CTS-mPEG/MMT Coated Nanoparticles for Treating *Helicobacter pylori*-Induced pH Dependent Gastritis

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Abstract

Due to difficulties with traditional treatment methods, gastritis, which is predominantly caused by an infection with *Helicobacter pylori (H. pylori)*, is a common stomach disease that requires novel therapeutic approaches. This project aims to develop a novel droplet-based drug delivery system to address the restrictions of existing therapies by magnifying the stability, efficacy, and bioavailability of eupatilin (5,7-dihydroxy-3,4,6-trimethoxyflavone), a flavone that plays a protective role against CagA-positive *H. pylori*-induced gastritis. Our approach uses poly(lactic-co-glycolic acid) (PLGA) to provide regulated release of eupatilin-encapsulated droplet-based chitosan-mPEG/MMT coated nanoparticles. Nanoparticles are often coated with chitosan for its bioadhesiveness, biocompatibility, and antibacterial properties, but its mechanical and thermal stability are limited. In this study, instead of modifying chitosan with mPEG and MMT separately, both were combined to enhance the nanoparticle's stability, biocompatibility, and mechanical properties, offering greater potential for gastritis treatment. The encapsulation process is optimized through microfluidic techniques to achieve uniform particle size and high drug-loading efficiency. Long-term medication release and defense against stomach acid breakdown are guaranteed by the nanoparticle coating. The system's performance in terms of cytotoxicity, surface morphology, and drug release kinetics will be validated by characterization techniques like as SEM, FTIR, XRD, and MTT assays, leading to next-generation targeted therapeutics in the treatment of gastric diseases.

Key words: *Controlled drug delivery, gastritis, Eupatilin, CTS-mPEG/MMT, PLGA nanoparticles.*

