# Oral Delivery of Glutathione: the Antioxidant Function, the Barriers and the Strategies

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# Abstract

**Background:** Glutathione (GSH) is a tripeptide, which is recognised as a potent antioxidant involving in numerous essential biological processes, and has been used for interventions in various degenerative diseases. However, as with all protein and peptide drugs, its oral delivery remains challenging due to the physical and enzymatical barriers existing in the gastrointestinal (GI) tract, leading to a low oral bioavailability. Although several approaches have been explored in the past to improve the oral bioavailability of GSH, an appropriate formulation with clinical therapeutic effects is still yet to be developed. This study explores an approach to develop an oral niosome-based GSH loaded delivery system that could provide protection against proteolytic degradation in the GI tract and enhance the molecular absorption across the epithelial membrane.

**Aim:** This project aims to improve GSH physical and chemical stability and explore the mechanism of GSH cellular uptake and transport using a cell culture model.

**Methods**: This study re-developed and validated a high-performance liquid chromatography (HPLC) method to qualify and quantify GSH, which was applied for GSH analysis in the entire project. A thin film hydration method was used to fabricate GLNs, whose composition was optimised by applying a 2 level 3 factors factorial design methodology using Design-Expert® software. The optimised formulation was then characterised for its particle size, zeta potential, entrapment efficiency (EE), morphology, physical stability, *in vitro* drug release and degradation. The mechanism of GLNs cellular uptake and transport was assessed using Caco-2 cells or co-cultured with Ht29 cells for transport study.

**Results:** The re-developed HPLC method was reliable and accurate, with the GSH calibration curve ranging from 1 to 100 µg/mL displaying excellent linearity ($R^2 = 0.9999$). The optimised formulation of GSH exhibited double-layered vesicular structure, with an average particle size at 253.3 ± 0.6 nm, PDI at 0.353 ± 0.028, zeta potential at -65.3 ± 3.5 mV and EE at 31.45 ± 0.46%. The physical stability study suggested that GSH incorporated with niosomes presented a better physical stability compared to pure GSH solution at the same temperature. The latter appeared to be more stable at 4 °C than at 25 °C or 40 °C. The release study suggested that GLNs demonstrated a two-phased release profile with sustained release behaviour as opposed to one-phased release of free GSH solution. Additionally, the *in vitro* degradation study

revealed the GLNs showed protective effect on GSH against enzymatic degradation in extracts from all intestinal regions of rats. Images taken by a confocal microscopy illustrated internalisation of fluorescent-labelled niosomes, suggesting that the mechanism of niosomes uptake and transport into Caco-2 cells might go through endocytosis, the common pathway for nanoparticles absorption across biological membrane and this machenism was confirmed from the transport study (1). The transport study was carried out to determine the effect of niosomes on the GSH transport across the monolayer of the Caco-2/HT29 cells, resulting to significant increases in the flux rates of GSH.

**Conclusion**: A reliable HPLC method was re-developed and validated for its accuracy and reliability based on ICH guideline. A niosomal formulation containing GSH was developed, optimised, and characterised, which has shown positive results in all studies. In addition, the degradation of luminal contents in intestinal areas of rats was superior to those of the mucosa. In terms of enzyme inhibitor, this study discovered that EDTA displayed a significant inhibition effect compared to bacitracin. *In vitro* studies of the formulation in terms of degradation, cellular uptake, and transport, suggest GSH loaded niosome (GLNs) significantly improve GSH's stability and cellular absorption across the intestinal membrane.

## Acknowledgements

Commencing this study has been an amazing and tremendous personal experience, which wouldn't have happened without the help, support and sacrifices from many selfless people whom I would like to acknowledge here.

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# Publications

### Review article

- **Tielan Wei**, Sachin Sunil Thakuu, Mengyang Liu, Jingyuan Wen. Oral delivery of glutathione: antioxidant function, barriers and strategies. Journal of Acta Materia Medica, 2022 May; 1 (2): 177-192

### Article in the process of publication

- **Tielan Wei,** Sachin Sunil Thakur , Mengyang Liu , Danhui Li, Jingyuan Wen. Glutathione-loaded niosomes: a promising nanoscaled formulation for oral delivery of GSH (Manuscript for submission to *Advanced drug delivery reviews*)

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# List of Abbreviations

| | |
|---|---|
| 3D | 3-Dimensional |
| ACN | Acetonitrile |
| ANOVA | Analysis of variance |
| AUC | Area under curve |
| BCS | Biopharmaceutical classification system |
| BCA | Bicinchoninic acid |
| BP | British pharmacopeia |
| BSA | Bovine serum albumin |
| CH | Cholesterol |
| Da | Dalton |
| DCP | Dihexadecyl phosphate |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediamine tetra-acetic acid |
| EE | Entrapment efficiency |
| FBS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| FITC | Fluoresce in isothiocyanate |
| GI | Gastrointestinal |
| GLNs | GSH-loaded niosomes |
| GSH | Glutathione |
| GPx | Glutathione peroxidase |
| GSSG | Disulphide-oxidized |
| HBSS | Hank's balanced salt solution |
| HPLC | High-performance liquid chromatography |
| ICH | International Conference on Harmonization |
| LC-MS | Liquid chromatography coupled with the mass spectrum |
| LOD | Limit of detection |
| LOQ | Limit of quantitation |
| M | Molar (mole per litre) |
| MEAN | Average |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NEAA | Non-essential amino acid |
| o/w | Oil-in-water |
| PBS | Phosphate buffered saline |
| PDI | Polydispersity Index |
| P-gp | P-glycoprotein |
| ROS | Reactive oxygen species |
| RSD | Relative standard deviation |
| SAG | S-allyl glutathione |

| | |
|---|---|
| SEM | Scanning electron microscopy |
| SLNs | Solid lipid nanoparticles |
| SLS | Sodium lauryl sulphate |
| Span® 60 | Sorbitan stearate |
| Span® 80 | Sorbitan monooleate |
| TEM | Transmission electron microscopy |
| TFA | Trifluoroacetic acid |
| Tween® 80 | Polyethylene glycol sorbian monooleate |
| U/mL | Unit per millilite |
| v/v | Volume per volume |
| w/o | Water-in-oil |
| w/w | Weight per weight |

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# Chapter 1

# General introduction

# 1 General introduction

## 1.1 Introduction

Glutathione (GSH) is a tripeptide, containing amino acids glutamic acid, cysteine and glycine (2). Endogenous GSH is a potent antioxidant, involved in many essential biological processes including protein and DNA synthesis, cell proliferation, and oxidation/reduction signalling (3). In the past decade, GSH has been used for various medical interventions in degenerative diseases such as Alzheimer's disease and Parkinson's disease (4-8).

Peptides, like GSH, are chemical compounds composed of two to fifty amino acids linked by peptide bonds (9,10). Endogenous peptides are involved in many physiological processes, acting as hormones, neurotransmitters, growth factors, ion channel ligands, or anti-infective agents (11,12). Their unique pharmacological profiles and intrinsic properties have made peptides excellent drug candidates with better tolerance and lower toxicity than traditional "small molecule" drugs (<500 Da). Their highly selective receptor binding properties ensure good clinical efficacy (13).

In recent years there has been an increasing interest in pharmaceutical research and formulation development for peptide therapeutics. By 2018, 7000 naturally occurring peptides had been identified. More than 60 peptide drugs have been approved by authorities across the United States of America, Japan, and Europe. There were also about 150 peptides in clinical trials and over 500 in preclinical trials at the time (12,14). Over the years, peptide-related patents have created financial potential for the pharmaceutical industry and led to remarkable profits. For example, Lupron$^{TM}$ Depot, a synthesised peptide drug mainly used to treat endometriosis and prostate cancer, achieved global sales of US $2.3 billion for Abbott Laboratories in 2011 (12,15). Moreover, the global peptide drug market has been predicted to grow further at a rate of 9-10% per annum (16).

In principle, peptides could have a lot of value in medicinal applications, however, they have been severely restricted by physical and chemical barriers in the gastrointestinal (GI) tract following oral administration, which leads to a low oral bioavailability (17). Physical barriers primarily comprise the biological characteristics of the GI tract including the intestinal epithelial membranes, tight junctions, unstirred water layer, and efflux system, which all restrict peptide transport across the intestinal epithelium. Chemical barriers include the extremely acidic environment in the stomach and various GI tract proteases, which cause

hydrolytic or enzymatic degradation of the peptide drug. These barriers have made peptides unsuitable to be administered using conventional oral formulations (18-20).

As a result, most peptide drugs are currently marketed as parenteral injections. Unfortunately, the injections' invasive nature, associated pain, and potential tissue damage have made these formulations unpopular for patient use. Therefore, strategies in developing peptide formulations with enhanced oral bioavailability have caught scientists' attention worldwide, exploring novel ground-breaking approaches to deliver peptide drugs in the most convenient and patient-friendly manner. Currently, there are only 13 oral form peptide drugs (tablets, capsules and solution) approved by the United States Food and Drug Administration (FDA, Table 1-1) (21).

**Table 1-1.** Oral Formulations of Peptide Drugs Approved by FDA.

| Brand | Form | Company | Name | Peptide Sequence | Absorption | Indications |
|---|---|---|---|---|---|---|
| Tekturna | Tablets | Physicians Total Care, Inc. | Aliskiren | N.A. | 2.50% | Hypertension, renal impairment, and hepatic impairment |
| Amturnide | Tablets (aliskiren/ amlodipine/ hydrochlorothiazide) | Novartis Pharmaceuticals Corporation | | | | |
| Tekamlo | Tablets (aliskiren/amlodipine) | | | | | |
| Tekturna HCT | Film-coated tablets | | | | | |
| Tekturna | Tablets | | | | | |
| Pertzye | Delayed-release capsule | Digestive care US, Inc. | Pancrelipase | Pancreatic alpha amylase | N.A. | Exocrine pancreatic insufficiency |
| Pancrecarb | Delayed-Release Capsules | Digestive care US, Inc. | | | | |
| Ultrase | Capsules | Axcan Pharma | | | | |
| Zenpep | Delayed-Release Capsules | Aptalis Pharma US, Inc. | | | | |
| Utresal | Delayed-Release Capsules | | | | | |
| Viokace | Tablets | | | | | |
| Ragwitek | Tablets | Merck Sharp & Dohme | Ragweed Pollen Extract | N.A. | N.A. | Allergic reaction |
| Sucraid | Solution | QOL Medical, LLC | Sacrosidase | N.A. | N.A. | Congenital sucrose-isomaltase deficiency |

In the last two decades, numerous papers have reported novel technologies for oral peptide delivery. Strategies to increase oral bioavailability have included adding enzymatic inhibitors and/or penetration enhancers in the formulation or chemical modification of the peptide to form analogues and pro-drugs. Many innovative techniques using nanocarrier systems have also

been evaluated, including polymeric nanoparticles, solid lipid nanoparticles, liposomes and niosomes (22-24).

As a peptide, GSH faces the same challenges as all other peptides in oral formulation development. Although some publications have promising potential to enhance GSH oral bioavailability using different strategies (22,23,25), further evaluation is still vital. In this review, we will highlight the physiological roles and molecular properties of GSH, the enzymatic and physical barriers of GSH uptake and transport across intestinal epithelial membranes in the GI tract, and the strategies used to enhance oral bioavailability of GSH and other peptide therapeutics (including using enzymatic inhibitors, permeation enhancers, chemical modification and formulation approaches).

## 1.2 Rationale for therapeutic GSH

Oxidative stress is a biological imbalance between the plasma concentration of reactive oxygen species (ROS) and the systemic ability to scavenge ROS and repair the resulting damage to proteins, lipids, and DNA (26). ROS (also referred to as free radicals) (27) are highly reactive and include superoxide radical $\cdot O_2^-$, peroxide $O_2^{2-}$, hydroxyl radical $\cdot OH$ and nitric oxide $\cdot NO$ (28). They are molecules whose outer shell has one or more unpaired electrons, which makes them highly unstable and ready to react with various organic substances including lipids, proteins and DNA (29,30).

Releasing free radicals is a mechanism of the human immune system to respond to the invasion of pathogenic microbes by destroying their structures (29). However, chronic accumulation of free radicals *in vivo* can be harmful, causing oxidative stress, which has proven to be responsible for the development of degenerative diseases (31). There are two pathways that result in a high concentration of free radicals in human body: the first is the accumulation of free radicals that are produced endogenously via normal cell metabolism, while the second is a build-up via environmental factors such as pollution, cigarette smoking, radiation or medications (32).

The human body has defensive mechanisms to counteract oxidative stress by either endogenously producing antioxidants such as GSH or by exogenously acquiring them through food and/or supplements. During the process, antioxidants act as a “free radical remover” to neutralise the excess oxidants/free radicals, protecting and repairing cellular damage, promoting enhanced immune function and lowering the risk of subsequent diseases (33). As

an antioxidant, the reduced form of GSH, is readily to be oxidised into GSH disulphide by free radicals and/or reactive oxidative species due to its cysteine residue. The intracellular balance of both forms of GSH determines the antioxidative state and capacity of cells (34).

## 1.3 GSH

### 1.3.1 Cellular structure and discovery of GSH

Glutathione (*N*-(*N*-L-γ-glutamyl-L-cysteinyl) glycine) is a tripeptide, composed of γ-glutamic acid, cysteine and glycine (35,36) (Figure 1-1). It was first discovered by Rey-Paihade in 1888 from extracts of yeast and many animal tissues including skeletal muscle, liver, intestine, brain, and fresh egg white (37). In 1929, Pirie and Pinhey reported the molecular structure of GSH as a tripeptide, which was confirmed by Harington and Mead in 1935 following successful chemical synthesis using *N*-carbobenzoxycysteine and glycine ethyl ester (37). Since then, GSH molecular structure has been well established: a γ-carboxyl peptide bond links the carboxyl group of glutamate side chain with cysteine whilst a normal peptide linkage bonds cysteine's carboxyl group with glycine (35,36). While exogenous GSH can come from many sources, endogenous GSH is mainly produced in the liver during normal cellular metabolism and is abundant in the cytoplasm, nuclei and mitochondria of all living cells (32,37-39).

Endogenous GSH is synthesised via two steps: the first step is the formation of γ-glutamylcysteine from glutamate and cysteine, catalysed by glutamate-cysteine ligase. The second step is the formation of GSH from the reaction between γ-glutamylcysteine generated at the first step and glycine, catalysed by GSH synthetase (40). GSH is hydrophilic and will be quickly degraded with an elimination half-life of 10 min in the human body (35) and 2 to 3 h in rat liver (41) that leads to extremely low GSH bioavailability via oral route. Cellular GSH is degraded via hydrolysis catalysed by γ-glutamyl-transpeptidase, breaking down the peptide bond linking glutamate and cysteine, generating glutamate and cysteinylglycine, which then will be further degraded into cysteine and glycine by dipeptidases (41).

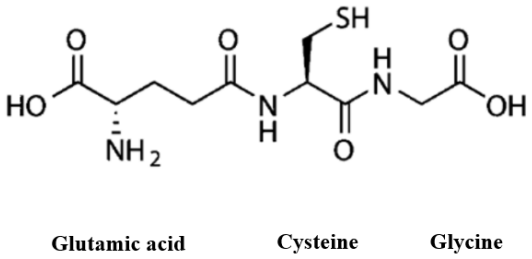


**Figure 1-1**: Molecular structure of GSH.

### 1.3.2 **Roles of GSH in preventing degenerative diseases**

GSH has been well studied and accepted as a potent antioxidant, participating in numerous basic cellular processes such as protein synthesis, DNA synthesis and repair, cell proliferation, and redox signalling (42). Additionally, it plays a significant role in detoxifying various electrophilic compounds such as heavy metals (43,44).

Naturally, glutathione exists in two forms in living cells, the thiol-reduced form (L-GSH) and the disulphide-oxidised form (GSSG). In healthy cells, L-GSH is the predominant form, accounting for >98% of total GSH (45,46). They are mostly stored in cytosol (80-85%), some in mitochondria (10-15%) and a small amount in the endoplasmic reticulum (45). The ratio of GSSG to L-GSH in cells represents the oxidative stress level (45). Namely, the higher the ratio of GSSG to L-GSH in cells, the greater the oxidative stress.

It is generally accepted that the antioxidant function of GSH is related to its scavenging activity towards free radicals accumulated during oxidative stress (47,48) and protecting living cells by neutralising excessive ROS from oxidative damage (2). The deficiency of GSH can cause excess oxidative stress and cellular dysfunction, leading to various degenerative and chronic diseases such as cancers, cardiovascular diseases, neurodegenerative diseases (Parkinson's disease and Alzheimer's disease) and glaucoma (4-6).

Studies have demonstrated that most human degenerative diseases and the general human aging process features deleterious free radical reactions, typically caused by ROS (49,50). For example, the cardiovascular condition atherosclerosis involves the build-up of fatty deposits on the endothelium of blood vessels whose structure has been damaged by ROS (49,50). Separately in cancerous diseases, the first mutagenic event is typically caused by ROS reactions. Interestingly, oxidative processes also help metastasised cancer cells attach to tissues (49,50). Finally, the eye has a high concentration of unsaturated lipids, and owing to poor clearance mechanisms, it is extremely defenceless against oxidative damage (49,50).

Unfortunately, there is no cure for most of these disorders, thus leading to the application of preventative strategies, such as using health supplements like GSH, which can slow down degenerative processes.

### 1.3.3 Mechanism of GSH as an antioxidant

The mechanism of GSH as an antioxidant can be explained as cellular oxidation and reduction (redox) reactions between the sulfhydryl group of the molecule and GSH-related enzymes (38). GSH contains a functional sulfhydryl group (also known as thiol group) on its cysteine moiety, consisting of sulphur bonded to a hydrogen atom. GSH's primary antioxidative role is to maintain the redox state of sulfhydryl groups of important proteins by forming a disulphide bridge to protect the structure of those important proteins (Figure 1-2).

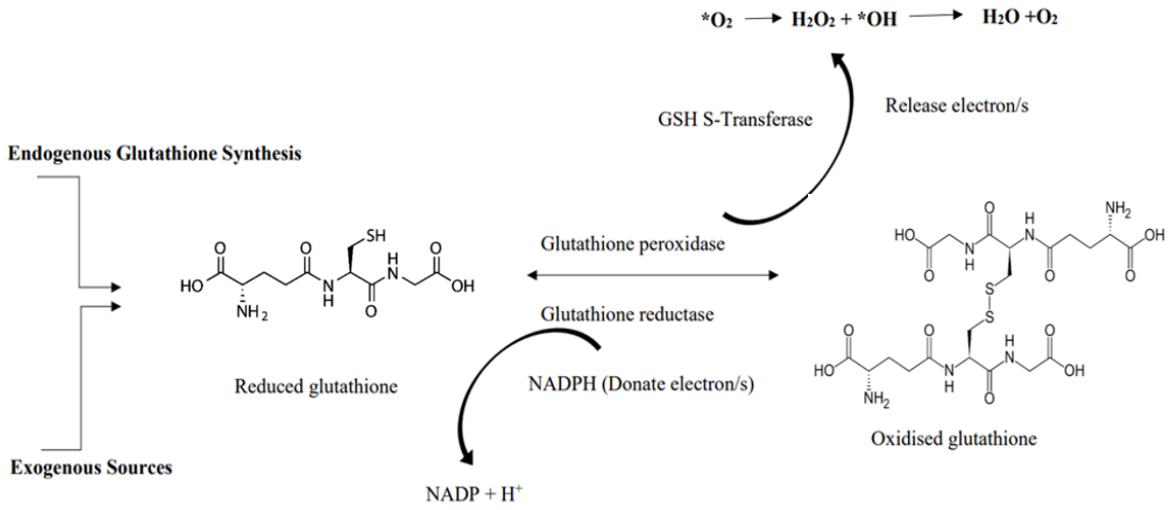


**Figure 1-2**: Antioxidant mechanism of GSH.

GSH can protect the body against oxidative stress both directly and indirectly. Directly, catalysed by glutathione peroxidase (GPx), GSH can scavenge ROS such as hydrogen peroxide ($H_2O_2$) by donating an electron, converting into GSSG and water (41). Indirectly, glutathione is involved in producing other critical cellular antioxidants such as vitamin C or E as the electron source (51,52).

### 1.3.4 Evidence of GSH medicinal function

In the last two decades, there has been increasing interest in studying GSH as a therapeutic. Many clinical trials and *in vitro* studies have been carried out to evaluate the clinical significance of GSH using different administration routes: intravenous, nasal, pulmonary and oral. Some encouraging results have been observed. For example, Cascinu *et al.* led one randomised double-blind placebo-controlled trial of 50 patients using GSH injection (50,53). This trial comprised of patients with advanced gastric cancer that were receiving weekly cisplatin treatment. In the treatment group, GSH was given intravenously at a dose of 1.5

mg/m$^2$ in normal saline solution immediately before cisplatin administration. Results showed that by the 9$^{th}$ week of the study, no patient treated with GSH showed signs of neuropathy, whereas 16 out of 18 patients in the control group did. By the 15$^{th}$ week, only 4 of 24 patients in the GSH group had developed neurotoxic symptoms.

