

ORIGINAL ARTICLE

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The effect of titanium dioxide particles of different shapes and sizes on the factor XII protein (intrinsic coagulation pathway) **Evren Kilinc***Acibadem Mehmet Ali Aydinlar University, School of Medicine, Department of Biophysics, Istanbul, Turkey*

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**Abstract**

Titanium dioxide (TiO₂) nanoparticles are widely used in food, cosmetics, biomedical implants, and pharmaceutical fields. Their effect on blood coagulation in human plasma and blood have been demonstrated. In this study, it is aimed to investigate whether the TiO₂ particles of different shapes and sizes directly activate an intrinsic coagulation pathway protein factor XII (FXII). Two different TiO₂ particles were morphologically characterized and prepared in physiological buffer solutions. The hydrodynamic radii and zeta potentials were measured using dynamic light scattering (DLS). The particles were incubated at 20, 10, 5 and 2.5 µg/ml (final concentrations) with 5 µg/ml FXII protein and enzymatic activity was measured with the help of a substrate of activated FXII (FXIIa). The enzyme activity was also measured in plasma in the presence and absence of corn trypsin inhibitor (CTI), a highly specific inhibitor of FXIIa. It has been found that FXII protein is activated in a dose-dependent manner by TiO₂ particles, and this activation has been determined to occur independently of the particle hydrodynamic radius. However, it has been found that the zeta potential values of the particles in the buffer solution are directly related to the FXII enzymatic activity. It has been shown that TiO₂ particles can activate the coagulation protein FXII based on surface charge distributions. Considering the possible effects of these materials on blood coagulation, zeta potential values should be considered when used in food and pharmaceutical production.

Keywords: Titanium dioxide, particles, intrinsic coagulation pathway, factor XII**Introduction**

Recently nanoparticles have become a subject of great interest that offers important advantages in many areas. Titanium dioxide (TiO₂) is one of the most commonly used nanoparticles which are produced in various size fractions as fine particles (0.1-2.5 µm) and nanoparticles (less than 0.1 µm), and have a wide range of uses, including food (gum, chocolate, milk powder, sugar, etc.), cosmetics (toothpaste, shampoo, hair styling, etc.), biomedical implants and pharmaceutical fields [1]. It is also used with the E171 code to whiten food as a nutritional supplement [1, 2]. Due to the fact that TiO₂ nanoparticles are used more in sugars and chocolates than other foods children are exposed to these particles more than adults [2]. The primary way of getting these particles into the body is through the consumption of food, drink and medicines through the digestive system. On the other hand, if these

nanoscale particles are inhaled, they can also enter the circulation by crossing the air-blood barrier in the alveolar region [2, 3]. A series of studies has shown that the nanoparticles accumulate in after inhalation or oral exposure in lungs, digestive system, liver, heart, spleen, kidneys and heart muscle [4-8].

TiO₂ nanoparticles have also been shown to have an effect on blood coagulation in human plasma and blood [9-11]. Shahramian et al., demonstrated that TiO₂ coatings on zirconia surfaces used in prosthetic dentistry promoted blood coagulation [12]. In another study, it has been shown that blood with added TiO₂ nanotubes and blood in contact with gauze pads embellished with TiO₂ nanotubes showed significantly stronger clot formation at reduced clotting times [10].

In the case of tissue damage, blood coagulation causes the production of the thrombin enzyme, converting the fibrinogen into fibrin and ensuring that the bleeding stops. The tissue factor that occurs with tissue damage initiates thrombin formation by complexing with activated factor VII (FVIIa). This pathway is known as the extrinsic and physiological pathway of coagulation [13]. On the other hand, coagulation can also start through the intrinsic pathway when the

***Corresponding Author:** Evren Kilinc, Acibadem Mehmet Ali Aydinlar University, School of Medicine, Department of Biophysics, Istanbul, Turkey
E-mail: evrenkilinc@gmail.com

FXII protein is activated by physiological and non-physiological factors such as negatively charged surfaces (kaolin, silica, glass, etc.), misfolded proteins and microparticles [14]. Although blood coagulation is vital in stopping bleeding, its pathology (excessive coagulation) causes thrombosis, leading to stroke and myocardial infarction.

Even though the intrinsic pathway has no physiological contribution to blood coagulation, its importance has been emphasized in recent years by showing active contributions of FXII to the ischemic and thrombotic processes [14]. Thus, there is a growing interest for the use of FXII inhibitors as a new generation of safe anticoagulants [15]. Although the global effect of TiO₂ nanoparticles on blood coagulation has been demonstrated in human plasma and blood, there is no study directly related to its effects on FXII protein [9-11].

