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# Effects of Nanoparticles in Cells Infected by *Toxoplasma gondii*

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Sprakel-Leyke Silja, Paulke Bernd-Reiner and Presber Wolfgang

Additional information is available at the end of the chapter

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## Abstract

Core-shell model drug carriers on two nanoscale size levels have been applied in cell culture studies and focused on Toxoplasmosis therapy. In synthesis, a seed of rhodamin B-labelled polystyrene latex particles was coated by polybutyl cyanoacrylate under physical inclusion of two different new drugs against Toxoplasmosis. Drug-loaded and drug-free core-shell model drug carriers were added to a cell culture of human macrophages, infected by *Toxoplasma gondii*, following an infection plan. Drug release from the carriers had been studied before by enzymatic degradation by means of pork liver esterase. Particle size decrease by degradation was investigated in a UV/VIS spectrometer via transmission measurements. Drug release profiles were obtained by HPLC studies. The dynamics in the population of infected human macrophages, *T. gondii* as well as model drug carrier numbers were registered by an FACS (fluorescence-activated cell sorter). As main result, the drug-free references in the two series of core-shell model drug carriers achieved ca.85% of the observed maximum in Toxoplasmosis therapy efficiency. These data were correlated with an immune stimulant effect on the side of the human macrophages, caused by the cell uptake of colloidal substrate, foreign to the body.

**Keywords:** toxoplasmosis, nanoparticles, model drug carriers, human macrophages, cell culture, infection, therapy efficiency, immune stimulation

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## 1. Introduction

Nanoparticles (NPs) are a tool for specifically targeting drugs or diagnostics to selected tissues or cell types [1–3]. Their use in cancer research and therapy is of special interest [4–9] as well as to overcome the blood-brain barrier [10, 11]. At least in theory, they are a tool to optimise pharmacological data such as drug release, tissue specificity and even cell specificity [2].

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In some cases, drug crystals have been used successfully as nanocarriers [12, 13]. NPs composed of polymers may be loaded with drugs which they release in a controlled manner, or they are coated with molecules which give them specific surface characteristics to bind and be taken up by certain cells. Despite these attractive goals and 30 years of research, there are no polymeric nanoparticles in practical, pharmaceutical use. There is hope to apply nanoparticles to deliver drugs across the blood-brain barrier [10, 14, 15].

The reasons for this obvious failure include following: first, the unresolved problem of cytotoxicity of several nanocarrier types [16], low drug loading and insufficient delivery of the bound drug and lack of production facilities to supply demand.

The problem of toxicity could be overcome at least partially by high selectivity with regard to the cells by which the NPs are taken up. Such a strategy is especially interesting in the case of cells, infected with microbes which only multiply intracellularly, such as viruses and some parasites, as these often alter the surface of the host cell, thereby offering the specific targets needed. In this case, even toxic NPs could be useful, as they would exert their toxicity mainly or even better only in or against infected cells which would inhibit the multiplication of the pathogens and destroy them.

At the moment, there are some data showing that tissue specificity may be achieved. One of the interesting targets is the endothelial barrier of the brain. Despite the big potential [17], there are no data concerning infected cells. As it is important to develop drugs against intracellular parasites such as *Toxoplasma* or *Leishmania*, therefore the goal is to establish a system which would allow to trace the fate of nanoparticles in infected cell cultures. It should be possible to evaluate the cell and parasite proliferation and the differential uptake of NPs.

A fluorescence-activated cell sorter (FACS) represents a useful tool to measure particles which differ in size and more importantly, in fluorescence [18]. Thus, it is possible to count extracellular parasites and their host cells separately, if they differ in size. To find the intracellular parasites and to differentiate between infected and non-infected cells, green fluorescent protein-transfected parasites were used. In the past, it was shown that it is possible to follow the fate of *Leishmania* in a cell culture which is conventionally treated (by adding drugs at the necessary concentration) [19].

Because the FACS is able to discriminate between fluorescences of different wave lengths, the purpose of this study was to establish a system with stained NPs as an additional reacting partner [20]. In the case of *Leishmania*, only phagocytic cells, mainly macrophages, act as host cells and the parasite becomes internalised through phagocytosis. There are some data that NPs without drugs may even interfere with normal white blood cell functions, including phagocytosis.