Cascinu then conducted a similar study of 52 patients who received a GSH infusion 1,500 mg/m2 over 15 min before treatment with oxaliplatin or saline (54,55). Clinical and electrophysiologic assessment was performed at baseline and after 4, 8 and 12 cycles of treatment. At the 4$^{th}$ cycle, from the GSH group, 7 out of 26 patients showed clinical signs of neuropathy (grade 1 or 2) compared to 11 out of 26 in the placebo group. After 8 cycles, 9 out of 21 patients in the GSH group suffered grade 1 or 2 neuropathy compared to 15 out of 19 in the placebo group. In terms of grade 3 or 4 neurotoxicity, there was 0 in the GSH group compared to 5 in placebo group. Only 18 patients completed 12 cycles of treatment for various reasons, of which only 3 of 10 patients in the GSH group developed neuropathy (grade 2 to 4) compared to 8 out of 8 in the placebo group. Therefore, from both studies they concluded that GSH might aid in preventing drug-induced neuropathy in platinum treatment without affecting the drug's chemotherapeutic activity (both cisplatin and oxaliplatin) (54,55).

Despite various routes being studied for GSH-containing formulations e.g., injections as anticancer agents (54,56) and eye drop used for glaucoma treatment (57), an oral formulation of GSH has always been the desirable administration route due to the low cost of production and excellent patient compliance. Recent studies suggest that oral administration of GSH may enhance both blood and tissue GSH levels of rats absorbed by the intestinal epithelial cell, leading to GSH restoration in intestinal mucosa under oxidative stress conditions (40,58). It is suggested that oral GSH supplements could provide a therapeutic strategy for disease treatment caused by the abnormality of ROS levels in the disease tissues.

### 1.3.5 **Preformulation parameters of GSH and their significance for oral formulation**

Liu (59) conducted a full preformulation study of GSH. According to his report, GSH was a needle-line crystal form, which had a melting point at 195.6°C and high water solubility at 252.7 ± 5.8 mg/mL. Based on the value of Log D (-3.6) and Log P (-3.1) of GSH obtained from the study, Liu stated that GSH should be categorized as class III chemical compound that naturaly has low permeability and high solubility. Moreover, GSH was unstable under forced degradation conditions of high temperature, oxitative, artificial light, acidic, basic and aqueous.

There were four resulting degradation products identified: cysteinyl glycine , glutathione disulfide, glutamic acid and pyroglutamic acid. Antioxidant effect of GSH (ranging 100 to 1000 μg/mL) and no significant cytotoxicity of GSH (ranging 5-10000 μg/mL) were reported(59).

Although there has not been a single GSH-containing preparation approved by FDA as a therapeutic agent, many GSH supplements have already been available on the current market in different forms, such as injections (60), lozenges (61), oral sprays (62), oral liquids (63) and oral capsules (64). Meanwhile a number of studies have been carried out to evaluate the therapeutic potential of GSH in different formulations like injections (for prevention of drug-induced neurotoxicity as discussed previously), eye drops (for glaucoma treatment) (57), dermal preparations (65) and oral formulations. GSH supplements may potentially provide a therapeutic strategy for diseases caused by the abnormality of tissue ROS levels.

Among all those formulations being studied, oral formulations have always been the desirable strategy for researchers due to their low cost of production, excellent patient compliance, the convenience of storage and transport, and good shelf life. However, the biggest challenge of the oral formulation of GSH is its extremely low bioavailability owing to the physical and enzymatic GI barriers. Therefore, this review will focus on investigating the strategies that may improve GSH oral bioavailability by using various pharmaceutical modifications.

## 1.4 Barriers of oral delivery of peptides

Orally administered peptides face several barriers in the GI tract. The GI tract has its predominant functions to digest food and absorb essential nutrients, electrolytes, fluids, and excrete waste. At the same time, it works as a physicochemical barrier to protect the human body from systemic invasion of toxins, antigens and pathogens (66).

To be absorbed into the blood stream, intact drug molecules, including peptides and proteins, must diffuse either between or through the intestinal epithelial cells. This process is hampered by physical and biochemical barriers in GI tract (18). The epithelial membranes in GI tract act as physical barriers with selective functions to allow the transportation of drug molecules. The phospholipid bilayer structure of the epithelial membrane restricts the transcellular transport of hydrophilic macromolecules (e.g. peptide and protein drugs), while the tight junctions are responsible for limiting paracellular transport (66). With absorption being a slow and difficult process, peptide and protein drugs remain vulnerable in the GI tract, with their enzymatic

degradation occurring at multiple sites along the GI tract, including brush border, the lumen, and intracellular environment.

### 1.4.1 Physical barriers

The physical barriers to oral delivery are considered to be the cell lining in the GI tract, which includes the intestinal cell membranes and tight junctions between neighbouring cells, as well as the unstirred water layer and efflux systems which play an important role in regulating drug absorption (66).

#### 1.4.1.1 Intestinal epithelial cell membranes

The anatomic structures of the intestine have been well described in other literature (67); here only the functional details relating to barriers to drug transportation and absorption are discussed. The wall of intestine primarily comprises a mono-layer of column-like epithelial cells, with goblet cells, enterocytes, endocrine cells, and Paneth cells interspersed in the architecture (67,68). Drug transportation and absorption after oral administration may depend on the physiochemical properties of bioactive molecules, including size, charge, lipophilicity, hydrogen bonding potential, and solution conformation, which are constrained by Lipinski's rule of five (69).

The phospholipid bilayer structure of the epithelial cells enables the semi-permeable properties of their membranes, allowing lipophilic drug molecules to be absorbed transcellularly via passive diffusion (Figure 1-3B). On the other hand, in principle, hydrophilic or highly charged molecules and macromolecules like peptide and protein drugs face great difficulty being transcellularly absorbed unless they are recognised and transported via a carrier-mediated pathway or endocytosis (Figure 1-3C and1-3E, respectively). Even though the size of molecules is recognised as the fundamental limitation for oral absorption of peptide and protein drugs, some successes regarding the development of oral dosage forms of polypeptides have been achieved, for instance, cyclosporin A and desmopressin (70).

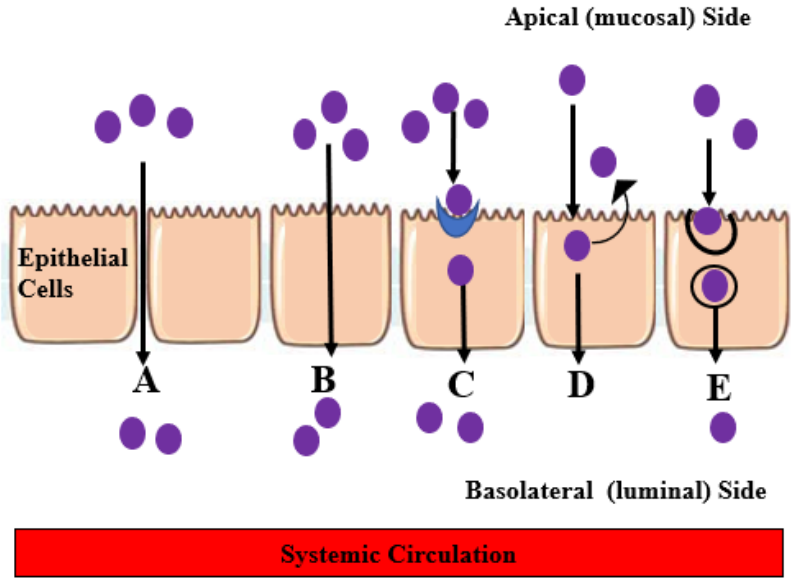


**Figure 1-3**. Pathways of intestinal absorption with example molecules that use this absorption method. A: Paracellular transportation, e.g., thyrotropin-releasing hormone (71); B: transcellular passive transportation, e.g., oestradiol, testosterone; C: carrier-mediated transcellular transport, e.g., amino acids, penicillins, ACE inhibitors (19); D: transcellular transportation modified by an efflux pathway; E: transcellular vesicular transportation(including endocytosis or receptor-mediated transcytosis), e.g., ciclosporin (19).

#### 1.4.1.2 Unstirred water layer

The unstirred water layer is described as an aqueous boundary layer covering the entire intestinal wall and consists of water, mucus, and glycocalyx. It is considered as the result of incomplete mixing of luminal contents. This unstirred water layer acts as an essential physical barrier for drug absorption with its thickness being controlled by the rate of mucus secretion and the rate of layer shedding. While this water layer is continuously being turned over, drug molecules have to move upstream through this structure in order to reach the epithelial surface (72). Additionally, the complexation/binding interactions between the diffusing drug molecules and mucins play a part in the barrier function (73).

#### 1.4.1.3 Tight junctions

Tight junctions are dense and hydrophobic intercellular structures that facilitate the paracellular pathway of GI drug absorption (73). From the apical to the basolateral epithelial membrane, junctional complexes are divided into three different layers: the apical tight junctions (zonula occludens), the underlying adherens junctions (zonula adherens), and the basal desmosomes (macula adherens) (74). The tight junctions form the continuous intercellular barrier amongst adjacent epithelial cells, creating a selective channel for solute movement across the epithelial

membrane. This selectivity of tight junctions can be regulated predominantly by claudins, which is a family of transmembrane proteins. It is capable of continuously sealing the spaces between neighbouring epithelial cells on the apical side and therefore creates a physical barrier for drug absorption (75).

Tight junctions primarily regulate the absorption of hydrophilic molecules across the epithelial membrane. Transport efficiency through this paracellular pathway is determined by the molecular size and polarity of the substances absorbed (76). Namely, tight junctions allow the intercellular diffusion of small hydrophilic molecules (e.g. ions, nutrients, and certain small drugs) while preventing large hydrophilic molecules (e.g. peptide and protein drugs) from passing through (77). Tight junctions are generally considered as the dynamic structures that can be affected by certain chemicals such as $Ca^{2+}$ chelators, surfactants and cationic polymers, resulting in an increase in their permeability (77). With the absence of peptideolytic and proteolytic activities in paracellular transportation, formulation design of peptide and protein drugs for oral applications via this route has increasingly drawn more attention from scientists.

#### 1.4.1.4 Efflux systems

The efflux systems are also considered an essential part of physical barrier in GI tract. Efflux systems consist of a protein transporter functioning via an intracellular pathway, and are responsible for the poor oral bioavailability of certain compounds, especially peptides and proteins (78-80) (Figure 1-3D). P-glycoprotein (P-gp), one of the efflux systems, is located on the apical side of the epithelial cell membrane and actively pumps drug molecules from inside of the epithelial cells back into the intestinal lumen (81). P-gp was first discovered amongst cancer cells (82), and since then high levels has been found in healthy tissues such as cells of the intestine, liver, kidney, blood-brain barrier and placenta (82,83).

### 1.4.2 Biochemical barriers

The biochemical barriers to oral peptide delivery include the acidic gastric environment and the presence of various metabolising enzymes and luminal bacteria (84). The pH of intestinal fluid varies considerably along the GI tract and consequently, the mechanism of pH-dependent hydrolysis varies in different parts of the intestine. The enzymatic barrier is the most daunting obstacle to oral peptide delivery. Enzymes catalysing proteolysis or peptidolysis are located on specific sites of the GI tract: pepsin in the stomach to elastase, carboxypeptidase A and B,

chymotrypsin, and trypsin in the intestine, which are all secreted by the pancreas. Due to the wide distribution of digestive enzymes, enzymatic degradation can occur at multiple sites throughout the GI tract. Meanwhile, degradation can also take place at multiple linkages of the peptide backbone (23). Microorganisms in the colon also secrete enzymes that are responsible for various reactions including decarboxylation, deglucuronidation, amide hydrolysis and dihydroxylation, and reduction of double bonds and esters (84).

Under the specific conditions of the GI tract, protein molecules are broken down into polypeptides, and polypeptides are further broken down into smaller units, such as bi-, tri-peptides and/or single amino acids via peptidolysis before being transported across the GI tract membrane into the bloodstream (85-87). These smaller units are the essential components for facilitating some crucial biological processes, for example, DNA synthesis. Unfortunately, the same mechanisms will become challenges to the oral delivery of peptide drugs, due to their chemical and structural similarities.

## 1.5 Prospective pharmaceutical strategies for improvement in GSH bioavailability

The physicochemical properties of GSH as a peptide drug have severely restricted its clinical development into oral dosage forms due to the limited membrane permeability and instability against enzymatic degradation mainly occurring in the jejunum (88). Studies suggested that the thiol group of GSH is susceptible to γ-glutamyltranspeptidase in the jejunum and is oxidised to GSSH (88), resulting in the loss of its antioxidant activity. Therefore, strategies focusing on improving GSH's physicochemical profiles and stability in GI tract could potentially make a breakthrough in formulation development, leading to enhanced oral bioavailability. These strategies include chemical modifications, formulation approaches and nanocarrier technologies.

### 1.5.1 Chemical modification strategies

Chemical modification is an approach to modifying the native structure of a peptide or a protein drug to enhance its stability and absorption across the epithelial membrane (89,90).

Application of prodrugs is one of these strategies. A prodrug is defined as a biologically inactive derivative that can be metabolised in the body and converted into a pharmacologically active drug. A prodrug protects the parent drug from enzymatic and/or chemical degradation in GI tract, resulting in increased permeability across the biological membrane and later

restoration of its pharmacological activity by systemic enzymatic cleavage before reaching the site of action (23,91). For example, one study reported a prodrug of GSH (L-cysteine-glutathione mixed disulfide) displayed better bioavailability in mice after oral administration, providing protection to mice from hepatic toxicity of acetaminophen compared to the control group treated with saline solution.

The application of an analog is another effective way of improving a parent drug's therapeutic effect. *S*-allyl glutathione (SAG) is an analog of GSH that is obtained by modifying the thiol group of GSH with the allyl group (92). It is proven that *S*-allyl group has anticancer effect by inhibiting topoisomerase activity, resulting in cell cycle arrest and cell death (93,94). One study investigated the anticancer effects of SAG using SAG-containing selenium nanoparticles. (92). The study reported that after 12 h of treatment with the formulation, SAG was released from the nanoparticles effectively into a hepatocarcinoma cell line in both acidic (pH 5.3) and neutral (pH 7.4) conditions with release rates of 72% and 67% respectively. Moreover, the SAG antiproliferation effect was improved by selenium nanoparticles as data showed the required concentration of SAG to achieve anticancer effect was lower than that of SAG alone *in vitro*.

### 1.5.2 **Absorption enhancers**

Absorption enhancers are a group of functional additives added into formulations to improve the permeability of drugs across biological membranes. This approach has been investigated for many years and applied in the development of oral formulations for protein and peptide drugs (95,96). Mechanisms include chemically opening tight junctions, decreasing mucous viscosity and changing intestinal membrane fluidity (96,97). Commonly used absorption enhancers and candidate drugs whose absorption they have enhanced are listed in Table 1-2.

**Table 1-2**. Commonly used absorption enhancers**.**

| **Absorption enhancers** | **Drug absorption enhanced** |
|---|---|
| Chitosan | Cyclosporine A (98) |
| Citric acid | Insulin (99,100) |
| Cyclodextrins | Limaprost (101) |
| Glycerides | DuP 532 (102) |
| Lauroyl carnitine chloride | Insulin (103) |
| Sodium lauryl sulphate (SLS/sodium dodecyl sulphate) | Cefazolin (104) |
| Sodium N-[8-(2-hydroxybenzoyl) aminocaprylate] | Semaglutide (105) |

Chitosan, a nontoxic biocompatible polymer, is a commonly used absorption enhancer for peptide drug formulations (106). A study conducted by Liu *et al*. illustrated that chitosan can enhance the permeation and absorption of cyclosporine A, an immunosuppressive agent, across the intestinal membrane *in vivo* in rats (98). To overcome the limitation of solubility of chitosan in a neutral pH environment (intestinal tract), the use of chitosan derivatives has led to more effective intestinal absorption enhancement. For example, trimethyl chitosan chloride has considerably increased the intestinal permeability of peptide analogues. Chitosan and its derivatives reversibly widen tight junctions, which enhances the biological penetration of peptide drugs (98). Surfactants and detergents are another group of absorption enhancers; these reversibly disrupt the phospholipid structure of the membrane, leading to the opening of the tight junctions. Examples include dodecyl sulphate, sodium caprate, and long-chain acylcarnitine, fatty acid and glycerides (107).

Interestingly GSH has been used as an absorption enhancer in some studies. One study reported that GSH significantly enhanced the permeability of sodium fluorescein across intestinal epithelium due to disruption of membrane integrity. Data collected from this study showed that significant increase in permeability of sodium fluorescein was observed when the concentration of GSH increased from 0.1% to 0.4% *in vitro* across guinea pig mucosa compared to the control medium without GSH (108). Permeation enhancement was also seen when GSH was used in combination with polycarbophil cysteine. These results have been proven by another study that used sodium caprate, a widely recognised absorption enhancer, as comparison (109).

Despite the favourable function of absorption enhancement, important disadvantages of these absorption enhancers have also been reported as these compounds may themselves penetrate the biological membrane and cause systemic toxicity. In addition, the disruption to the epithelial membrane structure may potentially have prolonged effects and compromise its biological functions (110,111).

### 1.5.3 **Enzymatic inhibitors**

Oral peptide drugs are degraded by various proteases in the GI tract such as trypsin, chymotrypsin, peptidases, and other proteolytic enzymes. Enzymatic inhibitors are molecules that bind to these enzymes and decrease their activity (23). As a promising approach, concomitant administration of enzyme inhibitors can restrict the metabolism of proteins and

peptides, leading to an increase in the availability of intact peptide drug molecules for absorption across the intestinal membrane (96).

Aprotinin (a small protein with a molecular weight of 6500 Da) is a competitive enzyme inhibitor for serine proteases such as trypsin and chymotrypsin (112). It has been employed as an enzyme inhibitor in various studies investigating protein and peptide drug absorption across the intestinal membrane. One study revealed that when concomitantly administered with aprotinin orally, insulin-containing microemulsions caused a significant reduction in plasma glucose levels between 90 and 120 min compared to those without aprotinin in both non-diabetic and diabetic rat models (113). Pechenkin *et al.* investigated the impact of several protease inhibitors (aprotinin, Soybean derived Bowman-Birk inhibitor and Kunitz soybean trypsin inhibitor) on oral delivery of insulin. The study found that insulin was well protected from proteolytic degradation (triggered by trypsin and chymotrypsin) when encapsulated with these enzyme inhibitors compared to the insulin solutions *in vitro* (114).

Bacitracin, a cyclic polypeptide antibiotic with a molecular weight of 1422.7 Da (115), is another enzyme inhibitor which can effectively inhibit various proteases including trypsin, pepsin and aminopeptidase. An *in vitro* study has reported that bacitracin, camostat mesilate, and sodium glycocholate, reduced insulin degradation in the large intestinal homogenate of rats (116). Although there are no studies published in relation to the use of enzymatic inhibitor of GSH, the mechanism would follow the same approaches applied to oral peptide drugs.

The limitations of using enzyme inhibitors in peptide drug delivery include systemic toxicity, digestive disorders and pancreatic islet cell hyperplasia (117), which need to be carefully considered for formulation development.

### 1.5.4 **Formulation approaches**

The properties of chemical materials change when their particle sizes approach atomic size. This is due to the increase in the ratio of surface area to the volume that may enable particles in nano-scale to exhibit different optical, physical, and chemical properties significantly compared to the ones in larger sizes (118). Nano-sized carriers offer many advantages for protein and peptide delivery, including high physical and chemical stability, high drug loading capacity, the capability of incorporation of both hydrophilic and hydrophobic drugs, and enhanced bioavailability with sustained release properties (119). In addition, nano-carriers can

be designed as formulations with various administration routes e.g. oral, nasal, dermal, pulmonary and parenteral routes (119).

There are numerous forms of nanocarriers that have been widely studied, in this review we will discuss microemulsions, nanoparticles, liposomes, niosomes and proniosomes. The transport mechanisms of these various formulation approaches over the barriers are illustrated in Figure 1-4. The mechanism, advantages and limitations of these strategies are also summarised in Table 1-3.

