Functional hybrid bionanomaterials based on titanium dioxide and cellulose, possessing antibacterial and drug delivery properties

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Abstract

The present work focuses on development and investigation of new functional hybrid nanomaterials based on titania nanoparticles and cellulose, possessing an important set of practical properties. The study includes two parts: first, nanocrystalline TiO_2 hydrosols produced by low-temperature sol-gel synthesis were successfully applied for cotton fabric modification. Comprehensive characterization of the cotton/TiO_2 composite was undertaken using such techniques as SEM-EDX analysis, FT-IR spectroscopy, X-ray diffraction, low-temperature nitrogen adsorption/desorption, Nanoparticle Tracking analysis, X-ray diffraction, and TGA. The interaction of TiO_2 nanoparticles with the functional groups of cotton fibers was achieved with the help of a cross-linking agent (1,2,3,4 –butanetetracarboxylic acid) through the formation of transverse ester bonds. The obtained TiO_2/composite demonstrated high bacteriostatic effect against gram-negative *Escherichia coli* bacteria after exposure to UV-irradiation for 10 minutes, and reduced bacteria survival by 70%.

In the second part of the thesis, the nanocomposites with potential for dermal drug delivery application were developed. They were produced using titania nanosol chemically grafted onto cellulose nanofibers as active ingredient for enhanced uptake and controlled release of model drug loads. Four different medicines, Diclofenac sodium, Penicillamine-D, Phosphomycin and Tetracycline were chosen as model drugs for the synthesis and further investigation of resulting drug release systems. Two different methods of medicine introduction were used to show that various interactions between TiO_2 and drug molecule could be used to control the kinetics of long-term drug release. The viability on the action of the released drug was examined for common and most widely tested pathogen micro-organisms: *Staphylococcus aureus* and *Escherichia coli.* The photocatalytic test showed that cellulose nanofibers–titania nanocomposites possessed high photocatalytic properties and could potentially be utilized in photovoltaic devices and photocatalysis. The influence of UV irradiation on the stability of the obtained nanocomposites loaded with drugs and their antibacterial properties was also investigated.

Keywords: titania, cellulose, cotton, drug delivery, hybrid material, nanocomposite, drug release, photocatalytic activity, cross-linking agent

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Dedication

To my wonderful mother and beloved fiancé for their unconditional support and believing in me

In the middle of difficulty lies opportunity
Albert Einstein

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I O.L. Galkina, A. Sycheva, A. Blagodatskiy, G. Kaptay, V.L. Katanaev, G.A. Seisenbaeva, V.G. Kessler, A.V. Agafonov (2014). The sol-gel synthesis of cotton/TiO_2 composites and their antibacterial properties. *Journal Surface and Coatings Technology,* 253, 171-179.

II O. L. Galkina, V. Ivanov, A.V. Agafonov, G.A. Seisenbaeva, V. G. Kessler (2015). Cellulose nanofiber–titania nanocomposites as potential drug delivery systems for dermal applications. *Journal of Materials Chemistry B*, 3(8), 1688-1698.

III O.L. Galkina, K. Önneby, A.V. Agafonov, G.A. Seisenbaeva, V. G. Kessler (2015). Antibacterial and photochemical properties of cellulose nanofibers–titania nanocomposites loaded with two different types of antibiotic medicines. (manuscript).

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Abbreviations

BTCA	1,2,3,4 – butanetetracarboxylic acid
CF	Pure cotton fibers
CF/TiO_2	Cotton fibers modified with TiO_2
CF/TiO_2-5W	Cotton fibers modified with TiO_2 after 5 cycles of washing
CF/TiO_2-CL	Cotton fibers modified with TiO_2 by using cross-linking agent BTCA;
CF/TiO_2-CL-5W	Cotton fibers modified with TiO_2 by using cross-linking agent after 5 cycles of washing;
CNF_TiO_2	Nanocomposite based on cellulose nanofibers modified with TiO_2
CNF_TiO_2_DS_M1	Nanocomposite based on cellulose nanofibers, modified with TiO_2 and DS by method #1
CNF_TiO_2_DS_M2	Nanocomposite based on cellulose nanofibers, modified with TiO_2 and DS by method #2
CNF_TiO_2_PCA-D_M1	Nanocomposite based on cellulose nanofibers, modified with TiO_2 and PCA-D by method #1
CNF_TiO_2_PCA-D_M2	Nanocomposite based on cellulose nanofibers, modified with TiO_2 and PCA-D by method #2
CNF_TiO_2_Phos_M2	Nanocomposite based on cellulose nanofibers, modified with TiO_2 and Phos by method #2
CNF_TiO_2_Phos_M3	Nanocomposite based on cellulose nanofibers, modified with TiO_2 and Phos by method #3
CNF_TiO_2_TC_M1	Nanocomposite based on cellulose nanofibers, modified with TiO_2 and TC by method#1
CNF_TiO_2_TC_M2	Nanocomposite based on cellulose nanofibers, modified with TiO_2 and TC by method #2

DS	Diclofenac Sodium
PCA-D	Penicillamine D
PCNF	Pure cellulose nanofibers
Phos	Phosphomycin
RC	Raw cotton
SHP	Sodium hypophosphite
TC	Tetracycline

1 Introduction

Over the past decade, the development of new functional polymer-inorganic nanocomposite materials has experienced a tremendous growth. Such materials synergistically combine the properties of organic substrates and inorganic modifiers. Among them, especially attractive are hybrid nanomaterials based on nanosized titanium dioxide and cellulose. Their development would allow to extend the range of the practical applications for cellulose, and, –will permit to obtain new fundamental knowledge about the influence of the structure of materials on their photocatalytic and biological activity. The approaches aimed at obtaining of "smart textiles" showing self-cleaning ability from organic pollutants induced by light, as well as high bactericidal activity, are considered to be highly important. An interesting challenge lies in the creation of bandage materials for advanced treatment of wounds, capable to deliver medicines in a controllable manner.

1.1 Cellulose: From Macroscopic Fibers to Nanocellulose

Cellulose is the most common, non-toxic, biodegradable, renewable natural biopolymer on Earth (Czaja *et al.*, 2007; Klemm *et al.*, 2005). This biopolymer consists of repeated β-D-glucopyranose units and contains three hydroxyl groups per anhydroglucose unit, thus giving the cellulose molecule a high degree of functionality. At the present moment, six polymorph modification of cellulose are known: I_α and I_β, II, III_I, III_{II}, IV_I and IV_{II} (French *et al.*, 2002; O'Sullivan, 1997). Cellulose I or native cellulose is found in natural materials: the I_α phase can be obtained from the primitive organisms whereas I_β phase from higher plants (Filson & Dawson-Andoh, 2009; Nishiyama *et al.*, 2003). The most intensively investigated form is cellulose II, which can be obtained from cellulose I by means of regeneration (dissolving with the subsequent reprecipitation) or mercerization (treatment in an alkaline solution) (Aulin *et*

al., 2009). Cellulose can be found in great amount in wood tissues (40-55%), linen fibers (60-85%) and in cotton (95-98%). Due to its molecular structure and large specific surface area, cellulose fibers can be a perfect matrix for development of bioactive, biocompatible and smart materials.

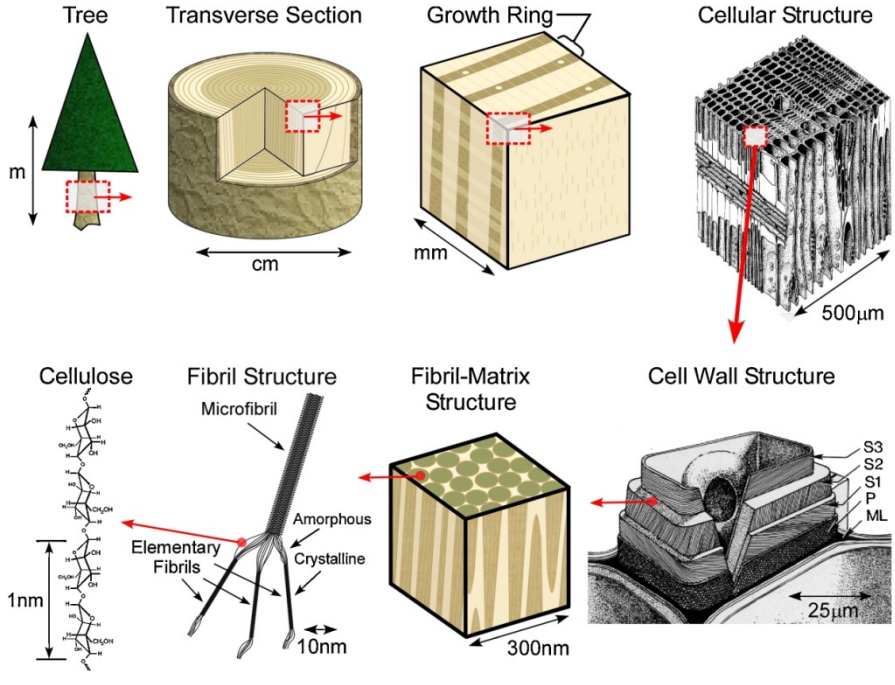

Figure 1. Wood hierarchical structure: from tree to cellulose (Dufresne, 2013)

At present, various methods are applied to provide functional properties to cellulose fibers (Zhou & Kan, 2015). Chemical modification of the natural fibers can improve their wettability, water or oil repellency, chemical affinity and reactivity, as well as increase competitivity of the textile products made of blends of cotton with chemical fibres. For example, photonic techniques (UV, IR, laser processing), or treatment of fibers by plasma discharge lead to insertion of functional groups on the polymer surface (Feng *et al.*, 2014; Nourbakhsh & Ashjaran, 2012). The above-mentioned methods have a great potential in modifying the fibers and textile, but their significant drawbacks are the high costs, difficulty in implementation, long duration of the processes and the expensive equipment required.

Sol–gel technology is a promising method for obtaining fibers and textile materials modified by functional nanoparticles of SiO_2, and of oxides of wide

range of metals (TiO_2, ZnO, Al_2O_3 and etc.) (Kalidindi & Subasri, 2015). It provides a new way of functionalizing fabrics by its processing in liquid-phase systems, leading to fixation of the nanoparticles on the surface of fibers and thereby providing fabrics with new specific properties. In particular, cotton fabrics have been treated by sol–gel method using different alkoxide precursors (tetraethyl orthosilicate, -titanate, -zirconate and aluminium isopropoxide), in order to improve their thermal and fire stability, as well as the mechanical behaviour and abrasion resistance of the obtained materials (Alongi *et al.*, 2012). Another research group improved the photostability and UV-protection of wool fabrics by its treatment with pure TiO_2 and TiO_2-based nanocomposite colloids (Pakdel *et al.*, 2015). Photocatalytic self-cleaning cotton fabric together with superhydrophobic properties were produced by using highly crystalline TiO_2 nanoparticles together with superhydrophobic SiO_2 (Xu *et al.*, 2015).

One of the areas of biomedical application of titania nanoparticle coatings is the so-called "smart" textiles, which are able to purify themselves from organic matter and fungal attack, as well as to destroy bacteria under the effect of soft UV-radiation (Radetić, 2013; Bozzi *et al.*, 2005). Montazer et al. prepared the antimicrobial textiles by treatment with TiO_2 and TiO_2/Ag (Montazer *et al.*, 2011). Strong self-cleaning properties of textiles functionalized with different nano-TiO_2 sols were assessed using the degradation of the Rhodamine B dye as a model reaction (Ortelli *et al.*, 2014). TiO_2-modified cotton fabrics modified using a polycarboxylic acid and chitosan phosphate, revealed excellent antibacterial properties (El-Shafei *et al.*, 2015). The application fields for such materials are quite broad – from the medical staff wear and bed linen to bandage materials.

1.1.1 Nanoscale cellulose

Through physico-chemical and mechanical effects the cellulose fibers can be converted to powder materials, for example, microcrystalline cellulose, which, in turn, can also be transformed into gelled dispersed systems with particle sizes of 40-400 nm (Alves *et al.*, 2015; Li *et al.*, 2015a; Abdul Khalil *et al.*, 2014; Liu *et al.*, 2014; Brinchi *et al.*, 2013; Dufresne, 2013).

