IDENTIFYING CHARACTERISTIC PROPERTIES AND ANTIOXIDANT EFFECT OF QUERCETIN LOADED TITANIUM DIOXIDE NANOPARTICLES

by
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ABSTRACT

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Molecular Biology, Genetics and Bioengineering, M. Sc. Thesis, June 2021

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Keywords: Quercetin, TiO_2 nanoparticles, antioxidant activity, DLS analysis

Quercetin (Que) is strong antioxidant but its poor solubility limits its applications. Que could be designed at the nanoscale level and using nanoparticles for this purpose brings improved solubility, drug release and bioavailability. In this study, we aimed to determine characteristic properties of quercetin-loaded-PEGylated titanium dioxide nanoparticles (Q-PEGTiO_2) and their biological effects. Firstly, TiO_2 NPs were modified with polyethylene glycol to increase biocompatibility. Next, SEM and DLS were used to characterize Q-PEGTiO_2 and results showed that bare TiO_2 NPs tend to agglomerate and have larger size in water. Additionally, cellular viability of cells was analyzed after the treatments and we observed that while Q-PEGTiO_2 showed lower toxicity, Que-treated cells lost their viability after 24 and 48 hour of treatment. Thereafter, cellular uptake of Q-PEGTiO2 and Que were detected by laser scanning confocal microscope and it was observed that they localized mainly in the cytoplasmic part. Penetration efficiency was determined by colocalization analysis and it was observed that the nanosystem reached its maximum levels at 12^{th} hour. Lastly, DPPH radical scavenging assay was used to see antioxidant effect of the nanosystem and we observed that Q-PEGTiO_2 has increasing scavenging activity over time. Here, we elucidated physicochemical identity of Q-PEGTiO_2 with its low toxicity and higher antioxidant effect.

ÖZET

QUERCETIN YÜKLÜ TİTANYUM DİOKSİT NANOPARTİKÜLLERİN KARAKTERİSTİK ÖZELLİKLERİNİN VE ANTİOKSİDAN ETKİSİNİN TANIMLANMASI

AYŞE NUR CIN

Moleküler Biyoloji, Genetik ve Biyomühendislik, Yüksek Lisans Tezi, Haziran 2021

Tez Danışmanı: Prof. Dr. Hüveyda Başağa

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Anahtar Kelimeler: Quercetin, TiO_2 nanopartiküller, antioksidan aktivite, DLS analizi

Quercetin (Que) güçlü bir antioksidan olarak bilinmesine rağmen hidrofobik doğası kullanım alanlarını sınırlamaktadır. Que'in nano-seviyede düzenlenmesi ve bu alanda nanopartiküllerin kullanımı arttırılmış çözünürlük, ilaç salınımı ve biyoyararlanım getirmektedir. Bu çalışmada, Que yüklü PEG-kaplı titanyum dioksit nanopartiküllerin (Q-PEGTiO_2) karakteristik özelliklerinin ve biyolojik fonksiyonlarının tanımlanması amaçlanmıştır. Öncelikle, TiO_2 NPs polietilen glikol (PEG) ile kaplanarak geliştirilmiş biyoyararlanım sağlanmıştır. Q-PEGTiO_2 sistemini karakterize etmek için SEM ve DLS teknikleri kullanılmış ve nanopartiküllerin sulu ortamda bir araya toplanma eğiliminde olduğu ve daha büyük boyutlara ulaştığı görülmüştür. Sonrasında, Q-PEGTiO_2 ve Que muamelesi sonrası hücre canlılığı incelendi ve Q-PEGTiO_2 grubunda düşük toksisite görülürken Que grubunda 24. ve 48. saatte hücre canlılığında büyük kayıplar gözlendi. Q-PEGTiO_2 ve Que'in hücreye girişi laser scanning confocal mikroskop ile incelendi ve bu yapıların hücre içinde çoğunlukla sitoplazmada yoğunlaştığı gözlendi. Colocalization analizi ile nano-antioksidanın 12. saatte hücre içinde en yüksek seviyeye ulaştığı görüldü. Son olarak, DPPH deneyi ile Q-PEGTiO_2'nin zamanla artan antioxidan aktivite gösterdiği görüldü. Bu çalışmada Q-PEGTiO_2'nin fizikokimyasal kimliğinin yanısıra düşük toksisitesini ve yüksek antioksidan özelliğini göstermiş olduk.

to my beloved nephews Emir and Eymen…

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LIST OF ABBREVATIONS

α	Alpha
β	Beta
μ	Micro
n	Nano
CO_2	Carbon dioxide
DAPI	4, 6-diamino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
H_2O	Water
HCl	Hydrochloric acid
NaCl	Sodium chloride
NP	Nanoparticle
PEG	Polyethylene glycol
PEG-TiO_2	PEGylated titanium dioxide nanoparticle
PBS	Phosphate-buffered saline
TiO_2	Titanium (IV) oxide
Q-TiO_2	Quercetin conjugated titanium dioxide NP
WST-1	Water Soluble Tetrazolium-1

1. INTRODUCTION

1.1. Flavonoids

The word ‘flavus’ means yellow in Latin and it is the origin of the word ‘flavonoid’. For this reason, yellow colored compounds with flavone nucleus were considered as flavonoids. However, nowadays the term flavonoid is used on a wide scale to include colorless (flavan-3-ol) and colored (flavanone) compounds [1].

Flavonoids and other phenolic compounds are synthesized by phenylpropanoid pathway that include phenylalanine as a starting compound [1]. Flavonoids could be found as aglycosides, glycosides, and methylated derivatives. The basic flavonoid structure is aglyconeis which contain six-carbon ring using α-pyrone or its dihydroderivative to condense with a benzene ring [2].

Flavonoids, defined as low-molecular-weight phenolic compounds, are produced by many plants as secondary metabolites. Since flavonoids have anticancer, antioxidant, anti-inflammatory and antimutagenic properties, they have many uses in pharmaceutical, cosmetic and medical fields. In addition to these important biological properties, polyphenolic compounds also have effects on important enzyme functions. For example, they have been shown to have inhibitory effect on xanthine oxidase (XO), cyclo-oxygenase (COX), and lipoxygenase.[3]

Plants produce flavonoids for development, pigmentation, protection against UV rays and defense against harmful microorganisms. [4] Flavonoids protect plants from biotic and abiotic stres conditions and acting as signal molecules. In addition to these, flavonoids may also function to protect the plant against climatic conditions such as drought or extreme cold [5]. Flavonoids are found in vegetables and fruits like apples, broccoli, onions and spinach; beverages of plant origin such as black tea, green tea and red wine [6].

1.2. Classification

Flavonoids could be presented as C6-C3-C6 chemical formula and named flavan nucleus as shown in Figure 1.1. Flavonoids are 2-phenyl-benzo-γ-pyrone derivative compounds and consist of many subgroups. This general flavonoid structure consists of two benzene rings, called A and B, and heterocyclic pyrane ring (C ring) that connects these rings [7]. B ring could be attached to the C ring at different positions on C ring. Binding through position 3 generates isoflavones while position 4 forms neoflavonoids. Binding via position 2 constitute various subgroups according to the properties of the C ring [3]. Flavonoids are divided into 6 subgroups according to their chemical structures especially extent of hydroxylation, saturation and oxidation of the C ring. These groups are flavanols, flavonols, flavanones, flavones, isoflavones and anthocyanins [8].

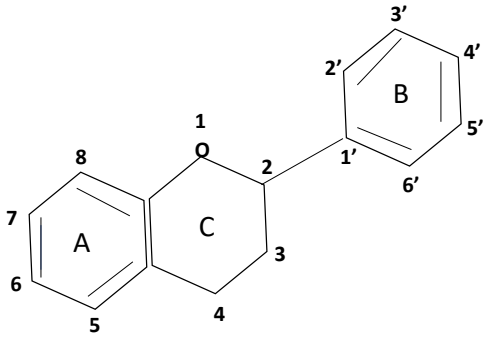


Figure 1. 1. Basic structure of flavonoids [3] (2-phenyl-benzo-γ-pyrone). Flavonoids contain two benzene rings which are named as A and B. Heterocyclic pyrane ring, C ring, is located between the A and B ring and connect them to produce flavonoid's chemical structure.

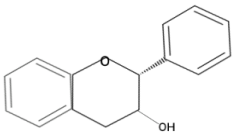
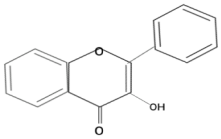
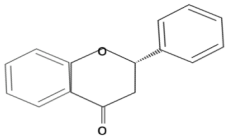
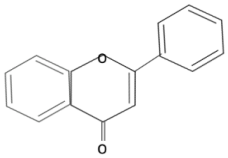
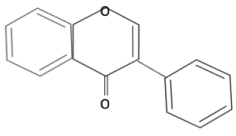
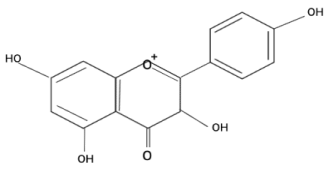
1.2.1. Flavanols

Flavanols are large and complex polyphenolic group containing many compounds ranged from monomeric flavan-3-ols (e.g. catechin, epicatechin, gallocatechin) to polymeric procyanidins. Flavanols are found especially in chocolate, cocoa, apples, tea, berries,

plum, peach, banana, grape and red wine. Flavanol content of vegetables and legumes other than lentil and broad beans is almost negligible. Seeds and peels of vegetables and fruits are rich in flavanol content but these parts are removed during eating or processing of foods. Removing flavanol rich parts of foods causes less dietary intake of flavanols [9].

Catechin, known as the major building blocks of tannins, are the most well-known members of the flavanol compounds. Green tea, back tea, red wine, chocolate, apples, and blackberry contain high amount of catechin [10]. Catethin shows radical scavenging activity by preventing protein oxidation and it also pretect proteins from covalent modification induced by ROS or oxidative stress related by-products [11].

Table 1.1. Classification of flavonoids and their basic structures [1 12]. Flavonoids are categorized into six different subgroups depending on their chemical structures and hydroxylation, saturation and oxidation level of the C ring. The groups have similar backbone with different functional groups which are located at specific positions.

Group of flavonoid	Structure backbone
Flavanols	
Flavonols	
Flavanones	
Flavones	
Isoflavones	
Anthocyanins	

1.2.2. Flavanones

Flavanones, that contain about 350 aglycones and 100 glycosylated forms identified up to know, are one of the well-known classes of flavonoids. Their structure consist of two aromatic rings (A and B) that are linked by a dihydropyrone ring (C). Hesperetin, naringenin, eriodictyol, isosakuranetin and taxifolin are the most common flavanones found in Citrus [13] [14]. Fresh fruits, Citrus juices made from blood oranges, grapefruits, sour orange, limes, mandarins, bergamots, kumquats, lemons, chinotto, tangors, and tangerins are known to have high flavanone content.

Citrus flavanones show both antioxidant and anti-inflammatory activities. Hesperidin and its aglycone form, hesperetin, catch the attention due to their important role in prevention of oxidative stress and inflammation related diseases, such as cardiovascular diseases and cancer. Number and organization of OH groups on the flavanone structure affect the antioxidant properties of these flavonoids [15] [16]. In addition, aglycone forms, such as hesperitin, show higher quenching activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals when compared to glycosylated forms, hesperidin and neohesperidin [17] [18]. Decreased number of hydroxyl groups causes reduction in antioxidant potential of flavanones and this decrease emphasize the direct role of hydroxyl groups in the radical scavenging reaction [19] [20]. Although it is not clear yet, some studies suggest that flavanones have anticancer properties by inhibiting the proliferation of cancer cells by inducing apoptosis [21].

1.2.3. Flavones

Flavones are originated from benzopyran class which create an important group of oxygen heterocycles. Flavones and flavonols share very similar structure but flavones have less OH group at the carbon-3 position. Flavones are presented by $C_{15}H_{10}O_2$ molecular formula. In this structure, they contain three different functional groups: hydroxyl, carbonyl, and conjugated double bond. Therefore they take part in reactions of

these three functional groups. Water and ethanol could be used as a solvent for flavones [12].

Flavones containing several substitution patterns are isolated from leafly vegetables and herbs. While protecting plants from diseases as secondary metabolite, flavones show certain useful biological activities such as anti-inflammatory, antioxidant, antitumor, antimicrobial and cytotoxic activities [22].

Flavones carry both lipophilic (non-polar) and hydrophilic (polar) groups so they have the ability to interact with membrane lipid bilayer. This intereraction brings some changes in terms of the rate of oxidation of membrane lipids and proteins. In addition to this, when polyphenols interact with membrane through hydrogen bonds, it could reduces access of deleterious molecules to lipid bilayer, thereby protect the structure and fuction of lipid membranes [23].

Polyphenolic flavones could also show antioxidant properties by free radical scavenging activity, chelation of oxidizing metal ions, inhibition of enzymes such as xanthine oxidase, and reducing free radicals by means of their lower redox potential [24] [25] [26].
Apigenin, luteolin and tricetin are the most known flavones found in vegetables and fruits. While broccoli, carrots, celery, onion leaves, parsley, peppers, cabbages, and apple skin mostly contain luteolin; onions, wheat sprouts, tea and oranges have higher amount of apigenin and tricetin [12].

Table 1.2. Bioactivities of flavonoids. Flavonoids, defined as low-molecular-weight phenolic compounds, are produced by many plants as secondary metabolites and they are known with their health-promoting effects.

BIOACTIVITIES OF FLAVONOIDS	
Anti-mutagenic	Inhibition of NF-kB
Antioxidant	Scavenging free radicals
BChE inhibition	Enzyme inhibition
AChE inhibition	Lipoxygenase inhibition
Anti-microbial	Xanthine oxidase inhibition
Insulin receptor activation	Aldose reductase inhibition
Anti-carcinogenic	Anti-inflammatory

1.2.4. Isoflavones

Nonsteoridal phenolic compounds phytoestrogens are classified into two main groups: flavonoids and non-flavonoids. While flavonoids include isoflavones, coumestans and prenylflavonoids; non-flavonoids contain lignans. Isoflavone structure shows 3-phenylchromen skeleton which is originated from 2-phenylchromen by an aryl-migration mechanism.

Legumes, especially soy, are known with their high amount of isoflavone content. This type of flavonoid also found in green split peas, chickpeas, split peas, lima beans, black beans, sunflower seeds, and clover sprouts.

