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Synthesis and antibacterial activity of novel titanium dioxide doped with silver

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Abstract Pure and silver doped nanoparticles of titanium dioxide (TiO_2) was prepared using novel, modified sol–gel method. The samples were characterized by transmission electron microscopy, X-ray diffraction, N_2 adsorption measurement, atomic absorption spectroscopy (AAS), UV–vis spectroscopy (UV–vis). The antibacterial activity of the prepared samples was indicated by minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) values according to the reference methods of Clinical and Laboratory Standards Institute for the determination of MIC of aerobic bacteria by broth microdilution. The results showed very good antibacterial activity of silver nanoforms to bacteria strains: Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* and *Klebsiella pneumoniae*. The sensitivity of the tested bacteria to silver nanoforms depends on the crystalline form of the carrier—TiO_2, its surface area, porosity, the content of silver, its particle size and oxidation state. The originality of this work is the synthesis of novel type of nanocomposites TiO_2 doped with silver and determination its excellent antibacterial activity.

Keywords Titanium dioxide · Silver · Nanoparticles · Bacteria

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1 Introduction

With regards to its unique photocatalytic activity, titanium dioxide (TiO_2) has received considerable attention in recent years. Since 1977, following Frank and Bard [1] confirming the possibility of using TiO_2 in the degradation of cyanide in water; there has been an increased interest in the environmental, medical and biological application of TiO_2 [1, 2]. Additional research has confirmed its good efficacy in the degradation of a wide variety of organic and inorganic pollutants [3–10]. Photocatalytic oxidation as a technique for microbial disinfection was first demonstrated by Matsanuga et al. [9]. Titanium white is, among other things, used in cosmetics as a white pigment in cream, face powder, lipstick and sunscreen [11]. TiO_2 is found in three main crystallographic forms in the environment: anatase, rutile and brookite [12]. The anatase type has been acknowledged as the highest in photocatalytic properties and may be used in various applications, also as a carrier of biologically active particles e.g. silver [13]. There is not much information about biological activity of anatase without UV irradiation which is necessary for photocatalytic activity. There is also no information about biological activity of amorphous TiO_2 as a carrier of silver ions or silver nanoparticles. We prepared various forms of TiO_2—amorphous and anatase (both doped with silver) and compared its antibacterial activity without UV irradiation.

Silver has been exploited for its medicinal properties for centuries. Its biological activity has been confirmed against Gram-positive and Gram-negative, aerobe and anaerobe bacteria, fungi and protozoan. Chemotherapeutic agents as opposed to antibiotic ones have shown an oligodynamic effect [14–18]. Involving metals by bacteria from culture medium can take place by physical adsorption of ions to

the surface of bacteria (van der Waals force) exchanging ions or connections between metal ion and groups of macro and macromolecules, especially in elements such as oxygen, sulfur and nitrogen [19]. Silver is toxic to microorganisms and poisons the respiratory enzymes and components in the microbial electron transport system. Additionally, silver can interact with structural proteins such as fimbriae—protein structures located on the cell's surface of bacteria, responsible for adhesion to the artificial surface or macroorganism cells. Other research connects silver accumulation in bacterial cells and its interaction with cytosolic proteins, mitochondrial enzymes and DNA or RNA synthesis [20]. Resistance of microorganisms to silver is rare [20, 21]. Bacteria can combat metal ions by decreasing the synthesis of outer membrane proteins, cellular efflux system (active or chemoosmotic) [21] and bound by cells in the form of an intracellular complex [20]. Bacteria can also transform ions to their less toxic form [21, 22]. It confirms very good properties of silver in biological and medical applications as alternative way of killing bacteria.

The originality of this work is the new method of synthesis of very small particles of TiO_2 doped with silver as a new type of nanocomposities, which exhibit excellent antimicrobial efficacy without additional factor such as UV irradiation. The tested bacteria strains (Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* and *Klebsiella pneumoniae*) are responsible for most hospital infections such as: wound infections and urinary track infections. We showed that the titanium dioxide doped silver nanocomposites can be used in numerous biomedical, pharmaceutical and industrial applications also without UV irradiations.

2 Experimental

2.1 Reagents

Titanium (IV) n-butoxide (Ti(O–Bu), 99%), ethyl alcohol (C_2H_5OH, 96%), methyl alcohol (CH_3OH), ammonium hydroxide ($NH_3 \times H_2O$, 25%), hydrofluoric acid (HF, 40%) and acetone were purchased from *POCh*. Glucose ($C_6H_{12}O_6$), potassium hydroxide (KOH) was bought from *Chempur*, and the silver nitrate ($AgNO_3$, 99%) was purchased from *Aldrich*. Deionized water was used in each of the experimental preparations.

