Development of a novel chlamydia growth-monitoring method and its application for screening anti- and pro-chlamydial compounds

Ph.D. Thesis

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1 Introduction

Sexually transmitted infections (STIs) are the most prevalent infectious diseases in the world. Among the STIs, *Chlamydia trachomatis* (*C. trachomatis*)-related infections are the most common. *C. trachomatis* serovars D-K cause pelvic inflammatory diseases and infertility, while the LGV serovars are the pathogens that cause lymphogranuloma venereum, an STI with systemic manifestations. *C. trachomatis* infections have also been linked to arthritis and spondyloarthritis. *C. pneumoniae* is a frequent cause of community-acquired pneumonia and is suspected of participating in the pathogenesis of chronic diseases, such as asthma and atherosclerosis.

Human herpes simplex virus-1 (HHSV-1) and preferentially HHSV-2 genital infections are common viral STIs. The number of HHSV-2 seropositive people (15–49 years) was estimated as 417 million in 2012, with an 11.3% prevalence in the population. Besides the vesicular lesions of the urogenital and anal regions, HSV infections may lead to severe complications including encephalitis, meningitis and neonatal herpes infections. *C. trachomatis* and HHSV-2 pathogens preferentially cause persistent or latent infections either locally (*C. trachomatis*) and/or from the site of the primary infection, such as in the sacral ganglia for HSV-2 and joints for *C. trachomatis*. Because of the long-term presence of the pathogens, there is a possibility that their infectious cycles, including the active and persistent growths, are influenced by locally or systematically applied compounds, including nanomaterials.

The standard method for counting chlamydiae is immunofluorescence staining and manual counting of chlamydial inclusions. High- or medium-throughput estimation of the reduction in chlamydial inclusions should be the basis of testing antichlamydial compounds and other drugs that positively or negatively influence chlamydial growth, yet low-throughput manual counting is the common approach.

Titanium dioxide (TiO_2) is the naturally occurring oxide of titanium. TiO_2 exists in three most common forms: rutile, anatase and brookite. Titanium dioxide, in the anatase form, is a photocatalyst under ultraviolet (UV) light. The positive holes oxidize water to create hydroxyl radicals, by the strong oxidative potential. TiO_2 nanoparticles have a strong bactericidal effect. The TiO_2 reacts by photocatalysis with water to release the hydroxyl radical with subsequent formation of superoxide. The reactive oxygen species can then synergistically act by attacking polyunsaturated phospholipids in bacteria and catalyzed site-specific DNA damage via generation of H_2O_2. TiO_2 NPs are applied as a food additive or a drug delivery vehicle and we wanted to test their interactions with *C. trachomatis* and HHSV-2 to test their efficacy in non-activated form.

2 Aims

The present study was designed to address the following aims:

***Aim 1*:** To develop an automatic system for counting the chlamydial inclusions.

***Aim 2*:** To investigate some novel compounds and nanomaterials whether they influence the growth of bacteria and viruses.

3 Materials and Methods

3.1 Cell strains

HeLa 229 (ATCC), McCoy (ATCC) and Vero cells (ATCC) were used in this study. The cells were cultivated on 96-well plates (Sarstedt, Nümbrecht, Germany) and chamber slides (Thermo Scientific™ Nunc™ Lab-Tek™,Waltham, MA, USA)

3.2 Chlamydial strains

Two *Chlamydia* species were used during my experiments: *C. trachomatis* (serovar D, UW3/CX reference strain, and serovar L2, strain VR-577; ATCC) and *C. pneumoniae* (CWL029; ATCC). The *Chlamydia* strains were propagated and partially purified according to methods described previously.

3.3 Human herpes simplex virus

Human herpes simplex virus type 2 (HHSV-2) reference strain (National Public Health Institute, Budapest) was used in this study. HHSV-2 strain was grown in Vero cells.

3.4 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

We determined the toxicological profile of NPs using a standard MTT assay. MTT assay was performed to characterize the maximum non-toxic concentration of the NPs on HeLa and Vero cells.

3.5 Preparation of silver nanoparticles (AgNPs)

The AgNPs were prepared according to our previously described procedure. Sodium borohydride and sodium citrate were used as reducing and stabilizing agents, respectively. The initial concentration of the prepared aquas AgNPs dispersion was 100 ppm (0.92 mM) and the average particle size determined by transmission electron microscope (TEM) was 20.2 ± 8.34 nm.

3.6 Preparation of the silver-modified TiO_2 particles (Ag-TiO_2)

Five grams of P25 TiO_2 (Degussa-Evonik) was dispersed in 100 ml of double distilled water; then, 40 ml of $57.9 \cdot 10^{-3}$ mM $AgNO_3$ (Reanal) solution was added to the suspension, and it was vigorously stirred. The pH was adjusted to 7.2, and then, 60 ml 38.6 mM $NaBH_4$ (Reanal) solution was added dropwise to the suspension. The obtained Ag-TiO_2 suspension was stirred for 60 min, washed with double distilled water, centrifuged, and dried.

3.7 Transmission electron microscopy measurements of TiO_2-, Ag- and TiO_2-Ag NPs

The morphology and the particle size of the prepared TiO_2-, Ag- and TiO_2-Ag NPs were examined by a TEM. For TEM measurements the samples were sonicated in distilled water before being dropped on a copper mounted lacy carbon film (200 Mesh) and dried.