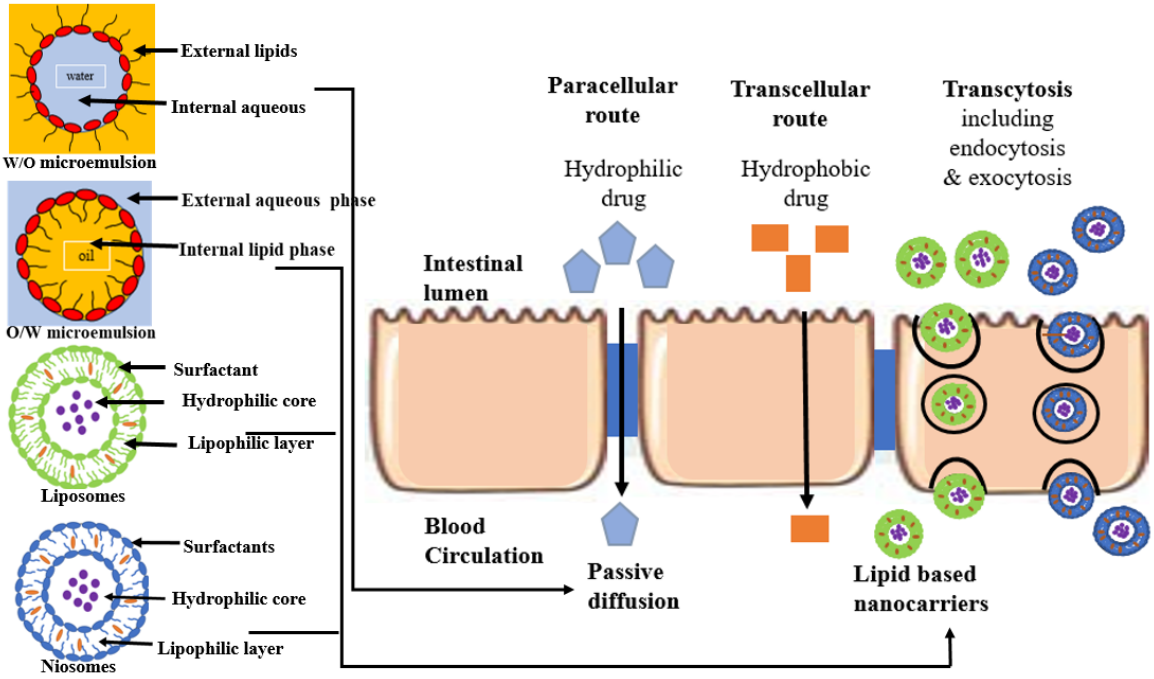


**Figure 1-4**. Nanocarriers and their transport mechanisms across the intestinal barriers.

**Table 1-3**. Mechanisms, advantages and limitations of various nanocarriers

| Nanocarrier | Mechanism | Advantages | Limitations | Applications | Ref |
|---|---|---|---|---|---|
| Microemulsion | Combination of passive diffusion and/or active transport and/or endocytosis | Thermodynamically stable;<br>Spontaneous formation;<br>Stable systems;<br>Improved drug solubility;<br>Long shelf life;<br>Ability to load large quantities of both hydrophilic/hydrophobic drug;<br>Enhanced bioavailability; | Toxicity due to the high concentration of surfactant/s | Oral administration of insulin on rats;<br>Cyclosporin oral formulation for human in treatment of rheumatoid arthritis and psoriasis | (120,121) |
| Nanoparticle | Transcytosis including | Long circulation half-life; | Cytotoxicity due to altered | Oral formulation of | (26,122) |

| | | | | | |
|---|---|---|---|---|---|
| | endocytosis and exocytosis transport | Reduced hepatic filtration;<br>Enhanced stability;<br>Enhanced bioavailability; | regulation function of endothelial cells | GSH for therapeutic effect on intestinal diseases caused by oxidative stress | |
| Liposome | Phosphate lipid based transcytosis pathway | Biocompatibility;<br>Biodegradability;<br>Non-immunogenicity;<br>Ability of entrapping with both hydrophilic and hydrophobic drug molecules;<br>Protection of drug molecules from GI tract;<br>Enhanced drug cellular uptake and transport;<br>Low toxicity;<br>Controlled or sustained drug release; | High manufacturing cost;<br>High time consumption;<br>Physical instability;<br>Inability of sterilisation;<br>Low drug entrapment;<br>Batch reproducibility ;<br>Leaking of entrapped medicine; | Oral GSH supplement for increase of GSH level in human body | (40,123) |
| Niosome | Surfactant-mediated transcytosis pathway | All advantages of Liposome;<br>Ease on scale-up production;<br>Low cost for preparation;<br>Improved physical stability compared to liposomes; | Physical instability<br>Inability of sterilisation;<br>Low drug entrapment;<br>Leaking of entrapped medicine; | GSH-loaded niosome oral formulation for hepatic protection, and enhanced hepatic cell uptake and GSH bioavailability | (124,125) |
| Pro-niosome | Transfer into niosome by hydration and surfactant-mediated transcytosis transport | All advantages of niosome;<br>Better physical and chemical stability than niosome;<br>Prolonged shelf life;<br>Better approach in dosing design; | Have to be compounded before use;<br>Limited volume of relevant studies available | Oral form of vinpocetine with improved oral absorption | (126) |

#### 1.5.4.1 Microemulsions

A microemulsion is defined as a dispersion of oil, water and surfactant (with co-surfactant). It is a spontaneously formed liquid mixture that is transparent, optically isotropic and thermodynamically stable with droplet sizes ranging from 10 to 200 nm (127). There are three types of microemulsions based on the internal and external phase: oil-in-water (o/w), water-in-oil (w/o) and bicontinuous (23) (Figure 1-5).

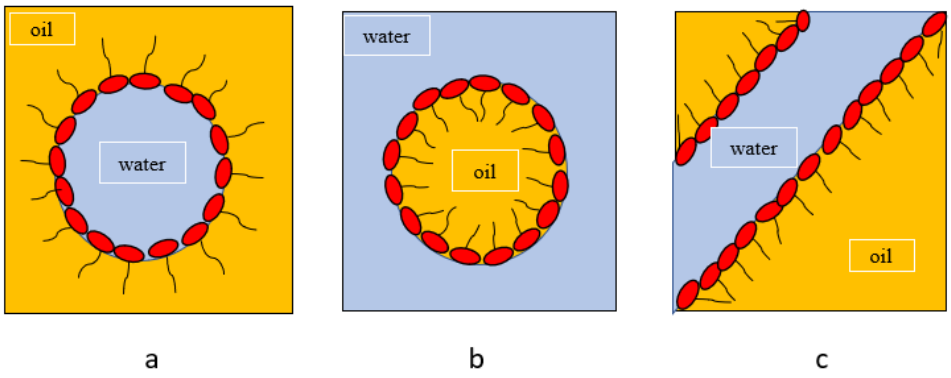


**Figure 1-5**. Structure of microemulsion droplets. (a) w/o, (b) o/w, (c) bicontinuous**.**

Compared to colloidal systems such as suspensions, microemulsions have several advantages as drug carriers, such as improved drug solubility, long shelf life, enhanced bioavailability and ease of preparation (128). Therefore, there has been great interest in the formulation design using microemulsions for oral peptide and protein drug delivery.

For example, Çilek *et al.* developed a lecithin-based microemulsion formulation of recombinant human insulin with aprotinin for oral administration, aiming to examine the hypoglycemic effects in non-diabetic and streptozotocin-induced diabetic rats (120). The study found that after oral administration, the insulin-containing microemulsion (with or without aprotinin) decreased plasma glucose levels by approximately 30% compared to unformulated oral insulin solution, and the effect lasted for about 90 min. In a study conducted by Wen *et al.* microemulsions applied as a glutathione delivery system achieved sustained release profiles of GSH compared to a colloidal emulsion system and glutathione alone. *In vitro* profiles from the study indicated that microemulsion may have provided sustained release of GSH after oral administration, therefore resulting to a promising oral delivery system with enhanced bioavailability for GSH (129).

A microemulsion oral solution of cyclosporin, Neoral, has been approved by FDA. This product is used for the prevention from organ rejection after organ transplantation (of liver, kidney and heart) and for the treatment of rheumatoid arthritis and psoriasis (121). This has proven that microemulsions can be promising drug carriers for oral protein and peptide drug delivery, therefore can be considered when designing oral formulations for GSH.

Despite being promising delivery systems, microemulsions have caused concerns in the potential toxicity due to the high concentration of surfactant/s, which needs to be addressed when designing oral delivery systems (130).

### 1.5.4.2 Nanoparticles

Nanoparticles have been extensively studied as peptide and protein drug delivery systems in the last decade. They are defined as colloidal particles (consisting of biodegradable or nonbiodegradable polymers) (131). The advantages of using nanoparticles as a peptide drug delivery system include long circulation half-life *in vivo* and reduced hepatic filtration, resulting in enhanced stability and bioavailability (131). The small size of nanoparticles allows higher cellular uptake of peptide drug molecules, resulting in improved drug absorption across the biological membrane. Furthermore, the use of nanoparticles as drug carriers may result in fast drug release because of the increased surface area corresponding to the small particle size (132). However, the limitations of such formulations have been reported e.g. cytotoxicity due to altered regulation function of endothelial cells (133), which may need to be overcom before they are applied as peptide drugs carriers (134).

Many studies have been conducted to evaluate the potential of using nanoparticles as delivery systems for oral GSH formulations. In order to examine GSH's therapeutic effect on intestinal diseases caused by oxidative stress, Bertoni *et al*. developed solid lipid nano-scaled particles (ranging from 250-355 μm) loaded with GSH (26). They discovered the encapsulation capacity of GSH was as high as of 20% w/w, whilst GSH's physicochemical properties were effectively retained during the process. They also found that varying the composition of the formulation may allow modulate the release of GSH; the more hydrophobic lipid contained in the particles, the longer the GSH release time in intestinal fluids. They concluded that these GSH containing formulations co-administered with another antioxidant (catalase) displayed excellent radical scavenging activity by decreasing the intracellular ROS's levels which was mimicked using hydrogen peroxide *in vitro* (26).

Another study conducted by Alobaidy investigated the impact of chitosan-formulated nanoparticles on the oral bioavailability of GSH. The study reported that GSH-loaded nanoparticles showed a rapid and prolonged release profile of GSH after being administered orally and was comparable to the profile of subcutaneously administered GSH *in vivo* in rats. The effect was dose-dependent, and the plasma concentration of GSH in rats was proportional to the GSH dose loaded in the nanoparticles (122). A separate study reported that the release of GSH from nanoparticles (composed of basil seed gum loaded with GSH) was pH-dependent. *In vitro* studies showed faster and more complete GSH release in pH 6.8 (mimics the intestinal environment) than in pH 1.2 (mimics the stomach environment) (135).

#### 1.5.4.3 Liposomes

Liposomes are defined as spherical particles composed of an aqueous core surrounded by one or more phospholipid bilayers, generally with a size ranging from 20 nm to 10 μm (23,136,137). Liposomes are capable of entrapping both hydrophilic drugs (in the aqueous core) and hydrophobic drugs (in the lipid bilayers) (Figure 1-6a) (138,139). Liposomes can be categorised into multilamellar vesicles and unilamellar vesicles, which can be further classified as small unilamellar vesicles and large unilamellar vesicles. A unilamellar liposome has a single phospholipid bilayer, while a multilamellar vesicle has an onion-type structure (140).

The use of liposomes for oral peptide and protein drug delivery has been investigated for many years due to their unique advantages, including biocompatibility, protection of drug molecules from the harsh environment of GI tract, and enhanced cellular uptake and transport. However, disadvantages such as high manufacturing cost, formulation instability, and long preparation time remain challenging during formulation development (139,141,142).

There have been many studies of liposomes for the oral delivery of GSH. A clinical study of 12 healthy adults revealed that oral administration of liposomal GSH supplements significantly increased the GSH levels in the body. Compared to the baseline, the maximum increase of GSH level was 40% in whole blood, 25% in erythrocytes, 28% in plasma (one week after administration) and 100% in peripheral blood mononuclear cells (two weeks after administration). Meanwhile, the enhancement in immune function markers and reduction of oxidative stress were also observed (40). Another study examined the impact of a proliposome formulation on the oral bioavailability of GSH and formulation stability. Data collected has shown that the structure of GSH was maintained in the proliposome formulation Compared to the commercially available capsules and pure GSH, proliposomes prepared in this study doubled the oral bioavailability of GSH in rats. Moreover, there were no significant changes in particle size and Zeta-potential of the formulation. Hence the study concluded that proliposome formulation might be applied as a novel delivery system for oral administration of GSH with better oral bioavailability and stability (123).

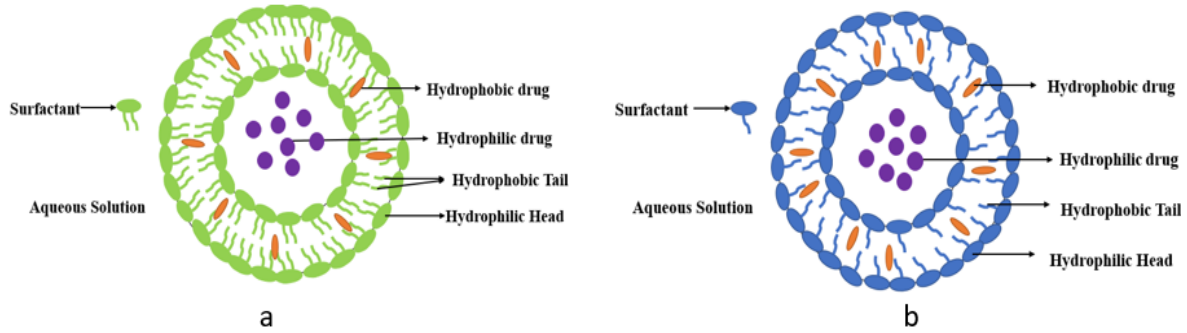


**Figure 1-6**. **a.** Structure of a unilamellar liposome showing the loading locations of hydrophilic and hydrophobic drugs. b. Structure of niosome showing the loading locations of hydrophilic and hydrophobic drugs.

### 1.5.4.4 Niosomes

Niosomes are defined as nano-structured vesicles with a size ranging from 10 nm to 3 µm (143) and produced from surfactants and cholesterol (CH) in an aqueous medium (Figure 1-6b) (22,144). They have similar structures to liposomes as small or large unilamellar or multilamellar vesicles and are prepared via similar production procedures (145,146).

As drug delivery systems, niosomes can accommodate both hydrophilic and hydrophobic drugs together and generate sufficient surface areas to facilitate targeted drug delivery to the site of therapeutic actions, therefore increasing drug efficacy and reducing side effects. In addition to all the advantages of liposomes (147), niosomes have their unique features to overcome the limitations associated with liposomes, such as difficulties in scale-up production, high cost of organic materials used for preparation, and low physical stability (22). Niosomes have been extensively studied as drug delivery systems, and their applications have been widely used in various pharmaceutical fields such as topical, oral, parental and transdermal application (22).

Owing to their biodegradability, biocompatibility, non-immunogenicity and low cost compared to liposomes, niosomes have increasingly drawn attention as nanocarriers in GSH oral delivery (147). For example, a recently published study evaluated GSH-loaded niosomes' (GLNs) hepatic protection, hepatic cell uptake and GSH bioavailability (125). The study reported that after oral administration, GSH-containing niosomes significantly restored rat liver damage (induced by $CCl_4$ administered intraperitoneally) compared to the pure GSH solution ($p < 0.05$). The GSH contents levels in liver tissues were detected at 15.90 µg/g for the GSH-containing niosomes group and at 9.91 µg/g for the GSH solution group, while the baseline of the damaged liver was at 8.15 µg/g. Stability studies showed no significant change in particle size, Zeta-potential, polydispersity index and encapsulation efficiency after 4-week storage in room

temperature and 4 °C. The study reported that this formulation exhibited GSH protective effects against stomach environment (pH 1.2) with release profile of 35.5% at pH 1.2 compared to that of 45% at pH 6.8 (mimic small intestine) after 6 h incubation *in vitro*. This pH-sensitive drug release profile of GSH-containing niosomes was proven by another study which further demonstrated this nanocarrier's non-toxicity effect on the cells *in vitro* even at a high concentration of GSH (400 µg/mL) (124). Anti-cancer effects and sustained protein alteration effects were also observed in this study, which lasted for 48 h (124). Therefore, niosomes could be the future drug carriers in GSH oral delivery for therapeutic purposes.

#### 1.5.4.5 Proniosomes

A proniosome is a dry free-flowing granular product that can be hydrated upon contacting aqueous media, forming a niosome dispersion immediately before use (148). Proniosomes have all the advantages that niosomes have, such as better chemical stability and lower cost in preparation compared to liposomes. Additionally, proniosomes exhibit better physical stability than niosomes due to their dry nature. Problems of niosome suspensions facing during storage, including aggregation and fusion of vesicles, leaking and hydrolysis of entrapped drug molecules could be addressed by proniosomes. (149-151). Consequently, with a prolonged shelf life, proniosome formulations may provide convenience in transportation, storage and distribution for large-scale pharmaceutical production. Furthermore, due to the dry state, proniosomes could be further processed into granules, tablets or capsules, leading to a better approach in unit dosing design than the liquid dosage form of niosomes (152).

There have been studies published to investigate proniosome preparation and the physicochemical characteristics, including particle size/distribution analysis and drug release profiles (152,153). Studies reported that compared to the conventional niosomes, the niosome dispersions derived from proniosomes were easier to be prepared without requiring a long time of agitation. Also, the proniosome dispersions tended to display better profiles in particle size uniformity. Meanwhile, drug entrapment efficiency and *in vitro* drug release profiles remained unchanged (149-151).

Another study evaluated the impact of proniosomes on the oral absorption of vinpocetine, a poorly water-soluble drug. The study reported that drug-entrapped niosomes illustrated significantly higher permeability *in vitro* in rats than unformulated vinpocetine suspension. The same increases in absorption *in vivo* had also been observed after the niosome formulations

were orally administered to rabbits. This study also investigated the stability of the proniosomes after being stored for 6 months, which presented sustained stability with the ease of preparation, unchanged drug entrapment efficiency and particle sizes (126). Therefore, proniosomes could be the ideal nanocarrier to deliver protein and peptide drugs with low bioavailability and poor stability in the GI tract.

## 1.6 Conclusion

The main challenge for oral delivery of protein and peptide drugs is their enzymatic degradation in the GI tract, which is the leading cause of low oral bioavailability. In order to address this problem, there have been many approaches studied by scientists, including chemical intervention, absorption enhancers, enzymatic inhibitors and formulation strategies e.g. microemulsions, nanoparticles, liposomes and niosomes, etc. In general, every strategy has its advantages and limitations as an oral drug carrier. Therefore, the best approach for a sufficient oral delivery system for protein and peptide drugs as well as GSH would be a comprehensive formulation combined with multiple strategies depending on the physicochemical characteristics of the drug molecules. In this project, niosomes have been selected to be the delivery carrier for GSH due to their unique advantages and cost-effectiveness.

# Chapter 2

# HPLC method re-development and validation

# 2 HPLC method re-development and validation

## 2.1 Introduction

As described in the previous chapter, GSH is a tripeptide, containing amino acids glutamic acid, cysteine and glycine (2). Endogenous GSH is recognised as a potent antioxidant, involved in many essential biological processes including protein and DNA synthesis, cell proliferation, and oxidation/reduction signalling (3). In the past decade, GSH has been used for various medical interventions in degenerative diseases such as Alzheimer's disease and Parkinson's disease.

Due to its poor stability and low bioavailability across the epithelium membrane of GI tract, the design of oral formulations of GSH is extreme challenging. Therefore, understanding GSH's physiochemical characteristics would provide a sound contribution toward a successful formulation design and development, which can be achieved through pre-formulation studies. Some publications have reported that preformulation studies of GSH can be successfully carried out by applying high performance liquid chromatography (HPLC) for GSH quantitative analysis (59) .

HPLC is an analytic tool for qualitative and quantitative analysis of chemical compounds. The mechanism of HPLC is to allow the separation of constituents in liquid mobile phase moving at different rate, leading to different retention time separating from each other. Because a testing sample in the mobile phase is forced through the stationary phase by a pump (rather than gravity), the process of each run can be completed very fast. Compared to other analytical techniques such as capillary electrophoresis, thin layer chromatography and gas chromatography, the HPLC method is fast, accurate, efficient and reproducible (154).

Many studies and publications have reported that the HPLC method had been applied for protein and peptide drug quantitative analysis, leading to reliable and accurate experimental data, therefore, in this project, a optimized HPLC method has been adopted to quantitative analysis of GSH (155,156).

## 2.2 Aim

- Re-development and validation of an HPLC analytical method.

## 2.3 Materials and method

### 2.3.1 Materials

Reduced L-glutathione and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile (ACN) and methanol (both of analytical reagent grade) were purchased from Merck (Kenilworth, NJ, USA). Trifluoroacetic acid (TFA) was purchased from Honeywell Research Chemicals Company (Portland, OR, USA). Milli-Q water was generated by Pharmaceutics laboratory at University of Auckland via a Millipore RO system (Bedford, MA, USA). Dulbecco's modified Eagle medium (DMEM), phosphate-buffered saline (PBS), non-essential amino acid solution (NEAA), heat inactivated foetal bovine serum (FBS), penicillin-streptomycin, trypsin and ethylenediaminetetraacetic acid (EDTA) were all purchased from Invitrogen (Auckland, New Zealand). All other reagents used were of analytical grade.

### 2.3.2 High performance liquid chromatography (HPLC) method validation

The HPLC method was obtained from Wen (129) and Liu (59,155), in which this analytical method had been initially developed and validated.

#### 2.3.2.1 HPLC conditions

The HPLC system was a combination of Agilent HP 1200/1260 series module (Santa Clara, CA, USA), connected with an Agilent vacuum degasser, quaternary pump, auto-sampler, thermos-stated column, photodiode array detector. Chemstation software was used for data acquisition. Analysis was conducted via a reverse phase HPLC assay at 25 °C, using a C18 column (250 x 4.60 mm diameter, 5 µm particle size**,** Hichrom) fitted with a C18 guard column. Mobile phase composition was 20:80 (v/v), 0.085% (v/v) trifluoroacetic acid (TFA) in acetonitrile (ACN): 0.100% (v/v) TFA in Milli-Q water at a flow rate of 0.7 mL/min with 20.0 µL injection volume. Ultraviolet detection was performed at the wavelength of 215 nm.

#### 2.3.2.2 HPLC method validation

Based on the British Pharmacopeia (BP) (157) and International Conference on Harmonization (ICH) guideline (158), the analytical method used in this study was validated using linearity, accuracy, intermediate precision, sensitivity expressed as the limit of detection (LOD) and the

limit of quantification (LOQ), and repeatability expressed as instrumental precision and intra-day precision (158).

#### 2.3.2.2.1 **Preparation of stock solution and standard solution of GSH**

GSH stock solution (500.0 µg/mL) was prepared by dissolving GSH in the mixed mobile phase described above. Seven standard solutions (1.0, 5.0, 10.0, 25.0, 50.0, 75.0 and 100.0 µg/mL) were prepared in triplicate by dilution of stock solution using mobile phase.