The nanoscale cellulose (NCC) possesses a whole complex of unique properties typical for nanomaterials in general, such as high specific surface area, enhanced chemical reactivity, and high mechanical durability together with biocompatibility, biodegradability, and non-toxicity, which makes it an excellent candidate for drug release applications (Lagerwall *et al.*, 2014). Due to these specific properties, bandage materials, transdermal patches, tablet binder, disintegrated vehicle for peptide and gene delivery can be obtained

based on nanoscale cellulose, and raw materials for their production are almost unlimited (Ng *et al.*, 2015; Lin *et al.*, 2012). In particular, nanocrystalline cellulose has been used as a matrix-former material for long-lasting sustained drug delivery and for food packaging materials (Akhlaghi *et al.*, 2013; Kolakovic *et al.*, 2013; Kolakovic *et al.*, 2012). NCC derived from softwood was used for binding tetracycline and doxorubicin, which could be released rapidly over one day time (Jackson *et al.*, 2011). Transdermal drug delivery system based on cellulose nanocrystals with chitosan oligosaccharide was also developed recently (Akhlaghi *et al.*, 2013).

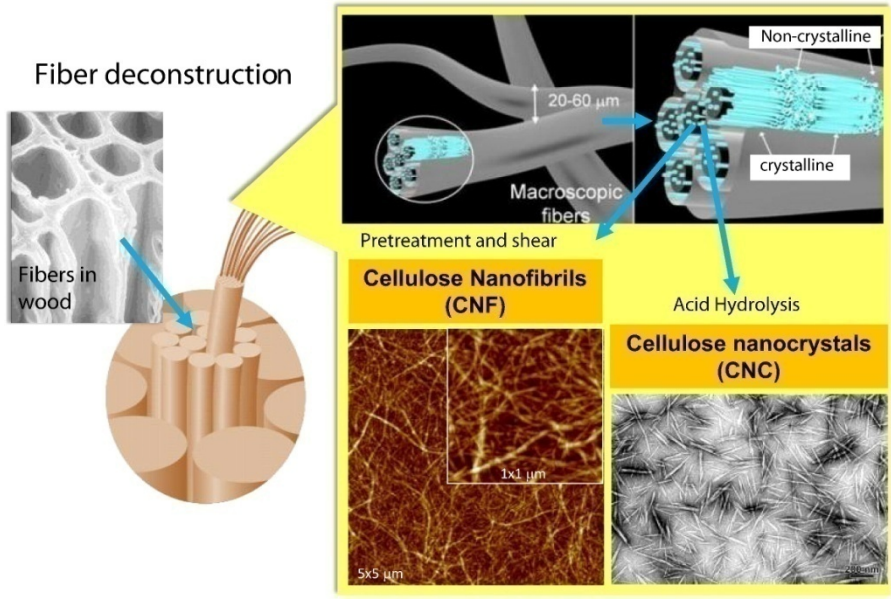

Figure 2. Schematic illustration of cellulose nanofibrils and cellulose nanocrystals production from fiber cell walls by mechanical and chemical treatments, respectively (Zimmermann *et al.*, 2004).

Preparation of bio-nanocomposites based on nanocellulose, nano- or microfibrilliar cellulose, cellulose nanofibers and inorganic nanoparticles is a hotspot of interest of the materials scientists in the relevant field (Wei *et al.*, 2014; Lam *et al.*, 2012). For instance, ternary nano-biocomposites containing modified cellulose nanocrystals and silver nanoparticles have been prepared and showed high potential in the development of new biodegradable materials for fresh food packaging applications (Fortunati *et al.*, 2014). Another example reports the formation of nanocellulose-titania nanoparticles hybrids with high inorganic content by adsorption of TiO_2 nanoparticles on wood-derived nanofibrillated cellulose (Schutz *et al.*, 2012). As transparent coatings, these

hybrids demonstrated high wear resistance and UV activity. Efficient strategy has been proposed to obtain bionanocomposites containing zinc oxide-silver nanoparticles stabilized by cellulose nanocrystals as multifunctional nanosized fillers in poly(vinyl alcohol)/chitosan matrices (Azizi *et al.*, 2014). The obtained films showed strong antibacterial activity against *S. choleraesuis* and *S. Aureus* and high protection against ultraviolet and visible light.

1.2 Titanium Dioxide

More than 40 years ago, Fujishima and Honda first demonstrated the potential of titanium dioxide as semiconductor material in photo electrochemical water splitting (Fujishima & Honda, 1972). Their research opened great possibilities for development of semiconductor photocatalysis for a wide range of environmental and energy applications (Ravelli *et al.*, 2009). Nowadays, TiO_2 is attracting more and more attention from both theoretical and practical point of view as nanomaterial with outstanding characteristics (Chen & Mao, 2007). The main advantages of nanoscaled titania are its long-term chemical stability, when exposed to acidic and basic compounds, non-toxicity, its high biocompatibility and relatively low cost. Due to these unique photophysical and chemical properties, TiO_2 can be used for self-sterilization, as self-cleaning surface (Shi *et al.*, 2015; Wang *et al.*, 2015a; Meilert *et al.*, 2005), or as photochromic (Djaoued *et al.*, 2013; Tobaldi *et al.*, 2013), and superhydrophobic/hydrophilic coatings (Kamegawa *et al.*, 2012; Zhang *et al.*, 2007) and just recently as matrices for bioencapsulation, drug delivery (Kessler *et al.*, 2008; Vallet-Regí *et al.*, 2007) and in tissue engineering (Akhavan & Ghaderi, 2013).

Numerous methods can be used to synthesize titanium dioxide particles with defined properties on the nanoscale (Cargnello *et al.*, 2014; Ouyang *et al.*, 2008; Wang *et al.*, 2014). For example, the well-known commercial titania nanoparticles such as Degussa P-25 are produced by flame hydrolysis of titanium tetrachloride ($TiCl_4$) in gas phase at high temperature over 1000 ºC. Major drawback of this method is that the nano-sized TiO_2 particles can easily aggregate into bigger particles with sizes varying in a wide range. Moreover, while TiO_2 is certified as both a food additive (E171 in EU) and as a solar protection factor in skin application (FDA approved concentration in sunscreen formulations is up to 25 wt%), and is often used as a negative control in the toxicity studies for nanoparticle materials (Karlsson *et al.,* 2014), they still reveal adverse bio-effects and cannot be applied for bioencapsulation and drug delivery application (Long *et al.*, 2006; Skocaj *et al.*, 2011).

The sol-gel method based on combination of hydrolysis and condensation reactions of metal alkoxide or halide precursors has been widely applied for preparing of TiO_2 with of narrow particle-size distribution (Bergamonti *et al.*, 2015; Matijević *et al.,*1977; Pinjari *et al.*, 2015). At the same time, non-aqueous sol-gel processes for the synthesis of titania nanoparticles offer attractive alternatives to their aqueous analogues. Among them, small-sized TiO_2 nanoparticles (4±1 nm) (see Fig.3) can be successfully produced by CaptiGel technique based on modification of metal precursors with chelating organic ligands resulting in facile formation of self-assembled micellar aggregates (MTSALs) (Kessler *et al.*, 2006). The major feature of the thus produced nano titania is its specific biodegradability, offering the possibility of enhanced chemically and bio-chemically triggered release (Kessler *et al.*, 2008).

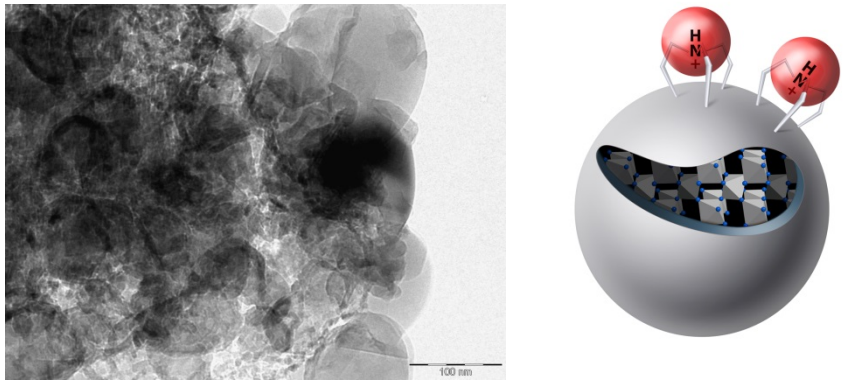

Figure 3. TEM image of titania CaptiGel nanoparticles (left) together with the structure of MTSALs (Kessler *et al*., 2008).

1.2.1 Application of nanosized TiO_2

In recent decades, major research efforts were focused on the investigation of the photocatalytic properties of TiO_2 due to its suitable bandgap energy (3.2 eV) and long life-time of photogenerated holes and electrons (Pelaez *et al.*, 2012; Hernandez-Alonso *et al.*, 2009; Thompson & Yates, 2006). It is a well-known that bulk titania exists in three different crystalline forms such as anatase (tetragonal), brookite (orthorhombic) and rutile (tetragonal). Among these, anatase exhibits the highest overall photocatalytic properties while rutile is the most stable phase (Kandiel *et al.*, 2010). On the other hand, a very interesting result was recently obtained by (Li *et al.*, 2015b). They developed a highly efficient visible active semiconductor photocatalyst based on sub-10 nm

rutile titanium dioxide nanoparticle with increased amount of surface/sub-surface defects.

As a photocatalyst, nanosized TiO_2 offers an effective destruction of pathogenic bacteria even under soft ultraviolet radiation, eliminating possibility of formation of xenobiotic-products and hazardous (carcinogenic, mutagenic) compounds (Koseki *et al.*, 2009; Kühn *et al.*, 2003). Moreover, low concentrations of nanosized titania also significantly damaged the cells of planktonic bacteria and algae in stream water (Battin *et al.*, 2009). O. Akhavana et al. synthesized TiO_2/multi-wall carbon nanotube heterojunction arrays based on Si substrate, possessing excellent antimicrobial properties against *E.coli* bacteria under visible light irradiation (Akhavan *et al.*, 2009).The observed bactericidal efficiency of titania nanoparticles can be explained by disruption of cell membranes activity or by induction of intercellular reactive oxygen species (ROS), such as hydrogen peroxide, superoxideanion (O_2^-) and hydroxyl radical, which are strongly oxidizing agents harmful to bacterial cells (Kumar *et al.*, 2011; Cho *et al.*, 2004).

Current research trend is to apply biocompatible nanostructured inorganic oxides for bioencapsulation and drug delivery with a controlled release profile. A substantial amount of literature has been published on the application of mesoporous silica nanoparticles as a drug delivery system (Wang *et al.*, 2015b). However, the combination of high porosity and slow biodegradability of silica nanoparticles can lead to immune rejection of, for example, encapsulated viruses before they are released in efficient amounts. On the other hand, there are very few reports on the application of titania based materials for the same purpose. In particular, titania nanotubes have been investigated as a possible source in the context of slow drug release (Arturo *et al.*, 2006). Recently, a new concept of local multi-drug delivery system based on nanoporous titania nanotubes and polymer micelles as drug carriers with sequential drug release was reported (Aw *et al.*, 2012). A common feature of these materials is that titanium dioxide has been used as container for small molecule and macromolecular drugs. At the same time, three-dimensional (3-D) porous structures would be more promising for such systems than one-dimensional nanotubes with their chemical inertness and cell adhesion (Tavangar *et al.*, 2011). For example, titania nanocrystals covalently tethered to the isotypical immunoglobulin were found to be very interesting as potential nano-biohybrid system for drug delivery vectors (Rozhkova *et al.*, 2009). Hybrid phosphonate–TiO_2 mesoporous nanoparticles as a novel controlled drug release system were produced and investigated by (Li *et al.*, 2013, Seisenbaeva et al. 2010). Another approach is to use photoactive nanosized titania for encapsulation of individual living cells and their further investigation as

biosensor circuits, novel bioreactors as well as for fundamental studies of cell molecular biology (Stojkovic *et al.*, 2015).

2 Research objectives

The main aim of this thesis is the development of new hybrid materials based on cellulose and titania nanoparticles with enhanced functionality. Synergistically combining the properties of organic substrate and inorganic modifier, these materials would acquire a complex of specific properties that could provide them with a wide range of practical applications such as desinfective cloth and bandaging materials. The latter can contribute to the treatment of non-healing ulcers and surgical wounds, exploiting their high photocatalytic, antibacterial and drug delivery properties.

