid is the larger will be the size of water clathrate surrounding its hydrophobic groups, thus increasing molecular associations between amino acids and ionic surfactants [86].

4.4. Surface tensiometry

Force and optical tensiometry are the most widely accepted methods to measure static surface and interfacial tension. In international standards, force tensiometry is the most common method to define surface tension.

4.4.1. Wilhelmy Plate method

The Wilhelmy Plate is an effective force tensiometry method applied to measure the static superficial and interfacial tension. This method yield values of the static surface tension, once the system has reached a stage of stable equilibrium [64].

It consists of a thin platinum plate as a probe placed vertically at the surface of the measured liquid. The force exerted on it is measured through a very sensitive electronic microbalance connected to it [62]. A schematic view of the apparatus used in this method can be seen in Figure ***4.14***.

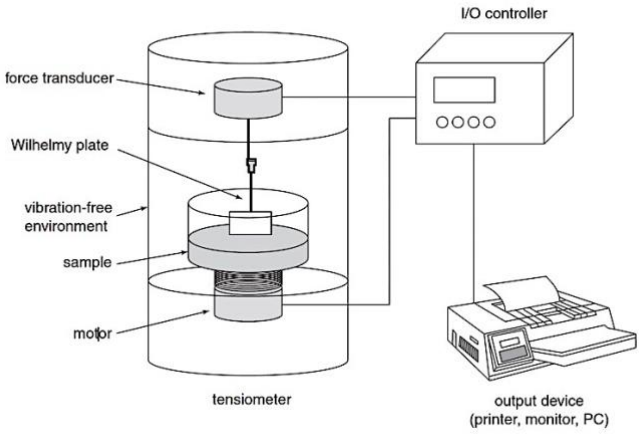


Figure 4.14. Schematic of the apparatus used in the Wilhelmy plate technique. Retrieved from [93]

Before immersing the plate in the liquid, only its weight is detected, and manually set to zero. It is only in the immersion and withdrawal of the plate from the liquid, in a determined depth of immersion, that the wetting and buoyancy forces are measured. Buoyancy forces are the upward forces exerted by the fluid opposing the weight of the platinum plate [62].

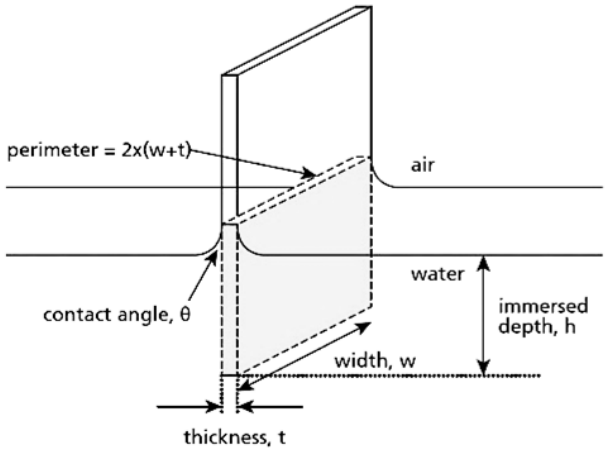


Figure 4.15. Schematic representation of the Wilhelmy plate method. Retrieved from [62]

The force exerted (F) on the Wilhelmy plate can therefore be expressed as:

$$F = \rho_p \cdot A \cdot l \cdot g + P \cdot \gamma \cdot cos\theta - \rho_l \cdot A \cdot h \cdot g \quad (4.8)$$

P being the perimeter of the plate, γ the static superficial tension, θ the contact angle, p the probe liquid density, A the cross-sectional area of the plate, h the immersion depth, l the total length of the plate and g the gravitational constant.

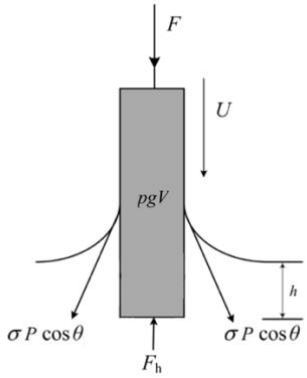


Figure 4.16. Schematic force diagram of the plate during immersion. Retrieved from [94]

The first term in equation (4.8) refers to the weight of the plate, the second is its wetting force and the third refers to the buoyance force – expressed as F_h in Figure ***4.16***. Superficial tension in the previous figure is referred to as σ. Both γ and σ are the most common symbols used in the literature as a reference to superficial tension. Since the weight of the plate is manually set to zero upon introduction on the tensiometer, this term in the force equation can be discarded.

The Wilhelmy method doesn't offer a direct measurement of the contact angle. However, it is very often considered to be negligible, due to the plate being platinum and therefore ensuring its complete wetting when immersed. This allows equation (4.8) to be rewritten to simply:

$$\gamma = \frac{F}{P} \quad (4.9)$$

The Wilhelmy method is also applicable to dynamic surface measurements by applying it in a flowing liquid, with the plate oriented in an inclined direction [95]. The experimental setup of which can be seen in the following figure:

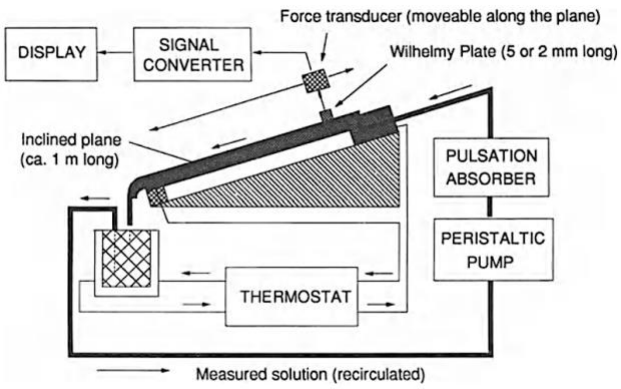


Figure 4.17. Inclined plane method of measuring dynamic surface tension. Retrieved from [95]

5. Experimental Development

As has been established, the surface tension of increased concentrations of l-tyrosine, as well as the surface tension of increased concentrations of Tween 20 with a constant concentration of l-tyrosine will be measured. Firstly, however, a few parameters need to be taken into consideration, such as: pH and solubility.

As mentioned in section 2.1., the solubility of tyrosine is dependent on the pH of the medium in which it is dissolved. Figure ***5.1*** displays the evolution of its solubility function of the pH.

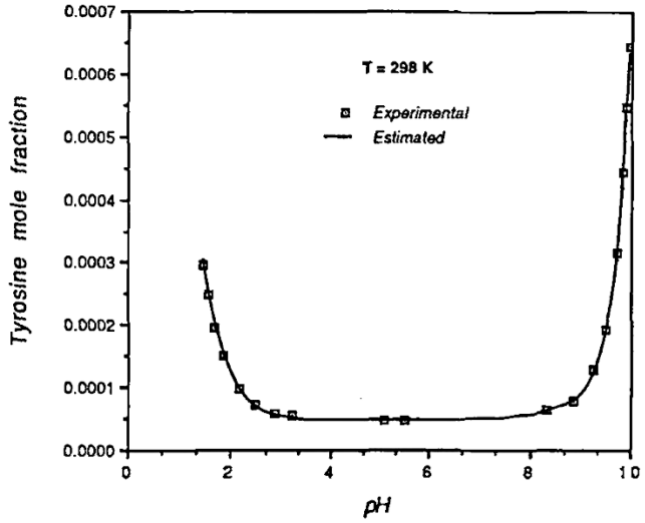


Figure 5.1. pH Effect on l-tyrosine solubility at 25ºC. Retrieved from [96]

In order for the chemical structure of l-tyrosine to have a net charge of zero, and thus be dissolved in aqueous solution as a zwitterion, the pH of the solution will need to be close to its isoelectric point. Therefore, establishing a range of pH between 5,50 to 6,10 approximately.

Since l-tyrosine is an amino acid, it would be advisable not to interfere unnecessarily with the pH values at which it is usually found in protein conformations. In other terms, trying to obtain values of higher solubility by submitting the amino acid to pH values at which protein denaturation would occur, would be undesirable and thus is discarded.

The solubility of tyrosine at said pH is of 0,45 g/L. Nevertheless, the highest concentration of tyrosine in solution will not exceed in any case approximately half of its solubility, so as to fully guarantee its complete dissolution in water.

5.1. Equipment and material

The surface tensiometer used in this study can be seen in Figure ***5.2***. As previously mentioned, this tensiometer used follows the Wilhelmy plate technique and therefore requires the use of a platinum plate, also seen in the figure above.

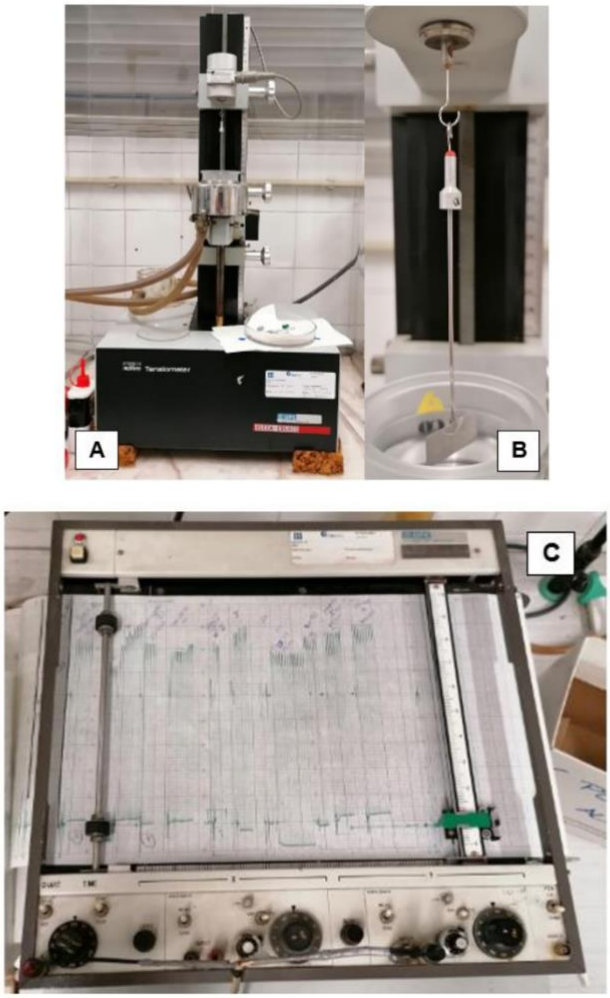


Figure 5.2. A: Tensiometer used for the measurement of superficial tension. B: Wilhelmy plate; C: Register. Retrieved from [97]

The chemical substances manipulated through this study are: L-tyrosine, which was purchased from *Merk KGaA*. Tween® 20 (USP-NF, BP, Ph. Eur.) and Glacial Acetic Acid (Reag. USP, Ph. Eur.), which were both purchased from *PanReac AppliChem*.