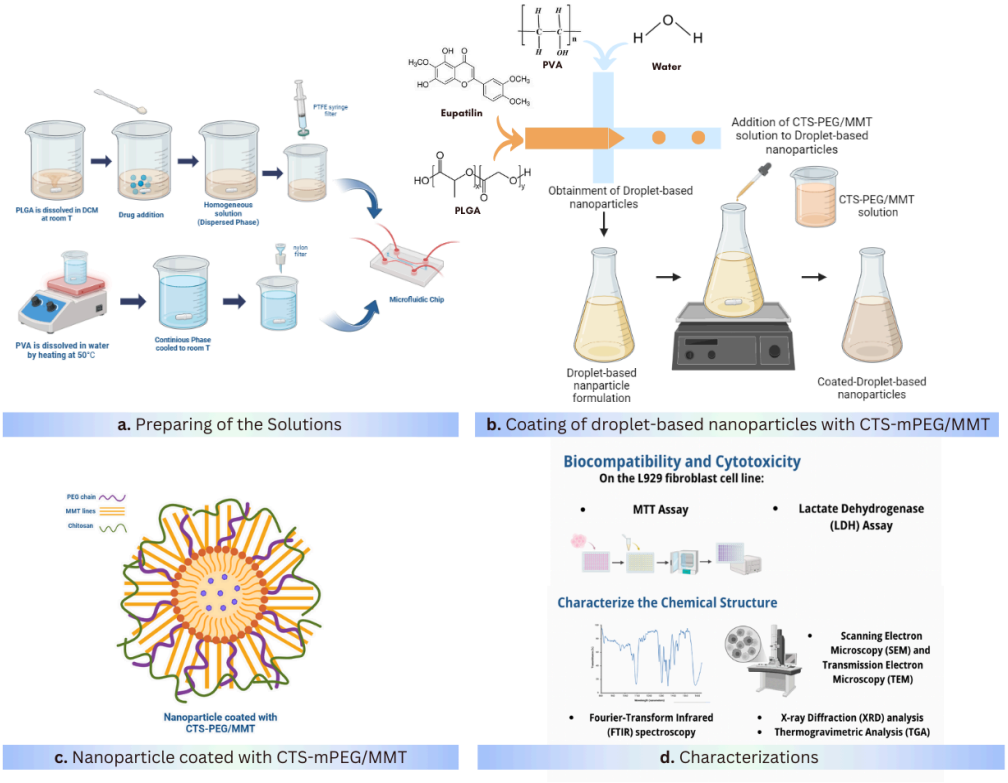

Fig. 1. Visualised summary of the project

Table of Contents

Project Value

Gastritis, often caused by *Helicobacter pylori*, is treated with proton pump inhibitors PPIs, H2-receptor antagonists, and antibiotics, but these methods face challenges such as poor gastric retention and side effects. Previous drug delivery systems, including single-polymer and basic chitosan-based designs, lack long-term efficiency and controlled release. Additionally, it can be stated that eupatilin, except for its coating with gold nanoparticles, has not been previously utilized for therapeutic purposes in a drug delivery system, highlighting a significant gap [1]. Even though chitosan is commonly used in gastritis treatments, which is a mucoadhesive molecule, objectives such as improvements to controlled release schemes, reducing drug degradation time and increasing bioactivity while reducing the dosage can be achieved with improved nanoparticle surface modification. While chitosan's mucoadhesive and pH dependent (below pH 6.5) properties improve retention, enhanced outcomes require advanced surface modifications. Our system combines chitosan with mPEG, boosting stability and hydrophilicity, and montmorillonite (MMT), offering high surface area and layered structure for controlled drug release. This synergistic system ensures mucoadhesion, stability, and prolonged drug activity, addressing limitations of existing methods and improving gastritis treatment by controlling dosage, side effects, efficiency [2-4]. Both academic and commercial researchers continue to place a high value on research in the domains of pharmacology, biomedical engineering, and drug delivery systems in order to enhance the prompt and simple diagnosis, management, and prevention of gastritis patients' conditions.

Background

Gastritis: Disease, Causes, and Pathophysiology

Gastritis is an inflammation of the stomach mucosa and is usually used to describe the abnormal appearance of it. Infectious or immunological inflammation of the stomach mucosa is commonly observed in gastritis. Gastritis can be classified according to the duration of the disease, histological features of the inflammation, or etiology. Gastritis is divided into two groups according to the rate of formation: acute and chronic gastritis. Acute gastritis is usually associated with a short-term attack caused by mucosal barrier disruption. Chronic gastritis occurs due to autoimmune disorders, antibodies attacking the gastric mucosa, or infection by *Helicobacter pylori* (*H. pylori*) [5].

Mechanisms and Pathophysiology of *H. pylori*

In 1982, Robin Warren and Barry Marshall discovered that the majority of gastritis was caused by *H. pylori* [6]. *H. pylori* is a Gram-negative, microaerophilic, curved, and S-shaped bacterium. The flagella-mediated motility of *H. pylori* allows it to enter the mucus layer. *H. pylori* produces a type of urease that is necessary for the activity of the urease holoenzyme. Urease provides the breakdown of urea into ammonia and carbon dioxide. Ammonia protects *H. pylori* from the excessive acidic environment it encounters in the stomach lumen by neutralizing it. *H. pylori* can adhere to gastric epithelial cells by binding surface molecules attached to its outer membrane to host cell receptors. *H. pylori* bacteria have adhesins on their surface that allow them to adhere to host cells. The bacteria have structural changes that prevent recognition by the immune system's danger-sensing receptors, such as TLR4 and TLR5. This allows *H. pylori* to escape the immune system and establish a long-term infection in the gastric mucosa. When the bacteria reach the gastric mucosa, it triggers inflammatory responses, especially through the gene region called cytotoxin-associated gene pathogenicity Island (cagPAI). This mechanism increases the release of cytokines that attract immune cells to the stomach causing chronic inflammation [7].

Drug-of-choice

5,7-dihydroxy-3,4,6-trimethoxyflavone (**Eupatilin**) is a flavone that is pharmacologically active (Fig. 2.). In a previous study the solubility of DA-6034, a synthetic derivative of eupatilin, was studied in buffer solutions with pH values ranging from 1.2 to 7.4 and concluded that its poor solubility in acidic media. It would have a limited gastroprotective effect if it was administered in a conventional tablet formulation [11]. For this reason, there has been a requirement for controlled release systems other than tablet form. It being one of the most abundant polymethoxy flavones has been isolated from a variety of medical plants, especially *Artemisia* species, specifically *Artemisia asiatica* [8] and *Artemisia argyi* [9]. It has pharmacological activities such as anti-inflammatory, anti-oxidant, anti-cancer effects [10]. These wide range effects and its abundance in *Artemisia* species makes eupatilin a highly valuable flavone which has been used in the treatment of many different diseases. Eupatiline and its derivatives such as DA-6034 (7-carboxymethyloxy, 3 ,4 ,5- trimethoxy flavone [11], DA-5204 (Stillen 2X) [12] have been studied in various experiments on gastrointestinal diseases.