The work has been divided in two parts:

- The first part is focused on the investigation of structure and functional properties of hybrid materials based on the nanocrystalline TiO_2 - modified natural cotton textile.

- The objective of the second part of the thesis is the development of nanocomposites based on synthesized cellulose nanofibers and grafted TiO_2 for drug delivery applications.

3 Experimental

3.1 Preparation of the nanosized TiO_2

In the present study, two types of nanosized titanium dioxide with different size and crystalline structure were prepared and used for cellulose modification.

3.1.1 Synthesis of nanocrystalline TiO_2 hydrosol (Paper I)

Nanocrystalline titania sol was obtained by low-temperature sol-gel synthesis in an aqueous medium. For this purpose, 16 ml of titanium tetraisopropoxide preliminarily dissolved in 12 ml of anhydrous isopropyl alcohol was added upon constant stirring to hot (70ºC) water acidified by 0.7 ml of nitric acid (ρ = 1,513 g/cm^3 at 20ºC) . The synthesis has been performed under conditions of continuous stirring at 80ºC for 8 h; as a result a transparent sol was formed.

3.1.2 Synthesis of TiO_2 nanosol, CaptiGel (Paper II & III)

The initial precursor solution was obtained by dissolving 4 ml of titanium alkoxide $Ti(OEt)_4$ in 6 ml of anhydrous EtOH and adding 1.0 ml of triethanolamine. Then, 1 ml of hydrolyzing solution, prepared by mixing 0.5 ml of 0.5M HNO_3 with 2.0 ml of EtOH, was added to provide the colourless and transparent organic sol (Kessler *et al.*, 2008).

3.2 Preparation of cotton/TiO_2 composites (Paper I)

3.2.1 Modification of natural cotton fibers by TiO_2 hydrosol

Before the TiO_2 modification, the raw cotton was boiled in 200 ml of distilled water solution containing Na_2CO_3 (2.8 g/l) at 100ºC for 2h for removal

of the most of the non-cellulosic constituents. After this, the pre-treated cotton fibers were thoroughly washed with distilled water and dried in air.

Then, the pre-treated cotton fiber samples were treated by freshly prepared surface treating solution. For this, 1 g of 1,2,3,4-butanetetracarboxylic acid (BTCA) was mixed with 1 g of NaH_2PO_2 and dissolved in 50 ml of doubly distilled water, and then the cotton fibers were kept in this solution at 70ºC during 2 h. Next, 10 ml of titania sol was added to this solution and the modification was carried out at 70ºC during 2 h. The modified fibers were washed with doubly distilled water and dried at 60ºC for 2 h for water removal. Laundry of the cotton fibers treated by the nanosized titania hydrosol was carried out according to the standard GOST 9733.4-83. The designations of the obtained materials are summarized in table I. A series of five samples was prepared and then tested for the bactericidal properties.

3.3 Preparation of cellulose nanofiber–titania nanocomposites (Paper II)

3.3.1 Synthesis of cellulose nanofibers

For obtaining cellulose nanofibers we have developed a method based on the use of copper ammonium complex for converting raw cotton (RC) into a molecular solution with subsequent regeneration by means of acid hydrolysis (see Fig.4).

5 g of copper (II) sulfate were dissolved in 100 ml of distilled water, and then sodium hydroxide (5M) was added until precipitation (eq.1). The copper hydroxide precipitate was thoroughly washed by distilled water to remove Na^+. Then, the precipitate was dissolved in 200 ml of ammonia (25 wt%) giving a deep blue solution of tetraamminediaquacopper dihydroxide (Schweitzer's reagent) (eq.2). Two grams of raw cotton were added to the cuprammonium solution. When cotton was completely dissolved in the Schweitzer's reagent (eq. 3), the solution was further used for obtaining aqueous suspension of cellulose nanofibers.

$$CuSO_4 \cdot 5H_2O + 2NaOH \longrightarrow Cu(OH)_2 + Na_2SO_4 + 5H_2O \quad (1)$$

$$Cu(OH)_2 + 4NH_4OH \longrightarrow [Cu(NH_3)_4(H_2O)_2](OH)_2 + 2H_2O \quad (2)$$

$$2(C_6H_{10}O_5)_x + 2x[Cu(NH_3)_4(H_2O)_2](OH)_2 \longrightarrow x[(C_6H_8O_5)_2Cu][Cu(NH_3)_4(H_2O)_2] + + 4xNH_3 + 4xH_2O \quad (3)$$

For this purpose, 25 ml of Schweitzer's reagent solution, containing the dissolved cotton, was added into 140 ml of the sulfuric acid solution (40 wt%)

and stirred vigorously at 60ºC for 4 hours. After that, hydrolysis was immediately quenched by adding 500 ml of cold distilled water to the reaction mixture. The sulfuric acid was removed from the resulting suspension by centrifuging with distilled water until pH=6.

$$x[(C_6H_8O_5)_2Cu][Cu(NH_3)_4(H_2O)_2] + 4xH_2SO_4 \longrightarrow 2(C_6H_{10}O_5)_x + CuSO_4 + (NH_4)_2SO_4 \quad (4)$$

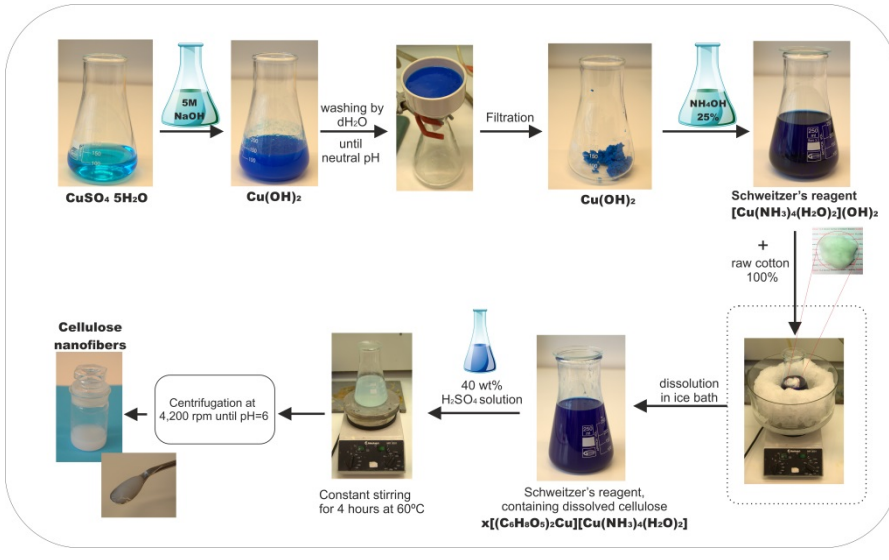

Figure 4. Scheme of cellulose nanofibers synthesis

3.3.2 Preparation of nanocomposite loaded with drugs

Firstly, to cross-link titania nanoparticles with cellulose nanofibers, 1,2,3,4-butanetetracarboxylic acid was used as a spacer in the presence of sodium hypophosphite (see Fig.5).

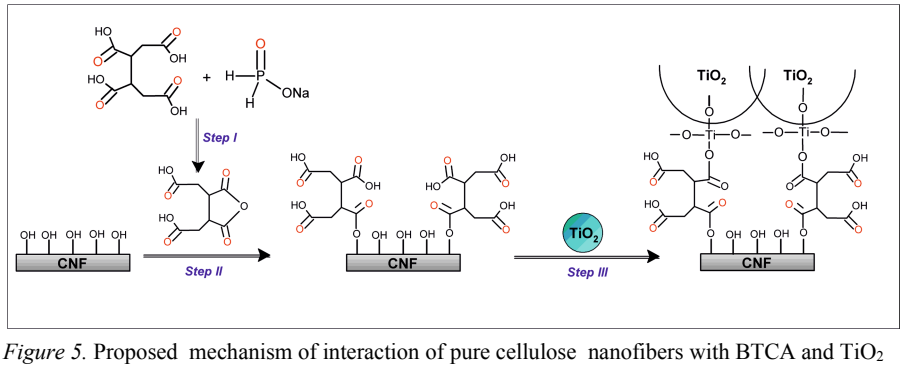

Figure 5. Proposed mechanism of interaction of pure cellulose nanofibers with BTCA and TiO_2

For this, the obtained aqueous suspensions of cellulose nanofibers were treated by BTCA (0,002 mol) with SHP (0,002 mol) aqueous solution at 70ºC during 2 h. Next, titania nanosol was added to the obtained solution and the modification was carried out at 70ºC during 2 h. The obtained nanocomposites were dried at 40ºC for 48 h.

Introduction of drugs

Method #0 (M0): The drug powder (TC or Phos) was initially dissolved in water, and then added to an aqueous suspension of cellulose nanofibers. The mixture was kept at 40ºC and 70ºC during 2 h for TC and Phos, respectively. The obtained nanocomposites were dried at 40ºC for 48 h.

The BTCA-treated cellulose nanofibers were modified by TiO_2 nanosol and drug by using two different methods.

Method #1 (M1): drug powder (DS, PCA-D, Phos or TC) was initially dissolved in titania nanosol and thereafter the obtained solution was added to an aqueous suspension of cellulose nanofibers and kept at 70ºC during 2 h. The obtained nanocomposites were dried at 40ºC for 48 h.

Method #2 (M2): The drug powder (DS, PCA-D, TC or Phos) was initially dissolved in water and then added together with the titania nanosol simultaneously to an aqueous suspension of cellulose nanofibers. The mixture was kept at 70ºC during 2 h. The obtained nanocomposites were dried at 40ºC for 48 h.

The composition of the obtained samples and the method of drug modification are presented in the Table 1. In order to obtain nanocomposites as a film, 3% of PVA was used (Fig.6).

Table 1.*Composition of the samples*

Sample	BTCA (mol)	SHP (mol)	TiO_2 (mol)	Drug (mol)	Method of modification
CNF_TiO_2	0,002	0,002	$1{,}5\cdot10^{-4}$	-	-
CNF_TiO_2_DS_M1	0,002	0,002	$1{,}5\cdot10^{-4}$	$4{,}74\cdot10^{-5}$	Method #1
CNF_TiO_2_DS_M2	0,002	0,002	$1{,}5\cdot10^{-4}$	$4{,}74\cdot10^{-5}$	Method #2
CNF_TiO_2_ PCA-D_M1	0,002	0,002	$1{,}5\cdot10^{-4}$	$4{,}74\cdot10^{-5}$	Method #1
CNF_TiO_2_PCA-D_M2	0,002	0,002	$1{,}5\cdot10^{-4}$	$4{,}74\cdot10^{-5}$	Method #2
CNF_TiO_2_Phos_M2	0,002	0,002	$1{,}5\cdot10^{-4}$	$4{,}74\cdot10^{-5}$	Method #2
CNF_TiO_2_TC_M1	0,002	0,002	$1{,}5\cdot10^{-4}$	$4{,}74\cdot10^{-5}$	Method #1
CNF_TiO_2_TC_M2	0,002	0,002	$1{,}5\cdot10^{-4}$	$4{,}74\cdot10^{-5}$	Method #2

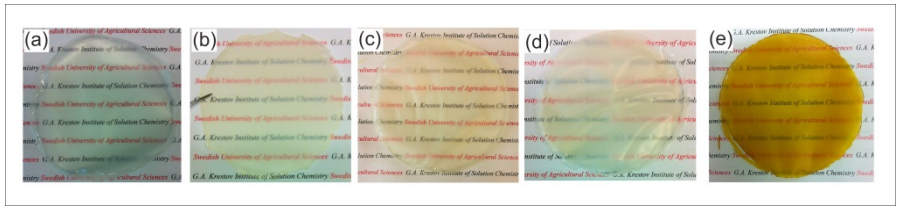

Figure 6. Visual images of the samples (a) PCNF, (b) CNF_TiO_2_DS_M1, (c) CNF_TiO_2_PCA-D_M1, (d) CNF_TiO_2_Phos_M2, (e) CNF_TiO_2_TC_M1

3.4 Material characterization

Several techniques have been used for characterization of the obtained materials.

The size of the obtained TiO_2 particles in the sol was determined by the dynamic light scattering method using Zeta Sizer Nano at 20ºC with a 10mV He-Ne laser, 633 nm wavelength and 90º fixed scattering angle (Paper I).

For the aqueous suspensions of PCNF and CNF_TiO_2 samples, nanoparticle tracking analysis measurements based on Brownian motion of nanoparticles were carried out using a NanoSight instrument, permitting to determine the size and particle size distribution in real time (Paper II).