In this study, it was investigated whether FXII protein, which has a key part in the intrinsic coagulation pathway, was activated by titanium dioxide. It is assumed that the effects of the nanoparticles vary depending on their size, morphology, and dosage. Therefore, two nanocrystalline forms of TiO₂ nanoparticles, anatase and rutile, were used in the study, where concentration, size and surface charges were evaluated, and it is aimed to investigate whether these particles activate FXII protein as a result of its interaction with pure FXII protein (>95% by SDS-PAGE) and human plasma.

Materials and Methods

Characterization of TiO₂ particles using scanning electron microscopy

TiO₂ particles were a kind gift of Dr. Ceyda Koyuncu from TUBITAK MAM, Turkey. It occurs in three different variants according to its crystal structure: rutile, anatase, and rarely brookite. Anatase compared to rutile and brookite has more industrial applications; however, it is the most toxic form [1]. Two types of TiO₂ nanoparticles, anatase and rutile were morphologically characterized using the scanning electron microscopy (SEM) technique, obtaining the images with (Thermo Fisher Scientific QUATTRO S ESEM) operating at an acceleration voltage of 20 kV for anatase and rutile images. The device was incorporated an energy-dispersive X-ray (EDS) spectrometer that was used to evaluate the aerodynamic radius, surface properties of the samples. SEM samples were prepared by the deposition of nanopowder on top of carbon conductive tabs.

Preparation of TiO₂ particles in different buffer solutions

TiO₂ particles, were prepared in phosphate buffered saline solution pH: 7.4 (PBS tablet Sigma Aldrich), commonly used in biological research, tris buffered saline solution pH: 7.8 (50 mM Tris HCl, 12 mM NaCl) and HEPES buffered saline solution pH: 7.7 (25 mM HEPES, 175 mM NaCl) and the hydrodynamic radii were measured in each buffer. Experiments were continued with PBS solution where the particle agglomeration was the least. In the literature, addition of 1% bovine serum albumin (BSA) has been shown to reduce clumping in solution [16]. A commercial PBS solution (Sigma Aldrich SRE0036-250ML pH 7.4) containing 10 % BSA was diluted with PBS to a final concentration of 1% BSA and the buffer solution. All the buffers used in the experiments were sterile filtered using 0.2 µm filter.

Hydrodynamic radius (dynamic light scattering; DLS) and zeta potential measurements

The hydrodynamic radii of TiO₂ particles in buffer solutions were measured using the zeta potential LiteSizer 500 (Anton Paar, Germany), an expression of the surface charges of the particles in the liquid.

FXII enzyme activity measurement

The coagulation protein FXII, which is a proenzyme, turns into an active enzyme form when it encounters artificial surfaces and this enzyme activity can be measured spectrophotometrically with the help of a specific chromogenic substrate. TiO₂ particles in different concentrations (final concentrations of 20, 10, 5, 2.5 µg / ml) were prepared in PBS with 1% BSA, were mixed with pure FXII protein (>95% by SDS-PAGE) to a final concentration of 5 µg / ml of FXII (HCXII-0155 Haematologic Technologies, Inc. USA). S2302 (Chromogenix-S2302, Diapharma Group, Inc. USA) substrate was added (final concentration 0.88 mM) at 37 degrees Celsius and kinetic reading performed at 405 nm for 30 minutes (BioTek microplate reader PowerWave HT). The results are presented as absorbance at 405 nm.

FXII enzyme activity measurement in plasma

In this part of the study, control plasma produced for coagulation tests was purchased and used (Biolabo S.A.S, control plasma, REF: 1361, LOT: 071833A1). Similar to experiments with FXII pure protein, TiO₂ particles prepared in different concentrations (final concentrations of 20, 10, 5, 2.5 µg/ml) in PBS with 1% BSA were added in the control plasma. S2302 (Chromogenix-S2302, Diapharma Group, Inc. USA) substrate was added (final concentration 0.88 mM) at 37 degrees Celsius and kinetic reading performed at 405 nm for 30 minutes.

Experiments with control plasma were repeated in the presence of corn trypsin inhibitor (CTI; final concentration 120 µg / ml; CTI-01 Haematologic Technologies, Inc. USA), an inhibitor specific for activated FXII (FXIIa). In this way, it has been investigated whether the enzyme activity caused by the TiO₂ particles in the plasma is due to the FXII protein in the plasma.