Inflammatory response that has been induced by *H.pylori* CagA has been shown to be a strong risk factor for these diseases. Inhibiting the chronic inflammatory response by suppressing pro-inflammatory cytokines (TNF-α, IL-6, IL-1ß) via the NF-κB-mediated signaling pathway is critical for managing gastric diseases associated with *H.pylori* [13]. There are studies that conclude eupatilin plays a protective role against CagA-positive *H. pylori*-induced gastritis [14]. Additionally, Eupatilin enhances the gastric mucosal defence. Similarly It has been shown in vitro studies that eupatilin protects gastric epithelial cells from oxidative cellular damage [15].

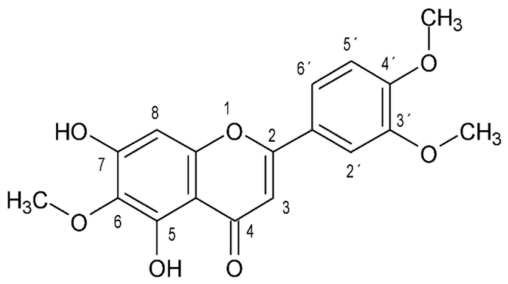

Fig. 2. Eupatilin chemical structure [16]

DA-5204 (Stillen 2X) is a medication primarily used for gastrointestinal complications. According to clinical studies, DA-5204 may help relax the digestive system and has the potential to be effective in treating various gastrointestinal disorders. However, in order for the medication to be used effectively and safely, it is crucial to apply the correct dosage. 1 tablet of Stillen 2X is 60 mg. Typically, treatment begins with 1 tablet per day. This dosage is used for the first few days, and the patient's response to the medication is assessed. After the initial dose, the dosage may be increased based on the drug's effectiveness and the patient's condition. For most patients, the ideal maintenance dose is 2 tablets per day. The maximum dosage should not exceed 3 tablets per day. Going beyond this limit could increase the risk of side effects. DA-5204 is generally well tolerated, but some patients may experience mild side effects such as nausea, dizziness, or digestive system complaints [12].

Droplet-Based Nanoparticles

Poly(lactic-co-glycolic acid) (PLGA) is a widely utilized biodegradable and biocompatible polymer for the fabrication of drug delivery systems. PLGA-based droplet nanoparticles, created using advanced microfluidic techniques, represent a significant advancement in precision medicine. These nanoparticles combine the controlled drug release properties of PLGA with the precise size control and reproducibility offered by droplet microfluidics. Through solvent evaporation, the polymer matrix solidifies around encapsulated drugs, enabling targeted delivery and sustained therapeutic effects. Their versatility allows for encapsulating a wide range of hydrophilic and hydrophobic drugs, making PLGA-based droplet nanoparticles a promising platform for applications in cancer therapy, inflammation management, and gastric disease treatment [17].

Coating

Chitosan, a linear polysaccharide, is composed of glucosamine and N-acetylglucosamine units - shown in Fig. 3. It is obtained from the deacetylation of chitin, the second most abundant biopolymer in nature that is derived from the exoskeleton of crustaceans.

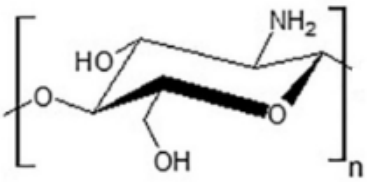

Fig. 3. Chemical structure of chitosan repeated unit [18]

Chitosan shows biocompatibility and biodegradability as a non-toxic material, thus is considered renewable, sustainable and affordable [18]. It also shows antibacterial activity as well as bioadhesiveness and film formation capability on skin and mucous membranes [18]. Although chitosan offers many advantages, it still has some limitations, such as the low mechanical properties and low thermal stability [19,20]. Previous studies have addressed these shortcomings by modifying chitosan with PEG and montmorillonite (MMT) separately [20, 21]. In this study, combining both mPEG and MMT to modify chitosan coating is expected to enhance the stability and biocompatibility of the nanoparticle drug carrier, improve mechanical properties, and make the system more suitable for a broader range of drug delivery applications for gastritis disease.