5.2. Methodology

In order to measure the surface tension, it is obviously required to first develop the solutions of which this parameter will be measured. The principles implemented when creating them, along with the basic outlines of the methodology can be seen in the following sections.

5.2.1. L-tyrosine solutions

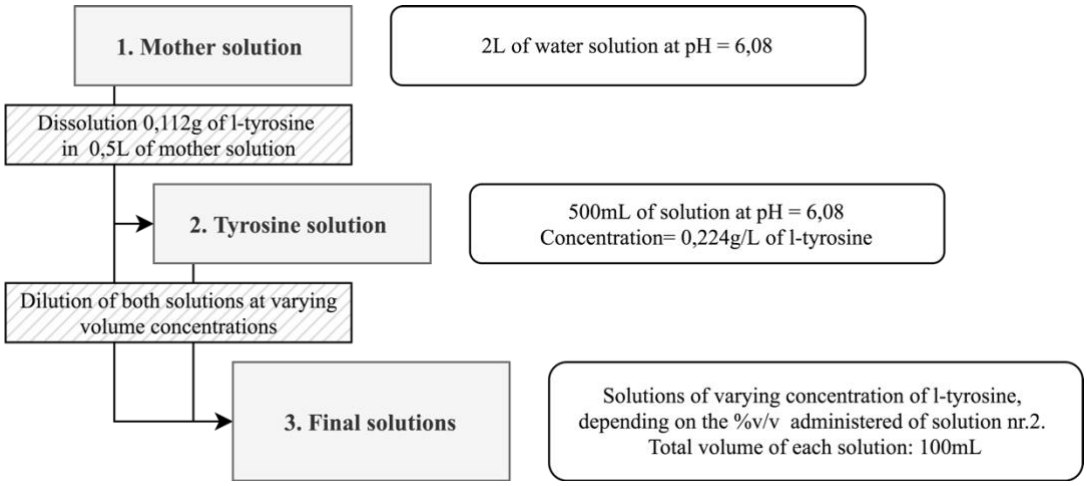


Figure 5.3. Schematic representation of the methodology employed in developing l-tyrosine solutions.

The basic outline of the process implemented when developing the solutions of varying concentrations of l-tyrosine can be seen in the figure above. The specific pH of the mother solution was obtained through the dilution of acetic acid in distilled water. Through 500mL of this first solution, a solution with a concentration of 0,224g/L of l-tyrosine was obtained by dissolving in it 0,112g of l-tyrosine. Finally, through the combination of varying volumes of each solution, as see in Table ***5.1***, the final solutions were obtained and thus available for the immediate measurement of their surface tension.

Table 5.1. Representation of the concentrations of tyrosine, along with the volumes of mother solution used.

No. of solution	*Volume of tyrosine solution [mL]*	*Concentration of L-tyrosine [g/L]*
1	5	0,011
2	10	0,022
3	20	0,045
4	30	0,067
5	40	0,090
6	50	0,112
7	60	0,134
8	70	0,157
9	80	0,179
10	100	0,224
11	N.A.(1)	0,302

(1)The last concentration was executed separately, a posteriori of the other solutions, in order to confirm the tendency of the measurements observed from the other solutions.

The choice to make the volume of the final solutions 100mL was made so that a second lecture of surface tension could be executed, in case the first reading of surface tension was somehow hindered. Since the tensiometer only required approximately 50mL of solution, its value was therefore duplicated. It must also be noted that the solutions were homogenized with the help of a magnetic stirrer.

5.2.2. L-tyrosine and Tween 20 solutions

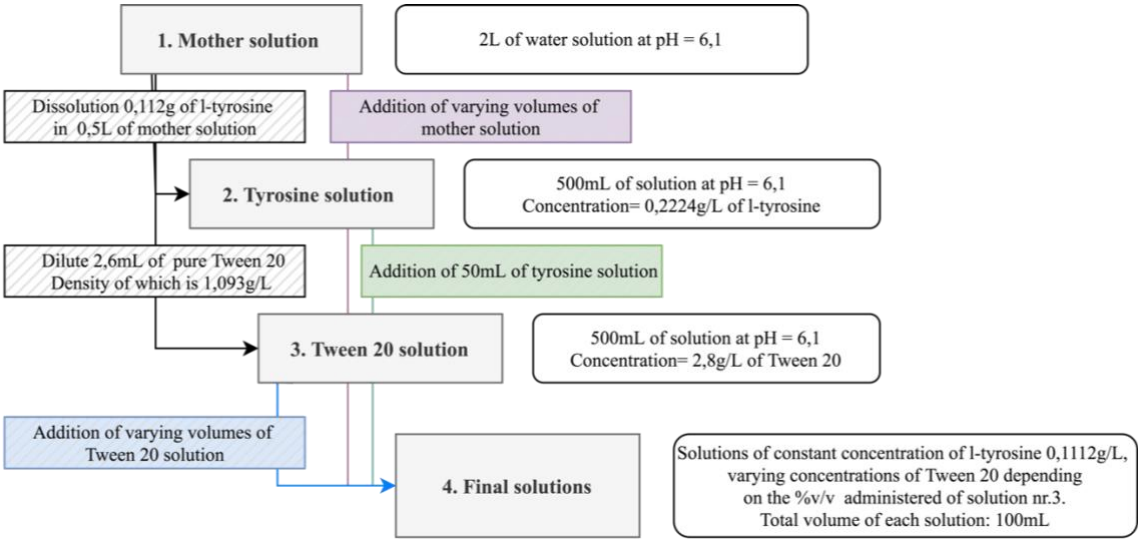


Figure 5.4. Schematic representation of the methodology employed in developing l-tyrosine and Tween 20 solutions.

As seen in the figure above, the addition of the surfactant in the solution causes a slight deviation in the process implemented elaborating the previous solutions. The only major difference in this methodology is that the concentration of l-tyrosine is constant, being Tween 20 the factor that varies in concentration. Any calculations that are not clearly stated in this document, such as the basic calculations involving solution concentrations, can be found in the annexed document.

It must be noted that the value at which tyrosine concentration remains constant was determined once the first study of surface tension was executed, along with the interpretation of the resulting data. Hence, the explanation behind this decision is justified in section 6.1. It must be

Table 5.2. Representation of the concentrations of tyrosine and tween, along with the volumes of mother solution used to make 100mL solutions, all of them containing 50mL of tyrosine at a concentration of 0,2224g/L.

No.	*Volume Mother Solution [mL]*	*Volume Tween Solution [mL]*	*Tween Concentration [g/L]*	*Tyrosine Concentration [g/L]*	*Molar relation Tyr:Tween*
1	49,5	0,5	0,014	0,1112	53,81
2	49,0	1,0	0,028	0,1112	27,37
3	48,0	2,0	0,056	0,1112	13,68
4	46,0	4,0	0,112	0,1112	6,84
5	42,0	8,0	0,224	0,1112	3,42
6	40,0	10,0	0,28	0,1112	2,74
7	36,0	14,0	0,392	0,1112	1,92
8	30,0	20,0	0,560	0,1112	1,35
9	10,0	40,0	1,120	0,1112	0,67
10	0,0	50,0	1,400	0,1112	0,54

5.2.3. Measurement of surface tension

As can be seen in the Figure ***5.5***, the measurement of surface tension followed a specific method., which had to be implemented in the reading of every solution, with exception of the third step.

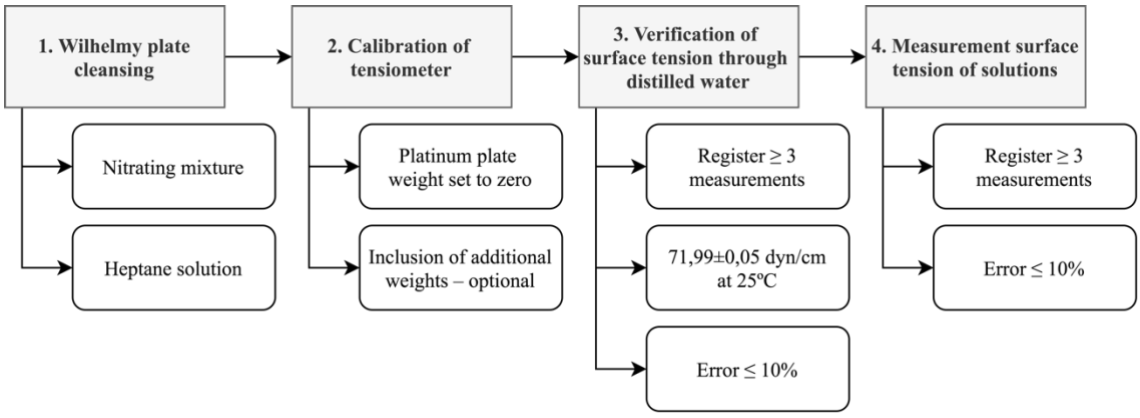


Figure 5.5. Methodology implemented when measuring the surface tension of solutions through the Wilhelmy plate technique.

Firstly, the Wilhelmy plate Wilhelmy platinum plate was cleaned to ensure that no contaminates would affect the measurements. A pair of tweezers was used to handle the plate since even oil from the hands could contaminate it. It was firstly rinsed with distilled water, and later dipped into a nitrating solution in order to eliminate any lingering residues. Lastly, the plate was immersed in a heptane solution, which was used to accelerate the drying of the plate, given its high volatility.

Once the plate was dry, again while using a pair of tweezers, the tensiometer, in particular its microbalance, was calibrated to obtain the most accurate results possible. In some cases, the inclusion of an additional weight was necessary in order to contain the scale of the surface tension measures within the graphing paper.

After calibration, a first solution containing only distilled water was tested for verification purposes. At least three measures of said superficial tension were taken, and the results were compared to the reported data in order to confirm their accuracy. For distilled water at room temperature (at 25 °C), the surface tension value should be 71,97 dynes/cm [98]. If there was a percentage difference greater than 10% between the recorded measurements and reported data, the measurements would be considered invalid and therefore would need to be repeated. In some cases, the calibration process would also need to be repeated. However, if the percentage difference was at or lower than 10%, it would enable to proceed to the next step: the preparation and measurements of the other solutions. Needless to say, this step was only executed in the beginning of the measurements., meaning it was only carried out when turning on the tensiometer at a given lab session.