#### 2.3.2.2.2 **Linearity**

The calibration curve was plotted with the peak area against the seven GSH concentrations (1.0, 5.0, 10.0, 25.0, 50.0, 75.0 and 100.0 µg/mL). The linear regression was determined by the slope, y-intercept and linearity of the curve (158).

#### 2.3.2.2.3 **Accuracy**

Accuracy was evaluated the true concentrations using three different concentrations (25.0, 50.0 and 75.0 µg/mL) in triplicate (158).

#### 2.3.2.2.4 **Intermediate precision**

Intermediate precision was assessed by using intra-day and inter-day repeatability. Intra-day precision was obtained by testing three different GSH concentrations at different times on the same day, while inter-day precision was evaluated at the same time on three consecutive days (158).

#### 2.3.2.2.5 **Sensitivity**

HPLC sensitivity was determined by using the values of LOD and LOQ. LOD and LOQ were measured according to the standard deviation of the response (σ) and the slop (S) of the calibration curve using equations (2-1) and (2-2), based on the ICH guideline (158).

$$LOD = 3.3 \times \frac{\sigma}{S} \qquad \text{Equation 2-1}$$

$$LOQ = 10 \times \frac{\sigma}{S} \qquad \text{Equation 2-2}$$

#### 2.3.2.2.6 **Repeatability**

Repeatability was determined by evaluating the system precision and method precision. System precision (also called instrumental precision) was conducted by analysing three replicate injections in three different GSH concentrations (25.0, 50.0 and 75.0 µg/mL) (158).

## 2.4 Results and discussion

### 2.4.1 HPLC method modification

The peak separation of GSH was well re-established by using a mixture of mobile phase: 0.1% TFA in Milli-Q water (pH 2.1) and 0.085% TFA in ACN with a ratio of 80:20 v/v. The retention time of GSH peak was 3.8 min (Figure 2-1), which significantly reduced the time interval of the experiment compared to the method developed by Liu (59,155). A very sharp peak with an excellent resolution was observed.

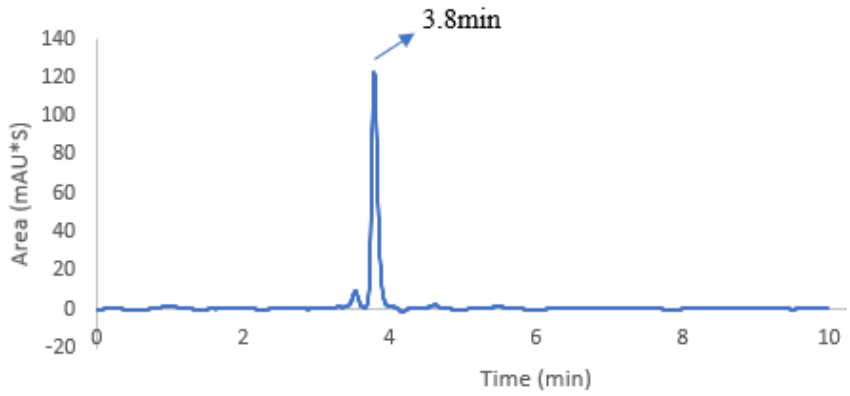


**Figure 2-1.** HPLC chromatogram of GSH with retention time at 3.8 min.

### 2.4.2 HPLC validation

#### 2.4.2.1 Linearity

The calibration results were shown in Figure 2-2 indicating excellent linearity within the investigated concentrations ranging from 1.0 to 100.0 µg/mL, resulting in a correlation coefficient ($R^2$) of 0.9999, which is within the range required by the International Conference on Harmonisation (ICH) guideline (158).

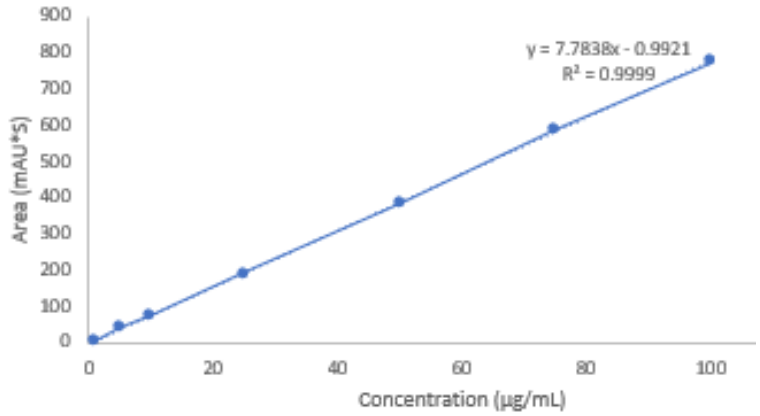


**Figure 2-2.** Calibration curve of GSH using the modified HPLC method (data points indicate Mean ± SD, n=3)

### 2.4.2.2 Accuracy

Accuracy was validated using the measured concentration of GHS against the actual concentration. All results are displayed in Table 2-1. All values of relative standard deviation (RSD) were less than 2%, indicating the method has excellent accuracy according to ICH requirements (158).

**Table 2-1.** Accuracy study data of GSH using HPLC method.

| Concentration (µg/mL) | Calculated Concentration (µg/mL) | Accuracy (%) | RSD (%) |
|---|---|---|---|
| 25.0 | 24.8 ± 0.1 | 99.3 | 0.5 |
| 50.0 | 49.6 ± 0.5 | 99.1 | 1.0 |
| 75.0 | 75.8 ± 0.4 | 101.0 | 0.4 |

### 2.4.2.3 Intermediate precision

Intermediate precision was evaluated by investigating intra-day and inter-day repeatability. The results are shown in Table 2-2. Both intra-day and inter-day have less than 2% RSD values in all three concentrations of 25.0, 50.0 and 75.0 µg/mL, which is within the range required by ICH guidelines (158).

**Table 2-2.** Intermediate precision study data of GSH using HPLC method.

| Concentration (µg/mL) | Intra-day precision | | Inter-day precision | |
|---|---|---|---|---|
| | Peak Area (mAU*S) Mean ± SD, n=3 | RSD | Peak Area (mAU*S) Mean ± SD, n=3 | RSD |
| 25.0 | 197.1 ± 3.6 | 1.8 | 194.9 ± 3.7 | 1.9 |
| 50.0 | 394.1 ± 3.8 | 1.0 | 391.1 ± 5.5 | 1.4 |
| 75.0 | 591.9 ± 4.2 | 0.7 | 589.9 ± 3.6 | 0.6 |

#### 2.4.2.4 Sensitivity

The result of LOD and LOQ were 0.136 µg/mL and 0.412 µg/mL, respectively, all meeting the requirement of ICH guidelines.

#### 2.4.2.5 Repeatability

Method precision (intra-assay precision) was determined by analysing three different concentrations with triplicate independent solutions (shown in Table 2-3). Table 2-4 displays intra-assay precision. All results of RDS for both studies were less than 2.0%, revealing that the results generated using the HPLC method applied in this study are precise.

**Table 2-3.** Instrumental precision**.**

| Concentration (µg/mL) | Instrumental Precision | |
|---|---|---|
| | Peak Area (mAU*S) Mean ± SD, n=3 | RSD (%) |
| 25.0 | 192.3 ± 0.8 | 0.4 |
| 50.0 | 384.7 ± 3.8 | 1.0 |
| 75.0 | 588.8 ± 2.9 | 0.5 |

**Table 2-4.** Intra-assay precision study**.**

| Concentration (µg/mL) | Intra-assay precision | |
|---|---|---|
| | Peak area (mAU*S) (Mean ± SD, n=3) | RSD (%) |
| 25.0 | 192.3 ± 0.8 | 0.4 |
| 50.0 | 384.7 ± 3.8 | 1.0 |
| 75.0 | 588.8 ± 0.8 | 0.5 |

## 2.5 Conclusion

In Chapter 2, an excellent HPLC method has been re-developed according to the literature reported. The method also was fully validated according to Liu *et al.* (155). The method adequately analysed the lowest concentration of GSH as 1μg/mL. The linearity, precision, accuracy and repeatability of the method had been proven to be excellent and fulfilled the requirements of ICH (158). A standard curve (ranging from 1 to 100 μg/mL) has been validated. It was used for the quantitative analysis of GSH-containing formulation in studies of stability, degradation and *in vitro* cell uptake and transport, which was described in the following two chapters.

# Chapter 3

# Formulation development and characterisation

# 3 Formulation development and characterisation

## 3.1 Introduction

According to the biopharmaceutical classification system (BCS), GSH is class III drug candidate with high aqueous solubility but low permeability across the biological membrane (via oral or topical route) (159,160). In addition, GSH shows poor stability under acidic, basic, and even neutral conditions, undergoing a certain degree of degradation (155). Therefore, an oral GSH formulation will request to solve the problems of low oral bioavailability and poor stability. Many researchers reported that nano-carriers that can address these problems would be ideal for developing GSH oral formulations (161-163). Niosomes, nano-scaled drug carriers, have been chosen for this project due to the advantages introduced in Chapter 1, such as relative high stability compared to the liposome, good biodegradability and biocompatibility, low toxicity, and low cost of production (164).

To achieve a promising GSH niosomal formulation, the physical and chemical stability of the carriers is essential to be investigated. Normally, the nanocarriers need to withstand the harsh acid environment of the stomach and the metabolism degradation caused by enzymes in the GI tract. In addition, the size of the niosomes is also important for efficient uptake and transportation across the epithelial membrane before releasing GSH to the targeting site of action.

In this chapter, a GSH-loaded niosomal formulation was developed and evaluated to enhance GSH stability against enzymatical degradation and improve the bioavailability via oral administration. The characterisation studies of the formulation started with factorial design considering the best formulation composition and optimal drug entrapment efficiency (EE), followed by the studies of particle size, zeta potential, morphology, stability and the in vitro drug release profile.

## 3.2 Aims

- To develop and optimise GSH-loaded niosomes using the factorial design approach
- To characterise the physicochemical properties of GSH-loaded niosomes, including particle size, zeta potential, morphology, entrapment efficiency, stability and drug release behaviour

## 3.3 Materials and methods

### 3.3.1 Materials

GSH, dihexadecyl phosphate (DCP), sorbitan stearate (Span® 60), polyoxyethylenesorbitan monooleate (Tween® 80), Triton™ X-100 and CH were purchased from Sigma-Aldrich (St Louis, MO, USA). Syringe filters (0.45 μm) were purchased from Membrane Solutions (Auburn, WA, USA). Milli-Q water was obtained from a Millipore RO system (Burlington, MA, USA). Other chemicals were all of analytical grade.

### 3.3.2 Formulation development and optimisation

#### 3.3.2.1 Niosome preparation

The preparation of GSH-loaded niosomes was carried out using the thin film hydration method. Briefly, three ingredients, CH, Span 60 and DCP in various ratios, were added to a 50 mL round bottom flask and dissolved with a 5 mL mixture of chloroform and methanol (4:1, v/v). The solvents were evaporated using a rotary evaporator (Rotavapor® R-215, Buchi, Flawil, Switzerland) at 48 °C on reduced pressure until the formation of a dry and thin film on the wall of the flask. To remove the residual organic solvents used, the flask with the film was then purged with nitrogen gas for 5 min. Afterward, the lipid film was hydrated by adding GSH solution (10 mg added into 10 mL water) for 1 h stirring at 52 °C. This yielded a GSH-loaded niosomal suspension, which was subsequently sonicated (Ultrasonic Homogeniser HD-2070, Bandelin Sonopuls, Berlin, Germany) for 3 min to obtain a homogeneous system of desired particle sizes.

#### 3.3.2.2 Factorial design

Factorial design is an approach to predict the best formulation composition for achieving maximum drug EE, which is more efficient and accessible in terms of different formulation parameters and the potential interactions between them. There are two steps involved in factorial design, including screening of formulation parameters and optimisation using central composite design and full factorial design (165). In this project, based on the literature reviewed, three independent factors (surfactant type, molar ratio of surfactant to CH, and GSH amount) were selected with two responses (EE and particle size). The design consisted of ten runs using a two-level full factorial design with one central point.

### 3.3.2.3 Characterisation of GSH-loaded niosomes

#### 3.3.2.3.1 Particle size, zeta potential and polydispersity index (PDI)

Size and surface charge properties of GSH-niosomes were evaluated using Zetasizer Nano series (Malvern Instruments, Malvern, Worcestershire, UK). Specifically, samples in triplicate were diluted 10-fold with Milli-Q water and tested at 25 °C. The average values of *Z*-average size, zeta potential and polydispersity index were then measured and evaluated.

#### 3.3.2.3.2 Entrapment efficiency (EE%)

EE is a crucial parameter for niosomal carriers, the EE% determination of GSH-loaded niosomes was carried out to separate the GSH-loaded niosomes from the free drug using an ultracentrifuge system (Sorvall WX80, ThermoFisher Scientific, MA, USA) at 41,000 rpm for 1 hour at 4 °C. After filtration through 0.45 µm, the supernatant was analysed quantitatively using the HPLC method introduced in chapter 2. The EE% was calculated using the following equation 3-1.

$$EE\ (\%) = \frac{total\ amount\ of\ drug\ added - amount\ of\ free\ drug}{total\ amount\ of\ drug\ added} \times 100 \qquad \text{Equation 3-1}$$

#### 3.3.2.3.3 Drug loading capacity (DL%)

DL% is another significant property for nanocarriers, the DL% determination of GSH-loaded niosomes was carried out according to equation 3-2 .

$$DL\ (\%) = \frac{total\ amount\ of\ drug\ added - amount\ of\ free\ drug}{total\ amount\ added\ (total\ drug + all\ excipients\ of\ niosome)} \times 100 \qquad \text{Equation 3-2}$$

#### 3.3.2.3.4 Morphology

Niosomal morphology was evaluated using an FEI Tecnai 12 transmission electron microscope (TEM, 3200 × magnification) (Hillsboro, OR, USA), operating at 120 kV accelerating voltage, which was connected to a camera (Ultrascan 1000, Gatan, Pleasanton, CA, USA). The samples of GSH-loaded niosomes were prepared on freshly glow-discharged R1.2/1.3 Cu 300 holey carbon grids (Quantifoil Micro Tools, Großlöbichau, Germany) and vitrified in liquified ethane using an FEI Vitrobot Mark IV.

#### 3.3.2.3.5 Physical stability study

The stability of GSH-loaded niosome suspension was evaluated at 4, 25 and 40 °C for 2 months. GSH-loaded niosomes were stored in a plastic tube with a screwed cap. Samples then were

withdrawn at pre-determined time points (30, 60 and 90 days) and subsequently studied for particle size and remaining GSH level.

Briefly, samples were ultracentrifuged to separate the free drug in suspension from the entrapped GSH in niosomes by ultracentrifugation as described in section 3.3.3.2. The niosome sediments were washed three times using Milli-Q waster, then dissolved by adding a solution of 10% v/v Triton X-100 in methanol using probe sonication for 10 minutes. After dilution and filtration, the dispersion was subjected to HPLC to measure the remaining GSH level.

Meanwhile, another set of samples of GSH-loaded niosomes powder were prepared. Specifically, after hydration with GSH, GSH-loaded suspension was ultracentrifuged at 41,000 rpm for 1 h at 4 °C. Then the sedimentations were stored at 4, 25 and 40 °C for 2 months. At each time point, niosomal powder was resuspended following the same protocol described above for suspension samples and subjected to HPLC analysis.

3.3.2.3.6 **In Vitro release study**

A 24-h *in vitro* release study of GSH-loaded niosomes was conducted using a dialysis membrane-based setup. Briefly, GSH-loaded niosomes were prepared using the method introduced previously in section 3.3.2.1. The suspension was centrifuged at 41,000 rpm for 1 h at 4 °C. The sediment was then resuspended using PBS (0.1 M, pH 6.8). A 2.5 mL aliquot of the suspension was added into a dialysis pouch made of synthetic dialysis tubing cellulose membranes (width 33 mm, pre-soaked in PBS overnight). After that, the dialysis bag was placed in a 50 mL tube (filled with 12 mL PBS as the release medium), which was then placed in a shaking water bath (GLS Aqua 18 Plus, Grant Instruments, Royston, UK) at 100 rpm and 37 °C. A sample of 0.4 mL was taken from the release medium at pre-determined time points (0, 0.5, 1, 2, 3, 5, 7, 10 and 24 h) that would be replaced with an equal amount of fresh PBS. The collected samples were then analysed using HPLC method. Controls were prepared by dissolving the equivalent amount of GSH in PBS before adding into a dialysis bag; then the same protocol would be followed. All niosome formulations and controls were tested in triplicates. Drug release was calculated using equation 3-3.

$$d\text{rug release (\%)} = \frac{amount\ of\ free\ drug\ in\ supernatant}{initial\ drug\ amount\ encapsulted\ in\ niosomes} \times 100 \qquad \text{Equation 3-3}$$

#### 3.3.2.3.7 Statistical analysis

Data was analysed using software Design-Expert® (called analysis of variance, ANOVA) for screening the impact of the three factors on EE% and particle size of those 10 GSH-loaded niosomal formulations.

## 3.4 Results and discussion

### 3.4.1 Effect of formulation parameters on particle size and EE%

During the development of new pharmaceutical formulations, there are various relevant factors involved that may affect the drug's EE, which is an important property of a formulation. Traditionally, a "one factor at a time" approach for screening factors has been utilised during this process, which means that only one formulation parameter would be altered at one time while other variables are kept constant to ensure the inclusion of all the possibilities. However, this manual process may result in a lengthy workload and a high chance of mistakes. In comparison, factorial design employs computer software to analyse the responses of the variables, providing the most efficient screening outcome without creating a significant increase in the number of runs (166,167). As a result, the cost of preparation of niosomes, the length of the time required and the effort of completing the screening will be considerably reduced.

Since high EE could potentially result in better therapeutic efficacy, EE% has been recognised as the main response for formulation screening process (168). However, the precise amount of drug loaded into the formulation should also be carefully considered. Particle size is another important property for formulation design, which will have a significant impact on the absorption across the biological membrane hence bioavailability. In this project, EE% and particle size were selected as the two responses to assess the impact of the three variable factors: GSH dose, type of surfactants and molar ratio of surfactant:CH. Using $2^3$ full factorial design, a total of 10 formulations were prepared including 2 central point runs. The results shown in Table 3-1 illustrated that EE% remained between 21.72 and 34.48% for all 10 formulations, and particle size was between 250.67 and 603.40 nm. Hence formulation #2 and #9 were the best compositions to achieve relatively high EE% and small particle size.

**Table 3-1.** Screening design of GSH-loaded niosomes and their response: EE (%), particle size (A: GSH amount for hydration, B: type of surfactant, C: molar ratio of surfactant:CH, total lipid load of 150 µmol) (Mean ± SD, n=3).

| | | Variable factors | | Response | |
|---|---|---|---|---|---|
| **Run** | A (mg) | B | C | EE (%) | Particle Size |
| 1 | 2 | Span60 | 1.5 | 23.42 ± 1.27 | 416.07 ± 5.28 |
| 2 | 10 | Span60 | 1.5 | **31.45 ± 0.46** | 250.67 ± 3.01 |
| 3 | 2 | Span80 | 1.5 | 27.35 ± 0.42 | 467.03 ± 5.91 |
| 4 | 10 | Span80 | 1.5 | 29.99 ± 0.76 | 566.40 ± 7.30 |
| 5 | 2 | Span60 | 4 | 27.14 ± 1.55 | 603.40 ± 1.44 |
| 6 | 10 | Span60 | 4 | 26.77 ± 0.89 | 586.27 ± 6.96 |
| 7 | 2 | Span80 | 4 | 27.96 ± 2.17 | 411.10 ± 3.58 |
| 8 | 10 | Span80 | 4 | 21.72 ± 0.68 | 455.27 ± 7.34 |
| 9 (central point) | 6 | Span60 | 2.75 | **34.48 ± 0.44** | 385.43 ± 5.30 |
| 10 (central point) | 6 | Span80 | 2.75 | 25.62 ± 2.02 | 557.57 ± 7.22 |

### 3.4.2 Optimisation of GSH-loaded niosomal formulation

Figure 3-1 displays how EE% responds in relation to the impact caused by variables A (drug amount) and C (molar ratio of surfactant:CH) when Span 80 (factor B) was selected, while Figure 3-2 displays their relationship when Span 80 was replaced by Span 60. Both figures indicate that a higher EE% appeared to be achieved when GSH amount range was 8-10 mg and the molar ratio of surfactant:CH range was 1.5-2:1 regardless of the surfactant type used. These results correlated to the data in the cube figure (Figure 3-3), which illustrated the relationship between EE% and all the three variables. The best value of EE% (31.16%) in the cube figure is observed when factors A, B and C were at 10 mg, Span 60 and 1.5, respectively, which reflected the second-best experiment result of EE % among all 10 runs.

In terms of particle size response, its relationship with two factors A and C are illustrated in Figures 3-4 and 3-5 when using Span 80 and Span 60, respectively. In Figure 3-6, a cube figure summarises the relationship between the particle size and all three variable factors A, B and C. Comparing the findings of both Figure 3-4 and 3-5, the best value of particle size was achieved when the composition consisted of Span 60 and molar ratio of surfactant:CH was between 3:2. This finding reflected the same results in the cube figure 3-6, which presented that the best value of the particle size of 250 nm was achieved when the value of the factors A, B and C were 10 mg, Span 60 and 1.5, respectively, which were confirmed by experimental results.

