



REPUBLIC OF TURKEY
MARMARA UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

**VITAMIN B₁₂-LOADED NANOPARTICLE-EMBEDDED SCAFFOLDS
PRODUCED BY THE 3D PRINTER FOR CONTROLLED DRUG
DELIVERY**

FATIMA KHALED MOHAMMED ABOBAKR
MASTER'S THESIS

SUPERVISOR
ASSOC. PROF. MUHAMMET EMIN CAM
PHARMACOLOGY MASTER'S PROGRAM

ISTANBUL-2025



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STATEMENT

I hereby declare that this thesis study is my own work and that I have not engaged in any unethical behavior during the planning and writing of the thesis. I have sourced all the information in this thesis in compliance with academic and ethical guidelines. I have cited all information and comments not obtained through this thesis study, and the list of references includes these sources. Additionally, I hereby declare that I have not infringed on any patents or copyrights during the study and writing of this thesis.

Fatima Abobakr

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And say, 'My Lord, increase me in knowledge.'" — Surah Taha (114).

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ABBREVIATIONS and SYMBOLS LIST

A β : Beta-amyloid

AD: Alzheimer's disease

APP: Amyloid precursor protein

APH-1: Anterior pharynx defective 1

ApoE: Apolipoprotein E

B6: Vitamin B6

B9: Vitamin B9 (Folate)

BACE1: Beta-site amyloid precursor protein cleaving enzyme 1

BBB: Blood-brain barrier

CDK5: Cyclin-dependent kinase 5

CLSM: Confocal laser scanning microscopy

CS: Chitosan

DHA: Docosahexaenoic acid

DDS: Drug delivery system

FTIR: Fourier-transform infrared spectroscopy

FAD: Familial Alzheimer's disease

HDL: High-density lipoprotein

Hcy: Homocysteine

Hcy/methionine cycle: The metabolic cycle involving homocysteine and methionine

IDE: Insulin-degrading enzyme

MCM: Methylmalonyl-CoA mutase

MAP: Microtubule-associated protein

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay)

NEP: Neprilysin

NMDA: N-Methyl-D-Aspartate

NCSTN: Nicastrin

PEO: Polyethylene oxide

PEN-2: Presenilin enhancer 2

PHF: Paired helical filaments

PP2A: Protein phosphatase 2A

PVA: Polyvinyl alcohol

PVP: Polyvinylpyrrolidone

PS: Pure scaffolds

PS1/2: Presenilin 1 and Presenilin 2

R²: Coefficient of determination (used in regression analysis)

SAM: S-adenosylmethionine

SAMe: S-adenosylmethionine (inferred from context)

SH-SY5Y: Human neuroblastoma cell line

TPP: Tripolyphosphate

VB₁₂: Vitamin B12

VB₁₂NPs: VB12 nanoparticles

VB₁₂NPS: VB12 nanoparticle-loaded scaffolds

XRD: X-ray diffractometry

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1. ÖZET

Tez Başlığı: Kontrollü İlaç Dağıtımı İçin 3D Yazıcıyla Üretilen Vitamin B12-Yüklü Nanopartikül Gömülü İskeleler

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Amaç: Bu araştırmanın amacı, 3 boyutlu yazdırılmış poli(etilen oksit)/polivinilpirolidon (PEO/PVP) iskelelerine yerleştirilmiş Vitamin B₁₂ (VB₁₂) yüklü kitosan-tripolifosfat (TPP) nanopartikülleri (VB₁₂NPs) kullanılarak Alzheimer hastalığı (AD) için bir dil altı ilaç dağıtım sistemi geliştirmektir. Amaç, bilişsel sağlıkla ilişkili nöroprotektif bir bileşik olan VB₁₂'nin mukozal dağıtımını, stabilitesini ve biyoyararlanımını iyileştirmektir.

Gereç ve Yöntemler: VB₁₂NPs iyonik jelasyon kullanılarak sentezlendi ve daha sonra PEO/PVP iskelelerine gömüldü. Üretilen malzemelerin fizikokimyasal özelliklerini araştırmak için SEM, FTIR, XRD ve DSC teknikleri, zeta potansiyeli, polidispersite indeksi, kapsülleme verimliliği ve parçalanma testi uygulandı. MTT testi, SH-SY5Y hücre hattında *in vitro* sitotoksiteyi değerlendirmek için kullanıldı ve hücre alım konfokal lazer tarama mikroskobu ile analiz edildi.

Bulgular: VB₁₂NP'ler $348,38 \pm 1,79$ nm'lik bir boyut, $1,39 \pm 0,04$ mV'lik bir zeta potansiyeli ve $0,317 \pm 0,02$ Mw/Mn'lik bir PDI gösterdi. Bu da kararlı ve monodispers parçacıkların üretilbildiğini ispatladı. İskeleler, NP'lerin dahil edilmesinden sonra ortalama gözenek boyutunun $337 \mu\text{m}$ 'den $248,72 \mu\text{m}$ 'ye düşmesiyle, birbirine bağlı gözeneklerden oluşan bir ağ sergiledi. FTIR, DSC ve XRD kullanılarak yürütülen analizler, polimer ve ilaç arasındaki etkileşimlerin yanı sıra karışık-fazlı bir yapının varlığını da doğruladı. Hücre canlılığı, tüm iskele örneklerinde sürekli olarak %85'ten daha yüksekti ve bu da dikkate değer bir biyoyumluluk sağladı. Konfokal mikroskopi, VB₁₂NPs'in başarılı hücre alımını göstererek bunların mukozal emilim potansiyelini doğruladı.

Sonuç: VB₁₂ yüklü NP iskelesi, avantajlı morfoloji, biyoyumluluk, hızlı parçalanma ve mukozal alım göstererek, etkili bir dil altı ilaç verme sistemi olarak umut vadetmektedir.

Anahtar kelimeler: Alzheimer hastalığı, vitamin b12, 3D baskı, kompozit iskelet, dil altı uygulaması

2. SUMMARY

Title of Thesis: Vitamin B₁₂-Loaded Nanoparticle-Embedded Scaffolds Produced By The 3D Printer for Controlled Drug Delivery

Student Name, Surname: Fatima ABOBAKR

Supervisor Name, Surname: Assoc. Prof. Muhammet Emin ÇAM

Program Name: Pharmacology (Pharmacy) Master's Program

Objective: The goal of this research is to develop a sublingual drug delivery system for Alzheimer's disease (AD) using Vitamin B₁₂ (VB₁₂)-loaded chitosan-tripolyphosphate (TPP) nanoparticles (VB₁₂NPs) incorporated into 3D-printed poly(ethylene oxide)/polyvinylpyrrolidone (PEO/PVP) scaffolds. The objective is to improve the mucosal delivery, stability, and bioavailability of VB₁₂, a neuroprotective compound associated with cognitive health.

Materials and Methods: VB₁₂NPs were synthesized using ionic gelation and then embedded into PEO/PVP scaffolds. To investigate the physicochemical characteristics of the produced materials, SEM, FTIR, XRD, and DSC techniques, zeta potential, polydispersity index, encapsulation efficiency, and disintegration test were applied. The MTT assay was utilized to assess *in vitro* cytotoxicity on the SH-SY5Y cell line, and cellular uptake was analyzed through confocal laser scanning microscopy.

Results: The VB₁₂NPs demonstrated a size of 348.38 ± 1.79 nm, a zeta potential of 1.39 ± 0.04 mV, and a PDI of 0.317 ± 0.02 Mw/Mn, suggesting the presence of stable, monodisperse particles. The scaffolds exhibited a network of interconnected pores, with the average pore size decreasing from 337 μ m to 248.72 μ m following the incorporation of nanoparticles. The analyses conducted using FTIR, DSC, and XRD provided confirmation for the interactions between the polymer and the drug, as well as the presence of a mixed-phase structure. Cell viability exceeded 85% for all NPs samples, indicating remarkable biocompatibility. Confocal microscopy demonstrated successful cellular uptake of VB₁₂NPs, validating their internalization into cells and potential for mucosal absorption.

Conclusion: The VB₁₂NPs scaffold demonstrates advantageous morphology, biocompatibility, rapid disintegration, and cellular internalization, indicating its promise as an efficient sublingual drug delivery system for AD.

Keywords: Alzheimer's disease, vitamin b₁₂, 3D printing, composite scaffold, sublingual administration

3. INTRODUCTION and OBJECTIVE

Alzheimer's disease (AD) is a neurodegenerative condition that compromises memory, cognitive abilities, and daily functioning (Cummings et al., 2015; Lauer et al., 2022). Approximately 50 million people are affected by this type of dementia worldwide, with projections indicating an increase to 100 million by 2050. The exact mechanism of AD's development is still under investigation, yet there is general agreement that the aggregation of extracellular beta-amyloid ($A\beta$) plaques and intracellular neurofibrillary tangles (NFTs), which are composed of hyperphosphorylated microtubule-associated tau proteins, causes neural dysfunction (Andrade et al., 2022; Aydin et al., 2022; Tiwari et al., 2019). Additionally, several studies have confirmed that elevated homocysteine (Hcy) levels, also known as hyperhomocysteinemia, have been identified as a major risk factor for AD. Hcy is increased in the cerebrospinal fluid and blood of AD patients compared to healthy controls, with meta-analyses and population studies such as those by Van Dam and Van Gool (2009) (Van Dam & Van Gool, 2009) and Seshadri et al. (2002) (Seshadri et al., 2002), indicating its predictive value for dementia years before onset. Increased levels of Hcy have been associated with increased tau protein phosphorylation, which results in the formation of NFTs, an independent risk factor for AD. Hcy negatively impacts blood-brain barrier (BBB) function, causes neuronal damage, affects $A\beta$ generation and toxicity, and causes oxidative stress, all of which contribute to AD pathology (Douaud et al., 2013; Seshadri et al., 2002; Zhang et al., 2008). Oxidative stress, neuroinflammation, and mitochondrial dysfunction are additional mechanisms that exacerbate neuronal damage (Butterfield & Halliwell, 2019; Castro-Gomez & Heneka, 2024; Wang et al., 2014). AD continues to impose a social and economic burden due to its incurability, despite the endeavors of the pharmaceutical and scientific sectors. Unfortunately, the medications that are currently available for AD only provide temporary relief from symptoms and do not cure the disease (Andrade et al., 2022; Massoud & Léger, 2011). As a result, there is a need for an urgent medicine that can delay or treat AD (Andrade et al., 2022).

Natural compounds have been the focus of recent studies as a potential substitute for synthetic chemotherapeutics, mainly due to the fact that they have a lower chance of causing negative side effects. In the view of Andrade, S., et al. the consumption of bioactive compounds can help reduce the mechanisms that cause neurodegenerative diseases, such as AD (Andrade et al., 2022). Vitamin B₁₂ (VB₁₂), also known as cobalamin, is a water-soluble vitamin that is essential for maintaining

neuron health and hematopoiesis (Green et al., 2017). In addition, emerging studies demonstrate that VB₁₂ improves cognitive performance, reduces inflammatory cytokines, and protects against A β -induced proteotoxicity through the SAMe cycle as well as reducing A β fibrillation and BACE1 expression (Andrade et al., 2022; Lam et al., 2021; Ma et al., 2019). Furthermore, VB₁₂ has a significant antioxidative mechanism that reverses oxidative stress caused by Hcy by functioning as a cofactor of methionine synthase, transforming Hcy into methionine (Lauer et al., 2022). This lowers plasma Hcy levels and consequently, stops tau phosphorylation, linked to AD. Furthermore, VB₁₂ makes a significant contribution to neuronal metabolism, such as transmethylation, energy production, and synaptic formation. It also aids in myelin formation by stimulating protein synthesis in myelin-producing oligodendrocytes (Lauer et al., 2022). These neuroprotective effects, which include its ability to reverse oxidative damage, reduce tau phosphorylation, and improve synaptic function, make it a promising therapeutic agent for AD treatment (McCaddon, 2013). However, there is no conclusive evidence that VB₁₂ supplementation improves memory or delays the onset of AD. The failure of VB₁₂ to cross the BBB is primarily due to its water-loving nature and significant molecular weight (1355 Da). The BBB exhibits a high level of selectivity, allowing only small, lipophilic molecules to pass through via passive diffusion. Hydrophilic molecules such as VB₁₂ cannot readily diffuse through the lipid-rich endothelial cells of the BBB, and its molecular weight surpasses the typical cutoff (400-600 Da) for passive permeability, thereby restricting its transport into the brain (Andrade et al., 2022).

VB₁₂ administered orally faces similar limitations as other oral medications, stemming from various challenges such as first-pass metabolism, reduced absorption in the gastrointestinal tract, and rapid clearance (Cano et al., 2021). These issues can be addressed by developing drug delivery systems (DDSs) that improve the transport of VB₁₂ across the BBB, avoid first-pass metabolism, enhance gastrointestinal absorption, and reduce rapid elimination through sustained release formulations (Andrade et al., 2022).

To solve these challenges, nanomedicine has demonstrated efficacy in delivering therapeutics to the brain (Andrade et al., 2022). Investigations have examined the utilization of drug-loaded nanocarriers as a viable strategy for addressing diverse neurodegenerative disorders, improving drug pharmacokinetics and pharmacodynamics while mitigating toxicity (Aydin et al., 2022; Cano et al., 2021; Elzayat et al., 2022; Guler, Abobakr, et al., 2024). Among nanocarriers, biodegradable polymer-based nanoparticles (NPs) are the most commonly utilized carriers in nanomedicine, owing to several benefits including enhanced stability, targeted delivery, and improved

bioavailability (Cano et al., 2021; Karuppusamy & Venkatesan, 2017). An exemplary case is the research conducted by Guler E. et al., who created chitosan (CS)-based VB12NPs, showcasing their ability to address delivery challenges and setting the stage for additional studies on VB12 loaded NP for AD treatment (Guler, Yekeler, et al., 2024).

CS is a semi-synthetic hydrophilic polymer recognized for its biodegradability, biocompatibility, and natural occurrence. It has been thoroughly investigated as a nanocarrier for various drug molecules, genes, and proteins. It is concurrently being examined as an independent intervention for the prevention and amelioration of AD's symptoms (Manek et al., 2020; Sarvaiya & Agrawal, 2015). CS possesses a pKa ranging from 6.2 to 7.0, rendering it insoluble in neutral and alkaline pH conditions. Nonetheless, it acquires a positive charge in acidic conditions, a crucial attribute for its role as a nanocarrier. The ionic gelation technique produces CS NP by utilizing the electrostatic interactions that occur between protonated amino groups of CS and an anionic cross-linker such as tripolyphosphate (TPP) (Desai, 2016). TPP is a low-toxicity, economical polyanion that facilitates the formation of well-defined nanoparticles under mild conditions while maintaining bioactive compounds (Di Santo et al., 2021). Polyvinyl alcohol (PVA), a biocompatible and biodegradable synthetic polymer, is commonly utilized in the formulation of NPs to enhance stability and structural integrity (Kyrychenko et al., 2017; Oyeoka et al., 2021).

Sublingual drug delivery is widely recognized for its advantages, which include precise dosing, simple administration, and improved patient compliance due to a reduction in the frequency of doses. These benefits make it perfect for chronic conditions like AD, where sticking to a medication schedule is crucial (Yekeler et al., 2024). However, it needs certain characteristics from the administered DDS, including rapid disintegration and dissolution, bioadhesivity, and a large surface area (Onal et al., 2024).

3D printing is an advanced manufacturing technology used in the pharmaceutical industry. It is a powerful tool for developing drug delivery systems (DDS) because it allows for the creation of drug delivery scaffolds that are highly customizable and precise (Agrawal et al., 2021). Unlike the traditional "one size fits all" approach, 3D printing allows for the creation of medications that can be released in a controlled way, supports multi-drug formulations, and results in tablets that dissolve quickly (Azad et al., 2020). Polymeric materials are often utilized in 3D printed products to stabilize medications within matrices (Deepak et al., 2018). The polymers utilized in this project were biocompatible, dispersible, and adhesive, fulfilling the criteria for transmucosal

administration. PEO functioned as the mechanical backbone of the system (Pereao et al., 2021), whereas PVP acted as a crystalline inhibitor, enhancing dispersion and facilitating disintegration processes (Chen et al., 2016). The components collaborated seamlessly to develop a DDS that is both efficient and suitable for sublingual drug delivery.

