Tannic acid-modified silver nanoparticles enhance anti-Acanthamoeba activity without increasing cytotoxicity of the contact lens solutions

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Research

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Abstract

Background: Free living amoebae of Acanthamoeba genus are cosmopolitan, widely distributed protozoans causing severe, vision-threatening corneal infection known as Acanthamoeba keratitis (AK). Majority of the increasing number of AK cases are associated with contact lenses use. Due to lack of effective therapies against AK, proper eye hygiene and effective contact lenses disinfection are crucial in prevention of this infection. Currently available multipurpose contact lens disinfection systems are not fully effective against Acanthamoeba trophozoites and cysts. There is an urgent need to increase the disinfecting activity of these systems to prevent Acanthamoeba keratitis infections. Synthesized nanoparticles have been recently studied and proposed as a new generation of anti-microbial agents. It is also known that plant metabolites, including tannins, present anti-parasitic activity. The aim of this study was to evaluate the anti-amoebic activity and cytotoxicity of the tannic acid-modified silver nanoparticles (AgTANPs) conjugated with selected multipurpose contact lens solutions.

Methods: The anti-amoebic activity of pure contact lens care solutions and nanoparticles conjugated with contact lens care solutions were examined in vitro by colorimetric assay, based on the oxido-reduction of AlamarBlue. The cytotoxicity assays were performed using a fibroblast HS-5 (ATCC CRL-11882) cell line. Results were statistically analyzed by ANOVA and Student-Newman-Keuls tests using the $p<0.05$ level of statistical significance.

Results: The obtained results showed that nanoparticles enhanced anti-Acanthamoeba activity of the tested contact lens solutions without increasing their cytotoxicity profile. The activity is enhanced within minimal disinfection time recommended by the manufacturer.

Conclusions: The conjugation of selected contact lens solutions with AgTANPs might be a novel and promising approach as part of preventive actions of Acanthamoeba keratitis infections among contact lens users.

Background

Amoebae of the *Acanthamoeba* genus are free-living, cosmopolitan protozoans with broad occurrence spectrum and various degrees of pathogenicity. They are ubiquitous in both natural and man created environment. As facultative human parasites, when transmitted form the environment into the eye surface, may cause progressive, sight-threatening corneal infection known as *Acanthamoeba* keratitis [1]-[5]. Unproper use and disinfection of contact lenses, corneal damages and exposure of eyes to the polluted water are the primary risk factors of the AK. Lack of specific symptoms in the early stage of infection, and co-infections with other microorganisms cause serious diagnostic difficulties and delay the treatment. The number of AK infections have been increasing worldwide. There is no dedicated effective therapy against AK. Current therapeutic approaches are limited to prolonged application of diamides and biguanides. The treatment is often unsuccessful and very toxic to the eye [6]-[9]. Amoebae trophozoites may attach to the surface of both, contact lenses and contact lenses storage cases. Multipurpose contact

lens disinfection systems are not effective against *Acanthamoeba* and need improvement on their anti-amoebic activity [10]-[12]. Summarizing, the prevention, including proper eye hygiene and effective contact lenses disinfection seem to be the best approaches to limit the AK incidence.

In the recent years fast development of nanotechnology has been observed. Synthetized nanoparticles (NPs) are currently proposed as a new generation of anti-bacterial, anti-viral and anti-fungal agents [13], [14]. Moreover, nanoparticles activity against different protozoans such as *Giardia intestinalis*, *Entamoeba histolytica*, *Cryptosporidium parvum* and *Leishmania* spp. has been already confirmed [15]-[17]. Plant metabolites, including tannins, present anti-microbial activity. Tannins are polyphenolic plants metabolites with confirmed anti-obesity, anti-diabetes, anti-oxidant and anti-microbial activity. They are capable to form insoluble complexes with nucleic acids, carbohydrates, proteins and to chelate metal ions. Tannic acid (penta-m-digalloyl glucose) is the simplest, hydrolysable tannin with confirmed anti-bacterial, anti-cancer and anti-oxidant activity [18]-[21]. In our previous studies we demonstrated that tannic AgTANPs were well absorbed and showed anti-amoebic activity against *Acanthamoeba* strains belonging to T4 genotype [22]. Other authors confirmed that nanoparticles enhance anti-amoebic effect of biguanides such as chlorhexidine digluconate and other therapeutic compounds [23]-[25]. The aim of this study was to evaluate the activity and cytotoxicity of AgTANPs conjugated with selected multipurpose contact lens solutions against the trophozoite stage of a strain of *Acanthamoeba castellanii* T4 genotype.

Methods

2.1. cultivation of the strain

ATCC 30010 type *A.castellanii* Neff strain was cultured axenically in 25 cm^2 culture tissue flasks (without shaking) at 27 °C in PYG medium [0.75% (w/v) proteose peptone, 0.75% (w/v) yeast extract and 1.5% (w/v) glucose] containing gentamicin 10 mg/mL in the Department of Medical Biology, Medical University of Warsaw, Poland. Amoebae were subcultured twice a month and observed for their growth under direct light microscope using a Bürker chamber (haemocytometer).