Genistein and daidzein are the most preferred isoflavones included in human diet. They can be exist in four different structures; aglycones, the 7-O-glucosides, the 6'-O-malonylglucosides, and the 6'-O-acetylglucosides [27].

Hormonal disorders such as many cancer types, cradiovascular diseases, osteoporosis, and menopausal symptoms can be cured by isoflavones [28]. In addition to this, isoflavones have lipid lowering effects. They also interact with estrogenreceptors-beta which take part in central nervous and cardiovascular systems. Soy isoflavones are considered as towardly dietary supplements to prevent brast cancer. Isoflavones bind estrogen receptors

and depending on the estrogen environment they show estrogen agonists or antagonists effect. Accordingly, studies demonstrate that breast cancer risk can be reduced by high intake of soy isoflavones [29] [30].

Genistein, is another type of isoflavone, have direct effect on osteoblasts and show anabolic effect so prevent bone loss [31] In addition to this, genistein consuming is related to decreased waist circumference, weight, and total body fat mass in postmenopausal women [32].

1.2.5. Anthocyanidins/Anthocyanins

Anthocyanidins are natural pigments found in many vegetables and fruits and give blue, red, purple and orange colors to plants [33]. Honey, teas, nuts, cocoa, cereals, berries, red wine and olive oil are plants which are known to have higher anthocyanidins content [34]. Anthocyanidins are found as aglycone form that is based upon 2-phenylbenzopyrilium or flavylium cation containing hydroxyl and methoxyl groups at different positions [35].

Glycosylated polyhydroxy and polymethoxy structures of flavilium salts are known as anthocyanins. In plants, stress factors such as UV light, drought, and cold temperatures induce the production of anthocyanins as a protective mechanism [36].

Cyanidin, pelargonidin, delphinidin, malvidin, petunidin and peonidin are main types of anthocyanidins widely distributed in fruits and vegetables. They are differ from each other according to the number and position of hydroxyl and methoxyl groups in their structures.

It is reported that anthocyanidins/anthocyanins have an important role in cardiovascular disease, visual acuity, cholesterol decomposition, antioxidant activity, and cytotoxicity. Edible berry anthocyanins have therapeutic and anti-carcinogenic properties. While anthocyanins in berries provide pigmentation to fruits, they also serve as natural antioxidants. Genomic DNA integrity is repaired and protected by anthocyanins. Previous studies have indicated that berry anthocyanins take part in reducing age-related oxidative stress, improving neuronal and cognitive brain function [37].

1.2.6. Flavonols

Flavonols (3-hydroxyflavones) have been the subject of many studies due to their antioxidant effects. Although the mechanism is not fully understood, they may show antioxidant effect by (i) acting as radical scavenger; (ii) chelating metal ions; (iii) converting α-tocopheroxyl radical to α-tocopherol. The most known members of this group of flavonoids are quercetin (Que), kaempferol and myricetin. Glycosides having many forms are the main flavonol compound found in plants [38]. Flavonol glycosides are found in higher concentrations in some parts of plants such as leaves, flowers and skin [39]. The richest sources in terms of flavonols are grapes, tomatoes, onion, broccoli, green tea, black tea and red wine [40 41].

Basically, flavonols can be grouped based on double-bond position on the C-ring between the 2 and 3 carbon atom, hydroxyl group in the 3-position and carbonyl group in the 4-position. Flavonols are generally found in the form of mono-, di-, and triglycosides in nature. The most widespread sugars attached to flavonols are glucose, galactose, rhamnose, arabinose, apiose, and glucuronic acid. In addition to sugars, phenolic acid residues, such as gallic acid, frequently acylate flavonol structures [42] .

Table 1.3. Main flavonoid classes, subclasses and natural sources. Six main flavonoid groups and their subclasses with natural sources are presented. Flavonoids show wide distrubition among vegetables and fruits as mentioned below.

Flavonoid classes	Flavonoid subclasses	Natural sources
Flavanols	Catechin, Epicatechin, Gallocatechin, Procyanidin	Chocolate, Cocoa, Apples, Tea, Berries, Plum, Peach, Banana, Grape, Red wine
Flavanones	Hesperitin, Naringin, Naringenin, Eriodictyol, Hesperidin	Citrus juices, Grapefruits, Limes, Mandarins, Bergamots, Lemons
Flavones	Apigenin, Tangeretin, Baicalein, Rpoifolin	Broccoli, Carrots, Celery, Onion leaves, Parsley, Peppers, Cabbages
Isoflavones	Genistin, Genistein, Daidzein, Glycitein, Daidzin	Legumes, Green split peas, Chickpeas, Black beans, Sunflower seeds
Anthocyanin	Cyanidin, Malvidin, Delphinidin, Peonidin	Honey, Tea, Nuts, Cocoa, Cereals, Berries, Olive oil, Red wine
Flavonols	Quercetin, Myricetin, Rutin, Kaempferol, Morin	Grapes, Tomatoes, Onion, Broccoli, Green tea, Black tea, Red wine

1.3. Quercetin (Que)

Quercetin (Que), an important subclass of flavonoids widely distributed in vegetables and fruits. This polyphenolic compound possesses beneficial biological properties that help to improve mental and physical performance and reduce the risk of development of diseases such as aging, cardiovascular and neurodegenerative diseases and cancer [43]. The well-known flavonoid quercetin has anti-carcinogenic, anti-viral, anti-inflammatory, antioxidant, and psychostimulant properties additionally the ability to inhibit lipid peroxidation, capillary permeability and platelet aggregation, and to stimulate mitochondrial biogenesis [44]. Due to its useful characteristics, quercetin catches the attention of many studies.

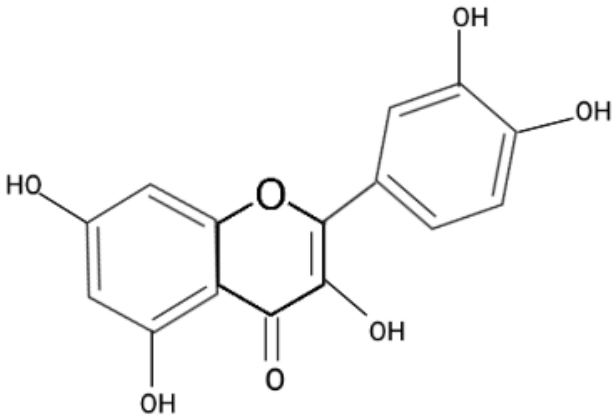


Figure 1. 2. Chemical structure of Que flavonol. Quercetin, as a well known flavonoid, is classified as flavonol and carries five hydroxyl groups which are responsible for the major biological activities of Que. The number and location of these functional groups make Que quite effective antioxidant.

1.3.1. Physicochemical properties of Que

Que is classified as a flavonol, one of the important subgroup of flavonoid polyphenols. The IUPAC (International Union of Pure and Applied Chemistry) nomenclature of quercetin is 3, 3', 4', 5, 7-pentahydroxyflavone (or 3, 3', 4', 5, 7-pentahydroxy-2-phenylchromen-4-one). This means that hydroxyl groups are located at 3, 5, 7, 3', and 4' positions on quercetin.

The name quercetin ($C_{15}H_{10}O_7$) represents the sugar-unbound form known as aglycone. It has brilliant yellow color and it is insoluble in cold water, poorly soluble in hot water, however quite soluble in lipids and alcohol. When a glycosyl group (sugars such as rutinose, rhamnose, or glucose) attached to Que, it becomes Que glycoside. Glycosyl groups take the place of one OH group which is generally located in position 3. Que glycosides show different absorption, solubility, and in vivo effects than aglycone forms [45].

1.3.2. Food sources of Que

Que is the most common flavonol in human diet. It is found in onions, apples, capers, grapes, berries, shallots, tea, tomatoes, and many seeds, nuts, and flowers. Medicinal botanicals such as *Ginkgo biloba*, *Sambucus canadensis*, and *Hypericum perforatum* also have Que glycosides. Onions are known as one of the compounds with the highest Que content [46] [47]. In onions, the parts closest to the root and outermost rings contain much more Que than other sections [48]. In addition to vegetables and fruits, certain types of honey from different plant sources contain Que, as well [49]. Among the defiend foods, caper (raw) has the highest quercetin content with 234 mg/ 100 g, while green and black tea have the lowest Que content with 2 mg/ 100 g [50].

1.3.3. Pharmacokinetics of Que

Que is known with its very low oral bioavailability after taking a single dose. It is estimated that, when 100 mg Que is consumed by healthy individuals, the absorption ratio is ranges from 3% to 17%. The relatively poor bioavailability of Que may be because of its low absorption, extensive metabolism and rapid elimination.

Que is mostly taken in the form of glycosides and glycosyl group is released by chewing, digestion, and absorption. Subsequently, by means of β-glycosidase enzymes glycoside forms are converted into aglycones in the intestine then absorbed into enterocytes. It is suggested that oral and intestinal bacteria take part in this enzymatic hydrolysis. Since Que is lipophilic, it can theoretically cross the intestinal membrane by passive diffusion [51] [52]. Attached sugar type might affect the absorption degree of Que glycosides [53]. Previous studies indicate that Que glycosides are better absorbed than its rutinosides [54]. Morover, this absorption level depends on parameters such as glycosylation type, the food matrix which Que is found, and the co-administration of other foods such as fat and fiber [55]. Que glycosides have ability to interact with sodium dependent glucose transport receptors located in the mucosal epithelium and this interaction may provide absorption by the small intestine [53].

After absorption, Que is metabolized by a variety of organs including the small intestine, liver, colon, and kidney. Methylated, glucuronidated and sulfo-substituted forms of Que metabolites are formed in the smal intestine and liver [56]. First and second phases of

metabolism occur in the liver and metabolic products enter the bloodstream for distribution into tissues. A study carried out on pig and rat shows that Que and its metabolites accumulate in rat lung and pig liver and kidney at the highest concentrations [57].

Previous studies indicate that Que and its metabolites mostly accumulate in organs which take place in its metabolism and excretion, and mitochondria might be the organelle for its accumulation. After absorption and metabolization of Que, it is removed from the body by means of kidney or lungs [58]. Que shows considerably short half-life and quick clearance in the blood. It is observed in the plasma 30 min after ingestion, however appreciable amounts are excreted over 24 h [59].

1.3.4. Antioxidant properties of Que

Polyphenolic flavonoids contain at least one hydroxyl group linked to benzene ring, which can interact with free radicals, for this reason used as a radical scavenger and show antioxidant properties [60]. These antioxidant molecules found in several medicinal plants, vegetables, fruits, and beverages. Que, which is classified as flavonol, is widely distributed among foods including vegetables, tea, wine and traditional Chinese medicine such as Forsythia [61].

Que takes attention especially for its antioxidant activity. It possesses five hydroxyl groups located at 3, 5, 7, 3', and 4'. Some hydroxyl groups are glycosylated to produce different Que derivatives [62]. Many studies have been conducted to reveal antioxidant and anti-inflammatory activities of Que and its derivatives. As a result, it is found that modification of Que causes remission in antioxidant capacity. The total activity is listed as follows: quercetin > tamarixetin=isorhamnetin > quercetin-3-O-glucuronide > isorhamnetin-3-O-glucoside > quercetin-3,5,7,3',4'-pentamethylether > quercetin-3,4'-di-glucoside, indicating that 3-hydroxyl group in quercetin basic skeleton has an important role in antioxidant activity [63].

It has been showed that Que is a splendid *in vitro* antioxidant. As a flavonoid, it shows efficient scavenging activity against ROS (reactive oxygen species), including $O_2^{\cdot -}$ and

RNS (reactive nitrogen species) such as $NO^{\cdot}$ and $ONOO^{-}$ [64] . These strong antioxidant properties come from two antioxidant pharmacophores within the structure which are optimally configured for free radical scavenging. For instance, the catechol group included by B ring and the OH group at position 3 in the A-C ring are important groups for radical scavenging. In addition to this, Que also increases the endogenous antioxidant protection by contributing to the total plasma antioxidant capacity [65].

Que has ability to prevent the spread of certain type of cancers such as lung, prostate, liver, breast, colon, and cervical cancers by modulating antioxidant ezymes and oxidative stress factors [66] [67]. Previous studies examined the antioxidant effect of Que in rats by comparing oxidative stress markers, such as lipid peroxidation, reduced glutathione, and hydrogen peroxide. It was showed that Que-treated rats had lower levels of lipid peroxidation and hydrogen peroxide but higher level of reduced glutathione. Furthermore, apoptosis proteins and antioxidant enzymes are increased by Que. Likewise, expression of some factors increased in cancer such as androgen receptors, protein kinase B, insulin-like growth factor receptor 1, and cell proliferation and anti-apoptotic proteins are regulated by this polyphenolic compound [66] [67]. Catalase and superoxide dismutase levels are increased by Que to protect the heart from oxidative stress so oxidative injury was prevented [68].

Basically, the flavonol Que performs antioxidant activity through its effect on glutathione (GSH), signal transduction pathways, enzymatic activity, and ROS caused by many factors such as environmental and toxicological factors. Que takes place in regulation of GSH levels. When free radicals are produced in the body, superoxide dismutase (SOD) converts O^{2-} into H_2O_2. After this, H_2O_2 is transformed into the non-toxic H_2O under favour of SOD. The reaction occuring to convert free radicals into non-toxic compounds requires GSH as a hydrogen donor. Studies carried with animals and cells showed that Que induces GSH synthesis [69] [70].

The hydroxyl groups located on the phenyl ring can interact with amino acid residues in active sites of two enzymes. When this binding occurs, Que inhibits the activity of acetylcholinesterase and butyrylcholinesterase which are associated with oxidative effcets [71].

Que has ability to activate/inhibite and upregulate/downregulate several molecules and ezymes which show important functions in many signaling pathways. Studies indicate that Que carries out its protective property against acute spinal cord injury through its inhibitory effect on p38MAPK/ iNOS signaling pathway, the upregulation of SOD activity, and the downregulation of malondialdehyde levels to increase antioxidant activity [72].

