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6 2 **Surface Interactions and Degradation of a Fluoroquinolone Antibiotic in the Dark in**
7 3 **Aqueous TiO₂ Suspensions**
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Abstract

Fluoroquinolone antibiotics (FQ) are important drugs used in human and veterinary medicine. Their detection in natural waters and waste water treatment plants, along with increased resistance to FQ among some bacteria, have generated an increased interest in the fate of these drugs in the environment. Partitioning of FQ between an aqueous solution and attendant substrates depends, in part, on the surface reactivity of the adsorbent, commonly a function of particle size, surface charge, and functional groups. This study investigated the surface interactions between the FQ drug ofloxacin (OFL) and titanium oxide (TiO₂), a common catalyst and widely-observed constituent in many consumer products. Raman and fluorescence spectroscopic techniques, as well as LC/MS, were used to determine the OFL moieties present on TiO₂ surfaces and in attendant solutions. Raman spectra indicate that the C=O (ketone) group of the quinolone core, the NH⁺ of the piperazinyl ring, and CH₃ of benzoxazine core are the most active in sorption onto the TiO₂ surface. Raman spectra also show that the sorbed benzoxazine-quinolone core and piperazinyl moieties are readily desorbed from the surface by re-suspending samples in water. Importantly, we found that OFL could be degraded by reacting with TiO₂ even in the dark. Complementary LC/MS analysis of the attendant supernatants indicates the presence of de-piperazinylated and de-carboxylated OFL breakdown products in supernatant solutions. Together, both Raman and LC/MS analyses indicate that TiO₂ breaks the compound into piperazinyl and carboxylate groups which attach to the surface, whereas de-carboxylated and hydroxylated quinolone moieties remain in solution. The present study thus identifies the sorption mechanisms and breakdown products of OFL during dark reactions with TiO₂, which is critically important for understanding the fate and transport of OFL as it enters the soil and aquatic environment.

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64	Key Words
65	Fluoroquinolone Degradation, Titanium Oxide Nanoparticles, Ofloxacin, Antibiotics in
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1. Introduction

Drugs used in both human and veterinary medicine have been detected in surface water (Zuccato et al., 2000; Kolpin et al., 2002) and ground water (Barnes et al., 2008; Fick et al., 2009) in concentrations ranging from ng/L to mg/L. These include antibiotics (Kummerer, 2009a,b), as well as their metabolites (Michael et al., 2014), which are a special concern because they can cause antibiotic resistance in certain bacteria. (Normark and Normark, 2002; Giger et al., 2003; Chander et al., 2005; Kim et al., 2007; Yu et al., 2009; Davies and Davies, 2010). This phenomenon is currently considered a major public health threat (CDC, 2013).

One group of antibiotics receiving considerable attention is the fluoroquinolones (FQ). Their detection in wastewater treatment plant effluent (Nakata et al., 2005; Vieno et al., 2007), surface waters (Kolpin et al., 2002), ground water (Fick et al., 2009) and soils (Pico and Andreu, 2007) highlights the need to understand the fate and transport of these drugs in natural soil/sediment-water systems.

Fate and transport of FQ in aqueous systems are controlled, in part, by sorption of the compounds to sediment minerals and attendant particles occurring in suspension. Some of the suspended particles will be nanoparticles of rock-forming oxides, including TiO₂. Though TiO₂ is not an abundant soil oxide (10th in crustal abundance at ~ 0.4% by weight) (Perkins, 2002), its importance is augmented by widespread industrial use and subsequent anthropogenic sources (Kaegi et al., 2008; Nowack and Bucheli, 2007), including processed food (Weir et al., 2012).