By considering all data and findings, the formulation for preparation of GSH-loaded niosomes for further development was chosen, which is composition #2, consisting of Span 60, molar ratio of surfactant:CH at 1.5 and 10 mg GSH. Although the achieved EE% (31.45 ± 0.46) was the second best, the total loading amount of GSH to niosomes was the highest among all 10 runs. Additionally, the particle size (250.67 ± 3.01 nm) of this composition was the smallest among all.

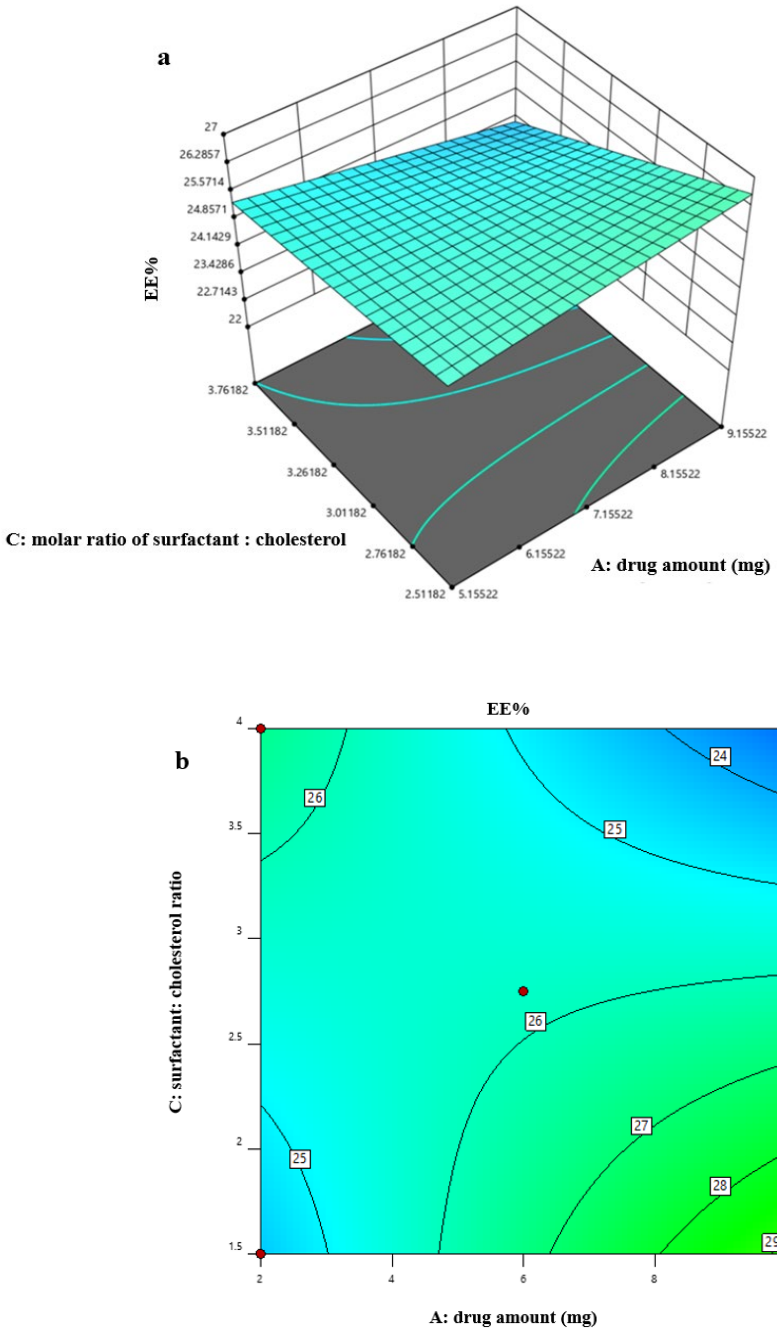


**Figure 3-1**.a) Three-dimensional surface plot for EE% as a function of GSH-loaded niosomes variables. b) Contour plot for EE% as a function of GSH-loaded niosomes variables when using Span 80**.**

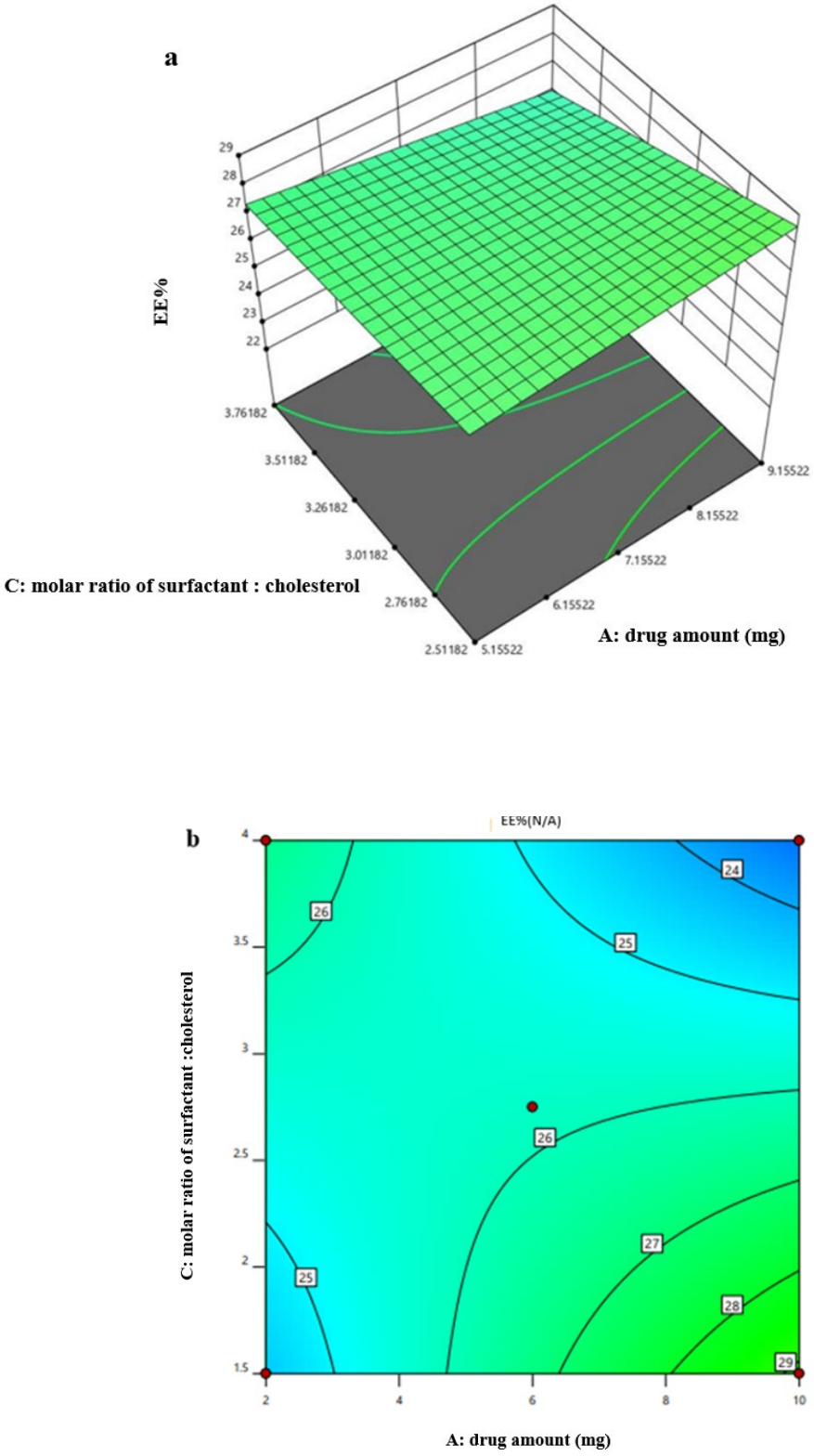


**Figure 3-2.** a) Three-dimensional surface plot for EE% as a function of GSH-loaded niosomes variables. b) Contour plot for EE% as a function of GSH-loaded niosomes variables when using Span 60.

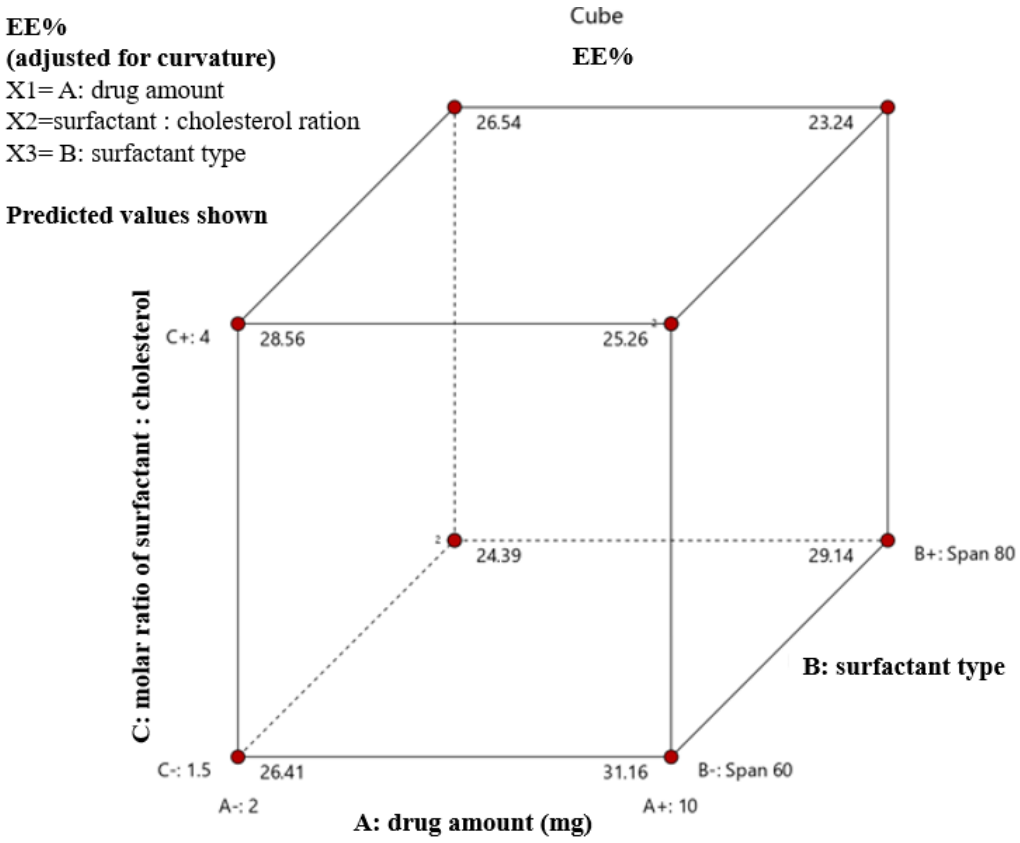


**Figure 3-3**. Cube plot for EE% as a function of the GSH-loaded niosomes variables (all factors (A: drug amount; B: surfactant type; C: molar ratio of surfactant:CH).

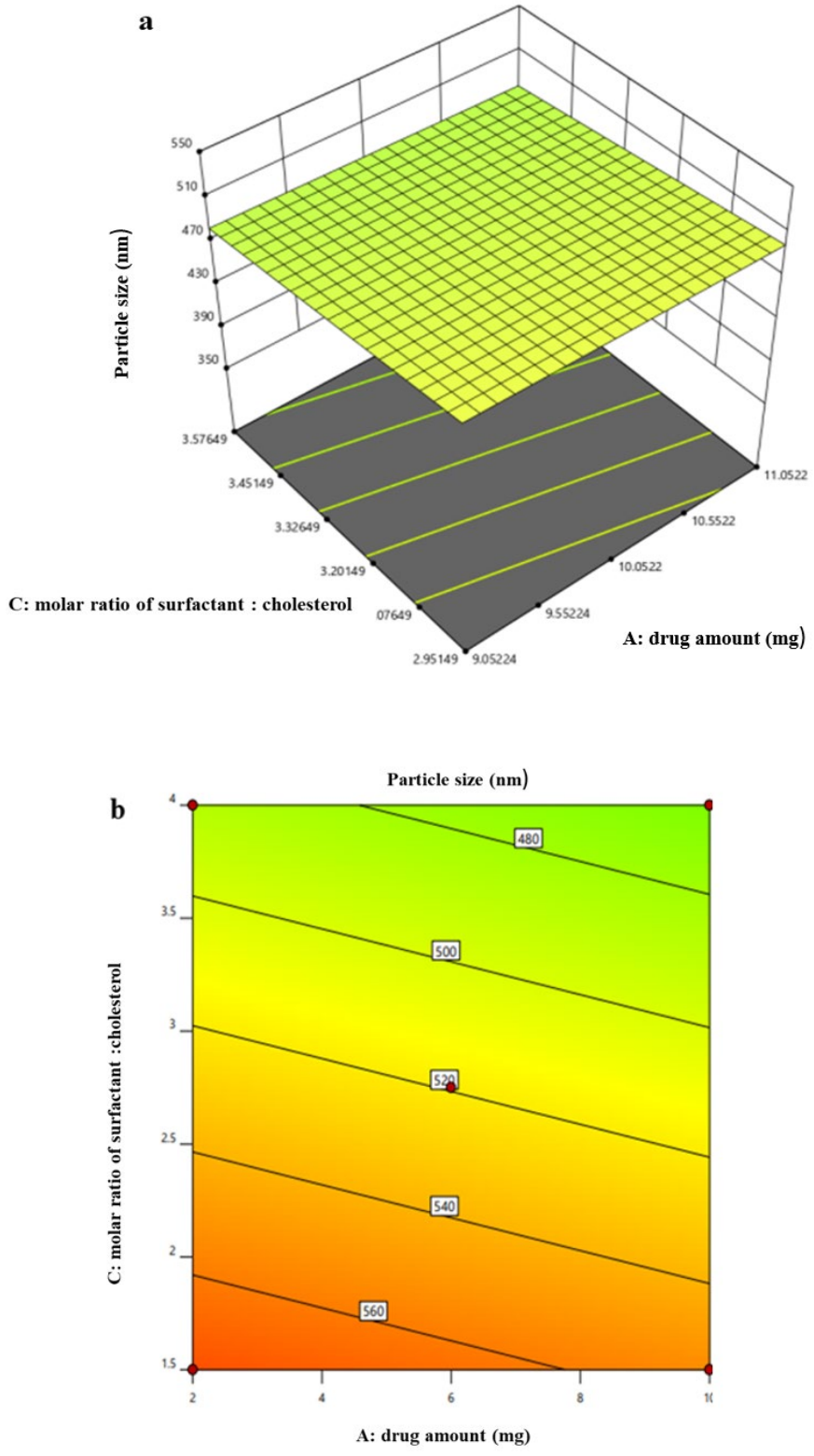


**Figure 3-4.** Three-dimensional surface plot for particle size as a function of GSH-loaded niosomes variables. b) Contour plot for particle size as a function of GSH-loaded niosomes variables when using Span 80.

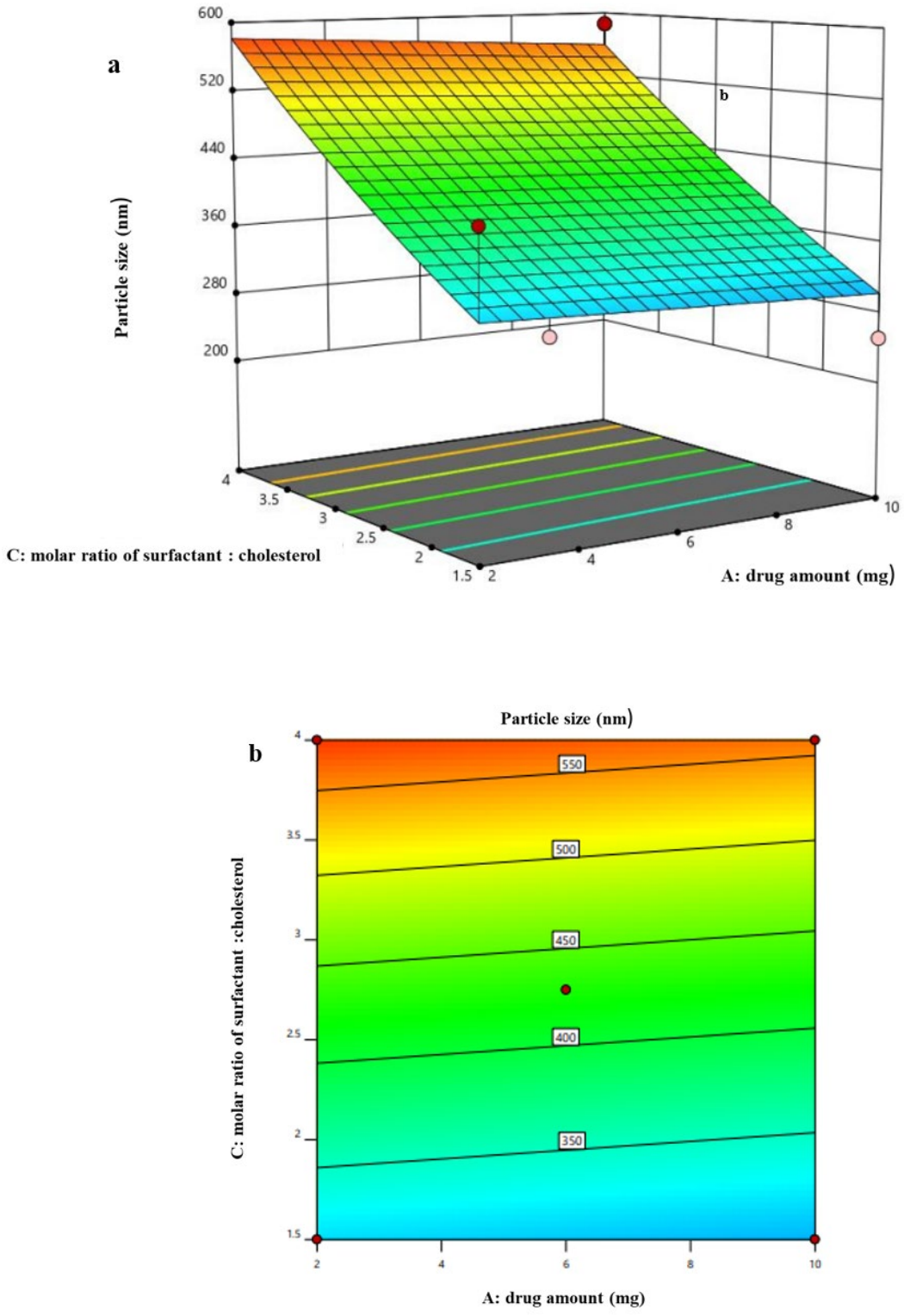


**Figure 3-5.** Three-dimensional surface plot for particle size as a function of GSH-loaded niosomes variables. b) Contour plot for particle size as a function of GSH-loaded niosomes variables when using Span 60.

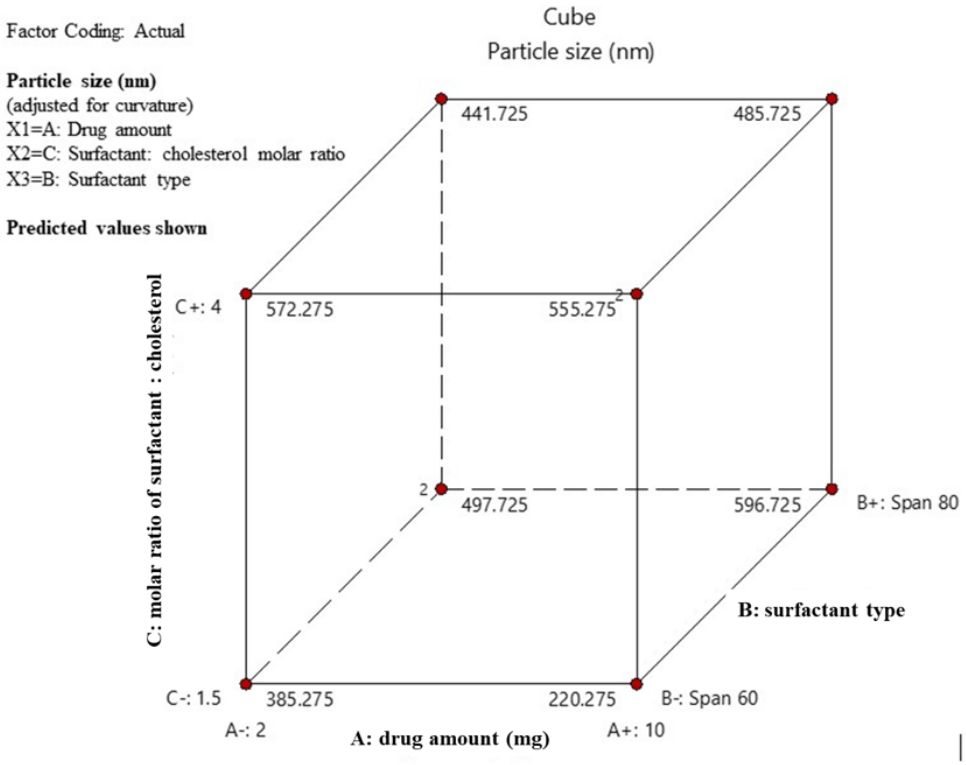


**Figure 3-6.** Cube plot for particle size as a function of the GSH-loaded niosomes variables (all factors (A: drug amount; B: surfactant type; C: molar ratio of surfactant:CH).