Statistical Analysis

The effects of TiO₂ particles in different concentrations on FXII activation were evaluated with ANOVA one-way variance analysis and subsequently, the post hoc Tukey HSD test were performed to specify where the significant differences come from. UNIANOVA (Univariate variance) analysis was performed to determine which particle was more effective on FXII activation for the same doses. Data were presented as mean ± standard deviation and p <0.05 was considered significant. For all statistical analysis, SPSS 18 software was used.

Results

Characterization aerodynamic properties of TiO₂ nanoparticles by SEM and EDS

Chemical composition of the samples obtained from the EDS spectra are shown in Figure-1A and 1B right panel. The analysis of anatase and rutile TiO₂ nanoparticles showed that only Ti and O

elements were detectable, and no other compounds were identified for the samples studied.

SEM images for both types of nanocrystalline structures are shown in (Figure-1A and 1B left panel). Nanoscale globular or nearly

spherical particles were identified both for anatase and rutile. Average particle sizes were estimated as 62 ± 14 nm for anatase and 200 ± 53 nm for rutile using SEM micrographs by counting a minimum of 50 particles.

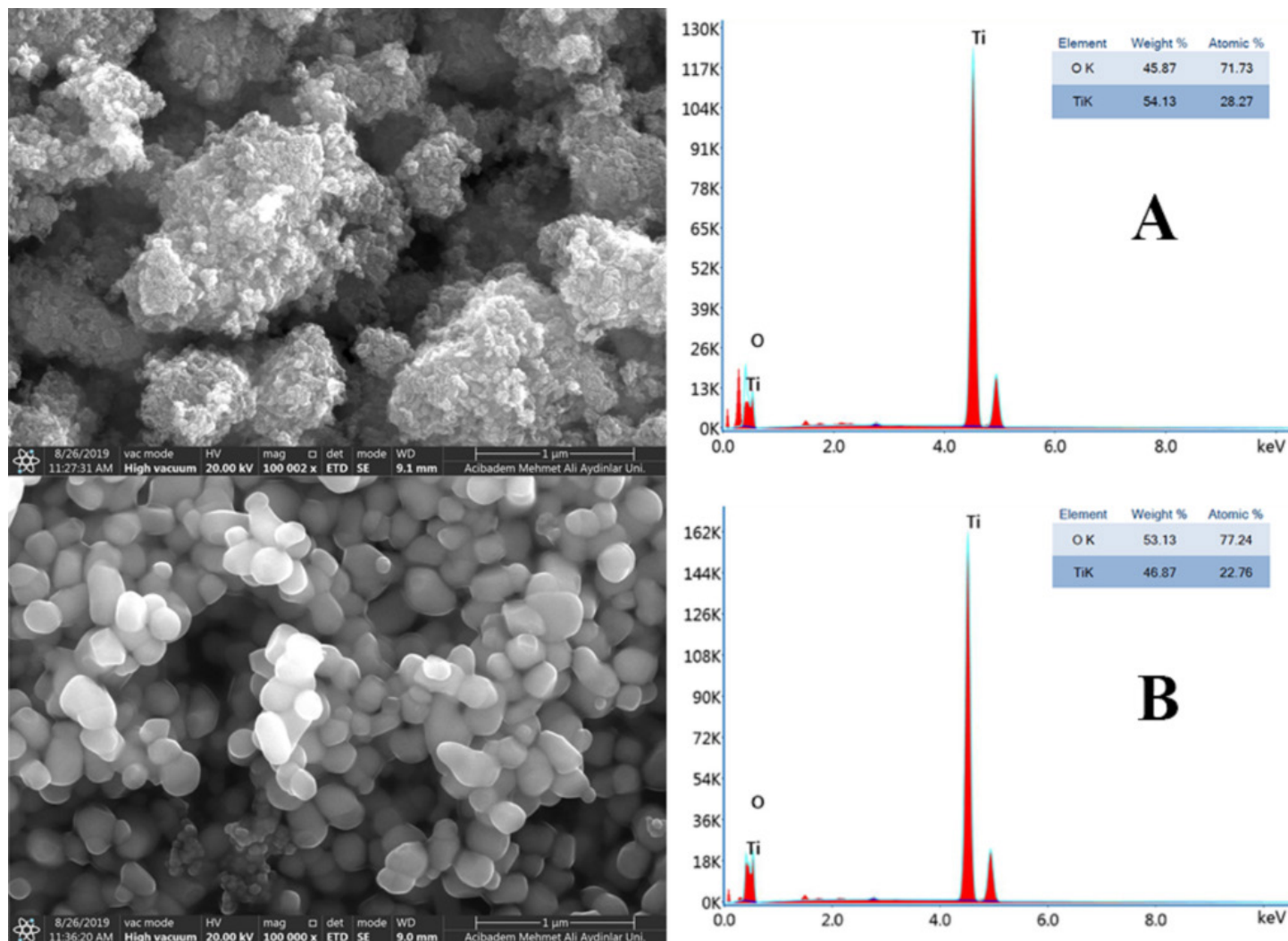


Figure 1. SEM and EDS images of TiO₂ particles A: No. 1 Anatase nanoparticles at magnification x50,000 B: No. 2 Rutile TiO₂ particles at magnification x 100,000

Characterization of hydrodynamic properties of TiO₂ nanoparticles by DLS

Hydrodynamic radii were measured for TiO₂ particles prepared in three different biological buffer solutions. The biological buffer solution, in which particle aggregation was generally the least, was found as the PBS buffer solution (Table 1).

Table 1. Hydrodynamic radius measurement results of TiO₂ particles 1 and 2 prepared in different buffer solutions

TiO ₂ Hydrodynamic radius (nm)		
Buffer	1	2
Tris-NaCl; pH: 7.8	2345	2915
HEPES-NaCl; pH:7.7	3082	2852
PBS; pH: 7.4	1875	2067

Addition of 1% BSA to the PBS buffer solution significantly reduced the particle aggregation for both nanoparticles in solution (Table 2).