Some previous studies have investigated the interactions between FQ and TiO₂ in aqueous solutions. For example, sorption and removal of the FQ, ofloxacin, have been demonstrated (Van Wieren et al. 2012). That study focused on isotherms at different pH and presented evidence based on LC/MS data for the removal of OFL parent molecule from solution, as well as the simultaneous generation of FQ degradation products, without detailed understanding of the mechanisms. Other studies have shown the TiO₂-enhanced photocatalytic degradation of FQ when exposed to UV radiation (Ohko et al., 2002; Calza et al., 2004; Doll and Frimmel, 2005; An et al., 2010). One study (Paul et al., 2007) demonstrated that some FQ degrade in visible light in the presence of TiO₂. While these studies have provided important information on the photochemical degradation of FQ in solution, little is known regarding the mechanisms and modes of FQ sorption on TiO₂, and whether TiO₂ also catalyzes the degradation of FQ in the dark. The goals of this study were to test whether or not TiO₂ enhances degradation of FQ without the input of light, and also to investigate FQ components in solution and the attendant FQ components sorbed to the TiO₂ surface, as the result of interaction in the dark.

2. Methods

2.1 Materials

The representative FQ drug used in this study was ofloxacin (OFL) (Sigma O8757, CAS 82419-36-1; MW = 361.4), a synthetic antibiotic. OFL forms cationic, zwitterionic, and anionic species in solution, depending on pH (Park et al., 2002). The structure of OFL and its speciation is illustrated in Supplementary Material A1.

TiO₂ used in experiments was 25-nm anatase with a nominal specific surface area of 200 m²/g, (Sigma 637254, CAS 1317-70-0), as provided by the manufacturer. This does not account for agglomeration into larger aggregates upon suspension in solution (French et al., 2009; Tso et al., 2010). Particle size distribution measurements were performed in suspensions using a Brookhaven 90 Plus Instrument (B1-MAS). At pH 3.2, TiO₂ occurs with an average particle size of 556 (+/- 4) nm, as a bimodal distribution about 7% (113 nm) and 93% (644 nm). At pH 6.4, TiO₂ occurs with an average particle size of 1719 (+/- 109) nm, as a bimodal distribution about 28% (755 nm) and 72% (3315 nm). This increase in particle size of one to two orders of magnitude for aggregates in aqueous suspension is consistent with previous observations (Fang et al., 2009). The surface charge of TiO₂ nanoparticles in water is characterized by the point of zero charge (pzc). The pzc is the pH below which the net surface charge of the oxide is positive (Drever, 1997). Zeta potential measurements in this study (Brookhaven 90 Plus, B1-MAS) indicate a pzc between pH 5.06-5.41. (Supp A1).

2.2 Protocol

Twenty-one experiments were performed by mixing nanoparticle suspensions of different pH with OFL solutions of known concentrations. TiO₂ stock suspensions of 194 to 258 mg/L (mass solid/volume liquid) were prepared in 18.2 MΩ cm⁻¹ deionized water, followed by pH adjustment with dilute NaOH or HCl. Stock OFL solutions were also prepared in deionized water at concentrations ranging from 94-387 μM. The pH values of stock solutions of TiO₂

suspensions and OFL solutions were measured directly. The pH of experimental mixes of TiO₂ and OFL were calculated based on dilutions. Experiments were performed at pH 3.2, 3.3, 6.1, 6.4, 6.6, and 11.2, with the objective of experimenting at cationic, zwitterionic, and anionic conditions for OFL. Experiments occurred by loading 1 mL of TiO₂ stock suspension and 1 mL of OFL solution into a 2 mL polypropylene centrifuge tube, followed by vigorous shaking for ~ 5 min. Experimental concentrations for substrate and solute ranged from 97-129 mg/L and 47-194 µM, respectively. Tubes were paired with a control sample to which no TiO₂ was added. Tubes were then left undisturbed for 3-14 days, in the dark, at room temperature, during which the nanoparticles settled out of suspension. Experiment duration (days) was selected to be on the same time scale as common precipitation events and their subsequent infiltration rates, with water movement from a loamy soil surface into the subsurface vadose zone (Dingman, 2002).