3.8 Surface charge measurements of the TiO_2-, Ag- and TiO_2-Ag NPs

Under a titration process the surface charge of the TiO_2-, Ag- and TiO_2-Ag NPs were compensated with hexadecylpyridinium chloride (HDPCl) as opposite charged surfactants with concomitant streaming potential measurements. All experiments were repeated three times.

3.9 Investigation of the impact of NPs on *C. trachomatis* growth in HeLa cells.

HeLa 229 (ATCC) cells were transferred into the wells of the 96-well plate (Sarstedt, Nümbrecht, Germany) at a density of 6 x 10^4 cells/well in 100 µl of minimal essential medium (MEM) with Earle salts supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Gibco; Germany), 2 mmol/l L-glutamine, 1x MEM vitamins, 1x non-essential amino acids, 0,005% Na-pyruvate, 25 µg/ml gentamycin, 1 µg/ml Fungisone.)

TiO_2-, Ag- and TiO_2-Ag NPs were prepared in a physiological salt solution and diluted in sucrose phosphate glutamate (SPG). *C. trachomatis* elementary bodies were incubated with the NPs for 1 hour at 37 °C. Incubations were performed in the dark in order to avoid the photocatalytic effect of TiO_2. Concentrations ranging from 100 to 0.024 µg/ml for TiO_2-, TiO_2-Ag- and 0.5-0.001 µg/ml for Ag NPs with 4-fold dilutions were tested. The cells were incubated with the treated and untreated *C. trachomatis* (multiplicity of infection (MOI) 8) for 1 hour, at 37 °C with a 5% CO_2 atmosphere. After infection the cells were washed twice with 200 µl/well of phosphate buffered saline (PBS), then the culture medium was supplemented with 1 µg/ml cycloheximide. The plates were incubated at 37 °C, 5% CO_2 for 48 h.

3.10 Investigation of the impact of NPs on HSV-2 growth in Vero cells.

Vero cells (ATCC) were transferred into the wells of the 96-well plate at a density of 6 × 10^4 cells/well in 100 µl of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma; USA) containing 5% fetal bovine serum (FBS) (Gibco; Germany), 0.14% sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 250 µg/mL amphotericin B. Preincubation and infection was performed as described for *C. trachomatis*. After the infection with HSV-2 (MOI 0,1) the cells were washed twice with PBS and the plates were incubated for 12 hours at 37 °C, 5% CO_2.

3.11 Monitoring the growth of *C. trachomatis* and HSV-2 by direct quantitative PCR (qPCR).

The supernatants of the cells were removed and the cells were washed with PBS twice. After the second wash 100 µl Milli-Q (MQ) water (Millipore, Billerica, MA, USA) was added to each well and subjected to two freeze-thaw cycles with a quick freezing (−80 °C, 15 min) and a quick thawing on a plate shaker at room temperature (RT). The cell lysates were thoroughly mixed including the edges of the wells using a multichannel pipette. The mixed lysates were used as a template in the qPCR.

The qPCR was performed using the Bio-Rad CFX96 real time system. The SsoFast EvaGreen qPCR Supermix (Bio-Rad, Hercules, CA, USA) master mix and *C. trachomatis pykF* gene specific primer pair were used. The primer sequences were the following: *pykF-F:*5′-GTTGCCAACGCCATTTACGATGGA-3′,
*pykF-R:*5′-TGCATGTACAGGATGGGCTCCTAA-3′.
Forty PCR cycles of 20 s at 95 °C and 1 min at 64 °C were performed with the first polymerase activation step of 10 min at 95 °C for.

The melting curve analysis was used to prove the specificity of amplification. For each PCR, the cycle threshold (Ct) corresponding to the cycle where the amplification curve crossed the base line was determined. For the quantitative measurement of HSV-2 growth a similar qPCR method was used as that applied for *C. trachomatis*, with HSV-2 gD2 gene specific primer pair: *gD2-F*:5′TCAGCGAGGATAACCTGGGA-3′, *gD2-R:*5′-GGGAGAGCGTACTTGCAGGA-3′. Student's t-test was used to evaluate the statistical differences between the samples (3 biological replicates for each condition).

3.12 Culture of chlamydiae on chamber slide

Chamber slides with 16 wells consisting of a removable, plastic chamber attached to a specially treated standard glass slide were used to culture host cells for infection with *Chlamydia*, as described previously. For *C. trachomatis* serovar D infection, the wells were washed with 200 µl/well of HBSS, and then a 1% DEAE-dextran solution (80 µl/well) was added to all wells and the slides were incubated for 15 min at RT. The DEAE-dextran solution was removed and the cells were infected at a MOI of 1 IFU/cell in each well or with serial 2-fold dilutions of stock in SPG starting with 1 IFU/cell. The cells were infected with *C. trachomatis* serovar L2 at MOIs ranging from 1 IFU/cell to 1:64 IFU/cell and with *C. pneumoniae* at MOIs ranging from 1:8 to 1:512 IFU/cell. The slides were incubated at 37 °C under 5% CO_2 for 24 or 48 h after infection with *C. trachomatis* serovars D and L2 or *C. pneumoniae*, respectively, and the cells were fixed for immunofluorescence staining.

3.13 Inhibition of chlamydial growth with antibiotics and IFN-γ

Concentration ranges of 0.25 to 0.004 µg/ml for moxifloxacin and of 0.04 to 0.0006 µg/ml for tetracycline with 2-fold dilutions were tested. The stock solution of antibacterial drug candidate PCC00213 (10 mg/ml) was prepared in dimethyl sulfoxide (DMSO) and was diluted 2-fold in DMSO to 0.156 mg/ml. After infection of McCoy cells with *C. trachomatis* serovar D (MOI 1), the culture medium with cycloheximide was supplemented with the serial 2-fold dilutions of the respective antibiotics and was added to duplicate wells. The chlamydia infected cells were cultured without adding any antibiotics served as controls; for PCC00213, 1% DMSO-containing medium was added to the control wells.