2.2. nanoparticles

AgTANPs were synthesized by chemical reduction method using silver nitrate ($AgNO_3$) purity 99.999% (Sigma-Aldrich, St. Louis, MO, USA). AgTANPs were prepared by mixing the heated aqueous solution of $AgNO_3$ (95.2 g, 0.017%) with the aqueous solution of tannic acid (0.6 g, 5% $C_{76}H_{52}O_{46}$ Sigma-Aldrich). The long-term stability of the colloidal dispersions of al tested NPs (zeta potential) was measured and confirmed by the electrophoretic light-scattering method with a Zetasizer Nano ZS, model ZEN3500 (Malvern Instruments, Worcestershire, UK) [24],[26]. The size and shape of AgTANPs were determined by using the high-resolution scanning transmission electron microscopy (HR-STEM) technique (Fig. 1). Measurements were taken with a scanning electron microscope (Nova NanoSEM 450, FEI) using transmission mode (STEM II) at an accelerating voltage of 30 kV. Samples for HR-STEM investigations

were prepared as follows: a drop of colloid was deposited onto carbon-coated copper grids (300 mesh) and left for 2 h for solvent evaporation. The well-dispersed nanofluids were used as a stock solution and were appropriately diluted to various concentrations ranging between 0.25–2.5 ppm and used in subsequent activity and cytotoxicity assays.

Figure 1. HR-STEM image of AgTANPs distribution and diameter.

2.3. contact lens solutions

The multipurpose solutions used in this study, represent the three most common types of solutions used for contact lens care in Poland, namely: Solo Care Aqua (SCA), Opti-Free (O-F) and ReNu MultiPlus (ReNu). The tested contact lens care solutions and their ingredients are listed in Table 1. All multipurpose solutions used in the study were purchased from authorized agents.

Table 1
Multipurpose contact lens solutions ingredients and minimum disinfection time recommended by the manufacturers.

Solution	Ingredients	Minimum disinfection time
ReNu MultiPlus (BAUSCH + LOMB)	HYDRANATE® (hydroxyalkylphosphonate) 0,03%, boric acid, edetate disodium, poloxamine 1%, sodium borate, sodium chloride preserved with DYMED™ (polyaminopropyl biguanide 0,0001%)	4 h
Opti-Free (repleniSH)	TEARGLYDE® (TETRONIC® 1304, nonannoyl ethylenediaminetriacetic acid), POLYQUAD (Polyquaternium-1) 0,001%, ALDOX (Myristamidopropyl Dimethylamine) 0.0005%	6 h
Solo Care Aqua (Menicon)	Polyhexanide 0,0001%, Hydrolock® (deksapenthenol, sorbitol), sodium phosphate, tromethamine, poloxamer 407, disodium edetate	4 h

2.4. activity assays

Pure contact lens solutions and nanoparticles at concentrations of 0.25, 0.5, 1.25, and 2.5 ppm conjugated with contact lens care solutions were examined *in vitro* and assessed for their anti-amoebic activity. To determine the anti-amoebic efficacy on trophozoites (log growth phase after 6 days following sub-culturing), a previously described colorimetric 96-well microtitre plate assay, based on the oxido-reduction of AlamarBlue was used [27]. Subsequently, the plates were analysed over a period of 6 h, 24 h, 48 h, 72 h and 96 h in the Synergy HTX Multi mode plate reader (BioTek) using the Gen5 software programme, a test wavelength of 570 nm and a reference wavelength of 630 nm in order to calculate the inhibition curves of the analysis. All experiments were performed three times, in triplicate. Amoebae growth and viability in both control and tested assays were visualized by Microscope Evos fl Cell Imaging System.

2.5. cytotoxicity

Briefly, the cytotoxicity assays were performed using a fibroblast HS-5 (ATCC CRL-11882) cell line as described in our previous studies [22]. A commercial kit for the evaluation of drug-induced cytotoxic effects based on the measurement of lactate dehydrogenase (LDH) activity released to the media (Pierce LDH cytotoxicity assay kit 88953, 88954) was used as per protocol. Fibroblasts were incubated with each of contact lens solution separately and contact lens solution + nanoparticles added in the same concentration as in the activity assays. To calculate the % of cytotoxicity, absorbance was measured at 490 nm and 680 nm.

2.6. statistical analysis

All the experiments were performed three times in triplicate. For all activity and cytotoxicity detailed results standard deviation (SD) and mean values were calculated. Results were statistically analyzed by ANOVA and Student-Newman-Keuls tests using the $p < 0.05$ level of statistical significance. For the not statistically significant results, “no activity” comment was added in the Tables 2 and 3.

Table 2
Anti-amoebic activity of the contact lens care solutions after 6-96hrs of incubation [% of inhibition].

	6 h	24 h	48 h	72 h	96 h
Solo Care Aqua (SCA)	31.95 ± 1,70	23.15 ± 3.93	37.68 ± 1.16	51.65 ± 2.75	47.15 ± 3.25
Opti Free (O-F)	-no activity	-no activity	-no activity	35.74 ± 0.95	47.35 ± 2.75
ReNu Multiplus (ReNu)	-no activity	-no activity	24.23 ± 4.88	43.99 ± 2.10	46.76 ± 0.64

Table 3
Anti-amoebic activity of the tannic acid-modified silver nanoparticles conjugated with the contact lens care solutions after 6–96 hrs of incubation [% of inhibition]. SCA – Sole Care Aqua, O-F – Opti Free, ReNu – ReNu Multiplus.