The objective of this work, given the background provided, is to develop and evaluate an advanced sublingual DDS aimed at treating AD. The ionic gelation method was used to synthesize VB₁₂NPs. Subsequently, these NPs were incorporated into the 3D-printed PEO/PVP scaffolds. The analysis of the two delivery systems employed a range of methods, such as scanning electron microscopy (SEM), Fourier transforms infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), and X-ray diffractometry (XRD). Additional analyses encompassed investigations into drug release, release kinetics, disintegration testing, encapsulation efficiency, and zeta potential. Furthermore, the systems underwent *in vitro* analysis for biological activity through polymerase chain reaction (PCR) to evaluate gene expression, MTT assays to measure cell viability and cytotoxicity, and *in vitro* cellular uptake studies to determine the system's efficacy in delivering its payload into target cells.

4. BACKGROUND

4.1. What is AD?

Dementia is a category of diseases characterized by the deterioration of memory and similar mental abilities, as well as the decline of daily cognitive functions. Approximately 50 million people live with dementia worldwide with AD being the most common type, constituting 50-75% of cases, is a neurodegenerative disease that causes functional impairments such as memory loss and cognitive decline, and constitutes 50-75% of dementia today. AD is a progressive neurodegenerative disease that leads to memory loss and cognitive decline (Ayodele et al., 2021; Pentkowski et al., 2021). According to the 2022 report of the American Alzheimer's Association, it is predicted that 6.5 million individuals aged 65 and over live with AD in the United States, and this number could reach 13.8 million by 2060. While the number of individuals with AD is expected to increase to 107 million worldwide by 2050, it is stated that approximately 321 billion dollars will be spent on health, long-term care, and nursing home services for these patients in 2022 alone (Calabrò et al., 2020). Dementia can be defined as a progressive disease that causes severe cognitive impairment and neuronal loss as a result of organic brain disease, and the most common cause worldwide is AD, known as primary brain damage. Although it has existed for thousands of years, the clinical syndromes of dementia and the associated neurodegenerative changes were only described in the early 20th century. In 1907, Alois Alzheimer meticulously documented the memory loss and cognitive impairments of Auguste Deter, a 51-year-old man who was being treated in a mental hospital and histologically analyzed his brain tissue using silver staining after his death. As a result of this analysis, he noted the progression of NFTs, neuritic plaques, and A β angiopathy, which are now considered the distinguishing pathological features of AD (Agrawal & Jha, 2020; Bondi et al., 2017).

4.2. Presentation and Progression of AD

AD's pathological process appears many years before clinical expression and is marked by the buildup of A β plaques and NFTs composed of tau protein in the brain (Jack et al., 2010). In this preclinical phase, patients do not exhibit any apparent symptoms, however, brain imaging and biomarker studies can detect pathological changes (Bateman et al., 2012; Fiandaca et al., 2014). The clinical course of the disease typically progresses in three main stages. In the mild cognitive

impairment (MCI) stage, patients often experience short-term memory problems in particular and exhibit symptoms that mildly affect daily life, such as forgetting appointments or misplacing things. During this period, family members often tend to attribute these changes to the natural aging process (Petersen et al., 1999).

As the disease advances to the middle stage, symptoms become increasingly evident and begin to show the characteristic "5A" symptom complex: Amnesia (pronounced memory loss), aphasia (difficulty in finding words and impaired language skills), apraxia (inability to perform complex motor tasks), agnosia (sensory perception disorders such as facial recognition) and abnormal executive functions (impairment in the ability to plan and organize). In this stage, patients increasingly need help with daily life activities (Jadhav et al., 2024).

In the advanced stage, patients become completely dependent. Emotional lability (unexplained crying or laughing spells), personality changes, hallucinations, and delusions are common. In the final stage, urinary and fecal incontinence, difficulty swallowing and complete loss of speech develop. Patients become bedridden and usually lose their lives due to complications such as infections (Honig & Mayeux, 2001).

Clinical evaluation, neuropsychological tests (e.g., Mini-Mental State Examination [MMSE], Montreal Cognitive Assessment [MoCA]), and neuroimaging methods such as magnetic resonance imaging (MRI) and positron emission tomography (PET) are used in the diagnosis process (Del Sole et al., 2017; Wang et al., 2022). Especially in the early stages, differential diagnosis with other neurodegenerative disorders, such as frontotemporal dementia or dementia with Lewy bodies, is important. Biomarker studies, including cerebrospinal fluid (CSF) assays and A β PET imaging, can support the diagnosis, but the routine use of these methods is still limited (Solje et al., 2021).

4.3. Pathogenesis and Hallmarks of AD

Pathologically, AD is identified by extracellular neuritic plaques and intracellular NFTs, which primarily accumulate in susceptible areas of the brain, including the hippocampus and cerebral cortex (Glenner & Wong, 1988; Grundke-Iqbal, Iqbal, Tung, et al., 1986). These plaques consist predominantly of peptides that impair cognitive functions, produced through the amyloidogenic processing pathway of the amyloid precursor protein (APP), a type I transmembrane protein (Dyrks et al., 1988). As shown in Figure 1, the process begins with β -secretase (BACE1) cleavage of APP to form N-terminus of A β peptides and γ -secretase-catalyzed cleavage of remaining C-terminal

fragments (Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). The γ -secretase complex comprising Presenilin 1 or 2 (PS1/2), anterior pharynx defective 1 (APH-1), presenilin enhancer 2 (PEN-2) and nicastrin (NCSTN) (Grimm et al., 2002; Haass, 2004; Kimberly et al., 2003), possesses the unusual intramembrane proteolytic activity generating multiple A β isoforms in which A β 40 constitutes 80-90% and the more pathogenic A β 42 accounts for around 10% of all A β generated. Heterogeneity of A β species is a result of the nonspecific cleavage of γ -secretase in the transmembrane domain of APP, a reaction influenced by various membrane lipids that regulate A β formation, aggregation, and clearance (Grimm et al., 2013; Grimm et al., 2011; Grimm, Mett, et al., 2016; Grimm et al., 2012; Rothhaar et al., 2012). The gradual accumulation of A β peptides in the brain, perhaps started decades before clinical onset indicates a disruption in the balance between pathways that produce and eliminate A β , which are managed by degrading enzymes like insulin-degrading enzyme (IDE) and neprilysin (NEP) (Farris et al., 2003; Iwata et al., 2001).

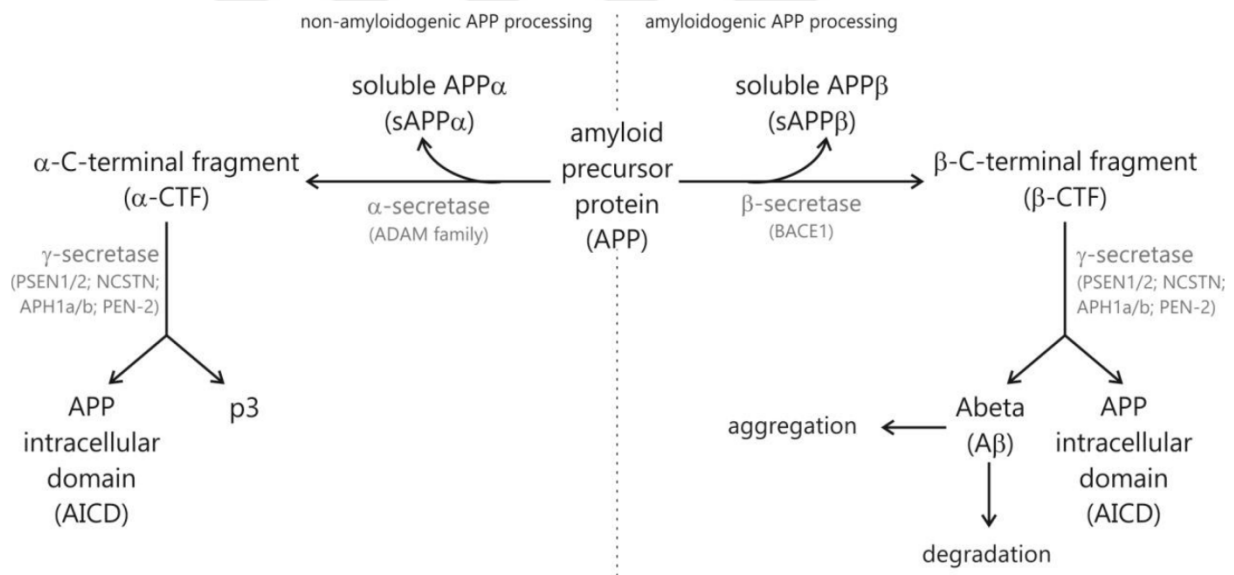


Figure 1. Schematic representation of the processing of APP, highlighting both amyloidogenic and non-amyloidogenic pathways, along with the resulting cleavage products (Lauer et al., 2022).

Importantly, accumulating A β peptides actively promote tau pathology by activating kinases like GSK-3 β and CDK5 while simultaneously inhibiting protein phosphatases such as PP2A. Such A β -induced disruption of phosphorylation homeostasis drives tau hyperphosphorylation and resultant aggregation (Singh et al., 2024). In contrast with amyloidogenic processing, APP may be processed non-amyloidogenically by α -secretases from the ADAM (a disintegrin and metalloprotease) protein

family, which cleave APP in the middle of the A β region and therefore prevent A β formation (Buxbaum et al., 1998; Kuhn et al., 2010; Lammich et al., 1999).

While most of the AD is sporadic late-onset type (LOAD) that occurs after age 65, approximately 5% are familial cases (FAD) caused by autosomal dominant mutations in APP, PS1, or PS2 genes predisposing to excessive production of highly amyloidogenic A β ₄₂ peptides. Progression of the disease follows BRAAK stages corresponding to the extent of tau pathology, which are NFTs of hyperphosphorylated tau protein that constitute insoluble paired helical filaments (PHF) in neurons (Goedert et al., 1992; Grundke-Iqbal, Iqbal, Quinlan, et al., 1986; Grundke-Iqbal, Iqbal, Tung, et al., 1986). Under normal conditions, tau acts as a microtubule-associated protein (MAP) that is essential for the assembly and stability of microtubules, supporting neuronal shape and mediating axonal transport (Weingarten et al., 1975), but in AD, the additive consequences of A β -mediated activation of kinases and inhibition of phosphatases result in abnormal hyperphosphorylation of tau by GSK-3 β and CDK5 and decreased PP2A activity (Martin, Latypova, Wilson, Magnaudeix, Perrin, & Terro, 2013; Martin, Latypova, Wilson, Magnaudeix, Perrin, Yardin, et al., 2013).

4.4. Risk Factors of AD

The aging process continues to be the most significant risk factor for LOAD, yet a variety of both non-genetic and genetic factors have been identified. Metabolic conditions including elevated cholesterol levels, increased Hcy levels, high blood pressure, hardening of the arteries, diabetes, and excessive body weight (Barnes & Yaffe, 2011; Polidori et al., 2012) are the most significant modifiable risk factors, followed by dietary components and micronutrient deficiencies (Luchsinger et al., 2007; Yusuf et al., 2017). Genetically, the apolipoprotein E (ApoE) ϵ 4 allele is the most potent risk factor for sporadic AD (Corder et al., 1993; Strittmatter et al., 1993). ApoE, a central nervous system lipid acceptor, enhances cholesterol clearance and high-density lipoprotein (HDL) production, with isoforms differentially regulating A β clearance (Castellano et al., 2011; Deane et al., 2008; Ma et al., 1994). The ApoE4 allele is strongly associated with hypercholesterolemia, which epidemiological studies correlate with elevated AD risk during midlife (Matsuzaki et al., 2011; Meng et al., 2014; Pappolla et al., 2003). Postmortem analysis detects increased cholesterol in AD brains, particularly in amyloid plaques (Burns & Duff, 2002), and cell studies demonstrate that cholesterol enhances A β production by multiple mechanisms: (1) direct activation of β - and γ -secretase activity (Ferrera et al., 2008), (2) APP internalization into acidic compartments that favor amyloidogenic processing (Cossec et al., 2010; Kojro et al., 2001),

and (3) lipid raft enrichment, membrane microdomains that promote APP cleavage (Hao et al., 2001; Simons et al., 1998). Cholesterol further accelerates A β aggregation and potentiates its neurotoxicity, an action reflected in animal models of increased A β levels under hypercholesterolemic conditions (Faßbender et al., 2001). Beyond cholesterol dysregulation, hyperhomocysteinemia has also been implicated in AD pathogenesis, wherein elevated Hcy levels are observed in cerebrospinal fluid and blood of AD patients (Zhang et al., 2022). Prospective studies correlate elevated Hcy with dementia risk years before symptom onset (McCaddon et al., 2001; Seshadri et al., 2002; Zylberstein et al., 2011), owing to its ability to destabilize BBB (Kamath et al., 2006), harm neurons (Maler et al., 2003), modulate A β (White et al., 2001), and induce oxidative stress (Chen & Zhong, 2014; Huang et al., 2016). Oxidative stress is itself an independent AD driver, indicated by neurons maintaining reduced glutathione levels combined with elevated PUFA content and susceptibility to lipid peroxidation (Ran et al., 2006). Paradoxically, oxidized docosahexaenoic acid (DHA) increases soluble A β (Grimm, Hauptenthal, et al., 2016), while more generic oxidative damage to proteins, lipids, and nucleic acids is well-established in AD brains (Shichiri, 2014). Such stress perpetuates a vicious cycle, increasing A β deposition (Apelt et al., 2004; Nunomura et al., 1999), tau hyperphosphorylation (Zhao & Zhao, 2013), and mitochondrial impairment—the latter witnessed by A β accumulation in neuronal mitochondria, which disrupts electron transport (e.g., reduced cytochrome-c oxidase activity) and elevated hydrogen peroxide (H₂O₂) generation (Manczak et al., 2006). Oxidative stress is also promoted by neuroinflammation (Lee et al., 2010), metal ion dysregulation (Ayton et al., 2013; Zhao & Zhao, 2013), and tau pathology (Dias-Santagata et al., 2007). Therapies targeting oxidative pathways are promising, such as antioxidants [96–100] and fat-soluble vitamins (A, D, E, K) with neuroprotective effects.

4.5. AD Treatment

Currently, AD is not curable, however, multiple medication therapies are employed to reduce symptoms and slow cognitive decline (Atri, 2019). Cholinesterase inhibitors, including donepezil, rivastigmine, and galantamine, are the most common medications to counteract the deficiency in acetylcholine resulting from neuronal degeneration, especially within the nucleus basalis of Meynert (Singh & Jha, 2021). Donepezil was the first cholinesterase inhibitor licensed in Europe in 1997 and remains a cornerstone of treatment (Wilkinson, 1999), with evidence of benefit in cognition at intervals of up to 240 weeks (Rogers et al., 2000). Nonetheless, patients with cardiac conduction abnormalities need to be treated cautiously due to the risk of bradycardia (Morris et al.,

2021). Memantine, Memantine, a non-competitive antagonist of the NMDA receptor, is used in moderate-to-severe AD and works by normalizing glutamatergic neurotransmission to prevent neurotoxicity, generally prescribed with cholinesterase inhibitors for established disease (Balazs et al., 2021; Kabir et al., 2019). Beyond pharmaceuticals, Souvenaid, a medical food that consists of precursors and cofactors of neuronal membrane synthesis, has been included to enable nutritional demands in AD patients (Ritchie et al., 2014). Furthermore, research into herbal treatments such as huperzine, which is derived from Chinese medicine, and bacopa suggests alternative mechanisms for managing cognitive symptoms (Zangara, 2003).

Therapies against oxidative pathways, such as antioxidants and fat-soluble vitamins (A, D, E, and K) with neuroprotective effects, are also emerging as promising adjunct therapies (Orywal et al., 2025). Furthermore, water-soluble Vitamin B₁₂ (cobalamin) also holds promise for delivering multi-target benefits in AD by combining its antioxidant effect with modulation of significant disease-related pathways (Halczuk et al., 2023; Lauer et al., 2022). These novel therapeutic avenues resonate with a growing recognition that an intermodal treatment strategy—blending pharmacologic, nutritional, and molecular therapy—is best suited to address the multifactorial pathology of AD.