On the day before infection, the cell layers were treated with serial 2-fold dilutions of murine IFN-γ over the concentration range of 100 to 0.046 IU/ml. Human IFN-γ was also added over a concentration range of 100 IU/ml to 1.5 IU/ml, as a control. After the infection procedure, IFN-γ diluted in cycloheximide-free medium was added at the same concentration as that used for the pretreatment of cells before infection.

3.14 Culture of *C. trachomatis* after TiO_2 NP treatment in HeLa cells in a chamber slide system.

*C. trachomatis (*MOI 8) was preincubated with TiO_2 NPs for 1 hour at 37 °C with a concentration range from 100 to 3.12 µg/ml with 2-fold dilutions. Incubations were performed in the dark in order to avoid the photocatalytic effect of TiO_2. As controls, two wells of HeLa cells infected with untreated *C. trachomatis* and two wells with uninfected but treated (100 µg/ml TiO_2, 1h, 37 °C) HeLa cells were included. The cells were infected/incubated with *C. trachomatis* or NPs for 1 hour, at 37 °C, 5% CO_2. After the infection, the cells were washed twice with 200 µl/well of PBS, and the culture medium was supplemented with 1 µg/ml cycloheximide. The plates were incubated at 37 °C, 5% CO_2 for 48 h.

3.15 Immunofluorescent labeling and scanning.

Cells in chamber slides infected with *C. pneumoniae* and *C. trachomatis* were examined after immunofluorescent staining. Anti-chlamydia LPS antibody was labeled with Alexa-647, and a 1:200 dilution was used for the detection of chlamydial inclusions. Fluorescence signals were analyzed with an Axon GenePix Personal 4100A DNA chip scanner and GenePix Pro (version 6.1) software (Molecular Devices, Sunnyvale, CA) using the Cy5 channel and a 5-µm resolution.

3.16 Image processing

The images were processed by ChlamyCount in two phases: preprocessing and analysis. In the preprocessing phase, after loading the image, the contrast was enhanced by performing the histogram normalization method of ImageJ.

In the analysis phase, the images of each well were processed independently. Finally, the result of the analysis was reported in txt, xls, and pdf files. The pdf file contains the numerical results and the processed images of the 16 areas. It should be noted that for easier visibility of the inclusions on the small-scale figures in this paper, the images of the 16 areas were further contrast enhanced using the duotone feature of PhotoFiltre image-processing software.

3.17 Confocal microscopy and imaging

Confocal laser scanning microscopy was performed using an Olympus FV1000 confocal laser scanning microscope. Alexa Fluor 647 was detected between 645 and 745 nm.

3.18 Transmission electron microscopy measurements of early interaction of TiO_2 NPs with HeLa and Vero cells

HeLa 229 and Vero cells were transferred to the wells of the 6-well plate at a density of 2×10^6 cells/well in culture medium. The cells were incubated with 100 µg/ml of TiO_2 (1 h, 37 °C, 5% CO_2). After the incubation 300 µl of Trypsin-Versen was added to each well. The detached cells were centrifuged and the cell pellets were fixed in 600 µl glutaraldehyde. Cell pellets were embedded in Embed 812. The 70-nm thin sections were prepared with an Ultracut S ultra-microtome. After staining with uranyl acetate and lead citrate, the sections were observed with a JEM-1400 plus electron microscope.

4 Results

4.1 ChlamyCount software.

McCoy epithelial cells grown on a 16-well chamber slide were used for chlamydial infection. We removed the chamber at 24 or 48 h postinfection (p.i.), depending on the chlamydial strain, fixed the host cells, and stained the chlamydial inclusions with Cy5 analogue Alexa 647-labeled anti-chlamydial LPS antibody. The stained inclusions were scanned with an Axon GenePix 4100 DNA chip scanner.

The ChlamyCount software was used to process the scanned image. The thresholds for the minimum intensity and size of the inclusion are changeable by the user; otherwise, fixed threshold values are applied; all the subsequent steps are automatic. In the next step, ChlamyCount automatically crops the 16 areas containing the host cells from the complete image. For each area, ChlamyCount processes the images with the same method.

After the image analysis, ChlamyCount provides a detailed txt and xls output of the 16 areas with the inclusion counts. The third output file is a pdf file with the numerical results and the processed images of the 16 areas. A usual scanning time is about 10 min, and the image analysis time is about 1 to 5 min.

4.2 Measuring the dynamic range of detection of *C. trachomatis* serovar D, *C. trachomatis* serovar L2, and *C. pneumoniae*

The ChlamyCount system was tested on McCoy cells infected with a 1:2 dilution series of *C. trachomatis* serovar D, *C. trachomatis* serovar L2, and *C. pneumoniae*. The highest MOI was 8, but between MOIs of 8 and 1, the infected areas were greatly confluent and ChlamyCount could not analyze these areas efficiently. For further experiments, the starting MOI was either 1 (*C. trachomatis* serovars D and L2) or 1:8 (*C. pneumoniae*), and 6 additional 1:2 dilutions were performed in duplicate.