It is known that human skin is the largest organ of the body and withstand several types of environmental insults. Nevertheless, when human skin exposed to UVB, it increases ROS levels and ruins endogenous antioxidant systems. It was showed that Que decreases the effect of UVB-induced radiation damage by removing ROS and protect the mitochondrion and cell membrane against ROS-induced damage [73]. Ionizing radiation also causes cell damage and death because of increased level of free radicals in cells. Que can scavenge free radicals and increase endogenous antioxidant levels and protect cells from genetic toxicity. Bioflavonoids have ability to reduce radicals by donating hydrogen to them, therefore they inhibit free radical toxicity and increase antioxidant properties [74].

It is known that redox homeostasis is crucial issue for living tissues. Human body needs antioxidants to maintain redox balance. Que is a well known strong antioxidant and can be used in medicinal field to protect cells from oxidative stress. Basically, it performs its antioxidant activity through different mechanisms as shown in Figure 1.3 [75].

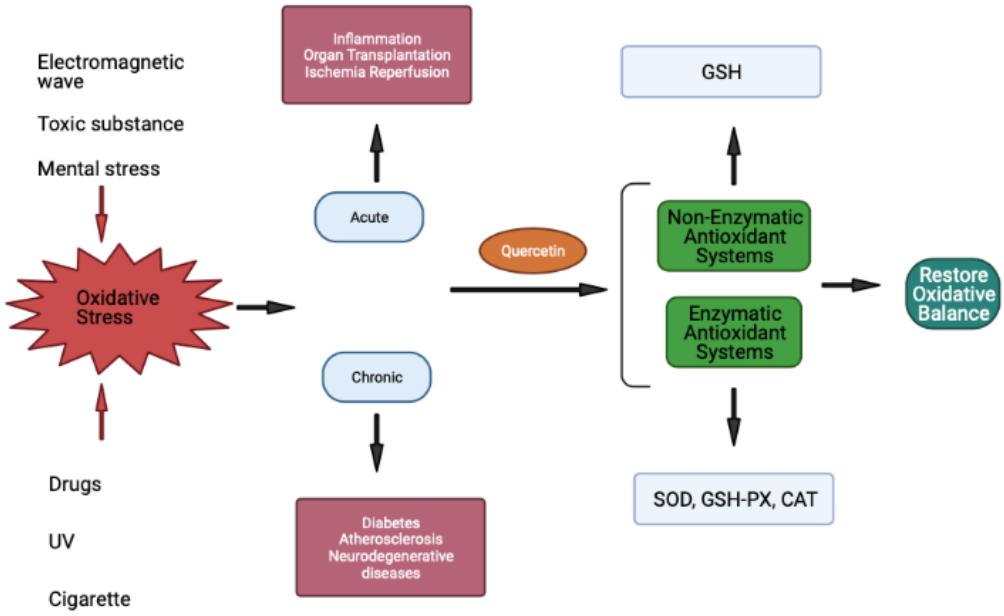


Figure 1. 3. Antioxidant effects of Que [75]. Environmental factors such as electromagnetic waves, drugs and cigarette come with disrupted redox homeostasis and oxidative stress. In this case, Que could restore oxidative balance in two ways: enzymatic antioxidant systems or non-enzymatic antioxidant systems.

Because of its poor permeability, bioavailability (5.3%), solubility, and stability it has limited application in the pharmaceutical industry. For this reason many studies have being carried out to modify its structure to enhance its bioavailability and water solubility and as a result increase its antioxidant activity [76]. Que can be included in some formulation techniques such as Que-loaded nanoparticles, Que-loaded gel, and Que-loaded polymeric micelle [75].

1.4. Free Radicals and Reactive Species

Atoms and molecules usually contain electrons in pairs which are found within defined an atomic or molecular orbital. A free radical term known as any species having the ability of independent existence and contains one or more unpaired electrons which are found alone in an orbital. If unpaired elecron is located on oxygen, it is called as oxygen-centered redicals, such as superoxide ($O_2\bullet^-$) and hydroxyl (OH•). While trichloromethyl ($CCl_3\bullet$) is an example of carbon-centered radical, thiyl radicals (RS•) are known as

sulfur-centered radicals. The unpaired electron in nitric oxide (NO•) radical is delocalized between nitrogen and oxygen atom. A dot used at the end of the atoms always represents free radicals. Biomoleculues are generally nonradicals, but when a radical species interact with them, this reaction produces a new free radical. For instance, hydrocarbons included by fatty acid side chains can react with OH• and then H• (hydrogen atom) is abstracted from hydrocarbons which generates a carbon-centered radical.

Figure 1. 4. The reaction of hydroxyl radical. Hydroxyl radical can reacts with hydrocarbons, which are contained by fatty acid side chains, and generates carbon-centered radicals. Radical chain reactions continue and causes cellular damage.

The reaction, which is showed in Figure 1.4, initiates the free radical chain reaction of lipid peroxidation. Carbon-centered radicals react with oxygen and produce peroxyl radicals. Adjacent fatty acid side chains and membrane proteins are the main targets of peroxyl radicals. Peroxyl radicals can oxidize many fatty acid side chains and produce lipid hydroperoxides [77].

ROS is a collective term that covers both oxygen-centered radicals (e.g. $O_2\bullet^-$ and OH•) and nonradical derivatives of oxygen such as hydrogen peroxide (H_2O_2) and singlet oxygen. These nonradical derivatives have the ability to oxidize biomolecules and/or they easily become radicals. Similarly, RNS contain radicals and and nonradical reactive molecules such as HNO_2 and N_2O_3 [77].

Free radicals and reactive species are formed in the human body to take part in vital processes such as inflammation reactions and neurotransmission, or they are produced as a byproduct that do not show any important role in the human body. During oxidative phosphorylation, most of the oxygen binds to hydrogen and they become reduced. In this process, a little amount of oxygen (1-3%) is not fully reduced and they are collectively called as ROS. Singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), ozone (O_3) and hypochlorous acid (HOCl) are the main ROS which have biological importance [78].

It is known that not all ROS and RNS show the same reactivity. While H_2O_2, $O_2^{\cdot -}$ and $NO^{\cdot}$ react with a few moelcules, $^{\cdot}OH$ reacts with almost all the molecules it encounters [79]. In addition to this property, reactive species have different site of reactivity. In general, free radical react with their target at the site of their formation, however non-radical species such as H_2O_2 can cross biological membranes and show their toxic effect even in the distant areas [61].

Within cells, many enzymatic pahways produce ROS, generally superoxide in the beginning. One-electron reduction of molecular oxygen generates superoxide which is converted to hydrogen peroxide by mitochondrial superoxide dismutase [80]. Hydrogen peroxide has poor reactivity but it has a role in Fenton reaction to produce hydroxyl radicals which are very harmful for cells. The electron transport chain included by oxidative phosphorylation is a major source of superoxide production. When electrons are leaked from electron transport complexes, they reduce oxygen and generate superoxide. Other reactions in mitochondria can be the site of superoxide production. Cytochrome P450 and α-ketoglutarate dehydrogenase are implicated in ROS production [81]. Reactions of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), xanthine oxidoreductase, and myeloperoxidase can produce ROS as well [82].

The presence of ROS in the human body is not completely harmful situation. ROS are important factor in redox homeostasis and it provides proper function in the immune and cardiovascular system. If ROS reach higher levels and the body cannot handle it, oxidative stress takes place. On the other part, if there are low levels of ROS within cell, reductive stress occurs and it can cause certain diseases such as cancer and cardiomyopathy [83]. ROS play important roles in particular signaling pathways. They can show their effects on signaling processes by reacting with cysteine residues of amino acids. Cell proliferation and apoptosis need ROS for proper functioning. MAPK signaling can be protected from inhibitory effect of phosphatases by ROS which causes cysteine oxidation on these enzymes. ROS inhibit tyrosine phosphatase by oxidizing it and provide suitable levels of growh factors. As an important role of ROS in antioxidant mechanism, ROS products are capable of activating antioxidant genes through certain mechanisms such as PI3K-NFE2-like2 (Nrf2)-antioxidant response element (ARE). Redox factor-1 (Ref-1) is an endonuclease and is regulated by p53, activator protein 1 (AP-1), nuclear

factor kappa B (NFkB), and hypoxia inducible factor 1. When cytoplasmic Ref-1 encounters with ROS, it travels to the nucleus and initiates antioxidant defense system. ROS play important roles in immune system as well. Macrophages, dendritic cells, and neutrophils are the main cells types in innate immunity. When they phagocytose foreign material, NADPH oxidases are triggered to produce ROS which will destroy the foreign entity [84]. In summary, ROS are generated during metabolism and included in oxidative phosphorylation, enzymatic reactions, activation of nuclear transcription factors, signal transduction, gene expression, and antimicrobial action in innate immunity [82].

1.5. Oxidative Stress and Antioxidant Mechanisms

Oxidative stress occurs when the balance between oxidants and antioxidants is disrupted and the level of oxidants exceeds antioxidants. This stress condition disrupts redox signaling and causes molecular damage [85]. Insufficient amount of antioxidants or overproduced oxidants may cause oxidative stress.

The human body contains antioxidant defence system against free radicals, ROS and other reactive species to protect cellular components from being oxidized. If a substance delays or prevents oxidation significantly, even at low concentrations, it is referred to as an antioxidant [86]. Antioxidants which react directly with reactive species are divided into two groups: enzymatic and non-enzymatic antioxidants. Enzymes such as catalase, superoxide dismutase and glutathione peroxidases are capable of reacting with reactive species, acting like catalyst and then they are recycled efficiently [87]. On the other hand, non-enzymatic antioxidants are sorted as hydrophilic and hydrophobic antioxidants as indicated in Table 1.4. Vitamin E (α-tocopherol), ubiquinol-10, and caretenoids are examples of hyfrophobic antioxidants which are usually found in membranes and lipoproteins. Glutathione, uric acid and ascorbate which are hydrophilic antioxidants are mainly located in mitochondria, cytosol and nuclear compartments [88]. These non-enzymatic antioxidants act as radical scavenger and donate an electron or proton to a radical to make it relatively stable compound when itself become oxidized. Only reduced form of antioxidants can perform scavenging activity, therefore oxidized form has to be converted into reduced status. The body contains antioxidant networks that reduce each other and maintain reduced form of antioxidants [89].

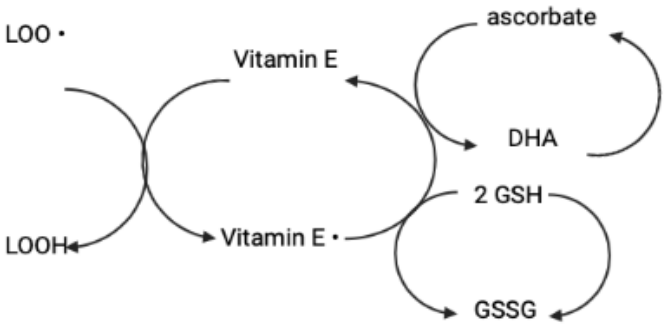


Figure 1. 5. Non-enzymatic antioxidant network. Lipid peroxyl radical (LOO•) is produced during lipid peroxidation. Vitamin E act as radical scavenger to reduce lipid peroxyl radical. Ascorbate and GSH are used in this network to regain reduced form of antioxidants as well [61].

Table 1. 4. Endogenous enzymatic and non-enzymatic antioxidants [61]. Antioxidant mechanisms consist of enzymatic and non-enzymatic antioxidants. SOD, GPx and Catalases are the major enzymes which take part in antioxidant defence. On the other hand, non-enzymatic antioxidants could be hyrophilic or hydrophobic.

Enzymatic Antioxidants	**Reaction**
Superoxide dismutases (SOD)	$2O^{\bullet -} + 2H^{+} \rightarrow H_2O_2 + O_2$
Catalases	$2H_2O_2 \rightarrow O_2 + 2H_2O$
Glutathione peroxidases (GPx)	$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$
Non-enzymatic, hydrophilic antioxidants	**Non-enzymatic, hydrophobic antioxidants**
Uric acid	α-Tocopherol (vitamin E)
Ascorbate (vitamin C)	Ubiquinol-10
Glutathione (GSH)	Caretenoids

Oxidative stress can be produced by exogenous factors, such as tobacco smoking, and endogenous factors including oxidative burst from activated macrophages [90]. Unhealthy lifestyle such as alcohol consumption, tobacco smoking, and medical drug abuse, as well as environmental pollutants including pesticides, xenoestrogens, and heavy metals increase ROS levels within cells. Overproduced ROS causes oxidative stress which brings many disorders. Abnormal ROS levels disrupt the structure of biomolecules, the

normal functioning of mitochondrial membranes and induce mitochondrial dysfunciton, and apoptosis [91]. Ruined mitochondria generate high amount of ROS, especially the superoxide anion and hydrogen peroxide, which activate mitochondrial signaling pathways and induce mitochondria-driven ROS propagation [92].

In a cell, ROS can be generated in mitochondria, cytosol, and extracellular spaces. Medium level of ROS take part in inflammatory response, activate mitogen-activated protein kinase (MAPK) and proinflammatory cytokines. However, excessive ROS levels are hazardous for cells because they may lead to apoptosis by activating apoptosome protein complex [93]. Certain enzymes may be the reason of overproduction of ROS. For instance, myeloperoxidase forms hypochlorous acid (HOCl) from hydrogen peroxide (H_2O_2) during an immune response. Oxidative stress, apoptosis, and inflammatory diseases may occur if HOCl is overproduced by myeloperoxidases [94].

Excessive amount of ROS comes with many disorders. All biomolecules including DNA, RNA, lipids, proteins, and carbohydrates inside the body are potantial target of ROS and RNS. A hydrogen atom is subtracted from these molecules and when they become oxidized, unpaired electron on ROS is converted into a more stable electron-pair. Instead of hydrogen atom, an electron can be transferred to reactive species. Because of this effect, reactive species and free radicals are usually named as pro-oxidants.

Cell membrane contains many polyunsaturated fatty acids which are important targets for ROS. Reactive species attack fatty acids and subtract a hydrogen atom from them, so leave an unpaired electron on the lipid. This new lipid radical needs to increase its stability therefore it reacts with oxygen and forms peroxyl radical. The peroxyl radical generate a lipid hydroperoxide and also a new lipid radical by removing a hydrogen atom from adjacent fatty acid. This chain reaction is called lipid peroxidation and it is very harmful for cell membrane [77].

