Adıyaman University Journal of Science
https://dergipark.org.tr/en/pub/adyujsci
DergiPark AKADEMİK
ISSN 2147-1630
e-ISSN 2146-586X

Effect of Solvent Type Approaches on Ester Formation in the Synthesis of Folic Acid N-Hydroxy Succinimide Ester

Sumeyye Idil CELİKKAYA[1, 2, 3,*], Şahin YILDIRIM[4], Ahmet ALTUN[4]

[1]*University of Strathclyde, Faculty of Chemistry, Department of Pure and Applied Chemistry, G1 1RD, Glasgow, United Kingdom*
sumeyye.celikkaya@strath.ac.uk, ORCID: 0000-0001-7036-7922
[2]*Sivas Cumhuriyet University, Health Sciences Institute, Department of Medical Pharmacology, 58070, Sivas, Türkiye*
idilkoc@cumhuriyet.edu.tr, ORCID: 0000-0001-7036-7922
[3]*Sivas Cumhuriyet University, Faculty of Pharmacy, Department of Pharmaceutical Sciences, 58140, Sivas, Türkiye*
idilkoc@cumhuriyet.edu.tr, ORCID: 0000-0001-7036-7922
[4]*Sivas Cumhuriyet University, Faculty of Medicine, Department of Medical Pharmacology, 58140, Sivas, Türkiye*
ysahin@cumhuriyet.edu.tr, ORCID: 0000-0003-1312-7273
aaltun@cumhuriyet.edu.tr, ORCID: 0000-0003-2056-8683

Received: 19.03.2025 **Accepted:** 11.08.2025 **Published:** 31.12.2025

Abstract

Folic acid is an essential B9 vitamin critical for various cellular functions. It is taken up into cells via folate receptors following pH-dependent Michaelis-Menten kinetics. Folic acid enables efficient intracellular drug delivery in diseases where these receptors are overexpressed. The ester form of folic acid is crucial in targeted therapies, particularly for its ability to be conjugated with carrier molecules. N-hydroxy succinimide (NHS) is a commonly used agent in ester formation, contributing to folic acid ester synthesis via esterification. Although solvents such as dimethyl sulfoxide (DMSO) and represents agents like N, N'-dicyclohexylcarbodiimide (DCC) are frequently used for efficient synthesis, the potential for by-product formation necessitates further analytical validation. Alternative solvents like dichloromethane (DCM) or

* Corresponding Author DOI: 10.37094/adyujsci.1661151

dimethylformamide (DMF) may offer advantages due to producing fewer impurities and allowing for easier removal of side products when using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as a coupling agent. Our results show that selecting a solvent significantly influences the synthesis's effectiveness and purity. Evaluation through TLC, UV-Vis, and ^{1}H NMR analyses showed that synthesis conducted in DMF resulted in a higher yield and more specific conjugate formation. The product synthesized in DMF displayed a UV spectrum closely resembling that of folic acid, including a slight hypochromic shift reported in the literature, thereby confirming successful ester formation. In contrast, the spectrum of the DCM-derived product showed similarities to free NHS, suggesting an inefficient reaction compared with DMF-derived esterification. Furthermore, the absence of characteristic ^{1}H NMR signals associated with EDC or potential intermediates in both syntheses supports the selectivity of the applied protocol. These results indicate that DMF is a more suitable solvent for such conjugation reactions and that FA-NHS conjugates can be synthesized with high yield and purity under these conditions.

Keywords: Esterification, N-hydroxy succinimide, Folic acid, Dichloromethane, Dimethylformamide, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide.

Folik Asit N-Hidroksisüksinimid Ester Sentezinde Farklı Çözücü Tiplerinin Ester Oluşumuna Etkisi

Öz

Folik asit, hücrelerin hayati fonksiyonları için önemli bir B_9 vitaminidir. Folik asit reseptörleri tarafından hücre içine pH'a bağlı Michaelis-Menten kinetiği ile alınan folik asit, bu reseptörlerin aşırı ifade edildiği hastalıklarda ilacın hücre içine kolay alınabilmesine imkan tanır. Folik asidin ester formunun hedefli tedavilerde kullanılması, folik asidin taşıyıcı ilaca bağlanması açısından önemlidir. N-hidroksi süksinimid (NHS), ester oluşturmak için kullanılan bir ajandır ve Steglich esterifikasyonu ile folik asitten ester oluşumuna katkıda bulunur. Birçok sentezde, çözücü olarak dimetil sülfoksit (DMSO) ve reaktif olarak ise N, N'-disiklohekzilkarbodiimid (DCC) kullanılır. Bu maddelerle etkili bir sentez gerçekleştirilmesine rağmen, olası safsızlıklar nedeniyle sonraki çalışmalarda başka analizler yapılmalıdır. Diklorometan veya dimetil formamid gibi çözücüler daha az safsızlık üretmeleri ve 1-Etil-3-(3-dimetilaminopropil) karbodiimid (EDC)'in reaktif olarak daha kolay bir şekilde uzaklaştırılabilen ara ürün oluşturması sebebiyle bu reaksiyon için daha uygun olabilir. Araştırmamız sonucunda, kullanılan çözücünün sentez verimliliği ve ürün saflığı üzerinde belirleyici bir rol oynadığı görülmüştür. TLC, UV-Vis ve ^{1}H NMR analizleri birlikte değerlendirildiğinde, DMF çözeltisinde gerçekleştirilen sentezin, daha yüksek ürün verimi ve daha spesifik konjugat oluşumu sağladığı anlaşılmıştır. DMF ortamında

elde edilen ürün, folik asit spektrumuna benzer bir UV profili sergilemiş ve literatürde bildirilen hafif hipsochromik kaymayı yansıtarak esterleşme reaksiyonunun başarıyla gerçekleştiğini doğrulamıştır. Buna karşın, DCM kullanılarak gerçekleştirilen sentezde ürün spektrumu serbest NHS'e benzerlik göstermiş, bu da reaksiyonun verimli gerçekleşmediğini düşündürmektedir. Ayrıca, her iki sentezde de EDC veya olası ara ürünlere ait herhangi bir ^{1}H NMR sinyali gözlenmemesi, protokolün seçici bir şekilde hedef konjugat oluşumunu sağladığını desteklemektedir. Bu sonuçlar, DMF'nin bu tür konjugasyon reaksiyonları için uygun bir çözücü olduğunu ve elde edilen FA-NHS konjugatının yüksek saflık ve verimle sentezlenebileceğini göstermektedir.

Anahtar Kelimeler: Esterifikasyon, N-hidroksisüksinimid, Folik asit, Diklorometan, Dimetilformamid, 1-Etil-3-(3-dimetilaminopropil) karbodiimid.

1. Introduction

Folic acid and its conjugates are widely utilized as targeting agents in cancer chemotherapy, with their mechanism of action recognized as a significant conjugation strategy in nanotechnology [1, 2]. Folic acid receptors (FRs) are frequently overexpressed in various cancer types, including ovarian cancer and breast cancer. These receptors represent an important target for therapy, as they serve as distinctive cellular markers for malignant cells [3].