	6 h	24 h	48 h	72 h	96 h
SCA + 2.5 ppm AGTANPs	61.18 ± 1.34	51.60 ± 8.50	59.79 ± 11.19	66.02 ± 5.42	61.9 ± 2.41
SCA + 1.25 ppm AGTANPs	61.67 ± 4.63	42.79 ± 19.26	52.03 ± 11.57	60.42 ± 4.25	54.50 ± 0.21
SCA + 0.5 ppm AGTANPs	54.91 ± 3.89	36.88 ± 20.08	47.33 ± 10.25	58.21 ± 3.07	72.77 ± 1.71
SCA + 0.25 ppm AGTANPs	52.49 ± 6.35	35.45 ± 10.65	45.35 ± 6.56	55.95 ± 1.15	51.31 ± 1.13
O-F 2.5 ppm AGTANPs	31.11 ± 3.09	21.83 ± 4.85	44.79 ± 4.92	59.58 ± 1.14	57.72 ± 0.55
O-F + 1.25 ppm AGTANPs	no activity	4.50 ± 11.51	33.32 ± 4.38	52.78 ± 0.98	52.29 ± 0.24
O-F + 0.5 ppm AGTANPs	no activity	no activity	16.63 ± 3.70	41.62 ± 3.08	41.15 ± 2.46
O-F + 0.25 ppm AGTANPs	no activity	no activity	no activity	36.82 ± 1.69	36.74 ± 1.77
ReNu 2.5 ppm AGTANPs	41.59 ± 2.18	36.63 ± 4.69	56.72 ± 3.58	67.45 ± 2.68	64.59 ± 2.98
ReNu + 1.25 ppm AGTANPs	39.58 ± 2.66	31.17 ± 5.28	50.07 ± 3.57	62.77 ± 2.79	58.85 ± 3.63
ReNu + 0.5 ppm AGTANPs	39.18 ± 0.87	22.60 ± 7.05	45.22 ± 5.33	61.90 ± 2.21	58.56 ± 3.39
ReNu + 0.25 ppm AGTANPs	32.99 ± 4.42	13.47 ± 0.14	43.52 ± 4.20	62.12 ± 7.68	57.55 ± 0.11

Results

3.1. activity

Obtained results confirmed insufficient anti-amoebic effect of the tested contact lens solutions against *Acanthamoeba* trophozoites. Anti-amoebic activity was revealed for SCA and reached 32% of inhibition after 6 h of incubation. ReNu and O-F did not show anti-amoebic effect on the tested *Acanthamoeba* strain within the first 24 h of incubation. The detailed data are shown in the Table 2.

AgTANPs significantly enhanced anti-*Acanthamoeba* activity of the tested contact lens solutions. Specifically, AgTANPs conjugated with SCA, after the minimal disinfection time recommended by the manufacturers (6 h), showed the most promising dose dependent increase of the amoebae inhibition (Fig. 2). Similar anti-amoebic effect was achieved for AgTANPs conjugated with ReNu (Fig. 3). The enhanced anti-amoebic effect of both conjugates last up to 96 h of incubation. O-F conjugated with the nanoparticles did not show any enhanced effect during the first 24 h of incubation (Fig. 4). The anti-amoebic effect was revealed just after 48 h of incubation. The detailed results are shown in the Table 3.

Figure 2. Anti-*Acanthamoeba* activity of AgTANPs conjugated with SCA contact lens solution after 6 h of incubation in relation to cytotoxicity.

Figure 3. Anti-*Acanthamoeba* activity of AgTANPs conjugated with ReNu contact lens solution after 6 h of incubation in relation to cytotoxicity.

Figure 4. Anti-*Acanthamoeba* activity of AgTANPs conjugated with O-F contact lens solution after 6 h of incubation in relation to cytotoxicity.

Compared to the control culture (Fig. 5. A), 6 h of incubation with AgTANPs did not influence the morphology and viability of the amoebae at this level of microscopic observation (Fig. 5. B). Incubation with SCA caused morphological changes in the trophozoites. More rounded cells and less acanthopodia number were visible comparing to the control cultures. Fragments of the disrupted cells were visualized between viable trophozoites (Fig. 5. C). After 6 h of incubation with AgTANPs conjugated with SCA, morphological degeneration of the trophozoites developed (Fig. 5. D). The size of the cells and number of visible acanthopodia were lower comparing to the Fig. 5. A), B) and C) assays. There were more disrupted cell fragments visualized. Some trophozoites started developing into rounded forms.

Figure 5. *Acanthamoeba* trophozoites after 6 h of incubation. A) control culture in PYG medium; B) incubation with AgTANPs; C) incubation with Solo Care Aqua; D) incubation with AgTANPs conjugated with Solo Care Aqua. The arrow shows rounded form. All images (x 40) are representative of the population of treated amoeba and are based on the live cell imaging microscope EVOS FL cell imaging system.

3.2. cytotoxicity

The overall cytotoxicity measured for SCA and O-F was similar and reached 36%. The cytotoxicity of ReNu reached 26%. Cytotoxicity values of nanoparticles conjugated with contact lens solutions comparing to pure contact lens solutions were not statistically significant. The cytotoxicity results are listed in Table 4.

Table 4
Cytotoxicity of the contact lens solutions and contact lens solutions conjugated with the tannic acid-modified silver nanoparticles - AgTANPs [%].

	contact lens solution	+ 0.25 ppm AgTANPs	+ 0.5 ppm AgTANPs	+ 1.25 ppm AgTANPs	+ 2.5 ppm AgTANPs
Solo Care Aqua (SCA)	35.3	24.2	20.7	19.2	23
Opti Free (O-F)	36	32.5	24.31	26.86	33
ReNu Multiplus (ReNu)	26.2	20.1	18.5	15	25.5

Discussion

In the recent years, AK incidents have been increasingly recognized worldwide. Available anti-amoebic therapies are not fully effective and results in high cytotoxicity to the human eye. The main key predisposing factor for AK is contact lens use. Effective contact lens disinfection is the best approach to minimize the number of AK incidences. In this study, we tested multipurpose contact lens disinfecting systems containing different active ingredients but characterized by similar mode of action, resulting in cell membrane perturbation (Table 1) The obtained results confirmed lack of amoebicidal activity of all tested multipurpose contact lens solutions against *Acanthamoeba* strain. Our results are in accordance with other publications and showed that disinfecting capabilities of market available contact lens solutions are insufficient [11],[12],[28]-[31].