As NPs are recognised as foreign structures, they are eliminated by phagocytising cells [21, 22]. Thus, it should be easier to treat microorganisms which multiply within macrophages, because the nanoparticles would be concentrated automatically in the desired target cells. An example for this strategy is *Leishmania* [23]. Another interesting goal of nanoparticle research is targeting NPs to the brain [11, 14, 24]. *Toxoplasma gondii* offers a possibility to combine these aims. *T. gondii* is a parasite which multiplies in different cell types including

macrophages and phagocytising cells in the brain [25]. The main problems in treatment of toxoplasmosis are infections during pregnancy and *Toxoplasma* encephalitis in the case of immunocompromised patients [25–28]. In normal *Toxoplasma* infections, the multiplication of the parasites is stopped by the immune system, but cysts remain in all formerly infected tissues including the brain, serving as a source for exacerbations. Therefore, efficient treatment of all infected cells to prevent cyst formation would be an interesting goal. Thus, we started our experiments with NPs in *T. gondii*-infected cultures, knowing that *T. gondii* has a completely different mode of penetration which is mostly independent of the support of the host cell [29].

To come closer to *in vivo* conditions and to make it easy to observe an effect, the culture should work for at least 3 days to allow several cycles of multiplication.

Initially, all experiments with FACS should be controlled by microscopy, to prove that the interpretation of the FACS data is consistent with the images seen under the microscope.

## 2. Results and discussion

The established and considered system consisted of three components: nanoparticles, parasites and cells which differed in size and shape. However, there were also infected cells, cells loaded with nanoparticles and infected cells with nanoparticles which should be differentiated as well. To trace the fate of parasites and nanoparticles with FACS, they had to be stained with dyes which are distinguishable from each other. Because the green fluorescent protein, *Toxoplasma* was transfected with, shows fluorescence in green and yellow [33, 34] a red dye to stain the nanoparticles gave sufficient results. Of the dyes tested, rhodamine B gave the best results. The intensity of fluorescence corresponds with the amount of dye, fixed inside the core of the NPs and should increase exponentially (to the third) with the increasing radius of the NPs. In larger particles, however, there seems to be a quenching, as light is unable to penetrate from the core of the particle to the surface.

Next, a broader spectrum of NPs of different sizes was tested to see, which gave the best results. To be able to compare the FACS data with microscopical counting, the NPs had to be larger than 100 nm; otherwise, microscopical identification, especially of intracellular particles, would be impossible. In **Table 1**, one of the two best candidates is shown. As the results obtained with the two different NPs in these experiments were identical, only data with MC81cs are shown here.

The nanoparticle suspensions were homogeneous in size, checked by electron microscopy—data not shown—and were shown to be non-toxic, by measuring growth of cell cultures in their presence.

**Figure 1A** shows an infected cell in the presence of NPs. NPs with diameters between 200 and 300 nm were focused on as they were perfect for these purposes, being visible under a microscope, and therefore allowing the opportunity to compare microscopically the results, obtained with FACS with the situation in the cell culture.

MC81			Core		Core-shell (cs)		Cs + pentamidine	
Z-ave (nm)			133.1		213.4		221	
PI			0.065		0.046		0.071	
ZP (mV)	pH	Distilled water	-93.20	5.52	-84.79	5.5	-79.70	5.55
		10E-3M NaCl	-70.33	5.53	-63.40	5.51	-61.66	5.51
		10E-2M NaCl	-50.85	5.54	-30.60	5.51	-29.00	5.45
Surface charge density ( $\mu\text{C}/\text{cm}^2$ )			-2.54		-0.93		-0.96	
<b>MC81c</b>			<b>MC81cs</b>					
			No drug		Pentamidine		Spiramycin	
Size (nm)			133.1		213.4		222.1	
PI			0.065		0.046		0.071	

**Table 1.** Nanoparticle properties (mean particle diameter [z-average] and polydispersity index from dynamic light scattering, zetapotential [ZP] from particle electrophoresis).

The same area photographed using different filters: the arrow points to a macrophage cell infected with one *Toxoplasma* cell.

- Three channel photography (FITC/PI/neutral): NPs are attached to the macrophage, and some are scattered around.
- Two channel photography (FITC/PI): the parasite inside the cell is clearly visible.
- One channel photography (FITC): only the parasite (green) is visible.
- One channel photography (PI): the parasite is invisible, the cell appears red as a result of uptaken NPs, and some extracellular NPs are visible.