2.2 Medium

Muller Hinton Agar (MHA), Muller Hinton Broth (MHB) was purchased from *Biocorp*.

2.3 Synthesis of amorphous and crystalline TiO_2 doped with silver (TiO_2:Ag^0, TiO_{2a}:Ag^0, TiO_2:Ag^0/Ag^+, TiO_{2a}:Ag^0/Ag^+, TiO_2:Ag^0/Ag^0 and TiO_{2a}:Ag^0/Ag^0 nanocomposites)

Method synthesis of TiO_2 doped with silver (TiO_2:Ag^0 and TiO_{2a}:Ag^0) consists in doping TiO_2 with silver during synthesis process.

Titanium dioxide doped with silver—amorphous TiO_2:Ag^0 and crystalline TiO_{2a}:Ag^0—were synthesized through modified sol–gel methods [23]. In our propose silver nanoparticles were incorporated in a TiO_2 matrix during the carrier synthesis. In the sol–gel process TiO_2 is usually prepared by the following steps: hydrolysis and polycondensation of titanium alkoxides (Ti(OR)n). Sol–gel reaction occurred in acetone environment, in presence of catalyst (ammonium hydroxide) and hydrofluoric acid. Titanium n-butoxide (6 ml) and hydrofluoric acid (250 µl) was added drop wise to acetone (50 ml) with stirring. Next, ammonium hydroxide (2 ml) and diamminesilver (I) ($[Ag(NH)_2]^+$) was added. The solution was stirred for 30 min. Preparation of diamminesilver (I) $[Ag(NH_3)_2]^+$ is described below.

The gel was then washed in methyl alcohol and water and centrifuged at 4,000 rpm. Ready gel was dried at 80°C. In preparing the crystalline form it was calcined at 400°C for 10 h at a ramp rate 5°C/min after drying.

Such prepared samples may be subjected to additional silvered. To prepare TiO_2 doped with additional silver (TiO_2:Ag^0/Ag^+, TiO_2:Ag^0/Ag^0, TiO_{2a}:Ag^0/Ag^+ and TiO_{2a}:Ag^0/Ag^0) the powder of amorphous and crystalline TiO_2 with silver (TiO_2:Ag^0, TiO_{2a}:Ag^0) were treated with diamminesilver (I) $[Ag(NH_3)_2]^+$ or diamminesilver (I) $[Ag(NH_3)_2]^+$ and reducing factor (glucose). The process consisted of two steps: impregnation and/or reduction. First, the powder of TiO_2:Ag^0 and TiO_{2a}:Ag^0—0.2 g—was impregnated with a silver solution $[Ag(NH_3)_2]^+$ at 22°C per 24 h (0.4 M) and distilled water (to final volume 22 ml). The products (TiO_2:Ag^0/Ag^+ or TiO_{2a}:Ag^0/Ag^+) was then washed with methyl alcohol and water to remove the excess of silver and centrifuged at 4,000 rpm. Next samples with additional silver ions were abandon or were submitted to a reducing chemical factor—glucose (6.8%) to prepared silvered TiO_2 with double silver nanoparticles—TiO_2:Ag^0/Ag^0 or TiO_{2a}:Ag^0/Ag^0 [24, 25]. The entire mixture was stirred for 15 min and stored at 70°C per 24 h. The product was then washed with methyl alcohol and distilled water, centrifuged at 4,000 rpm and suspended in a suitable amount of water. All prepared samples are described in Table 1.

2.4 Preparation of diamminesilver (I)$[Ag(NH_3)_2]^+$

The diamminesilver (I) nitrate solution (Tollen's reagent) was prepared by taking 0.1 M of the silver nitrate solution

Table 1 Characteristics of prepared samples

Sample	Kind of carrier	Size of carrier (nm)	Size of silver nanoparticles (nm)	Contents of silver (%)
TiO_2:Ag^0	Amorphous TiO_2	≤100 nm	≤5 nm	1.8
TiO_2:Ag^0/Ag^0	Amorphous TiO_2	≤100 nm	≤5 nm	11.6
TiO_2:Ag^0/Ag^+	Amorphous TiO_2	≤100 nm	n/a	11.6
TiO_{2a}:Ag^0	Crystalline TiO_2, anatase	≤50	≤5 nm	1.6
TiO_{2a}:Ag^0/Ag^0	Crystalline TiO_2, anatase	≤50	≤5 nm	2.2
TiO_{2a}:Ag^0/Ag^+	Crystalline TiO_2, anatase	≤50	n/a	2.2

and adding a solution of potassium hydroxide to form a brown precipitate [24, 25]. The sediment was then dissolved in ammonium hydroxide (25%), and stirred until all the precipitate disappeared.