4.6. Vitamin B₁₂ (VB₁₂)

B vitamins, particularly VB₁₂ (cobalamin), are essential hydrophilic micronutrients obtained through the diet. They play essential roles in neuronal integrity and hematopoiesis. Although clinical manifestations of VB₁₂ deficiency, myeloneuropathy and megaloblastic anemia, are relatively rare in the developed world, subclinical VB₁₂ deficiency is reasonably prevalent, in the range of 10–15% of individuals above 60 years of age and 25–35% of individuals above 80 years of age (Green et al., 2017). Subclinical deficiency with serum VB₁₂ levels ranging from 119 to 200 pmol/L often remains asymptomatic for long periods. Due to its antioxidant action, VB₁₂ deficiency exacerbates oxidative lipid, protein, and nucleic acid damage and thus predisposes individuals to the development of age-related disorders like AD, Parkinson's disease, and type 2 diabetes, where oxidative stress is known to act as a contributory pathogenic mechanism (McCaddon, 2013).

VB₁₂ possesses antioxidant activity via multiple mechanisms, including direct neutralization of ROS, most notably superoxide anions in the cytosol and mitochondria (Moreira et al., 2011), and indirectly through the preservation of cellular levels of glutathione (Karamshetty et al., 2016).

Additionally, VB₁₂ may prevent oxidative stress secondary to inflammation through its control of cytokine and growth factor secretion, e.g., interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α), and epidermal growth factor. Neuroinflammation, specifically, has been a well-documented promoter of the development of AD (Kinney et al., 2018). Impaired VB₁₂ status correlates with increased production of IL-6 and TNF- α (Politis et al., 2010); importantly, IL-6 induces hyperphosphorylation of the tau protein, while TNF- α induces A β aggregation through β -secretase upregulation and enhanced γ -secretase activity (Quintanilla et al., 2004).

Another significant antioxidant action of VB₁₂ is related to Hcy metabolism. VB₁₂ serves as an essential cofactor for methionine synthase, which facilitates the methylation of Hcy to methionine. VB₁₂ deficiency impairs this methylation, resulting in elevated intracellular levels of Hcy (Green et al., 2017). Hcy accumulation induces oxidative stress through multiple mechanisms, such as autooxidation, yielding hydrogen peroxide (H₂O₂), and inhibition of essential antioxidant enzymes such as glutathione peroxidase and superoxide dismutase (Weiss, 2005).

Beyond its antioxidative role, VB₁₂ plays a vital role in central and peripheral nervous system function, affecting cellular energy metabolism, myelin synthesis, and neurotransmitter regulation (Calderón-Ospina & Nava-Mesa, 2020; Evans & Lawrenson, 2017). Vitamin B9 (folate), vitamin B6, and VB₁₂ act synergistically in the Hcy/methionine cycle (Figure 2) (Miller, 2003). Methyl-folate donates a methyl group to activate VB₁₂ to its methylated form, which is an essential cofactor for methionine synthase. Methionine is subsequently metabolized to S-adenosylmethionine (SAM), a general methyl donor in DNA, RNA, protein, and lipid methylation and thus influencing genomic stability, gene expression, neuronal differentiation, and repair (Kennedy, 2016; Surtees et al., 1991). Deficiencies in VB₁₂ or folate compromise these methylation reactions with the potential to induce hippocampal atrophy, demyelination, and impaired nerve impulse transmission.

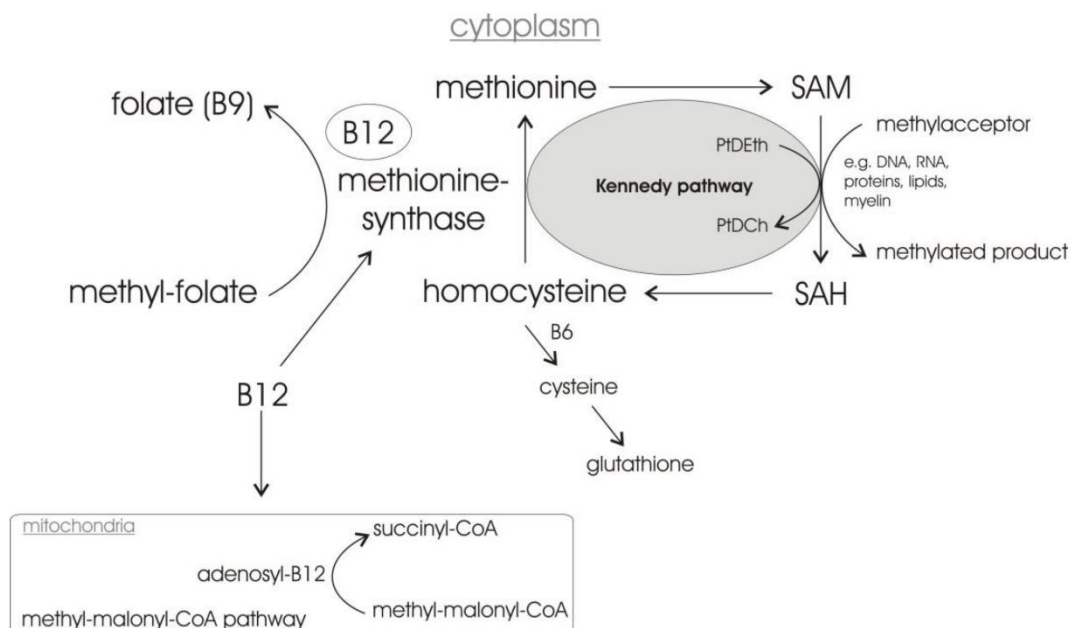


Figure 2. Depiction of both the homocysteine/methionine cycle and the biochemical mechanism of action of vitamin B12 in the homocysteine/methionine and methylmalonyl-CoA pathways (Lauer et al., 2022)

In parallel, vitamin B6 offers an alternative Hcy elimination pathway by metabolizing it to cysteine, a precursor to glutathione antioxidant. VB₁₂ also enters mitochondria to support methylmalonyl-CoA mutase (MCM), an enzyme that catalyzes methylmalonyl-CoA to succinyl-CoA, a vital citric acid (Krebs) cycle intermediate necessary for ATP synthesis.

Of particular significance is that S-adenosylmethionine (SAM) is necessary for the Kennedy cycle, which contributes to generating phosphatidylcholine, the prominent phospholipid constituent of neuronal membranes (Walker et al., 2011) (Figure 2). Dietary supplementation with lipid synthesis precursors has been shown to enhance neurite outgrowth and synaptogenesis (van Deijk et al., 2017). The medical food Souvenaid includes Fortasyn Connect (a nutritional mix that includes vitamins B₆, B₁₂, and folate) and has been shown to increase synaptogenesis in neuron-astrocyte co-cultures without influencing the number of astrocytes (van Deijk et al., 2017). Moreover, clinical trials have shown that Souvenaid improves the function of memory in early-stage AD patients (Soininen et al., 2017), pointing to its role as a synapse aid.

Beyond its function in Hcy metabolism, neurotransmitter synthesis, energy metabolism, and synaptogenesis, VB₁₂ also functions in fatty acid and nucleic acid synthesis. VB₁₂ plays a fundamental function in the development of myelin by regulating DNA synthesis by

oligodendrocytes (Briani et al., 2013; Calderón-Ospina & Nava-Mesa, 2020; Herrmann & Obeid, 2008). Interestingly, recent work indicates that demyelination may be one of the very early events during AD pathology, even before A β and tau pathology (Papuc & Rejdak, 2020). VB₁₂ supplementation has further been associated with nerve regeneration after damage (Adamo, 2014; Evans & Lawrenson, 2017).

4.7. Nanotechnology

Nanotechnology refers to the capacity to manipulate and reorganize materials at the molecular and atomic scale, specifically within the range of approximately 1-100 nanometers, and to utilize the novel properties that arise at these dimensions. Nanotechnology has produced diminutive structures and customized materials, devices, and systems exhibiting fundamentally novel properties (Roco, 2011). By tailoring material properties at the nanoscale in an economically feasible manner, it becomes possible to harness the most functionally advantageous dimensions for use in manufacturing and medicine (Jasmani et al., 2020). Since the early 2000s, this technology has increasingly replaced traditional industrial practices, particularly in advanced sectors (Bayda et al., 2019). While fields such as nanoelectronics have witnessed rapid innovation, adoption in more conventional industries like wood and paper has progressed more gradually (Jasmani et al., 2020). Nanomaterials are purposefully engineered to exhibit functional advantages that are unattainable in bulk or macro-scale forms. Both main approaches used in nanotechnology are the "bottom-up" and "top-down" approaches (Bayda et al., 2019). According to the bottom-up approach, macroscopic structures are built up by molecular-scale building block assembly; according to the top-down approach, ordinary materials are refined down to nanoscale and reorganized to possess desired properties (Halvacı et al., 2023). Nanotechnology in the medical field has made a significant contribution to the health sector with uses varying from biosensors, smart drugs, target drug carriers, diagnostic systems, to nanorobots. All these technologies, especially when combined with advances in biomaterials and biotechnology, have come together to form a new field of science known as "nanomedicine" for disease treatment and diagnosis (Patra et al., 2018). In cancer, Nanoparticles will accumulate in tumor sites due to the enhanced vascular permeability and impaired lymphatic drainage, which are wonderful advantages for targeted drug delivery (Yao et al., 2020). These systems enable the co-delivery of multiple therapeutic agents, allowing for both direct intracellular release and selective distribution to diseased tissues. For central nervous system disorders, where the BBB poses a significant obstacle, polymeric NP carriers offer promising

solutions by enhancing drug transport across the barrier, increasing drug loading capacity, and enabling the targeting of pathogenic proteins involved in diseases such as AD (Islam et al., 2025).

4.8. Nanoparticles (NP)

NPs are a novel platform for drug delivery targeting AD that offers solutions to the inherent limitations of conventional therapies, like poor BBB penetration, rapid drug clearance, and lack of selectivity. The unique pathophysiology of AD requires sophisticated delivery systems that can target these pathological components specifically with few systemic side effects (Islam et al., 2025).

In this regard, Cs based NP prepared via ionic gelation have piqued researchers' interest, owing to the natural biopolymer's high mucoadhesiveness, drug permeation enhancement ability, and biocompatibility across biological barriers (Manek et al., 2020). This preparative method takes advantage of the electrostatic attraction of positively charged CS amino groups and polyanionic tripolyphosphate (TPP), resulting in the spontaneous formation of stable, nanoscale particles in mild aqueous conditions that do not compromise the integrity of unstable therapeutic agents (Hussain & Sahudin, 2016). PVA is also a critical stabilizer in the system, preventing aggregation and offering a uniform size distribution that is necessary to obtain reproducible pharmacokinetics and biodistribution (Song & Cho, 2024). Such PVA-CS-TPP NP can be engineered precisely to release drugs in release profiles and to maintain the therapeutic levels of different types of AD drugs over extended periods for different types ranging from acetylcholinesterase inhibitors, NMDA receptor antagonists, A β disaggregating agents, to anti-inflammatories (Guler, Yekeler, et al., 2024). Their small size (typically 50-200 nm), combined with CS's positive charge, enables efficient transport across the BBB by mechanisms that might include adsorptive-mediated transcytosis but still enable retention within brain tissue on the basis of attraction to negatively charged cell membranes (Caprifico et al., 2020). Advanced systems have the ability to deliver more than one therapeutic drug at once, enabling combination therapies that target independent aspects of AD pathology, e.g., the co-delivery of anti-amyloid and anti-tau drugs along with neuroprotectants (Cheng et al., 2023). The modularity of this system enables surface engineering with targeting ligands like transferrin, lactoferrin, or peptides binding to receptors overexpressed on the BBB, further enhancing delivery specificity (Ghosh et al., 2019). Also being explored by ongoing research in the application of such NP for delivering new therapeutic agents like gene therapies, RNA interference medicines, and CRISPR-based strategies for AD treatments (Ahmed,

2024; Arora et al., 2022; Khan et al., 2025). While challenges associated with mass production and batch-to-batch consistency continue to persist; however, they are being alleviated through improvements in the quality-by-design approach and advanced characterization techniques (Yu et al., 2024). The future of CS-based NP therapy for AD looks particularly promising, with ongoing investigations into personalized medicine approaches that could tailor NP properties to individual patient characteristics and disease stages, potentially revolutionizing how we treat this devastating neurodegenerative disorder (Yu et al., 2024).

4.9. Three-Dimensional (3D) Printing

Additive manufacturing, or 3D printing, is transforming industries all over the world, with a significant impact on the pharmaceutical sector (Kulkarni et al., 2024). Unlike most traditional fabrication methods, which rely on subtractive or molding approaches, 3D printing builds products layer by layer, allowing for precise manufacturing of complex geometries (Ligon et al., 2017; MacDonald & Wicker, 2016). Originally designed for quick prototyping, the capability of 3D printing has increased, making it a viable, scalable technology suitable for both industrial and medical applications (Oleksy et al., 2023).

The introduction of 3D-printed pharmaceutical dosage forms, including orally disintegrating tablets (ODTs), marks a significant advancement in the pharmaceutical industry (Tang et al., 2025). These tablets stand out for their rapid dissolution in the mouth, which improves patient compliance and provides a faster onset of action. Aside from production efficiency, 3D printing reduces material waste and allows for lower active pharmaceutical ingredient (API) doses while maintaining efficacy, making it cost-effective for large-scale clinical and commercial applications (Alhnan et al., 2016; Prasad & Smyth, 2016).

The use of 3D printing in drug production enables personalized medicine, in which dosage forms can be tailored to the specific needs of individual patients. This is particularly useful in treating complex or unusual conditions, where generic forms of drugs would not work (Beg et al., 2020; Englezos et al., 2023). In addition, the technology can be used to deliver multiple drugs in one dosage form, which may make treatment protocols for patients with chronic illnesses or comorbidities easier to comply with (Beg et al., 2020; Norman et al., 2017). In industrial settings, 3D printing reduces human error and improves production consistency due to its precision. Its ability to reproduce intricate designs makes it ideal for biomedical innovations, such as controlled-release drug systems (Basit & Trenfield, 2022).

A critical factor in the fabrication of 3D-printed pharmaceutical products is the selection of appropriate polymers, which are accountable for delivering the structural integrity necessary for the final dosage form (Jain et al., 2018). Polymers such as polyvinylpyrrolidone (PVP) and polyethylene oxide (PEO) are widely utilized in 3D printing because of their superior film-forming properties and drug delivery potential (Peng et al., 2024). PVP, a water-soluble polymer, is commonly used in tablet formulations to improve drug dissolution and speed up the onset of action (Haaf et al., 1985). PEO, on the other hand, is recognized for its ability to release drugs in a controlled manner, making it suitable for use in applications that require long-term drug delivery over time (Salehi et al., 2022).

PVP and PEO are particularly appropriate for use in 3D printing applications because of their favorable rheological properties, which make them easy to extrude and mold into desired shapes (Peng et al., 2024). These polymers are non-toxic and biocompatible, hence printed dosage forms are appropriate for application in patients. Secondly, the regulation of blends and ratios of these polymers by 3D printing makes it possible to have multi-functional dosage forms that can address different therapeutic needs such as dose personalization, targeting, and bioavailability (Azad et al., 2020).

4.10. Sublingual Administration

The route of drug administration is the most important in identifying drug bioavailability, rate of absorption, and therapeutic effect (Benet, 1978). The most frequent routes of systemic drug administration are parenteral and enteral (Jain, 2008). Parenteral drug administration refers to the process of giving drugs directly into the blood or tissue, bypassing the gastrointestinal system through intravenous (IV), intramuscular (IM), and subcutaneous (SC) injections. This technique provides the benefit of quick and controlled drug delivery and is thus well suited for emergency situations or for situations where specific dosing is needed. However, it is associated with discomfort, potential tissue damage, and infection risks, making it less preferred for long-term or routine treatments (Bhattacharjee et al., 2019).

On the other hand, enteral drug administration through the oral route is most employed due to its ease of use, its cost-effectiveness, and the patient's relative ease of self-administration of the drug. Although it is very common, oral drug administration is not free from drawbacks due to mechanisms such as first-pass metabolism, where the drug is partially metabolized in the liver prior to systemic circulation, thus reducing its bioavailability. Additionally, chemical stability and

solubility of the drug can also impact the absorption rate in the gastrointestinal tract (Lou et al., 2023). Sublingual administration, wherein drug absorption is accomplished through the mucous membranes under the tongue, is an appealing option to oral delivery (Harris & Robinson, 1992). This route facilitates expedited absorption and enhanced bioavailability by circumventing the gastrointestinal tract and first-pass hepatic metabolism. Sublingual medication is typically utilized in the management of conditions where rapid onset of action is required, e.g., pain or cardiovascular disease. Secondly, it is a ready option for patients with pill-swallowing problems since it is easy to use and generally well-tolerated (Deepak et al., 2018).