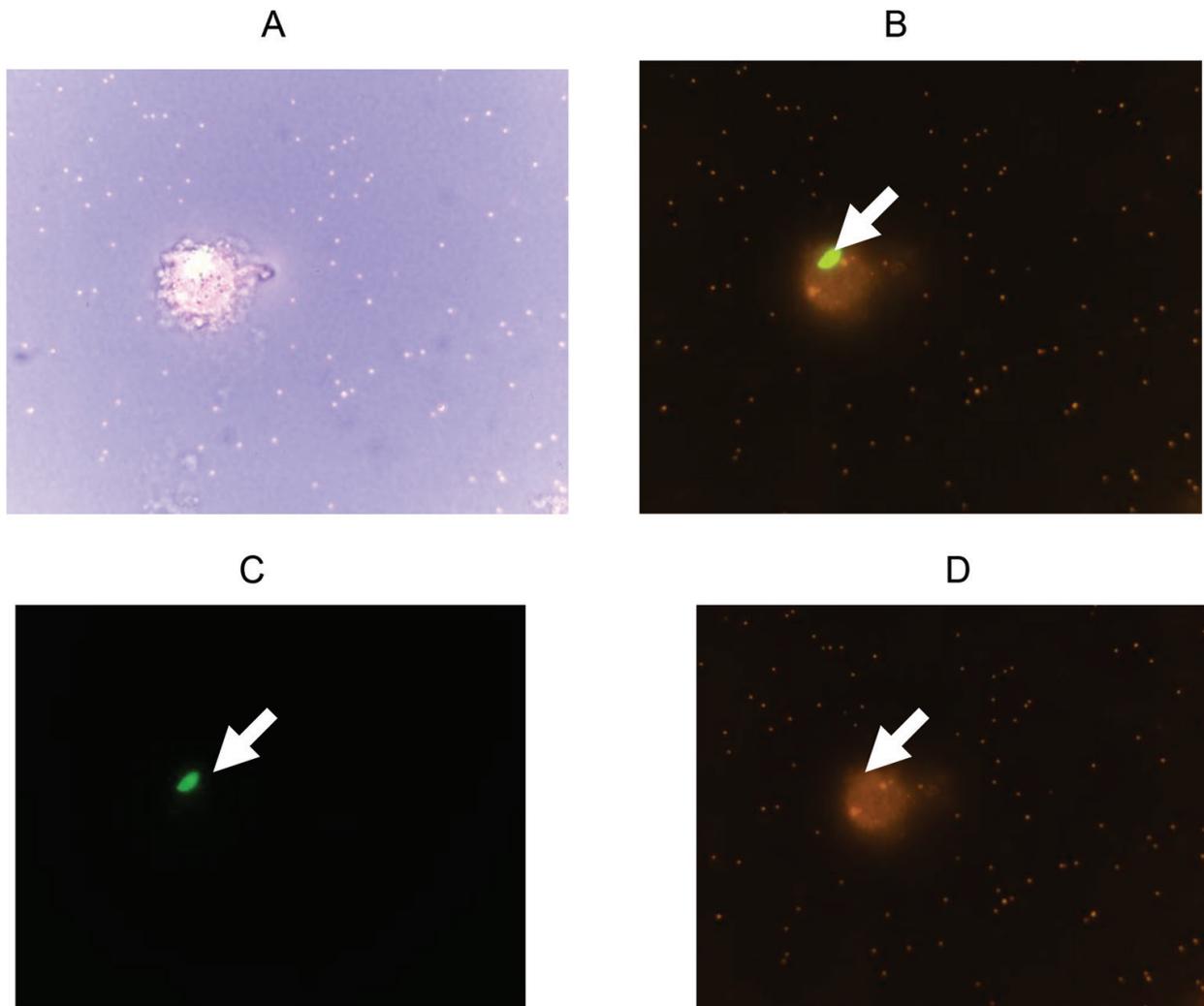
**Figure 1B–D** demonstrates how different filters are able to distinguish the dyes used. In a first series of experiments, different amounts of NPs added to cell culture were tested with the result that approximately 60,000 particles per cell gave measurable and reproducible results in this system. Most of the NPs were phagocytised by the cells within the first few hours.

As parasites not only infect cells and multiply—increasing in number in an unpredictable way—they will also destroy host cells, thereby diminishing their number.

To calibrate the exact increase or decrease in the different components, ‘Perfect Counting Beads’ (Caltag Laboratories) were added before FACS scan and all data were adjusted to the beads count.

The particle size (FSC-x-axis) and granularity (SSC-y-axis) were determined. In A, toxoplasmas’ and in B, macrophages were counted in relation to beads in order to calculate

absolute numbers. The beads are always seen in the same position. In parallel, the particles were characterised by fluorescence (see **Figure 3**).