Once all of the previous steps were completed, the measurement of surface tension of the solutions to be analyzed could proceed, following the procedure explained in section 4.4.1. The validation of the readings was executed using the same criteria applied for the surface tension of distilled water: three or more measurements were recorded and only if the error between them was lower or equal to 10% could said measurements be accepted, and thus considered valid.

6. Results and calculations

The results corresponding to each measurement in superficial tension are shown below.

6.1. Superficial tension of l-tyrosine

The specific readings obtained through the tensiometer can be observed in Table ***6.1*** and Table ***6.2***. All of them are expressed in [g].

Table 6.1. Direct measurements extracted from the tensiometer of solutions 1-5 of varying l-tyrosine concentrations.

Nº of Solution	**1**	**2**	**3**	**4**	**5**
1st Measurement	294,0	285,0	295,0	291,0	290,0
2nd Measurement	293,0	287,0	295,0	291,0	290,0
3rd Measurement	292,0	288,0	295,0	291,0	290,0
Average	293,0	286,7	295,0	291,0	290,0

Table 6.2. Direct measurements extracted from the tensiometer of solutions 6-11 of varying l-tyrosine concentrations.

Nº of Solution	**6**	**7**	**8**	**9**	**10**	**11**
1st Measurement	297,0	290,0	300,0	286,0	279,0	269,0
2nd Measurement	296,0	290,0	300,0	285,0	278,0	265,0
3rd Measurement	295,0	290,0	300,0	284,0	277,0	264,0
Average	296,0	290,0	300,0	285,0	278,0	266,0

The following figures show the resulting superficial tensions obtained in relation to the tyrosine concentration.

The conversion of these measurements into superficial tension was executed using the following equation:

$$\gamma = \frac{F}{P} = \frac{F[gf] \cdot 981{,}4}{4} [\frac{dynes}{cm}] \qquad (6.1)$$

The force measured is divided by four given it is the value of the perimeter of the plate which comes in contact with the solution. Since the units at which the force has been measured are gram-force, its conversion to dynes requires it be multiplied by 981,4. (1 gf is equal to 981,4 dynes).

Table 6.3. Results of the superficial tension measurements of increasing concentrations of L-Tyrosine.

Nº of Solution	Superficial Tension [dyn/cm]	Concentration [g/L]
1	71887,6	0,011
2	70333,7	0,022
3	72378,3	0,045
4	71396,9	0,067
5	71151,5	0,090
6	**72623,6**	**0,112**
7	71151,5	0,134
8	73605,0	0,157
9	69924,8	0,179
10	68207,3	0,224
11	65263,1	0,302

As mentioned in section 4.2.2, through the study of surface tension of polysorbate 20, the critical aggregation concentration of l-tyrosine can be obtained by equating the two linear behaviors exhibited by the amino acid in the figures below

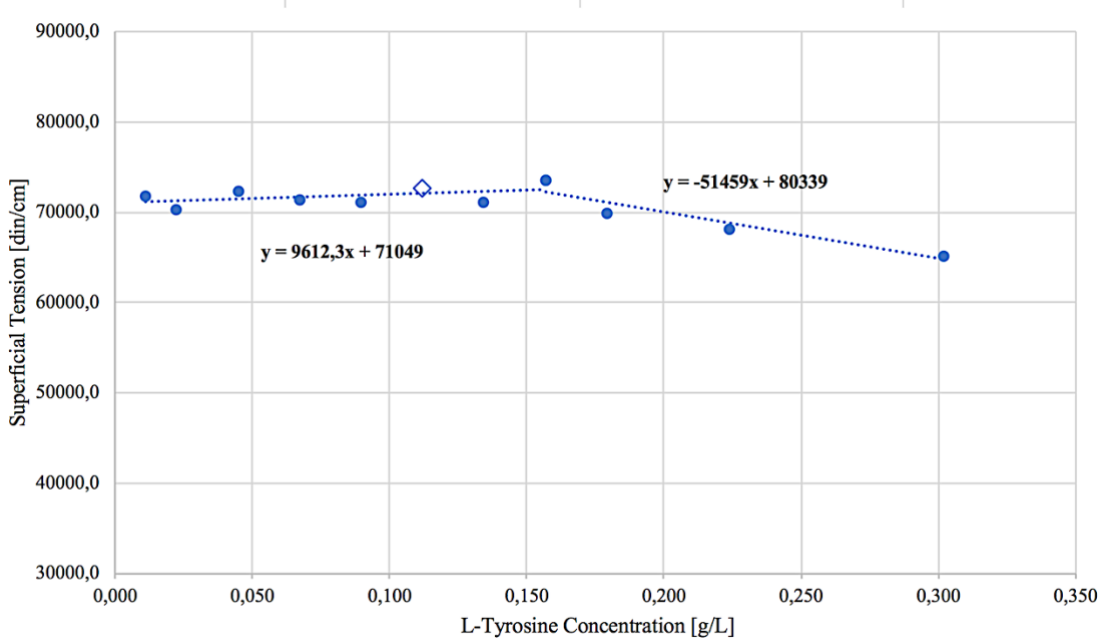


Figure 6.1. Superficial tension function of L-tyrosine concentration.

$$9612{,}3x + 71049 = -52459x + 80339$$

$$x = \frac{80339 - 71049}{9612{,}3 + 52459} = 0{,}152 g/L$$

Therefore, as can be through Figure ***6.1***, the surface tension of l-tyrosine in aqueous solution experiences a pronounced decrease in the concentration 0,152g/L. This could be attributed to the fact that from this concentration, l-tyrosine begins to assemble forming aggregates, thus decreasing the superficial tension of the solution, as predicted in section 4.3.

Before this phenomenon starts to happen, it is suggested that tyrosine is in isolated molecules, only after the critical aggregation concentration does it start to form aggregates.

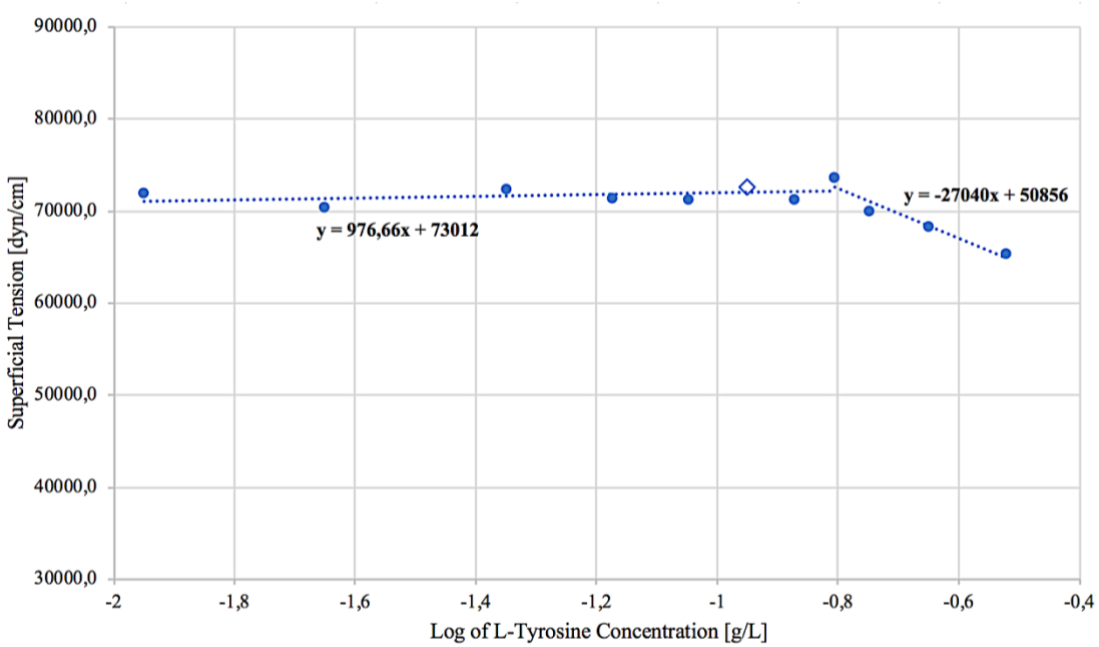


Figure 6.2. Superficial tension function of log(L-Tyrosine) concentration

Figure ***6.2*** shows the completely linearized behavior of the increase of concentration of l-tyrosine. Through this figure, the point at which tyrosine aggregation starts to occur can be seen clearly by the abrupt change of slope the graph exhibits past LogC=-0,8.

Having said this, the optimal concentration for l-tyrosine for later encapsulation would be at any value before the sudden decrease in superficial tension, given that passed this point l-tyrosine tends to agglomerate and its microencapsulation would result in larger particles. The chosen concentration is that of 0,112g/L, given that it is not at the verge of the change of slope.

6.2. Superficial tension of l-tyrosine in increasing surfactant concentrations

The specific readings obtained through the tensiometer can be observed in Table ***6.4*** and Table ***6.5***. All of them are expressed in [g].

Table 6.4. Direct measurements extracted from the tensiometer of solutions 1-5 of varying tween 20 concentrations.

Nº of Solution	**1**	**2**	**3**	**4**	**5**
1st Measurement	191,0	171,0	151,0	157,0	153,0
2nd Measurement	189,0	170,0	150,5	156,0	151,0
3rd Measurement	188,0	170,0	150,0	155,0	150,0
Average	189,3	170,3	150,5	156,0	151,3

Table 6.5. Direct measurements extracted from the tensiometer of solutions 6-11 of varying tween 20 concentrations.

Nº of Solution	**6**	**7**	**8**	**9**	**10**
1st Measurement	152,0	152,0	150,0	150,0	149,0
2nd Measurement	150,0	152,0	150,0	149,0	149,0
3rd Measurement	149,0	151,0	150,0	149,0	149,0
Average	150,3	151,7	150,0	149,3	149,0

Table 6.6. Results of the superficial tension measurements of increasing concentrations of Tween 20.

Nº of Solution	**Superficial Tension [dyn/cm]**	**Tween Concentration [g/L]**	**Tyrosine Concentration [g/L]**	**Molar relation Tyr:Tween**
0(1)	72623,6	N.A. (1)	0,112	N.A. (1)
1	46452,9	0,014	0,1112	53,81
2	41791,3	0,028	0,1112	27,37
3	36925,2	0,056	0,1112	13,68
4	38274,6	0,112	0,1112	6,84
5	37129,6	0,224	0,1112	3,42
6	36884,3	0,280	0,1112	2,74
7	37211,4	0,392	0,1112	1,92
8	36802,5	0,560	0,1112	1,35
9	36638,9	1,120	0,1112	0,67
10	36557,2	1,400	0,1112	0,54

(1) Data point from the surface tension study of l-tyrosine, added in order to contrast the superficial tension results.