5. MATERIALS and METHODS

5.1. Materials

The following materials were purchased from Sigma-Aldrich (Poole, UK): tripolyphosphate polyanion (TPP, $M_w \sim 367.86 \text{ g mol}^{-1}$), polyvinyl pyrrolidone (PVP, $M_w \sim 40,000 \text{ g mol}^{-1}$), polyvinyl alcohol (PVA, $M_w \sim 89,000\text{--}98,000 \text{ g mol}^{-1}$ with 99+ % hydrolyzation degree), polyethylene oxide (PEO, $M_w \sim \text{average } 600,000 \text{ g mol}^{-1}$), and vitamin B12 (VB_{12} , $M_w \sim 1355.4 \text{ g mol}^{-1}$). Acetic acid and phosphate-buffered saline (PBS) tablets ($\text{pH} = 7.4$) were acquired from Merk KGaA. Every item that was purchased was analytical grade.

5.2. Methods

5.2.1. Preparation of CS-TPP-PVA NPs containing VB_{12}

CS-TPP-PVA NPs were synthesized utilizing the ionic gelation technique (Calvo et al., 1997). Initially, 75 mg of CS was solubilized in 30 mL of 0.05% (v/v) acetic acid, diluted with double-distilled water using a magnetic stirrer, resulting in a concentration of 2.5 mg/mL. TPP was dissolved in 30 mL of double distilled water in a separate container to obtain a concentration of 2.5 mg/mL. After complete dissolution, 1 mg of VB_{12} was incorporated into the TPP solution and mixed until uniformly distributed. Subsequently, the TPP solution containing VB_{12} was incrementally added to the pre-prepared 0.1% (w/v) PVA solution, which was formulated by dissolving the requisite quantity of PVA in 30 mL of double-distilled water and thoroughly mixing with heat using a magnetic stirrer. The resultant PVA/TPP/ VB_{12} solution was subsequently amalgamated with the CS solution at a regulated drip rate while undergoing magnetic stirring for three hours. The final CS/TPP/PVA/ VB_{12} mixture was subsequently transferred to 50 mL sterile centrifuge tubes and centrifuged at 12,000 rpm for 15 minutes. The encapsulation efficiency percentage was ascertained by extracting 1 mL of supernatant from the upper layer post-centrifugation, with the residual solution being discarded. The pellet was resuspended in double-distilled water and subjected to centrifugation again, repeating this procedure three times. The resultant CS/TPP/PVA/ VB_{12} pellet was subsequently frozen overnight at -20°C , followed by an additional freezing period at -80°C for 24 hours (Figure 3). The subsequent day, the frozen pellet underwent lyophilization, yielding a powdered form. The powder was subsequently transferred to an Eppendorf tube and preserved in a moisture-free environment for subsequent analyses.

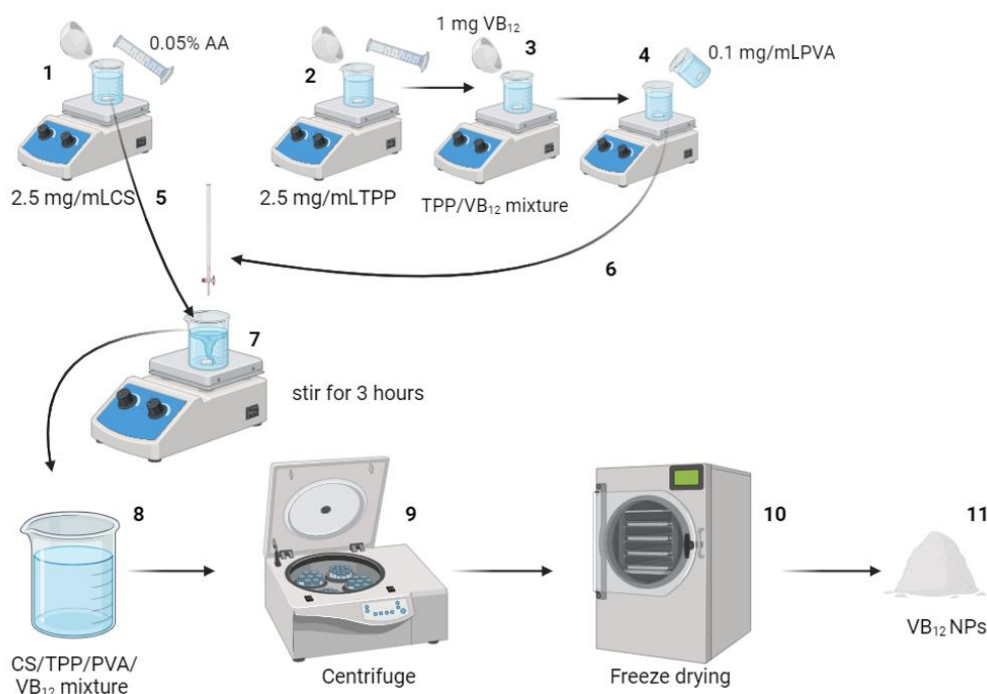


Figure 3. Illustration for VB₁₂NPs preparation (BioRender, 2024).

5.2.2. Physical and chemical characterization of VB₁₂ NPs

Various techniques were used to characterize the developed VB₁₂ NP. The Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., UK) was used to measure particle size, zeta potential, and polydispersity index (PDI) using dynamic light scattering (DLS).

5.2.3. Fabrication of scaffolds

PEO and PVP were prepared at room temperature using distilled water at 10% and 15% (w/v), respectively, and stirred continuously for 30 minutes with a magnetic stirrer (WiseStir®, MSH-20 A, Wertheim, Germany). Separately prepared polymeric solutions were then mixed in three different ratios (8:1, 9:1, and 10:1, v/v) and stirred for an additional 30 minutes until complete homogeneity was achieved. The pure scaffolds were printed with these solutions. Then, VB₁₂NPs were added to the optimal PEO/PVP mixture solution at a 0.5 mg/mL concentration (Figure 4).

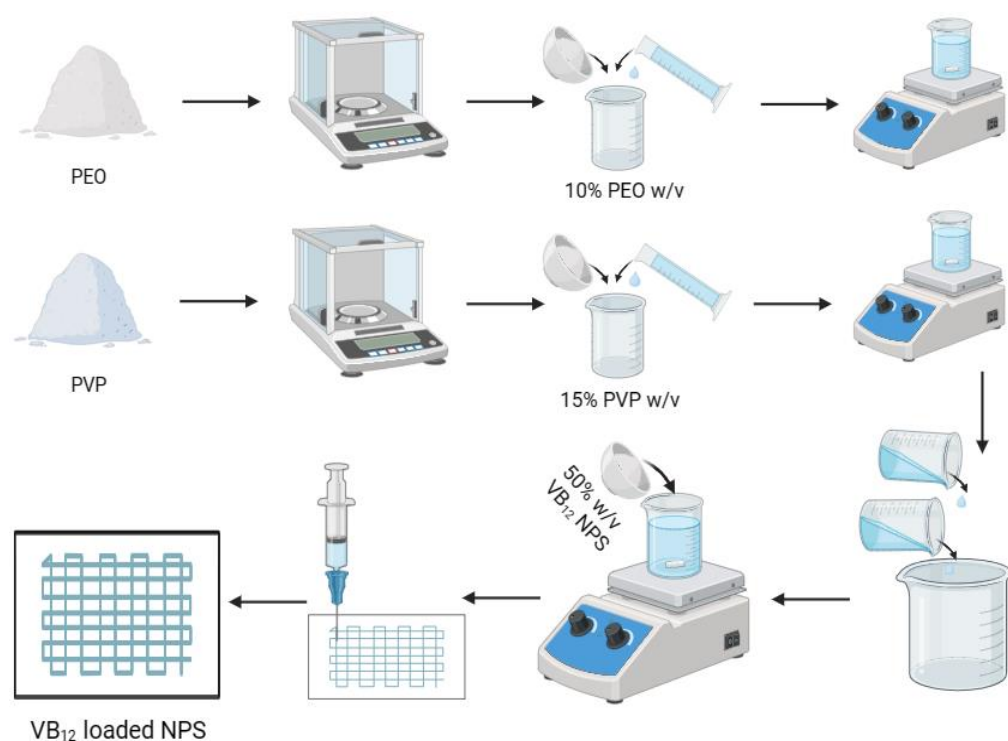


Figure 4. Illustration for scaffolds' fabrication (BioRender, 2024).

Table 1. Compositions of the solutions.

Prepared Solutions	PEO (wt.%)	Content	PVP (wt.%)	Content	VB ₁₂ (mg/mL)	Content
PEO	10		0		0	
PVP	0		15		0	
PEO/PVP (9:1)	10		15		0	
PEO/PVP/B12	10		15		0.5	

5.2.4. Scanning electron microscopy (SEM)

SEM (EVA MA 10, ZEISS, Istanbul, Turkey) was used to examine the external morphology of the nanoparticles and pore uniformity of each scaffold. Prior to imaging, the samples' surfaces were gold (Au) coated for 60 seconds with a sputter coating machine (Quorum SC7620, Laughton, UK). Scaffold pore sizes were measured with Image J (Brocken Symmetry Software) imaging software (Guler, Yekeler, et al., 2024). The average pore size was calculated and reported as mean $\pm$ SD.

5.2.5. Fourier transform infrared spectroscopy analyses (FT-IR)

A Thermo Nicolet 6700 FTIR spectrophotometer equipped with Smart Orbit diamond ATR accessories was used to analyze the molecular structures of both raw and processed materials at room temperature, with a wavelength interval of 400 cm⁻¹ to 4000 cm⁻¹, and in transmission mode. Finding peaks that demonstrate the encapsulation of VB₁₂, the existence of all utilized materials, and the development of intermolecular interactions between CS/TPP/PVA or PEO/PVP compositions is the aim of this test (Yekeler et al., 2024).

5.2.6. X-ray powder diffraction (XRD)

The crystalline forms and structures of the unrefined and refined materials were examined utilizing Cu K α radiation and a D/Max-BR diffractometer (Rigaku, Tokyo, Japan). The examination was conducted at 30 mA and 40 kV across a 2 θ range of 10-60 degrees, with a scanning speed of 2 degrees per minute. The diffractograms were generated utilizing OriginPro 7.0 software (OriginLab Corporation, MA, USA) from the analyzed data (Guler et al., 2023).

5.2.7. Differential scanning calorimeter (DSC)

The DSC analysis was carried out using PerkinElmer Inc.'s Jade DSC and Pyris software (MA, USA) to characterise the thermal properties of the loaded and pure samples. Between 0 and 300°C, the analysis was carried out at a heating rate of 10°C/min in a dynamic argon atmosphere (20 mL min⁻¹). The scaffolds and NPs' glass transition temperature (T_g) and melting temperature (T_m) were also examined. To take into consideration the effect of indium's melting enthalpy and melting point on temperature readings, the DSC calibration was modified (Aydin et al., 2022).

5.2.8. Oro-dispersion assay for the VB₁₂-loaded scaffolds

The oro-dispersion test was performed on the 3D-printed scaffolds made of VB₁₂ loaded material. In order to conduct the breakdown assessment, an artificial saliva consisting of Albumin (0.1%), sodium carboxymethylcellulose (0.5% weight-to-weight), methylparaben (0.2% weight-to-weight), potassium chloride (0.062% weight-to-weight), magnesium chloride (0.005% weight-to-weight), potassium phosphate (0.034% weight-to-weight), sodium fluoride (0.01% weight-to-weight), and dextrose (4.69% weight-to-weight) was prepared. First, Albumin, cellulose, potassium chloride, potassium phosphate, and sodium fluoride were dissolved in a small amount of water separately. The methylparaben, magnesium chloride, and dextrose were dissolved in warm water, and then, after being cooled, the two mixtures were mixed together at room temperature (Amal et

al., 2015). A 15 mL artificial saliva with a pH of 6.8 and a temperature of 37°C was used. During the experiments, a thermal shaker known as the BIOSAN TS-100 was employed. Additionally, the degradation properties of the scaffolds were recorded by a video camera (Canon Sx70 HS, Tokyo, Japan) with an average speed of 50 frames per second.

5.2.9. Drug entrapment efficiency

Through the utilization of UV-visible spectroscopy (Shimadzu UV-3600, Kyoto, Japan), the encapsulation efficiency (EE%) of VB₁₂-loaded NP and scaffolds was successfully determined. For NPs, the EE% was determined by analysing the supernatant that was collected after the initial centrifugation that took place during the preparation stage. For the purpose of determining the amount of VB₁₂ that is still present outside of the NPs, the absorption values of the supernatant were measured at 361 nm (Sugandhi & Mahajan, 2022) and then substituted into the equation. For scaffolds, 5 mg of the sample was dissolved in 10 mL of a solvent mixture, and the mixture was mixed for 24 hours to release VB₁₂. Additionally, 1 mL of the solution that was produced was analyzed at 361 nm (Sugandhi & Mahajan, 2022), and the measurements were repeated three times. For the purpose of calculating EE%, the following equation (1) (Zhang & Feng, 2006) was utilized:

$$EE\% = \frac{\text{The mass of actual drug loaded in the carrier}}{\text{Theoretical mass of drug used in carrier production}} \times 100$$

Equation (1)

5.2.10. *In vitro* drug release pattern

The *in vitro* drug release test was used to determine drug content release from VB₁₂NPs. A linear calibration curve was created using VB₁₂ solution in PBS at five different concentrations (50, 25, 13, 6.25, and 3.125 µg/mL). The experiment was conducted on a rotary shaker set to 250 rpm rotational speed and 37 °C temperature. Samples were weighed at 5 mg and placed in Eppendorf tubes until finished. The tubes were filled with 1 mL of PBS (pH 7.4 at 37°C) and shaken at predetermined times (1, 2, 3, 4, 6, 24 hours). The liquid portion of each set was collected for further analysis and replaced with 1mL of fresh PBS after each sample removal. The amount of drug released from the carrier was determined by measuring the removed solution at 361 nm with UV spectroscopy (Shimadzu UV-3600) (Sugandhi & Mahajan, 2022). This assay was repeated three times and presented as the average cumulative release percentage/time curve.

5.2.11. Kinetics of *in vitro* drug release

To interpret the drug release kinetics of VB₁₂NPs, five mathematical models were used: Korsmeyer-Peppas, zero-order, first-order, Higuchi, and Hixson-Crowell (Cam et al., 2021). The following equations apply to the Korsmeyer-Peppas (Equation (3)), zero-order (Equation (4)), first-order (Equation (5)), Higuchi (Equation (6)), and Hixson-Crowell (Equation (7)) models:

$$Q = Kt^n \quad \text{Equation (2)}$$

$$Q = K_0t \quad \text{Equation (3)}$$

$$\ln(1 - Q) = -K_1t \quad \text{Equation (4)}$$

$$Q = K_h t^{1/2} \quad \text{Equation (5)}$$

$$Q^{1/3} = K_{hc}t \quad \text{Equation (6)}$$

Q represents the fractional amount of drug released at time t, and the kinetic constants for the Korsmeyer-Peppas, zero-order, first-order, Higuchi, and Hixson-Crowell models are K, K₀, K₁, K_h, and K_{hc}, respectively. The diffusion exponent (n) indicates the mechanism of drug release.

5.2.12. *In vitro* cell culture assays

5.2.12.1. Culturing and treating cells

The human neuroblastoma cell line SH-SY5Y (CRL-2266™, ATCC) was maintained at 37 °C in a humidified atmosphere with 5% CO₂, utilising a culture medium composed of EMEM/Ham's F12 (1:1) (Capricorn Scientific GmbH) supplemented with 10% foetal bovine serum (Capricorn Scientific GmbH). The cells were trypsinised and passaged every three days (Alonso et al., 2021).