Aims of The Project

The 1st specific aim of this project is to **design and optimize a droplet-based nanoparticle system** capable of delivering therapeutic agents to treat *H. pylori*-induced gastritis. The contribution of this goal to the success of the project has been evaluated as 50%. In order to achieve this goal, it is necessary to implement the following sub-goals:

- To develop a microfluidic droplet production process to **create uniform nanoparticles** with controlled size and drug encapsulation efficiency.
- To **optimize the encapsulation of eupatilin** within the nanoparticles, ensuring stability and sustained release under gastric conditions.
- To **clarify the dosage** range in a way that **minimizes side effects.**
- To **enhance the efficacy of a single drug in the infected region**, rather than previously tested in triplicate drug combinations with proton pump inhibitors on gastritis.

The 2nd aim of this project is to **enhance the stability and functionality of the nanoparticles** through a chitosan-based coating. The contribution of this goal to the success of the project has been evaluated as 25%. In order to achieve this goal, it is necessary to implement the following sub-goals:

- To synthesize and characterize a **chitosan-mPEG/MMT coating to improve mechanical properties**, biocompatibility, and drug release control.
- To investigate the crosslinking of chitosan with tripolyphosphate (TPP) to **enhance the structural integrity of the coating under acidic conditions.**
- The therapeutic limitations of eupatilin due to its low solubility in acidic conditions are that to **increase the solubility** by designing chitosan-mPEG/MMT nanoparticles that will cause an oscillation **in gastric pH.**

The 3rd aim of this project is to ensure the nanoparticle system is **safe for biological applications**. The contribution of this goal to the success of the project has been evaluated as 25%. In order to achieve this goal, it is necessary to implement the following sub-goals:

- To achieve high efficacy in *H. pylori*-infected regions while **extending the drug's half-life and reducing potential toxic effects on cells.** To perform in vitro cytotoxicity tests (MTT and LDH assays) on L929 cells to evaluate the safety of the system.
- To use advanced microscopy techniques (SEM, TEM) and FTIR/XRD analysis to **verify the system's surface properties, chemical stability, and nanocomposite structure**.

Materials and Methods

Extraction, Isolation and Identification of Eupatilin

The air-dried leaves of *A. argyi* (10 kg) will be extracted with 95% ethanol under reflux for 2 hours and repeated three times. After the evaporation of the combined ethanol extracts in vacuo, the resultant residues will be suspended in water and extracted successively with petroleum ether, dichloromethane, ethyl acetate, and n-butanol to obtain CH_2Cl_2 extract (125.0 g), EtOAc extract (60.0 g), and n-BuOH extract (8.0 g), respectively. The CH_2Cl_2 extract will then purified via silica gel column chromatography eluting with a CH_2Cl_2–CH_3OH gradient (100:0–0:100). The fraction B (100: 2) will be further subjected to polyamide column chromatography with CH_3OH–H_2O (60: 100) as a mobile phase. The eluates will be separated on preparative HPLC with 58% MeOH/water as a mobile phase. Preparative HPLC will use YMC-ODS column (YMC-Pack ODS-A, 250 20 mm, 5 mm) with a refractive index detector (Shimadzu SPD-20A) to obtain eupatilin to afford >95% purity [1].

Droplet Generation

A dispersed phase, consisting of PLGA and eupatilin in dichloromethane (DCM), and the continuous phase, containing PVA in water, will be introduced into the microfluidic chip through separate pumps. At the flow-focusing junction of the microfluidic device, shear forces from the continuous phase will break the dispersed phase into uniform droplets. The size of the droplets can then be precisely controlled by adjusting the flow rate ratio between the continuous and dispersed phases. Formed droplets will be collected into a stirring aqueous bath and after solvent evaporation to solidify the PLGA matrix around the encapsulated drug, stable microparticles loaded with eupatilin will be afforded [17].

Characterization of microparticles

The droplet and particle sizes must be analyzed to confirm uniformity and ensure suitability for drug delivery. Droplets will be evaluated using dynamic light scattering (DLS) and laser diffraction techniques [17]. The average droplet size is expected to be 166 ± 7.21 nm [22], where the drug encapsulation efficiency is calculated from the following equation:

Encapsulatıon Efficiency = (Actual Drug Amount/Theoretical Drug Amount) x 100%

Coating the NPs with CTS-mPEG/MMT

Chitosan-mPEG solution will be treated with montmorillonite-water (MMT-water) solution followed by the application of sonication to ensure proper interaction. CTS-PEG/MMT solutions at different concentrations will be slowly poured into the aqueous formulation of droplet-based nanoparticles while gently stirring to facilitate coating. Reaction time here is an important parameter for coating, thus several time periods are planned to be tested to find the optimal reaction time. Due to chitosan's adhesive properties, the nanoparticles become covered with a shell of CTS-PEG/MMT. The morphology of the obtained product will be observed with SEM analysis, aiming to obtain a smooth surface and good shape at the end [18].