The superficial tension for the solution for the mixture of tyrosine and the surfactant, shown in Figure ***6.3***, illustrates that even in the solution where there is the least concentration of surfactant, the superficial tension is significantly lower than for any of the tyrosine/water solutions (Figure ***6.1*****Error! Reference source not found.**).

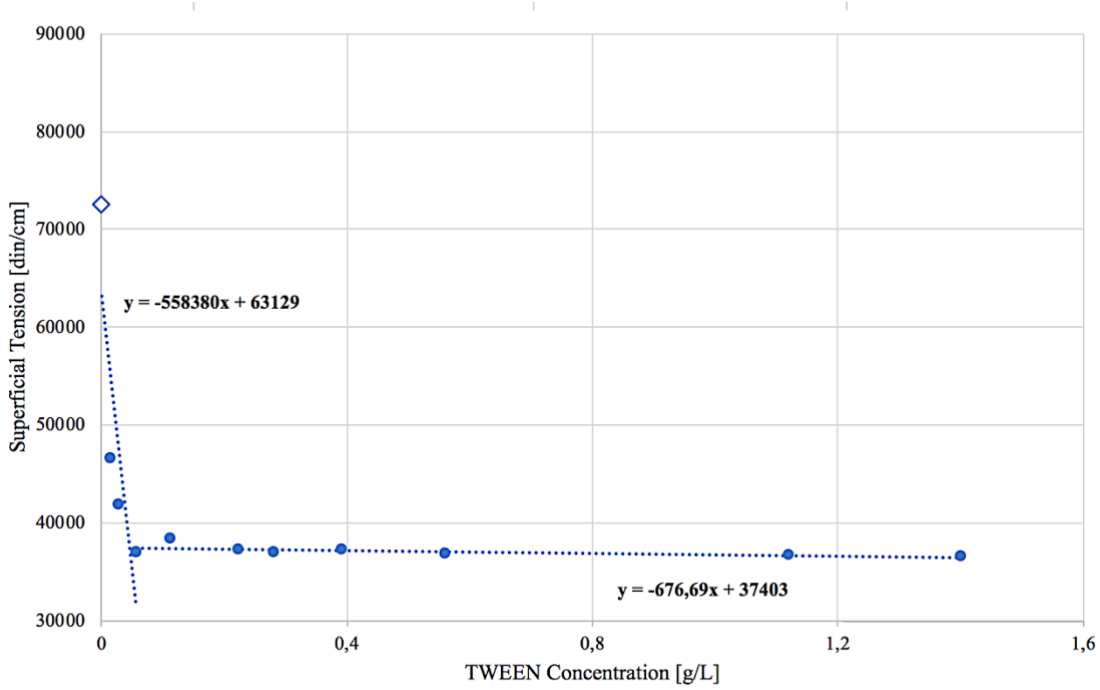


Figure 6.3. Superficial tension function of Tween 20 concentration, while maintaining a constant concentration of 0,112g/L of l-tyrosine.

Moreover, there is a further decrease in superficial tension with an even more prominent slope than in the previous graph, signifying that increasing concentrations of surfactant decrease superficial tension more rapidly than those of l-tyrosine alone.

Therefore, applying the same method as done for the previous values of surface tension, critical micelle concentration can be found by equating the two linear behaviors that the surfactant exhibits.

$$-558380x + 63129 = -676{,}69x + 37403$$

$$x = \frac{63129 - 37403}{558380 - 676{,}69} = 0{,}046 g/L$$

Therefore, the critical micelle concentration for the system of l-tyrosine and Tween 20 is at a concentration of the latter of 0,046g/L.

This value of critical micelle concentration is lower in comparison to the value of critical concentration of the surfactant on its own in aqueous solution, as seen in section 4.2.2, which was determined to be 0,06mg/m (0,06g/L).Therefore, as rationalized in section 4.3.2, the l-tyrosine and Tween 20 complex exhibits an increase of solubility compared to the compounds on their own, which is attributed to the previously discussed "hydrophobicity reversal".

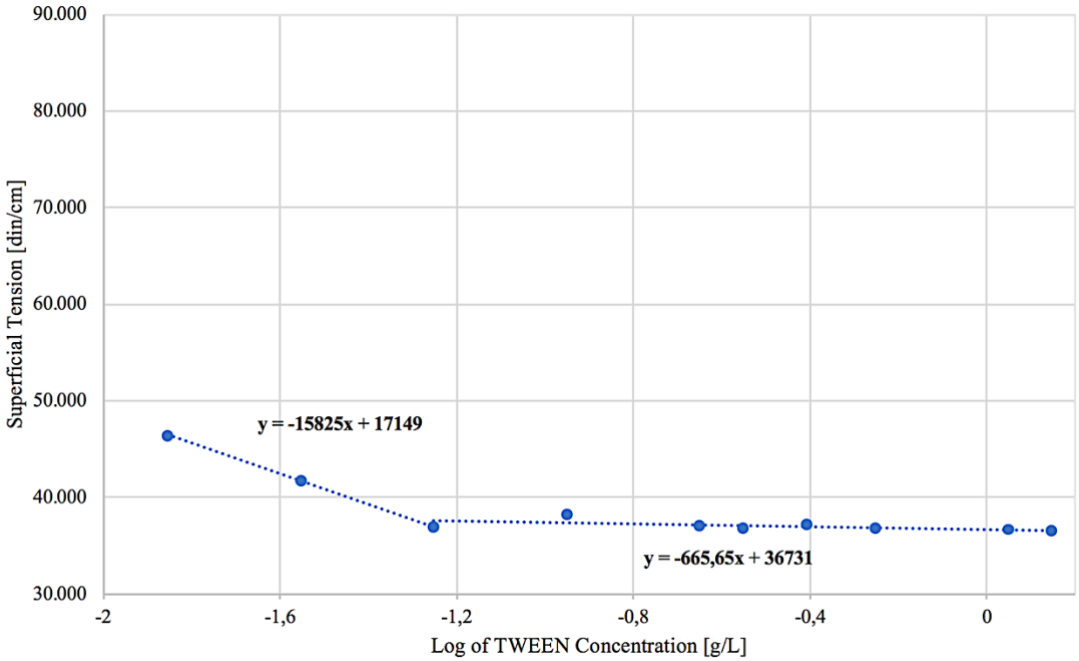


Figure 6.4. Superficial tension function of log of Tween 20 concentration, while maintaining a constant concentration of 0,112g/L of l-tyrosine.

Once again, Figure ***6.4***, shows the completely linearized behavior of the complex with the increase of concentration of Tween 20. Through this graph, and the equating of the two linear behaviors exhibited, the critical micelle concentration would be achieved at a concentration of 0,051g/L, which still confirms the effect the surfactant has on the amino acid and the implications the complex has on the CMC.

In conclusion, a value above the critical micellar concentration would be considered as a good option for the later microencapsulation of l-tyrosine. However, it is recommended the concentration of an excipient be limited to the minimal amount in a formulation [67], therefore amounts slightly above the critical micellar concentration determined by this study would be considered optimal.

The later step in this analysis would be to execute a particle size analysis on of the micelles created through this method, in order to determine the optimal concentration of surfactant to add so that the later microencapsulation would result in the minimal particle diameter.

6.3. Surface excess concentration and minimum area per molecule

As discussed in section 4.2, the Gibbs absorption equation can be applied to relate measurements of surface tension to the concentration of absorbed species at the air-liquid interface, only for binary systems. Therefore, an approximation of the excess surface evolution for the various readings of surface tension relating to the increasing concentration of l-tyrosine has been executed. The data for the surface excess concentrations of the solutions were estimated from Gibbs adsorption equation (4.5), and can be seen in the figure below:

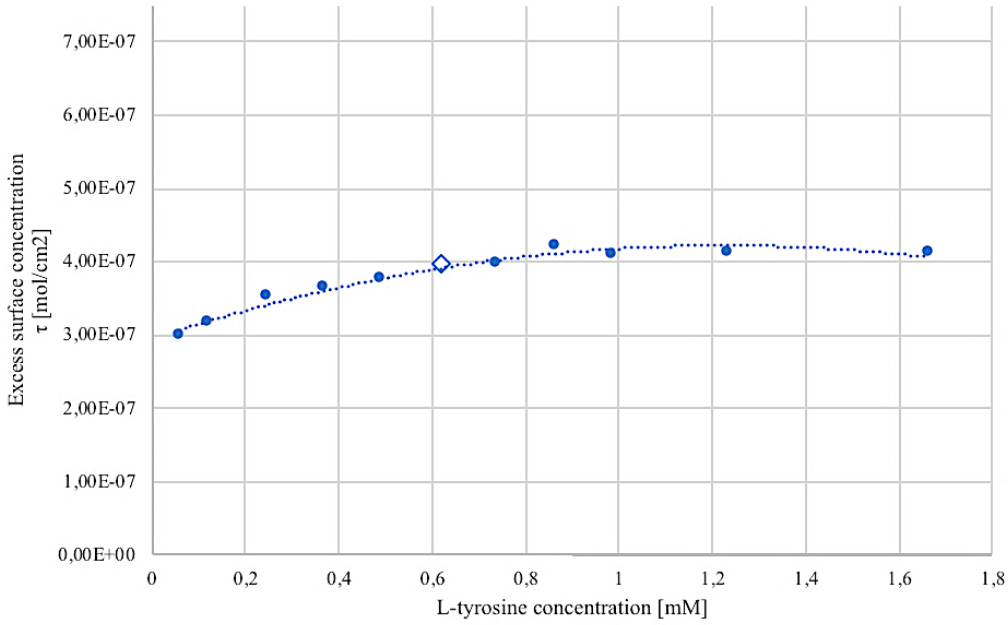


Figure 6.5. Influence of the tyrosine concentration on the excess surface concentration.

The calculations were made considering R = $8{,}31 \cdot 10^{7}$ ergs/mol /K and an ambient temperature of 25ºC (298K).

As can be seen in Figure ***6.5*****Error! Reference source not found.** , the values of the excess surface concentration, once having reached the critical aggregation concentration remain practically constant. The reason behind this phenomenon might be attributed once again to the formation of aggregates in solution, and once having reached the critical aggregation concentration at which the excess surface concentration is maximal ($4{,}21 \cdot 10^{-7}$ mol/cm^2), the surface becomes saturated.