The molecular formula of folic acid, also known as the synthetic form of vitamin B9, is $C_{19}H_{19}N_7O_6$ (Fig. 1). FA comprises pterin and L-glutamic acid groups, which are connected by a bridge of para-aminobenzoic acid [2, 4].

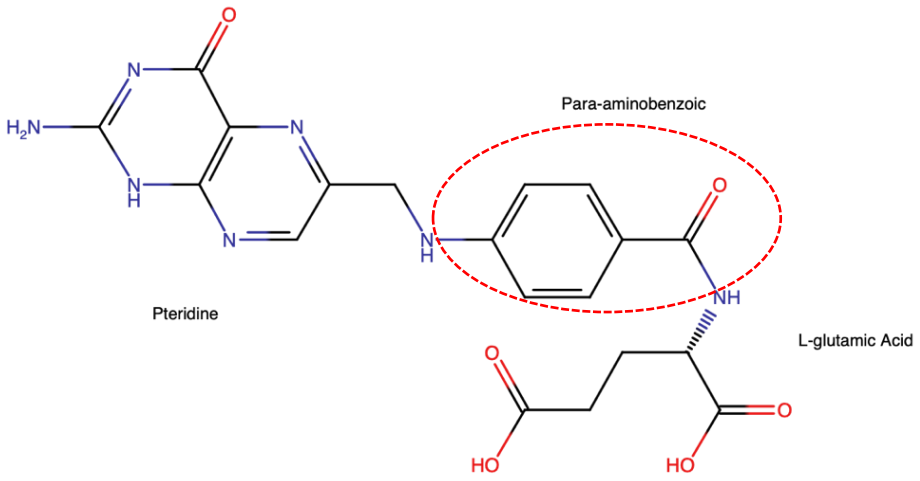


Figure 1: Molecular structure of folic acid, highlighting its three key structural components: a pteridine ring system, a para-aminobenzoic acid (PABA) moiety, and a terminal L-glutamic acid residue. The pteridine ring is a bicyclic heteroaromatic system crucial for the vitamin's biological activity. The PABA

unit acts as a linker between the pteridine and glutamate groups, contributing to the molecule's recognition and binding properties. The L-glutamic acid tail is essential for the cellular uptake and retention of folic acid through folate receptor-mediated transport and enzymatic processing in metabolic pathways.

Modifications to the functional groups on FA can be beneficial for modulating receptor activity aimed at targeting FRs. The terminal amino (NH_2) group in the pteridine region plays a crucial role in binding to FRs making alterations to this functional group significant for receptor interaction. Additionally, the carboxyl groups present in the L-glutamic acid component can be adjusted to enhance or modify receptor-ligand interaction [4].

The Biopharmaceutical Classification System (BCS) categorizes active pharmaceutical ingredients (APIs) into four classes based on their solubility and permeability. FA is classified as a Class IV substance, which signifies low solubility and low permeability (Fig. 2). As a result, the number of suitable solvents for FA's synthesis is considerably limited [5].

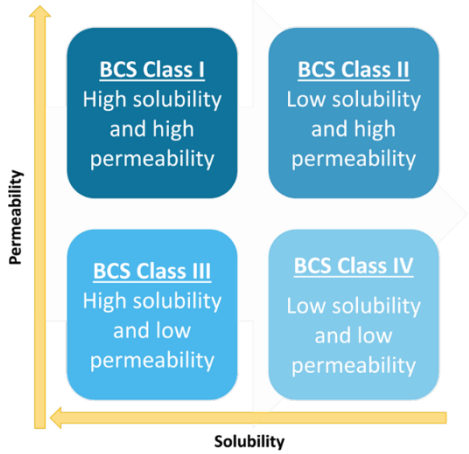


Figure 2: Biopharmaceutical classification system. According to this classification, folic acid, categorized as a Class IV compound, exhibits low solubility, which significantly limits its applicability in reactions such as esterification or other activation steps where sufficient solubility is essential for efficient synthesis.

The conjugation of FA onto nanoparticle surfaces is a crucial process in the targeted delivery of drugs via nanoparticle-folic acid complexes [1,6]. The ester form of FA efficiently binds nanoparticle structures, making the esterification is vital for creating stable nanoparticle assemblies [7]. Folic acid N-hydroxy succinimide ester (FA-NHS ester) is produced through esterification, utilizing N-hydroxy succinimide (NHS) (Fig. 3) [8].

FA features two carboxylic acid groups within the glutamic acid moiety: the α- and γ-carboxyl groups. During the formation of α and γ FA-NHS esters, either of these carboxylic acid groups has the potential to react with NHS when a coupling agent like 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) is present [9]. However, due to steric and electronic factors, FA-NHS esterification predominantly occurs at the γ-position.

Previous studies have corroborated this region selectivity, indicating that esterification at the γ-carboxyl group promotes more efficient subsequent conjugation reactions [10].

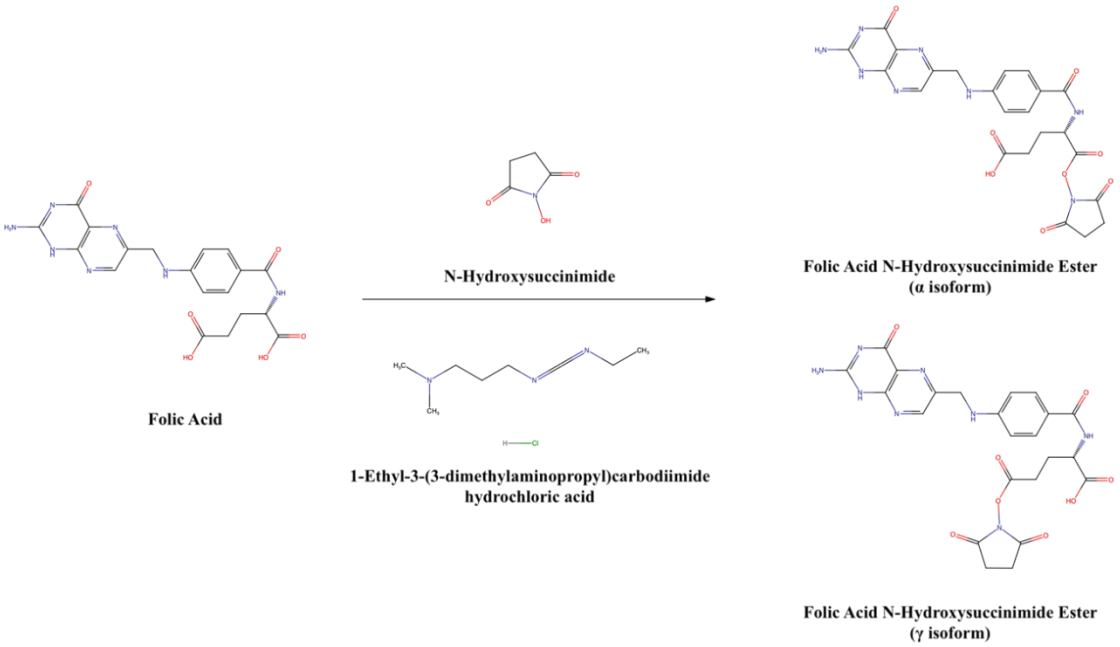


Figure 3: This schematic illustrates the synthesis of folic acid N-hydroxy succinimide (NHS) ester. The process begins with the activation of folic acid using N-hydroxy succinimide (NHS) in conjunction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) as coupling agents. This reaction produces two isomers of the folic acid NHS ester (α and γ), depicted on the scheme's right side.