2.5 Characteristics of TiO_2:Ag^0

The morphology of the samples was investigated by transmission electron microscopy (TEM, Philips CM20 Super Twin). The crystalline phase was identified using a X-ray diffraction (XRD, Stoe). Porosity and Brunauer-Emmett-Teller BET surface area were obtained from nitrogen adsorptions isotherms (Autosorb 1-C, Quantachrome Instruments). The contents of silver were measured using an atomic absorption spectroscopy (AAS—Perkin Elmer 1100).

2.6 Bacteria strains

The following bacteria strains from the American Type Culture Collection were tested:

Staphylococcus aureus ATCC 6538 (Gram-positive bacteria), *E. coli* ATCC 11229 (Gram-negative bacteria), *K. pneumoniae* ATCC 4352 (Gram-negative bacteria). Clinical isolates of bacteria (from urinary track infections and wounds) strains were also tested (from species of *S. aureus*, *E. coli* and *K. pneumoniae)*.

Before the test the purity of the colonies were verified. For the antibacterial test 16–20 h of bacteria incubation in MHB was used.

2.7 Antibacterial effect of amorphous and crystalline titanium doped with silver (TiO_2:Ag^0, TiO_{2a}:Ag^0, TiO_2:Ag^0/Ag^+, TiO_{2a}:Ag^0/Ag^+, TiO_2:Ag^0/Ag^0 and TiO_{2a}:Ag^0/Ag^0 nanospheres)

The antibacterial effect of amorphous and crystalline TiO_2:Ag^0, TiO_{2a}:Ag^0, TiO_2:Ag^0/Ag^+, TiO_{2a}:Ag^0/Ag^+, TiO_2:Ag^0/Ag^0 and TiO_{2a}:Ag^0/Ag^0 nanospheres was determined by minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values according to reference methods of Clinical and Laboratory Standards Institute (CLSI) for the determination of MICs of aerobic bacteria by broth microdilution. The MIC values were the lowest concentration of silver on a microplate that inhibited the growth of bacteria after 16–19 h of incubation at 37°C. MBC had the lowest concentration of the extract in which 99.9% of bacteria were killed [26, 27]. The lower MIC and MBC values the better antibacterial activity. In the experimental procedure, MHB and MHA were used as a medium. Stock of the antimicrobial agent's dilution was prepared according to the reference method and 200 ml per every spot were poured. Preparation of inoculum: adjusting the 0.5 McFarland standard contains approximately 1–2 × 10^8 CFU/ml (1) and diluted 0.5 McFarland suspension in sterile saline in the ratio of 1:10 (2). The final inoculum was 10^4 CFU per spot. Nanoparticles-free spots with medium and cultures were used as growth controls.

3 Results and discussion

3.1 Synthesis and characterization of silvered TiO_2

We have found that Ti(O-Bu) hydrolysis and sol–gel condensation in the presence of acetone is much better than in ethanol (data not described) and dropping small concentrations of hydrofluoric acid causes a decrease in the size of TiO_2 particles. The particle size of TiO_2 depends on composition, calcination temperature and the grain size [28]. Although the radius of silver ion (Ag^+) is much larger than that of Ti^{4+} ion and Ag^+ ion introduced by the impregnation process couldn't enter into the lattice of the anatase phase to form a stable solid solution [29], adding diamminesilver (I) to the prepared mixture of TiO_2 produced silver nanoparticles of size less than 5 nm embedded in the structure and on the surface of TiO_2. Silver embodied to the TiO_2 structure during synthesis (TiO_2:Ag^0 and TiO_{2a}:Ag^0) can also be a source for additional content of silver. It caused increase content of silver from 1.8 to 11.6% (for amorphous TiO_2:Ag^0) and from 1.6 to 2.2% (for crystalline TiO_{2a}:Ag^0). We indicated experimentally that the synthesis time can be reduced from 120 to 30 min, which does not impact the efficacy of technology.