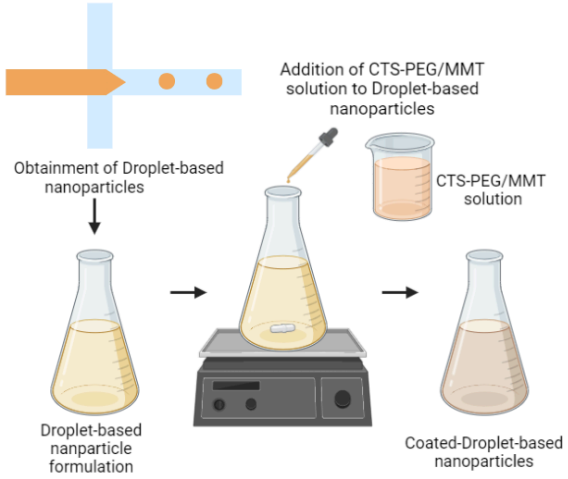

Fig. 4. Coating of droplet-based nanoparticles with CTS-PEG/MMT

Chitosan can be cross-linked with cross-linking agents such as TPP due to the presence of amine groups in its backbone. TPP solution is slowly added to the CTS-PEG/MMT solution under continuous stirring. The mixture will then be incubated at a controlled temperature to complete the cross-linking process [23].

Experimental Design and Expected Results

In studies examining drug release chitosan and PEG previously validated and providing a proper release profile, can be used as a positive control. Systems that are uncoated or contain only one carrier component can be preferred as a negative control. For the optimization of the reaction time, the positive control is made with a validated reaction time that provides an ideal release profile, while the negative control can consist of systems with insufficient or excessively long reaction times. In experiments examining the release rate of MMT-coated polymer micelles, MMT-coated systems with proven stabilization effects are used as the positive control, while the negative control can be micelles without MMT coating or containing insufficient MMT concentration. The positive control includes an effective TPP solution in which TPP and chitosan are successfully cross-linked. The negative control refers to systems without coating or containing only one carrier component and a situation where TPP is removed or used in insufficient amounts, thus not cross-linking. Also, depending on the pH, a system with audible acids is available as a positive control, and a system with release at neutral or basic pH is available as a negative control.

A. Summary of experimental approach

Previous studies have shown that the combination of chitosan (CTS) and PEG in coating of nanocomposite provides a prolonged release profile by slowing down the drug release.The majority of the drug is released within the first 3-5 hours, followed by complete release within 24-48 hours [24,25]. Additionally, while the polymeric micelles initially experience a rapid release due to the MMT coating, the stabilizing effect of MMT slows down the release rate at high loading rates [26]. This system is predicted to release the drug within 3-5 hours in the pH range of 1.5-3.

The biocompatibility and cytotoxicity of the CTS-PEG/MMT nanocomposite will be thoroughly evaluated through a series of toxicology tests will be conducted. On the L929 fibroblast cell line, the MTT assay will be employed to assess the metabolic activity of the cells. MTT (a yellow tetrazole) measures the reduction of MTT to formazan crystals by mitochondrial dehydrogenases in viable cells. Complementarily, the lactate dehydrogenase (LDH) assay will be utilized to evaluate cell membrane integrity by detecting LDH levels (LDH is an enzyme released into the culture medium when the plasma membrane is compromised.) Together, these assays will offer a comprehensive understanding of the cytotoxic effects of CTS-PEG/MMT on the cellular level [23].

To characterize the chemical structure and verify the success of the cross-linking reaction within the nanocomposite, Fourier-transform infrared (FTIR) spectroscopy will be performed. This technique will focus on identifying the characteristic functional groups, including –NH, –COOH, and –OH, which are associated with the molecular structure of chitosan and acetic acid [27]. Furthermore, X-ray diffraction (XRD) analysis will be conducted to investigate the crystallinity and structural organization of both the raw chitosan and the cross-linked chitosan within the CTS-PEG/MMT nanocomposite. XRD patterns will help to identify any changes in the nanocomposite's crystalline structure that result from the cross-linking process, providing deeper insights into its structural properties.