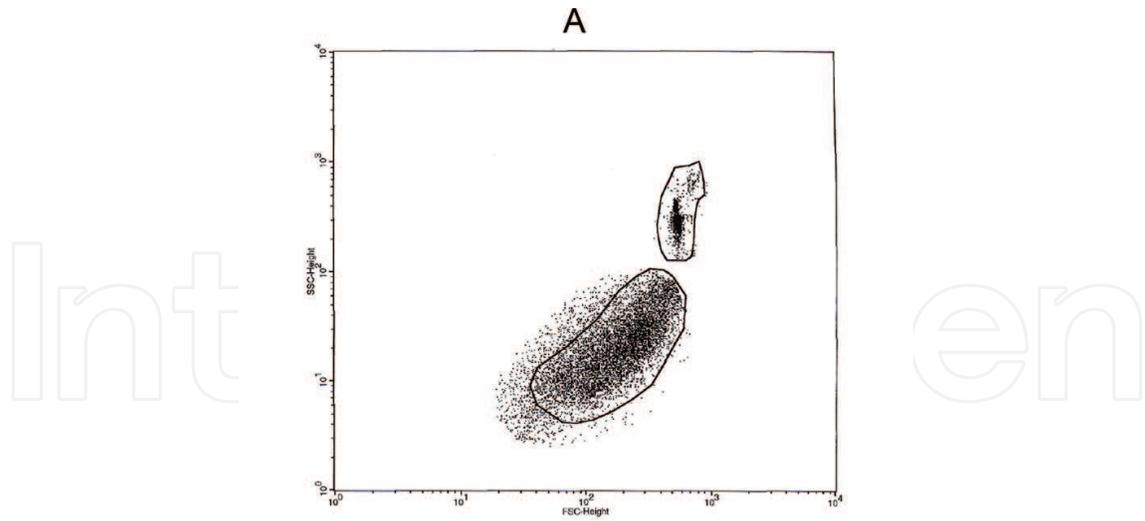


**Figure 1.** Situation in cell culture: macrophages, parasites and nanoparticles (NPs).

**Figure 2** shows an example of data obtained with a culture of J744-A1 cells infected with *T. gondii*. Each sample had to be measured twice: once to count the free parasites (**Figure 2A**) and a second time to count the cells (**Figure 2B**) separated by their size (FSC) and their granularity (SSC).

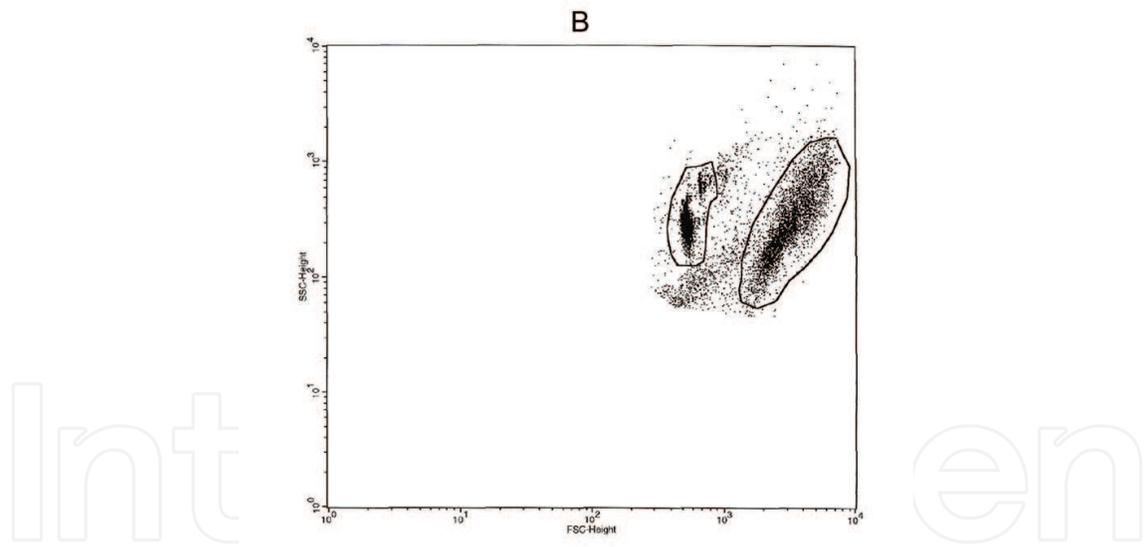
For analysis, the counted cells were sorted by fluorescence then. The series of dot blots in **Figure 3** compares uninfected, untreated control cells (A), infected cells (B), infected cells treated with unloaded nanoparticles (C), uninfected cells treated with unloaded NPs (D) and infected cells treated with pentamidine-loaded NPs (E).

The dot blots show changes in green fluorescence and granularity. In addition, the mean values of the particles in the gates are given in **Table 2**, as the numbers are hard to calculate from the images. The shift in the mean values is often more impressive than the density or position of the dots.