The minimum area per molecule at the interface can be obtained through the following equation:

$$A_{min} = \frac{10^{16}}{N \cdot \Gamma_{max}} = \frac{10^{16}}{(6{,}022 \cdot 10^{16}) \cdot (4{,}21 \cdot 10^{-7})} = 3{,}9 \cdot 10^{-2}\ cm^2 per\ molecule$$

The Gibbs adsorption equation cannot be applied for the values of surface tension regarding the complex of l-tyrosine and Tween 20, due to it not being a binary system, hence it would exhibit a more complex behavior. However, the maximum surface excess concentration of Tween 20, and its area per molecule have been determined through the previously cited study [82], the values of which are $3{,}44 \cdot 10^{-10}$ mol/cm^2 and a value of $4{,}84 \cdot 10^{-14}$ cm^2 per molecule.

7. Planning and programming of the next stage

Drug delivery encompasses the approach, formulation, technology and system involved in transporting drugs into the body. It ensures that the drug is presented in the correct form in which it will be administered, along with the release from its dosage form, adsorption and transportation to the targeted site. The evaluation and development of all of these properties would be implemented during the next stage of this study, which constitutes the design of the drug prototype. The different phases that constitute this process are illustrated in Figure ***7.1***.

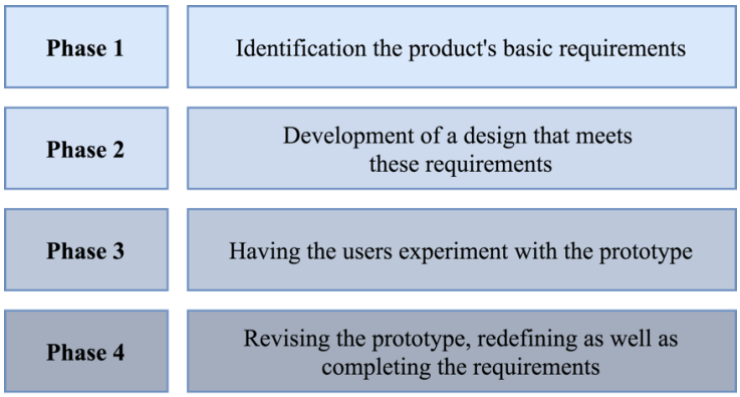


Figure 7.1. Phases of prototype development. Retrieved from[59]

The importance of the preformulation stage developed throughout this project can be seen in Figure ***7.2***, as the results obtained through this first stage have an influence in a great number of departments in charge of the prototype development process.

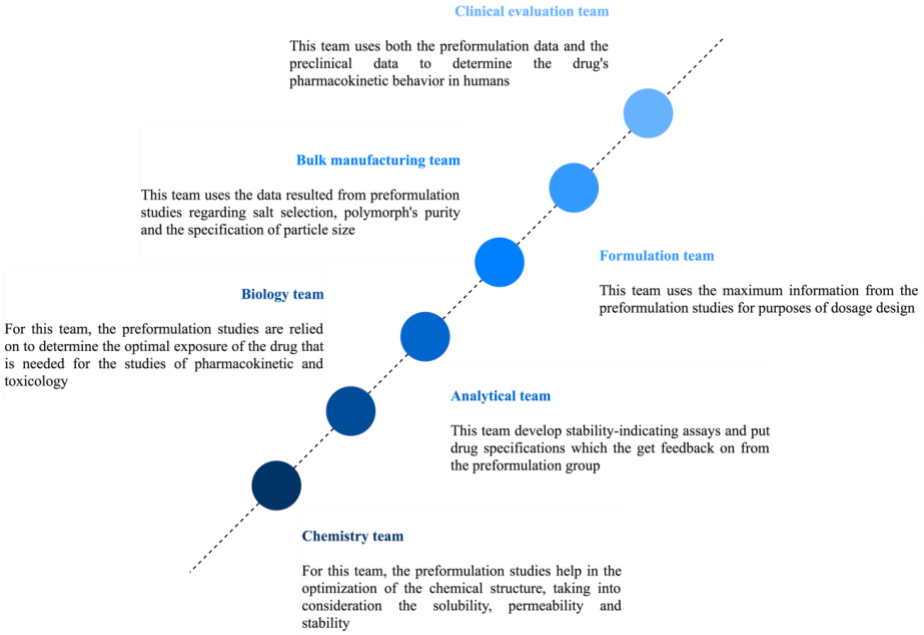


Figure 7.2. Different groups in a drug's discovery and development stages. Retrieved from [59]

7.1. Additional preformulation strategies

Another study on the surface tension of l-tyrosine could be conducted, only this time substituting the nonionic surfactant for an anionic, given anionic surfactants exhibit a higher solubilizing power than nonionic surfactants [65]. An additional one could be carried out using different concentrations of both, as often low concentrations of anionic surfactants are used alongside nonionic for stabilization purposes. Later, a particle size analysis of the resulting particles of all of the solutions could be executed, in order to determine the optimal size of the complex for microencapsulation.

7.2. Microencapsulation

An in-depth study into the physicochemical characteristics into the biopolymers of choice would be necessary. The recommended biopolymers would be gum Arabic and chitosan, the main physicochemical characteristics and applications of which can be seen in the table below.

Table 7.1. Examples of polysaccharides of various origin used in microencapsulation. Retrieved from [99]

Excipient	Physicochemical Properties	Applications and Benefits	Limitations	Ref.
Chitosan (deacylated chitin)	Soluble in weak acids Mucoadhesive reacts with negatively charged surfaces	Antifungal, antibacterial, reduces LDL (low-density lipoprotein), tissue regenerative, pulmonary delivery Ionotropic gelation, coacervation with anions, modified emulsification	pH dependence (insoluble above pH 6.5) Addition of electrolytes precipitates chitosan in solution, hygroscopic	[33–35]
Sodium hyaluronate	Anionic character, soluble in water, high viscosity at low concentration	In microspheres nasal, vaginal, ophthalmic delivery systems	Very hygroscopic, when heated, emits Na_2O	[36,37]
Starch (wheat, corn, potato, rice, tapioca)	Starch from different origins Differ in particle size and shape Soluble in hot water after a time of gelatinization	Spray drying, extrusion, molecular inclusion, coacervation with proteins, hydrocolloid-forming, release via swelling, diffusion, erosion	Hygroscopic	[38]
Guar gum	Water soluble, nonionic, galactomannan forms a thixotropic solution, stable at pH 4–10.5	Controlled-release, colon-targeted release, appetite suppressant, thermoreversible	Needs preservation, borate hinders swelling	[39]
Gum arabic/ Acacia gummi (Ph. Eur.)	Water soluble, clear solution of pH 4.5, protective colloid	Emulsifier, spray-dry encapsulation of flavor and of essential oils, 30% solution has a relatively low viscosity, Newtonian flow permeable coating dietary fiber	Variation dependent on source pH, ionic strength influences viscosity (max at ≈pH 6–7)	[48–50]

Chitosan has been extensively used in microencapsulation drug delivery system given that it is characterized by its high biocompatibility and also presents antibacterial qualities that might prove helpful to the formulation, in order to avoid infections[100][101].

There are a several studies that have employed these polysaccharides as their biopolymers of choice, the guidelines of which can be implemented in future projects as in order to have baseline of development [102][103][104].
The method employed in these studies is complex coacervation, although there are other methods of microencapsulation, as seen in the figure below. An assessment of the optimal technique could be executed in order to determine the best microencapsulation technique specifically for tyrosine.

Table 7.2. Different microencapsulation techniques. Retrieved from [105]

Chemical	Physical	
	Physico-chemical	Physico-mechanical
• Polymerization *In-situ* Emulsion, Suspension, Dispersion • Interfacial polycondensation	• Coacervation • Solvent evaporation, Solvent extraction • Layer-by-layer adsorption • Complex precipitation • Ionic gelation •Supercritical Fluid precipitation	• Spray-drying and congealing • Electrostatic encapsulation • Pan coating •Vacuum encapsulation • Extrusion • Air suspension • Multiorifice-centrifugal

The formation of microcapsules could be executed by combining different weights and relation of biopolymer concentration, so that with their later characterization the optimal polymer ratio could be determined. The characterization of the resulting microcapsules could be conducted through a series of analytical techniques once these were developed. Among the most commonly used techniques are zeta potential, thermogravimetric analysis, an assessment of their morphology through scanning electron microscopy and encapsulation yield Lastly, a study on the different mechanism of the compound's drug release would be required, as there are multiple ways in which the core materials can be delivered through the microcapsules. The stimulus that would cause the release of the active agent should be considered.

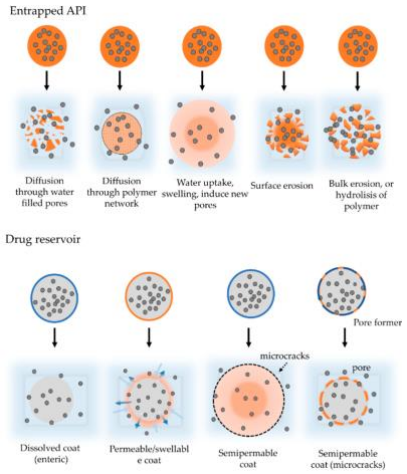


Figure 7.3. Most common release mechanisms of microencapsulated compounds. Retrieved from [99]

7.3. Gannt Diagram

The following Gannt diagram illustrates the planification of the next stages:

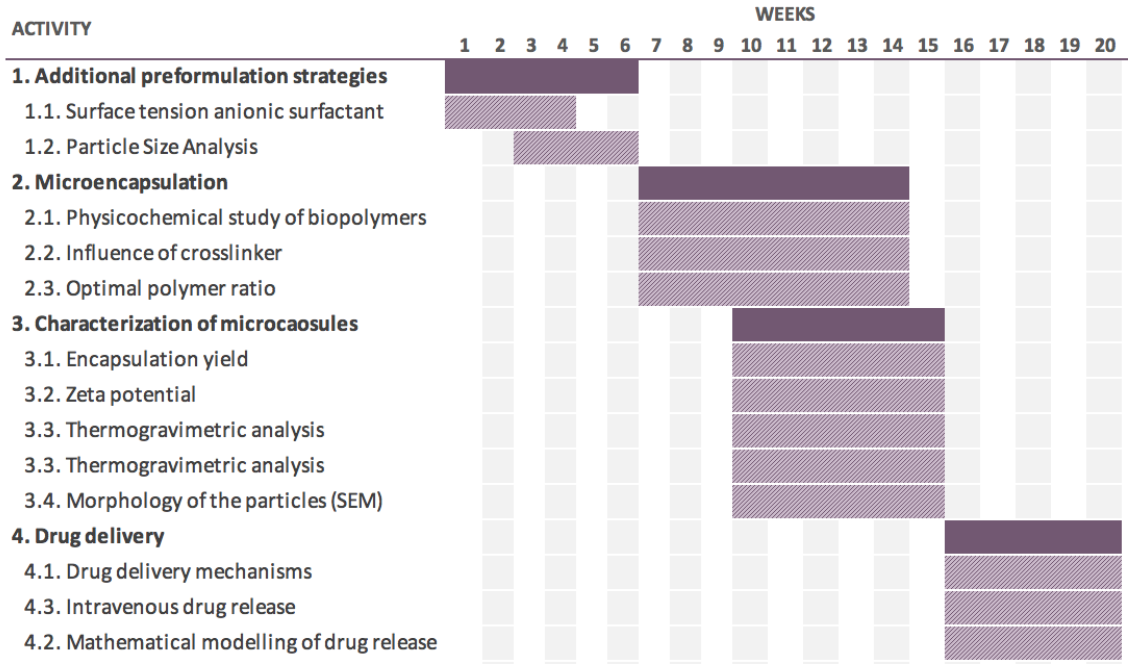


Figure 7.4. Gannt diagram

8. Budget summary

All of the reagents, materials and equipment used throughout this study has been supplied by the UPC-INTEXTER Laboratory. Therefore, the calculation of the budget is an approximation of the resulting cost of development of said study, in the case of not having had access to any of the supplied resources.