Based on the DLS analysis the hydrodynamic diameter of TiO₂ particle 1 and 2 were measured as 534 nm and 258 nm respectively, whereas the zeta potential was measured as -11.4 mV and -13.2 mV respectively. In contrast to the SEM analysis results, the hydrodynamic radius of particle 1 was found to be greater than particle 2 (Table 2). This could possibly be due to the tendency of particle 1, with its smaller aerodynamic radius, to form clusters in colloidal systems.

Table 2. Hydrodynamic radii and zeta potential values of TiO₂ particles 1 and 2 prepared in PBS solution with 1% BSA

	TiO ₂ Hydrodynamic radius (nm)	Zeta Potential (mV)
1	534	-11.4
2	258	-13.2

TiO₂ particles at different concentrations cause FXII to be activated both in the purified system and in the plasma

Anatase and rutile TiO₂ particles (in PBS with 1% BSA) prepared with final concentrations of 20, 10, 5 and 2.5 µg/ml were incubated with pure FXII protein and the enzyme activity was measured for each concentration. It was observed that the both TiO₂ particles caused the activation of the pure protein (Figure-2A and 2B). Activity measurements were also carried out in plasma to observe matrix effects associated with endogenous interactions (ie interactions of endogenous proteins with FXII, such as kalikrein). Accordingly, it has been shown that both particles cause FXII

activity in a dose dependent manner in plasma experiments (Figure-3A and 3C). As expected, the activity level measured for the same dose was higher in plasma than pure protein. In order to ensure that the measured enzymatic activity is purely dependent to FXII protein activation the measurements were performed in the presence of CTI. CTI is a highly specific inhibitor that only affects trypsin and FXIIa, but not FVIIa, FIXa, FXa or thrombin. For this reason, CTI is used to distinguish between intrinsic and extrinsic coagulation stages. It was observed that in the presence of CTI, FXII activity was completely disappeared at low TiO₂ doses (2.5 and 5 µg/ml) and significantly disappeared at higher doses (10 and 20 µg/ml) (Figures-3B and 3D).