FQ concentrations used in this study were higher than most environmental detections, but not all. Some surface waters have FQ concentrations 3-4 orders of magnitude lower (Kolpin et al., 2002), some wastewater treatment plant influent concentrations are 2 orders lower (Vieno et al., 2007), but some polluted surface waters and treatment plant effluent contain equivalent concentrations of FQ to those used in this study (Fick et al., 2009). Other workers have also used similar concentrations in FQ laboratory experiments (Figuerro-Diva et al., 2010; Conkle et al., 2010) for reliable quantitative analysis and to model long-term loading in natural systems. TiO₂ suspension concentrations were commensurate with OFL concentrations and were about the minimum level necessary for routine centrifugation, collection and spectroscopic analysis in this study.

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4 180 Samples were prepared for analysis in a series of steps, beginning with careful removal of
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6 181 approximately 90-95% of the liquid. The decanted supernatant was divided into 3 separate
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10 directly, and a separate aliquot was filtered first through a sterile PALL Acrodisc® PF 0.8/0.2
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12 183 µm syringe filter with a hydrophilic polyethersulfone matrix before analysis. These filters
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14 184 removed TiO₂ and parent OFL species. This was demonstrated by trials of filtering and analysis
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16 185 of control solutions, solutions with only OFL added or only TiO₂ added. The remaining
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18 186 supernatant suspension was centrifuged at 21,000g (rcf) and the clarified liquid was analyzed
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20 187 separately. The small amount of suspension remaining in the experiment sample tube was then
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22 188 wicked off with a lab tissue, leaving a TiO₂-substrate slurry, a portion of which was removed and
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24 189 analyzed by Raman spectroscopic techniques. Following analysis of the nanoparticle substrate,
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26 190 the remaining TiO₂ nanoparticles were re-suspended in the tube with water lacking OFL and
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28 191 TiO₂. This sample was then vigorously mixed for ~ 5 minutes, followed by centrifugation and
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30 192 decantation. Half of this supernatant was analyzed directly, and the other half filtered before
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32 193 analysis. The remaining TiO₂ substrate (post-re-suspension) was also analyzed by Raman
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34 194 spectroscopy. Supernatants from the initial experiments were diluted by factors of 50 or 100x
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36 195 and re-suspension liquids were diluted by 2x or 4x before analysis. Selected supernatants were
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38 196 separated for LC/MS analysis and loaded into 2 mL HPLC vials with a sterile syringe.
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Raman spectroscopy was used to investigate the sorption mechanisms of OFL onto TiO₂ nanoparticle substrates. A small sample of substrate slurry was placed on a gold-coated slide and mounted on the microscope stage of the Raman instrument for analysis. Raman spectra were collected with a Renishaw micro-Raman system (Renishaw Inc., New Mills, UK) equipped with a 300 mW near-infrared (NIR) diode laser (λ 785 nm) and a CCD array detector. Signal to noise ratios were low when the sample was a suspension, but improved continuously as the material dried, changing from a wet slurry, to a slurry, to a damp paste. In this drying sequence, spectra collected from damp pastes were considered the optimal representations of sorbed OFL. OFL peaks were compared to spectra collected for OFL stock precipitated from aqueous solution (Supplementary Material A2). Assignments for prominent Raman shift peaks in the current study were primarily based on ATR-FTIR (Goyne et al., 2005) and FTIR-Raman (Sahoo et al., 2012) analysis of OFL, with some minor peak assignments referenced to other sources.

2.3.2 Fluorescence Spectroscopy

Both the filtered clear supernatant and the unfiltered suspension from experiments were analyzed for parent OFL by fluorescence spectroscopy. Quantitative analysis measured the decrease in OFL fluorescence due to the presence of TiO₂. Data were collected with a HORIBA Scientific Aqualog-800C spectrometer/fluorimeter instrument equipped with a 150W continuous output xenon lamp and CCD detector. Signals were corrected for inner-filter effects and 1st order Rayleigh scattering with AquaLog® 3.6 software, and post-processed with Origin®. Excitation wavelength (λ_{ex}) of 293 nm was used to document fluorescence at 500.7 nm emission wavelength (λ_{em}) for all samples.