Fast development of nanotechnology showed significant anti-microbial potential of the nanoparticles, especially silver nanoparticles (AgNPs) [14],[32],[33]. Specific mechanism of action of AgNPs is still not entirely understood, however recent studies conducted on bacteria, shed more light on this process. We know that nanoparticles cause damages leading to disruption of membrane permeability and alteration of the respiratory functions of the cell. This process eventually leads to disruption of the cell integrity. After crossing the cell membrane, nanoparticles can penetrate inside the cell and interact with DNA, RNA and proteins altering both transcription and translation processes. Presence of nanoparticles in the cell matrix raises the oxidative stress. The intracellular damages and disruption of enzymatic pathways are done by the free radicals. Altogether, nanoparticles cause cytotoxic effects and finally lead to the cell death. Cytotoxicity of AgNPs depends on their physico-chemical properties such as size and density. Typically, smaller nanoparticles have relatively increased stability and enhanced anti-microbial activity. Similarly, higher concentrations of nanoparticles show increased anti-microbial activity. However, this property is strictly correlated to the tested microbial species and type of nanoparticles used. Shape of the nanoparticles has not been proved to be crucial factor influencing the anti-microbial activity. Some authors showed that truncated triangular or similar geometries such as hexagonal and octahedral shape of the AgNPs are more effective against bacteria while other authors reported that shape of AgNPs does not have any influence on their activity [34]-[36]. Recent publications showed, that nanoparticles can

prolong the ocular retention of some topical drugs, thus enabling treatment of eye diseases using reduced drug dosages [37],[38]. It was confirmed, that nanoparticles coated on the contact lenses caused significant reduction in microbial colonization on the contact lens surface [39]. Contact lenses impregnated with AgNPs, after 6 h of incubation, did not exhibit desirable anti-bacterial activity against *Staphylococcus aureus* while demonstrated excellent anti-bacterial effects against *Pseudomonas aeruginosa* [40]. Silver-impregnated lens cases showed lower proportion of microbial contamination compared to the control cases. Most microorganisms isolated from silver-impregnated cases were members of the normal skin flora [41].

There are just few studies that examined nanoparticles influence against *Acanthamoeba* spp. Cobalt nanoparticles have been studied for their anti-amoebic potential and confirmed that hexagonal microflakes showed the most promising anti-*Acanthamoeba* effects compared to nanoflakes and granular cobalt nanoparticles. Apart from the concentration and size, also composition and morphology of the tested noncompounds determined their anti-amoebic activity [42],[43]. AgNPs are well absorbed by the *Acanthamoeba* trophozoites and integrate in the cell matrix. The nanoparticles decrease trophozoites viability and alter their metabolic activity on the dose dependent manner [44]. In our previous studies we confirmed, that AgNPs conjugated with contact lens solutions showed dose dependent enhanced anti-amoebic activity [45]. Recently published studies confirmed enhanced anti-microbial effect of silver and gold (AuNPs) nanoparticles conjugated with commonly used drugs like chlorhexidine, fluconazole or amphotericin B as well as with some disinfectants [25],[46]. Guanabenz, a drug already approved for hypertension that crosses the blood – brain barrier, conjugated with AuNPs and AgNPs showed significant anti-amoebic activity against both *A.castellanii* and *Neagleria fowleri*. Significant reduction in the host cell cytopathogenicity, especially for silver nanoconjugates, was revealed and associated with negligible cytotoxicity against human cells [47].

In the 21st century, eco-friendly and cost-effective bio-nanotechnology techniques are used to prepare anti-microbial active conjugates as potential candidates to eradicate infections and reduce microbial contaminations of a healthcare equipment including contact lenses. The integration and conjugation of bioactive agents into nanomaterials was tested mainly for their anti-bacterial activities. Green synthesis of AgNPs, AuNPs and platinum (PtNPs) nanoparticles showed enhanced anti-bacterial activity after combining with different classes of antibiotics [48]. Biosynthesis of AgNPs with the plant extract of *Salvia spinosa* resulted in increased bactericidal activity against Gram-positive and Gram-negative bacteria [49]. Novel conjugates using biogenic AgNPs from *Convolvulus arvensi* extract and chitosan showed anti-microbial, anti-biofilm, and anti-cancer potentialities [50]. Extract of *Oscillatoria limnetica* conjugated with silver nanoparticles exhibited strong anti-bacterial activity against multidrug-resistant bacteria as well as cytotoxic effects against both human breast cell line and human colon cancer cell line [51]. Synthesis of silver chloride nanoparticles (AgCl-NPs), using walnut green husk extract as well as silver nanoparticles with *Peganum harmala* L leaf extract resulted in significant inhibitory effects against *Escherichia coli* and *S.aureus* clinical isolates [52],[53]. Bio-nanotechnology has been not studied on protozoan species extensively. There are just a few published studies focusing on the influence of nanoparticles conjugated with plants extracts on amoebae. Studies performed on the *Jatropha curcas,*

Jatropha gossypifolia and *Euphorbia milii* extracts combined with nanoparticles exhibited that such combination caused significant reduction of the *Acanthamoeba* trophozoites with low cytotoxic effect to human cells [23]. In our previous studies we confirmed that tannic acid-modified silver nanoparticles showed increased anti-amoebic activity and less cytotoxicity to human cells in comparison to the pure silver nanoparticles [22]. In this study we revealed that tannic acid-modified silver nanoparticles conjugated with contact lens solutions exhibited even better anti-amoebic activity in relation to the cytotoxicity than in results obtained in our previous studies where we tested pure silver nanoparticles conjugates [45]. We conclude that differences in the anti-amoebic activity of the tested conjugates may be mainly driven by the anti-amoebic activity of the pure contact lens solutions. Nanoparticles in the tested concentration seem to enhance the already existing anti-amoebic potential of the selected contact lens solution.