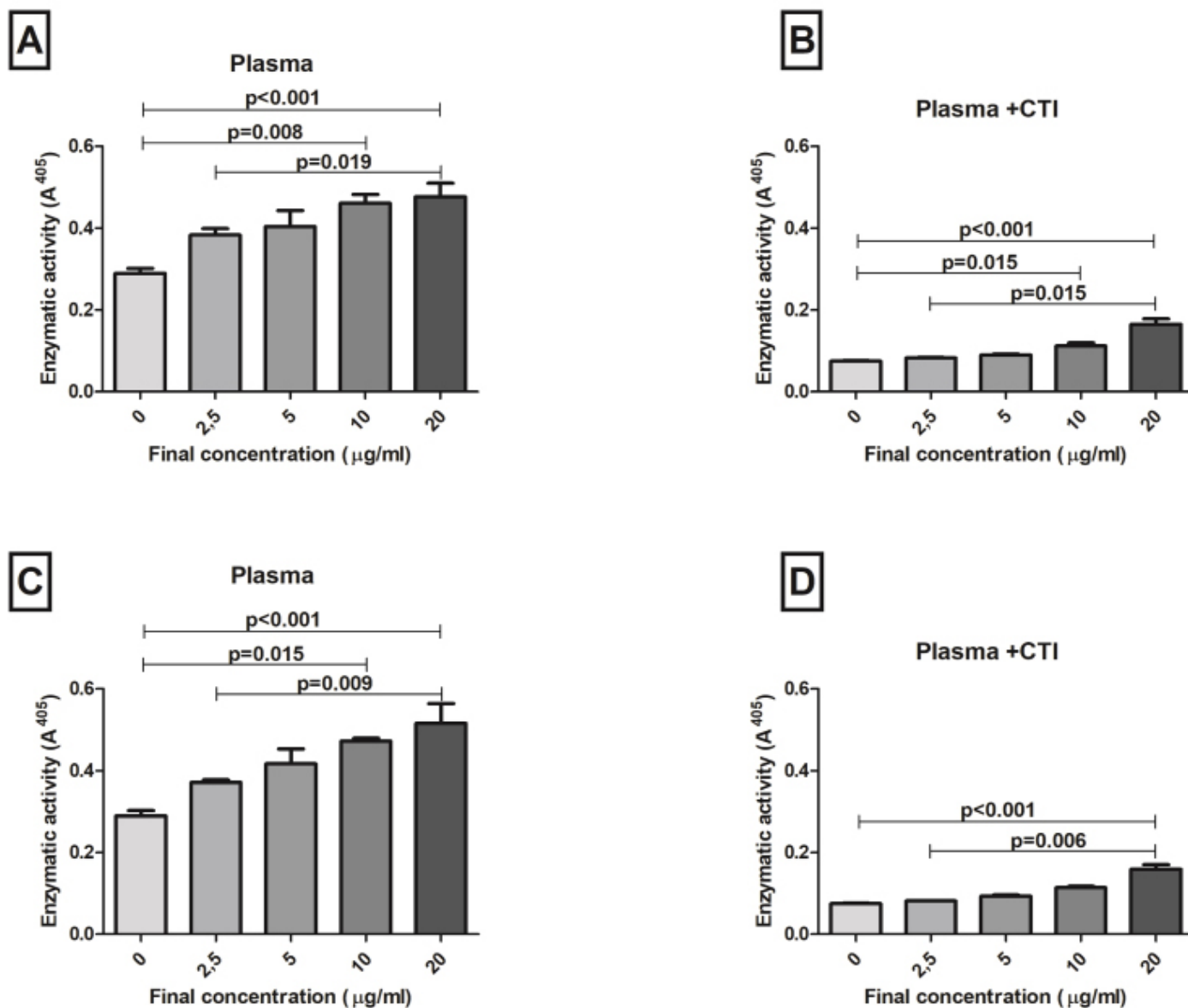


Figure 3. FXII Activity in the presence of TiO₂ in plasma as a dose-dependent manner.

A: No.1 in plasma, B: No.1 in CTI added plasma, C: No.2 in plasma; D: No.2 in CTI added plasma. Enzyme activity was measured at 405 nm and presented as mOD / minute.

(Regarding pairwise comparison of each concentration, adjusted p values are shown.)