We could measure a high correlation (R^2 = 0.95 to 0.98) between the measured *Chlamydia* inclusion count with all three *Chlamydia* species and the theoretical inclusion count calculated from the 1:2 dilution curve. The inclusion counts closely followed the theoretical inclusion counts between an MOI of 1 and MOIs of 1:8 to 1:16, when no centrifugation was used for infection (*C. trachomatis* serovars D and L2), resulting in an approximately 1-log-unit dynamic range of the ChlamyCount system. The centrifugation was more efficient and allowed us to use lower MOIs for *C. pneumoniae* infection. Nevertheless, we experienced leakage in about 25 to 30% of the chambers; therefore, this infection method was not used for other experiments.

4.3 Assessment of the minimal inhibitory concentrations (MICs) of moxifloxacin, tetracycline, and the novel antichlamydial compound PCC00213 for *C. trachomatis* serovar D growth.

We tested whether ChlamyCount was capable of determining the MICs of the known antichlamydial antibiotics moxifloxacin and tetracycline. The MIC of moxifloxacin for *C. trachomatis* serovar D was previously characterized to be 0.03 to 0.05 µg/ml. We performed *C. trachomatis* serovar D infections (MOI 1) in the presence of moxifloxacin at concentrations ranging from 0.25 µg/ml to 0.04 µg/ml. Our experiments showed that *C. trachomatis* could grow in the presence of moxifloxacin up to a concentration of 0.015 µg/ml but was inhibited at a concentration of 0.031 µg/ml, resulting in an MIC value of 0.031 µg/ml. The MICs of tetracycline and doxycycline for *C. trachomatis* serovar D were previously characterized to be 0.03 to 0.15µg/ml. We performed *C. trachomatis* serovar D infections (MOI 1) in the presence of tetracycline at concentrations ranging from 0.04 µg/ml to 0.0006 µg/ml. Our experiments revealed that *C. trachomatis* could grow in the presence of tetracycline up to a concentration of 0.01 µg/ml but was inhibited at a concentration of 0.02 µg/ml, resulting in a MIC value of 0.02 µg/ml. To determine the MIC of the novel antichlamydial compound PCC00213, ChlamyCount was used as well. *C. trachomatis* could grow in the presence of PCC00213 up to a concentration of 3.1 µg/ml but was inhibited at a concentration of 6.2 µg/ml, resulting in an MIC value of 6.2 µg/ml. We have to note that parallel MTT-based host cell viability assays showed that the antichlamydial effect of PCC00213 was partially due to the inhibition of host cell metabolism. Parallel to the ChlamyCount-based MIC determination, we investigated the same slides with fluorescence microscopy and determined the inclusion counts in each chamber. The absolute inclusion counts were generally higher when we applied ChlamyCount, but there was a high correlation between the two inclusion counts (R^2 = 0.94 to 0.98). Notably, the MIC values

determined by the ChlamyCount and manual methods were identical for all three tested compounds.

4.4 Assessment of the effect of IFN-γ on *C. trachomatis* serovar D and DEAE-dextran and cycloheximide on *C. trachomatis* serovar D and *C. pneumoniae* growth

The inhibitory activity of IFN-γ on chlamydial growth in human host cells via the degradation of the host tryptophan pool is a well-known phenomenon. We performed *C. trachomatis* infection (MOI 1) of McCoy murine fibroblastoid cells in the presence of murine and human IFN-γ. Our experiments showed a comparable extent of inhibition, albeit at a lower murine IFN-γ concentration: inhibition of chlamydial propagation was concentration dependent, and the maximum inhibition was approximately 3.8-fold at a murine IFN-γ concentration of 1.5 IU/ml (approximately 0.07 ng/ml) or higher. The human IFN-γ control did not show any inhibitory effect even at a concentration of 100 IU/ml**.** It was an early observation that the pretreatment of host cells with DEAE-dextran or treatment with cycloheximide could increase the number of chlamydial inclusions and the recoverable number of IFU. We applied ChlamyCount to detect these effects during *C. trachomatis* serovar D and *C. pneumoniae* infection. We pretreated HeLa cells with DEAE-dextran (1% DEAE-dextran, 15 min, RT) and/or applied 1 μg/ml cycloheximide during the infection and compared the direct inclusion counts to those for the untreated cells. Our results showed partially different effects of these drugs on the growth of the two *Chlamydia* species. For *C. trachomatis* serovar D, the application of DEAE dextran showed only a marginal effect, but the cycloheximide treatment increased the direct inclusion count 1.9- to 2.2-fold largely independently of the presence of DEAE-dextran. For *C. pneumoniae*, the application of dextran or cycloheximide alone increased the direct inclusion count 2.4- and 3.3-fold, respectively, but the coaddition of the two drugs did not show a further growth-promoting effect.

4.5 Morphological and surface charge properties of the TiO_2-, Ag- and TiO_2-Ag NPs

According to the TEM images the average particle size of the initial TiO_2 NPs was 18.4 ± 5.65 nm. The citrate stabilized Ag NPs obtained were nearly globular in shape, and their average particle size was 8.2 ± 3.34 nm. In the case of TiO_2-Ag NPs, the globular-shaped Ag NPs accumulating on the surface of the TiO_2 NPs were clearly seen, the average particle size was 21.4 ± 6.78 nm.