Microscopic techniques, including Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM), will be employed to examine the surface morphology and internal structure of the nanocomposite. SEM will reveal surface features while TEM will provide high-resolution images of the material's internal architecture. Additionally, the thermal stability and material composition of the CTS-PEG/MMT nanocomposite will be assessed using thermogravimetric analysis (TGA) [28]. Overall, these comprehensive characterizations aim to provide a deeper understanding of the physicochemical and biological properties of the CTS-PEG/MMT nanocomposite, ensuring its potential for biomedical or other applications.

B. Discussion and Risk Management

B.1. Drug Loading: Inconsistent drug loading efficiency can result in underloaded or overloaded particles, which compromises therapeutic efficacy. To address this, optimizing the drug-polymer ratio and encapsulation conditions, such as temperature and mixing speed, is essential. Regular quality control checks ensure that drug loading remains uniform, preventing deviations that could impact the performance of the delivery system.

B.2. Droplet Generating: Variability in droplet size can lead to uneven drug distribution and fluctuations in release rates. To mitigate this risk, flow rates, channel dimensions, and droplet formation parameters must be carefully calibrated and fine-tuned. Regular calibration of the equipment and robust quality assurance processes help minimize variability and maintain consistency in droplet size.

B.3. Flow Control System: Fluctuating flow rates can disrupt droplet formation, resulting in irregular encapsulation and non-uniform delivery. Implementing precise and automated flow control systems equipped with feedback mechanisms is crucial. Additionally, using microfluidic systems with integrated sensors ensures steady and consistent flow rates, improving reliability and encapsulation efficiency.

B.4. Preparation of Fluids: Impurities or incorrect concentrations in the preparation of solutions can compromise the drug delivery system’s performance, especially at the microfluidic level. Employing proper filtration methods, clean room practices, as well as high-precision weighing or measuring techniques will ensure accuracy and experimental success. Routine quality checks (e.g., NMR, MS, FTIR and DLS) for chemical purity and solvent quality further reduce the risk of errors during the preparation phase.

B.5. Droplet Formation and Encapsulation: Inadequate solvent evaporation or improper phase mixing can lead to incomplete

encapsulation of the drug. Proper solvent removal through controlled evaporation techniques ensures the elimination of residual solvents. Fine-tuning the phase mixing process and optimizing flow conditions significantly improve encapsulation efficiency, leading to a more reliable delivery system.

B.6. Solidification and Drug Entrapment: Incomplete solidification of droplets can result in poor encapsulation and drug leakage. Controlling the drying and solidification process, including temperature and evaporation rate, ensures complete solidification and stable drug entrapment. Utilizing controlled environments or advanced techniques like lyophilization can enhance the reliability of this stage.

B.7. CTS-PEG/MMT Preparation: Improper mixing or inadequate interaction between chitosan (CTS), polyethylene glycol (PEG), and montmorillonite (MMT) can lead to poor coating quality. Ensuring proper sonication, consistent magnetic stirring (at optimum RPM), and precise solvent ratios guarantee uniform preparation of the mixture and batch-to-batch reproducibility. Regular characterization using techniques such as FTIR or XRD helps verify the consistency and quality of the prepared solution.

B.8. Coating of Droplet-Based Nanoparticles: Inconsistent coating thickness or incomplete coverage can weaken the protection of nanoparticles, reducing the system's drug delivery efficiency. Slow and controlled addition of the CTS-PEG/MMT solution, combined with careful stirring, ensures a uniform coating. Monitoring the coating process using advanced techniques like SEM verifies proper coverage and thickness.

B.9. Crosslinking with Tripolyphosphate (TPP): Inadequate or excessive crosslinking can adversely affect the stability of the coating and the drug release characteristics. Optimizing the TPP concentration and reaction time is critical for achieving the desired crosslinking level. Monitoring the process under controlled conditions ensures balanced crosslinking, avoiding issues of over- or under-crosslinking that could compromise the system's performance.

Biosafety and Biosecurity Assessment

Our project integrates biosafety and biosecurity principles to ensure responsible research practices. We have identified potential risks associated with the development of CTS-mPEG/MMT-coated nanoparticles for the treatment of *H. pylori*-induced gastritis and implemented strategies to mitigate them. Importantly, the biocompatibility of CTS-mPEG/MMT-coated nanoparticles will be thoroughly assessed using cytotoxicity assays (MTT and LDH) to ensure minimal adverse effects on non-target cells.

The preparation and testing of the nanoparticles will be conducted in a controlled laboratory environment adhering to standard biosafety level protocols. Measures such as appropriate personal protective equipment (PPE) and chemical waste management systems were utilized to minimize exposure risks and prevent environmental contamination. Rigorous decontamination and quality control checks will be applied to prevent contamination between samples and to ensure the reproducibility of the experiments.