### 3.4.3 Characterisation of GSH-loaded Niosomes

#### 3.4.3.1 Particle size, Zeta potential, polydispersity index (PDI), EE% and DL%

The values of particle size, zeta potential and PDI of the GSH-loaded niosomes are displayed in Table 3-2. The average value of the particle sizes was 253.3 ± 0.6 nm and the PDI was 0.353 ± 0.028 (less than 0.5), indicating the size distribution of the particle was within the acceptable range. Figure 3-7 illustrates the size distribution of the tested GSH-loaded niosomes.

The value of zeta potential indicates the formulation's physical stability. It has been known that a formulation with a zeta potential value below -30 mV or above 30 mV would be stable during storage (169). The test result from the experiment was -65.3 ± 3.5 mV (< 30 mV), suggesting the resulting niosomal formulation was a stable system.

**Table 3-2**. Results of particle size, zeta-potential and PDI of GSH-loaded niosomes (Mean ± SD; n=3).

| Name | Particle size (nm) | PDI | Zeta potential (mV) |
|---|---|---|---|
| **GSH-loaded niosomes** | 253.3 ± 0.6 | 0.353 ± 0.028 | -65.3 ± 3.5 |

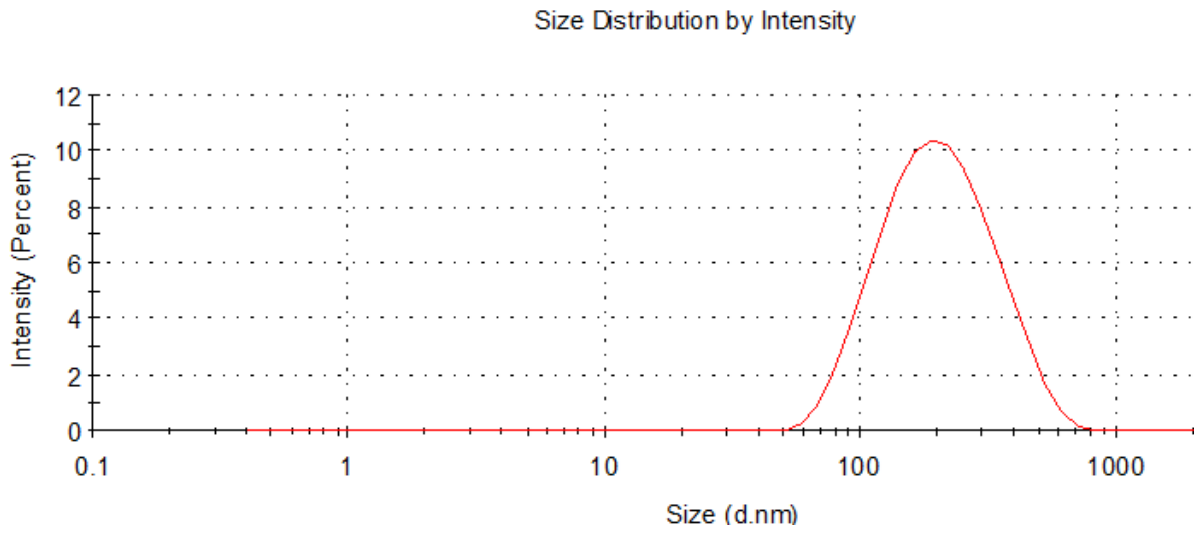


**Figure 3-7.** The particle size distribution of GSH-loaded niosomes**.**

Through the assessment of ten different formulations displayed in Table 3-2, it was found that the chosen formulation would produce an EE% value of 31.45% ± 0.46. DL% of the selected formulation was found to be 4.49 ± 0.24 % % for further characterisation. The data showed acceptable EE% and DL% for nanocarriers according to literature (59).

### 3.4.3.2 Morphology

The morphological features of the optimised GSH-loaded niosomes were observed using TEM (Figure 3-8). The obtained images showed closed spherical vesicular shape nanoparticles with a double-layered structure in a narrow size range between 100 to 350 nm. This result of particle size was supported by the previous particle size study.

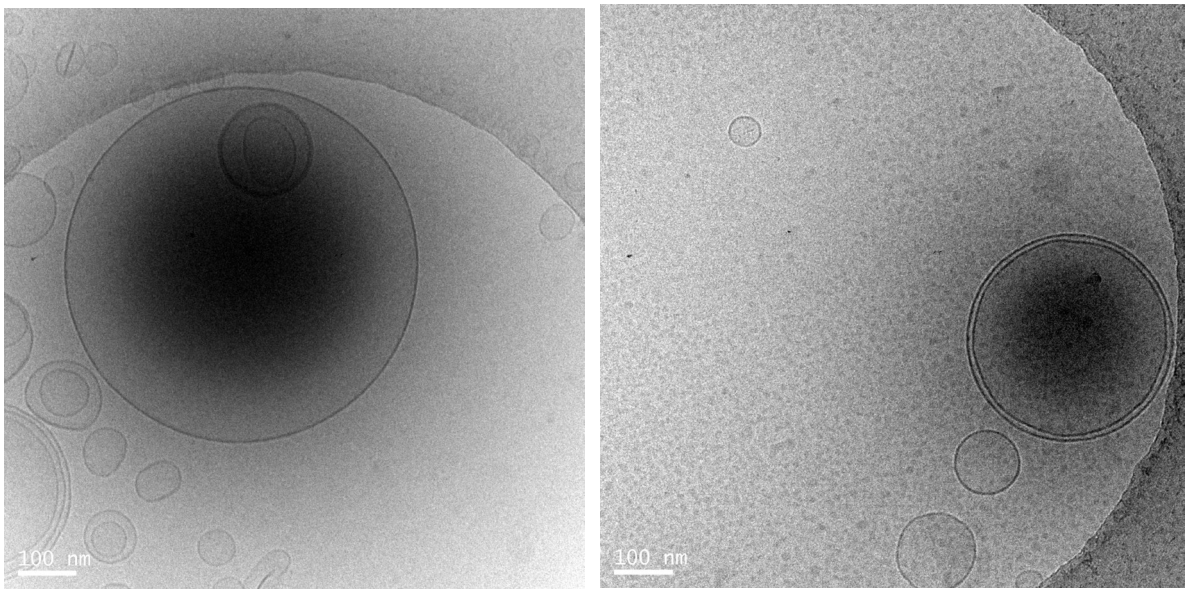


**Figure 3-8.** TEM images of GSH-loaded niosomes**.**

### 3.4.3.3 Physical stability

Over a 3-month period, the stability of GSH-loaded niosome suspensions stored at different temperatures was evaluated by their particle size and the remaining level of GSH in niosomes. The results were summarised in Tables 3-3 and 3-4.

Data in Table 3-3 indicates that the temperature had a significant impact on the particle size of GSH-loaded niosomes. Namely, the higher the temperature, the larger the particle size at any time point. This could result from increased thermal energy in the system, causing a higher rate

and force of collision between niosome particles compared to the ones at a lower temperature. Therefore, when vesicle aggregation occurred, it resulted in enlarged particle sizes. This suggested that a GSH entrapped in niosome was more stable at 4 ℃ than at 25 ℃ and 40 ℃.

The other important parameter of niosomal stability was the percentage of remaining GSH in formulations (Table 3-4). In general, there were decreases in the level of GSH retained in all formulations at all three temperatures over the 3-month period. In detail, within each group of formulations, the decrease in GSH level at 4 ℃ was the smallest among all three temperatures ($p<0.05$). Between groups, the change in remaining level of GSH of the two GSH-entrapped formulations (suspension and powder) appeared smaller than the one in the control group at each temperature, suggesting a significant protecting effect of niosomes on encapsulated GSH ($p<0.01$). Furthermore, the niosome powder form seemed to have slowed the leaking rate of GSH from the niosomes at the 2-month time point.

**Table 3-3.** Particle sizes (nm) of GSH-loaded niosomes in suspension were stored at different temperatures during 2 months (mean ± SD; n =3).

| Items | Conditions | Initial | 1 month | 2 months |
|---|---|---|---|---|
| **Particle size (nm)** | 4℃ | 247.4 ± 8.1 | 254.6 ± 4.2 | 258.3 ± 12.3 |
| | 25℃ | | 279.8 ± 3.9 | 293.5 ± 5.2 |
| | 40℃ | | 282.9 ± 19.7 | 314.1 ± 2.8 |

**Table 3-4.** GSH remaining (%) in niosomes stored as suspension, powder form and GSH solution (control group) at different temperatures during a 2-month period (mean ± SD; n =3)

| Items | Conditions | Initial (%) | 1 month | 2 months |
|---|---|---|---|---|
| GSH-loaded niosomal suspension | 4 ℃ | 100 | 89.6 ± 7.3 ** | 65.0 ± 3.8 ** |
| | 25 ℃ | | 72.5 ± 1.6 ** | 52.9 ± 5.4 ** |
| | 40 ℃ | | 68.1 ± 14.3 ** | 32.2 ± 1.6 ** |
| GSH-loaded niosomal powder | 4 ℃ | 100 | 87.9 ± 2.1 ** | 74.8 ± 1.8 ** |
| | 25 ℃ | | 64.8 ± 7.9 ** | 62.2 ± 4.0 ** |
| | 40 ℃ | | 66.2 ± 3.3 ** | 56.8 ± 5.0 ** |
| GSH solution | 4 ℃ | 100 | 60.6 ± 5.0 | 43.5 ± 3.6 |
| | 25 ℃ | | 26.2 ± 3.7 | 4.3 ± 1.0 |
| | 40 ℃ | | 0 | 0 |

** P < 0.01(compared to GSH control group)

### 3.4.3.4 *In Vitro* Release Study

*In vitro* release study is generally known as an important parameter for the development of new formulations, to ensure the adequate release profiles of the drug molecule could be achieved for the therapeutic purpose (170). Data of the 24-h release study of GSH-loaded niosomes and solutions are shown in Figure 3-9.

Data showed that GSH niosomal formulations presented a two-stage release profile: a fast release of GSH in the first 5 h, followed by a sustained release till the end of 24 h, with more than 45% cumulative GSH release. In contrast, GSH solution displayed different releasing behaviour: a rapid release of GSH in the first 3 h and reached maximum release (90%) at about 5 h, then levelled off till the end of 24 h. There was a drop in the concentration of GSH solution at the 24-h point, which may be due to the GSH degradation during the last few hours, whilst the GSH-niosomes showed a constant GSH-level over the 24-h period.

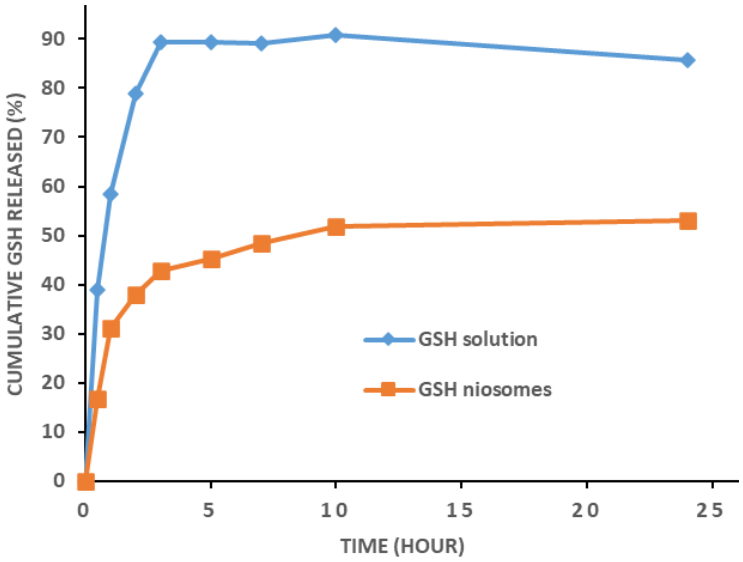


**Figure 3-9.** Release profiles of GSH-niosomes and GSH solution (mean ± SD, n=3).

Drug dissolution and release kinetics are well recognised as the important parameters for all forms of therapeutic formulations, such as solid forms (tablets and capsules), semi-solid forms (cream and ointment) and modified release forms (sustained and prolonged dosage forms) during the intended period of treatment. It is also crucial to understand the drug release patterns for the design and optimisation during the development stage of a new formulation (171).

To determine the mechanism of drug release, the data was collected and evaluated using different kinetic models. This was completed by assessing the goodness of fit of the coefficient

of determination ($r^2$) using linear regression analysis based on the chosen kinetic model. Four kinetic models, zero order, first order, Higuchi and Korsmeyer-Peppas model, were researched for this study (171,172). All the results are illustrated in Table 3-4.

Among all these four kinetic models, the data of this release study fitted Korsmeyer-Peppas model very well with a $r^2$ value of 0.93. The next highest value of $r^2$ (0.874) was obtained when using Higuchi model. The $r^2$ values of zero order and first order were 0.64 and 0.336, respectively. Therefore, findings suggested that the release profiles of GLNs complied with the Korsmeyer-Peppas model rather than the other three. Furthermore, the n value of 0.623 (between 0.43 and 0.85) indicated the sample followed non-Fickian release from non-swellable spherical vesicles. Hence the mechanism of GSH niosome release was a process of initial diffusion (due to a molecular gradient) followed by a relaxational release caused by niosome disentanglement and erosion (171).

**Table 3-5.** Parameters of GLNs using different kinetic models**.**

| Formulation | Zero order | | First order | | Higuchi model | | *Korsmeyer-Peppas model* | | |
|---|---|---|---|---|---|---|---|---|---|
| | ***r2*** | ***k0*** | ***r2*** | ***k1*** | ***r2*** | ***kh*** | ***r2*** | ***n*** | ***kk*** |
| **GSH-niosome** | 0.64 | 0.067 | 0.336 | 0.002 | 0.874 | 2.041 | ***0.93*** | *0.623* | *1.48* |

## 3.5 Conclusion

GLNs were fabricated using a thin film hydration strategy, which was then characterised and optimised to determine the best composition, aiming for the desired entrapment efficiency. In the selected formulation, Span 60 was chosen as the surfactant, with a molar ratio to the CH of 3:2 (total 150 µmol of Span 60 and CH), and DCP of 2 µmol was added to produce negatively charge niosomes. Then the resulting lipid film was hydrated for 1 h at 58 °C in 10 mL of distilled water containing 10 mg GSH. Through various characterisation studies, it appeared that the obtained GLNs has achieved acceptable parameters in terms of particle size (250-400 nm) and entrapment efficiency (31.45%). Electron microscopy revealed the double-layered structure of niosomes with spherical shapes. The release study has displayed a two-phased release profile: fast diffusion initial stage followed by sustained release erosion release. Data from the stability study provided evidence that temperature could broadly impact the stability of GLNs, on both particle size and remaining levels of GSH in formulations. Therefore, the proposed storage condition would be at a lower temperature such as at 4 °C at which GSH level

could be retained as high as 90% of the initial loading dose by the end of the first month of storage.

To sum up, niosomes could be applied as a potential oral drug delivery carrier with sustained release character. In the next chapter, the cellular uptake and transport profiles of GSH-entrapped niosomes will be evaluated.

# Chapter 4

# Cellular uptake and transport study of GLNs

# 4 *In vitro* cytotoxicity, degradation, and cellular uptake and transport studies of GLNs

## 4.1 Introduction

In Chapter 2, a comprehensive physicochemical characterisation was investigated, it provided critical information on the behaviour of a formulation in the physiological environment. In this chapter, the experiments used the human epithelial cell line (Caco-2), originally derived from a colon carcinoma, as a model of the intestinal epithelial barrier to investigate GLNs' safety, enzymatical degradation, uptake, and transport to obtain the essential information to improve GSH oral bioavailability.

GLNs was regarded as ideal formulation candidate and expected to deliver GSH via oral administration. Therefore, it is important to understand its behaviour inside the GI tract. The Caco-2 monolayers differentiated spontaneously in culturing environment, which has many characteristics mimicking to absorptive enterocytes in the small intestine (173). Hence, cytotoxicity and uptake studies using Caco-2 cell line are considered to be a suitable approach for the investigation either of the safety of the formulation or the mechanism of whether or not the internalisation of GLNs occurs.

Moreover, as the most cost-effective strategy, *in vitro* study, which includes cytotoxicity study and degradation study, may provide valuable data on GLNs in the safety assessment with a final goal of assessing its risk to a human at a fundamental level of cells. It can also provide critical information of its key component in the assessment of stability under controlled conditions (174) when influenced by pH and enzymes (e.g. trypsin, elastase, chymotrypsin and carboxypeptidase produced by the pancreas, and dipeptidase and aminopeptidase produced by brush border membranes) (175).

In this chapter, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay was applied to quantify the toxicity of GLNs, which is a tool to estimate the metabolic activity of living cells. The mechanism of this assay is based on the enzymatic reduction of tetrazolium salt (light colour) to its formazan (purple-blue colour), which can be evaluated using a spectrophotometer, of which the value of absorbance is correlated to the amount of living cells (176). *In vitro* degradation study of GLNs was conducted using luminal and mucosal extract of rats from four different regions (duodenum, jejunum, ileum and colon).

The cellular uptake and transport and its mechanism studies of GLNs were carried out using Caco-2 cell model and the results were analysed using HPLC and flueresenct microscopy.

## 4.2 Aims

- To investigate the *in vitro* cytotoxicity of the GLNs on Caco-2 cells
- To investigate the *in vitro* degradation activity of GLNs under simulated gastrointestinal conditions
- To evaluate the cellular uptake and transport of GLNs using cell culture models in the absence or presence of variable inhibitors

## 4.3 Materials and method

### 4.3.1 Materials

The Caco-2 cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). FBS, DMEM, PBS, 0.25% trypsin-EDTA, NEAA, penicillin-streptomycin (10,000 U/mL), and 0.4% trypan blue solution were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Pierce$^{TM}$ BCA protein assay kit was obtained from Thermo Fisher Scientific (USA). Fluorescein isothiocyanate (FITC), trifluoroacetic acid, CH (Sigma grade, ≥99%), and bacitracin were purchased from Sigma-Aldrich (St. Louis, MO, USA). EDTA, methanol, and acetonitrile were purchased from Merck (Whitehouse Station, NJ, USA). All other reagents and chemicals were of analytical grade.

### 4.3.2 Method

#### 4.3.2.1 Cell culture

Caco-2 cells were cultured in a complete DMEM (with additional 10% FBS, 1% penicillin-streptomycin and 1% NEAA) in a tissue culture flask and then incubated at 37 °C (5% $CO_2$ and 95% relative humidity). The DMEM was changed daily until 80% cell confluence was observed with an EVOS™ XL Core Imaging System (Thermo Fisher Scientific, Waltham, MA USA).

#### 4.3.2.2 Cytotoxicity study

MTT assay was employed to identify the toxic threshold of GSH. Caco-2 cells in DMEM were seeded into a 96-well plate (BD Falcon ™, BD, New Zealand) at a rate of 20.000 cells per well and then incubated for 24 h at 37 ℃ in an incubation chamber. Next, the medium in each well was then replaced by GSH solution (200 µL dissolved in DMEM) at concentrations of 0, 0.01, 0.05, 0.10, 0.50, 1.00 and 5.00 mg/mL. Once incubated for 12 and 24 h at 37 ℃, GSH solutions were then replaced using 200 µL MTT (0.5 mg/mL) followed by 4 h incubation at 37 ℃. Then MTT was discarded before cells were washed with PBS (pre-warmed). Then 200 µL dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals, followed by 10 min incubation at 37 ℃. After being gently shaken, absorbance was obtained using a microplate reader (Varioskan™ LUX, Thermo Fisher Scientific, Waltham, MA, USA) at 570 nm.

Cell viabilities were calculated for each concentration of GSH using equation 4-3.

$$V = \frac{A}{A_0} \times 100\% \qquad \text{Equation 4-3}$$

(Where V represents the cell viability, A represents the absorbance of the GSH solution and $A_0$ is the absorbance of the control).

#### 4.3.2.3 *In vitro* degradation study

##### 4.3.2.3.1 Isolation of luminal extracts and mucosal homogenates

Adult male Wistar rats (from the Vernon Jansen Unit, the University of Auckland, New Zealand) were euthanised by inhalation of highly concentrated $CO_2$. An operation of a midline incision was performed, and the intestine was removed. Once identified, duodenum, jejunum, ileum, and proximal colon were then sectioned. The luminal contents of each region were collected and diluted with 0.125 M NaCl solution before centrifuged at 21,000 *g* for 15 min at 4 °C. After adding 5 mL of phosphate-buffered saline (PBS; 50 mM, pH 7.4), the supernatants were divided into small aliquots of 1 mL and stored at 80 °C. A similar protocol was applied for obtaining mucosal extracts. After washing with PBS (50 mM pH 7.4), the mucosal surface was removed with the edge of a microscope slide, then mucosal tissue containing epithelial cells layers was collected immediately. Once the tissue contents were suspended (5 mL of PBS)

and centrifuged (21.000 *g* for 15 min at 4 °C), the supernatants were divided into small amounts (1 mL each) and stored at 80 °C (177).

#### 4.3.2.3.2 **Determination of enzyme concentration in luminal and mucosal homogenates**

Pierce® BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine enzyme concentration in luminal and mucosal extracts followsing the manufacturer manual. Specifically, nine albumin (BSA) standards were prepared by dilution with concentrations ranging from 0 to 2000 µg/mL. Then the BCA (bicinchoninic acid) working reagent (WR) was prepared by mixing reagents A and B at a ratio of 50:1, followed by adding 25 µL of each standard or testing sample into a 96-well plate (as three replications). 200 µL of the prepared WR was then added to each well, shaking for 30 s. After the cover was replaced, the plate was incubated for 30 min at 37 °C, then cooled down to room temperature. Then the absorbance at 562 nm was determined using a microplate spectrophotometer (Varioskan™ LUX, Thermo Scientific, Waltham, MA, USA) (178). The BSA standard curve was then generated by plotting the readings of absorbances and used to calculate the total amount of protein (enzyme) of all samples.