Conclusions

In this study we showed dose dependent enhanced anti-amoebic effect of the tannic acid-modified silver nanoparticles (AgTANPs) conjugated with SCA and ReNu solutions against *Acanthamoeba* T4 strain. The promising results were obtained within the minimal disinfection time recommended by the manufacturers (6 h) and without increased toxicity to the human cells. Summarizing, conjugation of selected contact lens solutions with AgTANPs might be a promising approach to prevent *Acanthamoeba* keratitis infections among contact lens users. Nevertheless, further studies should be conducted to elucidate stability of the conjugation and activity against *Acanthamoeba* spp. cysts.

Abbreviations

AK
Acanthamoeba keratitis
AgTANPs
tannic acid-modified silver nanoparticles
NPs
nanoparticles
SCA
Solo Care Aqua
O-F
Opti-Free
ReNu
ReNu MultiPlus
SD
standard deviation
AgNPs
silver nanoparticles

AuNPs
gold nanoparticles
PtNPs
platinum nanoparticles
AgCl-NPs
silver chloride nanoparticles

Declarations

7.1. Ethics approval and consent to participate

Not applicable.

7.2. Consent for publication

Not applicable.

7.3. Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

7.4. Competing interests

The authors declare that they have no competing interests.

7.5. Funding

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Not applicable.

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Figures

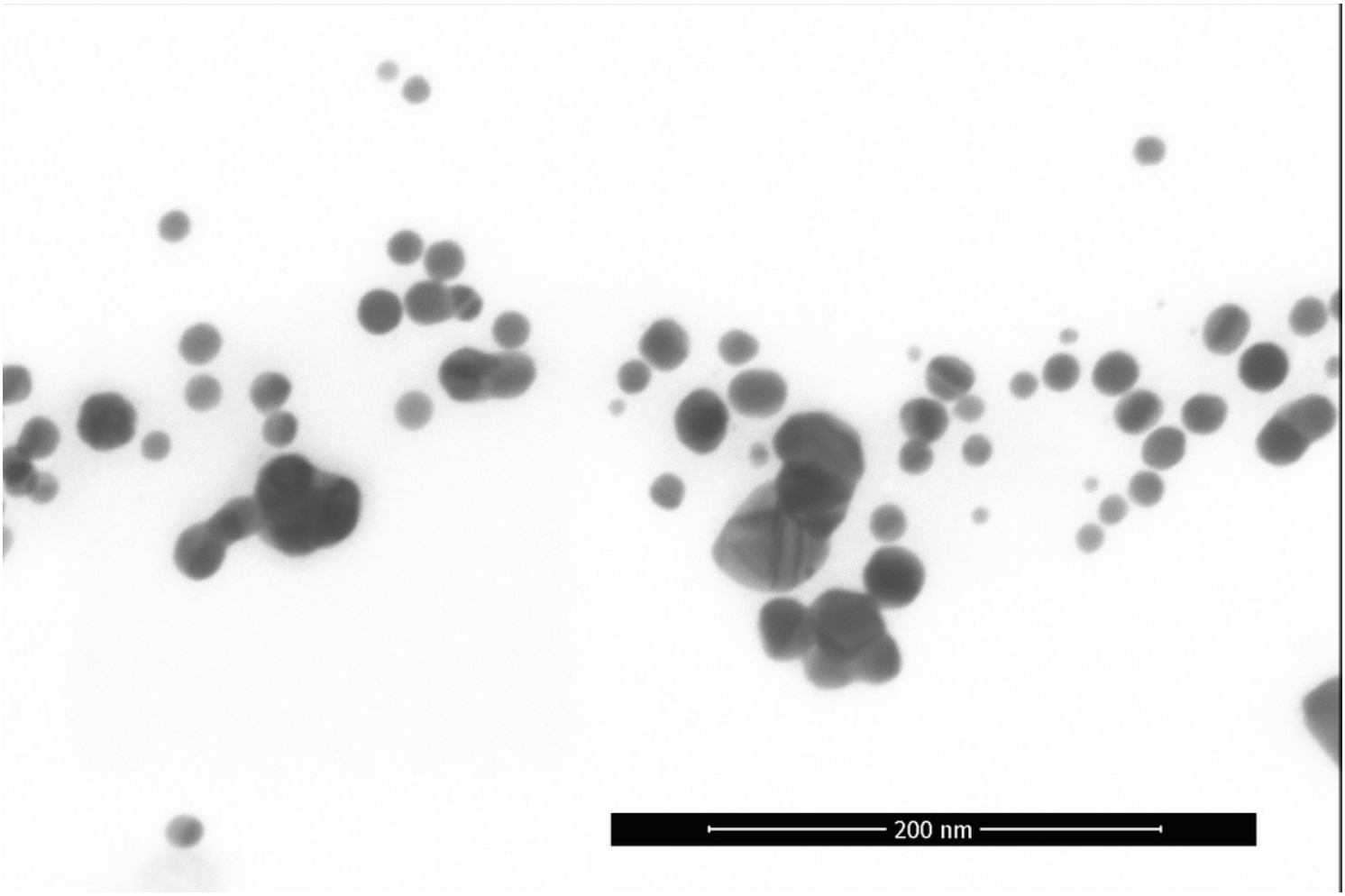

Figure 1

HR-STEM image of AgTANPs distribution and diameter.