It is well known from the literature that the TiO_2 has a pH-dependent surface charge with a point of zero charge (p.z.c.) value of 6.2. Above this pH value, the surface of TiO_2 is negatively charged. Accordingly, the initial -250 mV surface charge of the TiO_2-Ag was continuously increased during the titration process due to the concomitant loss of surface charge. The specific surface charge of TiO_2-Ag NPs was -3.54 meq/100 g at pH = 7.4. Similar to TiO_2-Ag NPs, the initial P25 TiO_2- and Ag NPs were also titrated and -19.3 and -184.35 meq/100 g values were obtained, respectively. The results clearly showed that the Ag NPs had the highest surface charge value, while the surface charge of TiO_2-Ag NPs and TiO_2 NPs were negligible.

4.6 Cytotoxicity of the TiO_2-, Ag- and TiO_2-Ag NPs

MTT assay was used to assess the cytotoxicity of the NPs applied. We found that TiO_2- and TiO_2-Ag NPs did not produce significant toxicity in the applied concentration range. Maximum cytotoxicity was observed at concentrations of 8-2 μg/ml for Ag NPs, the viability reached its maximum at 0.5 μg/ml. We considered 0.5 μg/ml as the

maximum non-toxic concentration for Ag NPs. The maximum non-toxic concentration of TiO_2- and TiO_2-Ag NPs was 100 µg/ml. Vero cells showed similar toxicity profiles. TiO_2- and TiO_2-Ag NPs were not toxic at the concentrations applied. 0.5 µg/ml Ag NPs treatment resulted a ~70% viability, and 0.125 µg/ml was the maximum non-toxic concentration for Ag NPs. To be able to compare the two cell lines, the subsequent growth inhibitory experiments were started with 100 µg/ml TiO_2- and TiO_2-Ag NPs, and 0.5 µg/ml Ag NPs.

4.7 Assessment of the impact of the TiO_2-, Ag- and TiO_2-Ag NPs on *C. trachomatis* and HSV-2 growth by direct qPCR

We used the direct qPCR method to determine the antimicrobial activity of the NPs on *C. trachomatis* and HSV-2. qPCR measurement of the control, nanoparticle-free *C. trachomatis* growth resulted in a Ct value of 26.81 +/- 0.58. Interestingly, the TiO_2 NPs increased the growth of *C. trachomatis* relative to the control. The growth stimulation was concentration-dependent, with a Ct value of 24.85 +/- 0.64 at the maximum TiO_2 NP concentration. The difference of 1.96 qPCR cycles (ΔCt=26.81-24.85) DNA concentration between the TiO_2 NP- treated and the control *C. trachomatis* means ~3.89 fold (~$2^{1.96}$) growth increase.

Ag NPs had a strong inhibitory activity against *C. trachomatis* between the 0.5-0.031 µg/ml concentrations. On the other hand, TiO_2-Ag NPs showed reduced antimicrobial activity compared to the Ag NPs against *C. trachomatis* despite the fact that the Ag content of the TiO_2-Ag NPs was the same as that of the Ag NPs. The difference in antichlamydial activity was the most pronounced at the 25 µg/ml and 6.25 µg/ml TiO_2-Ag NP concentrations, where the growth difference between the Ag NP and TiO_2-Ag NP treated chlamydiae was 15.59 and 27.92 fold respectively.

4.8 Estimation of the time dependence of the TiO_2 growth enhancing effect on *C. trachomatis*

To identify the exact time window when the TiO_2 NPs alter *C. trachomatis* growth, we performed an experiment where *i*, the TiO_2 NPs were preincubated with the *C. trachomatis* elementary bodies one hour before infection and coincubated during the infection for an additional hour; *ii*, the TiO_2 NPs were coincubated with the *C. trachomatis* elementary bodies during the one-hour-long infection; *iii*, the TiO_2 NPs were added to the *C. trachomatis* infected HeLa cells at various time points (0-32 hours) post infection. No centrifugation was used for the infection. Data showed that the growth enhancing effect of TiO_2 NPs was detected at the coincubation + infection treatment (~2 qPCR cycles, 4 fold growth increase), and when the TiO_2 was applied during the one hour infection (~2 qPCR cycles, 4 fold growth increase).

4.9 Estimation of the direct impact of the TiO_2-, Ag- and TiO_2-Ag NPs on the qPCR

Since the growth-related chlamydial DNA concentrations were measured by a direct qPCR method, we wanted to test the potential impact of the NPs on the DNA polymerase of the qPCR. A qPCR enzyme inhibitory effect would appear as a false antichlamydial activity, while a stimulatory effect would appear as a false chlamydial growth enhancing effect. If there was no direct impact of NPs on the qPCR, then the Ct levels of the mixture of the infected and uninfected but NP containing cell lysates would have been similar to the above-mentioned controls.

4.10 Quantitative immunofluorescent measurement of the impact of TiO_2 NPs on *C. trachomatis* growth

An independent immunofluorescent growth measurement method was used to validate the qPCR results. HeLa cells cultured on a 16-well chamber slide were infected with *C. trachomatis* (MOI 8) after preincubation at various concentrations of TiO_2 NPs. No centrifugation was used for the infection. Infected but untreated and uninfected + TiO_2 NP-treated cells (100 µg/ml) were also included as controls. The chlamydial inclusions were labeled with an Alexa-647-labelled anti-chlamydia LPS antibody. As described previously, the slide was scanned with a DNA-chip scanner, and the ChlamyCount software was used to enumerate the chlamydial inclusions. ChlamyCount inclusion number counts supported the qPCR results.