#### 4.3.2.3.3 **Proteolytic activity of GSH in luminal and mucosal extracts**

For the preparation of protein substrate, firstly GSH was dissolved in PBS (0.1M, pH 7.4) to make a solution (1 mg/mL). Then GSH solution and frozen luminal and mucosal contents of each intestinal region (stored at -80 °C) were warmed to 37 °C in a water bath. Enzyme-substrate mixture (1/5 w/w) was prepared and incubated at 37 °C. Samples (250 µL) were withdrawn at predetermined time points (0, 30, 60, 120, 180, 270 and 360 min), of which the enzymatic activity was terminated by adding 50 µL of HCl (0.1M). After centrifugation at 21,000 *g* for 5 min at 4 °C, the supernatants were then evaluated using the HPLC method described in Section 2.3.2.1.

#### 4.3.2.3.4 **Inhibition of proteolysis of GSH by enzymatic inhibitors and by niosomal carrier system**

The evaluation of enzymatic inhibition *in vitro* was conducted by incubation of GSH solution with or without inhibitors. Two inhibitors were chosen: bacitracin and EDTA. Specifically, luminal, or mucosal extracts (50 µL) from each rat intestinal region (duodenum, jejunum, ileum, and colon) was mixed either with PBS (0.1 M, pH 7.4, 50 µL) as control or with enzymatic inhibitor solution (dissolved in PBS, 50 µL) then incubated for 30 min at 37 °C. The

concentration of bacitracin and EDTA solutions were 2.0 mM and 1 mg/mL respectively. Then the mixture was added to the GSH solution (1 mg/mL) to generate an enzyme-substrate (at a ratio of 1:5 w/w) and incubated for 6 h at 37 °C. Then a sample of 250 µL was withdrawn and added to 50 µL of 0.1 M HCl to terminate the enzymatic activity. After centrifugation at 21,000 *g* for 5 min at 4 °C, the supernatant was analysed using HPLC to determine the remaining concentration of GSH.

The inhibition of proteolysis of GSH by niosomal carrier system study was conducted by using the mixture of equal portions of luminal and mucosal contents to mimic the real intestinal environment. Briefly, the same volume of luminal and mucosal extracts from four rat intestinal regions was mixed and warmed at 37 °C for 30 min. The four mixtures were then either added to pure GSH solution (1 mg/mL) as control or GLNs (equal to 1mg/mL). Following 6 hincubation at 37 °C, an aliquot of 250 µL was taken from each sample, with the addition of 0.1 M HCl (50 µL) to terminate the enzymatic activity. 10% Triton-X-100 (200 µL) was then used to destroy the structure of niosomes, releasing entrapped GSH. Samples were centrifuged (21,000 *g* for 5 min at 4 °C), and the supernatants were then analysed using HPLC to determine the concentration of remaining GSH.

#### 4.3.2.4 Investigation of niosomal uptake into Caco-2 cells

A fluorescent microscope (CLSM, Olympus FV1000; Olympus, Heidelberg, Germany) was employed to investigate whether the labelled niosomes (with FITC) were intracellularly absorbed or superficially adsorbed onto the cell surface. 8-well chamber slides were seeded with Caco-2 cells (with a density of $5 \times 10^4$ cells/cm$^2$) that were cultured in DMEM. Meanwhile, FITC-labelled niosomes were produced by replacing GSH with FITC, which is the same method used for preparation of GLNs (introduced in section 4.3.2.1). After adding the FITC-labelled niosomes (0.2 mL), Caco-2 cells in the slides were incubated for 1 h at 37 °C. The cells were then washed 3 times with ice-cold Hank's balanced salt solution (HBSS) and incubated for 15 min. Once equilibrium was achieved, cells were stained by CellTracker™ Red CMPTX (cytoplasm dye, Invitrogen, Thermo Fisher, USA) (0.2 mL, 5µM in HBSS) and incubated for a further 5 min at 37 °C. After being washed with HBSS again, paraformaldehyde solution (4% w/v, freshly prepared) was added to the Caco-2 cells and left on for 15 min at 25°C. After being rinsed twice with PBS, the Caco-2 cells (nuclei) were stained with 4,6-diamidino-2-phenylindol (DAPI, nuclei dye, Invitrogen, Thermo Fisher, USA, 300 nM in HBSS) and incubated for 5 min at 37 °C. To prevent bleaching effects when analysing using

high-powered laser fluorescent microscopy, the slides were removed from the chamber under darkness, washed with HBSS, and mounted with CitiFluor™ AF1 solution. Then the slides were covered with coverslips and then sealed with nail polish. The slides were observed using fluorescent microscopy with 405 nm (blue), 473 nm (green) and 559 nm laser lines. 3D images of the cells were captured using an oil immersing lens (60/1.35) and processed by the FV10-ASW 4.2 Viewer software (Olympus, Heidelberg, Germany). Through visualising at different planer sections of the cells, it can confirm whether the FITC-labelled niosomes were internalised by the cells.

#### 4.3.2.5 Transport study of GSH with or without niosomes using Caco-2/HT29 cells model

To carry out transport study, the Caco-2 and HT29 cells with the ratio of 9:1 were co-cultured and seeded into a 6-well Corning® Transwell® culture plate (0.4 μm in diameter per pore and 4.67 $cm^2$ per well) (Corning, New York, USA). Then in an environment of 5% $CO_2$ and 95% relative humidity at 37°C, cells were incubated in a complete medium (1.5 mL on the apical side and 3.0 mL on the basolateral side of the well). The complete medium was changed in a three-day interval. The transepithelial electrical resistance (TEER) of the co-cultured cells was measured to monitor its integrity by using a device consisting of an EVOM™ Volt-Ohmmeter and a set of Evom STX3 electrodes (World Precision Instruments, FL, USA) until values reached 350 Ω.$cm^2$. TEER value can be determined by using the equation 4-4:

$$TEER = (R_{cell} - R_{blank}) \times A \qquad \text{equation 4-4}$$

Where $R_{cell}$ and $R_{blank}$ are the TEER value of the test and blank group, respectively, and A is the area value of the insert in $cm^2$.

Caco-2/HT29 cells were stabilized at 37°C for 30 min by adding HBSS before the experiment. Selected chemicals (EDTA and sodium taurocholate) were first dissolved in DMSO before diluting to a certain concentration using HBSS, which were then mixed with GSH or GLNs. Then HBSS on the apical side of the transwell insert was replaced either with 1.5 mL of GSH (1mg/mL in HBSS), GLNs (equal to 1mg/mL in HBSS) or chemical-treated GSH/GLNs (equivalent to 1mg/mL), while the basolateral side was filled with 3 mL of HBSS. At the predetermined time points (0.5, 1, 2 and 3 h), an aliquot of 400 μL sample from the basolateral side was withdrawn and replaced immediately with the same amount of pre-warmed HBSS.

The samples collected (triplicates) were then analysed for the determination of GSH concentration using HPLC method introduced in chapter 1.

## 4.4 Results and discussion

### 4.4.1 Cytotoxicity

The cell viability in all groups were above 100% (Figure 4-1). After 12 h of incubation, the Caco-2 cell viability appeared to have similar results regardless of the concentration of free GSH ($p>0.05$). However, after 24 h of incubation, there was an increase in Caco-2 cell viability in all groups. The highest increase was observed in the group with a concentration of 5.0 mg/mL at about 300% ($p<0.01$). These findings indicated that GSH did not show any toxic effect on Caco-2 cells within 24 h of incubation when the concentrations of GSH were between 0.01 to 5.0 mg/mL. This result agrees on the theory mentioned in section 1.3.1 (179) that endogenous GSH plays an important role as an antioxidant in cell survival and health.

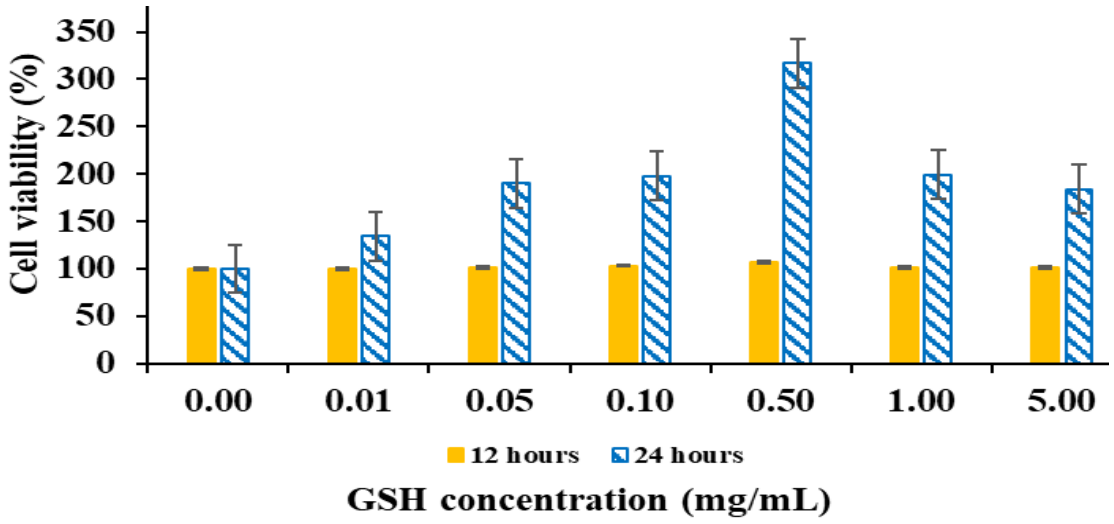


**Figure 4-1.** Impact of GSH solution (of different concentrations) on the Caco-2 cell viability after 12 and 24-h incubation at 37 °C.

### 4.4.2 *In vitro* degradation study

#### 4.4.2.1 Proteolytic activity of GSH in luminal and mucosal extracts

The enzyme concentrations of both luminal and mucosal contents (as defined as total protein concentration) collected from duodenum, jejunum, ileum, and colon regions are displayed in

Table 4-1. The enzyme concentrations of luminal and mucosal contents in the jejunum were the highest among the regions at 12.23 ± 0.15 and 5.36 ± 0.02 mg/mL, respectively. Compared to other studies reported by other authors using the same luminal and mucosal contents (177), the quantity of enzymes in all other luminal and mucosal extracts (except extracts in jejunum) was significantly lower ($p<0.01$). It could be due to the long period of storage, which may contribute to the inactivation to the luminal or mucosal enzymes except those ones from jejunum. However, because GSH degradation mainly takes place in jejunum (88) and the ratio of GSH-enzyme subtract was considered based on the concentration of enzymes in each extract to achieve comparable results, the impact of the low concentration of enzymes on the data collected from this study would remain minimum.

**Table 4-1.** Enzyme concentrations of luminal and mucosal samples in different regions of rat intestine (mean ± SD, n=3).

| **Samples** | **Estimated enzyme concentration (mg/mL)** |
|---|---|
| **Luminal extracts** | |
| Duodenum | 0.96 ± 0.01 |
| Jejunum | 12.23 ± 0.15 |
| Ileum | 0.58 ± 0.01 |
| Colon | 0.29 ± 0.01 |
| **Mucosal homogenates** | |
| Duodenum | 0.93 ± 0.04 |
| Jejunum | 5.36 ± 0.02 |
| Ileum | 0.09 ± 0.01 |
| Colon | 0.27 ± 0.03 |

Data (Figure 4-2 and Figure 4-3) indicated the enzymes that existed in luminal content in the ileum displayed the most active enzymatic behaviour (less than 10% GSH remaining after 6 h) followed by luminal contents in the jejunum and duodenum. The most inactive enzymatic activity was seen in mucosal contents in the colon with 84.45 ± 0.83 % GSH remaining after 6 h incubation, which was similar to the control of 89.54 ± 0.39 %.

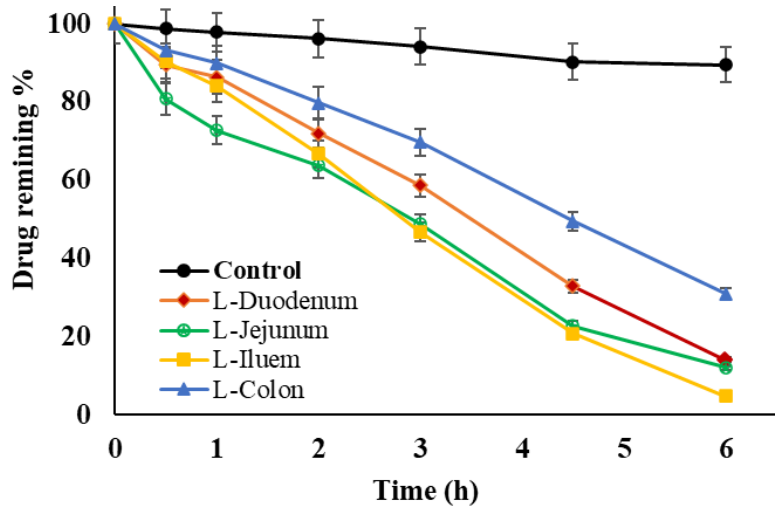


**Figure 4-2.** Degradation study of GSH in the absence or presence of the luminal contents from four intestinal regions of rats.

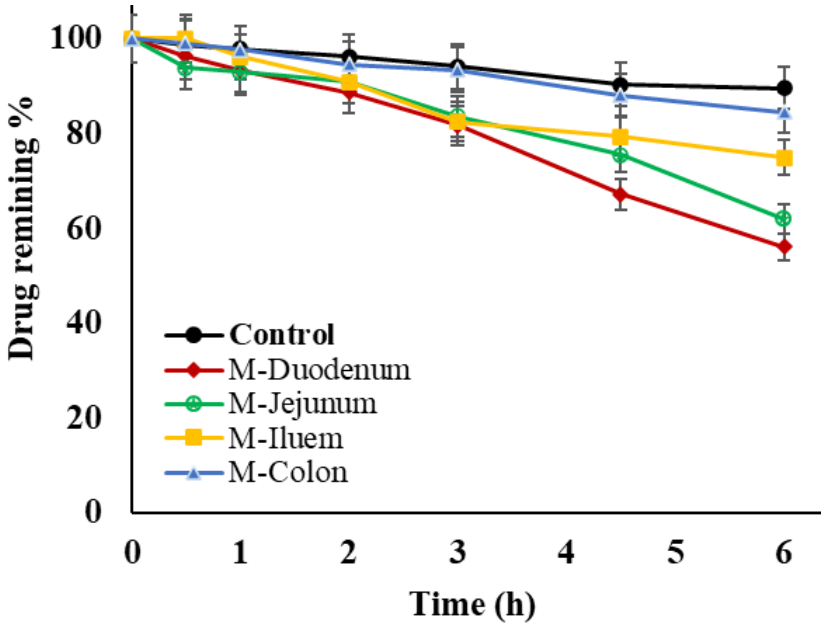


**Figure 4-3.** Degradation study of GSH in the absence or presence of the mucosal contents from four intestinal regions of rats.

#### 4.4.2.2 Inhibition of proteolysis of GSH by enzymatic inhibitors

The effects of chosen enzyme inhibitors on the enzymatic degradation of GSH in luminal and mucosal contents of each intestinal region are summarised in Figure 4-3. Compared to the control group, EDTA exhibited a significant enzymatic inhibition ($p < 0.05$) to protect GSH degradation in all luminal and mucosal extracts from all regions except mucosal extracts from colon. In contrast, no significant inhibition effects on GSH degradation were observed by

bacitracin compared to the control group ($p > 0.05$). Hence, EDTA may have the potential to increase the enzymatic stability of GSH when GSH is administered orally. This result is consistent with other authors' research (180).

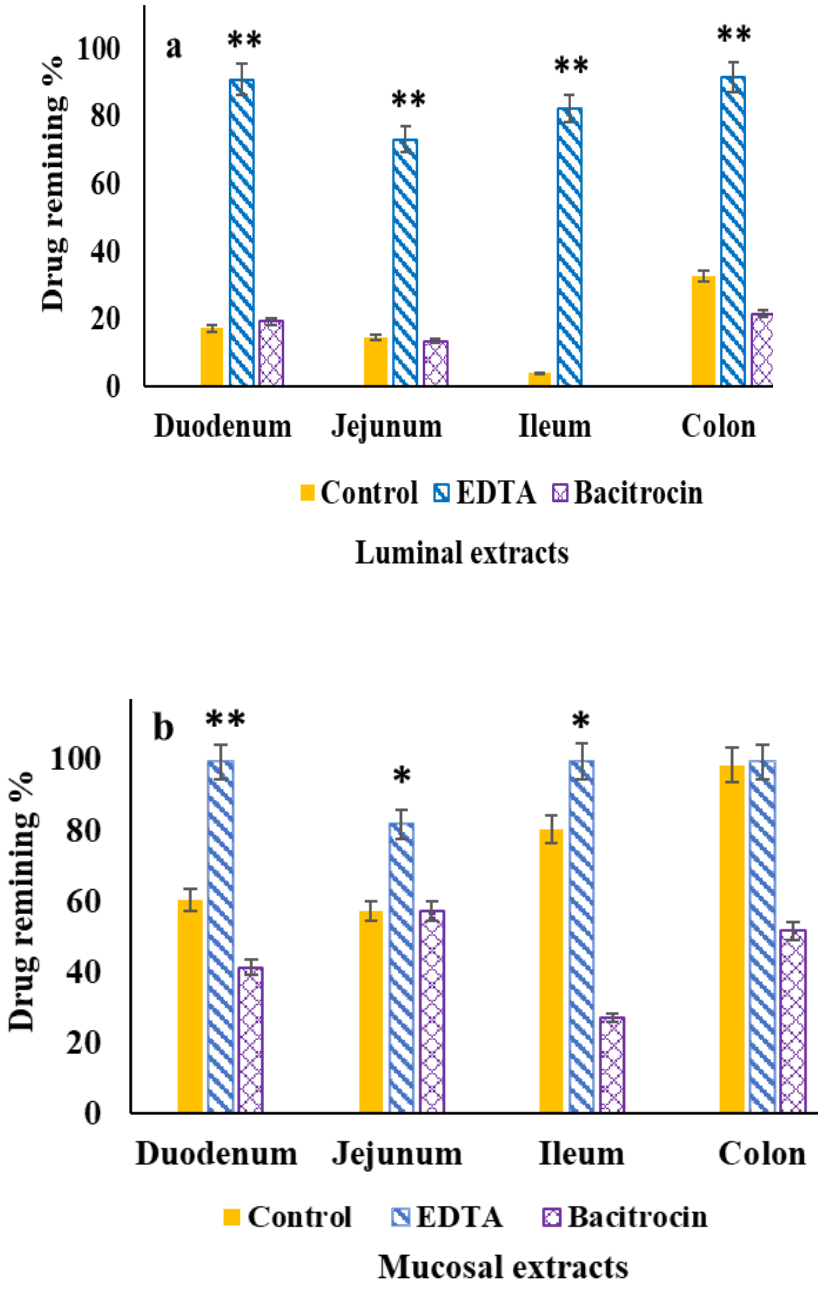


**Figure 4-4**. Effects of inhibitors on GSH proteolysis in (a) luminal extracts and (b) mucosal extracts in four intestinal regions of rats (** $p<0.01$, * $p<0.05$).

#### 4.4.2.3 Inhibition of peptidolysis of GSH by niosomal carrier system

The protective effects of niosomal carrier system on enzymatic degradation of GSH are illustrated in Figure 4-4. Compared to the free GSH (control group), GLNs displayed inhibitive effects against the enzymatic degradation of GSH in all four intestinal regions of rats. The highest protective effect could beis seen in the ileum followed by jejunum and duodenum while the effect on the colon region was insignificant. Therefore, niosome carrier system may be able to provide significant protection against the enzymatic degradation of GSH when uptaken/transported through the intestinal membrane, potentially enhancing the oral bioavailability of GSH.

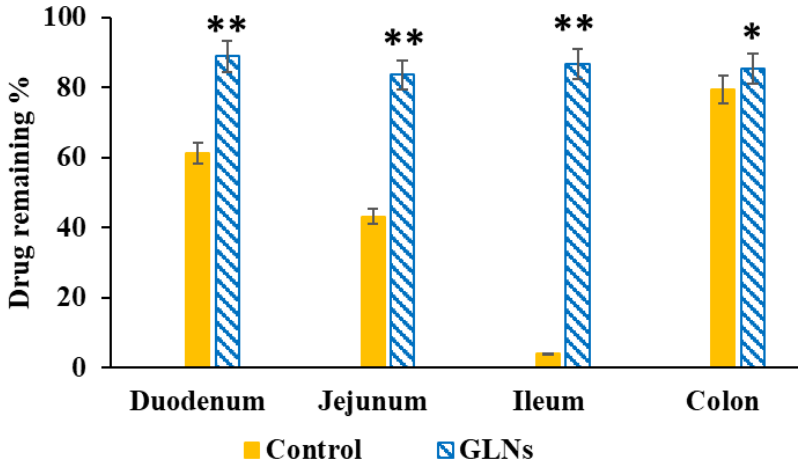


**Figure 4-5**. Inhibition effects of niosomal carrier system on proteolysis of GSH (** $p<0.01$, * $p<0.05$).

### 4.4.3 Uptake of FITC-labelled niosomes by Caco-2 cells

Figure 4-6 (A-D) illustrate the cellular uptake of the FITC-labelled niosomes. Figure 4-6 A and B show the cell's cytoplasm and nuclei, which were stained with fluorescence in red and blue colour, respectively. In Figure 4-6 C, FITC-labelled niosomes were displayed as green fluorescence, which were internalised in Caco-2 cells in Figure 4-6 D, showing the merged image of green and blue. Hence, the images obtained in this study demonstrate the location of the FITC-labelled niosomes is intracellular through Caco-2 cells uptake, indicating niosomes were internalised across Caco-2 cells. This may suggest that GLNs could be taken up across the intestinal epithelium membrane. The uptake mechanism may occur via endocytosis pathway as reported by other researchers (1) .