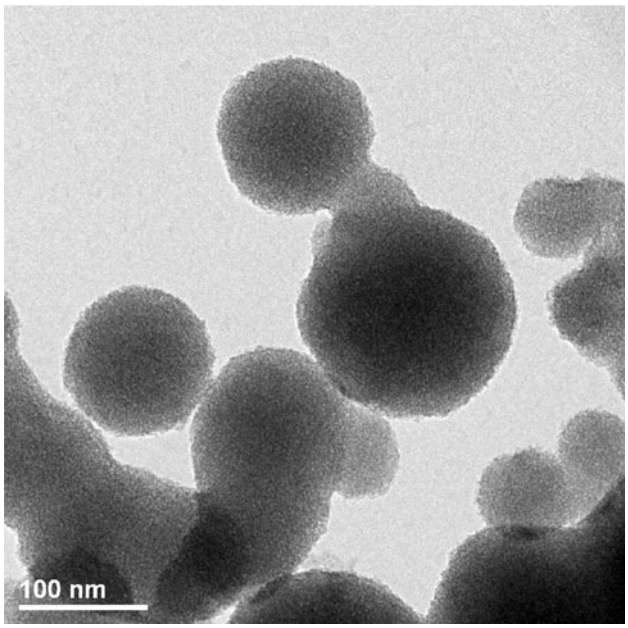

Fig. 1 TEM image of an amorphous TiO_2:Ag^0

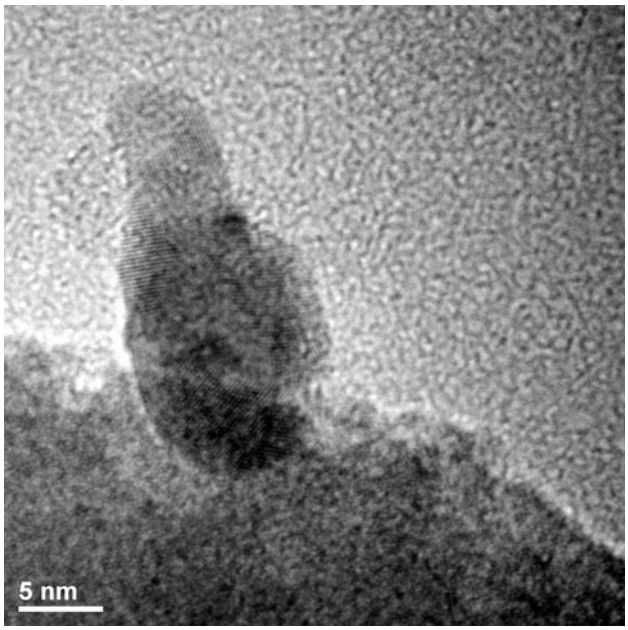

Fig. 2 TEM image of silver nanoparticles located at the surface of the amorphous TiO_2 (TiO_2:Ag^0)

Figures 1–3 show TEM images of the prepared basic samples. Figure 1 shows amorphous TiO_2:Ag^0 and silver nanoparticles on the amorphous TiO_2 are presented on Fig. 2. We have indicated that TiO_2 size is smaller than 100 nm and particle size of silver embodied to the structure of TiO_2 is smaller than 5 nm. Figure 3 shows crystalline TiO_2 doped with silver during the synthesis (TiO_{2a}:Ag^0). There are seen particles of anatase (with size about 50 nm)

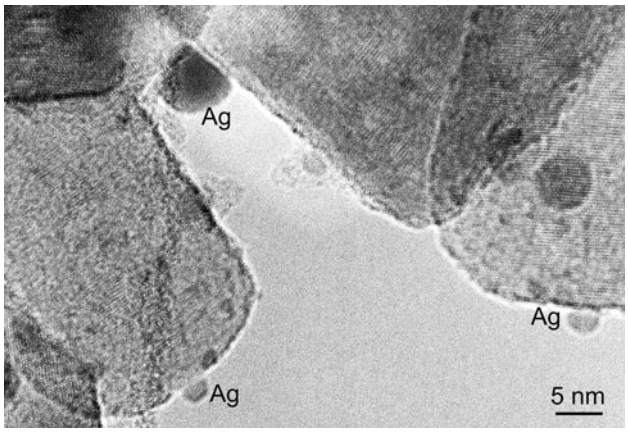

Fig. 3 TEM image of crystalline TiO_{2a}:Ag^0

Table 2 BET surface area and pore size of amorphous TiO_2:Ag^0 and crystalline TiO_{2a}:Ag^0

Sample	S_{BET} (m^2/g)	Pore size (nm)
TiO_2:Ag^0	464 ± 5	3
TiO_{2a}:Ag^0	37.5 ± 0.2	8

and silver nanoparticles with size below 5 nm. Calcination of the amorphous TiO_2:Ag^0 caused an increase of TiO_2 particles size and didn't change the silver crystallite size. In result the specific surface area of the powder decreased from 424 ± 8 m^2/g (for amorphous TiO_2:Ag^0) to 37.5 ± 0.2 m^2/g (for crystalline TiO_{2a}:Ag^0) (data shown in Table 2).