5.2.12.2. Cell viability assay

Upon achieving 80% confluency, the cells were transferred to 96-well plates (Corning®) at a density of 0.5×10^4 cells per well after trypsinization. VB₁₂NP were synthesised at three distinct concentrations: 2.5, 5, and 10 nM. Following a 24-hour incubation period, the cells were subjected to treatment and incubated for an additional 48 hours. At the conclusion of this period, cell viability was evaluated utilising the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which quantifies overall metabolic activity. Subsequently, 10 µL of 5 mg/mL MTT reagent was introduced into each well. The 96-well plates were subsequently incubated for 3 hours at 37 °C in a humidified environment with 5% CO₂ and 95% relative humidity. Subsequent to incubation, the medium was meticulously extracted, and 100 µL of DMSO was introduced to each well to

solubilise the formazan crystals. Following 5 minutes of agitation, absorbance values were recorded at 570 nm utilising a multi-plate reader (ELISA reader, BioTek Epoch). Cell viability (%) was determined using the absorbance values (A) and the formula:

$$\text{“Cell Viability (\%)} = [(A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}})] \times 100.”$$

Equation (7)

The lyophilised A β 1–42 peptide (Genscript, USA) was reconstituted in 1 mL of DMSO and incubated at 37 °C for 72 hours. To establish an AD model, the cells were treated with A β 1-42 at a concentration of 5 μ M for 24 hours (Pillot et al., 1999). Subsequent to this induction, the cells were subjected to treatments from the stock solutions, evaluating them at various concentrations. The IC₅₀ values for the VB₁₂NP and A β 1-42 were ascertained utilising CompuSyn software (Paramus, NJ). The MTT assay was subsequently repeated utilising the IC₅₀ value (Sucu et al., 2019). To achieve this, the cells were inoculated, and A β 1-42 was administered 24 hours subsequently. After a 24-hour exposure, the medium was extracted from the cells. VB₁₂NP and pure NP were utilised. Following a 48-hour period, a viability assessment was performed to evaluate the anti-Alzheimer efficacy. All experiments were conducted thrice.

5.2.12.3. *In vitro* cellular uptake of rhodamine b-labeled VB₁₂NPs

Rhodamine B-labeled VB₁₂NPs were synthesised with alterations to the procedure outlined by Wang et al. (Wang et al., 2017). Initially, 0.5 mg of Rhodamine B was solubilised in 1 mL of DMSO. Subsequently, 0.2 mL of the dye solution was incorporated into 2 mL of the NP solution, which was agitated in the absence of light for 12 hours. The solution was then centrifuged at 10,000 $\times$ g for 10 minutes. The VB₁₂NPs conjugated with the dye were purified and re-suspended in 2 mL of distilled water. The washing procedure was conducted thrice. The cells were inoculated into a 24-well plate at a density of 0.05×10^6 and incubated for 24 hours. Subsequently, 200 μ L of Rhodamine B-labeled VB₁₂NPs were introduced to each well and co-cultured with the cells for a duration of 4 hours. Subsequent to incubation, the cells were rinsed with fresh PBS to eliminate residual free NP. In accordance with the protocol (Del Turco et al., 2013), fluorescence staining utilising DAPI was conducted with the actin cytoskeleton and focal adhesion staining kit (FAK100, Millipore, USA) to assess cellular uptake. The cells were observed using confocal laser scanning microscopy (CLSM) (Zeiss, Germany).

5.2.12.4. ELISA Analysis

To evaluate the effect of treatments on extracellular A β_{1-42} levels, SH-SY5Y cells were seeded at a density of 400,000 cells per well in 6-well plates and incubated overnight. Four experimental groups were established: Control, VB₁₂, VB₁₂NPs, and TPP CS NPs. After incubation, treatment groups were administered, except the untreated control group. The cells were then incubated for 48 hours under standard conditions. Subsequently, 5 μ M A β_{1-42} was added to all wells, including the control group, followed by 48 hour incubation. At the end of the treatment period, culture supernatants were carefully collected for analysis. Quantification of A β_{1-42} levels was performed using the Human A β_{1-42} ELISA Kit (AFG Bioscience, USA), following the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader, and A β_{1-42} concentrations were calculated from a standard curve. Results were presented as percentages relative to the control group (Sharma et al., 2024).

5.2.12.5. Real-time Polymerase Chain Reaction (RT-PCR)

For the experimental procedures, SH-SY5Y cells were seeded into 6-well plates at a density of 4×10^5 cells per well. Following a 48-hour incubation period with A β_{1-42} to establish the AD's model, the cells were subsequently treated with the designated agents and maintained under standard culture conditions for an additional 48 hours. After treatment, total RNA was extracted using the Cistron Biologics RNA Kit (Istanbul, Turkey). The quantity and purity of the RNA were assessed using a nanodrop spectrophotometer. Following this, cDNA synthesis and qPCR analysis were performed according to the manufacturer's instructions. SYBR Green-based detection was utilized, and relative expression levels were calculated using the $\Delta\Delta C_t$ method, with β -actin as the reference gene for normalization (Livak & Schmittgen, 2001).

Each target gene was amplified using specific primers as listed below:

Genes	Forward Primers (5'→3')	Reverse Primers (5'→3')
GSK-3 β	AGTGGTGTGGATCAGTTGGT	ACGGCTACACAGTGCAATTG
MAPT	ACTGAGAACCTGAAGCACCA	TCCTGGCTTGTGATGGATGT
Cyclin D1	TACACCGACAACCTCCATCCG	ATGGAGGGCGGATTGGAAAT
β -Catenin	CTTACACCCACCATCCCACT	CCTCCACAAATTGCTGCTGT
Presenilin-1	TGGCTCATCTTGGCTGTGAT	CGGGTCTCCTTCTGCCATAT
Akt	CTGCCCTTCTACAACCAGGA	ATGATCTCCTTGGCGTCTC
β -Actin	CATGGAATCCTGTGGCATCC	CACACAGAGTACTTGCGCTC

5.2.13. Statistical analysis

Statistical outcomes obtained from GraphPad Prism 9.0 software were expressed as mean $\pm$ standard error of the mean, except for morphological and *in vitro* drug release studies, which were reported as mean $\pm$ standard deviation (SD). The cross-group comparison examination utilised analysis of variance (ANOVA) and the Tukey post-hoc test. Significance levels of 0.05, 0.01, and 0.001 were indicated by (*), (**), and (***) respectively.



6. RESULTS

6.1. Characteristics of Physical Parameters

Table 2 delineates the physical parameters of both the unadulterated scaffolds and those infused with VB₁₂NPs. The addition of VB₁₂NP to the PEO/PVP polymeric solution led to a decrease in viscosity, surface tension, and density relative to the pure PEO/PVP solution. This reduction did not adversely impact the speed or quality of the 3D printing process.

Table 2. Physical parameters of the solutions.

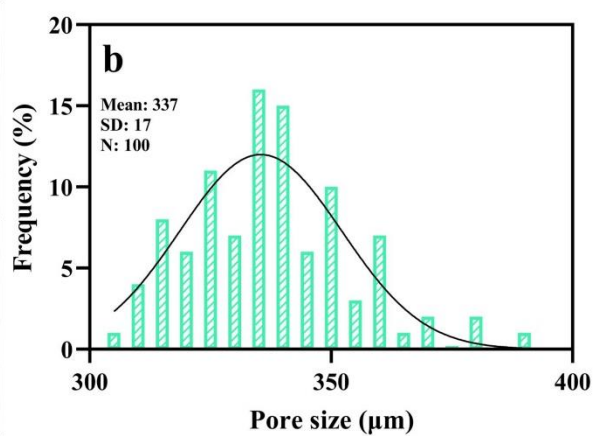
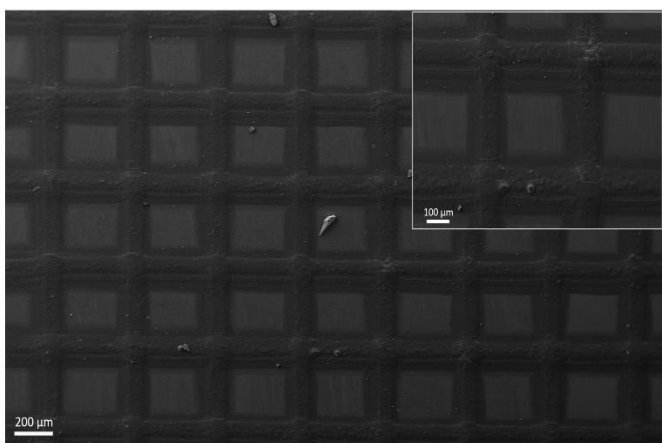
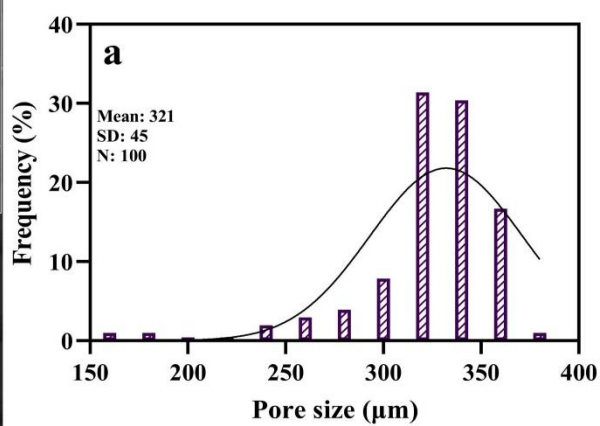
Composition	Viscosity (mPa s)	Density (g/mL)	Surface Tension (mN /m)
Pure PEO/PVP solution	10420	1.1987	291.27
VB ₁₂ NP-loaded PEO/PVP solution	5980	0.7923	82.92

6.2. Morphological Examination of 3D-Printed Scaffolds

The morphology of the 3D-printed scaffold was assessed according to average pore size, uniformity of pore distribution, and surface structure. SEM images (Figures 5a–5d) and associated histograms were utilized to evaluate these attributes. Scaffolds constructed with PEO:PVP ratios of 8:1, 9:1, and 10:1 demonstrated average pore sizes of 321 μm , 337 μm , and 271 μm , respectively. The 9:1 ratio was chosen for further analysis because of its comparatively larger average pore size and superior pore uniformity relative to the other formulations. The 8:1 ratio exhibited comparable pore dimensions but demonstrated reduced homogeneity and surface smoothness.

Upon the integration of VB₁₂NPs into the chosen scaffold formulation, the mean pore size diminished from 337 μm to 249 μm . The final scaffold exhibited consistent pore distribution and a well-defined surface morphology, making it suitable for controlled drug release applications.

SEM images of (e) CS TPP NP and (f) VB₁₂NPs. Compared to the pure form, VB₁₂NPs exhibit a more spherical and compact morphology with smoother surface features, as evident in the inset (2 μm scale).



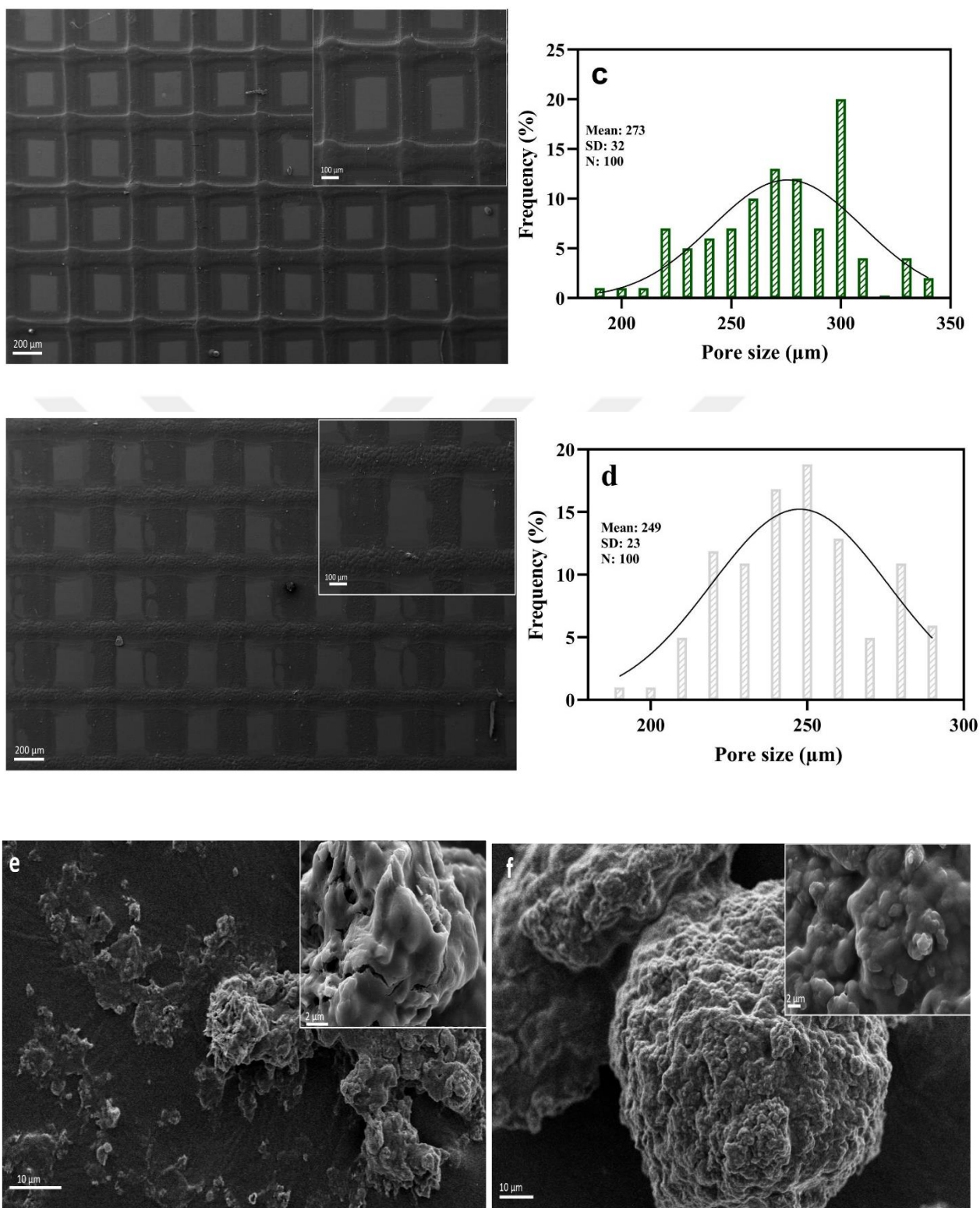


Figure 5. SEM results of a) PEO: PVP scaffolds (8:1) b) PEO: PVP scaffolds (9:1) c) PEO: PVP scaffolds (10:1) d) VB₁₂ loaded NPs e) TPP CS NPs f) VB₁₂NPs

6.3. Chemical Composition Analysis via FTIR

FTIR spectroscopy was employed to explain the molecular structures and assess the interactions between scaffold components. The FTIR spectra of the individual materials, specifically CS, TPP, PVA, PEO, PVP, and VB₁₂, as well as the composite formulations of TPP-CSNPs, VB₁₂NPs, pure scaffolds (PS), and VB₁₂NP-loaded scaffolds (VB₁₂NPS), were recorded (Figure 6). Distinct peaks corresponding to each component were identified. CS exhibited characteristic OH and NH stretching bands near 3355 cm⁻¹, as well as C–O stretching bands at 1060 cm⁻¹ and 1024 cm⁻¹. TPP displayed peaks at 1122, 1074, and 865 cm⁻¹, indicative of phosphate group vibrations. PVA exhibited significant bands at 3268, 2915, and 1649 cm⁻¹, whereas PEO showed absorption at 2877 cm⁻¹ and C–O–C stretching at 1143 and 1058 cm⁻¹. PVP exhibited a maximum at 2956 cm⁻¹ along with pronounced carbonyl and C–N-related absorptions. VB₁₂ exhibited several peaks at 3291, 1147, 1076, 995, and 759 cm⁻¹.

The FTIR spectra of VB₁₂NPs and VB₁₂NPS displayed bands indicative of their constituent materials, thus confirming the presence of VB₁₂ within the NP and scaffold matrices. The notable finding was the lack of distinct characteristic VB₁₂ peaks in the 3000–3500 cm⁻¹ range in the final scaffold formulation, indicating changes in molecular interactions during NP integration.