Tube 2 Infection control

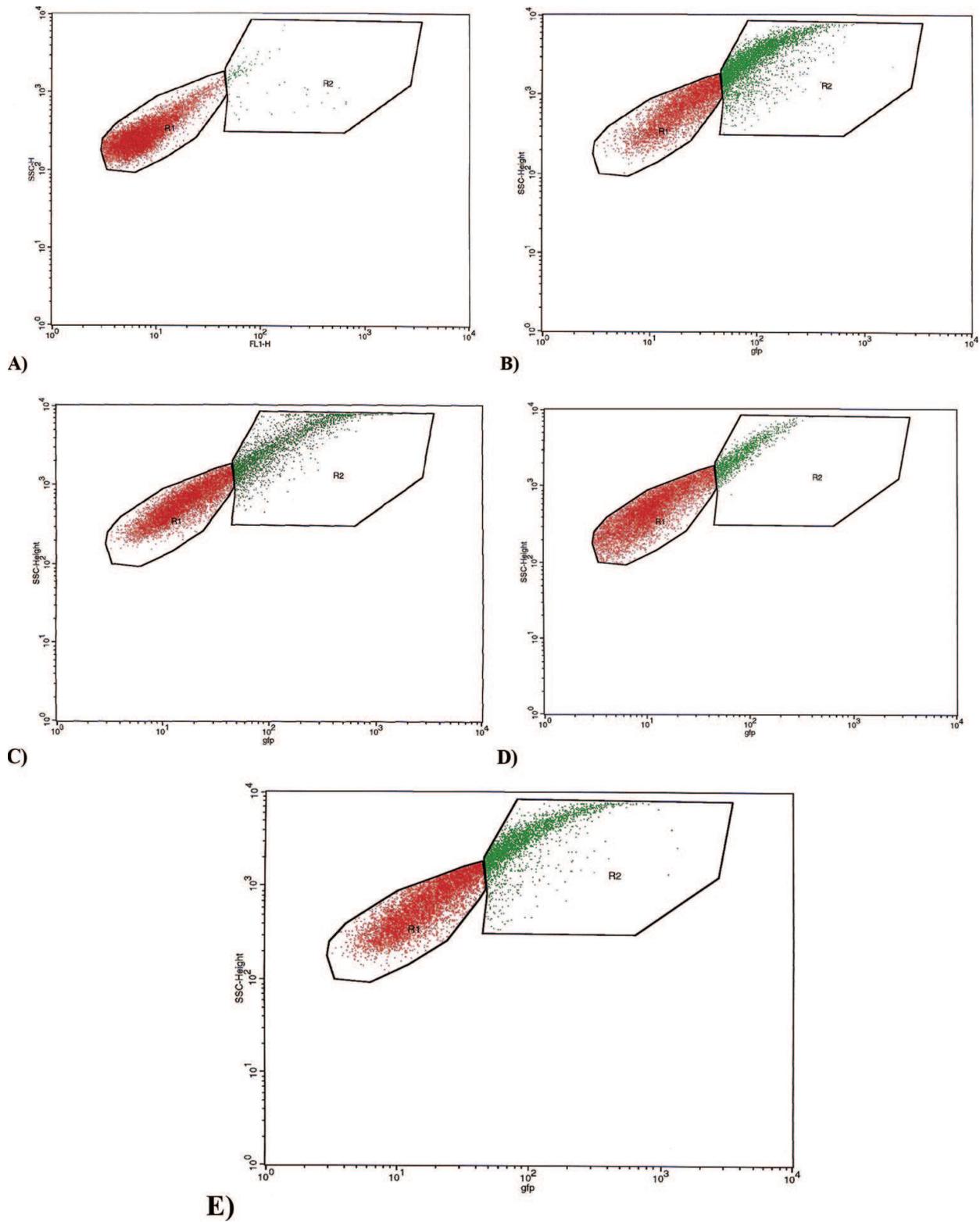
Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
R1	1846	18.46	18.46	546.01	543.77	329.01	313.71
R2	7183	71.83	71.83	214.94	178.95	24.96	19.97



Tube 1 Cell control

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
R1	4933	49.33	49.33	540.11	537.92	344.53	329.27
R2	3968	39.68	39.68	3343.07	3134.86	362.81	286.31

Figure 2. Particle size (FSC-x-axis) and granularity (SSC-y-axis) of (A) Toxoplasma cells and (B) macrophages population.



**Figure 3.** Granularity (SSC) and green fluorescence (FL1) of non-infected, untreated cells (A), infected, untreated cells (B), infected and treated with pure nanoparticles (C), uninfected cells, treated with nanoparticles (D) and infected cells, treated with pentamidine-loaded nanoparticles (E), given after 1 day of incubation.

**Figure 3A** illustrates the fluorescence of the macrophages of the cell control with its characteristic unspecific autofluorescence. The presence of *Toxoplasma* and/or NPs induces a movement into the area of higher fluorescence (**Figure 3B–E**). Whereas the *Toxoplasma* induces a stronger shift into the green fluorescence (**Figure 3B**), the NPs induced a smaller one (**Figure 3D**) which is an effect of an unspecific stimulation of the cells resulting in an increased autofluorescence [30].