2.3.3 LC/MS

Selected supernatants were also analyzed by liquid chromatography/mass spectroscopy (LC/MS) to identify OFL degradation products. Product m/z values were determined by a single quadrupole mass selective detector (Agilent 1100 LC/MSD SL). Sample injections of 20 µL were separated on Kinetex EVO 150 C-18 column (100 x 4.6 mm, 5 µm diameter particle size) at a flow rate of 0.8 mL/min using an isocratic mobile phase of 5% acetonitrile/95% water with 0.3% v/v formic acid. UV absorbance was monitored by diode array detection at 242, 254, 287, 293, and 329 nm wavelength. Analytes that did not absorb at the selected wavelengths were distinguishable at different retention times on the total ion count (TIC) spectra. The MSD was operated in the atmospheric pressure ionization – electrospray mode with positive ion detection, and a mass fragmentor voltage of 40 V. Compounds were identified in both scan mode (100-800 m/z) and selected ion mode (SIM) with 24 separate m/z intervals (± 0.1) ranging from 119.1-119.2 to 363.2 m/z.

3. Results

3.1 Sorption of OFL

Fluorescence spectroscopic analysis of samples of OFL mixed with a TiO₂ suspension at pH 6.1 indicates a nominal partitioning of 1071 L/kg, or 5.4×10^{-3} L/m². This value is consistent with previously-published results (1288 ± 217) L/kg (Van Wieren et al. 2012) from an isotherm at nearly the same pH (6 vs. 6.1). Example spectra are illustrated in Supplementary Material A3.

3.2 OFL on TiO₂-pH Comparison

Figure 1 shows representative Raman spectra of OFL on TiO₂ collected from damp pastes from experiments performed at pH 3.3, 6.4, and 11.2. These represent conditions where OFL is mostly cationic, zwitterionic, and anionic, respectively. A spectrum from OFL stock precipitated at pH 6.6 (Supp. A2) is shown for comparison. While the overall appearance of the spectra is similar, some distinct differences are evident. Several peaks are blue-shifted for OFL on TiO₂, compared to the stock OFL precipitate. These are peaks assigned to C=C stretching (1545 cm⁻¹) in the quinolone moiety, plus C-H₃ stretching (1444 cm⁻¹) and C-O-C ether stretching (1042 cm⁻¹) in the benzoxazine moiety. These shifts are relatively independent of pH. A red-shift of the C-C stretching/C-H deformation peak (1142 cm⁻¹) is also observable and is slightly greater at pH 6.4. The pH 6.4 spectrum also shows the emergence of a peak at 1299 cm⁻¹, probably attributable to C-H₂/C-H₃ twisting mode (Nyquist, 2001) of the benzoxazine core component. The peak assigned to symmetric stretching of COO⁻ (1340 cm⁻¹) is present in all samples.

3.3 Desorption of OFL

Figure 2 compares the Raman spectrum of OFL on TiO₂ substrate after an initial mixing experiment with a spectrum of the same substrate after re-suspension in OFL-free solution (rinsing) and subsequent decantation of the supernatant. Two distinct peaks are present in the spectrum of OFL attached after initial mixing. These are a C=O stretching (pyridine)/NH⁺ deformation (piperazinyl) peak at 1626 cm⁻¹ and the peak assigned to the protonation and CH₂/CH₃ deformation of the piperazinyl ring at 1401 cm⁻¹. Three smaller peaks are also present after initial mixing, the C=C stretching (quinolone) peak at 1559 cm⁻¹, a C-H₃ stretching peak from benzoxazine at 1454 cm⁻¹, and a peak assigned to the symmetric stretching of the

carboxylate anion at 1342 cm^{-1} . After re-suspension/rinsing of the substrate, the spectrum shows a diminishing of the pyridine and piperazinyl components and an increase of the CH_3 components on the surface. The carboxylate anion peak is also relatively less distinct after re-suspension. Those parts of the OFL molecule attributable to the diminished peaks must have been removed/desorbed from the substrate during re-suspension, while CH_3 groups remained attached to the surface. Based on fluorescence spectroscopic analysis of the rinsate, a maximum of ~18% of the sorbed OFL could be removed from the TiO_2 substrate.