Table 8.1. Global budget

Concept	Cost
Reagents	429,53
Equipment and material	384,77€
Working hours	4.800,00€
Total	5.614,3€

It is worth noting that the cost regarding the hours of work has been calculated through multiplying the workload related to this project (24 ECTS = 600h) by 8€/h, as it is the recommended hourly compensation for undergraduate students, as advised by UPC [107]. A more detailed explanation and calculation of the cost of the reagents, materials and equipment can be found in the document regarding the whole budget.

9. Environmental implications

The development of this project involved the handling and utilization of several chemical substances, the environmental impact of which is not assessed given that their respective chemical residues were all disposed of following the protocol established by the general guidelines specified by the Spanish Ministry of Labor and Social Affairs along with the National Institute of Workplace Safety and Hygiene [108].

It must be specified that most of the quantities of the reagents used were so insignificant and diluted in distilled water that they qualified to be poured directly down the drain. The only exception were the quantities of pure acetic acid used (5-10mL) to make the first solutions, which were disposed of in the jugs pertaining to organic acid waste in order to minimize its environmental impact.

In addition, the use of the articles used such as gloves, Pasteur pipettes and pipette tips, among other items has strictly been limited to only when absolutely essential, and the collection of the waste of such objects was always disposed of at the waste container identified as "contaminated plastic". The specific containers referenced were later managed and ultimately disposed of by an external waste management company.

The carbon footprint that the development of this project has is very minimal. The only parameters that can be taken into account and quantified are the indirect ones, pertaining to the electric consumption of equipment such as the tensiometer, magnetic stirrer and even the computer. The direct parameters, such as vehicle transport, are not applicable since they cannot be verified and directly quantified.

Therefore, the table below offers a brief approximation of the impact of the electric consumption that the use of the equipment has, using as a guideline the documentation generated by the Spanish Office for Climate Change [109].

Table 9.1. Assessment of the environmental impact of the study.

Equipment	**Hours of use [h]**	**Consumption [kW]**	**Impact factor [kg of CO_2/kWh]** [110]	**Carbon footprint [kg of CO_2]**
Magnetic stirrer	10	0,090[111]	0,37	0,33
Microbalance	1	0,003 [112]	0,37	0,00
Tensiometer	10	0,040 [113]	0,37	0,15
Computer	300	0,065 [114][1]	0,37	7,22
		Total		**7,70**

[1]At maximum capacity

It must be noted that it is an estimate of the time of usage of the resources employed throughout this study, and therefore not an exact representation.

10. Conclusions

The preformulation stages within the development of a drug delivery system are crucial to the process of drug design. It is through this stage that the physicochemical properties of a compound are investigated and therefore characterized, so that in the later stage of prototype development through various methods of chemical manipulation they can be optimized to fit the specific needs of the target in question.

After an in-depth analysis of the challenges surrounding the free delivery of the amino acid as a therapeutic agent, involving not only l-tyrosine's physicochemical properties and self-assembly, but also considering the characteristics of the blood-brain barrier and the agents regulating its transport, microencapsulating the compound was concluded to be the most effective option as a drug delivery system.

In order to develop said system, as previously mentioned, a preformulation study of all of the components involved was essential so as to determine the optimal parameters for its microencapsulation specifically. Since the l-tyrosine is one of the 20 standard proteogenic amino acids, its physicochemical properties had already been extensively characterized and analyzed in previous studies, hence an in-depth review was conducted of the pertaining literature to enable the interpretation of the study on its superficial tension. The same methodology was implemented for the study of the surfactant, along with the study of its effect on intermolecular forces and interactions with amino acids.

Finally, a successful interpretation was derived from the study of the surface tension of the amino acid l-tyrosine, along with the addition of a surfactant. Hence, it was determined that the optimal concentration at which to microencapsulate the amino acid in order to avoid its aggregation was at 0,112g/L. Through the study of the complex comprised of l-tyrosine and the surfactant, the optimal range of concentration of latter was able to be determined.

Through the realization of this study it would now be possible to execute the next stage of drug development: the actual microencapsulation process and assessment of its drug delivery. However, it must be considered that although the direct administration of tyrosine to the brain is presented as the optimal solution for a number of diseases, further research on the molecular, pharmacokinetic and pharmacodynamic properties of amino acid transport across the blood-brain barrier would have to be conducted. This would enable the assessment of the optimal parameters of its drug design, as well as obtain the most efficient results after drug administration.

11. Bibliography

[1] "Tyrosine | Infoplease." [Online]. Available: https://www.infoplease.com/encyclopedia/science/biochemistry/concepts/tyrosine.

[2] "Tyrosine - Wikipedia." [Online]. Available: https://en.wikipedia.org/wiki/Tyrosine#Biosynthesis.

[3] C. Pommié, S. Levadoux, R. Sabatier, G. Lefranc, and M. P. Lefranc, "IMGT standardized criteria for statistical analysis of immunoglobulin V-Region amino acid properties," *J. Mol. Recognit.*, vol. 17, no. 1, pp. 17–32, Jan. 2004.

[4] M. R. Barnes, *Bioinformatics for Geneticists: A Bioinformatics Primer for the Analysis of Genetic Data: Second Edition*. John Wiley and Sons, 2007.

[5] "HSDB : 2003 - PubChem." [Online]. Available: https://pubchem.ncbi.nlm.nih.gov/source/hsdb/2003.

[6] "Amino Acids - Tyrosine." [Online]. Available: http://www.biology.arizona.edu/biochemistry/problem_sets/aa/Tyrosine.html.

[7] "Can tyrosine form dianionic form since it has acidic carboxyl group and also hydroxyl group at the phenyl group? | Socratic." [Online]. Available: https://socratic.org/questions/can-tyrosine-form-dianionic-form-since-it-has-acidic-carboxyl-group-and-also-hyd.

[8] "Tyrosine | C9H11NO3 - PubChem." [Online]. Available: https://pubchem.ncbi.nlm.nih.gov/compound/Tyrosine.

[9] "biochemistry - How do I calculate the isoelectric point of amino acids with more than two pKa's? - Chemistry Stack Exchange." [Online]. Available: https://chemistry.stackexchange.com/questions/55683/how-do-i-calculate-the-isoelectric-point-of-amino-acids-with-more-than-two-pkas.

[10] K. Molčanov, B. Kojic´-, and K.-P. Prodic´, "Towards understanding p-stacking interactions between non-aromatic rings."

[11] K. L. Copeland, S. J. Pellock, J. R. Cox, M. L. Cafiero, and G. S. Tschumper, "Examination of tyrosine/adenine stacking interactions in protein complexes," *J. Phys. Chem. B*, vol. 117, no. 45, pp. 14001–14008, Nov. 2013.

[12] R. Chelli, F. L. Gervasio, P. Procacci, and V. Schettino, "Stacking and T-shape competition in aromatic-aromatic amino acid interactions," *J. Am. Chem. Soc.*, vol. 124, no. 21, pp. 6133–6143, May 2002.

[13] C. E. Humphrey *et al.*, "Optimized synthesis of L-m-tyrosine suitable for chemical scale-up," *Org. Process Res. Dev.*, vol. 11, no. 6, pp. 1069–1075, Nov. 2007.

[14] G. Litwack, "Metabolism of Amino Acids," in *Human Biochemistry*, Elsevier, 2018, pp. 359–394.

[15] "NCI Thesaurus." [Online]. Available: https://ncit.nci.nih.gov/ncitbrowser/pages/concept_details.jsf?dictionary=NCI_Thesaurus&version=20.02d&code=C915&ns=NCI_Thesaurus&type=properties&key=null&b=1&n=0&vse=null.

[16] S. Paravati and S. J. Warrington, *Physiology, Catecholamines*. StatPearls Publishing, 2019.

[17] M. E. Gnegy, "Catecholamines," in *Basic Neurochemistry*, Elsevier, 2012, pp. 283–299.

[18] R. Kvetnansky, E. L. Sabban, and M. Palkovits, "Catecholaminergic systems in stress: Structural and molecular genetic approaches," *Physiological Reviews*, vol. 89, no. 2. American Physiological Society, pp. 535–606, Apr-2009.

[19] "Tyrosine." [Online]. Available: http://www.russelllab.org/aas/Tyr.html.

[20] N. A. Campbell and J. B. Reece, *Biology*. Benjamin Cummings, 2002.

[21] R. J. Wurtman, F. Hefti, and E. Melamed, "Precursor control of neurotransmitter synthesis.," *Pharmacol. Rev.*, vol. 32, no. 4, pp. 315–335, Dec. 1980.

[22] D. D. RASMUSSEN, B. ISHIZUKA, M. E. QUIGLEY, and S. S. C. YEN, "Effects of Tyrosine and Tryptophan Ingestion on Plasma Catecholamine and 3,4-Dihydroxyphenylacetic Acid Concentrations*," *J. Clin. Endocrinol. Metab.*, vol. 57, no.

4, pp. 760–763, Oct. 1983.

[23] L. S. Colzato, L. Steenbergen, R. Sellaro, A. K. Stock, L. Arning, and C. Beste, "Effects of L-Tyrosine on working memory and inhibitory control are determined by DRD2 genotypes: A randomized controlled trial," *Cortex*, vol. 82, pp. 217–224, Sep. 2016.

[24] C. R. Mahoney, J. Castellani, F. M. Kramer, A. Young, and H. R. Lieberman, "Tyrosine supplementation mitigates working memory decrements during cold exposure," *Physiol. Behav.*, vol. 92, no. 4, pp. 575–582, Nov. 2007.