TiO_2 NP pretreatment of the chlamydial elementary bodies induced an increase in chlamydial inclusion numbers, with a 400%-500% increase at the 100 and 50 µg/ml TiO_2 NPs concentrations, and a gradual, concentration-dependent decrease in growth enhancement in the 25-3.12 µg/ml TiO_2 NPs concentration range. Uninfected but TiO_2 NP treated wells displayed only marginal positivity, indicating that the observed increase of chlamydial immunofluorescence was not due to the aspecific binding of the anti-chlamydial LPS antibody to TiO_2 NPs.

5 Discussion

5.1 Aim 1: To develop an automatic system for counting the chlamydial inclusions: ChlamyCount Software

We designed a low-cost, medium-throughput method for the rapid enumeration of chlamydial inclusions. Chlamydial inclusions on a 16-well chamber slide were labeled by a fluorescently labeled genus-specific antibody and scanned by a commercial DNA chip scanner. In this detection system, the DNA chip scanner is the most expensive component; however, these scanners are easily available in core facilities and in many cases, the new-generation sequencing technology makes these scanners infrequently used or redundant. Our technology reuses these scanners in a novel role, when the high-resolution images produced by these scanners are used to visualize chlamydial inclusions. The images are processed either completely automatically or after small intensity and area threshold adjustments on a single desktop computer with an average or low-average year 2013 hardware configuration.

The “gold standard” test of inclusion counting is the infection of host cells with serial dilutions of *Chlamydia* and the subsequent counting of inclusions. Our method was capable of counting 1:2 dilutions of inclusions of three different chlamydial species and provided a high correlation with the theoretical estimates. The system was capable of measuring the inclusion counts over a 1-log-unit range, which is comparable to or better than that of other previously described methods. As with the other methods, a limitation of the ChlamyCount method at higher MOIs is the accurate dissection of the high number of confluent or nearly confluent fluorescent areas that originate from close inclusions. Also, ChlamyCount detects a higher number of inclusions than manual counting by microscopy, likely because the software detects smaller fluorescent areas as inclusions. Therefore, the current version of ChlamyCount may not be used for the absolute quantitation of inclusions. ChlamyCount was designed to measure the effect of various treatments on chlamydial growth, and for this task, it is enough to follow the changes in inclusion counts with a high degree of accuracy and it is not necessary to determine their absolute number.

Indeed, we could demonstrate that the inclusion counts detected by ChlamyCount and manual microscopy closely correlated and therefore could be applied to tasks where the detection of changes in bacterial (inclusion) counts are important, such as MIC determination. We used ChlamyCount to determine the MICs of two well-characterized antichlamydial antibiotics with different mechanisms of action: the ribosome inhibitor tetracycline and the gyrase inhibitor moxifloxacin. In both cases, ChlamyCount was able to determine that the MIC values were identical to the MIC values determined by manual microscopy and also close to the previously described values. ChlamyCount was also able to reproducibly determine the MIC value of the novel antichlamydial compound PCC00213. Besides antibiotics, various chemicals and cytokines can affect chlamydial growth in a positive or a negative manner. ChlamyCount was also able to determine the previously described inhibitory effect of IFN-γ and the growth-promoting effect of DEAE-dextran and cycloheximide. Importantly, these data show that ChlamyCount can be used to quantitatively measure the fold changes in inclusion counts between treated and control samples. This type of relative quantitation makes our method applicable in chlamydia basic biology experiments where the effect of a given treatment should be quantitatively measured.

Considering the previously described methods, the rapid estimation of chlamydial growth can be achieved via two approaches. The first approach uses either a fluorimeter or a spectrophotometer to measure the total intensity of a *Chlamydia*-specific fluorescently labeled antibody or indirectly measure *Chlamydia* growth by measuring decreased host cell metabolism after *Chlamydia*- induced lysis. These methods are rapid and inexpensive but do not rely on counting the individual inclusions; hence, the possibility of aspecific antibody binding or an aspecific change in host cell metabolism cannot be excluded. The second approach mostly but not exclusively uses automatic microscopes to take a certain number of images per well, followed by computer image analysis for the specific detection and enumeration of inclusions. ChlamyCount relates to these methods. Compared to the recently described automatic microscope-based inclusion counting method, our method has certain advantages and disadvantages. The automatic microscope-based method uses 96-well plates and obviously produces images with a higher resolution. Since analyzing the images requires significant computational power, the image analysis is performed by a computer cluster with 16 processors. In contrast, our method has a lower throughput, but the analysis time is significantly shorter; therefore, the processing time for 96 samples (6 × the 16 wells in the chambers) is comparable, at least for the first 96 samples. The ChlamyCount system set-up cost is generally lower, and the computational support required is significantly simpler. Although it has a lower resolution, ChlamyCount also preserves a major advantage of the automatic microscope-based methods; namely, it provides topological information about the inclusions. Since DNA chip scanners can scan at two different wavelengths, our method can potentially be applied to provide colocalization information, allowing, e.g., testing of the effect of anti- or prochlamydial proteins recombinantly expressed in the host cells.