Lesions which include oxidized bases, abasic sites, and DNA single and double-strand breaks are produced because of oxidative stress. These lesions can be mutagenic and lead to mismatched incorporation of DNA bases during replication [95].

It is known that ROS can cause protein oxidation. Thiol oxidation and generation of protein carbonyls is an example of oxidative protein modification. Cysteine and methionine amino acids have sulfur on their structure therefore they are very sensitive to oxidative attact of ROS. While oxidation of cysteine forms thiyl radicals and disulfides, reaction of ROS with methionine produces methionine sulfoxide. As a reulst of oxidative modification, enzyme activities and protein binding properties are change in an undesirable direction [82].

Excessive level of ROS may be controlled by exogenous antioxidant supply. Que is well-known flavonoid with its strong antioxidant capacity but it needs to be constructued to improve its poor permeability, solubility, stability, and bioavailability. Nanoparticles are promising drug carriers for their advantageous properties. For this reason, Que can be combine with nanoparticles to enhance its bioavailability and antioxidant effect.

1.6. Nanoparticles (NPs)

As a scientific field, nanotechnology contains modification and control of matter on the nanoscale dimension and nanoparticles are the fundamental components of this field. Particulate substances which own one dimension less than 100 nm are called as nanoparticles. They are able to show specific colors and properties which depend on their size and shape [96].

Nanoparticles possess three layers in their structure, they are: (a) The surface layer which contains functional materials such as metal ions, ligands, surfactants and polymers. (b) The shell layer, chemical material different from the core, and (c) The core, which is the main component of the nanoparticle and generally stands for the nanoparticle itself [97].

Nanoparticles are promising drug carriers due to their advantageous properties, such as : (1) They are easily modified to obtain desired particle size and surface characteristics (2) Targeted drug delivery by nanoparticles increases drug therapeutic efficacy and reduces side effects. (3) While nanoparticles provide controlled drug release, they also have high drug loading capacity and drugs can can be loaded to nanoparticles without any chemical

reaction so drug activity is protected. (4) Ligand binding to surface or magnetic guidance can be used for site-specific targeting in nanoparticles [98].

Nanoparticles are categorized into several groups according to their size, shape, morphology, chemical and physical characteristics. Depending on their physical and chemical properties, nanoparticles are divided into six classes which are: carbon-based nanoparticles, metal nanoparticles, ceramics nanoparticles, semiconductor nanoparticles, polymeric nanoparticles, and lipid-based nanoparticles [99].

Nanoparticles can be synthesized by several methods such as bottom-up and top-down syntheses. Modifiying process parameters such as temperature, time, solvent ratio, and substrates give a chance to obtain nanoparticles with desired physicochemical properties, such as morphology, photoactivity, size, surface area and uniformity in size distribution [100].

1.6.1. Titanium (IV) Oxide Nanoparticles

Titanium (Ti) is widely distributed in nature because it is the ninth most abundant element in the earth. This element shows high affinity against oxygen and some other elements. Ti can be found in $^{+}3$ and $^{+}2$ oxidation states but the most common state is $^{+}4$. Titanium dioxide or titanium (IV) oxide, also known as titania, titanic acid anhydride, titanic anhydride, or Ti white is chemically inert and semiconducting metal oxide which show photocatalytic properties in the presence of light and it occurs naturally. In addition to these advantegeous characteristics, titanium dioxide has been calling attention over recent decades due to its low price and easy processing. As an odorless, noncombustible white powder, TiO_2 has 79.9 g/mol molecular weight, 2972 ^{0}C boiling point, 1843^{0}C melting point, and relative density of 4.26 g/cm^3 at 25^{0}C [101].

TiO_2 is classified as biologically inert and natural material as well [102]. It has been used in many industrial areas such as paints, papers, and cosmetics by the reason of its ability to give whiteness and opacity to various products. It possesses light scattering properties and very high refractive index. These properties make available to a white and opaque coating with low amount of TiO_2. Nano-sized TiO_2 has been using for self-cleaning and

antifogging on glass, for building facades, in plastics and in confectionary industry as well. TiO_2 has been also allowed to use in food and pharmaceutical products as an additive (E171) [103]. It has currently been involved in cosmetics, sunscreen products, ceramics and used as food, drug and plastic colorant [104]. Crystalline TiO_2 include three natural polymorphs which are rutile, anatase and brookite. Rutile form is the most stable one among other forms.

The major reason of the wide application of TiO_2 is its low toxicity. TiO_2 has been currently using in cosmetic formulations such as sunscreens, eyeshadows, and powders and it is suggested that its crystal forms (anatase and rutile) and size affect the safety of its use.

Depending on the nature of TiO_2 nanoparticle, when they dispersed in aqueous solutions, they tend to form relatively strong bonded aggregates or soft agglomerases [105]. Agglomerated particles have decreased surface area and they can lower their concentration and prevent steady dosage. Therefore, it is necessary to modify TiO_2 nanoparticle's surface to eliminate this undesired property [106]. Functionalized TiO_2 surface may provide non-aggregating, stable formulations in aqueous solutions.

Polymers have wide range of uses in many applications. In medical field, developed materials and systems need polymers to become more suitable with biological systems [107]. Polymers have been using as diagnostic and therapeutic systems such as fluorescent loaded polymeric nanoparticles and drug carriers for years [108]. Biocompatibility is the most important factor for polymers to can be used. Synthetic and natural polymers are two classes of polymers using in biomedical engineering. As a synthetic polymer, polyethylene glycol (PEG) has various applications, especially in medicine. Sometimes polyetheylene oxide (PEO), another name of PEG, is used instead of PEG. As a general rule, while ethylene oxide macromolecules which have less than 20.000 g/mol molecular weight are named as PEG, others with molecular weitght above 20.000 g/mol are called PEO. Ethanol, water, benzene, acetonitrile, and dichloromethane are suitable solvent for PEG, however, PEG can not be solved in hexane and diethyl ether. PEG can be found in different structures such as star, branched, and comb-like macromolecules. PEGylation stand for bonding of PEG to another molecule and it is promising method for nanoparticles [109]. When the surface of nanoparticle is coated with PEG, it improves the

efficiency of drugs delivery to target cells. As a foreign matter, nanoparticles are removed from systemic circulation by the mononuclear phagocyte system (MPS) cells such as dendritic cells, monocytes, and macrophages. On the contrary, it was observed that PEGylation protects nanoparticles from aggregation, opsonization, and phagocytosis, therefore enhance their circulation time. Nanoparticles having uncharged, hydrophobic nature are easily aggregate via hydrophobic and van der Waals forces in aqueous solutions. However, negatively or positively charged nanoparticles show repulsive forces and stay stable in colloidal solutions with low ionic strength. But in the case of high ionic strength like the blood, nanoparticles interact with oppositely charged molecules and form aggregates. PEG has hydrophilic nature and produce a hydrated cloud around nanoparticles which protect nanoparticles from interaction with blood components or other nanoparticles. Increasing circulation time by PEGylation increases the interaction of ligand, which is presented on nanoparticles, and receptor on cell surface. PEGylation also improve drug delivery by improving the penetration of biological barriers. Decreased interaction with extracellular matrix, biological fluids, and cellular barriers lead to improved penetration [110].

1.6.2. Nanoparticles as drug carrier systems

Nanoparticles are quite small particles thefore they can travel more frely in the human body when compared to bigger materials. They show unique chemical, structural, electrical, mechanical, magnetic, and biological features. Nanomedicine is a subtype of medicine using nanotechnology to prevent or cure various diseases. This field of medicine employs nanostructures such as nanoparticles and nanorobots for several applications. Nanomedicine has been taking attention due to fact that nanomaterials can be employed as drug delivery systems by attaching or encapsulating therapeutic drugs and deliver them to desired tissue with a controlled release. Nanostructures which are unified with therapeutic drugs can stay in the blood circulatory systems for long a long time and they are able to release drugs as per the determined dose. When they are directed to target tissue, they cause reduced adverse effects. Low soluble drugs have many drawbacks including low bio-accessibility after drug intake, less diffusion level into membrane, require more quantity and unwanted adverse effects. Nanotechnology can overcome all of these limitations. Drugs can be designed at the nanoscale level and using nanoparticles

for this purpose brings many advantages including improved solubility, drug release properties, diffusivity, bioavailability and immunogenicity. These modifications on drug systems lead to development of lower toxicity, less side effects, proper administration routes, upgraded biodistribution and prolonged drug life cycle. Nanostructures combined with drugs can deliver their cargo in two ways: passive or active targeting. In active targeting, antibodies or peptides are combined with drug carrier system and at the target site they recognize receptors. Cell membrane receptors, lipids of the cell membrane, antigens or proteins on the cell surfaces are the major targets for drug carrier systems. On the other hand, passive targeting does not include specific ligands on it. Prepared drug delivery system circulates through the blood stream and it is transported to desired location by binding or affinity which are affected by a number of parameters including pH, molecular shape and temperature [111].

1.6.3. Cellular uptake of nanoparticles

In terms of toxicology size, shape, surface chemistry, charge, surface area, crystallinity, solubility and agglomeration or aggregation are the important properties of nanoparticles. Nanoparticles can be lipophilic or lipophobic, hydrophilic or hydrophobic, catalytically active or passive. These caharacteristics of nanoparticles affect their cellular uptake, subcellular localization, and toxic effects [112].

Nanoparticles are taken in cells by highly regulated mechanisms which contain complex molecular interactions. Cellular membrane is composed of lipid bilayer and embedded proteins and they cause overall negative charge with few cationic domains. Because of that membranes are selectively permeable to ions, biomolecules, and nanoparticles. Thus, nanoparticles have to overcome cellular membrane to reach inside the cell. Nanoparticles are taken into cells by different pathways which determine nanoparticle's intracellular fate, function, and biological effects [113].

Interestingly, nanoparticles with the same surface chemistry and different morphology use different cellular uptake pathways to get into the cell. Cells have ability to take nanoparticles with the help of two main pathways: active uptake by endocytosis or passive uptake by free diffusion. Endocytosis-based pathways are categorized into five

classes: clathrin-dependent endocytosis, caveolin-dependent endocytosis, clathrin- and caveolin-independent endocytosis, phagocytosis, and macropinocytosis. Various lipids and transport proteins such as clathrin, caveolin, dynamin, and receptors are employed in endocytosis. After endocytosis, nanoparticles are kept within intracellular vesicles such as phagosomes, endosomes, and macropinosomes. Nanoparticles which are taken in vesicles such as endosomes might fuse with lysosomes. As a result, cellular uptake of nanoparticles is followed by lysosomal breakdown which leads to drug release from nanoparticles because of the low pH.

Another way, direct diffusion, does not require energy and provides direct entry into the cell by engaging with lipid bilayer molecules. Direct cytoplasmic entry include four different ways: direct translocation, lipid fusion, electroporation, and microinjection. In this way, nanoparticles cross the cell membrane under favour of biochemical or physical means and then move to the cytoplasm directly. Nanoparticles taken in cytoplasm can interact with subcellular organelles or intracellular structures and express their biological effects [114].

2. AIM OF THE STUDY

In human body there is homeostatic adjustment between oxidants and antioxidants for proper function of metabolism. In some cases, environmental factors disrupt redox homeostasis and cause oxidative stress. Que is well known flavonoid and it expresses strong antioxidant capacity. However, in physiological conditions, it is known that due to its hydrophobic nature Que shows low solubility, permability, stability and bioavailability which limit its possible applications in the pharmaceutical industry. TiO_2 NPs were accepted as biologically inert materials and they have been using in nanotechnology for years because of their desirable properties such as high refractive index, low toxicity, antibacterial property, and high level of biosafety. As a drug carrier, TiO_2 NPs could be conjugated with Que for better penetration, improved bioavailability, and efficient drug delivery. In this study, we aimed to prepare Que conjugated TiO_2 NPs (Q-PEGTiO_2). which were characterized with SEM and DLS. Next, drug conjugation ratio of nanosystem was checked spectrophotometrically and we have seen that Q-PEGTiO_2 has ability to conjugate with high level of Que. With the intention of checking cellular toxicity of the Q-PEGTiO_2, WST-1 assay was performed. As the next step, cellular penetration and colocalization analysis were carried through laser scanning confocal microscope and proper analysis software. In the last experiment, we aimed to examine *in vitro* antioxidant activity of Q-PEGTiO_2 by DPPH radical scavenging assay.

By depending on our knowledge so far, we suggest that Q-PEGTiO_2 nanosystem could be a good carrier for Que and be used in topical cream formulation for protection against oxidative damage in skin.

3. MATERIALS & METHODS

3.1. MATERIALS

3.1.1. Chemicals

All chemicals used in this thesis are listed in Appendix A.

3.1.2. Equipment

Equipment that were used in this thesis are listed in Appendix B.

3.1.3. Solutions and Buffers

All solutions and buffers used in this thesis are listed in Appendix C.

3.1.4. Growth Media

DMEM: DMEM growth medium was supplemented with 1% Pen/Strep (100 Units/mL Penicilin and 100 ug/mL Streptomycin) antibiotics and 10% fetal bovine serum (FBS) was used for cell culture.
Freezing Media: Freezing medium that contains 80% FBS and 20% DMSO was used to freeze mammalian cell lines.

3.1.5. Molecular Biology Kits and Reagents

Molecular biology kits and reagents used in this thesis are listed in Appendix D.

3.1.6. Mammalian Cell Lines

ATCC 3T3-Swiss albino (ATCC® CCL-92™) was used in all experiments in this thesis.

3.1.7. Software and Programs

All software programs used in this thesis are listed in Table 3.1.

Table 3.1. Table of software programs used in the thesis. Softwares for statistical analysis, imaging as well as image post-processing were listed below.