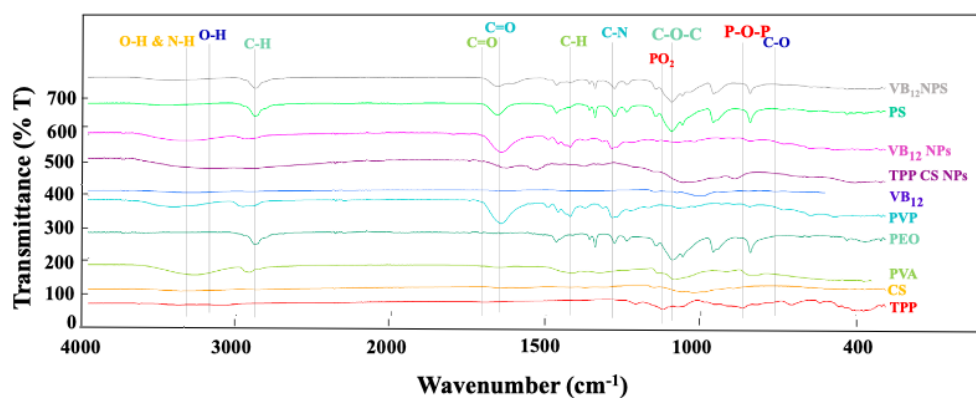


Figure 6. FTIR spectra of TPP, CS, PVA, PEO, PVP, VB₁₂, TPP CS NPs, VB₁₂ NPs, PS, and VB₁₂NPS

6.4. Crystal Structure Determination Using XRD

XRD analysis was performed to assess the crystallinity of the individual components and the final scaffold formulations (Figure 7). TPP powder displayed pronounced sharp peaks at $2\theta = 11.4^\circ$, 18.6° , 22.8° , 31.8° , 32.5° , and 36.5° , indicative of its crystalline structure. CS exhibited a pronounced peak at $2\theta = 20.38^\circ$, indicative of its amorphous characteristics. PVA exhibited peaks at $2\theta = 19.46^\circ$ and 40.98° , signifying partial crystallinity, whereas PEO demonstrated semi-crystalline peaks at $2\theta = 19.15^\circ$ and 23.35° . PVP and VB₁₂ exhibited extensive halos at $2\theta = 11.4^\circ$ – 21.2° and 19.4° , respectively, indicating their amorphous characteristics. A peak at $2\theta = 19.72^\circ$ was observed in VB₁₂NPs, indicating modified crystallinity relative to the individual components. The PS and VB₁₂NPS formulations exhibited overlapping crystalline and amorphous characteristics in their diffractograms.

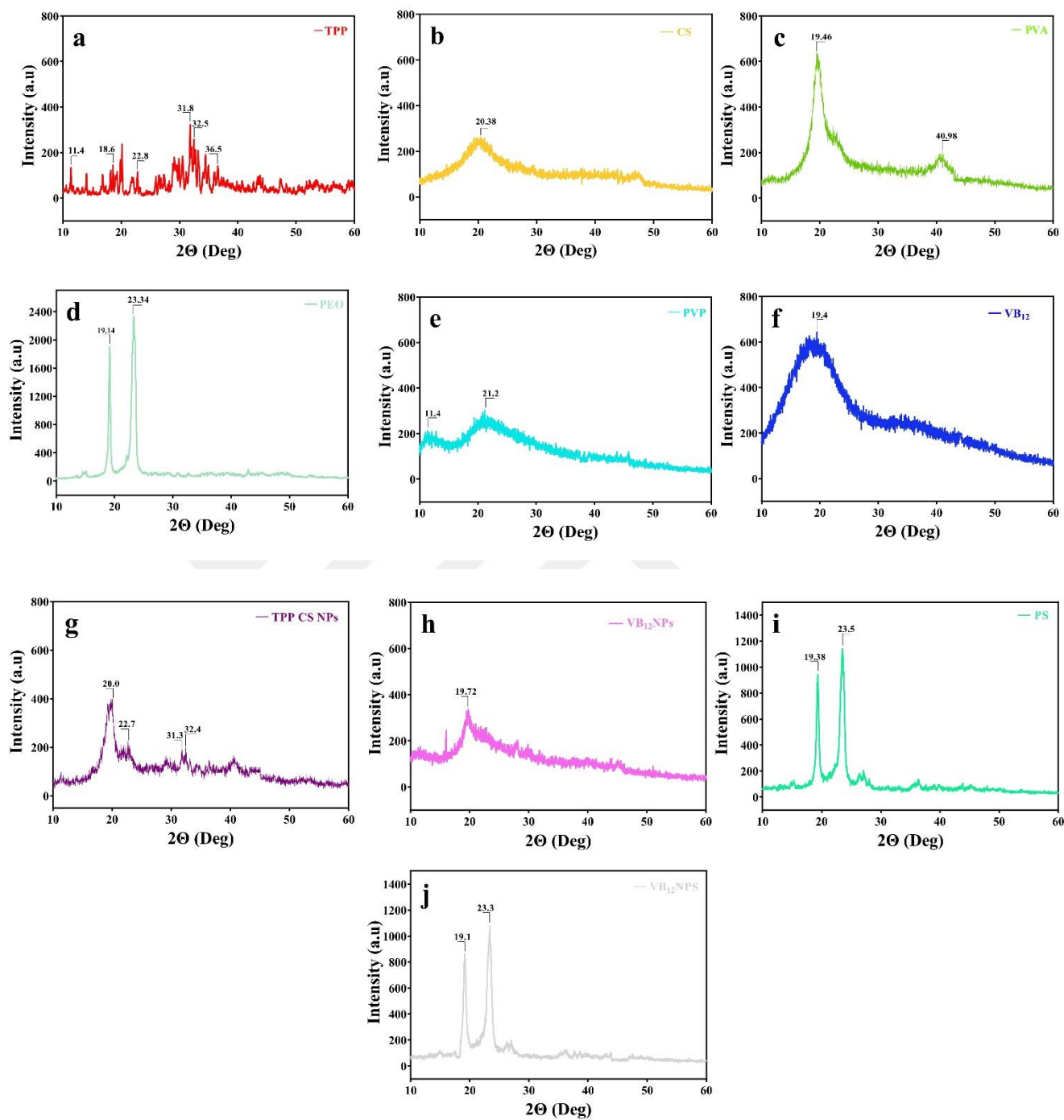


Figure 7. XRD patterns of (a) TPP, (b) CS, (c) PVA, (d) PEO, (e) PVP, (f) VB₁₂, (g) TPP CS NPs, (h) VB₁₂NPs, (i) PS, and (j) VB₁₂NPS.

6.5. Thermal Characterization by DSC

The thermal characteristics and interactions between the materials were evaluated using DSC analysis. The thermograms of TPP CS NPs, VB₁₂NPs, PS, and VB₁₂NPS are shown in Figure 8. The glass transition (T_g), which is achieved by a melting point (T_m) of 228.1 °C, was indicated by the endothermic peak that TPP CS NPs showed at 75.6 °C. The introduction of VB₁₂ resulted in a minor alteration of T_m to around 223.45°C, indicating an interaction among CS, TPP, and VB₁₂, presumably through hydrogen bonding or electrostatic interactions, in accordance with the FTIR results. The changes observed between 230-280°C were ascribed to the thermal degradation of VB₁₂, characterized by a broad peak that signifies its partially amorphous structure and complexity. The PS thermogram exhibited a crystallization peak at 65.1°C, signifying that PEO crystallized later than anticipated due to PVP's influence on the nucleation process, which impeded molecular mobility and led to an elevated crystallization temperature. The phase relaxation transition of PVP occurred at 187°C, and the peak's broadening and attenuation upon the addition of VB₁₂ suggested a drug-polymer interaction. A notable thermal peak at approximately 278°C in the VB₁₂NPS thermogram, which signifies VB₁₂ degradation, further confirms the effective incorporation of VB₁₂.

The DSC results validate the miscibility of PEO and PVP in the polymer blend, the successful incorporation of VB₁₂ into the polymeric systems, and the thermal stability of these systems at body temperature, highlighting their appropriateness for biomedical applications.

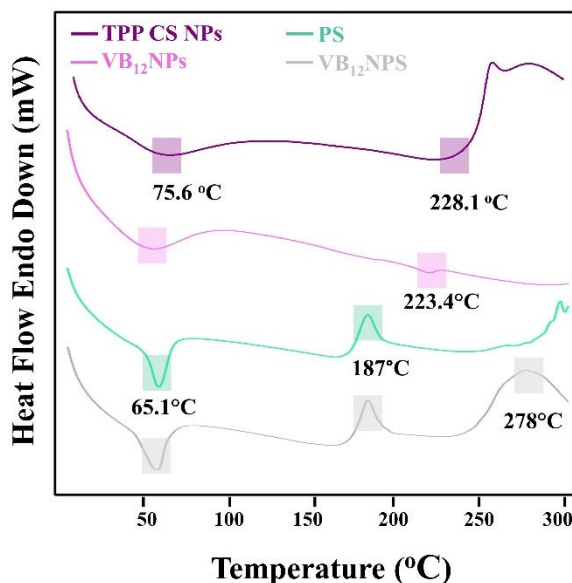


Figure 8. Thermograms of TPP CS NPs, VB₁₂ NPs, PS, and VB₁₂NPS.

6.6. Wettability and Disintegration Studies

A crucial factor in sublingual drug delivery that enhances patient adherence is the swift wetting and disintegration of drug carriers. According to the United States Pharmacopeia (USP) guidelines, a sublingual tablet must disintegrate within 2 minutes or less to be deemed acceptable for use. In this study, the VB₁₂NPs completely dissolved within five seconds when placed in artificial saliva, as shown in Figure 9. This rapid dissolution is well within the permissible time limit of the USP, indicating that the developed formulation is highly suitable for sublingual drug delivery.

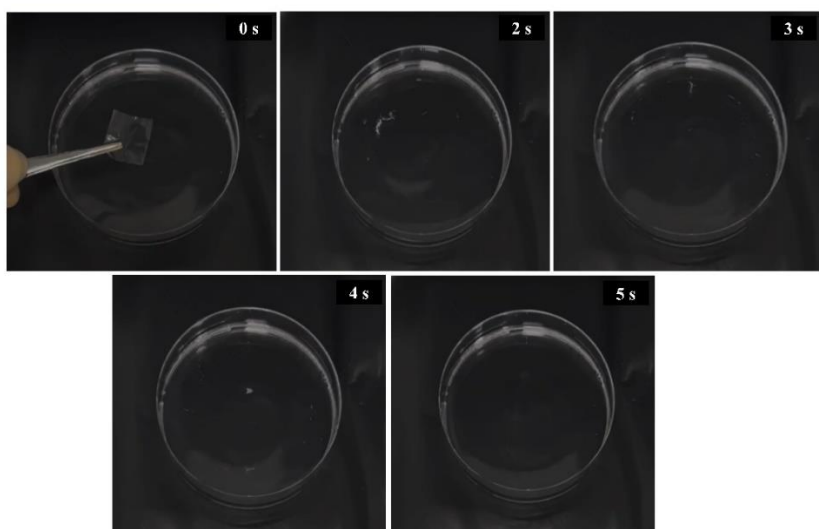


Figure 9. The disintegration of the VB₁₂NPS per time.

6.7. Physicochemical Characterization

The zeta potential (ζ) of CS TPP NPs was measured at 1.65 ± 0.05 mV, while the zeta potential of VB₁₂NPs was recorded at 1.39 ± 0.04 mV. The diminished ζ value for VB₁₂NPs is attributed to the measurement being conducted at a physiological pH of 7.4, which approximates the isoelectric point (IEP) of the CS TPP NP, resulting in a minimized surface charge. The moderate molecular weight of CS and the low weight ratio of CS to TPP may lead to a diminished surface charge. The PDI values of 0.291 for TPP CS NPs and 0.317 for VB₁₂NPs indicate acceptable uniformity in particle size distribution. Additionally, the particle sizes for both types of NP fall within the optimal nanometric range (1–1000 nm), ensuring suitability for biomedical applications, especially in drug delivery.

Table 3. Physicochemical properties of pure and VB₁₂NPs.

Samples	ζ potential $\pm$ SD (mV)	PDI $\pm$ SD (Mw/Mn)	Size $\pm$ SD (nm)
CS TPP NPs	1.65 $\pm$ 0.03	0.319 $\pm$ 0.01	362.75 $\pm$ 2.39
VB ₁₂ NP	1.39 $\pm$ 0.04	0.317 $\pm$ 0.02	348.38 $\pm$ 1.79

6.8. *In Vitro* Release Kinetic Analysis and Evaluation of Encapsulation Efficiency

The encapsulation efficiency (EE%) of VB₁₂ was assessed for both VB₁₂NPs and the associated VB₁₂NPS. The EE was determined to be 82.6% for VB₁₂NPs and 79.9% for the VB₁₂NPS, as illustrated in Figure 10a. These values confirm the successful and efficient encapsulation of VB₁₂ in both delivery systems.

A standard calibration curve was created utilizing VB₁₂ concentrations (50, 25, 13, 6.25, and 3.125 μ g/mL) to quantify drug content and assess release kinetics. The derived linear regression equation is $y = 0.022x + 0.0217$, with a coefficient of determination (R^2) of 0.9997 (Figure 10b), validating its suitability for drug quantification in release studies.

The *in vitro* release profile of VB₁₂ from the NP demonstrated a biphasic pattern (Figure 11a). A preliminary burst release phase was observed, resulting from the rapid diffusion of drug molecules affixed to the NP surface. During the first 3 days a significant burst release was achieved and 71% of the drug were release. The following phase demonstrated a more gradual release: at day 6, the cumulative drug release reached 77.66%, indicating a substantial initial release phase. This was followed by a gradual increase, with the release reaching 84.95% on day 8 and 86.08% on day 10. By day 12, the cumulative release rose to 92.19%, approaching completion. Ultimately, a maximum release of 100% was achieved by day 14, reflecting the sustained and controlled release behavior of the formulation over the evaluated period. The extended release is likely influenced by the incorporation of PVA in the NP matrix, which establishes a steric barrier that impedes drug diffusion, thereby slowing the release rate in the later phase.

In vitro release testing was not conducted for the VB₁₂NPS, as they are intended for sublingual drug delivery. Their appropriateness for this route was validated through expedited disintegration testing.

In this study, Five different mathematical models were used to analyze the release kinetics of drugs encapsulated in VB₁₂ NPs (figure 11 b, c, d, e, f). The regression coefficients (R^2) and kinetic constants of these models are presented in Table 4, while Figure 11 provides a comprehensive overview of the details and characteristics of each model. Notably, the first order model demonstrated the highest R^2 value, indicating its superior fit to the release profile of VB₁₂. Furthermore, the calculated n value using the Korsmeyer-Peppas equation for VB₁₂ was determined to be 0.4512, indicating that the drug release mechanism follows non-fickian mechanism.