3.4 OFL Components in Solution

Supernatants from OFL- TiO_2 experiments were analyzed by LC/MS techniques to identify OFL degradation products in solution. Figure 3 shows mass spectra from the liquids attendant to the substrates analyzed in Figure 2. Figure 3a is a spectrum of the centrifuged supernatant from the experiment of $55\text{ }\mu\text{M}$ OFL and 129 mg/L of TiO_2 , at pH 6.1, after 139 hours (about 6 days) of interaction. This liquid was present with the substrate analyzed by Raman spectroscopy, the spectrum of which is indicated by a dashed line in Figure 2. In addition to OFL parent ($m/z = 362.2$, not shown), OFL breakdown products are present. These include quinolone core components which are missing the piperazinyl group and are also de-carboxylated and hydroxylated and/or de-methylated. An m/z value corresponding to a succinylated piperazinyl group is also present. Figures 3b-d are spectra from the rinsate after re-suspension of the same substrate attendant to the liquid of Figure 3a. These mass spectra correspond to the Raman spectrum of the desorbed substrate indicated by a solid line in Figure 2. OFL breakdown products desorbed from the TiO_2 substrate include masses assignable to de-methylated quinolone cores and ethyl-1 piperazine carboxylate (Figure 3b), de-carboxylated/hydroxylated parent (Figure

3c), and intact OFL parent molecule plus parent with a de-methylated piperazinyl ring (Figure 3d).

4. Discussion

Raman spectra collected in this study (Figs. 1 and 2) indicate that the dominant OFL moieties involved in the TiO_2 surface interactions are functional groups of both the benzoxazine-quinolone core, and the protonated N_4 of the piperazinyl group. Indirect support for benzoxazine-quinolone core activity can be seen in the spectra at low and high pH (Fig. 1). At pH 3.3, OFL is cationic and the TiO_2 surface is also positively charged (Supp. A1) so electrostatic repulsion exists between the substrate surface and the ionization sites of OFL. The opposite repulsive effect exists at pH 11.2, where OFL is anionic and the TiO_2 surface is negatively charged. Therefore at low and high pH, involvement of the piperazinyl and carboxylic acid groups would not be expected. However, the ketone group of the quinolone and/or the methyl group of the benzoxazine are candidates for surface interaction. Ketone complexation has been observed for OFL- Al_2O_3 interactions (Goyne et al., 2005), ciprofloxacin complexation to hydrous iron oxide (Gu and Karthikeyan, 2005), and other FQ adsorption to TiO_2 (Paul et al., 2007). De-alkylated transformation products of several FQ, including OFL, have been reported (Zhang and Huang, 2005; 2007), suggesting methyl group adsorption, and are consistent with Raman observations in this study.

Spectra from near pH 6 are important because here OFL is cationic and zwitterionic and the TiO_2 surface is negatively charged (Supp. A1). A comparison (Fig. 2) of what OFL components attach during the initial mixing versus what remains after desorption directly indicates the role of

the benzoxazine-quinolone core functional groups and the protonated piperazinyl at the substrate surface. This evidence is the decrease of the C=O/NH⁺ peak at 1626 cm⁻¹, and the NH⁺/CH₂/CH₃ peak at 1400 cm⁻¹. Others (An et al., 2010) have proposed that the first step in TiO₂-catalyzed photodegradation of FQ is partial or complete elimination of the piperazinyl ring, followed by hydroxylation reactions with the parent quinolone core, as part of an adsorption/desorption process. If elimination of the piperazinyl ring and hydroxylation of the quinolone is caused by attachment (Goyne et al., 2005), or at least close proximity to the oxide surface (Zhang and Huang, 2007), the Raman data support the interpretation.