The crystalline character of TiO_2 was checked by electron diffraction. The electron diffraction pattern in Fig. 4 shows that the samples are composed of an amorphous or a crystalline form. In the case of the amorphous sample TiO_2:Ag^0 diffuse rings in diffraction pattern are visible (Fig. 4a). The diffraction pattern of the crystalline sample TiO_{2a}:Ag^0 confirms its anatase form (Fig. 4b). Results obtained by electron diffraction coincide with the XRD results. XRD of the amorphous and crystalline TiO_2 doped with silver (TiO_2:Ag^0 and TiO_{2a}:Ag^0) are shown on the Fig. 5. All reflections in the diffraction patterns of TiO_2 shown in Fig. 5b were identified as belonging to anatase. In the pattern of the amorphous TiO_2, no reflections are observed (Fig. 5a). However, similar as in [30], there are no obvious peaks showing the presence of silver in the XRD of the silver doped titania samples (Fig. 5a, b). It probably results from very small size of silver nanoparticles (<5 nm) and its low content (below 2%). In comparison to Chao et al. [29] TiO_{2a}:Ag^0 powder calcined already at 400°C is well crystallized.

The room temperature UV–vis diffuse reflectance spectra of amorphous and crystalline samples doped with

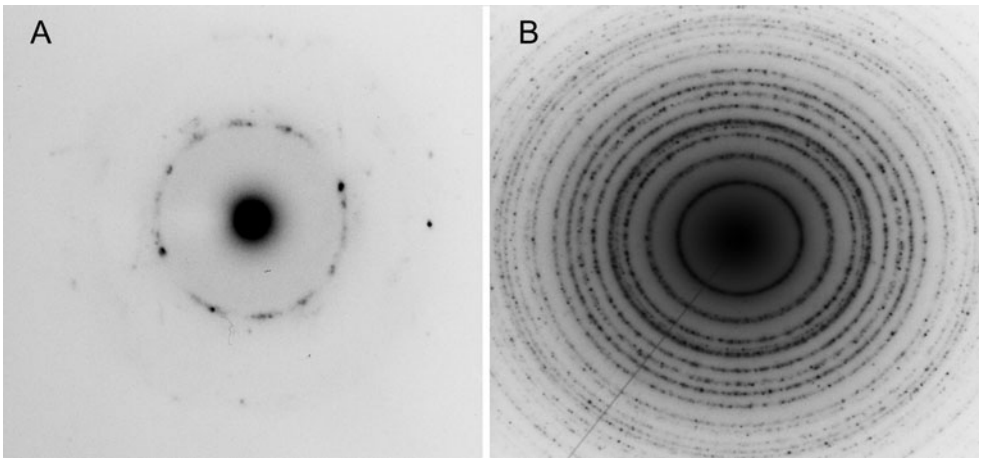

Fig. 4 Electron diffraction patterns of: (**a**) amorphous TiO_2:Ag^0 and (**b**) crystalline TiO_{2a}:Ag^0