Conclusions

We mainly propose this project to increase the low solubility of drugs such as eupatilin in acidic pH with chitosan/mPEG-MMT coating PLGA nanoparticles of the pH dependent diseases like *H. pylori* infected gastritis. Nanoparticle drug delivery system technology is mainly sustainable and innovative for enhancing the release of chemicals. A study concluded that Eupatilin-loaded gold nanoparticles are a promising targeted drug delivery system which is worthy of application in other natural products. We argue that this project has a solubility-enhancing effect of eupatilin and its synthetic derivatives, which are effective in the treatment of gastritis. In the following stages, if success is achieved in cytotoxicity and cell tests, we recommend that the drug delivery and solubility of the nanoparticle be evaluated in human cell cultures and animal experiments with gastritis models.

References

[1]: Sun, Y. W., Liang, H., Zong, K. Q., Che, X., & Meng, D. L. (2021). Green and facile preparation and dual-enhancement cytotoxicity of eupatilin loaded on hollow gold nanoparticles under near-infrared light. New Journal of Chemistry, 45(34), 15676-15681.

[2]: Suzuki, M., Suzuki, H., & Hibi, T. (2008). Proton pump inhibitors and gastritis. *Journal of Clinical biochemistry and nutrition*, *42*(2), 71-75.

[3]: Zhao, S., Lv, Y., Zhang, J. B., Wang, B., Lv, G. J., & Ma, X. J. (2014). Gastroretentive drug delivery systems for the treatment of Helicobacter pylori. *World journal of gastroenterology: WJG*, *20*(28), 9321.

[4]: Lin, Y. H., Chang, C. H., Wu, Y. S., Hsu, Y. M., Chiou, S. F., & Chen, Y. J. (2009). Development of pH-responsive chitosan/heparin nanoparticles for stomach-specific anti-Helicobacter pylori therapy. *Biomaterials*, *30*(19), 3332-3342.

[5]: Azer, S. A., & Akhondi, H. (2019). Gastritis.

[6]: Pennelli, G., Grillo, F., Galuppini, F., Ingravallo, G., Pilozzi, E., Rugge, M., ... & Mastracci, L. (2020). Gastritis: update on etiological features and histological practical approach. Pathologica, 112(3), 153.

[7]: Malfertheiner, P., Camargo, M. C., El-Omar, E., Liou, J. M., Peek, R., Schulz, C., ... & Suerbaum, S. (2023). Helicobacter pylori infection. Nature reviews Disease primers, 9(1), 19.

[8]: Seo, H. J., & Surh, Y. J. (2001). Eupatilin, a pharmacologically active flavone derived from Artemisia plants, induces apoptosis in human promyelocytic leukemia cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *496*(1-2), 191-198.

[9]: Du, K., Zheng, C., Kuang, Z., Sun, Y., Wang, Y., Li, S., & Meng, D. (2024). Gastroprotective effect of eupatilin, a polymethoxyflavone from Artemisia argyi H. Lév. & Vaniot, in ethanol-induced gastric mucosal injury via NF-κB signaling pathway. *Journal of Ethnopharmacology*, *318*, 116986.

[10]: Nageen, B., Sarfraz, I., Rasul, A., Hussain, G., Rukhsar, F., Irshad, S., ... & Ali, M. (2020). Eupatilin: a natural pharmacologically active flavone compound with its wide range applications. *Journal of Asian natural products research*, *22*(1), 1-16.

[11]: Jang, S. W., Lee, J. W., Park, S. H., Kim, J. H., Yoo, M., Na, D. H., & Lee, K. C. (2008). Gastroretentive drug delivery system of DA-6034, a new flavonoid derivative, for the treatment of gastritis. *International journal of pharmaceutics*, *356*(1-2), 88-94.

[12]: Cho, J. H., Yoon, H., Shin, C. M., Park, Y. S., Kim, N., & Lee, D. H. (2020). Efficacy of DA-5204 (Stillen 2X) for patients with gastroesophageal reflux disease: A randomized, double-blind, placebo-controlled pilot study. Medicine, 99(44), e22729.

[13]: Choi, E. J., Lee, S., Chae, J. R., Lee, H. S., Jun, C. D., & Kim, S. H. (2011). Eupatilin inhibits lipopolysaccharide-induced expression of inflammatory mediators in macrophages. *Life Sciences*, *88*(25-26), 1121-1126.