SOFTWARE-PROGRAM NAME	COMPANY	INTENDED PURPOSE
CoLocalizer Pro 3.0.2	CoLocalization Research Software	Colocalization analysis of confocal images
GraphPad Prism 5	GraphPad Software	Design of graphics
ZEN Blue edition 2.3	Carl Zeiss Microscopy	Post-processing of confocal images

3.2. Methods

3.2.1. PEGylation of Titanium dioxide nanoparticles (TiO_2 NPs)

TiO_2 NPs (Sigma, Cat. No: 637262) used in this thesis have <100 nm primary size. These TiO_2 NPs were surface modified with PEG-6000 (Sigma, Cat. No: 81255). In order to achieve this aim TiO_2 NPs were suspended in Milli-Q water then sonicated (500 W) on ice for 30 min with 34% amplitude in a 10 s pulse with 20 s intervals. 1% PEG-6000 solution was prepared in Milli-Q water and mixed with sonicated TiO_2 suspension for surface modification in a 1:1 mass ratio (PEG: TiO_2 particles). The mixture was allowed to stir overnight at 750 rpm at room temperature (RT). After the overnight stirring, PEGylated TiO_2 NPs (PEG-TiO_2) were obtained by centrifugation at 18,000 rpm at 10^0 C for 1h. The supernatant was removed and the PEG-TiO_2-containing pellet was washed with Milli-Q water. PEG-TiO_2 NPs were frozen by using liquid nitrogen and inserted into freeze-dryer to obtain PEG-TiO_2 powder.

3.2.2. Characterization of PEG-TiO_2 and Q-PEGTiO_2

3.2.2.1. SEM analysis

The morphology of bare TiO_2 and Q-PEGTiO_2 were examined by scanning electron microscopy (SEM) analysis. Bare TiO_2 and Q-PEGTiO_2 were dispersed in Milli-Q water and subjected to a 10 minutes ultrasonic dispersion to obtain homogeneous suspension. The sample was mounted on a silicon wafer chips then sputter coating of thin Pd-Au layer for SEM imaging (Cressington Asputter Au/Pd Coater) was done. SEM operator at 3 keV beam (Carl Zeiss, LEO Supra 35 V P) was used to record SEM images.

3.2.2.2. DLS analysis

Dynamic light scattering (DLS) measures dynamic properties of materials by using single scattering light. Each detected photon is scattered by the sample exactly once. Sample containing colloidal particles is irradiated by laser and the scattered light is recorded as a function of time. This spectral technique is widely used to estimate the particle size in the submicron range. Hydrodynamic diameter of bare TiO_2 and PEG-TiO_2 were determined by DLS by using Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). Bare TiO_2

and PEG-TiO_2 nanoparticle powders were dispersed in Milli-Q water then sonicated (500 W) on ice for 30 min with 40% amplitude in a 10 s pulse with 20 s intervals. Suspensions containing 100 μg/ml PEG-TiO_2 and bare TiO_2 were filtered by 0,45 μm filter to remove dust and artifacts from the sample. DLS measurement duration was set to automatic, and three repeat measurements were taken at 25°C. 1.33 was used as a solvent refractive index because the major component is water. Sample cell was filled with suspension of nanoparticles and irradiated by a laser.

3.2.3. Conjugation of PEG-TiO_2 with Que

To prepare 1 mg/mL Que solution, absolute (100%) ethanol was used as solvent for Que powder. The concentration of Que was measured at its maximum absorption wavelength of 380 nm. The solution was mixed with 1mg/mL PEG-TiO_2 suspension, which was prepared in Milli-Q water, in a mass ratio of 1:1 then the mixture was stirred overnight at 22 rpm at RT. To obtain Que-conjugated PEG-TiO_2 (Q-PEGTiO_2) the mixture was centrifuged at 2000 rpm for 10 min at RT. The supernatant was measured spectrophotometrically to calculate residual concentration of Que in the supernatant. The pellet containing Q-PEGTiO_2 was resuspended in the same volume of DPBS. The % Que conjugation was calculated according to the Equation 1.

$$\text{Que conjugation \%} = \left[\frac{A-B}{A}\right] x\ 100 \qquad (3.1)$$

In this equation, ‘A’ and ‘B’ represents the concentration of Que, before and after the conjugation process, respectively.

3.2.4. Mammalian Cell Culture

3.2.4.1. Maintenance of cell lines

3T3 mouse fibroblast cell line was grown in DMEM complete medium and incubated at 37^0 C in the presence of 5% CO_2. When the cells reached 80% confluency they were

subcultured. Subculturing protocol was performed by washing the cells with sterile DPBS and detaching them with appropriate amount of Trypsin-EDTA. To neutralize trypsin, proper amount of complete medium was added on the cells and depending on the experiment, the cells were seeded in sterile 6-well, 96-well, T-25, T-75 or 100 mm cell culture plates.

3.2.4.2. Cryopreservation of the cells

Freezing medium was prepared by using 80% FBS and 20% DMSO. The cells were frozen in freezing medium and stored in liquid nitrogen for further use. After medium aspiration, the cells were washed with sterile DPBS and harvested by trypsin. Cell pellet was obtained by centrifugation at 300 g for 5 min. The pellet was resuspended with 1 mL freezing medium and transferred into a cryovial which are stored in a freezing container at -80^0 C for one day to allow the temperature decrease gradually. The day after, cryovials were inserted into liquid nitrogen for long term storage.

3.2.4.3. Thawing mammalian cells

Frozen cells were taken out of liquid nitrogen and quick-thaw method was followed. Cells were thawed in a 37^0 C water bath for 1 min and transferred into 15 mL centrifuge tube which contains 9 ml complete medium (DMEM). To remove freezing medium, centrifugation was performed at 300 g for 5 min. The cell pellet was resuspended with 5 mL growh medium and transferred into T-25 cell culture plate and incubated at 37^0 C in the presence of 5% CO_2.

3.2.5. Treatments

Que (Sigma, Q4951, MW: 302,24 g/mol) was freshly prepared in absolute ethanol. The cells were treated with PEG-TiO_2, Q-PEGTiO_2 or Que at final concentration of 100

ug/mL. Untreated cells was grown under identical test conditions and accepted as the untreated control group (UC).

3.2.6. Cell Viability Assay

Cell Proliferation Reagent WST-1 Assay (Roche) manufacturer's protocol was followed to determine cellular viability. 3T3 cells were counted and seeded into 96-well cell culture plate then treated with Q-PEGTiO_2, PEG-TiO_2 or Que. After 24h or 48h incubation, 10 uL WST-1 reagent was added on the cells and the cells were incubated at 37^0 C for 4 hours. Absorbance reading was done at 450 nm by using microplate reader (Biorad). The absorbance of UC was set to 100 %. Absorbance results obtained from treated groups and UC were presented.

3.2.7. Cellular Uptake of Q-PEGTiO_2 and Que

3.2.7.1. Staining of mitochondria

3T3 cells ($1,3x10^5$ cells/well) were seeded on sterile cover slides (Jena Biosciences circular cover slide 0.22mm, CSL104) that were inserted in 6-well plate. When cells were atttached on the slide, they were treated with Q-PEGTiO_2 or Que. After the treatment, mitochondria was stained with Mito-Tracker Red at a final concentration of 50 nM and incubated in 37^{o}C for 5 minutes.

3.2.7.2. Fixation

After mitochondria staining, cells were washed three times with 1X PBS for 10 minutes. Later on, 4% PFA (paraformaldehyde) solution was used to fix cells. Cells were incubated on ice for 10 minutes then washing steps (3 times, 10 min) were followed to remove excess PFA.

3.2.7.3. Nuclei staining and mounting

DAPI was prepared in 1X PBS and staining of cell nuclei was performed on a shaker for 10 minutes. After the necessary washes, the coverslips containing cells were mounted with mounting solution on the microscope slides and sealed with transparent nail polish.

3.2.7.4. Confocal imaging

Cells were observed by Zeiss LSM 710 Confocal Microscope. 63x oil immersion objective was utilized to observe cells and capture image. Channels, lasers and excitation-emission values were given in Table 3.4.

Table 3. 2. Excitation-emission maxima, lasers and fluorophere types used in confocal imaging.

Channel/Stain	**Excitation maxima**	**Emission maxima**	**Laser**
DAPI	405nm	459nm	UV Laser
Que / EGFP	488nm	509nm	Argon Laser
Mito-Tracker Red	560nm	630nm	DPSS 561-10 Laser

3.2.7.5. Post-processing and colocalization analysis

Images saved as .lsm5 format were converted into .tiff format with the help of ZEN software (Blue edition 2.3). Colocalization analysis was performed by CoLocalizer Pro (3.0.2) software by using .tiff images. Briefly, signal intensity of pixels in channels, Pearson's coefficient (R_r) and Manders' overlap coefficient (R) were calculated to estimate colocalization of two channels (red-green) [115].

3.2.8. DPPH Radical Scavenging Assay

As a final concentration, 0,1 mM DPPH (D9132, Sigma) solution was prepared in methanol. Q-PEGTiO_2 and Que were prepared as a final concentartion of 100 μg/ml and 50 μg/ml then they were 5-fold diluted with DPPH solution and placed in 96-well culture plate in 250 μl final volume. The plate was inserted in Tecan microplate reader (Life Sciences, Switzerland) and absorbance reading was made at 24°C at a wavelength of 517 nm and the instrument was set to 15 minutes interval with 48 kinetic cycles. The % Scavenging activity was calculated according to Equation 2.

$$\% \text{ Activity} = [(Abs_{blank} - Abs_{sample}) / Abs_{blank}] \times 100 \quad (3.2)$$

In the equation, the blank solution represents absorbance value at zero hour and sample solutions indicate absorbances at certain time intervals such as 15-120-180-360-700 minutes.

3.2.9. Statistical Analysis

Acquired data are presented as mean ± SD. Student's t-tail was performed to detect statistical significance of the results and $^{*}P<0,05$ was considered statistically significant.

4. RESULTS

4.1. SEM analysis of Nanoparticles

The morphology of nanoparticles were examined by means of SEM imaging. It was observed that bare TiO_2 nanoparticles tend to agglomerate over time although intense and

long time ultrasonication (figure 4.1). This agglomeration could be because of inherent physicochemical properties of the TiO_2 NPs (A). Agglomeration was decreased by PEGylation (PEG-TiO_2) as seen in Figure 4.1B. PEG-TiO_2 was conjugated with Que and Q-PEGTiO_2 was presented in Figure 4.1B. Conjugation ratio of Q-PEGTiO_2 was calculated according to Eq. 1 and it is found as >95 % .

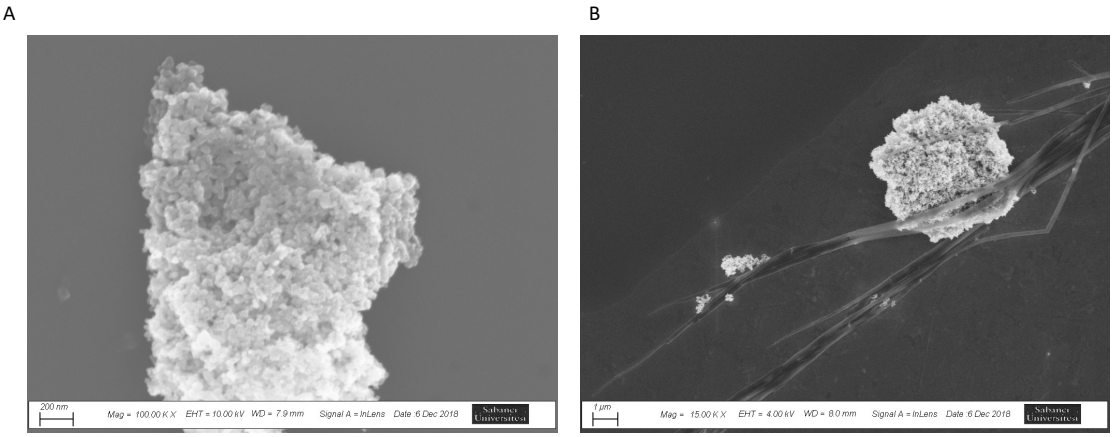


Figure 4. 1. SEM analysis of bare TiO_2 and Q-PEGTiO_2. (A) Bare TiO_2 NPs tend to form agglomerates before PEGylation. (B) PEG-TiO_2 was conjugated with and used as a drug carrier for Que. The SEM images of bare TiO_2 and Q-PEGTiO_2 at 100.00 KX and 15.00 KX magnification is captured by SEM, respectively.

4.2.Dynamic Light Scattering (DLS) Analysis of Nanoparticles

DLS was performed to determine the hydrodynamic diameter of bare TiO_2 and PEG-TiO_2 nanoparticles. Bare TiO_2 and PEG-TiO_2 nanoparticle powders were dispersed in Milli-Q water then sonicated (500 W) on ice for 30 min with 40% amplitude in a 10 s pulse with 20 s intervals. Suspensions containing 100 µg/ml bare TiO_2 and PEG-TiO_2 were filtered by 0,45 µm filter to remove dust and artifacts from the sample. Sample cell was filled with stable, nonsettleable nanoparticle suspension and irradiated by a laser. Non-dispersed nanoparticle clusters were observed even after extensive ultrasonication and the subsequent use of 0.45 µm filter. Hydrodynamic diameter of bare TiO_2 and PEG-TiO_2 were recorded as 307.0 and 193.0, respectively. Their size as a function of intensity is presented in Figure 4.2.