The corresponding **Table 2** shows the percentage of particles in the gate for non-infected cells (R1) and the percentage in the gate of infected cells (R2) as well as the corresponding mean values (mean) and geometrical mean values (G-M).

Green fluorescence is represented on the x-axis and granularity on the y-axis.

Macrophages, activated by nanoparticles, have a basic fluorescence which is at least partially an indicator of their activity [36]. Thus, controls of cells, treated only with nanoparticles, are essential. For data processing, other combinations may be selected, such as granularity or red fluorescence. There is always an overlap of data however, and the interpretation of the blots must be proofread against the mean values of the particles within the gates—here given in the corresponding tables.

In the following series of experiments, a cell system was established in a manner that after infection on day zero, there is a continuous multiplication of *Toxoplasma* at least until day three. Defined concentrations of cells were put into wells, and parasites in different ratios were added for this purpose. The ratio parasite to cell is given as MOI (multiplicity of infection). All tests were carried out in three wells in parallel, and all experiments were carried out at least twice. The most promising results were obtained with MOI's of 1–10.

**Figure 4** shows the results of one of these experiments. MOI's of 10, 5, 2 and 1 were used, and free parasites (A), total cells (B) and infected cells (C) were determined at the first, second and third day after infection. There was a large increase in free parasites over the 3 days with MOI's of 10 and 5, a moderate increase with an MOI of 2 and almost no increase with an MOI of 1 (**Figure 4A**). In parallel, there was no such strong increase in total cell number as with the uninfected controls (**Figure 4B**). With the high MOI's of 10 and 5, there was a decrease at day two and an increase at day three. Only with MOI's of 1 and 2, there was a slight increase in cells over the 3 days. The number of infected cells indicates that with a MOI of 10 and 5, almost all cells were infected, whereas with MOI of 2 and 1, many cells remained uninfected until day three (compare **Figure 4B** and **C**).

It was shown that MOI 2 is most appropriate for these tests, because there was an increase in the parasites and of the cells over the 3 days.

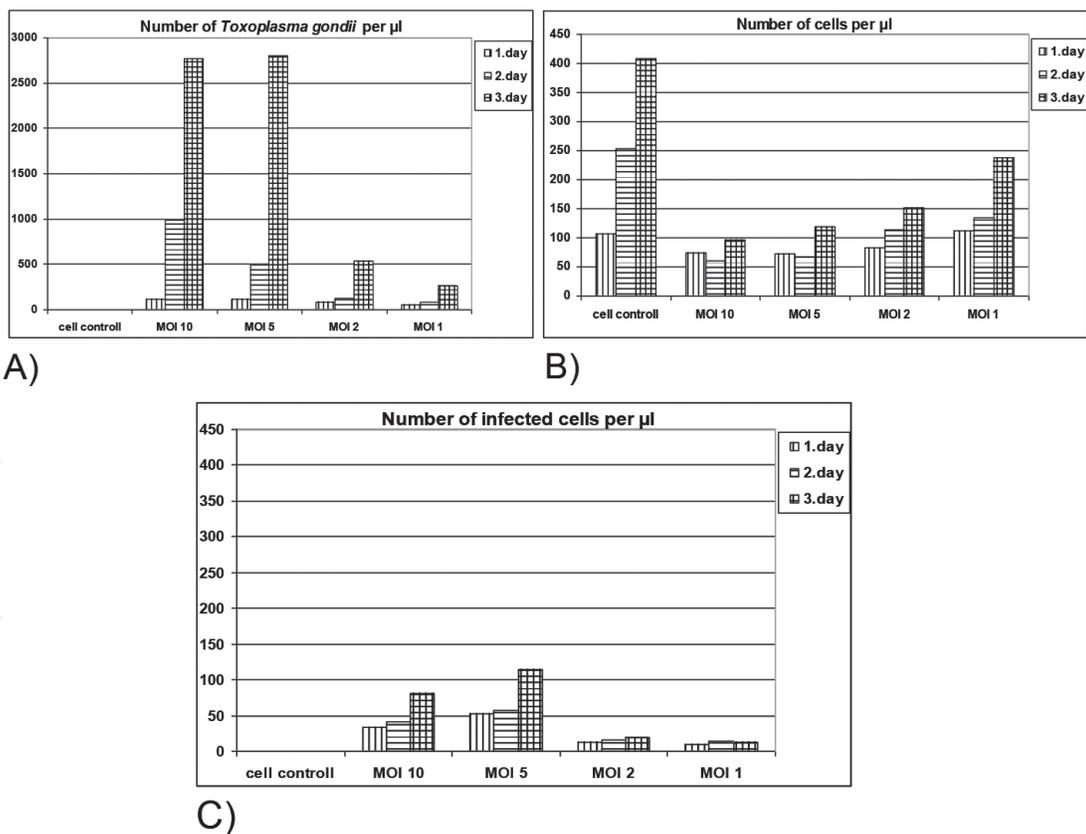
In a culture of infected cells, running for at least 3 days, there will be individual death of cells and parasites. Some of the free *Toxoplasma* in the culture will die and—as the infected cells disintegrate after liberating multiplied parasites—will shift into the debris. Thus, there will be an unpredictable increase or decrease in cell counts. To differentiate between an increase of non-infected cells and a loss of infected cells, both of which result in an increase in the percentage