[14]: Lee, B. E., Park, S. J., Kim, G. H., Joo, D. C., & Lee, M. W. (2024). Anti-inflammatory effects of eupatilin on Helicobacter pylori CagA-induced gastric inflammation. *PloS one*, *19*(11), e0313251.

[15]: Choi, E. J., Oh, H. M., Na, B. R., Ramesh, T. P., Lee, H. J., Choi, C. S., ... & Jun, C. D. (2008). Eupatilin protects gastric epithelial cells from oxidative damage and down-regulates genes responsible for the cellular oxidative stress. *Pharmaceutical research*, *25*, 1355-1364.

[16]: Palacios-Espinosa, J. F., Núñez-Aragón, P. N., Gomez-Chang, E., Linares, E., Bye, R., & Romero, I. (2021). Anti-Helicobacter pylori activity of Artemisia ludoviciana subsp. mexicana and two of its bioactive components, Estafiatin and Eupatilin. *Molecules*, *26*(12), 3654.

[17]: Yonet-Tanyeri, N., Amer, M., Balmert, S. C., Korkmaz, E., Falo Jr, L. D., & Little, S. R. (2022). Microfluidic systems for manufacturing of microparticle-based drug-delivery systems: design, construction, and operation. ACS biomaterials science & engineering, 8(7), 2864-2877.

[18]: Frank, L. A., Onzi, G. R., Morawski, A. S., Pohlmann, A. R., Guterres, S. S., & Contri, R. V. (2020). Chitosan as a coating material for nanoparticles intended for biomedical applications. Reactive and Functional Polymers, 147, 104459.

[19]: Tsai, W. B., & Ahmed, I. N. (2023). The Impact of Polyethylene Glycol-Modified Chitosan Scaffolds on the Proliferation and

Differentiation of Osteoblasts. International Journal of Biomaterials, 2023(1), 4864492.

[20]: Bhagath, S., Vivek, A., Krishna, V. V., Mittal, S. S., & Balachandran, M. (2021). Synthesis and characteristics of MMT reinforced chitosan nanocomposite. Materials Today: Proceedings, 46, 4487-4492.

[21]: Zhang, M., Li, X. H., Gong, Y. D., Zhao, N. M., & Zhang, X. F. (2002). Properties and biocompatibility of chitosan films modified by blending with PEG. Biomaterials, 23(13), 2641-2648.

[22]: Diefenthaeler, H. S., Bianchin, M. D., Marques, M. S., Nonnenmacher, J. L., Bender, E. T., Bender, J. G., ... & Külkamp-Guerreiro, I. C. (2020). Omeprazole nanoparticles suspension: Development of a stable liquid formulation with a view to pediatric administration. International Journal of Pharmaceutics, 589, 119818.

[23]: Casettari, L., Vllasaliu, D., Castagnino, E., Stolnik, S., Howdle, S., & Illum, L. (2012). PEGylated chitosan derivatives: Synthesis, characterizations and pharmaceutical applications. Progress in Polymer Science, 37(5), 659-685.

[24]: Najafabadi, A. H., Abdouss, M., & Faghihi, S. (2014). Synthesis and evaluation of PEG-O-chitosan nanoparticles for delivery of poor water soluble drugs: Ibuprofen. *Materials Science and Engineering: C*, *41*, 91-99.

[25]: Ngawhirunpat, T., Wonglertnirant, N., Opanasopit, P., Ruktanonchai, U., Yoksan, R., Wasanasuk, K., & Chirachanchai, S. (2009). Incorporation methods for cholic acid chitosan-g-mPEG self-assembly micellar system containing camptothecin. *Colloids and Surfaces B: Biointerfaces*, *74*(1), 253-259.

[26]: Hou, D., Gui, R., Hu, S., Huang, Y., Feng, Z., & Ping, Q. (2015). Preparation and characterization of novel drug-inserted-montmorillonite chitosan carriers for ocular drug delivery. *Advances in Nanoparticles, 4*(3), 84–94.

[27]: Bhagath, S., Vivek, A., Krishna, V. V., Mittal, S. S., & Balachandran, M. (2021). Synthesis and characteristics of MMT reinforced chitosan nanocomposite. Materials Today: Proceedings, 46, 4487-4492.

[28]: Bhumkar, D. R., & Pokharkar, V. B. (2006). Studies on effect of pH on cross-linking of chitosan with sodium tripolyphosphate: a technical note. Aaps Pharmscitech, 7, E138-E143.