In the synthesis of FA-NHS esters, dimethyl sulfoxide (DMSO) is frequently used as a solvent. At the same time, N,N'-dicyclohexylcarbodiimide (DCC) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) are prevalent as coupling agents based on carbodiimide chemistry (Table 1) [2, 8, 11]. Although DCC and EDC are used for the similar purposes in facilitating ester bond formation, they exhibit significant differences in their physicochemical properties, particularly concerning solubility and by-product formation [12]. DCC is characterised by its water-insolubility and the generation of N, N'-dicyclohexylurea (DCU) and N-acylurea as by-products, which can accumulate within the reaction medium. These by-products may impede the reaction progression and cause challenges during purification [13]. In contrast, EDC is water-soluble, permitting the straightforward removal of the reagent and its urea-based intermediate products from aqueous environments, thus resulting in a more efficient and cleaner reaction profile [14].

Table 1: This summary compiles selected literature findings on synthesising folic acid NHS esters through esterification

Solvent	Represents Reagent	Synthesis Time and Synthesis Environment Conditions	Molar Ratio (Folic acid:NHS:DCC/EDC)	Literature
Dry DMSO	DCC	Overnight at room temperature (25 ˚C)	1:2:2	[8]
DMSO	DCC	Overnight at room temperature (25 ˚C)	1:2:2	[11]
DMSO	EDC	Dark for 3 h	1:5:5	[2]

The accompanying table 1 outlines essential reaction parameters, including the solvent system, coupling reagents (either N, N'-dicyclohexylcarbodiimide (DCC) or EDC), reaction duration, environmental conditions (such as temperature and light exposure), and the molar ratios of the reactants. Notably, dry dimethyl sulfoxide (DMSO) is frequently employed as the solvent medium, with DCC or EDC acting as the coupling agents. Reaction times can vary, ranging from 3 hours under dark conditions to overnight reactions at ambient temperature. The molar ratios of folic acid to NHS to coupling agent range from 1:2:2 to 1:5:5, depending on the specific methodology used. These protocols offer foundational guidance for optimising the esterification of folic acid under mild conditions while considering its solubility limitations.

In this study, based on previously established FA-NHS ester synthesis protocols, the reaction was performed by adding all reagents (FA, NHS and EDC) into the reaction vessel, followed by stirring the mixture in an ice bath (below 0 °C) for 30 minutes in the dark. The reaction was then allowed to reach room temperature and continued stirring under dark conditions for 24 hours [2]. The purpose of varying the solvent system was to investigate its role in the formation of undesired by-products and its overall impact on the efficiency of NHS ester formation.

Despite the common usage of DMSO (commercial form (Biosynth®, Synchem®, Clinivex®) the elevated boiling point presents a significant challenge. The challenge of removing DMSO from the final product can lead to the retention of undefined impurities potentially compromising product purity and diminishing yield [15]. Furthermore, deionised H_2O utilisation in forming an ester from carboxylic acids inadvertently shift the reaction equilibrium toward hydrolysis, per Le Chatelier principle. Consequently, water-containing systems may detrimentally affect reaction efficiency [16, 17].

In light of these considerations, this study aims to investigate the implications of different solvent systems on the synthesis of FA-NHS esters. Specifically, alternative organic solvents—

anhydrous dichloromethane (DCM) and anhydrous dimethylformamide (DMF)—have been selected due to their advantageous properties, and assessed their contribution of yield of esterification reaction. DCM has been chosen for its low boiling point, facilitating solvent removal, while DMF has been selected for its compatibility with coupling reactions and more straightforward purification steps compared to DMSO [18, 19].

To this end, two distinct FA-NHS ester synthesis routes were conducted utilising DCM and DMF as solvents, with EDC as the coupling agent. The primary objective of this investigation was to elucidate the influence of solvent on the production of intermediate products, reaction impurity, and overall yield of the conjugation process.

2. Materials and Methods

2.1. Materials

In this study, all reagents were used as received, without further purification. N, N-Dimethylformamide (DMF, ≥99.8%, anhydrous), dichloromethane (DCM, ≥99.8%, anhydrous, stabilized with 40–150 ppm amylene), ethyl acetate (ACS reagent grade, ≥99.5%), hexane (≥99%, suitable for GC/MS and EPA 1613 analysis, ReagentPlus®) were supplied by Sigma-Aldrich. Dimethylsulfoxide (DMSO, ACS reagent grade, ≥99.9%) and deuterated DMSO (d_6-DMSO, 0.06% v/v TMS, CAS No: 2206-27-1) were obtained from Sigma-Aldrich™ and Eurisotop™, respectively. Folic acid (assay ≥95.0%, MW: 441.404 g/mol) was purchased from Fisher BioReagents™. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, commercial grade, powder form, MW: 191.70 g/mol), N-hydroxysuccinimide (NHS, 98%, MW: 115.09 g/mol), magnesium sulphate (anhydrous, ReagentPlus®, ≥99.5%) were acquired from Sigma-Aldrich™. Acetone (extra dry, 99.8%, AcroSeal™, CAS: 67-64-1; C_3H_6O; MW: 58.08 g/mol) was provided by Thermo Scientific Chemicals™. Thin layer chromatography (TLC) plates with silica layers on Alugram™ aluminium sheets (Xtra SIL G, UV254) were obtained from Macherey-Nagel™.

2.2. Solubility Study

Folic acid (1 mg) was accurately weighed and dissolved separately in 1 ml of anhydrous DCM and 1 ml of anhydrous DMF at a fixed concentration of 1 mg/mL. Each solution was left to stand at room temperature (25 °C) for 10 minutes after gentle mixing to assess solubility. The presence of suspended particles or precipitation was visually observed in dissolution period.

2.3. Synthesis

Folic acid was initially dissolved in anhydrous DCM, and the resulting solution was stirred under a nitrogen atmosphere at 0 °C. Following this, NHS (1.1 equivalents) and EDC hydrochloric acid (2.5 equivalents), previously dissolved in anhydrous DCM, were introduced to the reaction mixture while keeping the pH around 6. The mixture was stirred at 0 °C for 30 minutes before gradually allowing the temperature to increase to room temperature. Stirring continued at room temperature for another 24 hours.

This method was then repeated using anhydrous DMF as the solvent for the reaction.

2.4. Purification

The reaction mixture was concentrated using a rotary evaporator (Büchi® Model R-200) to remove excess anhydrous DCM, after which the resulting crude product was weighed. To eliminate water-soluble byproducts such as EDC, NHS and urea-based intermediate products, a liquid-liquid extraction was performed, 50 ml of anhydrous DCM and 25 mL deionized water for each gram of crude product [20, 21]. The organic phase was subsequently dried with magnesium sulphate and filtered to yield a white solid.

In the reaction conducted in anhydrous DMF, ethyl acetate was employed to selectively precipitate FA-NHS ester, as it is insoluble in ethyl acetate, while anhydrous DMF readily dissolves it [22]. The solid was collected through centrifugation at 5000 rpm for 15 minutes and then washed three times with anhydrous acetone. The sample was concentrated using a rotary evaporator and finally lyophilized to produce an orange dry product [23]. Both final products were stored at -20 °C.