The specific surface area of cotton/TiO_2 composites was calculated using the BET method on a Quantachrome Nova 1200 Series-E analyzer at 77K at the laboratory of hybrid materials and supramolecular system, G.A. Krestov Institute of Solution Chemistry of RAS, Ivanovo, Russia. Using low-temperature nitrogen adsorption/desorption data the pore size distribution according to the BJH method was also calculated (Paper I)

3.4.1 SEM and AFM microscopy

Surface morphology and composition of pure and modified cotton fabrics were investigated by Zeiss EVO MA scanning electron microscope (SEM) equipped with the EDAX Genesis APEX 2 system for energy-dispersive X-ray microanalysis (Paper I). Before the analysis the fabric samples (size of about 10x10 mm) were sputtered with thin gold layer to avoid electrostatic charging during examination. The micrographs with a magnification of 500 times were obtained by back scattered electron detector (BSE) in order to register both topography and compositional contrast. The average chemical composition was determined from a 4.87 mm x 3.61 mm area.

Scanning electron microscopy images of the cellulose nanofiber–titania nanocomposites were obtained using Carl Zeiss NVision at the Department of Chemistry and Biotechnology, Swedish University of Agricultural Sciences, Uppsala, Sweden (Paper II). The elemental analysis of pure cellulose

nanofibers and nanocomposites was carried out by a HITACHI TM-1000 scanning electron microscope equipped with an EDX detector. For analysis a drop of each of the diluted suspensions was deposited in the holder with a carbon pad and allowed to dry in air.

Atomic force microscopy (AFM) studies were carried out using a Bruker MultiMode 8 microscope (Paper II). Pure cellulose nanofibers (PCNF) and nanocomposite based on cellulose nanofibers modified with TiO_2 (CNF_TiO_2) were applied on carbon pad having clean surface.

3.4.2 IR-spectroscopy

Analysis of chemical bonds in cotton fibers before and after TiO_2 treatment was carried out using an Avatar IR-spectrophotometer in the range of 500–4500 cm^{-1} (Equipment of the Center for Collective Use, Ivanovo State University of Chemistry and Technology, Ivanovo, Russia).

IR spectra of cellulose nanofiber–titania nanocomposites were obtained with a Perkin Elmer FT-IR spectrometer Spectrum-100 (Swedish University of Agricultural Science, Uppsala, Sweden). A total of 8 scans were carried out on wavenumbers from 400 cm^{-1} to 4000 cm^{-1}, in transmittance mode. All spectra were smoothed and baseline corrected.

3.4.3 X-ray diffraction

Crystal structure of the cotton/TiO_2 composites was determined using X-ray diffraction on a Bruker Nanostar U diffractometer (MoKα radiation, λ = 0.71073 Å) operating at 45kV voltage and 40 mA current at the Department of Inorganic Chemistry, Ivanovo State University of Chemistry and Technology, Ivanovo, Russia (Paper I). The crystallite size was calculated using the Scherrer equation:

$$D = \frac{k\lambda}{B\cos\theta}$$

Where D is a crystallite size (nm); k is a Scherer's constant equal to 0.94 (Langford *et al.*, 1978), λ is the X-ray wavelength, B is the full width at half maximum of the diffraction line (FWHM) in rad, and θ is the diffraction angle of the phase under investigation (rad).

The X-ray powder diffraction (XRD) studies of the obtained cellulose nanofiber–titania nanocomposites were carried out at room temperature using a Bruker APEX II CCD diffractometer (Mo Kα radiation, graphite-monochromator) at the Department of chemistry and Biotechnology, Swedish University of Agricultural Sciences, Uppsala, Sweden. The main diffraction peak was integrated and used to calculate the crystalline index (CrI,%) of the samples:

$$\mathrm{CrI}\ (\%) = \frac{(\mathrm{I_{total}} - \mathrm{I_{am}})}{\mathrm{I_{total}}} \times 100$$

Where I_{total} is the scattered intensity at the main peak of cellulose I or II, and I_{am} is the scattered intensity due to the amorphous portion evaluated as the minimum intensity between the main and secondary peaks.

3.4.4 Thermal analysis

The thermal analysis of the cotton/TiO_2 composites was performed using a Netzsch Derivatograph in the temperature range from 20 to 500ºC, at the heating rate of 10ºC/min in the Ar medium. For the purpose of the estimation of total titania concentration the pure and modified cotton fibers were calcined for 2 h at 650ºC.

Thermo-gravimetric analysis was carried out for nanocellulose-based materials in air at a heating rate of 10ºC/min, using a Perkin-Elmer TGA-7 or Pyris 1 device.

3.4.5 Radiological studies of model drug adsorption (Paper II)

The measurements of uptake and release from pure cellulose nanofibers and nanocomposite based on titania and cellulose nanofibers were performed by Ridgeview Instrument AB, exploiting Ligand Tracer™ White technology (Fig.7), using ^{33}P-labeled (ATP) (adenosine 5"-triphosphate, substitution on γ-phosphorus atom, 1 ml of solution with 1mCurie total β-activity) as model compound (Seisenbaeva *et al.*, 2010; Björke & Andersson, 2006). A part of sample was deposited on a PMMA Petri dish as dispersion in toluene and immobilized by drying in air (Björke & Andersson, 2006). Association solution (3.33ml of MQ with 0.1% Tween 20) was added to the dish that was mounted in an inclined position into the instrument and subjected to rotation so that the immobilized material was periodically wet by solution and its β-emission was registered immediately afterwards. 1µl of ^{33}P-marked ATP solution (resulting in 1nM of ^{33}P-ATP in the dish) was added after 40 min and one more portion of 2 µl of ^{33}P-marked ATP solution of ^{33}P-marked ATP solution (resulting in ~ 3 nM of ^{33}P-ATP in the dish) – after 23 hours, corresponding to the start of saturation in the observed adsorption. When the adsorption equilibrium was achieved (after 43 hours), the mother liquor was replaced by the dissociation solution (phosphate buffer solution (pH 7.4) with 0.1% Tween 20), and the decrease of radioactivity in the material was followed in the same way.

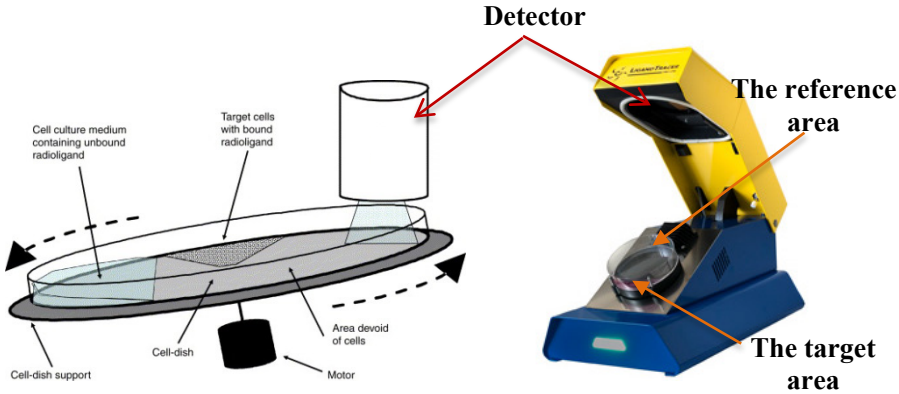

Figure 7. The LigandTracer instrument (Björke *et.al*., 2006)

3.4.6 *In vitro* drug release (Paper II)

The in vitro release studies of DS were carried out by placing the nanocomposites containing TiO_2 and DS in definite volume (300 ml) of releasing medium (10-fold isotonic NaCl solution) at constant temperature (37±0.5ºC) on constant stirring at 100 rpm (Kessler *et al.*, 2008). To investigate the release profile of PCA-D and TC, the nanocomposites containing TiO_2 and PCA-D or TC were incubated in 300 ml of citrate buffer (0.02M, pH=6) at constant temperature (37±0.5ºC) on constant stirring at 100 rpm (Walash *et al.*, 2004). At determined time intervals, 1 ml of each solution was taken out for analysis, and the same volume of fresh medium was added to maintain a constant volume. DS, PCA-D and TC content in each aliquot was determined by spectrophotometry. All the experiments were made three times to get an indication about the reproducibility. UV–Vis quantitative analysis of drugs was performed on a UV/Vis spectrophotometer UV-1800. A linear calibration curve for DS, PCA-D and TC was obtained at 278 nm, 270 nm and 375 nm, respectively. Released drug was determined by using the following equation:

$$\text{Drug release (\%)} = \frac{\text{(released drug)}}{\text{(total drug)}} \times 100$$

where released drug was calculated from the drug concentration measured in the total volume and total drug was the amount loaded in the obtained sample.

3.4.7 Photocatalytic Activity Determination (Paper III)

Photocatalytic activity of pure cellulose nanofibers and nanocomposites were tested by the rate of decomposition of Rhodamine B under the effect of UV irradiation. The source of UV radiation was a high-pressure 250W mercury

lamp with radiation maximum at 365 nm. Then 2 ml of Rhodamine B solution (0.04 g/l) was added to the samples. The colored samples were dried and subjected to UV irradiation for 30 min. Difference in colour for each sample before and after UV irradiation is the corresponding factor for determination of TiO_2 photocatalytic effect on decomposition of dye stains.

3.4.8 Bacterial inoculation of cotton/TiO_2 composites (Paper I)

Before testing the bactericidal properties of the TiO_2 treated cotton fibers, produced with or without cross-linking agent, they were activated by UV-irradiation using Philips TUV-W30/G30T8 Lamp during 10 minutes. The treated fibers were placed subsequently in a tube with the *Escherichia coli* (*E. coli*) bacterial culture for 1 minute and their bactericidal activity was investigated. For cultivation in the liquid phase the overnight culture of E.coli DH10B, having optical density at 600 nm (OD_{600}) equal to 0.9 was diluted 100 times by media LB100. The tube with the fibers and the bacteria was incubated on a shaker at 37ºC taking a sample every half an hour to control the OD_{600}

3.4.9 Bactericidal activity of cellulose nanofiber–titania nanocomposites modified with different drugs (Paper III)

Minimum inhibitory concentration analysis

Minimum inhibitory concentration (MIC) was determined for pure drugs by broth micro dilution using Mueller-Hinton (MH) broth (OXOID, England) loaded with Tetracycline (0.5-100 μg/mL) or Phosphomycin (1-200 μg/mL). McFarland 0.5 dilutions from overnight cultures of the Gram-positive bacteria *Staphylococcus aureus* and the Gram-negative *Escherichia coli* were used as inoculum. After 18 h of incubation at 37ºC the MIC was defined as the lowest concentration of antibiotic with no visible growth and by viable count (VC). VC was determined by 10-fold serially dilutions plated on MH agar (OXOID, England) and incubated at 37ºC overnight. Colonies were counted and colony forming units (cfu) per ml were calculated.

Disk diffusion tests

The disk diffusion method (EUCAST, 2014) was used to assay the antibacterial activity of the of the cellulose nanofibers and nanocomposite loaded with TC or Phos (1-100 μg/disk) against test strains *S. aureus* and *E. coli* on MH agar plates. Pure cellulose nanofibers and nanocomposite with TiO_2 were used as controls. Inhibition zone diameters were measured after incubation at 37ºC for 18 h. Experiments were performed with biological triplicate. In addition, bacterial susceptibility testing was carried out on CNF_TC_M0 and CNF_TiO_2_TC_M1 samples with 1-100 μg of TC after UV-

irradiation for 30 and 60 min, using high-pressure 250W mercury lamp with radiation maximum at 365 nm.

Antibacterial growth inhibition assay

CNF_TC_M0, CNF_TiO_2_TC_M1 samples with 1-10 μg/ml of Tetracycline and CNF_Phos_M0, CNF_TiO_2_Phos_M2 samples with 100 μg/ml of Phosphomycin were added to MH broth 0, 12, 18 and 24 h before inoculation with McFarland 0.5 dilutions of *S. aureus*. After 18 h of incubation at 37ºC the growth inhibiting effect was determined by VC and expressed as % in relation to cfu of the control without addition of CNF. To simulate body fluid conditions, citrate buffer (0.75 ml, 0.03M, pH=6) was added to MH broth. Experiments were performed with biological triplicate.