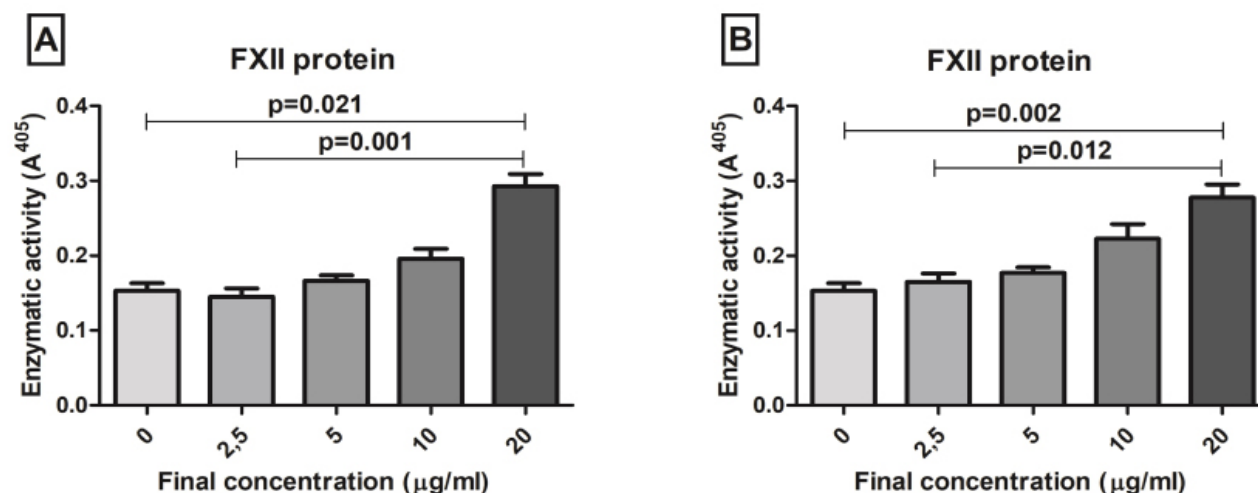


Figure 2. FXII activity in the presence of different doses of TiO₂.
A: No.1 in pure FXII B: No.2 in pure FXII. Enzyme activity was measured at 405 nm and presented as mOD / minute.
(Regarding pairwise comparison of each concentration, adjusted p values are shown.)

On the other hand, no significant difference was found when comparing the enzymatic activities of both particles in pure FXII protein, plasma and CTI added plasma for the same doses. The enzymatic activity results in CTI added plasma were extracted from the related results in plasma, and thus the enzymatic activity of the pure FXII protein found in the plasma was obtained. These data were compared for both particles using univariate analysis of variance, and no significant difference was found in terms of enzymatic activity.

Discussion

The aggregation in solutions containing TiO₂ particles was shown to be the least in PBS buffer with 1% BSA. Considering the use of particles in biological studies, this physiological solution can be used. In the study, the hydrodynamic radii and zeta potentials of TiO₂ particles were measured and then it was shown that they activate the FXII protein at doses (final concentration) above 5 µg / ml. When FXII activation by TiO₂ particles is evaluated together with hydrodynamic radius and zeta potential values, each parameter has been shown to have an effect on FXII activation. Considering its hydrodynamic radii, TiO₂ particles number 1 have a larger hydrodynamic radius than particles number 2, but their zeta potential value is lower (Table-2). When both FXII and plasma activities are evaluated, the absence of significant difference in enzymatic activity for same doses indicates that both the hydrodynamic radius and the zeta potential value should be evaluated together.

It is known that FXII protein is a proenzyme and when it meets negatively charged surfaces, this protein is activated and shows enzymatic activity [17]. Recently, the FXII protein and its activation in the body has been associated with inflammatory-based diseases such as Alzheimer's disease, allergy, anaphylaxis, and thrombotic diseases such as atherothrombosis, ischemic stroke, and deep vein thrombosis [18]. Pele et al. treated the volunteers whom they found to have normal intestinal permeability with TiO₂ capsule by mouth (50 mg in 2 doses) found that the amount of particles in the blood of the volunteers reached the highest level in the 6th hour [19]. In another volunteer study, TiO₂ particles administered

orally were detected in the blood and it was emphasized that the amount determined showed personal differences [20]. Considering that TiO₂ is frequently used in food additives and drug content, and Ti is used in medical implants, this element has an important place in our lives.

The doses used in the study are higher than the doses detected in the blood after single dose of TiO₂ treatment reported in the literature. However, given the contribution of blood cells, longer systemic biological survival times and repeated doses, the FXII protein and thus blood coagulation may become active if these doses are reached. Supporting our assumption, in a study it was reported that TiO₂ particles were detected in 15 post-mortem human liver and spleen, the dose in some livers was at a level that could cause liver damage [21].

Consequently, considering the repeated doses, accumulation in organs and their transition from the gastrointestinal tract to blood; TiO₂ particles might affect the course of inflammation and thrombotic diseases by triggering blood coagulation. Studies are needed where these risks are carefully evaluated.

Further studies are needed focusing on understanding whether the zeta potential modifications on TiO₂ particle surfaces with same hydrodynamic diameter modulates activity of FXII protein.

Conflict of interests

The author declares that there is no competing interest.

Financial Disclosure

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Ethical approval

No ethical approval is needed for this research.

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