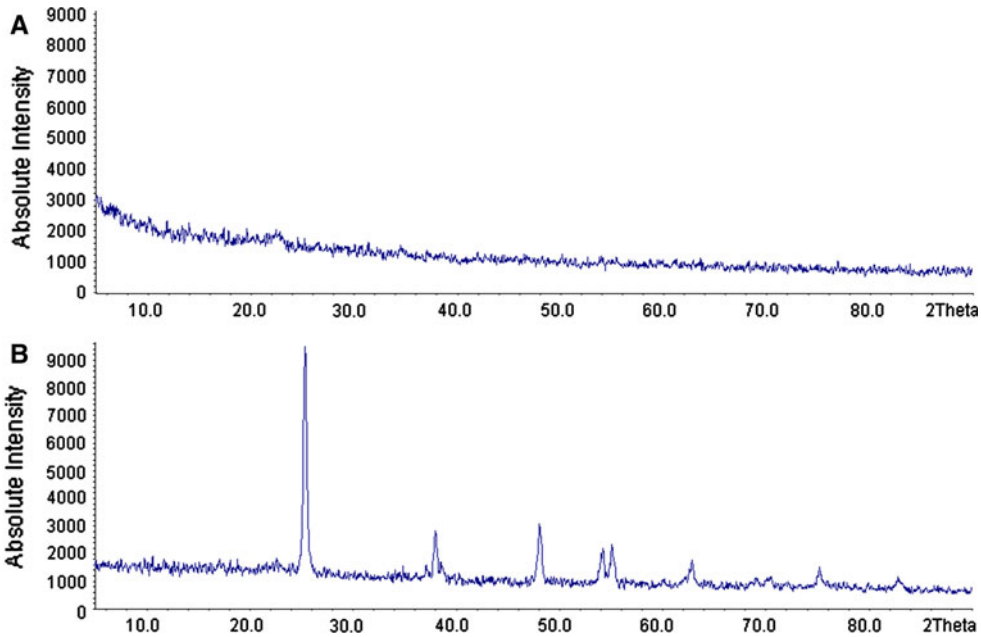

Fig. 5 XRD patterns of: (**a**) amorphous TiO_2:Ag^0 powder and (**b**) crystalline TiO_{-2a}:Ag^0 powder

silver are presented in Fig. 6. It is well known that the UV–vis absorption spectra of the Ag nanoparticles with a size range from 3 to 20 nm exhibit a single absorption peak at 410 nm [6]. As can be seen from curve A in Fig. 6 nanocomposites of crystalline TiO_2 have a broad intense absorption below ca. 400 nm. This is the characteristic absorption of TiO_2 corresponding to the charge transfer process form the valence band to conduction band in anatase [31]. Lack of visible silver absorption peak in samples TiO_2:Ag^0 (B) and TiO_{2a}:Ag^0 (A) can be due to small size or low concentration of the silver nanoparticles in the TiO_2 powder (Fig. 6).

Figure 7 illustrates the N_2 adsorption–desorption isotherm of the powders. The isotherm of TiO_2:Ag^0 powder before annealing is a typical type IV with hysteresis and isotherm of TiO_{2a}:Ag^0 powder is typical for mesoporous adsorbent (pore diameter from 2 to 50 nm). Effect of the drying and calcination temperature on the surface area and pore size of the both prepared samples are summarized in Table 2. The surface area of the powders decreased drastically while their pore size increased after calcination at 400°C [32].

3.2 Antibacterial activity

The originality of this work is the synthesis of novel type of TiO_2 and determination antibacterial activity its crystalline and non crystalline forms. Results of the MIC and MBC values obtained for all samples are summarized in Tables 3, 4. We tested antimicrobial activity of basic forms—TiO_2 doped with silver (TiO_2:Ag^0 and TiO_{2a}:Ag^0) and samples with additional silver count (TiO_2:Ag^0/Ag^+,

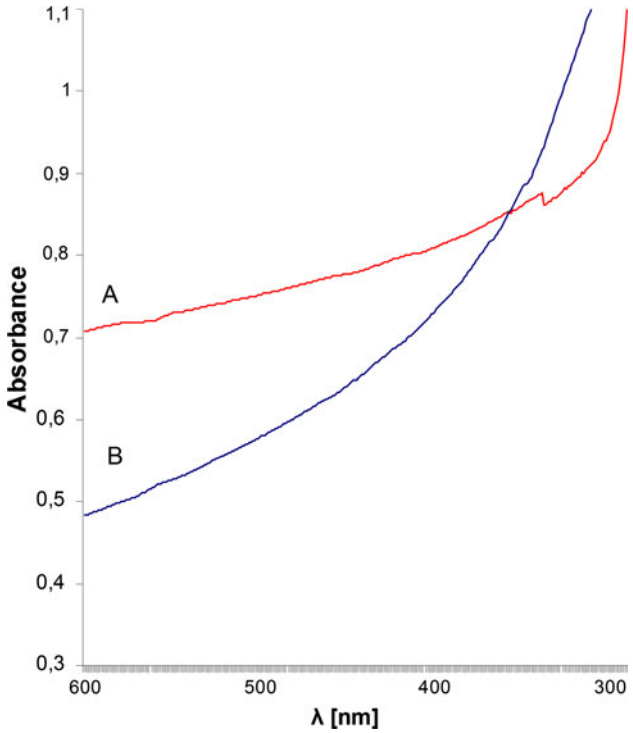

Fig. 6 UV–vis spectra of: (**a**) crystalline TiO_{2a}:Ag^0 and (**b**) amorphous TiO_2:Ag^0