4 Results and Discussion

4.1 Investigation of cotton/TiO_2 composites – Paper I

The main objective in Paper I was to obtain composites based on natural cotton fibers and nanocrystalline TiO_2 and investigate their bactericidal properties. A previously developed method of obtaining nanocrystalline titania sols using titanium tetraisopropoxide as precursor and nitric acid as peptizing agent via low-temperature sol-gel synthesis in aqueous medium was applied and used for cotton modification (Galkina *et al.*, 2012). It is important to note that among wide range of possible modifications of textile fibers the main challenge is the absence of strong bonds between titania nanoparticles and the polymer. TiO_2 can be attached to the fibers mainly by physical adsorption. Thus, such materials can give rise to aerosol of titanium dioxide nanoparticles, which enter the respiratory system and open wounds, while they are not able to withstand prolonged washings and subsequently lose their properties. In this aspect, we used the 1,2,3,4-butanetetracarboxylic acid (BTCA) as a spacer to cross-link the titania nanoparticles with the functional groups of cotton fibers and evaluated the ways of their interaction. It was demonstrated that the cotton fibers modified by TiO_2 nanosol exhibit high self-cleaning properties and maintain them even after five cycles of washing. It should also be mentioned that a large number of works are devoted to the investigation of bactericidal properties of nanocrystalline titania under uninterrupted UV-radiation exposure (Cai *et al.*, 2013). In our experiments titania modified cotton fibers were continuously exposed to UV light for 10 minutes only.

4.1.1 Characterization of TiO_2/composites

According to SEM-EDX analysis, the pure cotton fibers did not contain any contaminations like Na^+ and Ca^{2+} ions after pre-treatment with Na_2CO_3 removed by thorough washing process (Fig.8(a,1)). After modification by

titania hydrosol, the cotton fibers in the CF/TiO_2 sample (cotton fibers modified with TiO2) are unevenly covered by TiO_2 nanoparticles (Fig.8(b,2)). The cotton fibers modified with TiO2 by using cross-linking agent BTCA (CF/TiO_2-CL) contain titanium dioxide particles more uniformly distributed through fibers in comparison with the sample CF/TiO_2 (Fig.8(c,3)).

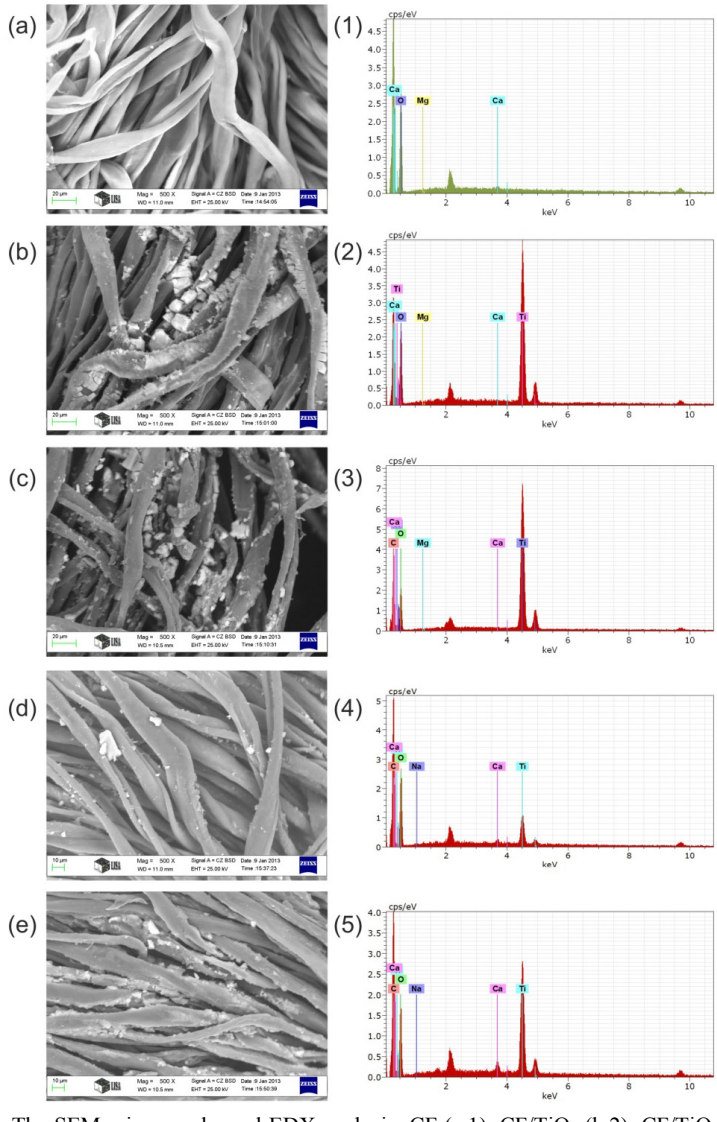

Figure 8. The SEM micrographs and EDX analysis: CF (a,1), CF/TiO_2 (b,2), CF/TiO_2-CL (c,3), and the same composite samples after 5 cycles of washing CF/TiO_2-5W (d,4) and CF/TiO_2-CL-5W(e,5).

It has to be noted that the Ti peak is present even in the analyses of the darker parts, not bearing visible bright aggregates, indicating that smaller primary particles are more-or-less evenly coating the fibers and that the bright aggregates are secondary formations on the surface of the coating resulting from primary particles. The elemental compositions obtained from EDX analysis are listed in the Table 1 in Paper I, in which the atomic fractions (%) of Ti, C, and O are used to give the quantitative evaluation in the samples.

To identify the crystal structure of TiO_2 on the surface of cotton fibers, XRD analysis has been used. Two peaks are characteristic for pure cotton fibers: one at 2θ = 10.9º and 2θ = 16.4º which refers to the cellulose-I (Fig.9(1)a). For the pure titania quite strong peaks of anatase and brookite phases, 2θ = 12.2º, 17.6º, 24.7º and 2θ = 14.3º, 22º, respectively, were found (Fig.9(1)b). For CF/TiO_2 sample, additional new peaks have been found at 2θ = 7.2º and 2θ = 12.2º characteristic of the anatase crystalline phase (Fig.9(1)c). For CF/TiO_2-CL sample, a decrease in intensity of the peaks related to cellulose, and an increase in intensity of the anatase peak are observed at 2θ = 12.2º, 17.6º and the brookite peak at 2θ = 22º with crystallite size of 9.1 nm (Fig. 9d). Interestingly, the cross-linking agent allows to increase the degree of crystallinity of the CF/TiO_2-CL sample compared to the cotton fibers treated by nanocrystalline titania only.

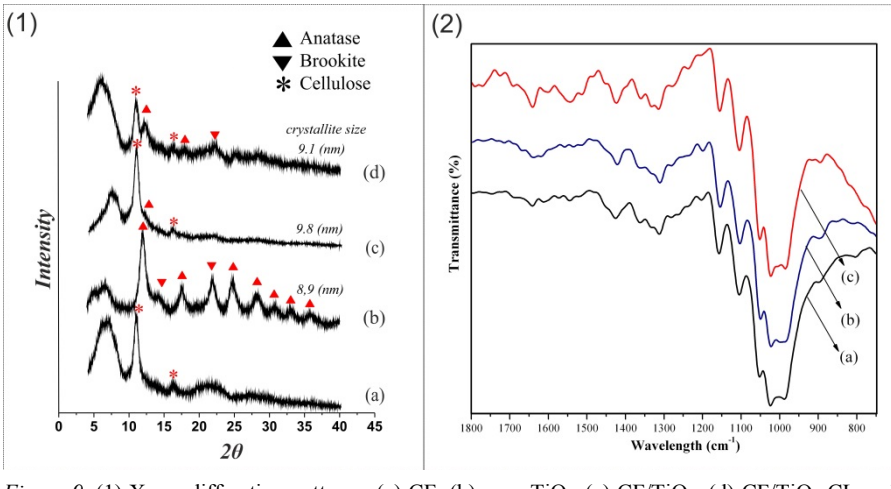

Figure 9. (1) X-ray diffraction patterns: (a) CF; (b) pure TiO_2; (c) CF/TiO_2; (d) CF/TiO_2-CL and (2) FTIR spectra of (a) CF, (b) CF/TiO_2,(c) CF/TiO_2-CL

An insight into interaction of BTCA with titania nanoparticles on the surface of cotton fibers is provided by the FTIR spectroscopy (Fig.9(2)). The absorption peaks assigned to carboxylic groups are present in the spectra of

CF/TiO_2-CL sample after TiO_2 modification with the cross-linking agent BTCA in the 1800–1600 cm^{-1} region (Fig.9(2)c). The carbonyl adsorption peak at 1730 cm^{-1} is attributed to C=O stretching and confirmed the formation of ester bonds in the CF/TiO_2-CL sample. On the basis of observed morphology, it is possible to assume that TiO_2 nanoparticles are cross-linked to the surface of a cotton fiber by formation of transverse ester bonds with BTCA (see Fig.6 in Paper I). Sodium hypophosphite, in this case, acts as a catalyst of the reaction, increasing the rate of cross-linking of cellulose macromolecules to BTCA and TiO_2.

According to SEM and IR analyses and the results of calcination process (see Table 2 in Paper I), in the case of using the cross-linking agent, the mechanism of grafting of titania nanoparticles includes two stages: first, chemical bonding and then – adsorption of nanoparticles on the fiber surface (see Fig.6 in Paper I). The effect of this mechanism can be seen in the materials obtained with a high content of fibers deposited on the surface of titanium dioxide (Galkina *et al.*, 2012). The TiO_2 content in the CF/TiO_2 sample decreases practically twofold after 5 cycles of washing compared to the CF/TiO_2-CL sample (see Table 2 in Paper I). So, the use of a spacer in the samples CF/TiO_2-CL and CF/TiO_2-CL-5W allows binding of titania to the functional groups of cellulose 52% more effectively than without it. Also it can be concluded that only unfixed adsorbed titanium dioxide is washed off during laundry both from the CF/TiO_2 and CF/TiO_2-CL samples.

4.1.2 Antibacterial properties of cotton/TiO_2 composites

It is well known that bactericidal activity of the samples is closely related to its photocatalytic properties and the crystalline structure of synthesized titania (Cai *et al.*, 2013; Gamage McEvoy *et al.*, 2013). As a result of TiO_2 UV-irradiation the reactive oxygen species (OH•, O_2•, HO_2•) are generated on its surface (Chen *et al.*, 2014). Among them OH• hydroxyl radical is considered to be the most important oxidizing agent providing inactivation of bacterial cell (Daoud *et al.*, 2005). Previously, in our research work (Galkina *et al.*, 2012) high photocatalytic activity of the cotton fabric, modified with titania and chemically bonded titania with the usage of the cross-linking agent was investigated in the reaction of decomposition of Rhodamine B as a model dye applied on the material. According to the obtained data, the maximum amount of active oxygen forms and hydroxide radicals is accumulated during this UV-irradiation, which is sufficient for long-term inactivation of *E. coli* bacterial culture. We have been evaluating these properties only after 10 minutes of UV light exposure.

The conducted investigations have shown that the cotton fabrics treated by TiO_2 with or without the cross-linking agent decrease the survival of microorganisms *E. coli* in comparison with the control sample (D = 1.18) and demonstrate high bacteriostatic effect. The interaction of titania nanoparticles with the surface of the *E. coli* cell is quite complicated. To kill *E. coli* completely, the direct attack of hydroxyl radicals is necessary (Ishibashi *et al.*, 2000).

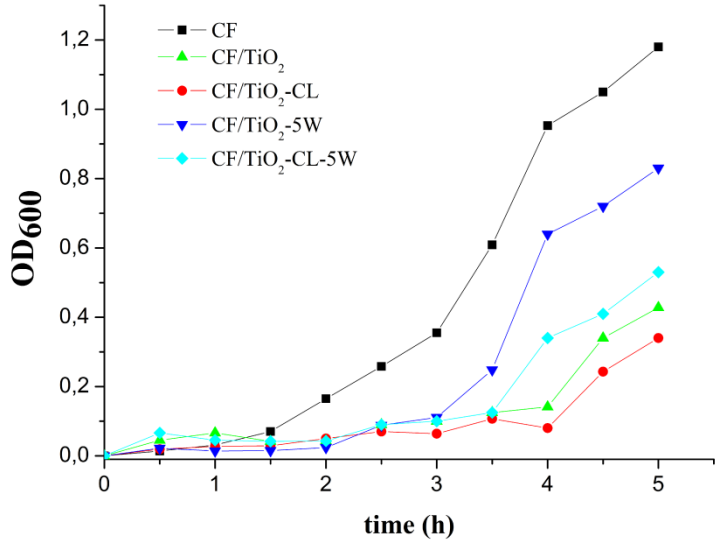

Figure 10. Dependencies of optical density OD_{600} of the gram-negative bacteria *E. coli* solution on the time of contact with the samples showing the antibacterial effect.