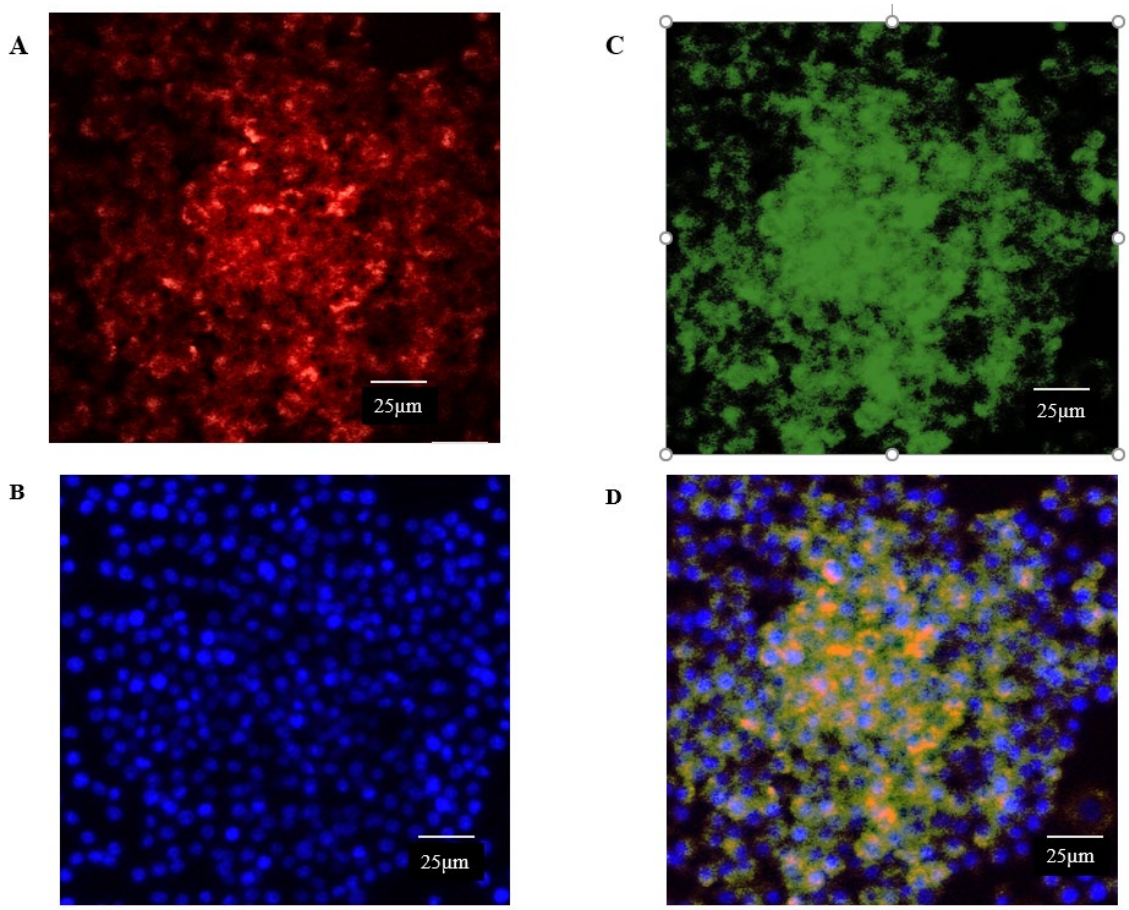


**Figure 4-6.** Cross-section image (3D) of Caco-2 cell (after incubation with FITC-labelled niosomes at 37 ºC for 1 h) using fluorescent microscopy, showing an intracellular accumulation of FITC-labelled niosomes (A) Cytoplasm stained with CellTracker Red; (B) Nuclei stained with DAPI (blue); (C) FITC-labelled niosomes (green), and (D) Merged image.

### 4.4.4 Transport study of GSH with or without niosomes using Caco-2/HT29 cells

The apparent permeability coefficient Papp of GSH can be calculated using the equation 4-5:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0} \qquad \text{equation 4-5}$$

Where $\Delta Q/\Delta t$ represents the GSH amount cumulated per unit time (µg /min) on the basolateral side, $C_0$ is the initial concentration of GSH (µg/mL) in the apical chamber, and A is the membrane area value of the transwell insert in $cm^2$.

The results of $P_{app}$ in all groups were shown in table 4-2 with the ranking of the $P_{app}$ values as: GLNs-EDTA > GLNs-sodium taurocholate > GLNs > GSH-EDTA > GSH-sodium taurocholate > GSH.

**Table 4-2.** The $P_{app}$ values of GSH with/without niosomes in the absence or presence of EDTA and sodium taurocholate (mean ± SD, n=3)

| | $P_{aap}$ ($10^{-6}$ μg/cm/s) | |
|---|---|---|
| **Groups** | **GSH** | **GLNs** |
| Control | 4.78 ±1.97 | 14.27 ± 3.55** |
| EDTA | 11.49 ±5.3** | 17.21 ± $5.3^{++}$ |
| Sodium Taurocholate | 9.67 ± 3.23** | 15.53 ± $2.38^{+}$ |

** $p < 0.01$ compared with GSH control group; + $p < 0.05$, ++ $p < 0.01$ compared with GLNs group

The flux rates of transport of GSH were summurized in Figure 4-7. Compared to the control group of GSH, GLNs group displayed significant improvement in transport rate from apical to basolateral side of the transwell, indicating the permeability of GSH significantly increased across the monolayers of Caco-2/HT29 cells over the 3-hour period in the presence of niosomes ($p<0.01$). Meanwhile, the other two GSH groups treated with penetration enhancers, EDTA and sodium taurocholate (a derivative of bile salt), exhibited higher value of permeability than the control group, owing to the permeation enhancing effect by opening the tight junctions of the monolayer (107) ($p < 0.01$ for both groups). In GLNs groups, both absorption enhancers (EDTA and sodium taurocholate) displayed further significant improvement in transport rate of GSH ($p < 0.01$ in EDTA group and and $p < 0.05$ in sodium taurocholate group).

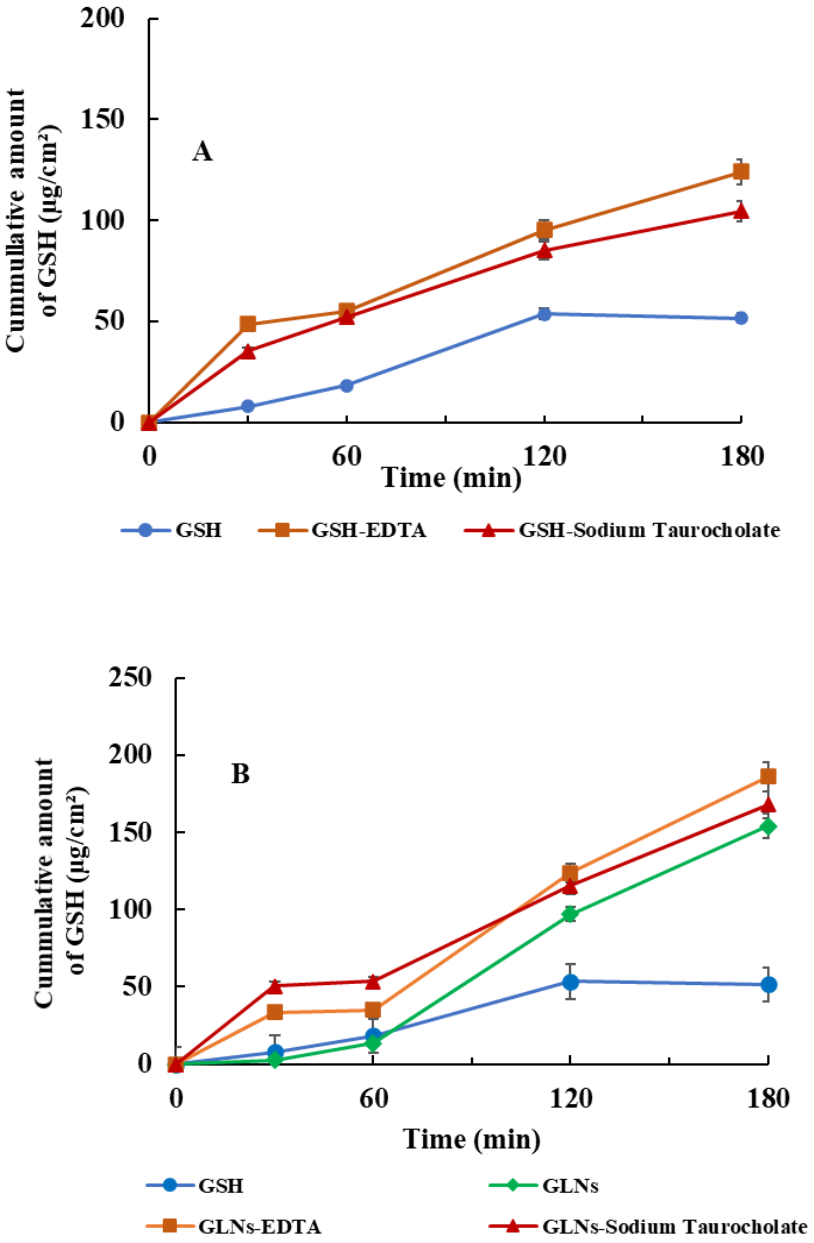


**Figure 4-7.** The effects of EDTA or sodium taurocholate on GSH transport (1mg/mL) across the monolayer of Caco-2/HP29 cells over 3 h at 37°C. A. GSH groups and B. GLNs groups (mean ± SD, n=3)

## 4.5 Conclusion

In this chapter, *in vitro* cytotoxicity, degradation, and cellular uptake and transport of GSH were evaluated. By employing MTT assay on Caco-2 cells, the toxicity of GSH was studied, revealing that no *in vitro* cytotoxicity was observed after 24 h incubation with GSH ranging from 0.01 to 5.00 mg/mL. The *in vitro* degradation study summarised that the presence of EDTA significantly inhibited GSH breakdown in all luminal and mucosal extracts of rats (except mucosal extracts from colon) after

6 h incubation when compared to bacitracin. Meanwhile, niosomes displayed significant protective effects on GSH enzymatic degradation in duodenum, jejunum and ileum of rats. Therefore, compared to enzyme inhibitor-EDTA, the advantage of using niosomal carrier system is obvious. The carrier system not only can provide GSH similar protection against enzymatic degradation, but also build up a safer cellular environment for living cells due to niosomes' less toxic physiochemical characteristics. These findings may indicate that niosomal carriers have the potential to develop a new oral formulation of GSH with enhanced oral bioavailability compared to unprotected GSH preparations. Moreover, the microscopic analysis demonstrated that the FITC-labelled niosomes were internalised in the Caco-2 cells after incubation, which means that niosomes could be taken up across the cellular membrane, leading to an increase in bioavailability after being administered orally (181). In addition, the transport study demonstrated that GLNs can significantly improve the permeability of GSH across the Caco-2/HT29 cells, exhibiting the similar permeation-enhancing effect promoted by absorption enhancers such as EDTA and other surfactants (e.g. sodium taurocholate).

# Chapter 5

# General discussion and future perspectives

# 5 General discussion and future perspectives

## 5.1 Overview

As a tripeptide, GSH is recognised as a potent antioxidant, involved in many fundamental biological processes such as protein and DNA synthesis, cell proliferation, and oxidation/reduction signalling, and is used for various medical interventions in degenerative diseases such as Alzheimer's disease and Parkinson's disease (4-8). However, due to physical and chemical barriers in GI tract, the oral preparations of GSH for therapeutic purposes have always faced challenges due to its low bioavailability (18-20). Moreover, the physicochemical properties of GSH have severely limited its clinical efficacy as an orally administered treatment due to its low membrane permeability and instability (88) (discussed in Chapter 1). Consequently, most formulations of GSH with clinical effects are currently marketed as parenteral injections, which unfortunately affects patient compliance owing to the invasive nature and associated pain and potential tissue damage. Therefore, as the most convenient and patient-friendly dosage form, the oral preparation of GSH with enhanced bioavailability has always attracted scientists' attention in pharmaceutical research.

To date, many strategies have been developed to enhance the bioavailability of GSH. Approaches focusing on improving the physicochemical profiles and stability of GSH in GI tract have been investigated and applied in many studies, such as chemical modifications, absorption enhancers, enzymatic inhibitors, and formulation approaches (including nanocarrier technologies e.g. microemulsions, nanoparticles, liposomes and niosomes) (Chapter 1). In this study, niosomes, nano-scaled carriers, were selected as the drug delivery carriers for the development of GSH-containing formulation because of their unique advantages such as relatively high stability compared to liposome, good biodegradability and biocompatibility, low toxicity, and excellent cost-effectiveness for preparations (161). Many publications have revealed drug formulations using niosomes as delivery carriers could improve stability and bioavailability of protein or peptide compound (182-184), which agreed the findings from the present study.

The aim of this project was the development and characterisation of GSH-loaded niosomes (GLNs) (Chapter 3) using an optimised HPLC method (Chapter 2); and the evaluation of GLNs in terms of *in vitro* toxicity, degradation and cellular uptake and transport (Chapter 4).

To quantify the concentration of GSH in the studies of this project, HPLC, as an economical and well-recognised analytical tool, was adopted and validated based on the ICH guideline (155) (Chapter 2). Its linearity, accuracy, precision, sensitivity, and repeatability were determined, showing excellent compliance with the standards of the guideline. A very sharp peak of GSH with an excellent resolution was achieved using this method with a retention time of GSH at 3.8 min. A standard linear curve was obtained with GSH concentration ranging from 1 to 100 μg/ml, which was sufficient for multiple applications in the project.

GLNs in this project were fabricated using a thin film hydration strategy (in Chapter 3), a simple technique that is commonly applied in practice (185). A factorial design method was employed in the GLNs development, aiming to achieve the best composition with the minimum particle size and the maximum EE. Three independent variables (surfactant type, molar ratio of surfactant to CH, and GSH amount) were selected with two responses (EE and particle size). The entire study consisted of ten runs using a two-level full factorial design with one central point, which was optimised using Design Expert® software and analysed via a statistic model. Upon evaluating the importance of each response, the optimised formulation was confirmed, which consists of Span 60 and CH (with a molar ratio of 3:2), with 10 mg GSH per 10 mL for hydration.

The characterisation studies of the optimised formulation revealed that the average value of the particle sizes was at 253.3 ± 0.6 nm with PDI of 0.353 ± 0.028, indicating the size distribution of the particles was within the acceptable range. The value of zeta potential was at -65.3 ± 3.5 mV, suggesting the GLNs were a stable system. The images from the morphology study displayed closed spherical vesicular-shaped nanoparticles with a double-layered structure with a narrow size ranging between 100 and 350 nm approximately. The physical stability study evaluated the change of the remaining level of GSH in two GSH-entrapped formulations (suspension and powder) over a 3-month period. Findings suggested a significant protecting effect on the entrapped GSH (suspension of powder form) was observed compared to the control group of free GSH. By testing the remaining level of GSH at three different temperatures, it was revealed that the ideal storage condition of GLNs should be at 4 °C and the remaining level of GSH in GLNs could be retained up to 90% of the initial loading dose by the end of the first month of storage.

*In vitro* release study (for 24 h) was conducted and the collected data was assessed using four different kinetic models: zero order, first order, Higuchi and Korsmeyer-Peppas model

(171,172). The study findings aligned with the Korsmeyer-Peppas model. Data illustrated that GLNs displayed a two-stage release profile: a fast release of GSH (in the first 5 h), followed by a sustained release (till the end of 24 h), with approximately 45% cumulative GSH release. However, the control group of the GSH solution behaved differently with a rapid release of GSH (in the first 3 h), levelling off at around 5 hours and then releasing very slowly for the remainder of the study, with approximately 90% cumulative release. These findings suggested that GLNs may be able to provide a delivery system with a sustained release profile, which agrees the findings from other publications relating to peptide- or protein-loaded niosomes. For example, similar release kinetics have been reported for delivery of insulin encapsulated with niosomes, stating the biphasic release profiles consisted of a rapid initial insulin release followed by a sustained release phase (183). This was confirmed by another study when evaluating the release profiles of bioactive peptides in milk formulated in niosomes (184).

Being internalized into the target cells is the first step of the delivery process for nanomedicines to achieve the intended therapeutical purpose; therefore, proper delivery of carriers by their cargo on the plasma membrane into the interior of the cells is vital to the formulation uptake (1). To gain access entry and be delivered into the cells, nanocarriers are taken up mainly via endocytosis, the carrier-mediated pathway, which was discussed in chapter 1 (1.4.1.1). In this project, the uptake of FITC-labelled niosomes was assessed by using Caco-2 cells. The results demonstrated that FITC-labelled niosomes were internalised by Caco-2 cells, which was confirmed using confocal fluorescent microscopy. It suggested the uptake mechanism of niosomes was endocytosis which is the dominant pathway for nanoparticle uptake across the intestinal epithelial membrane (1,186). The transport study revealed that GLNs could significantly increase the permeability of GSH across the monolayer of Caco-2/HT29 cells, which was similar to the effects induced by those absorption enhancers for protein and peptide drugs reported by other publications (107).

## 5.2 Limitations and future directions

The aim of this project is to develop a nano-scaled carrier system for oral delivery of GSH with enhanced permeability and stability. Studies have confirmed that the GLN developed in this project is able to fulfil most of the objectives, which suggests GLNs could be a potential candidate for further development.

However, there has been an issue regarding the low value of the EE (30%) that needs to be addressed in future studies. There are some strategies that could be applied for the improvement of EE. For example, since only a single surfactant was used in the preparation of GLNs, the addition of a cosurfactant could be a possible approach for an enhanced EE. Other options include could be to find a replacement for the current surfactant. In the current project, only Span 60 and Span 80 were evaluated, hence the selection of other products (e.g., Span®40 and Tween 80) could be further tested for a higher value of EE. A study reported that better values of EE (> 60%, almost doubled the result of this project) was achieved by using the combination of two surfactants selected from Span®40, Span®60 and Tween®80 (125). The HPLC method for GSH quantification assay in the current study has basically achieved the goals. However, there is a limitation caused by sample flow rate in HPLC method. The set flow rate was increased from 0.5 to 0.7 mL/min in HPLC method in comparison to the original publication (129), aiming to reduce the total analysis time (from 10 to 8 min per run). This allowed a substantial time reduction when a large number of samples were required for analysis, which has been undoubtedly beneficial when there was time constraint. However, since the retention time of GSH peak appears at 3.8 min, and nearby this time zoom, there are peaks produced by the solvent when water (as solvent) was replaced by PBS for analysis, which could have potentially interfered and compromised the accuracy of GSH quantitative analysis. In the future study, this flow rate setting should be reconsidered through increasing GSH retention time by decreasing the flow rate. This will allow the resulting peak of GSH moves further away from the interrupting peaks caused by PBS.

## 5.3 Conclusion

The present project has achieved the objectives of development and optimisation of nano-scaled vesicular carriers for GSH oral delivery, which improved the permeability of GSH in an *in vitro* model. It also investigated the mechanism of the formulation's intestinal uptake and transport, which confirmed the internalisation of GLNs in Caco-2 cells and indicated an endocytosis pathway which is the common mechanism for nanoparticle's absorption in the cellular level (1). The results of this project are as follows:

1. An HPLC method was redeveloped, which appeared to be fast, accurate and reliable. It was validated based on the ICH guideline and then applied for the quantitative analysis of GSH throughout the entire project.

2. GLNs was successfully formulated using a thin film hydration method and optimised by a $2^3$ factorial design methodology to find the best composition. The formulation was then characterised for its EE, particle size, zeta potential, morphology, and physical stability. The data showed that the average value of particle size was about 250 nm with a narrow range of distribution index; EE was around 30%; and the preferred storage condition was suggested as 4 °C.

3. The release study revealed that the formulated GLNs in the study displayed prolonged two-phased *in vitro* release. There was no cytotoxicity to Caco-2 cells observed induced by GLNs. Therefore, GLNs could be a potential candidate for developing an oral formulation of GSH that is safe to use and can be released in a sustained manner.

4. Through *in vitro* uptake study and observation through a fluorescent microscopy, FITC-labelled niosomes were revealed to be internalized in Caco-2 cells, which might indicate that the mechanism of the cellular uptake of niosomes was via endocytosis pathway, the common pathway for nanoparticles' cellular uptake as reported by other studies. Moreover, the transport study revealed that GLNs could significantly increase GSH permeability across the Caco-2/HT29 cells, which was similar to the effect achieved by oral absorption enhancers for protein and peptide drugs with safer profiles.

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