TiO_2:Ag^0/Ag^0, TiO_{2a}:Ag^0/Ag^+ and TiO_{2a}:Ag^0/Ag^0). All samples were tested against Gram-positive and Gram-negative bacteria. The best efficacy indicate basic sample based on anatase TiO_{2a}:Ag^0. MIC value of this powder is very low for all bacteria strains: 0.4 μg/ml! However Keleher et. al. [33] in their research obtained MIC values for the crystalline TiO_2/Ag^0 particles at the level of 6.4 μg/ml for *E. coli* and 3.9 μg/ml for *S. aureus* strains. Very good efficacy also indicates a sample with additional silver nanoparticles form (Ag^0) of the same samples (TiO_{2a}:Ag^0/Ag^0): MIC values from 0.4 to 3.2 μg/ml (depending on bacteria strains). It is interesting that antibacterial activity is inversely proportional to surface area (lower in crystalline forms). Results confirm that affecting factor is also silver count and size; the lower count of silver particles, the better antibacterial efficacy (Tables 3, 4). It is also interesting that antibacterial activity dependence on silver oxidation state. In case of amorphous and crystalline TiO_2 is better for sample with additional silver ions (TiO_2:Ag^0/Ag^+ and TiO_{2a}:Ag^0/Ag^+) than additional silver nanoparticles (TiO_2:Ag^0/Ag^0 and TiO_{2a}:Ag^0/Ag^0). This may be related to the ionization efficacy of Ag^+ on the surface of the particles and its ability to be released from the particles and diffuse to the bacterial cell wall. In case of amorphous and crystalline form of TiO_2 doped with additional silver ions count (samples signed as TiO_2:Ag^0/Ag^+ and TiO_{2a}:Ag^0/Ag^+) we have indicated MIC values approach to MBC values. It prove high antibacterial efficacy We suggest that samples, which already has silver present in the form of Ag^+, can directly react with cell membranes, whereas the Ag metal bound to the TiO_2 particles surface must first dissolution to form Ag^+ and then migrate to the bacteria cell. Keleher et al. [33] suggested that the smaller amounts of silver ions are needed to produce the same antibacterial effect as Ag metal.

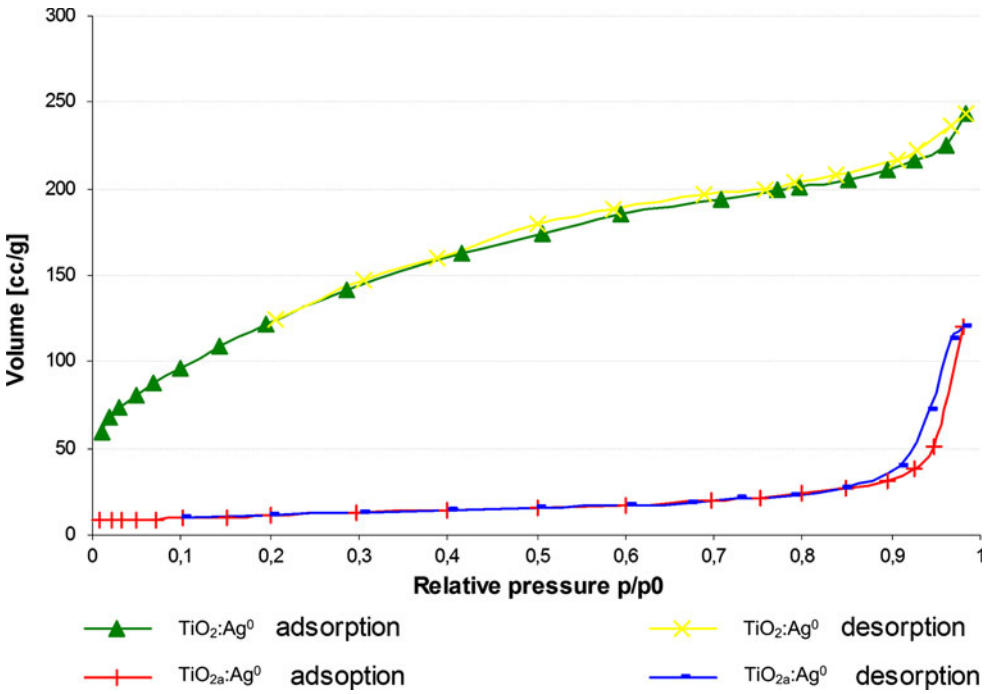

Fig. 7 BET adsorption–desorption of amorphous TiO_2:Ag^0 and crystalline TiO_{2a}:Ag^0

Table 3 MIC and MBC values of amorphous TiO_2:Ag, TiO_2:Ag^0/Ag^+ and TiO_2:Ag^0/Ag^0 of references and clinical bacteria strains