It can be seen from Fig.10 that the relative density of the culture after 5 hours of contact is decreased by more than 70% for the CF/TiO_2 and CF/TiO_2-CL samples compared to the control one. Although the lifetime of active oxygen and hydroxyl radicals OH•, resulting after TiO_2 photocatalytic activation on the surface of cotton fibers was insufficient to penetrate the cell and cause its complete destruction. The bacteriostatic activity of titanium dioxide for the CF/TiO_2-CL sample is 20% as low in comparison with the CF/TiO_2 sample. This fact may be explained by the acidic character of the cross-linking agent. It is worth noting that the deviation from monotony of the growth curves in Fig.10, first of all, is related to the non-uniform distribution of the titania hydrosol coating the cotton fibers.

The bactericidal properties of the samples were also tested after five washing cycles. The presence of titania covalently bonded with the cotton fibers leads to inhibition of the bacteria growth even after numerous washing cycles. The relative optical density of the CF/TiO_2-5W sample is equal to 0.83 (see Table 3 in Paper I). On the other hand, using the spacer, the same parameter OD_{600} of the CF/TiO_2-CL-5W sample goes down to 0.53. In this case, the bactericidal activity of the fabric with unfixed titania is reduced by a factor of 2.5 in a result of mechanical action (laundry) in comparison with CF/TiO_2 sample. Thus, the application of 1,2,3,4-butanetetracarboxylic acid as cross-linking agent allows to maintain the bactericidal effect of the modified fibers at approximately half of the level of CF/TiO_2-CL sample after 5 washing cycles.

4.2 Investigation of specific properties of cellulose nanofiber–titania nanocomposites (Paper II & III)

In first paper, the major focus in application of TiO_2 nanoparticles to textile materials was set on cotton fabrics. The modified TiO_2 cotton fibers have self-cleaning and bacteriostatic effects, inhibiting the growth and the development of pathogenic bacteria in the fight against hospital infections. However, the main drawback was the non-uniform distribution of TiO_2 hydrosol in the material and as result non monotonic behaviour of bactericidal properties. Taking into account all benefits that TiO_2 offer to textile materials, it was worth trying to achieve uniform single layer distribution of nanoparticles and expand the scope of practical application of these materials. To the best of our knowledge, no attempts have been made in combining the advantageous characteristics of nanocrystalline cellulose with the specific properties of nanosized titania by chemical modification. According to this, the main objective of Paper II was the development new types of nanocomposites based on cellulose nanofibers and grafted TiO_2 for drug delivery application. This approach has potential to open endless possibilities for creation of new efficient drug delivery systems with a whole range of functional properties. In this part, in order to modify the synthesized cellulose nanofibers, highly bio-digestible TiO_2 nanosol (CaptiGel AB) was used (Cai *et al.*, 2013; Seisenbaeva *et al.*, 2013; Groenke *et al.*, 2012; Seisenbaeva *et al.*, 2010; Kessler *et al.*, 2008). The next step was to bring an insight into how the chemical binding of the two chemically different drugs, Tetracycline and Phosphomycin to the carrier is influencing the antimicrobial efficacy of nanocomposites based on cellulose nanofibers and grafted titania.

4.2.1 Characterization of pure cellulose nanofibers and the nanocomposite based on cellulose nanofibers modified with TiO_2

The SEM study showed that the surface of pure cellulose nanofibers exhibited smooth, homogeneous structures. Moreover, elemental analysis confirmed that the PCNF sample didn't contain any impurity arising from the preparation of the nanocomposites (see Fig.11a). As expected, copper Cu^{2+} and sulphur SO_4^{2-} ions were washed out after acid hydrolysis. According to TEM analysis, pure cellulose nanofibers have a rod shape, and their rough average length and diameter were about 15 and 5 nm, respectively (Fig.11b). After modification with TiO_2, the CNF_TiO_2 sample has uniform morphology and contains TiO_2 nanoparticles evenly distributed over the whole surface. NTA analysis showed particle size distribution of PCNF and CNF_TiO_2 samples centred at around 260 nm and 160 nm respectively for the hydrodynamic size, involving also water in primary hydration layers (Fig.11). A larger hydrodynamic size for pure cellulose nanofibers in comparison with those in CNF_TiO_2 sample is connected with their aggregation via strong hydrogen bonding, which is apparently decreased after their coating by titania.
The results obtained from AFM analysis also confirmed that pure cellulose nanofibers had homogeneous topography. CNF_TiO_2 sample was uniformly coated by titania nanoparticle with a size of around 10 nm (Fig.12(b,c)).

Thus, the results of SEM, TEM and AFM analyses showed that the use of CaptiGel nanosol allowed obtaining of the uniform single layer of TiO_2-coalesced nanoparticles onto the surface of cellulose nanofibers.

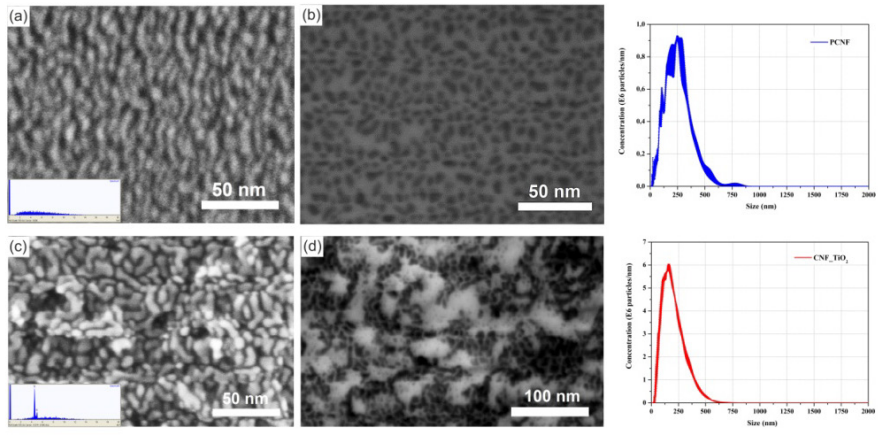

Figure 11. SEM and TEM images of (a,b) PCNF and (c,d) CNF_TiO_2 respectively together with the hydrodynamic size of pure cellulose nanofibers and nanocomposite based on cellulose nanofibers modified with TiO_2

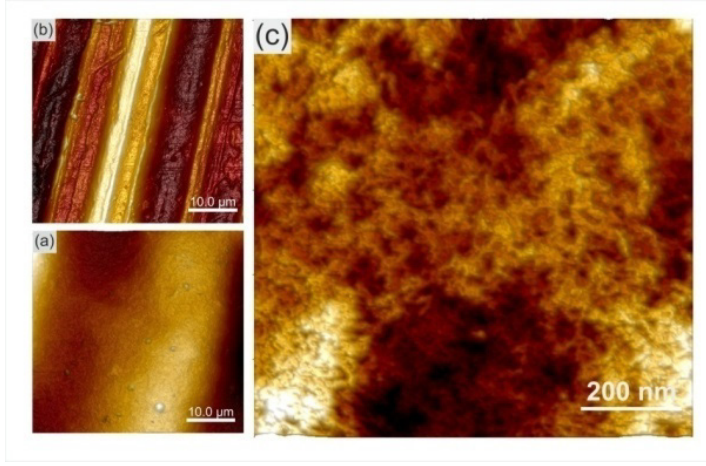

Figure 12. AFM images of (a) PCNF and CNF_TiO_2 samples (b,c)

XRD measurements were performed in order to verify any alternations of cellulose crystallinity due to chemical treatment (Fig.13(1)). From the diffractograms it is clear that the structure of PCNF and CNF_TiO_2 samples refers to cellulose II crystalline structure with characteristic peaks at 2θ = 5.5º and 2θ=9.3º in comparison with raw cotton. The absence of the TiO_2 diffraction peaks in the sample CNF_TiO_2 should be remarked, as it shows that the size of TiO_2 nanoparticles is smaller than the coherence domain required for the X-ray reflection. The cellulose crystallinity of the synthesized samples is 86-98%, which is much higher than the value for the starting material due to removal of amorphous parts from cellulose composition during the acid hydrolysis as well as cellulose recrystallization (see Table 2 in Paper II).

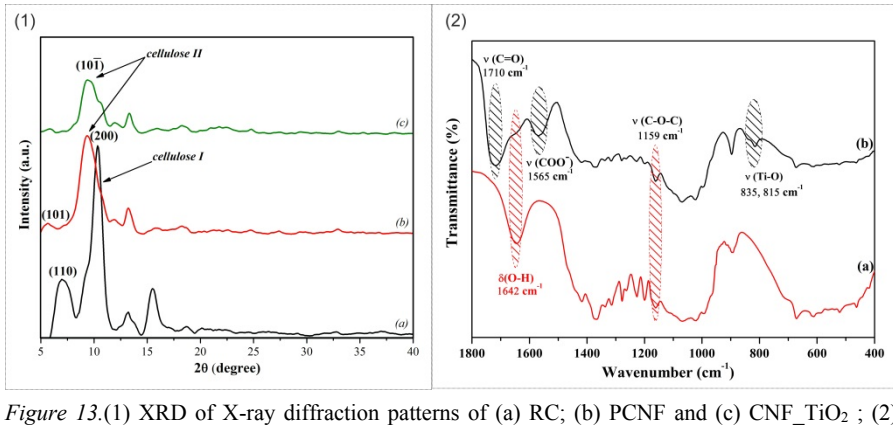

Figure 13.(1) XRD of X-ray diffraction patterns of (a) RC; (b) PCNF and (c) CNF_TiO_2 ; (2) FTIR spectra of (a) PCNF and (b) CNF_TiO_2

As in Paper I, BTCA was used to cross-link titania nanoparticles with cellulose fibers. In this case, the chemical interaction of BTCA with titania nanoparticles on the surface of cellulose nanofibers was also confirmed by IR-spectroscopy. The spectra (Fig.13(2)) show characteristic peaks of carboxylic groups in CNF_TiO_2 sample after TiO_2 modification when using the cross-linking agent BTCA in the 1800–1600 cm^{-1} region. The carbonyl adsorption peak at 1710 cm^{-1} is attributed to C=O stretching and confirmed the formation of ester bonds in the CNF_TiO_2 sample. The weak absorption peaks at 835 cm^{-1} and 815 cm^{-1} are attributed to Ti-O-Ti vibration (Fig.13(2)).

4.2.2 Preparation and characterization of nanocomposite TiO_2-nanocellulose loaded with different drugs

Four different medicines, Diclofenac sodium, Penicillamine-D, Phosphomycin and Tetracycline were chosen as model drugs for modification of cellulose nanofibers and further investigation in resulting drug release system. DS is a potent non-steroidal compound with pronounced analgesic, anti-inflammatory and antipyretic properties. PCA-D is used as a medicinal agent against rheumatoid arthritis, and other chronic autoimmune diseases (Czlonkowska *et al.*, 1996). Phos is a natural broad spectrum antibiotic compound which is mainly used for the treatment of uncomplicated urinary tract infections and meningitis, pneumonia, and pyelonephritis (Castañeda-García *et al.*, 2013). TC was selected because of its wide applications as antibiotic in the treatment of skin bacterial infections, arising from its antimicrobial activity against numerous medically relevant aerobic and anaerobic bacterial genera, both Gram-positive and Gram-negative (Karuppuswamy *et al.*, 2015). Moreover, TC is the most commonly prescribed antibiotics in outpatient care within Swedish human medicine after betalactams and is also widely used in veterinary medicine, especially for therapeutic group treatment of pigs (SWEDRES-SVARM, 2012).