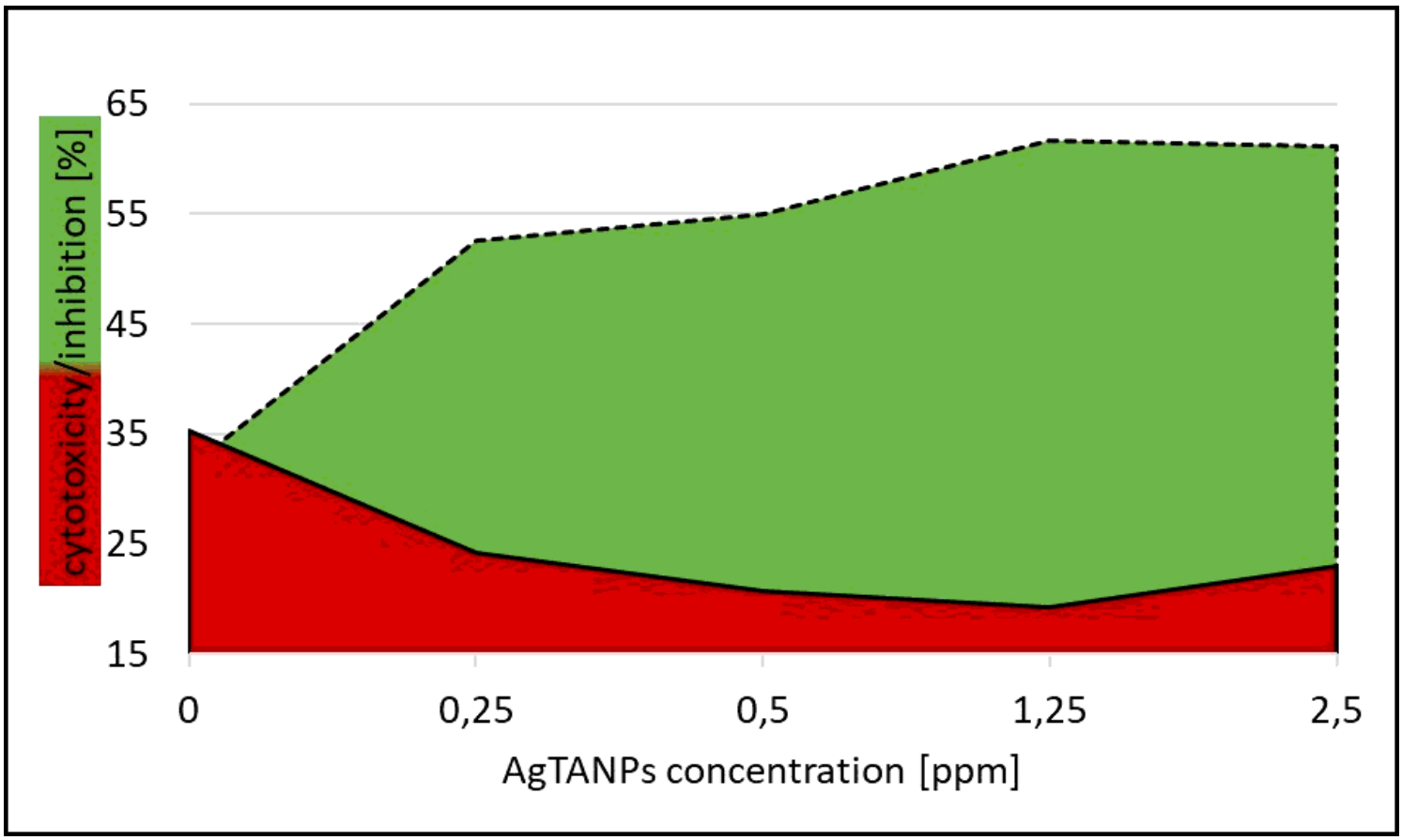

Figure 2

Anti-Acanthamoeba activity of AgTANPs conjugated with SCA contact lens solution after 6h of incubation in relation to cytotoxicity.