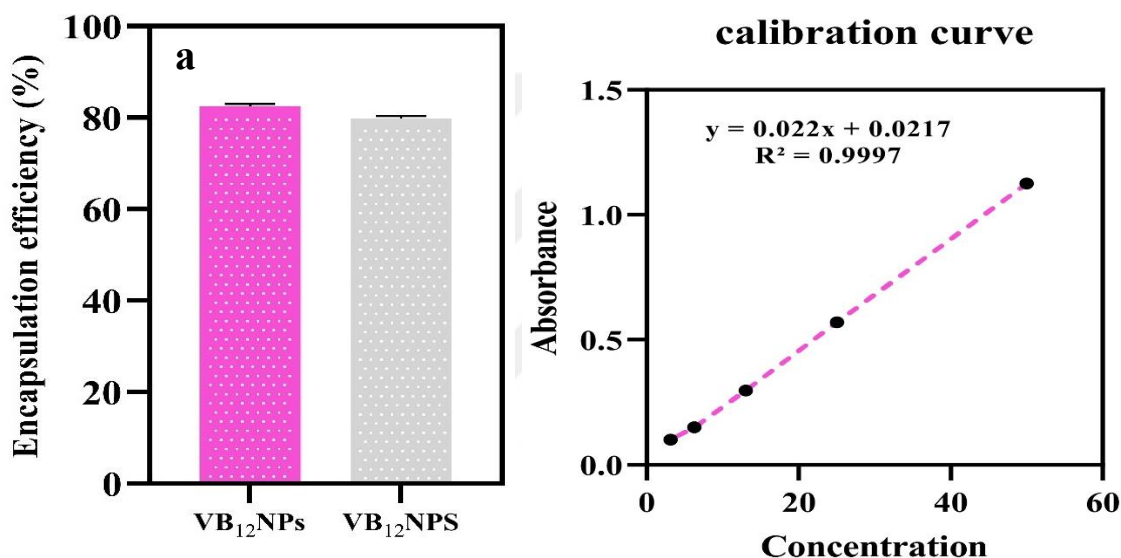


Figure 10. a) The encapsulation efficiency for VB₁₂NPs and VB₁₂NPS, b) the calibration curve of VB₁₂

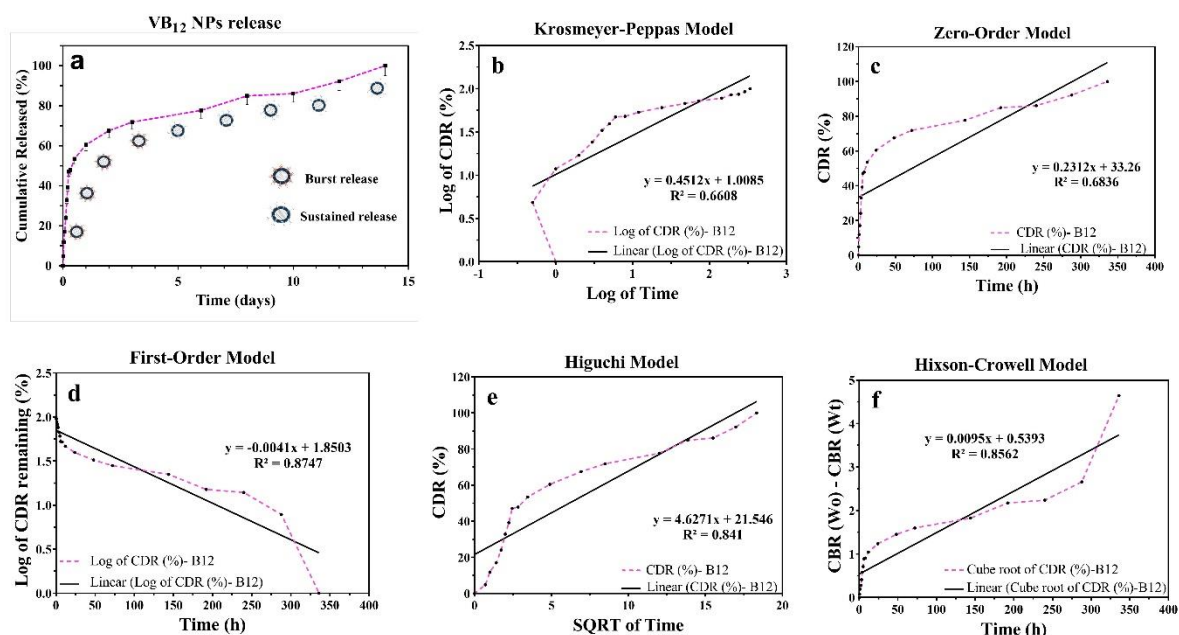


Figure 11. a) VB₁₂ release profiles from NP. The release kinetic models of VB₁₂ for b) Korsmeyer-Peppas, c) Zero-Order, d) First-Order, e) Higuchi, and f) Hixson–Crowell models. CDR (%) (Cumulative Drug Release), Log of CDR (%) (Logarithm of Cumulative Drug Release (%)), W₀ (Initial weight (or amount) of the drug), W_t (Remaining weight (or amount) of drug at time), CBR (Cumulative B12 Released), R² (Coefficient of determination), SQRT of Time (Square root of time).

Table 4. Mathematical modeling and drug release kinetics of VB₁₂ from NP.

Sample	Korsmeyer-Peppas Model		Zero-Order Model		First-Order Model		Higuchi model		Hixson–Crowell model	
	R^2	n	R^2	K_0	R^2	K_1	R^2	K_h	R^2	K_{hc}
VB ₁₂	0.6608	0.4512	0.6836	0.2312	0.8747	-0.0041	0.841	4.6271	0.8562	0.0095

6.9. Assessment of Cellular Viability and Anti-Alzheimer Efficacy in SH-SY5Y Cells

IC₅₀ values were ascertained to determine suitable dosages for both VB₁₂NPs and Aβ_{1–42}. VB₁₂NPs were evaluated at concentrations of 2.5, 5, and 10 nM over a duration of 48 hours, yielding cell viability rates of 93.4%, 90.1%, and 85.9%, respectively (Figure 12a). The IC₅₀ (Dm) value was determined to be 197.2 nM, with a R² value of 0.999, utilizing CompuSyn software.

Cell viability for A β ₁₋₄₂ was evaluated after 24 hours at concentrations of 2.5, 5, and 10 μ M, resulting in viability rates of 69.37%, 50.40%, and 33.13%, respectively (Figure 12b). A concentration of 5 μ M was chosen for subsequent experiments as it aligned with the IC₅₀ value.

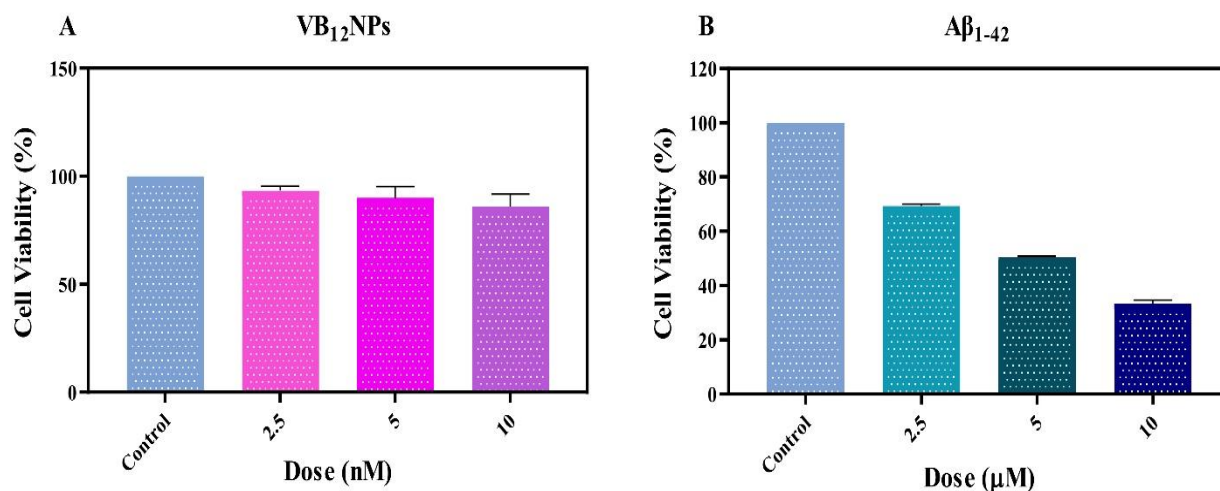


Figure 12. The dose-dependent effects of different concentrations of NPs and A β ₁₋₄₂ on cell viability. (A) VB₁₂ NPs and (B) A β ₁₋₄₂.

To assess neuroprotective effects, VB₁₂NPs were administered at concentrations of Dm, Dm/10, and Dm/100 alongside 5 μ M A β ₁₋₄₂. The viability percentages were 73.95% (Dm), 65.34% (Dm/10), and 59.78% (Dm/100), indicating a dose-dependent response (Figure 13).

Conversely, pure NPs at comparable concentrations did not markedly enhance viability, with recorded values of 50.92% (Dm), 47.02% (Dm/10), and 45.09% (Dm/100).

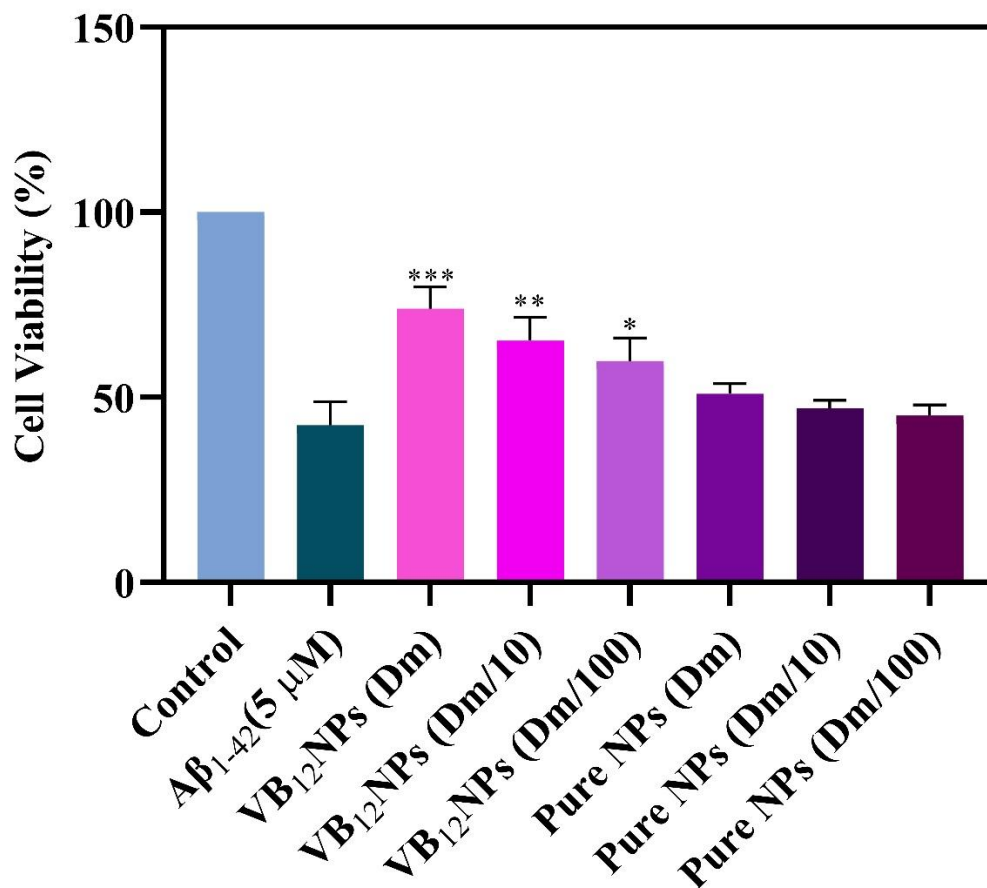


Figure 13. Evaluation of anti-Alzheimer activity in Aβ₁₋₄₂ induced SH-SY5Y cells. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Data are expressed as mean ± standard deviation. Comparisons to the (–) Control group showed significance: * p < 0.05, **p < 0.01, ***p < 0.001. Dm: median effective dose (197.2 nM)

6.10. *In vitro* Cellular Uptake of Rhodamine B-labeled VB₁₂ NPs

Confocal laser scanning microscopy (CLSM) images validated the successful internalization of Rhodamine B-labeled VB₁₂NPs by SH-SY5Y cells (Figure 14). The red fluorescence signal, indicative of Rhodamine B, was predominantly localized in the cytoplasmic region, whereas the nuclei stained with DAPI exhibited blue fluorescence. No red fluorescence was observed in the nuclear region, signifying that the VB₁₂NPs were restricted to the cytoplasm. The results validate the cellular absorption and cytoplasmic positioning of the NP in SH-SY5Y neuroblastoma cells.

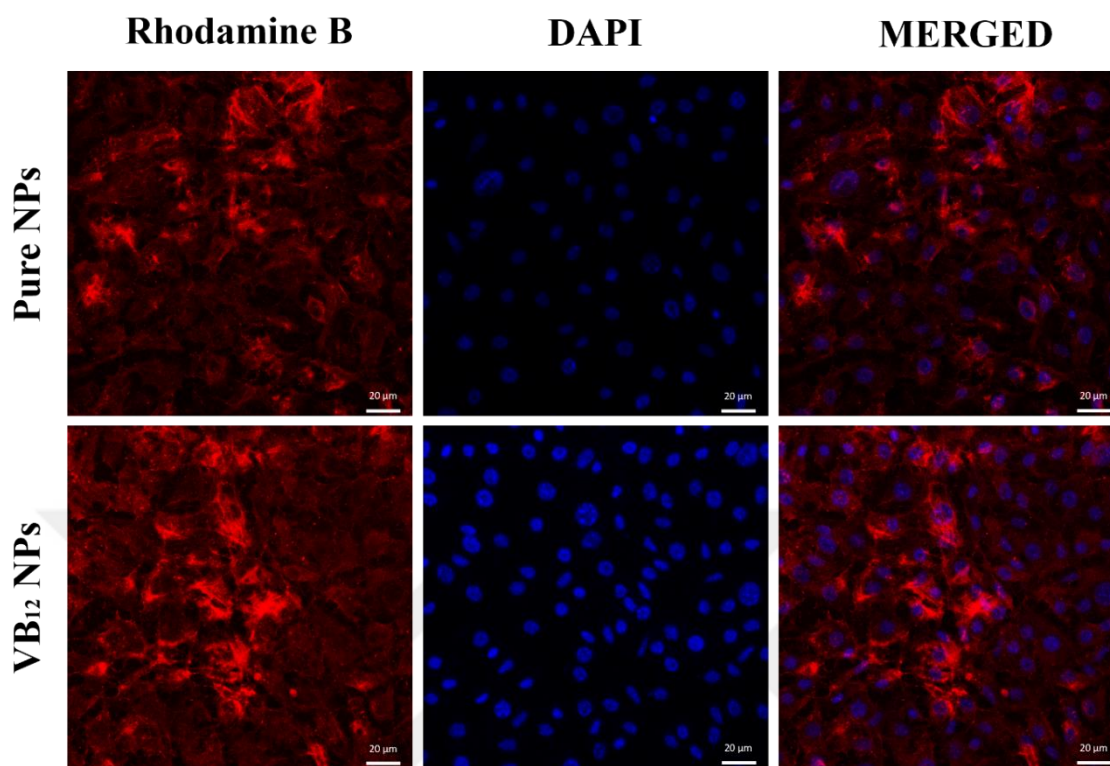


Figure 14. Confocal laser scanning microscope images of SH-SY5Y cells. (The scale bar represents 20 μm .)

6.11. Assessment of ELISA results

In this study, we evaluated the effects of VB₁₂, VB₁₂ NPs, TPP CS NPs on the accumulation of A β in an *in vitro* AD model using SH-SY5Y cells (Figure 15). The group treated solely with A β ₁₋₄₂, without any additional interventions, was designated as the “(-) Control,” and the A β ₁₋₄₂ level in this group was set at 100%.

Compared to the (-) Control group, we observed significant decrease in A β ₁₋₄₂ levels for the VB₁₂ group (68.79%, $p < 0.001$) and the VB₁₂NPs group (75.48%, $p < 0.01$). Although a decrease was also noted in the TPP CS NPs group (93.34%), this change was not statistically significant.

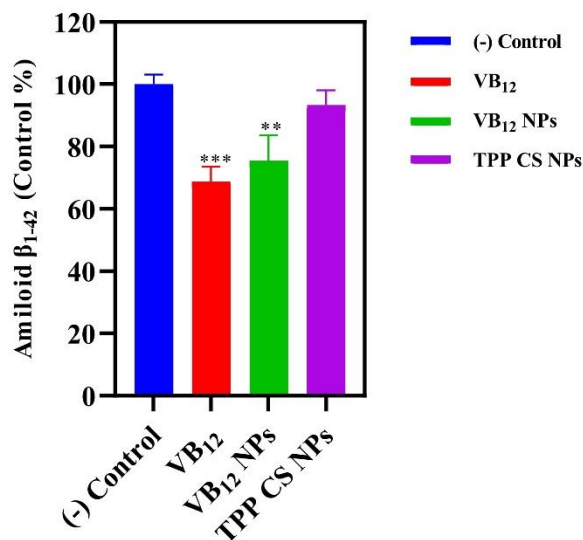


Figure 15. Effects of VB₁₂, VB₁₂NPs, and TPP CS NPs on Aβ₁₋₄₂ levels in Aβ-induced SH-SY5Y cells. VB: Vitamin B12; NP: Nanoparticle. Statistical analysis was performed using one-way analysis of variance (ANOVA), with results presented as mean ± standard deviation. Comparisons to the (-) Control group showed significance: **p < 0.01, ***p < 0.001.

6.12. Assessment of RT-qPCR results

In this study, the SH-SY5Y neuroblastoma cell line was utilized to investigate the potential effects of VB₁₂, VB₁₂NPs, and TPP CS NPs on the PI3K/AKT/GSK-3β and Wnt/β-catenin signaling pathways. RT-qPCR analysis was conducted using specific primers for MAPT, Presenilin-1 (PSEN1), Cyclin D1, GSK-3β, Akt-1, and β-catenin. Cycle threshold (Ct) values were obtained for each target gene, and the relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. The control group served as the calibrator, with its fold change set to 1 (Figure 16).