The purpose of chemical modification was to synthesize a novel transdermal drug delivery system by binding a drug molecule to the biopolymer through interaction with TiO_2 grafted onto it (Fig.14). In order to obtain uniform distribution of the drug within the cellulose nanofibers film and to bind drug with cellulose nanofibers, drug modification was performed by two different methods, which are described in the Experimental part and named M1 and M2. Drug grafting was performed in amounts calculated assuming the formation of a uniform, single layer coverage on TiO_2-modified cellulose nanofibers' surface (Pązik *et al.*, 2011). Thus, total amounts of DS, PCA-D, Phos and TC in the obtained nanocomposites were 7.7 wt%, 3.8 wt%, 4.7 wt% and 4.6 wt%, respectively.

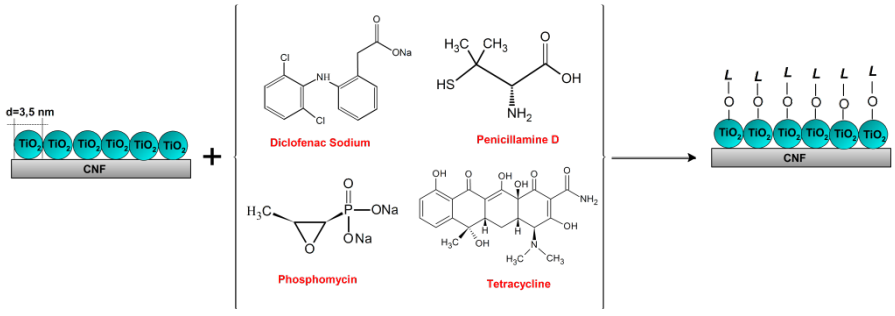

Figure 14. Proposed scheme for the interaction of nanocomposite based on cellulose nanofibers and TiO_2 with different types of drugs

From results of the UV-Vis spectroscopic investigation for CNF_TiO_2_DS_M1 and CNF_TiO_2_DS_M1, CNF_TiO_2_PCA-D_M1 and CNF_TiO_2_PCA-D_M2 samples the complexation of drugs on the surface of titania was confirmed (Fig. 15). The intensity of maximum absorption peak at 224 nm was observed for pure titania nanoparticles and used as a reference (Kessler *et al.*, 2008). The spectrum of the resulting solution from reaction between titania nanosol and a drug shows an absorption peak at 375 nm and 474 nm for DS and PCA-D, respectively (Fig.15). Tetracycline is supposedly binding to the titania surface through formation of phenoxide complexes, which are also considered as quite stable (Shavit *et al.*, 2007). The FT-IR spectra of CNF_TiO_2_TC_M1 and CNF_TiO_2_TC_M2 samples shows the appearance of a new absorption peak at 1620 cm^{-1} corresponding to the stretching vibration of C=O aromatic ring carbonyl groups in TC (Myers *et al.*, 1983) (see Fig.1 in Paper III). Thus, titania binds to cellulose nanofibers via formation of ester bonds and with drug molecules due to formation of chelating complexes.

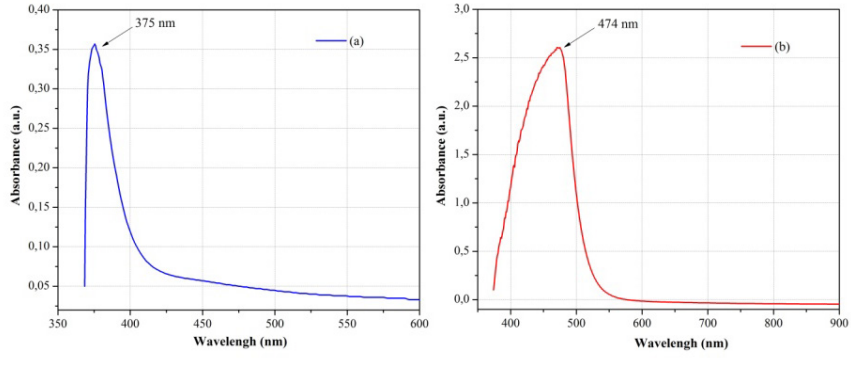

Figure 15. UV spectrum of the resulting solution from the reaction between titania and diclofenac sodium (a); between titania and penicillamine D (b)

4.2.3 *In vitro* drug release

The drug delivery matrices reported in the present work were employing nanotitania as the active component for drug binding and retention. Comparison between release profiles of drugs from the nanocomposites showed that DS and TC are released much faster than PCA-D regardless of the modification method (Fig.16)

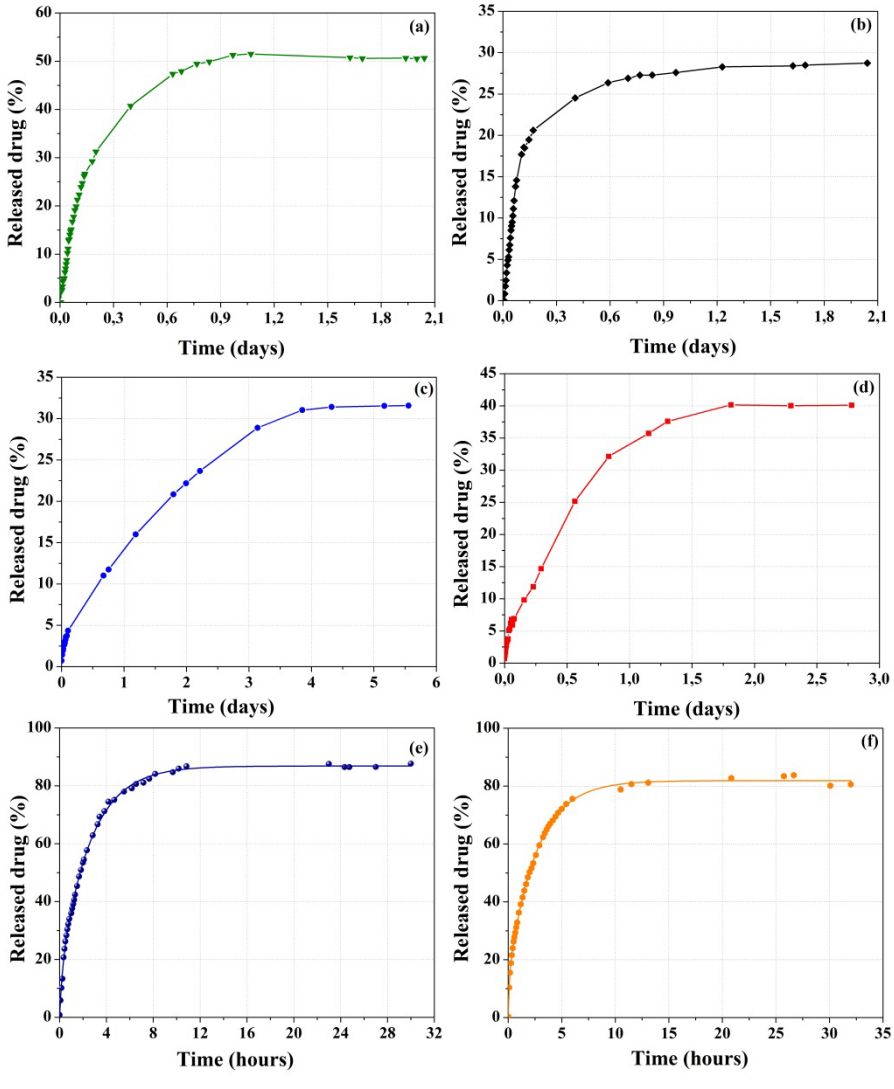

Figure 16. In vitro drug release profiles from the obtained nanocomposites: (a) CNF_TiO_2_DS_M1, (b) CNF_TiO_2_DS_M2, (c) CNF_TiO_2_PCA-D_M1, (d) CNF_TiO_2_PCA-D_M2, (e) CNF_TiO_2_TC_M1, (f) CNF_TiO_2_TC_M2

For the CNF_TiO_2_DS_M1 sample, the release of DS is carried out with a constant speed for more than 10 hours, reaching the equilibrium in 16 hours with a total amount of DS released around 50% (Fig. 16a). A similar behaviour of slow DS release was observed for the CNF_TiO_2_DS_M2 sample, but only 28% of DS was released over 15 h (Fig. 16b). The nanocomposites with Penicillamine D obtained by method #1 and #2 released the drug more slowly. The CNF_TiO_2_PCA-D_M1 sample displayed a sustained long-term release profile of PCA-D where around 31% of drug released in a controlled manner over 96 hours. Method #2 for incorporation of PCA-D into the nanocomposite allows the reduction of the release time to 43 hours with a total amount of ~40% (Fig.16d). In case of TC, around 80% of the drug was released in a controlled manner over 7 hours for CNF_TiO_2_TC_M1 and CNF_TiO_2_TC_M2 samples (Fig.16(e,f)). Thus, these studies clearly demonstrated that using different methods of binding drug molecule to the biopolymer through interaction with TiO_2 provide slow release, and most importantly, the level of released drug remains constant over a long time. It is important to note that using cross-linking agent for binding TiO_2 with cellulose nanofibers has important implications for the release process. In particular, we have demonstrated burst release of DS from nanocomposites obtained without using BTCA (see Fig.10 in Paper II). The drug was lost together with the non-grafted titania, simply washed from the material to which, in the absence of the cross-linking agent, it was not chemically bound.

To study the *in vitro* kinetics of Phosphomycin release from the obtained nanocomposites, were performed by radio-labeling analysis using phosphorylated drug ^{33}P-marked ATP as a model for Phos.

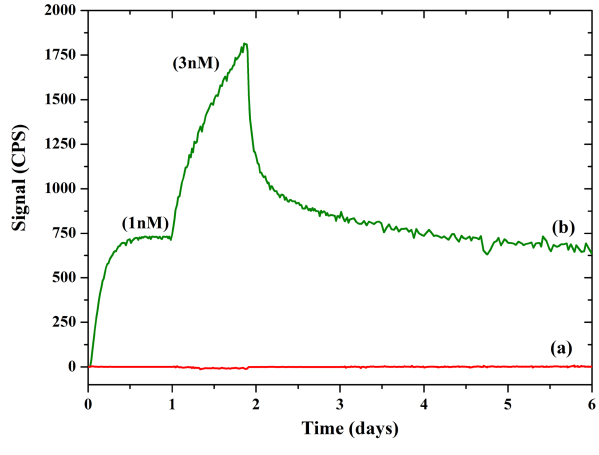

Figure 17. Real-time binding and release curve of 1 nM and 3 nM ^{33}P-marked ATP to (a) PCNF and (b) CNF_TiO_2 sample

The studies of immobilization and release ^{33}P-marked ATP from mesoporous titania microparticles have been reported previously (Seisenbaeva *et al.*, 2010). In this work, strong binding of ^{33}P-marked ATP with the surface of TiO_2-modified nanocomposites was also observed. Namely, the release of ^{33}P-marked ATP from the CNF_TiO_2 sample occurred in two steps: first, 40% of the ^{33}P-marked ATP dissociated after first 4 h and only an additional 25% dissociated in the following 100 h. No uptake was observed for the CNF sample (Fig.17a). The obtained results showed very long-term release for the strongly chemisorbed antibiotic.

The release kinetics data were in good agreement with the medicinal properties of drugs. It should also be mentioned that antibiotic formulations are usually applied topically three of four times a day in order to achieve the recommended dosage (Boateng *et al.,* 2008; Hurler *et al.,* 2012). In comparison with them, the benefits on the use of the developed nanocomposites as antibiotic patches with ability of sustained drug release offer an enhancement of local concentration of the antibiotics without necessity of frequent dressing changes. Thus, the nanocomposites produced by this technology have promising performance as wound-dressing materials and, as shown recently, even as anesthetics and analgesics.

4.2.4 Antibacterial activity of cellulose nanofibers - titania nanocomposites

The purpose of the bacteria susceptibility tests was to get an insight into how the discovered different release kinetics could influence the antibacterial effects of the drugs after release. The disk diffusion method and liquid broth assay were used to evaluate the susceptibility of two of the most common and important pathogens, *S. aureus* and *E. coli*, against two different types of antibiotic drugs such as TC and Phos immobilized into the nanocomposites.