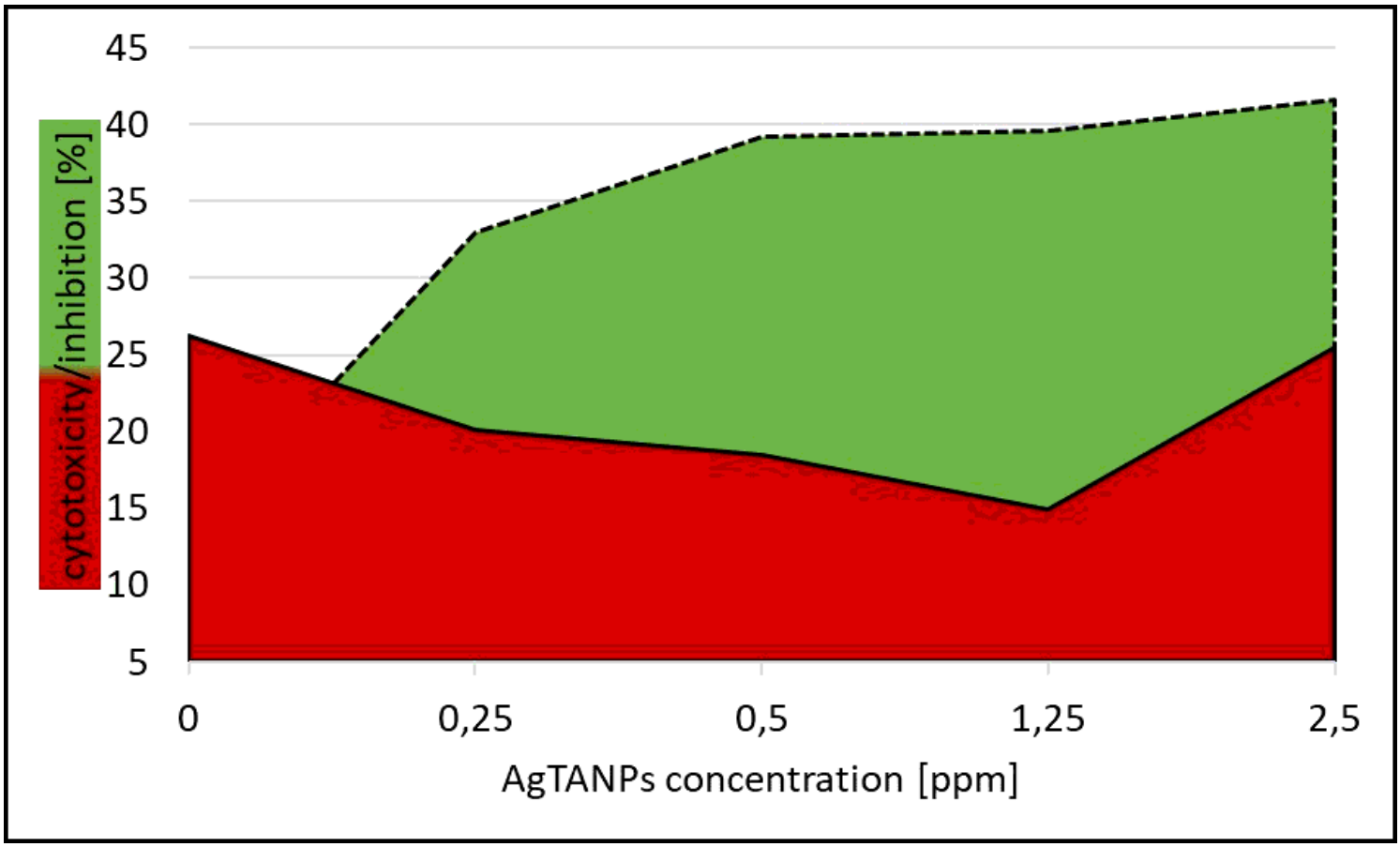

Figure 3

Anti-Acanthamoeba activity of AgTANPs conjugated with ReNu contact lens solution after 6h of incubation in relation to cytotoxicity.