[25] B. J. Jongkees, B. Hommel, S. Kühn, and L. S. Colzato, "Effect of tyrosine supplementation on clinical and healthy populations under stress or cognitive demands-A review," *Journal of Psychiatric Research*, vol. 70. Elsevier Ltd, pp. 50–57, 01-Nov-2015.

[26] A. Alabsi, A. C. Khoudary, and W. Abdelwahed, "The antidepressant effect of L-tyrosine-loaded nanoparticles: Behavioral aspects," *Ann. Neurosci.*, vol. 23, no. 2, pp. 89–99, Jul. 2016.

[27] A. J. Gelenberg and C. J. Gibson, "Tyrosine for the Treatment of Depression," *Nutr. Health*, vol. 3, no. 3, pp. 163–173, 1984.

[28] J. J. Schildkraut, "The catecholamine hypothesis of affective disorders: a review of supporting evidence.," *The American journal of psychiatry*, vol. 122, no. 5. American Psychiatric Publishing, pp. 509–522, 01-Apr-1965.

[29] B. S. Glaeser, E. Melamed, J. H. Growdon, and R. J. Wurtman, "Elevation of plasma tyrosine after a single oral dose of L-tyrosine," *Life Sci.*, vol. 25, no. 3, pp. 265–271, Jul. 1979.

[30] C. Tomaro-Duchesneau, S. Saha, M. Malhotra, I. Kahouli, and S. Prakash, "Microencapsulation for the Therapeutic Delivery of Drugs, Live Mammalian and Bacterial Cells, and Other Biopharmaceutics: Current Status and Future Directions.," *J. Pharm.*, vol. 2013, p. 103527, 2013.

[31] P. Patel, "Preformulation Studies: An Integral Part of Formulation Design," in *Pharmaceutical Formulation Design - Recent Practices*, IntechOpen, 2020.

[32] R. A. Razzak, G. J. Florence, and F. J. Gunn-Moore, "Approaches to cns drug delivery with a focus on transporter-mediated transcytosis," *International Journal of Molecular Sciences*, vol. 20, no. 12. MDPI AG, 02-Jun-2019.

[33] "Tyrosine - DrugBank." [Online]. Available: https://www.drugbank.ca/drugs/DB00135.

[34] A. Macdonald, R. H. Singh, J. C. Rocha, and F. J. Van Spronsen, "Optimising amino acid absorption: essential to improve nitrogen balance and metabolic control in phenylketonuria," *Nutrition Research Reviews*, vol. 32, no. 1. Cambridge University Press, pp. 70–78, 01-Jun-2019.

[35] S. Chevalier, R. Gougeon, S. H. Kreisman, C. Cassis, and J. A. Morais, "The hyperinsulinemic amino acid clamp increases whole-body protein synthesis in young subjects," *Metabolism.*, vol. 53, no. 3, pp. 388–396, Mar. 2004.

[36] G. Loch-Neckel and J. Koepp, "[The blood-brain barrier and drug delivery in the central nervous system].," *Rev. Neurol.*, vol. 51, no. 3, pp. 165–74, Aug. 2010.

[37] A. Khaskel, P. Barman, and U. Jana, "L-Tyrosine loaded nanoparticles: An efficient catalyst for the synthesis of dicoumarols and Hantzsch 1,4-dihydropyridines," *RSC Adv.*, vol. 5, no. 18, pp. 13366–13373, Jan. 2015.

[38] B. Yu and T. M. S. Chang, "IN VITRO ENZYME KINETICS OF MICROENCAPSULATED TYROSINASE," *Artif. Cells, Blood Substitutes, Biotechnol.*, vol. 30, no. 5–6, pp. 533–546, Jan. 2002.

[39] R. Aluri and M. Jayakannan, "Development of l-Tyrosine-Based Enzyme-Responsive Amphiphilic Poly(ester-urethane) Nanocarriers for Multiple Drug Delivery to Cancer Cells," *Biomacromolecules*, vol. 18, no. 1, pp. 189–200, Jan. 2017.

[40] A. Contino *et al.*, "Synthesis and characterization of new tyrosine capped anisotropic silver nanoparticles and their exploitation for the selective determination of iodide ions," *Colloids Surfaces A Physicochem. Eng. Asp.*, vol. 529, pp. 128–136, Sep. 2017.

[41] Z. Zhang, X. Zhao, X. Jv, H. Lu, and L. Zhu, "A Simplified Method for Synthesis of l -Tyrosine Modified Magnetite Nanoparticles and Its Application for the Removal of

Organic Dyes," *J. Chem. Eng. Data*, vol. 62, no. 12, pp. 4279–4287, Dec. 2017.
[42] K. Dubey *et al.*, "Tyrosine- and tryptophan-coated gold nanoparticles inhibit amyloid aggregation of insulin," *Amino Acids*, vol. 47, no. 12, pp. 2551–2560, Jul. 2015.
[43] M. A. Gatoo, S. Naseem, M. Y. Arfat, A. M. Dar, K. Qasim, and S. Zubair, "Physicochemical properties of nanomaterials: implication in associated toxic manifestations.," *Biomed Res. Int.*, vol. 2014, p. 498420, 2014.
[44] L. Bjerkenstedt, L. Farde, L. Terenius, G. Edman, N. Venizelos, and F. A. Wiesel, "Support for limited brain availability of tyrosine in patients with schizophrenia," *International Journal of Neuropsychopharmacology*, vol. 9, no. 2. pp. 247–255, Apr-2006.
[45] L. F. Rich, M. E. Beard, and R. P. Burns, "Excess dietary tyrosine and corneal lesions," *Exp. Eye Res.*, vol. 17, no. 1, pp. 87–97, 1973.
[46] D. E. Raymond E Kirk, Donald F Othmer, Martin Grayson, *Encyclopedia of Chemical Technology, 5th Edition*, vol. 22. 2007.
[47] S. K. Bardal, J. E. Waechter, and D. S. Martin, "Chapter 2 - Pharmacokinetics," *Appl. Pharmacol.*, pp. 17–34, Jan. 2011.
[48] L. L. Brunton, R. Hilal-Dandan, and B. C. Knollmann, *Goodman & Gilman's the pharmacological basis of therapeutics*. .
[49] S. Rehman, B. Nabi, S. Ahmad, S. Baboota, and J. Ali, "Polysaccharide-based amorphous solid dispersions (ASDs) for improving solubility and bioavailability of drugs," in *Polysaccharide Carriers for Drug Delivery*, Elsevier, 2019, pp. 271–317.
[50] M. N. Singh, K. S. Y. Hemant, M. Ram, and H. G. Shivakumar, "Microencapsulation: A promising technique for controlled drug delivery," *Research in Pharmaceutical Sciences*, vol. 5, no. 2. pp. 65–77, 2010.
[51] N. K. Pandit and R. P. Soltis, *Introduction to the pharmaceutical sciences : an integrated approach*. 2012.
[52] "Protein Catabolism - Lipid and Amino Acid Metabolism - MCAT Biochemistry Review." [Online]. Available: https://schoolbag.info/chemistry/mcat_biochemistry/74.html.
[53] E. H. Kerns and L. Di, "Lipophilicity," in *Drug-like Properties: Concepts, Structure Design and Methods*, Elsevier, 2008, pp. 43–47.
[54] *IUPAC Compendium of Chemical Terminology*. IUPAC, 2009.
[55] "(17) How do solubility, permeability and bioavailability of drugs relate to each other?" [Online]. Available: https://www.researchgate.net/post/How_do_solubility_permeability_and_bioavailability_of_drugs_relate_to_each_other.
[56] H. Pajouhesh and G. R. Lenz, "Medicinal chemical properties of successful central nervous system drugs," *NeuroRx*, vol. 2, no. 4, pp. 541–553, 2005.
[57] A. Dahan and J. M. Miller, "The solubility-permeability interplay and its implications in formulation design and development for poorly soluble drugs," *AAPS J.*, vol. 14, no. 2, pp. 244–251, Jun. 2012.
[58] P. N. O. Kasimbeg, F. C. Cheong, D. B. Ruffner, J. M. Blusewicz, and L. A. Philips, "Holographic Characterization of Protein Aggregates in the Presence of Silicone Oil and Surfactants," *J. Pharm. Sci.*, vol. 108, no. 1, pp. 155–161, Jan. 2019.
[59] R. K. Tekade, *Dosage Form Design Considerations: Volume I*. Elsevier, 2018.
[60] M. Gibson, *Pharmaceutical Preformulation and Formulation: A Practical Guide from Candidate Drug Selection to Commercial Dosage Form*, vol. 9, no. 6. 2005.
[61] "Effects of Drug Carrier Geometry on Drug Delivery - Inquiries Journal." [Online]. Available: http://www.inquiriesjournal.com/articles/597/effects-of-drug-carrier-geometry-on-drug-delivery.
[62] S. Laurén and B. Scientific, "Surface and interfacial tension-What is it and how to measure it?"
[63] J. Weiss, "Key Concepts of Interfacial Properties in Food Chemistry," *Curr. Protoc. Food Anal. Chem.*, vol. 5, no. 1, p. D3.5.1-D3.5.22, Aug. 2002.
[64] E. D. (Evgeniĭ D. Shchukin, *Colloid and surface chemistry*. Elsevier, 2001.

[65] M. J. Rosen and J. T. Kunjappu, *Surfactants and Interfacial Phenomena: Fourth Edition*. Hoboken, NJ, USA: John Wiley and Sons, 2012.

[66] "Dynamic surface tension | KRÜSS." [Online]. Available: https://www.kruss-scientific.com/services/education-theory/glossary/dynamic-surface-tension/.

[67] J. F. Carpenter, B. S. Chang, W. Garzon-Rodriguez, and T. W. Randolph, "Rational Design of Stable Protein Formulations," *Pharm. Biotechnol.*, vol. 13, pp. 109–133, 2002.

[68] M. de Almeida Bezerra, M. A. Zezzi Arruda, and S. L. Costa Ferreira, "Cloud point extraction as a procedure of separation and pre-concentration for metal determination using spectroanalytical techniques: A review," *Applied Spectroscopy Reviews*, vol. 40, no. 4. pp. 269–299, Oct-2005.

[69] B. A. Kerwin, "Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: Structure and degradation pathways," *Journal of Pharmaceutical Sciences*, vol. 97, no. 8. John Wiley and Sons Inc., pp. 2924–2935, 2008.

[70] J. D. Shosa and L. L. Schramm, "Surfactants: Fundamentals and Applications in the Petroleum Industry," *Palaios*, vol. 16, no. 6, p. 614, Dec. 2001.