The minimal inhibitory concentration (MIC) against the two strains differs for TC and Phos. To be able to directly compare the antibacterial effect of the cellulose nanofibers loaded with the two drugs, their MIC-values have to be taken into account. TC showed higher activity against the S. aureus strain with MIC-value of 1 μg/ml compared to the *E. coli* strain of 5 μg/ml. Phos exhibited a MIC-value of 10 μg/ml against *S. aureus* and > 100 μg/ml for *E. coli.* The disk diffusion test showed that both bacterial strains responded to the TC released from CNF_TiO_2_TC_M1 sample (Fig.18(d,f)). The inhibition zones, for both bacteria strains, were similar for CNF_TC_M0 and CNF_TiO_2_TC_M1. However, a trend that CNF_TC_M0 sample without titania resulted in a higher inhibition zone was observed, especially for *E. coli* (please, see Supplementary for Paper III, Table S1).

In contrast, the too slow release of Phos from nanocomposite with titania (CNF_TiO_2_Phos_M2) did not permit the drug to achieve a concentration high enough to inhibit bacteria growth, rendering the related nanocomposite formulations inactive (Fig.18e). Compared to it, CNF_Phos_M0 sample obtained without using TiO_2 as a binding agent between the biopolymer and Phos showed good antibacterial activity against both bacteria strains (Fig.18b). These results were in good agreement with release kinetics of ATP studies (Paper II) and clearly demonstrated that the usage of titania provides much slower release of Phos.

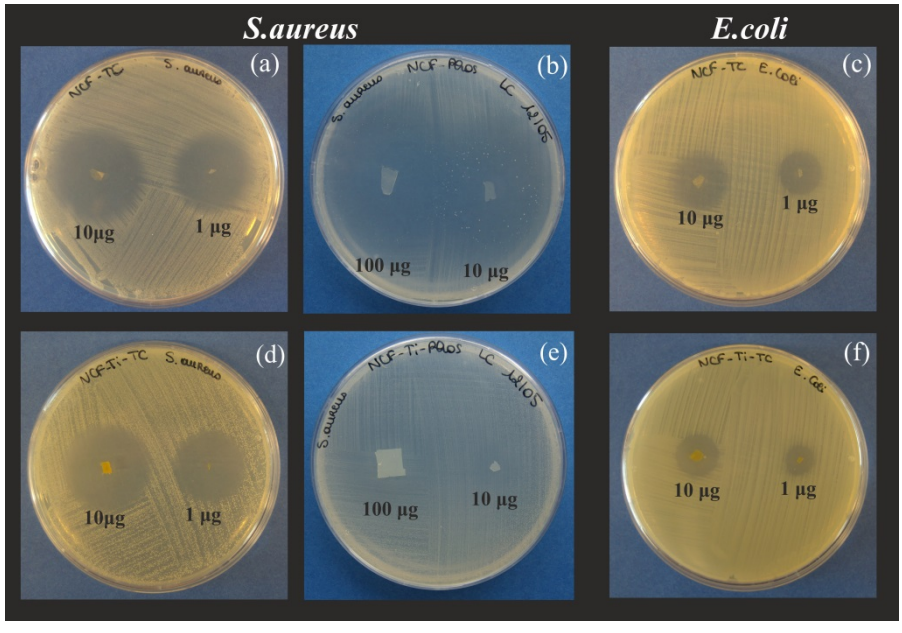

Figure 18. Inhibition zone of the (a,c) CNF_TC_M0, (b)CNF_Phos_M0, (d,f)CNF_TiO_2_TC_M1 and (e) CNF_TiO_2_Phos_M2 against *S. aureus* and *E.coli*

The results from the liquid broth assay also demonstrated the effectiveness of TC released from samples in inhibiting bacterial activity (please, see Supplementary for Paper III, Table S2). Addition of CNF_TiO_2_TC_M1 or CNF_TC_M0 giving a final concentration of TC in the broth 10 µg/ml, i.e. 10 x MIC, simultaneously as bacteria inoculation resulted in complete growth inhibition of *S. aureus*. The same results were revealed when decreasing the concentration of TC to 1 µg/ml, i.e. the MIC value for *S. aureus*. This implies that all TC molecules loaded on the cellulose nanofibers are released to the media in a bio-active form.

In the view of the long-term release profile of Phosphomycin, we further investigated the effects of inoculation delay of CNF_TiO_2_Phos_M2 sample against *S. aureus* in MH broth (see Supplementary for Paper III, Table S2). It was found that CNF_TiO_2_Phos_M2 sample in concentration of 10 x MIC reduced bacterial growth compared to control without cellulose nanofibers, but did not result in a completely growth inhibition. This implies that less than 10% of the Phoshomycin is released in a bio-active form under these experimental conditions. Not even delaying the bacteria inoculation with 24 hours resulted in a complete growth inhibition.

On the other hand, important difference in antibacterial activity of CNF_TiO_2_Phos_M2 sample with immediate inoculation of *S. aureus* has been found after addition of citrate buffer (0.03M, pH=6) using as a model for body fluid conditions as well as to dissolve titania binding to Phosphomycin by chelating complexes. Consequently, the enhanced speed of drug release resulted in increased antibacterial activity of CNF_TiO_2_Phos_M2. The obtained results from broth culture test with immediate inoculation of *E. aureus* demonstrated that Phos released from the nanocomposite possess high antibacterial activity and reduce the bacterial growth to 100% (please, see Supplementary for Paper III, Table S2). Thus, our results proved that in spite of strong binding by intermolecular interactions between drug molecule and the nanocomposite, the released drug retains its medicinal properties against pathogen microorganism and confirmed that the drug molecule and the nanocomposite are compatible with each other.

4.2.5 Photochemical properties of cellulose nanofibers - titania nanocomposites

Photocatalytic activity of pure cellulose nanofibers and the nanocomposite based on cellulose nanofibers and TiO_2 was assessed on Rhodamine B degradation under the UV irradiation (Fig.19).

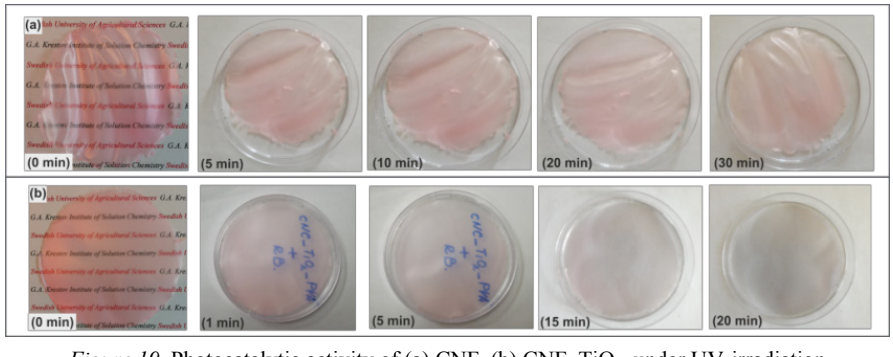

Figure 19. Photocatalytic activity of (a) CNF, (b) CNF_TiO_2, under UV-irradiation

The obtained results showed that no decomposition of an organic dye on PCNF took place (Fig.19a). Unlike untreated fabrics, it was interesting to observe UV-activity of the CNF_TiO_2 nanocomposite with complete degradation of Rhodamine B after 20 minutes (Fig.19b).

As could be noticed, titania nanoparticles, which are used for cellulose nanofibers modification, have a crystalline core covered with an amorphous layer containing triethanolamine. Previously, it has already been demonstrated that initial titania nanosol did not display any noticeable photochemical activity according to the photocatalytic test with Rhodamine B (Kessler *et al.*, 2008). However, such behaviour of the CNF_TiO_2 nanocomposites can be explained by the presence of carboxylic groups in the butane-tetracarboxylate linker, which activates the crystalline core of TiO_2 under UV-irradiation (Fujishima *et al.,* 2008; Moustakas *et al.,* 2013; Ohtani *et al.,* 1997).

Another important observation is that the photocatalytic properties of nanocomposites modified with TiO_2 and the drugs were found to be completely different in comparison with pure and TiO_2-modified cellulose nanofibers. Specifically, CNF_TiO_2_Phos_M2 displayed photochromic effect, i.e. colour change from pink to purple under UV-light for 10 minutes (please, see Fig.5c in Paper III). We suggested that this change in colour can be attributed to transition of Ti^{4+} to Ti^{3+} under UV-irradiation and the following formation of the complexes with the drug molecule (Sanchez *et al.,*2010). Moreover, the reverse transition of colour shows that the formed complexes are not redox stable and are re-oxidized over time. At the same time, Tetracycline modified nanocomposites did not display any photocatalytic activity even after 3 hours of UV irradiation (see Fig.5d in Paper III). A possible explanation for this effect is that the complexation of the titania with the extended aromatic system of the Tetracycline drug results in a system acting as a trap, causing immediate recombination of the electron-hole pairs created by irradiation. Moreover, the nanotitania is in this case acting for protection of Tetracycline against UV-rays. In the view that Tetracycline as effective broad-spectrum antibiotic is widely used for wound healing, its protection against UV-rays is also a very important goal in the development of bactericidal patches. According to this, the results of the disk diffusion tests of CNF_TiO_2_TC_M1 showed that the released TC retains its medicinal properties without decreasing of biological activity after 30 and 60 minutes of UV treatment against *S.aureus* (Table 2). In case of CNF_TC_M0 sample obtained without using titania as a binding agent, a significant drop of antimicrobial activity was observed.

Table 2. *Antimicrobial activity of samples after UV-irradiation against S.aureus*

Sample	Concentration (μg/disk)	Inhibition zone (mm)		
		without UV-irradiation	after 30 min of UV- irradiation	after 60 min of UV- irradiation
CNF_TC_M0	1	23.5±2.9	13.8±4.0	12.3±3.8
	10	30.5±0.6	26±1.4	23.3±0.5
	100	38.3±2.6	33.5±1	31.8±1.3
CNF_TiO_2_TC_M1	1	19.3±3.4	11.3±1.7	10.5±1.7
	10	25.8±3.7	27.0±2.1	25.5±1.3
	100	32.3±4.4	33.2±1.0	32.3±1.7

Thus, the relatively weaker binding between Tetracycline and modified biopolymers makes possible the use of these nanocomposites as a very efficient antibacterial patches against most pathogens bacteria with protection against UV exposure.

5 Conclusion

In perfect agreement with the main goal of the thesis, new functional hybrid nanomaterials based on titania nanoparticles and cellulose were successfully produced by "green chemistry" approach in aqueous media. One of the most important advantages of this work is that the composite material contains titania strongly bound to the matrix, which makes it possible to use for different potential applications with prolonged action of specific properties.

In the first part, we demonstrated the application of nanocrystalline titania hydrosol produced by low-temperature sol-gel method for modification of natural cellulose fibers with usage of 1,2,3,4 –butanetetracarboxylic acid as a cross-linking agent. The obtained composites exhibited highly bactericidal properties after UV irradiation and maintained them even after five cycles of washing.

Second part of work demonstrated the unique properties of titania-based system. New efficient drug delivery systems based on cellulose nanofibers – titania nanocomposites grafted with four different type of model drugs such as Diclofenac Sodium, Penicillamine D, Phosphomycin and Tetracycline were successfully synthesized. The results of SEM and TEM analyses demonstrated that titania nanoparticles and drugs were uniformly distributed within the cellulose nanofiber films. IR and UV-Vis spectroscopies confirmed that titania binds to cellulose nanofibers via the formation of ester bonds and with drug molecules due to formation of chelating complexes.

According to drug release studies, the chemical structure of the medicines and as a result, difference in their binding to nanocomposite has important implication for drug release process. In particular, the relatively weaker phenoxide binding between TC and TiO_2-modified cellulose nanofibers leads to faster release compared to DS and PCA-D. In case of DS, the complexation with the titania through carboxylic groups produced relatively slow drug release. PCA-D, on the other hand, was much slower released from

nanocomposite due to strong binding to titania surface via complexation through amino groups. Finally, due to formation of highly dissociation-stable complex between Phos and titania, the longest release kinetics were observed for nanocomposites modified with Phos.

In vitro results of microorganism susceptibility tests proved that released TC was highly effective against both bacteria strains with MIC value of 1 μg/disk. Moreover, TC retains its medicinal properties without decreasing of any biological activity after 60 minutes of UV treatment against *S.aureus*. Due to long-term release profile of Phos, high antibacterial activity of nanocomposite modified with Phos against *S.aureus* was observed only after addition of citrate buffer helping to dissolve titania.

In addition, a photocatalytic test showed that nanocomposites modified only with TiO_2 possess strong photocatalytic properties with complete degradation of model dye Rhodamine B after 20 minutes.

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