2.5. Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was conducted using Macherey-Nagel™ Xtra SIL G silica gel plates on Alugram™ aluminium sheets (UV254, measuring 4 × 8 cm). A total of 3 µL of samples were placed with a micropipette located 1 cm from the bottom edge of the plate. DMSO served as the solvent for spotting. The plates were developed with a mobile phase made of hexane and ethyl acetate in a 1:4 ratio, and the visualization was carried out under UV light at 254 nm. The spots were stable and clearly detectable under UV light [24].

2.6. Nuclear Magnetic Resonance (NMR)

^{1}H NMR spectra were recorded employing a Bruker Ascend™ 500 MHz spectrometer (AV500) at 25 °C, situated at the NMR-BASE facility at the University of Strathclyde, Glasgow. Sample solutions were prepared in deuterated DMSO (d_6-DMSO) at a concentration of 10 mM. Tetramethylsilane (TMS) served as an external reference standard, with chemical shift values determined relative to the coupling constants of the TMS molecule [25,26]. Furthermore, as an external standard, TMS was employed in quantitative NMR (qNMR) measurements, utilizing appropriate programming (MestReNova™) for quantitative analysis [27].

2.7. Quantitative NMR (qNMR) Analysis

The reaction yields were calculated using qNMR based on the integrated intensities of the ^{1}H NMR spectra [27]. To ensure accurate quantification, a constant concentration of TMS was added as an external standard to each NMR sample [28, 29]. For monitoring the esterification reactions and yield determination, signals observed at δ 2.74 ppm in DCM and δ 2.72 ppm in DMF were used. These signals correspond to non-amine-bonded protons within the succinimide ring. In the NHS spectrum, a similar signal is observed at around δ 2.8 ppm, while no such signal appears in the folic acid spectrum due to the absence of this structural motif [30]. Concentrations were calculated using the equation shown in Equation 1.

In this equation, C represents the concentration, I denotes the integrated signal intensity, n is the number of protons responsible for the signal, and P is the pulse length. Subscript A refers to the analyte, and R refers to the reference standard [27].

$$C_A = C_R \times \frac{I_A}{I_R} \times \frac{n_R}{n_A} \times \frac{P_A}{P_R} \quad (1)$$

Eqn (1) was used to calculate the concentrations of the ester products based on the reference standard (TMS), followed by yield determination from the corresponding molar quantities.

2.8. UV-Visible Spectrophotometry

UV-Visible (UV-Vis) spectroscopic analysis was conducted using an Agilent™ Cary 60 UV-Vis Spectrophotometer to compare the absorption profiles of both final products in the range of 200–800 nm. For reference, the spectra of pure FA and NHS were also received. All samples were prepared in 100% DMSO [25].

3. Results and Discussion

3.1. Qualitative Solubility Observations

The solubility of folic acid in different organic solvents at fixed concentration (1 mg/mL) and temperature (25 °C) was evaluated qualitatively. After the addition of folic acid to the solvents, the dissolution process was completed in an ultrasonic bath (Bandelin SONOREX™ SUPER without heating Ultrasonic baths). For the qualitative determination of solubility, after being taken from the ultrasonic bath, it was kept for 10 minutes at 25 °C and checked for precipitation. Since folic acid did not precipitate in anhydrous DCM and anhydrous DMF solvents, these solvents were selected.

3.2. TLC

Thin Layer Chromatography (TLC) revealed that the product synthesized in DCM displayed Rf values that differed from those of FA and NHS, indicating successful conjugation (Table 2). However, the further validation through NMR spectroscopy and UV-Vis spectrometry was necessary.

The product which has been synthesized with DMF exhibited as the same Rf value as FA, which suggesting incomplete reaction or co-elution (Table 2). This result implies the presence of a significant amount of unreacted FA in the final mixture, likely due to suboptimal reaction efficiency.

None of the TLC profiles revealed a spot corresponding to EDC detected, and no additional impurity spots were detected in any reaction mixtures. TLC results suggest that no detectable side products were formed during the reaction and that EDC probably decomposed or was fully consumed under the reaction conditions without leading to side reactions. Consequently, using both synthetic route (DCM and DMF) has been completed without any impurity at the final mixture, including EDC.

Table 2: TLC analysis results of FA-NHS ester synthesis using different solvents (DCM and DMF)

Sample Name	Rf	Distance (cm)
FA-NHS ester (synthesis with DCM (DCM-FA-NHS))	0.292	1.9
FA-NHS ester (synthesis with DMF (DMF-FA-NHS))	0.474	2.7
Folic Acid	0.474	2.7
NHS	0.439	2.5
EDCI	0.298	1.7

The Rf values indicate that synthesis in DCM yielded a distinct product compared to folic acid and NHS, supporting successful conjugation. In the case of DMF, the synthesized product shared an Rf value with NHS, suggesting possible co-elution or incomplete separation on TLC; however, subsequent qNMR analysis confirmed a conjugation yield of 48.2%, indicating partial reaction success.

3.3. NMR

The ^{1}H NMR spectra of the DCM-FA-NHS and DMF-FA-NHS conjugates exhibited new signals that were absent in the spectra of either folic acid or NHS alone (Figs. 4-5). The ^{1}H NMR spectra of the esters synthesized using DCM and DMF did not show the emergence of new peaks at δ 2.8 and δ 3.5 ppm compared to the control spectra of FA and NHS [31]. While this absence does not confirm the presence of EDC, the detection of doublets at δ 1.1 ppm suggests its presence. EDC can be converted to the final product by utilizing excess FA in the synthesis process. Furthermore, the absence of any peak at δ 0.7 ppm indicates that no urea-based intermediates from EDC were formed, suggesting minimal possible impurities [32].

The characteristic NHS methylene protons in both NMR spectra can be observed in the δ 0–3 ppm region. The absence of residual peaks from DCM and DMF indicates that the synthesized products were properly purified before NMR analysis. Only the d_6-DMSO solvent peak is detected, and a small *J*-coupling constant of approximately 1.9 Hz is noted, which aligns with previously reported values (Figs. 6-7) [33].

Notably, distinct peaks were detected at δ 2.72 ppm for the DCM-FA-NHS conjugate and δ 2.74 ppm for the DMF-FA-NHS conjugate, corresponding to the methylene protons of the succinimide ring that are not bound to the amine group [34]. These chemical shifts are indicative of ester bond formation and confirm the successful conjugation of folic acid to NHS in both solvents (Fig. 8).

The NMR findings, along with the results from TLC analyses, present strong evidence for the stable formation of FA-NHS ester conjugates, particularly in DCM. This solvent displayed a unique Rf value (0.292) and a more defined spectral profile.

While the presence of both α and γ-NHS esters is possible, the NMR spectra did not clearly distinguish between the two isomers due to signal overlap and the potential formation of mixed products. Further analysis, such as chromatographic separation or 2D-NMR, would be necessary to achieve a definitive assignment.