[71] A. C. Mitropoulos, "What is a surface excess?," *J. Eng. Sci. Technol. Rev.*, vol. 1, no. 1, pp. 1–3, 2008.

[72] L. Martínez-Balbuena, A. Arteaga-Jiménez, E. Hernández-Zapata, and C. Márquez-Beltrán, "Applicability of the Gibbs Adsorption Isotherm to the analysis of experimental surface-tension data for ionic and nonionic surfactants," *Advances in Colloid and Interface Science*, vol. 247. Elsevier B.V., pp. 178–184, 01-Sep-2017.

[73] A. A. Atia and N. R. E. Radwan, "Adsorption of different surfactants on kaolinite," *Adsorpt. Sci. Technol.*, vol. 15, no. 8, pp. 619–626, 1997.

[74] "Critical micelle concentration (CMC) and surfactant concentration - KRÜSS." [Online]. Available: https://www.kruss-scientific.com/services/education-theory/glossary/critical-micelle-concentration-cmc-and-surfactant-concentration/.

[75] I. W. Hamley, *Introduction to Soft Matter: Synthetic and Biological Self-Assembling Materials*. Chichester, UK: John Wiley and Sons, 2007.

[76] D. Coelho *et al.*, "Biosurfactant Production from Unconventional Resources: a Short Overview Development of an Annular Centrifugal Extractor for Bromelain Extraction using Aqueous Two-Phases Systems View project Therapeutic l-asparaginase for Acute Lymphoid Leukemia (ALL) purification employing alternative bioproces View project Biosurfactant Production from Unconventional Resources: a Short Overview," *Int. Rev. Chem. Eng.*, vol. 4, no. 2, 2012.

[77] K. Karim *et al.*, "Niosome: A future of targeted drug delivery systems," *Journal of Advanced Pharmaceutical Technology and Research*, vol. 1, no. 4. Wolters Kluwer -- Medknow Publications, pp. 374–380, Oct-2010.

[78] I. F. Uchegbu and S. P. Vyas, "Non-ionic surfactant based vesicles (niosomes) in drug delivery," *Int. J. Pharm.*, vol. 172, no. 1–2, pp. 33–70, Oct. 1998.

[79] S. Moghassemi and A. Hadjizadeh, "Nano-niosomes as nanoscale drug delivery systems: An illustrated review," *Journal of Controlled Release*, vol. 185, no. 1. Elsevier, pp. 22–36, 10-Jul-2014.

[80] "Polysorbate 20 - DrugBank." [Online]. Available: https://www.drugbank.ca/drugs/DB11178.

[81] H. Rabiee, S. M. S. Shahabadi, A. Mokhtare, H. Rabiei, and N. Alvandifar, "Enhancement in permeation and antifouling properties of PVC ultrafiltration membranes with addition of hydrophilic surfactant additives: Tween-20 and Tween-80," *J. Environ. Chem. Eng.*, vol. 4, no. 4, pp. 4050–4061, Dec. 2016.

[82] K. L. Mittal, "Determination of CMC of polysorbate 20 in aqueous solution by surface tension method," *J. Pharm. Sci.*, vol. 61, no. 8, pp. 1334–1335, Aug. 1972.

[83] "Polysorbate 20 | 9005-64-5." [Online]. Available: https://www.chemicalbook.com/ChemicalProductProperty_EN_cb3462544.htm.

[84] K. L. Zapadka, F. J. Becher, A. L. Gomes dos Santos, and S. E. Jackson, "Factors affecting the physical stability (aggregation) of peptide therapeutics," *Interface Focus*, vol. 7, no. 6. Royal Society Publishing, 06-Dec-2017.

[85] J. Wang, K. Liu, R. Xing, and X. Yan, "Peptide self-assembly: Thermodynamics and kinetics," *Chemical Society Reviews*, vol. 45, no. 20. Royal Society of Chemistry, pp. 5589–5604, 21-Oct-2016.

[86] N. A. Malik, "Surfactant–Amino Acid and Surfactant–Surfactant Interactions in Aqueous Medium: a Review," *Applied Biochemistry and Biotechnology*, vol. 176, no. 8. Humana Press Inc., pp. 2077–2106, 01-Aug-2015.

[87] "Zwitterion @ Chemistry Dictionary & Glossary." [Online]. Available: https://glossary.periodni.com/glossary.php?en=zwitterion.

[88] M. C. Brick, H. J. Palmer, and T. H. Whitesides, "Formation of colloidal dispersions of organic materials in aqueous media by solvent shifting," *Langmuir*, vol. 19, no. 16, pp. 6367–6380, Aug. 2003.

[89] A. N. Ganesh, E. N. Donders, B. K. Shoichet, and M. S. Shoichet, "Colloidal aggregation: From screening nuisance to formulation nuance," *Nano Today*, vol. 19. Elsevier B.V., pp. 188–200, 01-Apr-2018.

[90] G. M. Whitesides and M. Boncheva, "Beyond molecules: Self-assembly of mesoscopic and macroscopic components," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 99, no. 8, pp. 4769–4774, Apr. 2002.

[91] D. Reker, G. J. L. Bernardes, and T. Rodrigues, "Computational advances in combating colloidal aggregation in drug discovery," *Nature Chemistry*, vol. 11, no. 5. Nature Publishing Group, pp. 402–418, 01-May-2019.

[92] N. G. Arutyunyan, L. R. Arutyunyan, V. V. Grigoryan, and R. S. Arutyunyan, "Effect of aminoacids on the critical micellization concentration of different surfactants," *Colloid J.*, vol. 70, no. 5, pp. 666–668, Oct. 2008.

[93] J. Weiss, "Static and Dynamic Interfacial Tension Analysis," *Curr. Protoc. Food Anal. Chem.*, vol. 7, no. 1, p. D3.6.1-D3.6.16, Feb. 2003.

[94] X. Wang, Q. Min, Z. Zhang, Y. Duan, Y. Zhang, and J. Zhai, "Influence of head resistance force and viscous friction on dynamic contact angle measurement in Wilhelmy plate method," *Colloids Surfaces A Physicochem. Eng. Asp.*, vol. 527, pp. 115–122, Aug. 2017.

[95] Y.-M. Tricot, "Surfactants: Static and Dynamic Surface Tension," in *Liquid Film Coating*, Springer Netherlands, 1997, pp. 99–136.

[96] C.-C. Chen, Y. Zhu, and L. B. Evans, "Phase Partitioning of Biomolecules: Solubilities of Amino Acids," *Biotechnol. Prog.*, vol. 5, no. 3, pp. 111–118, Sep. 1989.

[97] B. Salas Vidal, "FUNCIONALITZACIÓ DE CEL·LULOSA MICROCRISTAL·LINA MEMÒRIA Autor," Universitat Politècnica de Catalunya, Jan. 2020.

[98] "Water | H2O - PubChem." [Online]. Available: https://pubchem.ncbi.nlm.nih.gov/compound/Water#section=Corrosivity.

[99] M. Lengyel, N. Kállai-Szabó, V. Antal, A. J. Laki, and I. Antal, "Microparticles, microspheres, and microcapsules for advanced drug delivery," *Scientia Pharmaceutica*, vol. 87, no. 3. MDPI AG, p. 20, 01-Sep-2019.

[100] C. Peniche, W. Argüelles-Monal, H. Peniche, and N. Acosta, "Chitosan: An Attractive Biocompatible Polymer for Microencapsulation," in *Macromolecular Bioscience*, 2003, vol. 3, no. 10, pp. 511–520.

[101] A. Bernkop-Schnürch and S. Dünnhaupt, "Chitosan-based drug delivery systems," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 81, no. 3. Elsevier, pp. 463–469, 01-Aug-2012.

[102] H. Rajabi, S. M. Jafari, G. Rajabzadeh, M. Sarfarazi, and S. Sedaghati, "Chitosan-gum Arabic complex nanocarriers for encapsulation of saffron bioactive components," *Colloids Surfaces A Physicochem. Eng. Asp.*, vol. 578, p. 123644, Oct. 2019.

[103] H. Espinosa-Andrews, J. G. Báez-González, F. Cruz-Sosa, and E. J. Vernon-Carter, "Gum Arabic−Chitosan Complex Coacervation," *Biomacromolecules*, vol. 8, no. 4, pp. 1313–1318, Apr. 2007.

[104] C. Butstraen and F. Salaün, "Preparation of microcapsules by complex coacervation of gum Arabic and chitosan," *Carbohydr. Polym.*, vol. 99, pp. 608–616, Jan. 2014.

[105] "(PDF) Microencapsulation techniques and its practices." [Online]. Available:

https://www.researchgate.net/publication/279498086_Microencapsulation_techniques_and_its_practices.

[106] C. Saraiva, C. Praça, R. Ferreira, T. Santos, L. Ferreira, and L. Bernardino, "Nanoparticle-mediated brain drug delivery: Overcoming blood-brain barrier to treat neurodegenerative diseases," *Journal of Controlled Release*, vol. 235. Elsevier B.V., pp. 34–47, 10-Aug-2016.

[107] "Pràctiques acadèmiques externes — Pràctiques acadèmiques externes — UPC. Universitat Politècnica de Catalunya." [Online]. Available: https://www.upc.edu/cce/ca/estudiants.

[108] "NTP 276: Eliminación de residuos en el laboratorio: procedimientos generales."

[109] G. EL Para Cálculo De La Huella De Carbono Y Para La Elaboración De Un Plan De Mejora De Una Organización, "Ministerio para la Transición Ecológica."

[110] "FACTORES DE EMISIÓN REGISTRO DE HUELLA DE CARBONO, COMPENSACIÓN Y PROYECTOS DE ABSORCIÓN DE DIÓXIDO DE CARBONO."

[111] "Description - IKA Plate (RCT digital)." [Online]. Available: https://www.ika.com/en/Products-Lab-Eq/Magnetic-Stirrers-Hot-Plate-Lab-Mixer-Stirrer-Blender-csp-188/IKA-Plate-(RCT-digital)-cpdt-25004601/.

[112] "Analytical and semimicro balance RADWAG series AS - Labbox Export." [Online]. Available: https://ien.labbox.com/product/analytical-and-semimicro-balance-radwag-series-as/.

[113] "Force Tensiometer – K20." [Online]. Available: https://www.kruss-scientific.com/products/tensiometers/force-tensiometer-k20/.

[114] "Mac Pro: información de consumo energético y potencia térmica (BTU/h) - Soporte técnico de Apple." [Online]. Available: https://support.apple.com/es-cl/HT201796.