According to the qRT-PCR analysis, a comparison of the Aβ₁₋₄₂-treated group with the control group revealed a decrease in the expression levels of β-catenin (0.80-fold) and Akt1 (0.99-fold). Conversely, the expression levels of PSEN1 (1.65-fold), MAPT (1.20-fold), GSK-3β (1.64-fold), and Cyclin D1 (1.60-fold) were significantly elevated. In the group treated with Aβ₁₋₄₂ and VB₁₂, the expression of β-catenin and Akt1 genes increased to 1.27-fold and 1.03-fold, respectively, while there were mild increases in PSEN1 (1.30-fold), GSK-3β (1.10-fold), and Cyclin D1 (1.20-fold). Notably, MAPT expression decreased to 0.82-fold. In the Aβ₁₋₄₂ + VB₁₂ NPs group, the expression levels of β-catenin (1.02-fold), PSEN1 (1.40-fold), GSK-3β (1.30-fold), and Cyclin D1 (1.36-fold) were elevated, whereas Akt1 (0.89-fold) and MAPT (0.94-fold) levels showed slight reductions. In

the A β_{1-42} + TPP CS NPs group, β -catenin expression decreased to 0.94-fold, while Akt1 (1.06-fold), PSEN1 (1.42-fold), MAPT (1.02-fold), GSK-3 β (1.55-fold), and Cyclin D1 (1.51-fold) all exhibited increases. When comparing the treatment groups to the A β_{1-42} group, VB₁₂ treatment resulted in a significant increase in β -catenin expression ($p < 0.001$) and a notable reduction in PSEN1 ($p < 0.05$), MAPT ($p < 0.01$), GSK-3 β ($p < 0.001$), and Cyclin D1 ($p < 0.01$) gene expression levels. Additionally, treatment with VB₁₂NPs led to a significant downregulation of GSK-3 β expression ($p < 0.05$), while no statistically significant changes were observed for the other genes.

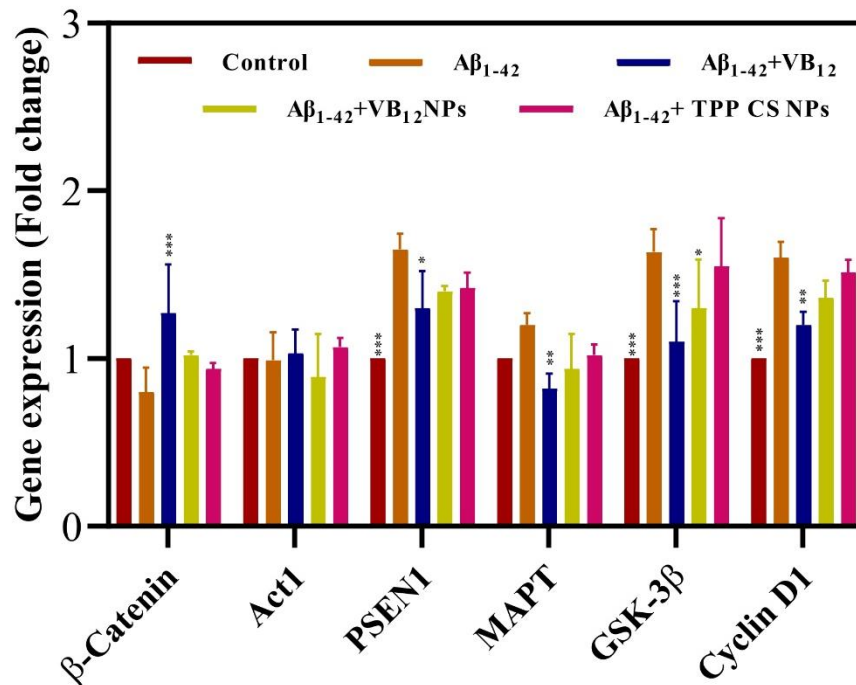


Figure 16. Relative gene expression levels (fold change) of β -Catenin, Act1, PSEN1, MAPT, GSK-3 β , and Cyclin D1 in different treatment groups. Data are presented as mean $\pm$ SD. Statistical significance was determined. Comparisons to the Control group showed significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

7. DISCUSSION and CONCLUSION

The physicochemical characterization of the developed materials confirmed the successful fabrication of a stable and efficient drug delivery system. FTIR analysis for the synthesized carriers ensured the proper embedding of the polymers and drug into the carrier systems. XRD patterns demonstrated the semi-crystalline nature of TPP and the amorphous characteristics of chitosan, PVP, and VB₁₂, with noticeable peak broadening or disappearance in the composite formulations, suggesting that VB₁₂ was physically encapsulated within the polymer matrix without forming new crystalline phases (Onal et al., 2024; Othayoth et al., 2015). XRD patterns demonstrated the semi-crystalline nature of TPP and the amorphous characteristics of CS, PVP, and VB₁₂. The comparison of TPP CS NPs and VB₁₂NPs shows a peak at $2\theta = 19.72^\circ$, indicating successful loading of VB₁₂. The sharp crystalline PEO peak superimposes the XRD result for PS and VB₁₂NPS, owing to the amorphous nature of PVP and VB₁₂ (Muhammed et al., 2020). These results confirm that the current polymer blend system is multiphase, with both crystalline and amorphous regions. DSC supported the effective encapsulation by detecting negligible changes in melting points and no significant exothermic or endothermic phenomena in the thermograms of the NP and scaffold formulations compared to the pure components, indicating good compatibility and stability (Baskakova et al., 2016). The structural and thermal analyses together indicate that the incorporation of VB₁₂ into the nanoparticulate system and scaffold matrix preserved the integrity of the individual components, resulting in a thermally stable and uniform formulation.

The analysis of the VB₁₂NPS in artificial saliva indicated an average disintegration time of less than five seconds, significantly shorter than the USP standard of 2 minutes for sublingual formulations, thereby validating its appropriateness for swift oromucosal delivery (Rawas-Qalaji et al., 2006). The ζ values of the CS TPP NP and VB₁₂NPs were 1.65 ± 0.04 mV and 1.39 ± 0.04 mV, respectively, indicating marginally positive surface charges. The relatively low values are likely due to the formulation pH being near the particles' isoelectric point, in conjunction with the moderate molecular weight of CS and the specific CS-to-TPP mass ratio used (Athavale et al., 2022; Benamer Oudih et al., 2023; Gan et al., 2005; Li et al., 2021; López-León et al., 2005; Ngan et al., 2014). The incorporation of PVA can further diminish surface charge owing to steric stabilization and the shielding effect of acetate groups (Wiśniewska et al., 2016). The findings

indicate that the formulated NP exhibits stability in suspension and possesses advantageous characteristics for mucosal adhesion and cellular uptake.

The NP size of 362.75 ± 2.39 nm and the polydispersity index of 0.319 ± 0.01 suggest a relatively uniform distribution, which is beneficial for intracellular and mucosal delivery systems. Particles smaller than 500 nm are typically regarded as ideal for cellular absorption and traversal through mucosal barriers (Danaei et al., 2018; Takechi-Haraya et al., 2022). Encapsulation efficiency of VB₁₂ was 82.6% in NP and 79.9% in the scaffold system, indicating that the formulation process efficiently encapsulated the active compound through both NP development and scaffold incorporation.

The findings indicate the reproducibility of the fabrication process and ensure the maintenance of the therapeutic dose required in the delivery system for uniform and effective drug release upon administration..

The *in vitro* drug release profile of the VB₁₂NPs exhibited a biphasic pattern, marked by an initial burst phase that remained for 3 days followed by an extended sustained release phase till the 14th day of the release. This sustained release profile demonstrates a controlled and prolonged drug release from the NP. 100% of the VB₁₂ was released on the 14th day. The release mechanism corresponds with the surface desorption of drug molecules, followed by matrix degradation and diffusion-controlled release, which is further hindered by the barrier effect of PVA that slows down diffusion (Mohammed et al., 2017; Song & Cho, 2024). The analysis of release data against multiple kinetic models revealed that the first order model yielded the optimal fit, evidenced by the highest correlation coefficient, suggesting that the drug release is primarily related to the concentration. The release exponent (n) obtained from the Korsmeyer–Peppas model was 0.4512, indicating that the release mechanism follows non-fickian diffusion. These findings validate the formulation's capability for regulated, predictable release kinetics appropriate for prolonged therapeutic uses.

The *in vitro* cytocompatibility and anti-amyloidogenic efficacy of VB₁₂NPs were assessed utilizing SH-SY5Y cells. VB₁₂NPs demonstrated dose-dependent cytocompatibility, with viability rates of 93.4%, 90.1%, and 85.9% at concentrations of 2.5, 5, and 10 nM, respectively. The IC₅₀ (Dm) value was established at 197.2 nM ($r^2 = 0.999$) utilizing CompuSyn software. Aβ_{1–42} was employed to create a cellular model of AD, resulting in a significant decrease in cell viability to 50.4% at a concentration of 5 μM, which was subsequently chosen for model establishment.

Administration of VB₁₂NPs markedly enhanced cell viability in a dose-dependent fashion: 73.95% at Dm ($p < 0.0001$), 65.34% at Dm/10 ($p < 0.01$), and 59.78% at Dm/100 ($p < 0.005$). Treatment with unloaded NP resulted in minimal restoration of cell viability, suggesting that the therapeutic effects were due to the presence of VB₁₂. These findings endorse the prospective neuroprotective function of VB₁₂NPs in alleviating amyloid-induced cytotoxicity and reinstating cellular function in pathological conditions.

The cellular uptake study employing confocal laser scanning microscopy (CLSM) confirmed the internalization of Rhodamine B-labeled VB₁₂NPs by SH-SY5Y cells. The red fluorescence was primarily observed in the cytoplasm, while the nuclei stained with DAPI showed no evidence of NP infiltration, thus confirming that the NP were confined to the cytoplasmic region. The presence of cytoplasmic localization lends credence to the hypothesis regarding endocytic uptake mechanisms, frequently observed in TPP CS NP, attributed to their mucoadhesive and positively charged surfaces (Shen et al., 2016; Wang et al., 2017). The observed intracellular distribution indicates that the NP effectively navigated extracellular barriers and delivered their payload to the correct cellular compartments, thus improving therapeutic efficacy.

At the molecular level, VB₁₂ has a significant impact on reducing A β ₁₋₄₂ levels. This observation aligns with prior studies that suggest VB₁₂ can directly interact with A β structures, thus inhibiting fibril formation (Andrade et al., 2021). While adding VB₁₂ into a NP-based delivery system may enhance its intracellular uptake, the limited release of the compound under short-term *in vitro* conditions likely diminishes its overall efficacy (Ravichandran, 2009). As a result, the reduction in A β ₁₋₄₂ levels observed in the VB₁₂NPs group appears to be less pronounced than that in the free VB₁₂ group. As anticipated, the TPP CS NPs group—lacking any active pharmaceutical ingredient—exhibited A β ₁₋₄₂ levels similar to those of the untreated AD's model. This is consistent with previous reports indicating that empty nanocarriers do not provide significant therapeutic benefits in neurodegenerative disease models (Vilella et al., 2018).

Further mechanistic exploration of quantitative gene expression analysis revealed that A β ₁₋₄₂ significantly dysregulated several AD-related genes; however, treatment with VB₁₂ partially or fully reversed these alterations in key targets. Notably, Cyclin D1, a crucial regulator of the cell cycle, exhibited a 1.6-fold increase following A β ₁₋₄₂ exposure, indicating an aberrant re-entry of postmitotic neurons into the cell cycle—a phenomenon that often leads to apoptotic cell death due to the inability of neurons to complete mitosis under genotoxic stress (Tsai et al., 2019).

Correspondingly, VB₁₂ treatment significantly reduced Cyclin D1 expression to 1.2-fold, suggesting that VB₁₂ may prevent the abnormal reactivation of the cell cycle and protect neuronal viability ($p < 0.01$). Furthermore, levels of GSK-3 β , a kinase associated with tau hyperphosphorylation and Wnt pathway inhibition, were markedly elevated in the A β ₁₋₄₂ group (1.64-fold). This finding aligns with evidence linking GSK-3 β overactivation to increased tau pathology and neuronal degeneration (Abid et al., 2019; Krugluger et al., 2011). Treatment with VB₁₂ resulted in a significant decrease in GSK-3 β levels (1.10-fold, $p < 0.001$). At the same time, VB₁₂ NPs also achieved a statistically significant reduction (1.30-fold, $p < 0.05$), indicating that VB₁₂ may help restore pathway balance and mitigate neurotoxicity. Concerning the MAPT gene, which encodes the microtubule-associated protein tau, exposure to A β ₁₋₄₂ resulted in a moderate increase in its expression (1.20-fold), consistent with previous findings that indicate A β -induced upregulation and aggregation of tau in the brain (Abid et al., 2019). The application of VB₁₂ reduced MAPT expression to 0.82-fold ($p < 0.01$), highlighting its potential to alleviate tau-associated toxicity through transcriptional regulation. PSEN1, a key component of the γ -secretase complex that is responsible for A β generation, exhibited a significant upregulation following A β ₁₋₄₂ treatment (1.65-fold). In contrast, VB₁₂ treatment lowered PSEN1 expression to 1.30-fold ($p < 0.05$), suggesting a link to reduced amyloidogenic cleavage of APP and a subsequent decrease in A β burden. Notably, similar effects have been observed in studies indicating that B vitamin deficiency can lead to hypomethylation and overexpression of PSEN1, which can be reversed through nutritional intervention (Andrade et al., 2021; Fuso et al., 2011). Additionally, β -Catenin, a pivotal molecule in the Wnt signaling pathway, was suppressed by A β ₁₋₄₂ exposure (0.80-fold), reflecting the pathway inhibition commonly seen in AD models (Krugluger et al., 2011). VB₁₂ significantly restored β -catenin expression to 1.27-fold ($p < 0.001$), indicating that VB₁₂ may counteract A β -induced pathway suppression, thereby promoting synaptic maintenance and neuroprotection. Akt1 is an important protein in the PI3K/Akt signaling pathway. After treatment with A β ₁₋₄₂, its levels slightly decreased to 0.99-fold. Adding VB₁₂ brought its expression back up to 1.03-fold. However, VB₁₂NPs showed a small drop in levels to 0.89-fold. This indicates that VB₁₂ may help support cell survival, though different forms may work differently. There isn't much direct evidence about how VB₁₂ affects Akt1 levels in SH-SY5Y cells, but our results suggest that B12 can influence other pathways that impact Akt1 activity. These results underscore the therapeutic potential of VB₁₂ in restoring intracellular signaling homeostasis in models resembling

AD. However, additional mechanistic studies are necessary to elucidate causal relationships and confirm these effects at the protein level as well as in *in vivo* systems.

Our findings corroborate the existing literature that supports the neuroprotective attributes of VB₁₂. Prior studies by Lauer et al. (2022) and Theiss et al. (2022) (Lauer et al., 2022; Theiss et al., 2022) demonstrated that VB₁₂ mitigates oxidative stress and preserves membrane integrity in neuronal cells. Moreover, research by Andrade et al. (2021) (Andrade et al., 2021) demonstrates that NP based delivery systems improve drug transport across the BBB, thereby validating our approach. The integration of VB₁₂ into a nanoparticulate sublingual system provides multiple advantages. It circumvents gastrointestinal degradation and first-pass hepatic metabolism, potentially allowing for elevated systemic and CNS concentrations with diminished dosages. This is particularly advantageous in chronic conditions such as AD, where sustaining long-term compliance, guaranteeing non-invasiveness, and reducing systemic side effects are crucial. However, it is essential to acknowledge several limitations of this study. The data presented are confined to *in vitro* analyses, and while they exhibit potential, they may not fully represent *in vivo* results. The penetration of the BBB, systemic pharmacokinetics, and the long-term safety and efficacy of VB₁₂NPs require validation in animal models.

Finally, the results of this study highlight the successful development and testing of VB₁₂-loaded NP and scaffolds as a potential treatment for AD. The physicochemical characterization confirmed the stability and suitability of the formulations for sublingual drug delivery, resulting in controlled and sustained release of VB₁₂. *In vitro* studies provided compelling evidence of the neuroprotective properties of VB₁₂NPs against amyloid-induced cytotoxicity, thereby underscoring the potential of this nanoparticulate system to improve AD's treatment. Despite certain limitations inherent to the *in vitro* methodology of the study, these results warrant additional investigation into the *in vivo* efficacy and safety of VB₁₂NPs, particularly concerning BBB permeability and long-term therapeutic effects.

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