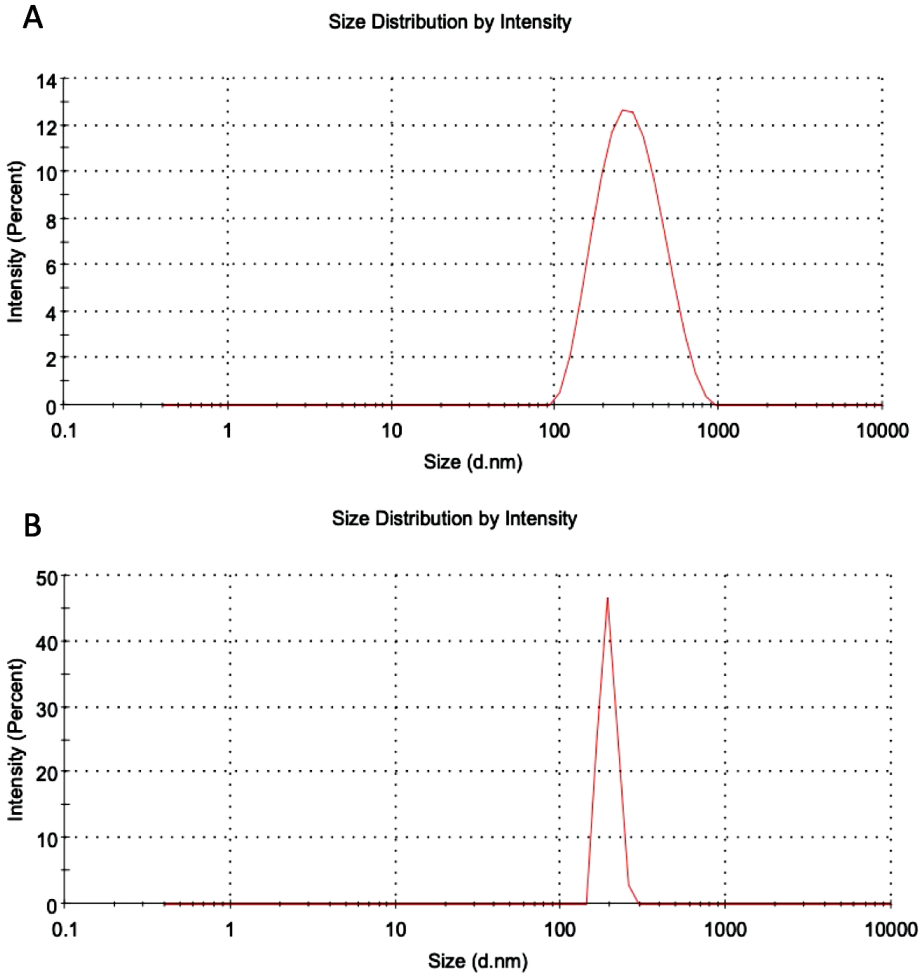


Figure 4. 2. DLS analysis of nanoparticles. DLS measurement was performed to determine hydrodynamic diameter of nanoparticles, which are specified as <100 nm by provider, in aqueous environment. (A) Hydrodynamic diameter of bare TiO_2 was measured by DLS and 307.0 nm was recorded. (B) PEG-TiO_2 was characterized with 193.0 nm hydrodynamic diameter, (n=3).

4.3. Que conjugation to PEG-TiO_2

Que could be combined with PEG-TiO_2 to improve its bioavailability [116]. For this purpose, it was mixed with PEG-TiO_2 in a ratio of 1:1 and incubated overnight at room temperature (RT) under constant stirring (22 rpm). After that, this Que suspension was centrifuged at 2000 rpm for 5 min at RT to obtain pellet as Q-PEGTiO_2. Supernatant was measured spectrophotometrically to calculate residual Que in it. The % Que conjugation was calculated according to Equation 1 and it was found as > 95%.

4.4. Aggregation of Nanoparticles

It is usually observed that nanoparticles are prone to aggregate in aqueous solutions. Many studies have shown that TiO_2 nanoparticles form aggregation and agglomeration when dispersed in water [117]. While PEG-TiO_2 is dispersed in MilliQ, Q-PEGTiO_2 was prepared in DPBS. As shown in Figure 4.3, PEG-TiO_2 and Q-PEGTiO_2 were observed before vortex and it could be seen that they aggregated at the bottom of the falcon (A). Then 15 sec vortex was performed to disperse the nanoparticle clusters, and uniform suspension was obtained (B). 2 minutes after the vortex, nanoparticles accumulated at the bottom part once again (C).

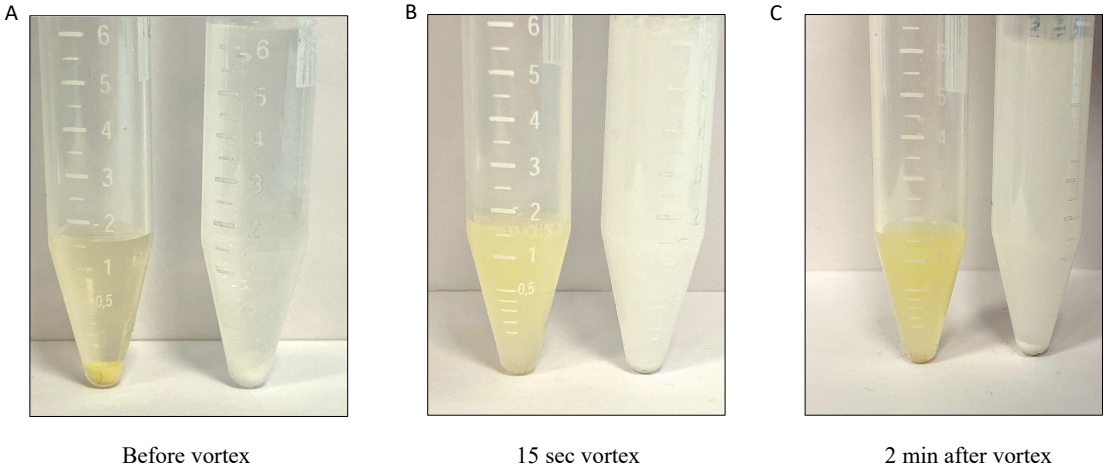


Figure 4. 3. Image of Q-PEGTiO_2 (left) and PEG-TiO_2 (right). Aggregation behaviour of Q-PEGTiO_2 (left) and PEG-TiO_2 (right) are presented. Nanoparticles show aggregation before vortex (A), however, when they are subjected to 15 sec vortex they have uniform dispersion (B). 2 min after the vortex, they aggregated again (C).

4.5. Cell viability assessment after nanoparticle treatment

3T3 cell line was treated with 100 µg/ml concentration of Q-PEGTiO_2, Que or PEG-TiO_2 in time-dependent manner. The effect of the treatments on cell viability was determined

by WST-1 Cell Proliferation Assay (Roche). Mean values of cell viability results are presented in Figure 4.4 as a function of obtained absorbance from the cells. Viability results are compared with untreated control group (UC). In the 24h treatment groups, while Que-treated cells showed the lowest viability, Q-PEGTiO$_2$-treated cells protected their viability when compared to UC group. Increasing time of treatment (48h) came with reduction in viability. The decrease in viability could be seen in all treatments groups, however, quercetin could be easily noticed with significant decrease in viability (Fig. 4.4). The cell viability results indicate that while Que at the concentration of 100 µg/ml revealed significant decrease in viability, Q-PEGTiO$_2$ at the same concentration did not show reduction in cell viability. However, increased time exposure (48h) came with decreased viability in both Que and Q-PEGTiO$_2$-treated cells. According to this results, 24h exposure time was determined as the safe condition for future experiments.

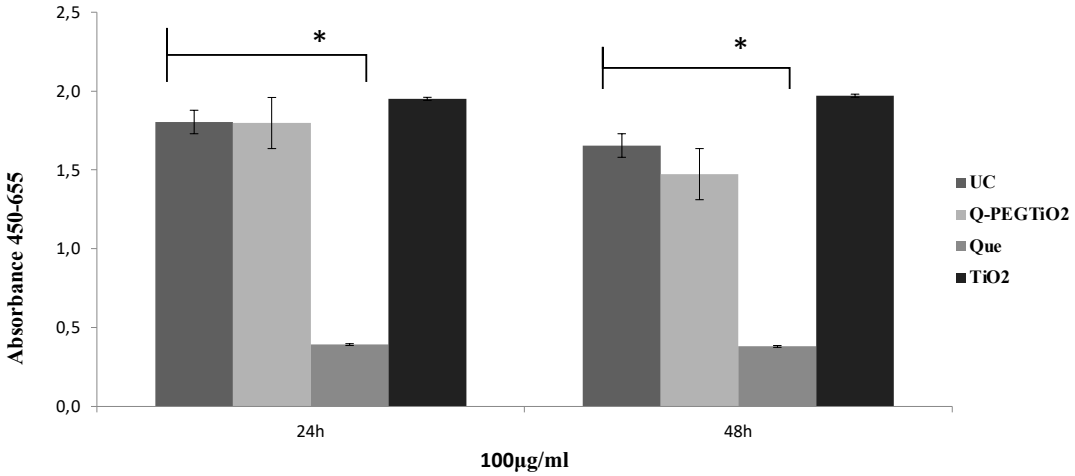


Figure 4. 4. Time-dependent effect of Q-PEGTiO$_2$, Que, and PEG-TiO$_2$ treatment on cell viability. 3T3 cell line was seeded as 25.000 cell/well and treated with compound indicated for 24 or 48h at a concentration of 100 µg/ml. Cell viability was determined by WST-1 Cell Proliferation Assay by following manufacturer's protocol. Results represents the mean values ± SD and asterisks state significant variations compare to control group (*P < 0.05). Three independent experiment were carried out to determine the viability of cells after each treatment.

4.6. Cellular Uptake and Intracellular Localization of Q-PEGTiO$_2$ and Que

It is known that nanoparticles and Que have ability to penetrate into cells. Some studies propose that Que and its metabolites might accumulate organs which take part in its metabolism and excretion. According to this suggestion small intestine, kidney, colon, and liver are the major organs in terms of Que accumulation. Additionally, mitochondria is suggested to be an organelle for Que accumulation [118]. Laser scanning confocal microscope with 63X objective was used to observe cellular entrance of Q-PEGTiO_2 and Que. The autofluorescence properties of Que was utilized to track Que and nano system within cells. 3T3 mouse fibroblast cells were seeded in 6-well plate then treated with Q-PEGTiO_2 or Que for different time intervals (1, 3, 6, 12, and 24h) . After the mitochondria staining, cells were fixed with PFA and necessary washing steps were followed. As a last step, DAPI staining of nuclei was performed and cover slips were prepared for confocal imaging. Compatible filter sets were employed for DAPI, Que, and MitoTracker Red. As indicated in Figure 4.5 and 4.6, untreated cells were used as a control group and expressed as 0 h while treated groups were represented by their incubation time with Q-PEGTiO_2 or Que. Obtained results pointed out that both Que and nanosystem could effectively penetrate into cells and localized primarily in the cytoplasmic part of the cell. It was noticed that, Q-PEGTiO_2 treated cells remained healthy and conserved their morphology even until the end of 24 h of treatment. However, Que-treated cells lost their characteristic morphology and viability after the treatment. Additionally, some Q-PEGTiO_2 did not enter into cells and they accumulated between cells. When all the above results come together, it could be stated that Que molecules conjugated with nanosystem are stable inside cells and show homogeneous distribution.

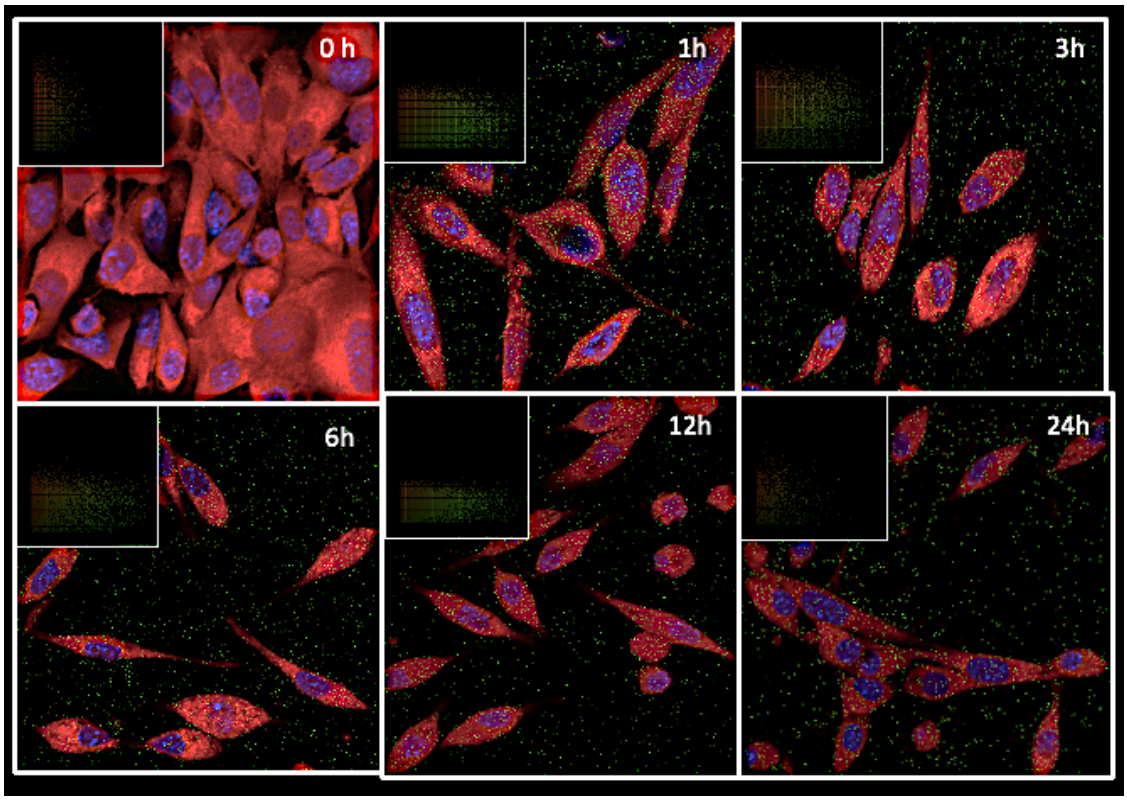


Figure 4. 5. Cellular uptake of Q-PEGTiO$_2$. 3T3 cells were incubated with Q-PEGTiO$_2$ for 1, 3, 6, 12, and 24h and Que signals coming from the nanosystem were captured by means of laser scanning confocal microscope with 63x objective. Nuclei and mitochondria were stained with DAPI (blue) and MitoTracker Red (red), respectively. Green fluorescence represents Q-PEGTiO$_2$. Pixel scattergram and incubation time were expressed on the upper-left and upper-right part of each image, (n=3).