Bacteria strains	MIC (μg/ml) TiO_2:Ag^0	MBC (μg/ml)	MIC (μg/ml) TiO_2:Ag^0/Ag^+	MBC (μg/ml)	MIC (μg/ml) TiO_2:Ag^0/Ag^0	MBC (μg/ml)
S. aureus ATCC 6538	1.6	25.6	1.6	25.6	6.4	102.4
S. aureus 173[a]	1.6	25.6	1.6	25.6	6.4	102.4
S. aureus 187[a]	1.6	25.6	1.6	25.6	6.4	102.4
S. aureus 298[a]	1.6	12.8	1.6	12.8	12.8	51.2
E. coli ATCC 11229	3.2	6.4	3.2	6.4	51.2	51.2
E. coli 475[a]	3.2	6.4	3.2	6.4	51.2	51.2
E. coli 555[a]	3.2	6.4	3.2	6.4	6.4	25.6
E. coli 574[a]	3.2	3.2	3.2	6.4	12.8	25.6
K. pneumoniae ATCC 4352	3.2	3.2	6.4	6.4	6.4	12.8
K. pneumoniae 626[a]	3.2	3.2	3.2	3.2	12.8	51.2

[a] Clinical isolates

Table 4 MIC and MBC values of crystalline TiO_{2a}:Ag^0, TiO_{2a}:Ag^0/Ag^+ and TiO_{2a}:Ag^0/Ag^0 of references and clinical bacteria strains

Bacteria strains	MIC (μg/ml) TiO_{2a}:Ag^0	MBC (μg/ml)	MIC (μg/ml) TiO_{2a}:Ag^0/Ag^+	MBC (μg/ml)	MIC (μg/ml) TiO_{2a}:Ag^0/Ag^0	MBC (μg/ml)
S. aureus ATCC 6538	0.4	0.4	1.6	3.2	0.8	12.8
S. aureus 173[a]	0.4	0.4	1.6	3.2	0.8	12.8
S. aureus 187[a]	0.4	0.4	1.6	3.2	3.2	12.8
S. aureus 298[a]	0.4	0.4	1.6	3.2	0.8	12.8
E. coli ATCC 11229	0.4	0.8	3.2	3.2	1.6	12.8
E. coli 475[a]	0.4	0.8	1.6	3.2	1.6	12.8
E. coli 555[a]	0.4	0.4	1.6	1.6	0.4	6.4
E. coli 574[a]	0.4	0.4	1.6	1.6	0.8	6.4
K. pneumoniae ATCC 4352	0.4	0.4	1.6	1.6	0.8	3.2
K. pneumoniae 626[a]	0.4	0.8	1.6	3.2	0.8	3.2

[a] Clinical isolates

Thiel et al. [8] suggested that efficient bactericidal behavior of silver nanoparticles results from the amounts of silver: the higher number of silver atoms on the surface, the higher the antibacterial effect. We indicated that the contents of silver have an influence on the antibacterial effect but is inversely proportional to bactericidal efficacy.

Received results indicate differences between microorganisms belonging to two groups. From our test data results that Gram-negative bacteria (tested *E. coli* and *K. pneumoniae*) are more sensitive to silver nanoforms than Gram-positive *S. aureus*. This may result from a difference in the building of the cell-wall. Guillard et al. [7] propose that bacteria which have more fimbriae are more quickly inactivated in the presence of TiO_2/Ag^0 than those without fimbriae. These protein surface structures help adhere the bacteria cell to TiO_2 carriers. It is well known that fimbriae are produced more by Gram-negative bacteria than Gram-positive [34], so we suggest that the higher sensitivity of *E. coli* and *K. pneumoniae* results from the presence of fimbriae on the surface of these bacteria.

4 Conclusions

Modified sol–gel technology has made it possible to synthesize nanoparticles of TiO_2 with a diameter about 50 nm. Preparation of silver-coated TiO_2 is possible by the impregnation and chemical reduction of silver ions during the synthesis of TiO_2. An amorphous TiO_2 heated at 400°C transformed into crystalline anatase. The surface area of TiO_2 decreases while pore size increases during calcination. Sensitivity of tested bacteria strains to silver impregnated TiO_2 depends on the crystalline form of carrier, silver particle size, powder surface area, its porosity, the content of silver, and its oxidation state. We have proven that crystalline TiO_2 may be very good carrier for

silver. Samples synthesized by us may be used as very attractive antimicrobial agents excluding additional factor such as UV irradiation.

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