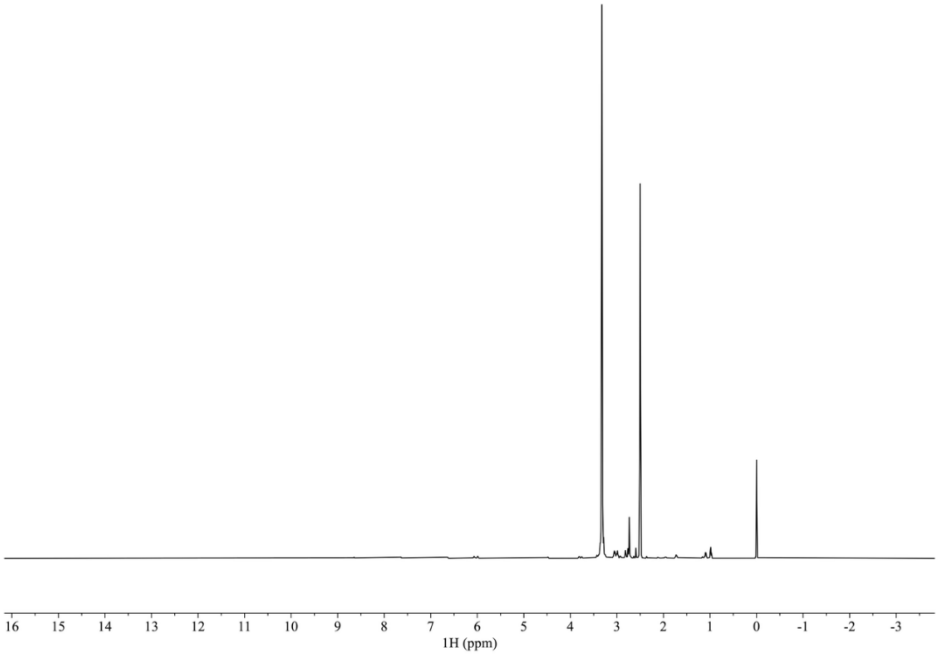


Figure 4: The full ¹H-NMR spectrum of folic acid-NHS ester synthesized in DCM. The singlet observed at ~ δ 2.72 ppm corresponds to the succinimide ring's methylene protons (–CH_2–), confirming successful NHS ester formation. All characteristic peaks of folic acid are preserved, with minor shifts due to esterification.

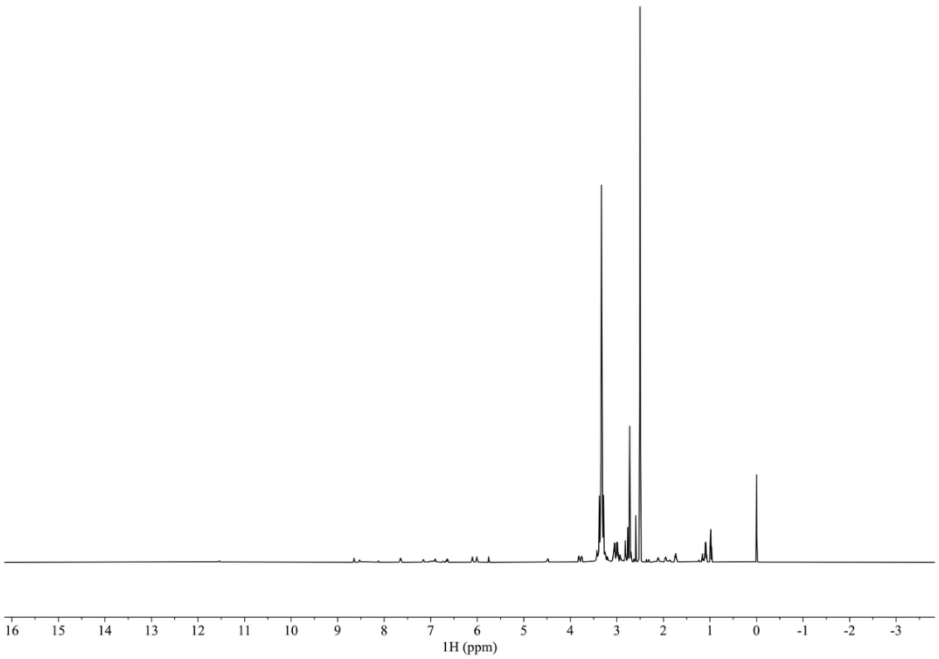


Figure 5: The complete ¹H-NMR spectrum of folic acid-NHS ester synthesized in DMF shows a singlet at approximately δ 2.74 ppm, corresponding to the NHS moiety of methylene protons. This finding supports the successful activation of the carboxylic acid group. The slight differences in chemical shifts observed compared to the DCM-based synthesis can be attributed to solvent effects.

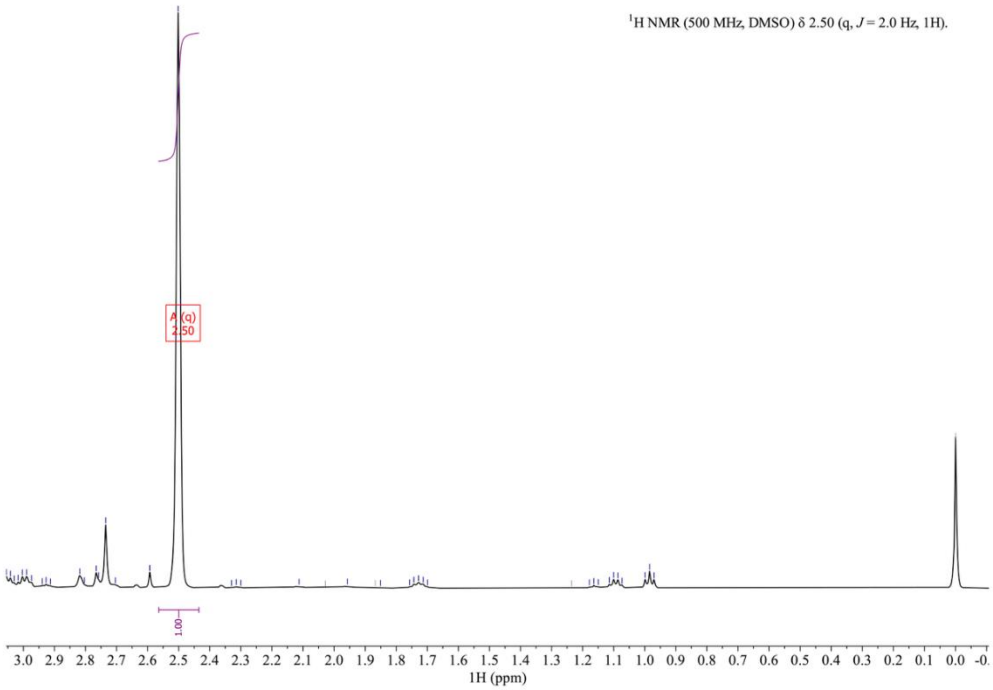


Figure 6: The magnified ¹H-NMR spectrum (δ 0–3 ppm) of folic acid-NHS synthesized in DCM reveals a singlet at approximately δ 2.72 ppm, corresponding to the NHS ring's methylene protons. The residual solvent peak of d_6-DMSO is observed at δ 2.50 ppm, with a measured *J*-coupling value of 2.0 Hz. Notably, no residual signals from DCM are detected, indicating clear purification prior to NMR analysis.