Half of the carboxylic acid groups of OFL are deprotonated to the carboxylate anion at pH 6.1 (Supp. A1), and the Raman spectra do indicate some interaction with the TiO₂ substrate (Figs. 1 and 2). Previous work on FQ sorption and degradation presented evidence for COO⁻ substrate complex formation (Goyne et al., 2005; Gu and Karthikeyan, 2005; Paul et al., 2007), COO⁻ complexation as a precursor to redox reactions (Zhang and Huang, 2005; 2007), and intact carboxyl groups on quinolone rings of FQ degradation products at near-neutral pH (An et al., 2010). The lack of strong Raman evidence for COO⁻ interaction is most likely because of the TiO₂ pzc (Supp. A1), which renders a negatively-charged surface, which would repel COO⁻.

LC/MS results complement the Raman spectra in terms of surface interactions and OFL degradation. OFL degradation products remaining in solution after mixing (Fig. 3a) lack the piperazinyl ring, are de-carboxylated, and lack the quinolone ketone group. Desorbed degradation products (Figs. 3b-d) include de-methylated moieties and carboxylated piperazinyl rings. Previous LC/MS analysis of degradation products at these conditions (Van Wieren et al.,

2012; Maia et al., 2014) detected two de-carboxylated and hydroxylated quinolone compounds. This is consistent with the current interpretation. Succinylation of the piperazinyl ring, desorbed from the substrate, has been reported previously (Terzic et al. 2011), and is recognized as an important biotransformation product of FQ in the overall study of pharmaceuticals in aquatic environments (Michael et al., 2014).

5. Conclusions

Experiments were performed to investigate surface interactions and OFL degradation in aqueous TiO₂ suspensions in the dark. OFL components sorbed at the substrate surface were detected by Raman spectroscopy, and OFL species were detected in the attendant solutions by LC/MS. Raman spectra indicate sorption occurs via the C=O (ketone) and CH₃ group of the benzoxazine-quinolone core, and the NH⁺ of the piperazinyl ring. Raman spectra also show that benzoxazine-quinolone ketone groups and piperazinyl groups are most readily removed/desorbed from the surface. Complementary information from analysis of the attendant supernatants indicates the presence of quinolone and piperazinyl groups in solution. Sorption breaks the compound into piperazinyl and quinolone groups which attach to the substrate surface. Decarboxylated and hydroxylated quinolone moieties do not attach to TiO₂ and stay in solution. Altered piperazinyl and quinolone moieties which attach, are readily desorbed from the substrate upon rinsing. Methyl groups from these moieties stay attached to the substrate surface. The resultant fate and toxicity of these FQ fragments in the environment is uncertain. Degradation of FQ in this study occurred in the dark during experiments on the order of 100 hours in length. UV-mediated

degradation occurs on the order of 10s of minutes to 1 hour (Paul et al., 2007; An et al., 2010).
More study is needed to understand the kinetics and sequence of FQ-TiO₂ interactions under
low-light conditions.

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Appendix A. Supplementary Materials

Supplementary figures to this article (A1, A2, and A3) can be found online.

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FIGURE CAPTIONS

Fig. 1. Raman spectra for OFL on TiO₂ (interaction time ~144 hr.) and OFL stock precipitated from pH 6.6 solution.

Fig. 2. Raman spectra of OFL components attached to TiO₂ substrate after initial mixing (pH = 6.1) compared to OFL components remaining after re-suspension/desorption in OFL-free solution.

Fig. 3. Mass spectra of OFL and OFL degradation products in solution: (a) After initial mixing with TiO₂ suspension, parent OFL (m/z =362.2) not shown; (b-d) After re-suspension/desorption of TiO₂ substrate. RT=retention time.

Figure 1

Figure 2

Figure 3