of non-infected cells, it was necessary to determine the total number of cells and the proportion of infected and non-infected cells in relation to the cell input on day zero. Before measuring, cells were fixed with paraformaldehyde, and after 'Perfect Counting Beads' (Caltag Laboratories) were added, the samples were counted.

Experiment	R1 %	R1 X mean	R1 X G-M	R2%	R2 X mean	R2 X G-M
A C	98.84	8.35	7.35	1.16	102.68	77.60
B C+T	50.83	<b>23.42</b>	<b>20.62</b>	49.17	<b>108.24</b>	<b>91.94</b>
C C+T+ NPs	73.05	<b>18.68</b>	<b>16.16</b>	26.95	<b>165.08</b>	<b>116.78</b>
D C+ NPs	85.46	13.72	11.16	14.54	88.33	80.10
E C+T+ NPs+ P69.55		<b>19.58</b>	<b>16.84</b>	30.45	<b>103.65</b>	<b>86.43</b>

Exp.: part of the experiment corresponding to A to E; R%: percent of counts in this gate; R1 X mean: mean value of counts in gate 1; R1 X G-M: geometrical mean value of counts in gate 1; R2 X mean: mean value of counts in gate 2; R2 X G-M: geometrical mean value of counts in gate 2.

**Table 2.** Granularity (SSC) and green fluorescence (FL1) with the corresponding values of **Figure 3**.



**Figure 4.** Influence of multiplicity of infection (MOI)  $1.36 \times 10^5$  cells per ml was infected with different multiplicities of infection (10, 5, 2 and 1). A number of free parasites (A), total cell number (B) and number of infected cells (C) were determined over 3 days.

There was an indication that even unloaded NPs have an inhibitory effect. To prove this, the influence of pentamidine-loaded and unloaded nanoparticles on *Toxoplasma*-infected cell cultures was determined. **Figure 5** shows the histograms in each case for day 2 of non-infected cells (A), infected untreated cell controls (B), cells only treated with NPs (C), infected cells treated with unloaded NPs (D) and pentamidine-loaded NPs (E). The histograms show the changes in green fluorescence (FL1) and number of counts. One table with all data obtained (**Figure 5A**) is shown as an example of how CellQuest® analyses data. Data of corresponding tables, with the mean values of the gates for all 3 days, are given in **Table 3**. It is shown that the presence of *Toxoplasma* induced a shift to higher fluorescence. Comparing the data of infected cells, treated with pentamidine-loaded nanoparticles with those treated with unloaded nanoparticles, demonstrates that nanoparticles alone had an effect on *T. gondii* multiplication.

Simply comparing the mean values of M2 (the gate with the infected cells) would lead to a misinterpretation: the geometrical mean value would indicate that NPs even enhance *Toxoplasma* multiplication (106.69 vs. 86.41-labelled with '1' in **Table 3**), and the median would also suggest this (84.26 vs. 77.04 labelled with '2' in **Table 3**).

By looking at the percentage, one sees that with NPs, there was an increase in cells in the gate of non-infected cells (M1: 71.0 vs. 49.5% labelled with '3' in **Table 3**) and as the mean value of all cells indicates, there was an inhibition by unloaded NPs visible as reduction in green fluorescence (57.48 vs. 63.41 labelled with '4' in **Table 3**). The median indicates that the inhibition was substantially (22.27 vs. 43.32 labelled with '5' in **Table 3**).