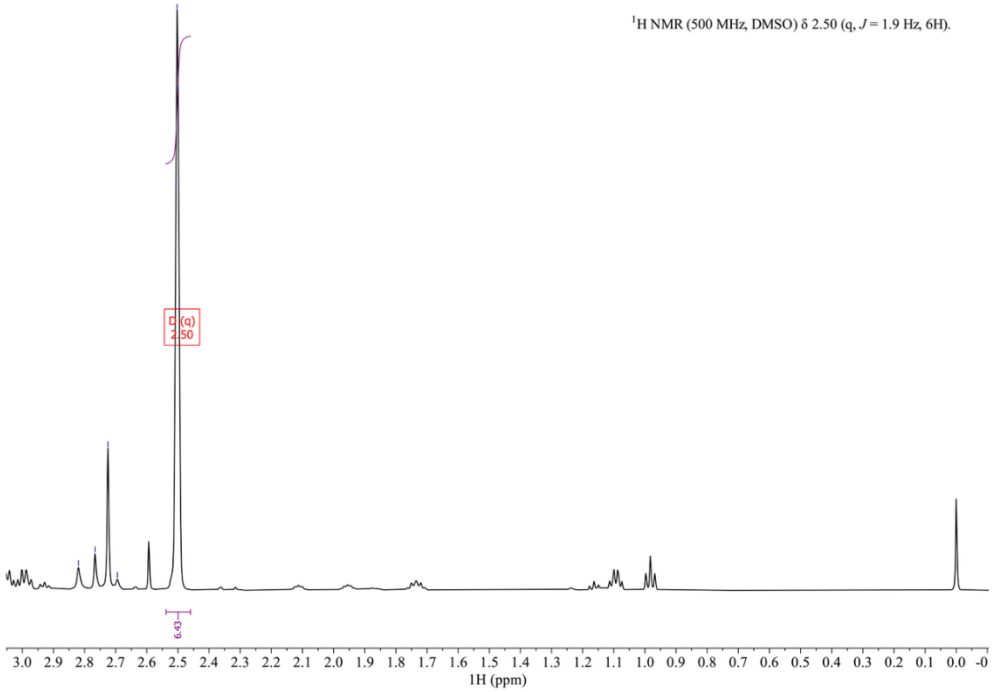


Figure 7: The magnified ¹H-NMR spectrum (δ 0–3 ppm) of FA-NHS synthesized in DMF reveals the NHS methylene peak at approximately δ 2.74 ppm. The d_6-DMSO solvent peak also appears at δ 2.50 ppm, accompanied by a *J* value of 1.9 Hz. Notably, no detectable peaks from DMF are present, which confirms the successful removal of unreacted solvents.

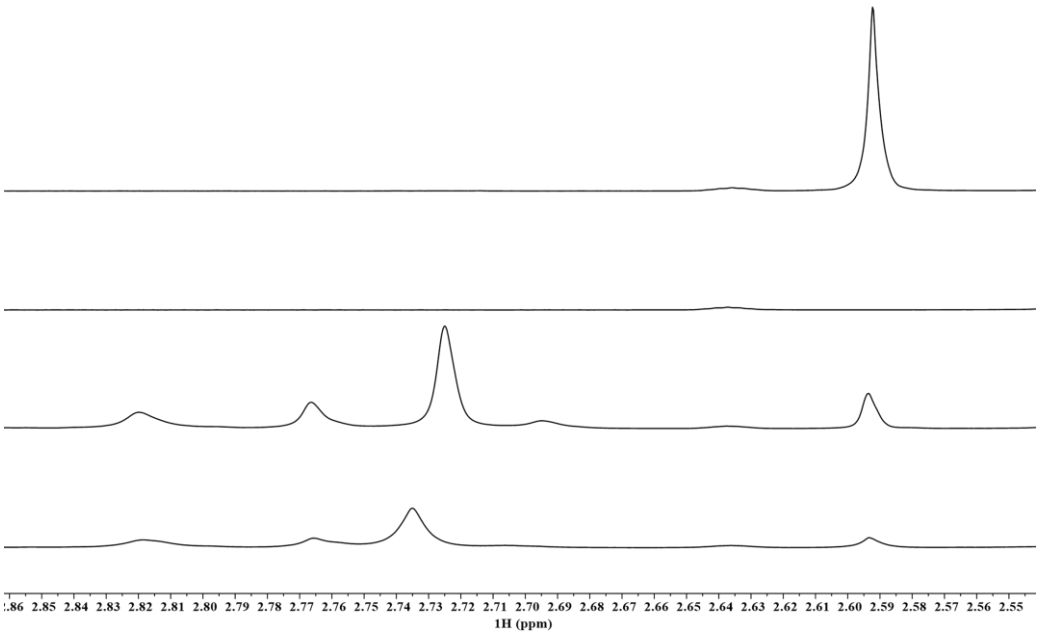


Figure 8: The ^{1}H NMR signals observed between δ 2.55 and δ 2.86 ppm for NHS, FA, DCM-FA-NHS, and DMF-FA-NHS, respectively, from top to bottom. The characteristic peaks observed at δ 2.72 ppm for DCM-FA-NHS and δ 2.74 ppm for DMF-FA-NHS correspond to ester functionalities. These signals are attributed to the ring protons of the succinimide moiety (excluding the amine-bound protons), indicating the successful formation of the ester bond in the conjugated structures.

3.4. Quantitative NMR (qNMR)

qNMR analysis facilitated the estimation of reaction yields by examining characteristic signals at δ 2.74 ppm (DCM) and δ 2.72 ppm (DMF), which are associated with the succinimide moiety of the ester product. The calculated yields were 26.80% for the DCM-based synthesis and 48.20% for the DMF-based synthesis, revealing a significantly higher conversion efficiency in the DMF reaction medium (According to the Fig. 4 equation).

3.5. UV-Visible Spectrophotometry

The UV–Vis absorption spectrum of the product synthesized using DCM as the reaction solvent displayed a peak corresponding to NHS rather than the anticipated FA or FA-NHS ester profile. This finding contradicts the NMR and TLC data, which indicate that conjugation was successfully achieved. According to the literature, the FA-NHS ester should exhibit absorbance peaks similar to FA spectrum but with a slight hypochromic shift for the primary absorption band [35, 36]. In contrast, the UV–Vis spectrum of the product obtained from the synthesis in DMF closely matched that of FA, suggesting stable FA-NHS ester formation. These results are consistent with the NMR analyses. The lower yield and UV–Vis discrepancies noted with DCM as a solvent may stem from an incomplete reaction, potentially due to the suboptimal

stoichiometry of NHS and the coupling reagents used. Consequently, the adjusting of the molar ratios of the reactants—particularly by increasing amount of NHS and coupling agents relative to FA—may enhance the efficiency and consistency of ester formation in DCM-based synthesis (Fig. 9).