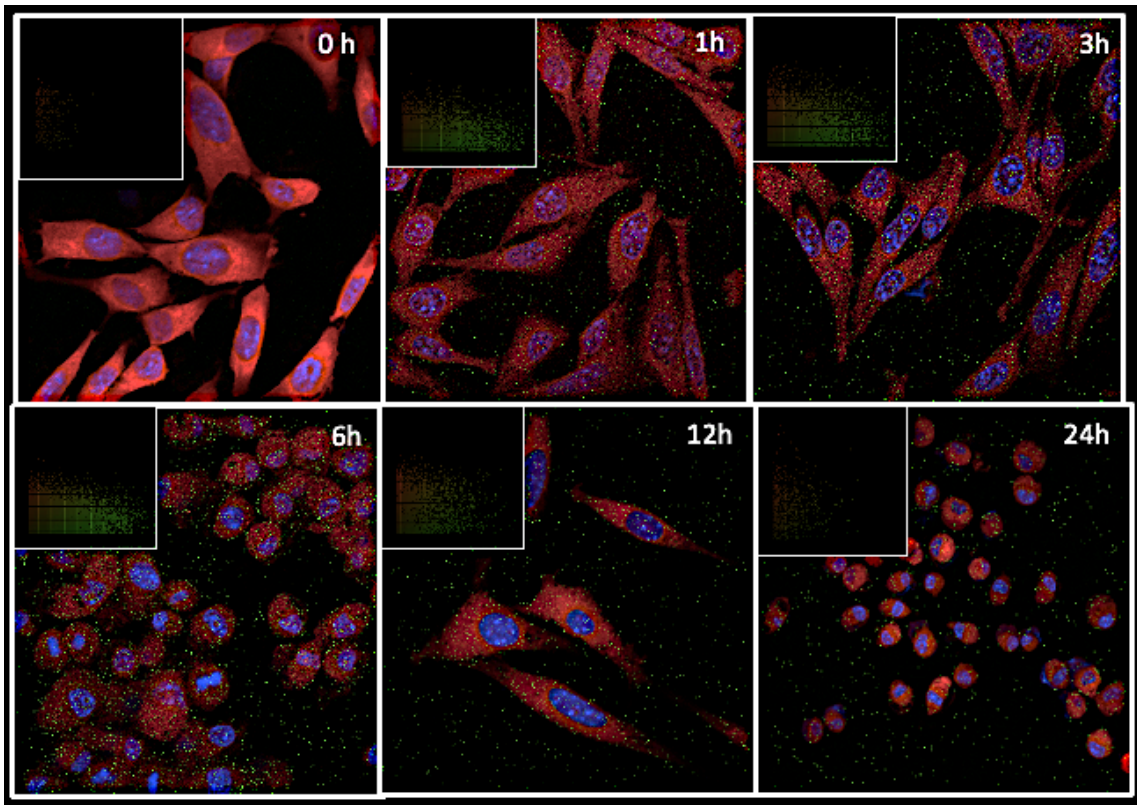


Figure 4. 6. Cellular uptake of Que. 3T3 cells were incubated with 1, 3, 6, 12, and 24h for 100 µg/ml Que. Penetrance of Que into cells were observed by laser scanning confocal microscope with 63x objective. Nuclei and mitochondria were stained with DAPI (blue) and MitoTracker Red (red), respectively. Green fluorescence represents Que molecules. Pixel scattergram and incubation time were presented on the upper-left and upper-right part of each image, respectively, (n=3).

Confocal imaging results showed that both Que and Q-PEGTiO$_2$ effectively penetrated into cells. To understand what time the Que and nanosystem reach their maximal penetration into cells, colocalization analysis was carried out. Images, which were captured by confocal laser scanning microscope, were imported in CoLocalizer Pro software and analyzed with the help of two channels, green channel for Que and red channel for mitochondria. Merged images with their scattergrams are presented in Figure 4.5 and Figure 4.6. Colocalization ratio was expressed as Manders' Overlap Coefficient (R) which calculate pixel densities of channels. Figure 4.8 represents mean values ± SEM of Manders' Coefficient (R) for Que and Q-PEGTiO$_2$-treated cells . Although Que indicated better penetration, the accumulation of both Que and Q-PEGTiO$_2$ within cells has shown increase over time and reached its maximum accumulation level at the 12th

hour (Figure 4.8). After the maximal peaks, a slight decrease was observed in both groups' cellular penetrance.

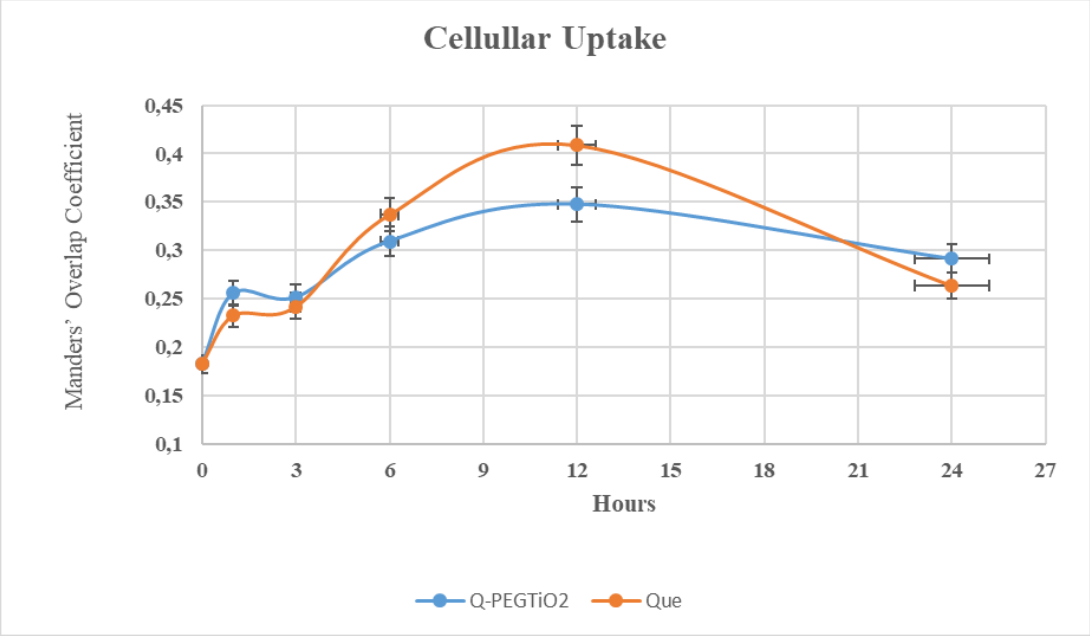


Figure 4. 7. Cellular uptake of Que and Q-PEGTiO$_2$. Cellular penetration of Que and Q-PEGTiO$_2$ showed similar trend during 24h according to Manders' overlap coefficient values. Maximum penetrations were determined after 12 hours of incubation in both treatment groups. However, Que uptake is slightly effective than Q-PEGTiO$_2$ by the fibroblast cells (n=3).

4.7. DPPH Radical Scavenging Assay

DPPH is a free radical and in the presence of antioxidant, it is converted from oxidized form to reduced form. During this redox reaction, color change also occurs. While oxidized form has purple color, reduction of DPPH come up with yellow color. This changes could be tracked by spectrophotometer [119]. To evaluate antioxidant effect of Q-PEGTiO$_2$ and Que, DPPH radical scavenging assay was performed. 0.1 mM DPPH solution was prepared in methanol. Que was prepared at two different concentrations which are 100 µg/ml and 50 µg/ml and they were used as an internal control. In order to see antioxidant effect, 100 µg/ml Q-PEGTiO$_2$ was prepared and placed into the reaction medium. DPPH solution and Que or Q-PEGTiO$_2$ were mixed in 96-well plate well then the plate was inserted into the Tecan microplate reader and absorbance reading was made

at 24°C at a wavelength of 517 nm and the instrument was set to 15 minutes interval with 48 kinetic cycles. Obtained results were indicated in Figure 4.8, as a function of time to radical scavenging activity. Que shows strong antioxidant property, therefore it is usually used as a standard on DPPH assays [120]. It could be easily seen that Que was able to scavenge radical species in a matter of minutes. In the case of Q-PEGTiO$_2$, we see increase in scavenging activity, but it took a while compared to Que. When Que showed its maximum activity at 2nd hour, Q-PEGTiO$_2$ expressed only 20% of its activity. As expected, half dose of Que showed half activity of 100 µg/ml Que.

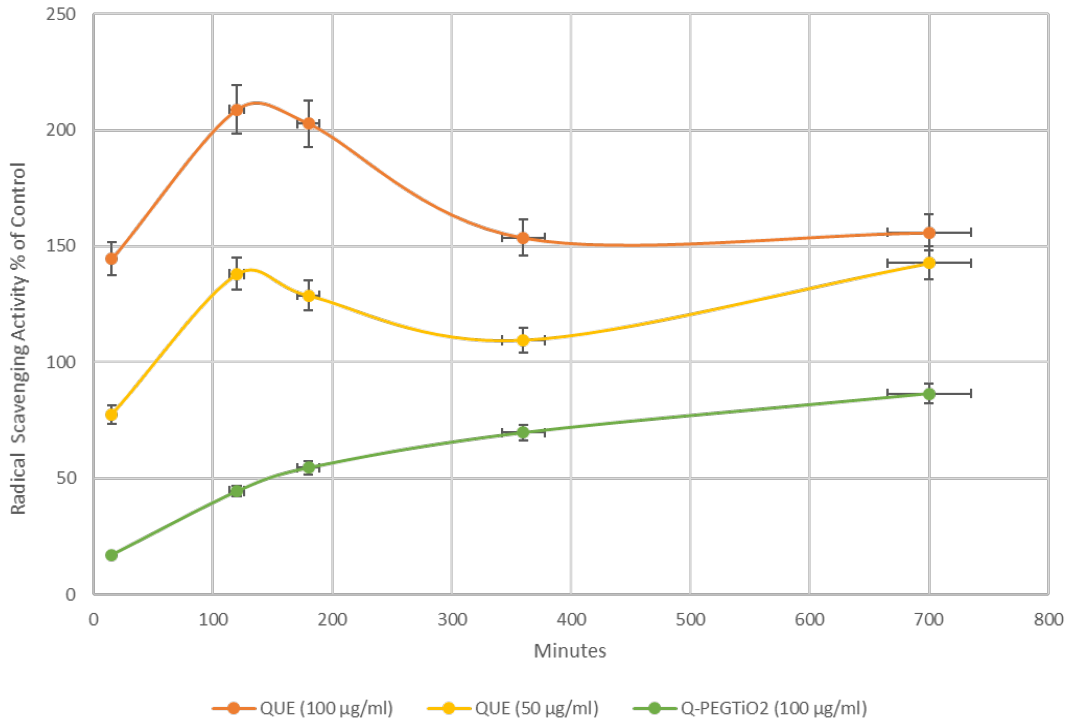


Figure 4. 8. DPPH radical scavenging activity of Que and Q-PEGTiO$_2$. Radical scavenging activity of Que/ Q-PEGTiO$_2$ was determined by DPPH assay during 12 hours at 517 nm wavelength. Zero-hour groups serve as control groups and all values were corrected to controls. Que showed maximum activity at the 2nd hour wheras Q-PEGTiO$_2$ showed only 20 % of its activity. Half dose of Que group was used as an internal control and demonstrated half of the its activity. Values represent the means of three independent measurements and % of SD bars are shown on the chart.

5. DISCUSSION

Reactive oxygen species (ROS) is a collective term that covers both oxygen-centered radicals (e.g. $O_2\bullet^-$ and OH•) and nonradical derivatives of oxygen such as hydrogen peroxide and singlet oxygen. These nonradical derivatives have the ability to oxidize biomolecules and/or they easily become radicals [77]. Free radicals and reactive species are formed in the human body to take part in vital processess such as inflammation reactions, neurotransmission [78] , many signaling pathways, cell proliferation and apoptosis. Normal metabolism needs ROS to maintain the proper function of oxidative phosphorylation, enzymatic reactions, activation of nuclear transcription factors, signal transduction, gene expression, and antimicrobial action in innate immunity [82] .

Oxidative stress occurs when the balance between oxidants and antioxidants is disrupted. This stress condition disarranges redox signaling and damage to biological molecules [85] . Normally, human body contains antioxidant defence against ROS to protect cellular components being oxidized [86] . Unhealthy lifestyle such as alcohol consumption, tobacco smoking, and medical drug abuse, as well as environmental pollutants including pesticides, xenoestrogens, and heavy metals increase ROS levels within cells [91].

Que is well known flavonoid due to its strong antioxidant properties. It can scavenge free radicals, increase endogenous antioxidant levels, inhibit lipid peroxidation, chelate metal ions and protect cells from genetic toxicity. However, Que has many unfavorable properties such as great hydrophobicity, poor permeability, bioavailability (5.3%) [76] , solubility, and stability which limit Que's applications in pharmaceutical industry.

Nanoparticles are quite small particles thefore they can travel more frely in the human body when compared to bigger materials. Hydrophobic drugs can be designed at the nanoscale level and using nanoparticles for this purpose, as a drug carrier, brings many advantages including improved solubility, drug release properties, diffusivity, bioavailability and immunogenicity. TiO_2 nanoparticles are classified as biologically inert

materials and they are known with their low toxicity [102] . Due to their desired properties, TiO_2 nanoparticles have been using in many areas such as paints, papers, cosmetics, and pharmaceuticals [103] .

In this study, we proposed that hydrophobic Que could be conjugated with TiO_2 NPs to improve its stability, bioavailability and thus its antioxidant effect. This Que-including nanosystem could be employed as a biologically inert topical skin agent and might be formulated in a cream formulation.

In order to achieve this aim, the surface of TiO_2 nanoparticles were modified with PEG and more dispersed and biocampatible nanosystem was produced (Figure 4.1). Next, PEG-TiO_2 were conjugated with Que and a fairly high conjugation ratio has been obtained (>95%). In Figure 4.1, it could be seen that bare TiO_2 exhibits aggregation/agglomeration behavior while PEG-TiO_2 is more dispersed. Additionally, the conjugation ratio which was expressed as >95% show that this nanostructure (Q-PEGTiO_2) could be a good delivery system for Que [116].

The size of nanoparticles is important paremeter for them because it affects nanoparticles' behaviour in clinical applications. Uptake, deposition, and clearance of nanoparticle are determined by the size [114]. There are few techniques measuring particular size in aqueous environment. DLS, or photon correlation spectroscopy (PCS) (also known as quasielastic light scattering- QELS), is one of the most popular technique which provide an accurate result about hydrodynamic size of nanoparticle. It would be very useful to know hydrodynamic size of bare TiO_2 and PEG-TiO_2, therefore DLS analysis was performed. Bare TiO_2 and PEG-TiO_2 , which were specified by the supplier as <100 nm, were suspended in suitable dispersant and their size were recorded by means of DLS. Hydrodynamic size of bare TiO_2 and PEG-TiO_2 were found as 307.0 nm and 193.0 nm, respectively (Figure 4.2). These outcomes confirm SEM (Figure 4.1) data, bare TiO_2 tends to agglomerate and form clusters. We obtained decreased size in PEG-TiO_2 compared to its bare counterparts, therefore it could be indicated that PEG modification decreases aggregation tendency of TiO_2. It was showed in the literature that TiO_2 nanopowders produce aggregates and agglomerates when dispersed in water [121] [122] [123]. Bendixen et al. used TiO_2 nanoparticles with a primary size of about 21 nm but provider declared that nanoparticles could be presented as agglomerates with a size of <150 nm.