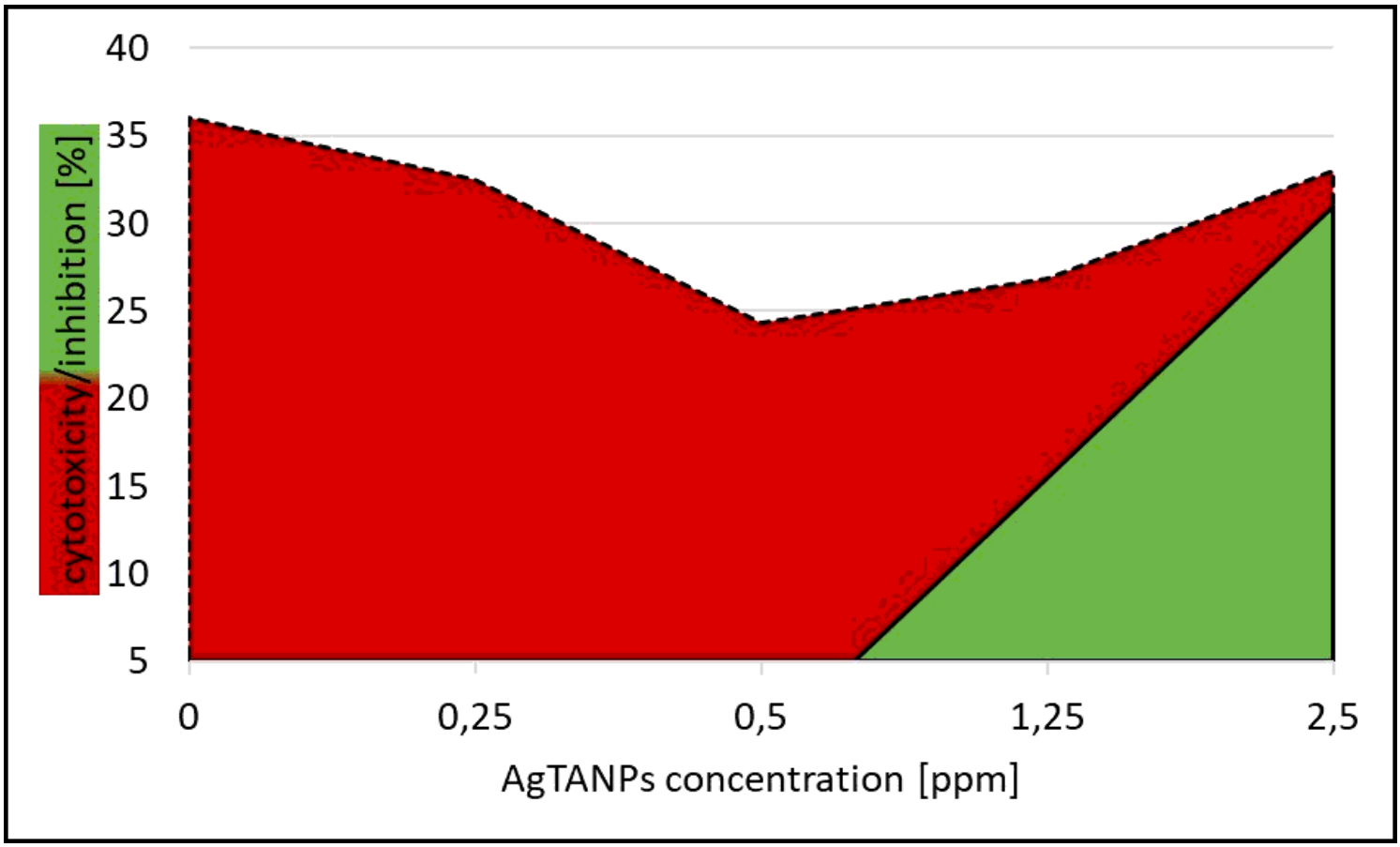

Figure 4

Anti-Acanthamoeba activity of AgTANPs conjugated with O-F contact lens solution after 6h of incubation in relation to cytotoxicity.

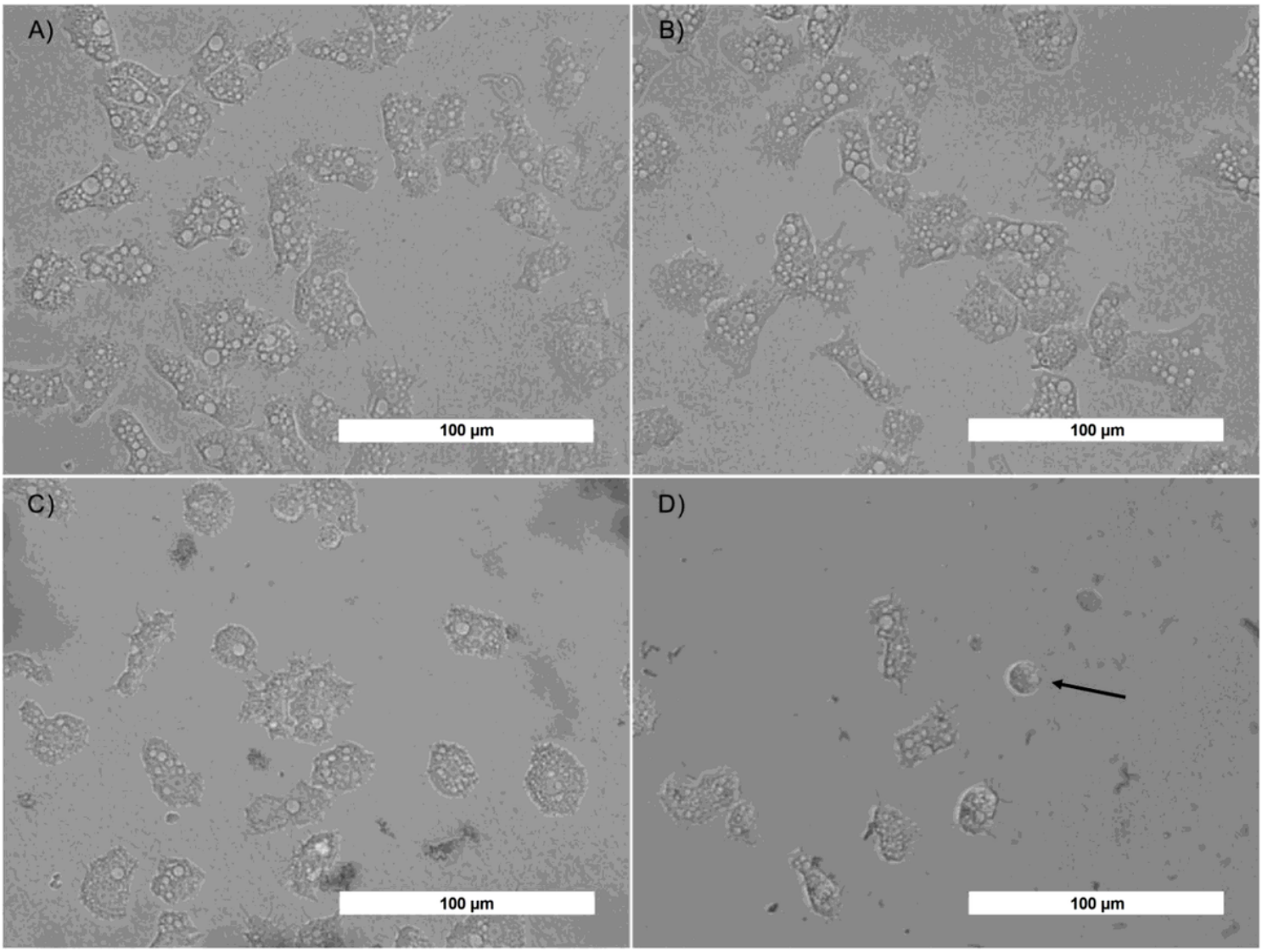

Figure 5

Acanthamoeba trophozoites after 6h of incubation. A) control culture in PYG medium; B) incubation with AgTANPs; C) incubation with Solo Care Aqua; D) incubation with AgTANPs conjugated with Solo Care Aqua. The arrow shows rounded form. All images (x 40) are representative of the population of treated amoeba and are based on the live cell imaging microscope EVOS FL cell imaging system.