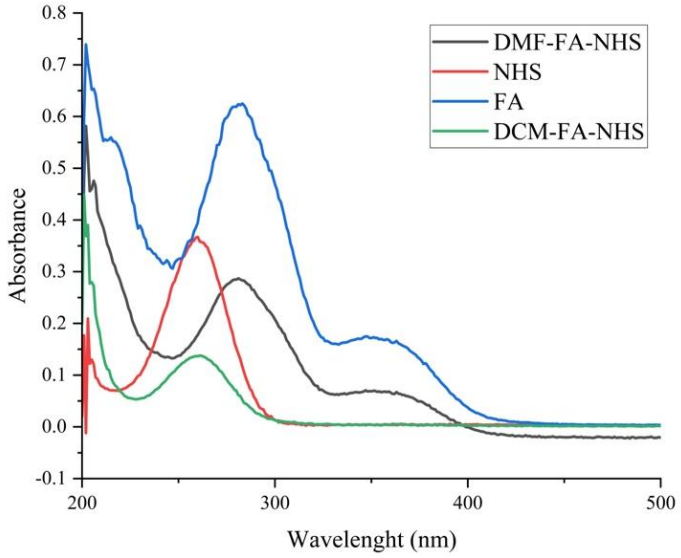


Figure 9: UV-Vis absorbance spectra of FA-NHS, folic acid (FA), and N-hydroxy succinimide (NHS) synthesized using dichloromethane-(DCM) and-dimethylformamide (DMF) as reaction solvents. DMSO was used as the solvent for sample preparation in all spectrophotometric measurements. The observed spectral shifts and absorbance changes support the successful formation of the ester product (FA-NHS) and provide additional evidence for the conjugation process.

4. Conclusion

TLC and ^{1}H NMR spectroscopy analysis revealed significant information about the synthesis of FA-NHS ester with different solvent types. FA-NHS ester synthesized in DCM showed different Rf values in TLC and specific ester peaks in ^{1}H NMR. FA-NHS ester produced in DMF showed similar Rf values to FA in TLC, while ester peaks in the ^{1}H NMR spectrum were less pronounced than those observed with DCM. This may indicate that the product synthesized in DMF yielded less; however, the quantitative analysis showed that the product derived from DMF was associated with a higher yield. This difference in yield observed according to TLC and ^{1}H NMR results may probably be due to different physicochemical properties of the solvents used. In a qualitative solubility analysis, FA formed a cloudy, orange-coloured solution in DCM, while a bright, light, yellow-coloured solution was observed with DMF. In addition, DMF increases the solubility of FA and other reagents due to its high dielectric constant and polar aprotic properties, resulting in a more consistent reaction medium and increasing the chance of a successful esterification process. In addition, the lower polarity of DCM compared to DMF may have negatively affected the kinetics of the reaction by causing a more turbid FA solution.

The absence of distinctive signals related to EDC in the ^{1}H NMR spectra of the synthesized products reveal the protocol's efficiency by intermediate impurity. In addition, the urea-based intermediate products that might be formed due to EDC is not evident in ^{1}H NMR and TLC, and minimal byproduct formation occurs in the reaction. These findings emphasize the protocol's reliability in receiving the pure final product, FA-NHS, reinforcing the confidence in the research methods used.

According to the data obtained by UV-Vis spectrophotometry, the UV-Vis spectrum of the product formed in DCM did not show the expected spectral changes associated with FA [35,36]. It showed an identical spectrum profile with free NHS. This indicates that the synthesis was inefficient or resulted in the product containing unreacted starting materials. This result is inconsistent with the TLC and NMR findings and suggests that the limited solubility in DCM may have caused insufficient reaction yield. In contrast, the UV-Vis spectrum of the FA-NHS ester synthesized in DMF reveals the similar spectrum to FA with a slight hypochromic shift, as reported in the previous literature. This suggests that the carboxyl group of FA is esterified with NHS. The spectrum also supports the hypothesis that the synthesis is more efficient in DMF medium, producing higher yield FA-NHS ester than DCM medium. The quantitative ^{1}H NMR spectrum confirms this observation and highlights that the DMF is a more suitable solvent for this synthesis reaction.

The final products (FA-NHS esters) resulting from these syntheses can be existed both α- and γ-NHS esters. Due to signal overlap, the ^{1}H NMR spectra does not differentiate between the two isomers. Further techniques, such as chromatographic separation or 2D-NMR, can be required to achieve a definitive analysis.

Acknowledgements

This study was run within the scope of the PhD thesis of the corresponding author, Sümeyye İdil Çelikkaya, titled "In Vitro Investigation of the Activity of Folic Acid Coated Hybrid Nanoparticles on Paclitaxel Resistant Ovarian Cancer Cells" at Sivas Cumhuriyet University, Health Sciences Institute. The authors gratefully acknowledge the support of the Scientific and Technological Research Council of Türkiye (TÜBİTAK), BIDEB, under the 2211-National Graduate Scholarship Programme, and Health Institutes of Türkiye (TÜSEB) Group A Rapid R&D Project Support Program A3-36121. This study was conducted at the Bionanotechnology Lab, Technology and Innovation Centre, University of Strathclyde, Glasgow. The authors would like to thank Prof Clare Hoskins for her theoretical and experimental contributions to the study.

References

[1] Angelopoulou, A., Kolokithas-Ntoukas, A., Fytas, C., Avgoustakis, K., *Folic Acid-Functionalized, Condensed Magnetic Nanoparticles for Targeted Delivery of Doxorubicin to Tumor Cancer Cells Overexpressing the Folate Receptor*, ACS Omega, 4(26), 22214–27, 2019.

[2] Bhushan, B., Gopinath, P., *Tumor-targeted folate-decorated albumin-stabilised silver nanoparticles induce apoptosis at low concentration in human breast cancer cells*, RSC Advances, 5(105), 86242–53, 2015.

[3] Fernández, M., Javaid, F., Chudasama, V., *Advances in targeting the folate receptor in the treatment/imaging of cancers*, Chemical Science, 9(4), 790–810, 2018.

[4] Chen, C., Ke, J., Zhou, X.E., Yi, W., Brunzelle, J.S., Li, J., Yong, E.-L., Xu, H.E., Melcher, K., *Structural basis for molecular recognition of folic acid by folate receptors*, Nature, 500(7463), 486–9, 2013.

[5] Siengalewicz, P., Mulzer, J., Rinner, U., 6.09 Synthesis of Esters and Lactones. In: Knochel, P., (Ed.), *Comprehensive Organic Synthesis Second Edition*, Elsevier, Amsterdam, 355p., 2014.

[6] Pérez-Carreón, K., Martínez, L.M., Videa, M., Cruz-Angeles, J., Gómez, J., Ramírez, E., *Effect of Basic Amino Acids on Folic Acid Solubility*, Pharmaceutics, 2544, 15(11), 2023.

[7] Shulpekova, Y., Nechaev, V., Kardasheva, S., Sedova, A., Kurbatova, A., Bueverova, E., Kopylov, A., Malsagova, K., Dlamini, J.C., Ivashkin, V., *The Concept of Folic Acid in Health and Disease*, Molecules, 3731, 26(12), 2021.

[8] Singh, S.K., Singh, R., *Surface Plasmon Resonance, a Novel Technique for Sensing Cancer Biomarker: Folate Receptor and Nanoparticles Interface*., Cancer Biomarkers: Methods and Protocols, Springer US, 2413, 211–28, 2022.