5.2 Aim 2: To investigate some novel compounds and nanomaterials whether they influence the growth of bacteria and viruses

As the usage of TiO_2 NPs is significant and the prevalence of *C. trachomatis* and HSV-2 is high, it is important to study their interactions. Therefore, we performed an *in vitro* study where we evaluated the impact of non-activated TiO_2 NPs on the growth of *C. trachomatis* and HSV-2. Since the activated TiO_2 NPs have well described antimicrobial activity, we hypothesized, that the non-activated TiO_2 NPs would not have any effect on

the growth of these two intracellular pathogens. Indeed, TiO_2- and TiO_2-Ag NPs had no effect on HSV-2 growth in the tested concentration range, and Ag NPs only displayed a minimal inhibition (about two-fold) at the highest concentration. It is worth to note, that the addition of TiO_2 to Ag NPs eliminated completely this minimal HSV-2 inhibitory activity. On the other hand, qPCR growth measurements showed that the TiO_2 NPs significantly promoted the chlamydial growth at the 100 µg/ml concentration. Albeit did not reach the significance threshold, the growth promoting effect could also be detected at 50 µg/ml and 25 µg/ml concentrations. Since the growth promoting effect of TiO_2 NPs was unexpected, and chlamydial DNA synthesis can be observed in the absence of active growth (e.g. in persistence), we applied an independent, immunofluorescence based method to validate the data. We applied the ChlamyCount system to quantitate chlamydial inclusions. ChlamyCount measurements supported the qPCR data with a prominent growth increasing effect at the 100 µg/ml and 50 µg/ml TiO_2 NP concentration range. The observed *C. trachomatis* growth increasing effect of TiO_2 was unexpected, but not without precedent in the literature. A recent study by Xu et al. showed that non-activated TiO_2 NPs increased the attachment/internalization of *Staphylococcus aureus* (*S. aureus*) to HeLa cells. HeLa cells treated with 100 µg/ml TiO_2 NPs (the same concentration that increased the growth of *C. trachomatis* by about 400% in our study) for 24 hours resulted in a 250-350% increase of *S. aureus* attachment/internalization. In contrast to the chlamydial infection, TiO_2 NPs did not alter HSV-2 growth, therefore the HSV developmental cycle does not benefit from the cellular process(es) that was induced by TiO_2 NPs, or the TiO_2 NPs could not induce the growth promoting cellular effects in Vero cells. Our data support the latter: since TiO_2 NPs were not able to increase chlamydial growth in Vero cells, the TiO_2 NP-related growth promoting effect had a cell-type dependent component.

Chlamydia has a complex developmental cycle, starting with the attachment of the infectious form, the so-called elementary body to the plasma membrane of the target cells. After attachment, the elementary body enters the cell, and differentiates to the non-infectious, but replicating form, the reticulate body. Reticulate bodies grow in a membrane bound vacuole, the so-called inclusion in the cytoplasm of the host cell. The reticulate bodies then redifferentiate to elementary bodies and exit the host cells 48-72h post infection. Theoretically, this complex developmental cycle can be influenced by the TiO_2 NPs at various stages. Our kinetic experiments revealed that the TiO_2 NPs promoted chlamydial growth when they were added to the elementary bodies before the infection or added during the infection. This result indicates that TiO_2 NPs facilitated the attachment/ entry of the chlamydial elementary bodies to the host cells. *Chlamydia* enters into the target cell via multiple mechanisms including phagocytosis, caveolae-mediated endocytosis and clathrin-mediated endocytosis. Among these processes, clathrin-mediated endocytosis seems to be important for *C. trachomatis* entry to epithelial cells. TiO_2 NPs can also enter via clathrin-mediated endocytosis, and thus there is a possibility that the TiO_2 NP entry co-stimulates the entry of the chlamydial elementary bodies. On the other hand, our TEM images showed TiO_2 NP incorporation after 1 hour post incubation in both HeLa and Vero cells, while the chlamydial growth promoting effect could not be detected in Vero cells (data not shown), therefore a mechanistic co-uptake is not likely the source of growth promotion. The net charges of the *C. trachomatis* elementary bodies are negative and the infectivity of the *C. trachomatis* urogenital serovars (D-K) can be enhanced by polycations such as DEAE-dextrane and poly-L lysine and can be inhibited by polyanions such as dextrane-sulphate. The observed chlamydial growth-promoting effect cannot be explained by the TiO_2 NP-mediated bridging of the negatively charged *C. trachomatis* elementary body and the negatively

charged host cell plasma membrane, since the net charges of the TiO_2 NPs were close to zero. Altogether these data indicate that the TiO_2 NPs binding/incorporation itself is not a key factor in chlamydial growth promotion, rather the incorporated TiO_2 NPs may induce a unique early signal transduction or plasma membrane alteration in HeLa cells that are beneficial to chlamydial growth.

Silver-containing antimicrobials were commonly used before to treat *C. trachomatis* conjunctival infections and were shown to inhibit HSV-2 replication. Interestingly, while the pure TiO_2 NPs did not influence HSV growth, the TiO_2-Ag NPs showed reduced antimicrobial activity against both *C. trachomatis* and HSV-2 than the Ag-NPs. The reduction of antichlamydial activity in certain concentrations was close to 30 fold. It is possible that the antichlamydial effect of Ag NPs –at least partially- is due to their high negative charges. As we showed, the TiO_2-Ag NPs have a more positive net charges compared to Ag NPs, which may contribute to the lower antichlamydial effect.

Our study is one of the few, where the impact of non-activated TiO_2 NPs on the growth of intracellular pathogens has been measured. Because of the high prevalence and debilitating sequelae of *C. trachomatis* infections, the TiO_2 NP-induced growth promotion is a significant finding which requires further animal model/epidemiology investigations. An important application of NPs is the drug delivery of antimicrobials. It is generally accepted that the antichlamydial effect of the first-choice antibiotic azithromycin is augmented by its intracellular accumulation. Theoretically, the uptake and intracellular accumulation of TiO_2 NPs make them particularly amenable for use as antimicrobial compound delivery vehicle to combat intracellular pathogens. The fact that addition of TiO_2 greatly reduced the antichlamydial activity and reduced the antiviral activity of Ag NPs highlights the need for further testing of TiO_2 NPs in this application.