In another study, two different TiO_2 NPs, having 21 nm (Sigma Aldrich) or 25 nm (Aeroxide® P25) primary size, were measured by DLS [122] , AF4-MALS [122] and TEM [121] , the size ranges obtained from these measurements were 70-100 nm, 49-87 nm and 25-85 nm, respectively. In addition to this, other studies found 100-600 nm sized aggregates of TiO_2 by performing AF4-MALS-ICP-MS [124]. According to Ji et al., TiO_2 P25 nanoparticles show ~200 nm size under the optimum sonication conditions [117]. They claimed that TiO_2 P25 sample contains some hard aggregates that are not seperated by ultrasonication. Some earlier studies support the idea that nanoparticles are found as large aggregates [125] [126]. When TiO_2 nanoparticles were dispersed in cell culture media without any dispersing agent, they observed decreased dispersion and high throughput DLS measurement indicated that TiO_2 suspension had agglomerate size between 770 nm to 1052 nm [117]. Aggregation of PEG-TiO_2 and Q-PEGTiO_2 are evident in Figure 4.3, as well. Nanoparticles were photographed before vortex, right after 15 sec vortex, and 2 min later, respectively. During storage, nanoparticles aggregates over time (Figure 4.3A) but vortex could be a transient solution to make them homogeneous (Figure 4.3B) however 2 minutes after the vortex, nanoparticles accumulated at the bottom part once again (Figure 4.3C).

On the purpose of determining cellular viability after treatment regimes, 3T3 cells were exposed to Q-PEGTiO_2, Que, or PEG-TiO_2 for 24 or 48h and viability results were given as absorbance values (Figure 4.4). In the case of 24h treatement, cellular death was not observed in Q-PEGTiO_2-treated groups however Que showed a great lost in viability. Then, 48h treatment was done to see the response of the cells to the increased time of incubation. This time of incubation brought viability lost in all groups even in the UC group. 3T3 cells have 18 h doubling time and during 48h incubation they may become crowded in limited area. Therefore, it is normal to see slightly decrease in the viability. In the case of 48h Q-PEGTiO_2 treatment, cells were detected with reduced viability compared to 24h treated counterparts. 48h-treated Que group is still the group which has the lowest viability. All these results revealed that 100 μg/ml Q-PEGTiO_2 treatment for 24h was the safest condition in terms of viability.

Que is well known flavonoid and it has been taking attention due to its highly desirable antioxidant effect by scavenging free radicals and chelating metal ions. However,

antioxidant property of Que could shift to pro-oxidant effect at high concentrations or longer incubation time [127]. Que-induced cytotoxicity on rat thymocytes was studied and it was observed that Que at 3 μM or less did not have any cytotoxic effect but when its concentration is increased to 30 μM, significant cell death has begun to appear [128]. Additionally, other studies indicated that Que also exhibit mutagenic activity if there is no liver-mediated metabolism [129]. In accordance with the literature, cellular toxicity was observed in Que-treated cells. On the other hand, almost no toxicity was seen in the 24h treatment of Q-PEGTiO$_2$. In the case of TiO$_2$, studies presented that TiO$_2$ nanoparticles reduce viability in a mass-based concentration and size-dependent manner [130]. Similarly, other studies showed that TiO$_2$ nanoparticles induced cytotoxicity in a time-dependent manner after 24h exposure [131]. In the case of our study, we did not observe any cytotoxic effect in Q-PEGTiO$_2$-treated cells.

Studies show that nanoparticles are taken in cells by highly regulated mechanisms which contain complex molecular interactions. Cellular membrane is composed of lipid bilayer and embedded proteins. Nanoparticles have to overcome cellular membrane to reach inside the cell. Cellular uptake of nanoparticles is achieved by different pathways determining nanoparticles' intracellular fate, function, and biological effects [113]. Nanoparticles taken in cytoplasm can interact with subcellular organelles or intracellular structures and express their biological effects [114]. In order to check whether Q-PEGTiO$_2$ and Que penetrate into cells, laser scanning confocal microscope with 63X objective was used. The autofluorescence properties of Que was utilised to track Que and nanosystem within cells. Our results showed that both Q-PEGTiO$_2$ and Que have ability to enter into cells and they locate mainly in the cytoplasmic compartments in the cells (Figure 4.5 and 4.6). When Q-PEGTiO$_2$-treated cells were observed for their morphology after the treatment, it was seen that cells protect their viability and characteristic shapes. On the other hand, Que-treated cells lost their typical shape after long exposure time. This finding is convenient with viability results which showed that Que at that concentration could become toxic for the cells while Q-PEGTiO$_2$ at the same concentration does not show any toxicity. In literature, studies examined cellular internalization of TiO$_2$ NPs and they found that NPs were present within cytoplasmic vesicles in the form of agglomerates [132]. Additionally, another study analyzed cell enterance of TiO$_2$ NPs by means of Raman spectroscopy and atomic force microscopy (AFM) and revealed that nanoparticles, which

had about 100 nm size themselves, transported into cells via endosomes and they became aggregated into cytoplasm [133].

When we found out that both Que and Q-PEGTiO$_2$ penetrated into the cells, we wanted to determine the time when they reached the maximum level inside cells. With the purpose of this, mitochondria of 3T3 cells were stained with MitoTracker Red and colocalization analysis was performed. In figure 4.5 and figure 4.6, confocal images of Q-PEGTiO$_2$- or Que-treated cells with MitoTracker Red staining as merged channels with scatter grams were shown. Confocal images were subjected to colocalization analyses and obtained coefficients were analyzed to see if the cellular uptake of nanoparticles changes upon the treatment are significant or not. Our results showed that entrance of both Que and Q-PEGTiO$_2$ into cells increased by time. Additionally, both of them showed their maximum entrance inside the cells at 12th hour. Althought Que indicated better penetration, the accumulation of both Que and Q-PEGTiO$_2$ within cells has shown increase over time and reached its maximum level at the 12th hour (Figure 4.8). After the maximal peaks, a slight decrease was observed in both groups' cellular penetrances. Previous studies showed that silica and polystyrene nanoparticles have entered into cells in minutes and their enterance increased by time until they reach the saturation point [134] **.** Additionally, other studies analyzed size- and time-dependent cellular uptake of silver nanoparticles (AgNPs) which show different diameters (5, 20, 50, and 100 nm). According to their ICP-MS measurements, after 12h exposure to AgNPs, 100 nm-sized nanosystem showed 76.2 % uptake efficiency but this ratio decreased to 66.1 % at 24th hour. When they interpreted the results as regards to size dependency, they showed that 100 nm sized AgNP had the highest uptake efficiency compared to smaller size NPs. Also, they found that 20, 50, and 100 nm AgNPs were mainly located in endocytic vesicles like lysosomes and early endosomes. In terms of 100 nm AgNPs, they could not found them in nucleus even after 12th h of treatment but the nanosystem uniformly dispered in the cytoplasm. In this study, it was specifically indicated that when estimating cellular uptake efficiency, exocytosis should be considered. Their findings showed that, exocytosis efficiency may change accordingly size of AgNP, therefore it is normal to expect effective penetrance in larger AgNPs because smaller NPs could might penetrate into cells less effectively but leave the cells easily [135]. In another study, it was showed that cellular uptake of NPs are time-dependent. Cells were exposed to NPs for 0 to 6h and fluorescence signals from the NPs were detected at 30 min, near the cell membrane. When

the time of exposure increased, fluorescence signals became more intense and NPs were observed inside the cell mostly. However, after 4h treatment, no increase in fluorescence signal was observed. Therefore, they suggested that NP penetrance inside cells is time-dependent and it increases until the limited saturation level [136]. Similarly, our results demonstrated that Q-PEGTiO$_2$ shows time-dependent cellular uptake and the saturation point of the Q-PEGTiO$_2$ is 12th hour. After that hour, decreasing signals were detected inside the cells.

In the next step, we examined Q-PEGTiO$_2$'s in vitro antioxidant effect against to DPPH free radical. Q-PEGTiO$_2$ and Que could donate electron or hydrogen atom to DPPH and this activity was measured from the bleaching of a purple-colored methanol solution of DPPH. During this experiment, dissapperance of purple color of DPPH might be due to presence of antioxidant in the reaction medium [119]. Que is well known antioxidant and, as expected, acted radiply at scavenging electrons. It showed maximum antioxidant activity at 2nd hour of treatment (Figure 4.8). In literature, it was indicated that Que at high concentrations react with DPPH radical rapidly and reach the steady state conditions almost immediatelly [137]. In our case Que exhibited two steps activity, firstly, Que donates its labile hydrogen atoms to DPPH and exhibits rapid scavenging activity. Then, the oxidation-degredation products remaining from Que shows the second scavenging activity and finally reach a constant value (Figure 4.8). The results obtained Que radical scavenging action was consistent with the literature [138]. Previous studies indicate that the degree of disappearance of purple color is related to the concentration of antioxidant [139]. The 50 μg/ml of Que group was used as an internal control and it showed half level of 100 μg/ml dose activity with a same pattern. On the other hand, Q-PEGTiO$_2$ showed its effect gradually and quite slowly compared to Que activity. However, Que activity has decreased by time after 2 hours but Q-PEGTiO$_2$ showed slightly increase on its radical scavenging activity (Figure 4.8). Increasing radicals scavenging activity of Q-PEGTiO$_2$ emphasize that Que retained its antioxidant activity after conjugation to nanosystem [140]. In the case of evaluation that dramatic difference in radical scavenging activities of Que and Q-PEGTiO$_2$, it must be considered that Que shows lethal effect at high doses (Figure 4.4).

In summary, we prepared a nano system which is a good carrier for Que molecules. In the literature, the number of studies have been trying to optimise best nanocarrier for Que

and this study might be an approach for it. We found that, our nano system shows suitable characteristic properties, high Que conjugation and almost no toxicity. The nanosystem expresses in vitro antioxidant activity and have ability to penetrate into cells and demonstrate homogenous distribution in cytoplasmic compartments. As a future prospect, this Q-PEGTiO_2 nanosystem could be formulated in topical cream formulation to improve skin's antioxidant capacity.

6. REFERENCES

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APPENDIX A- Chemicals

Chemicals	Supplier Company
DAPI	Life Technologies, USA
Distilled water	Milipore, France
DMSO	Sigma, Germany
DMSO.	AppliChem, Germany
DMEM.	Thermo Fisher Scientific,
DPBS	PAN, Germany
DPPH	Sigma, Germany
Ethanol	Merck, Germany
FBS	Pan, Germany
Glycerol	Sigma, Germany
Glycine	Molekula, UK
HCl	Merck, Germany
Liquid nitrogen	Karbogaz, Turkey
Methanol	Sigma, Germany
Mito-Tracker Red	Life Technologies, USA
NaOH	Merck, Germany
Paraformaldehyde	Sigma, Germany
PBS	PAN, Germany
PEG6000	Sigma, Germany
Penicilin-Streptomycin	Life Technologies, USA
Quercetin	Sigma, Germany
TiO_2	Sigma, Germany
Trypan Blue	NanoEnTek, Korea
Trypsin-EDTA	PAN, German

APPENDIX B- Equipment

Equipment	Supplier Company
Autoclave	Hirayama, Hiclave HV-110, Japan
Balance	Sartorius, BP221S, Germany
Cell Counter	NanoEnTek, Korea
Centrifuge	Kendro Lab. Prod., Multifuge 3S-R, Heraeus, Germany
	Eppendorf, 5415D, Germany
	Eppendorf, 5424R, Germany
	Eppendorf, 5430R, Germany
	ScanSpeen Mini, Labogene, Denmark
CO_2 Incubator	Binder, Germany
Computer Software	CoLocalizer Pro 3.0.2
	GraphPad Prism 5
Depfreeze	-80°C, Kendro Lab Prod., Heraeus Hfu486Basic, Germany
	-20°C, Bosch, Turkey
Distilled Water	Millipore, Elix-S, France

Hemocytometer	Hausser Scientific, Blue Bell Pa., USA
Ice Machine	Scotman Inc., AF20, USA
Laminar Flow	Kendro Lab. Prod., Heraeus, HeraSafe HS12, Germany
Liquid Nitrogen Tank	Taylor-Wharton, 3000RS, USA
Magnetic Stirrer	VELP Scientifica, ARE Heating Magnetic Stirrer, Italy
Microliter Pipettes	Gilson, Pipetman, France
	Eppendorf, Germany
Microscope	LSM 710, Confocal Laser Scanning Microscope, Zeiss, Germany
	Olympus CKX41, Japan
Microscope Slides	0.17mm, Thermo Fisher Scientific, USA
Microtiter Plate Reader	Microplate Reader 680, Biorad, USA
pH Meter	WTW, pH540 GLP MultiCal, Germany
Refrigerator	Bosch, Turkey
Shaker-Rotater	Gyro-rocker SSL3, Stuart, UK
	IKA KS 260 Basic, USA
	Labquake, ThermoScientific, USA
Spectrophotometer	Tecan Microplate Reader, Life Sciences, Switzerland

Vortex	Velp Scientifica, Italy
Water Bath	Huber, Polystat ccl, Germany

APPENDIX C- Solutions

4% PFA

4 g Paraformaldehyde

70 g dH_2O

200 µl 5M NaOH

10 ml 10X PBS

Complete to 100 ml with dH_2O

DPPH solution

0.003 g DPPH

750 µl methanol

Mounting medium

50% Glycerol in 1X PBS

PBS (10X)

80 g NaCl

2.25 g KCl

23.27 g $Na_2HPO_4.12H_2O$

2.05 G KH_2PO_4

Complete to 1000 ml with dH_2O

Adjust pH to 7.4

APPENDIX D- Molecular Biology Kits and Reagents

Commercial Kit	Supplier Company
Cell Proliferation Reagent WST-1	Roche, Germany