[9] Alexander, C.M., Hamner, K.L., Maye, M.M., Dabrowiak, J.C., *Multifunctional DNA-gold nanoparticles for targeted doxorubicin delivery*, Bioconjugate Chemistry, 25(7), 1261–71, 2014.

[10] Figliola, C., Marchal, E., Groves, B.R., Thompson, A., *A step-wise synthetic approach is necessary to access γ-conjugates of folate: folate-conjugated prodigiosenes*, RSC Advances, 9(25), 14078–92, 2019.

[11] Atkinson, S.F., Bettinger, T., Seymour, L.W., Behr, J.-P., Ward, C.M., *Conjugation of Folate via Gelonin Carbohydrate Residues Retains Ribosomal-inactivating Properties of the Toxin and Permits Targeting to Folate Receptor Positive Cells*, Journal of Biological Chemistry, 276(30), 27930–5, 2001.

[12] Hermanson, Greg T. *Bioconjugate techniques*. Academic press, 3 ed., 1146p. 2013.

[13] Jordan, A., Whymark, K.D., Sydenham, J., Sneddon, H.F., *A solvent-reagent selection guide for Steglich-type esterification of carboxylic acids*, Green Chemistry, 23(17), 6405–13, 2021.

[14] Grumezescu, Alexandru Mihai, ed. *Organic materials as smart nanocarriers for drug delivery*. William Andrew, 738p. 2018.

[15] Clark, T., Murray, J.S., Lane, P., Politzer, P., *Why are dimethyl sulfoxide and dimethyl sulfone such good solvents?*, Journal of Molecular Modeling, 14(8), 689–97, 2008.

[16] Ha, N.T.N., Gopakumar, T.G., Yen, N.D.C., Mende, C., Smykalla, L., Schlesinger, M., Buschbeck, R., Rüffer, T., Lang, H., Mehring, M., Hietschold, M., *Ester formation at the liquid–solid interface*, Beilstein Journal of Nanotechnology, 8, 2139–50, 2017.

[17] Parekh, V. J., V. K. Rathod, and A. B. Pandit. *Substrate Hydrolysis: Methods, Mechanism, and Industrial Applications*. Academic Press, 2 ed., 655p. 2011.

[18] Wang, R., Chen, C., Yang, W., Zhou, P., Zhu, F., Xu, H., Hu, G., Sun, W., Shen, W., Hu, Y., *Solubility determination and thermodynamic characterization of orotic acid in twelve pure solvents and four binary mixed solvents*, Journal of Molecular Liquids, 341, 117335, 2021.

[19] Yang, N., Dichloromethane. In: Wexler, P., (Ed.), *Encyclopedia of Toxicology Third Edition*, Academic Press, 3 ed., 5220p. 2005.

[20] Klykov, O., Weller, M.G., *Quantification of N-hydroxysuccinimide and N-hydroxysulfosuccinimide by hydrophilic interaction chromatography (HILIC)*, Anal. Methods, 7(15), 6443–8, 2015.

[21] Pottorf, R.S., Szeto, P., Srinivasarao, M., *1-Ethyl-3-(3'-dimethylaminopropyl)carbodiimide Hydrochloride, Encyclopedia of Reagents for Organic Synthesis*, John Wiley & Sons, Ltd, 1–5, 2017.

[22] Wang, R., Chen, C., Yang, W., Zhou, P., Zhu, F., Xu, H., Hu, G., Sun, W., Shen, W., Hu, Y., *Solubility determination and thermodynamic characterization of orotic acid in twelve pure solvents and four binary mixed solvents*, Journal of Molecular Liquids, 341, 117335, 2021.

[23] Nam, C.T., Yang, W.-D., Duc, L.M*., Solvothermal Synthesis of TiO2 Photocatalysts in Ketone Solvents with Low Boiling Points*, Journal of Nanomaterials, 2013(1), 627385, 2013.

[24] Wilkinson, T.J., *Recycling Solvent Mixtures of Ethyl Acetate and Hexanes*, Journal of Chemical Education, 75(12), 1640, 1998.

[25] Oluwasanmi, A., Lindsay, S., Curtis, A., Perrie, Y., Hoskins, C., *Chain length impact on the retro Diels-Alder mediated release of gemcitabine from hybrid nanoparticles towards pancreatic cancer therapy*, International Journal of Pharmaceutics, 644, 123304, 2023.

[26] Becker, E.D., *High Resolution NMR Third Edition*, *Chapter 4-Chemical Shifts,* Academic Press, 3 ed., 441p. 2000.

[27] Lee, Y., Matviychuk, Y., Holland, D.J., *Quantitative analysis using external standards with a benchtop NMR spectrometer*, Journal of Magnetic Resonance, 320, 106826, 2020.

[28] Pauli, G.F., Jaki, B.U., Lankin, D.C., *A routine experimental protocol for qHNMR illustrated with taxol*, Journal of Natural Products, 70(4), 589–95, 2007.

[29] Pinciroli, V., Biancardi, R., Colombo, N., Colombo, M., Rizzo, V., *Characterization of small combinatorial chemistry libraries by 1H NMR. Quantitation with a convenient and novel internal standard*, Journal of Combinatorial Chemistry, 3(5), 434–40, 2001.

[30] Bai, M.-Y., Tang, S.-L., Chuang, M.-H., Wang, T.-Y., Hong, P., *Evaluation of chitosan derivative microparticles encapsulating superparamagnetic iron oxide and doxorubicin as a pH-sensitive delivery carrier in hepatic carcinoma treatment: an in vitro comparison study*, Frontiers in Pharmacology, 9, 1025, 2018.

[31] Castillo, J.J., Torres, M.H., Molina, D.R., Castillo-León, J., Svendsen, W.E., Escobar, P., Martínez, F., *Monitoring the functionalization of single-walled carbon nanotubes with chitosan and folic acid by two-dimensional diffusion-ordered NMR spectroscopy*, Carbon, 50(8), 2691–7, 2012.

[32] Motsch, S., Tremmel, P., Richert, C., *Regioselective formation of RNA strands in the absence of magnesium ions*, Nucleic Acids Research, 48(3), 1097–107, 2020.

[33] Budavari, S., O'Neil, M.J., Smith, A., Heckelman, P.E., *The merck index. vol. 11*, Merck & Company Incorporated, 938p. 1989.

[34] Bai, M.-Y., Tang, S.-L., Chuang, M.-H., Wang, T.-Y., Hong, P., *Evaluation of chitosan derivative microparticles encapsulating superparamagnetic iron oxide and doxorubicin as a pH-sensitive delivery carrier in hepatic carcinoma treatment: an in vitro comparison study*, Frontiers in Pharmacology, 9, 1025, 2018.

[35] Zu, Y., Zhao, Q., Zhao, X., Zu, S., Meng, L., *Process optimization for the preparation of oligomycin-loaded folate-conjugated chitosan nanoparticles as a tumor-targeted drug delivery system using a two-level factorial design method*, International Journal of Nanomedicine, 3429–41, 2011.

[36] Jain, S., Rathi, V. V., Jain, A.K., Das, M., Godugu, C., *Folate-decorated PLGA nanoparticles as a rationally designed vehicle for the oral delivery of insulin*, Nanomedicine, 7(9), 1311–37, 2012.