6 Summary

Chlamydial species are Gram-negative, obligate, intracellular pathogens. They have to use their host cell's energy resources, because they are unable to synthesize their ATP. For this reason, chlamydiae were once considered viruses. The species of chlamydia, *Chlamydia trachomatis* and *Chlamydia pneumoniae* are known human pathogens and *Chlamydia psittaci* is the pathogenic agent of ornithosis or psittacosis, a primarily avian respiratory disease which can manifest as a zoonotic disease in humans. *C. trachomatis* has several serovariants based on the features of their major outer membrane protein. Trachoma is caused by serovars A, B and C. Serovars D to K infect ophthalmic, genital and rectal columnar epithelial cells leading to conjunctivitis, urethritis, cervicitis and proctitis, respectively. These serovars also infect respiratory epithelial cells and cause infant pneumonitis. Serovars L1-L3 cause lymphogranuloma venereum (LGV). As *C. trachomatis* and *C. pneumoniae* are frequent pathogens development of systems allowing high-throughput evaluation of chlamydial growth influencing bioactive agents is desirable.

Discovery of novel antimicrobial compounds for treatment and possible chemoprevention is a medically important research task. High-throughput testing of potentially antichlamydial compounds is hampered by the small size and obligate intracellular propagation of the bacterium. After infecting the host cell, the chlamydia propagates in a distinct cellular space called an inclusion. Since, at a low multiplicity of infection (MOI), one chlamydia can form one inclusion, the original chlamydia count is indirectly measured by labeling and manual microscopy counting of inclusions. To circumvent the labor-intensive and subjective manual counting, we designed a relatively low-cost, easy-to-use system that automatically counts chlamydial inclusions. The system consists of a commercial DNA chip scanner and custom made image analysis software.

We applied this system to detect fluorescently labeled chlamydial inclusions in host cells propagated in a 16-well chamber slide. We designed ChlamyCount, a custom ImageJ plug-in for the completely automatic detection of fluorescently labeled chlamydial inclusions on the scanned image. The image processing with ChlamyCount is almost fully automatic, including the extraction of the areas of the 16 wells, the automatic detection of inclusions in each well, dissection and counting of individual inclusions, and automatic reporting. ChlamyCount was successfully used to determine the MICs of the known antichlamydial antibiotics, tetracycline and moxifloxacin and the novel antimicrobial compound PCC00213. ChlamyCount was also applicable to evaluate of the effect of compounds that indirectly influence the chlamydial growth cycle, such as gamma interferon (IFN-γ), DEAE-dextran, and cycloheximide.

In conclusion, we developed an easily useable, accurate system for measuring the antichlamydial effects of known and novel antibiotics and for measuring the effects of various compounds on chlamydial growth. We think that ChlamyCount has the potential to be further optimized. An extended dynamic range of detection and absolute inclusion number estimation may be achieved by applying new raw image-processing methods and an improved confluent area dissection algorithm, goals we are currently pursuing.

Titanium-dioxide (TiO_2) is a frequently used whitening agent and food additive (E171), with an average daily consumption of 0.2-2 mg/body weight (kg). E171 contains various sizes of TiO_2 particles ranging from 60 to 300 nm. Apart from the larger particles, approximately 5-15% of E171 and E171 containing foods contain below 100 nm diameter nano-sized TiO_2 particles. Some study showed that the orally administered TiO_2 NPs could be detected in the liver, spleen, kidneys and lung tissues. In a study, 54-86% of the TiO_2 was found in various organs 90 days after intravenous administration. Besides the above-mentioned applications, TiO_2 NPs can also be used intravenously as drug delivery vehicles. TiO_2 NPs have been used to deliver various drugs, including paclitaxel, 5-fluorouracil and antisense oligonucleotides. Although UV-activated TiO_2 NPs have a strong oxidative potential responsible for its well-described antimicrobial activity, the food additive and drug delivery application do not require activation.

We assessed the antimicrobial effects of non-activated TiO_2 NPs against *C. trachomatis* and HSV-2. We demonstrated that non-activated TiO_2 NPs increased *C. trachomatis* growth in a concentration-dependent manner, with an approximately four-fold increase at 100 μg/ml concentration. This effect was pathogen-specific, since TiO_2 NPs did not increase HSV-2 replication. Our above results point to the potential side effect of food or drug additives.

The following results are considered novel:

- We designed ChlamyCount, a low-cost, medium-throughput *Chlamydia* growth-monitoring method. ChlamyCount is based on the cultivation and infection of host cells in chamber-slides, immunofluorescent labeling, scanning and computer-assisted counting of chlamydial inclusions. The ChlamyCount method was suitable to rapidly determine the growth of both *C. trachomatis* and *C. pneumonia* over a 1-log-unit dynamic range.

- ChlamyCount was also suitable to identify the MICs of the well-characterized antichlamydial antibiotics tetracycline, moxifloxacin and also the MIC values of novel antichlamydial compounds.

- Based on ChlamyCount and qPCR measurements, we demonstrated that the non-activated TiO_2 NPs significantly increased the *C. trachomatis* growth in a concentration-dependent manner. The growth-promoting effect was pathogen-specific, since TiO_2 NPs did not increase HSV-2 replication.

- We found, that the incorporation of TiO_2 significantly decreased the antimicrobial activity of TiO_2-Ag NPs against both *C. trachomatis* and HSV-2 compared to the Ag-NPs.

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