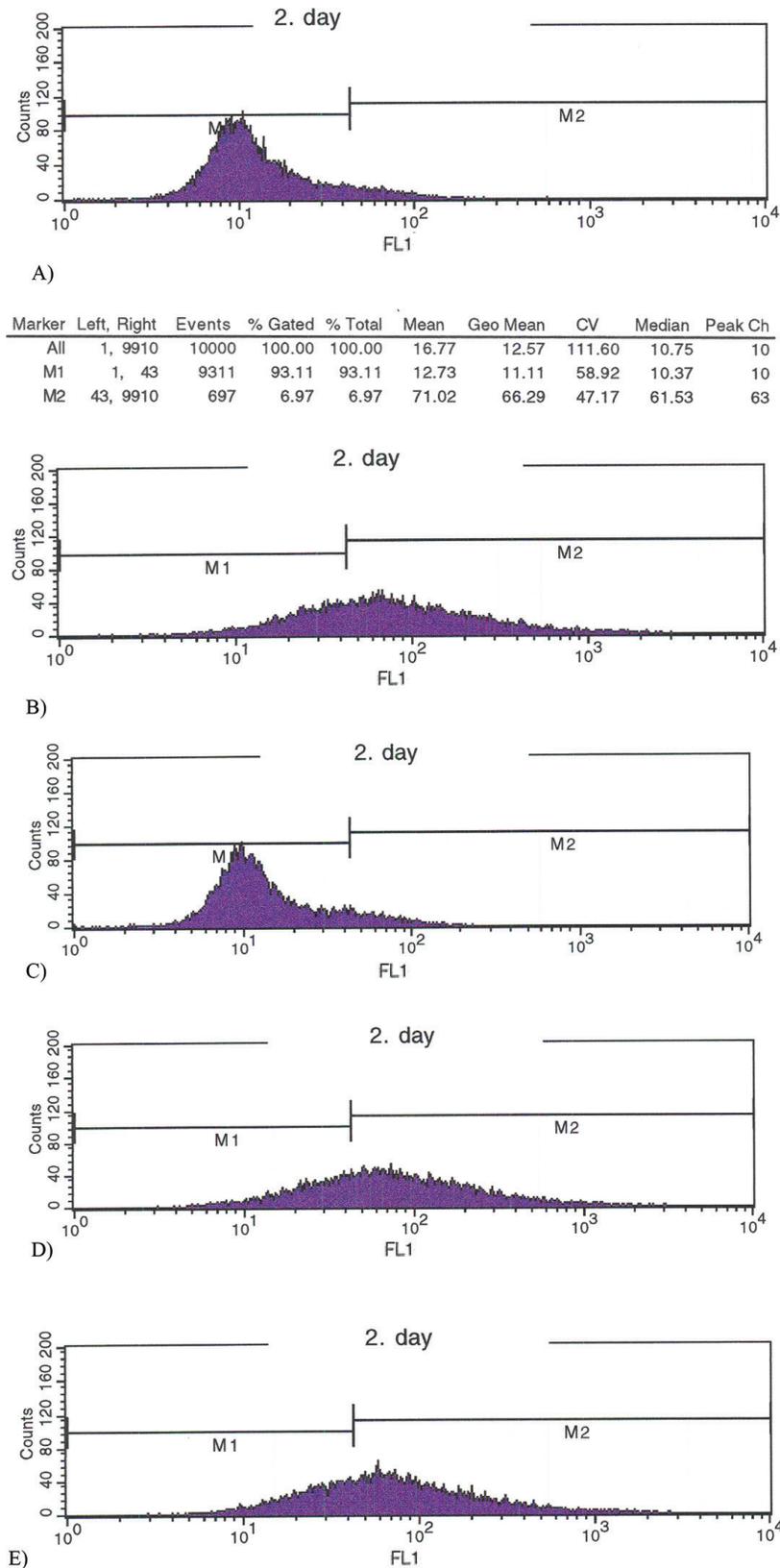
The mean value of the green fluorescence marker reflects the parasitic load, as fluorescence is proportional to parasite number. Thus, few cells with high numbers of parasites may increase the mean value despite the fact that there were lots of non-infected cells. The median indicates the shift of the population towards infected or non-infected cells. The closer the median of infected, treated cells come to non-infected control cells, the better the treatment success. The geometrical mean gives some information, concerning the heterogeneity, but avoids the overestimation of exceptionally heavy infected single cells.

By measuring 10,000 cells, FACS offered the opportunity to statistically treat these data in different ways, resulting in a more complex interpretation. In addition to this evaluation of data, one had to take into account that nanoparticles are known to stimulate the macrophages [30–32]. As an increase in unspecific fluorescence was seen, an inhibition of *Toxoplasma* growth by unloaded nanoparticles was not surprising.

The explanation was that the nanoparticles counteracted the suppression of activities caused by the parasites [33], and this unspecific stimulation of macrophages by nanoparticles was more effective than the inhibition of parasites within downregulated host cells.

As cells, treated with nanoparticles, were always used as controls to estimate the unspecific increase in fluorescence, it was realised that there was a substantial inhibition by unloaded nanoparticles in all experiments.

In an additional series of experiments, the effect of nanoparticles without any drug was specifically proven. Data of an experiment to determine the effect of particle number on the multiplication of *T. gondii* during a period of 3 days are given in **Figure 6**.

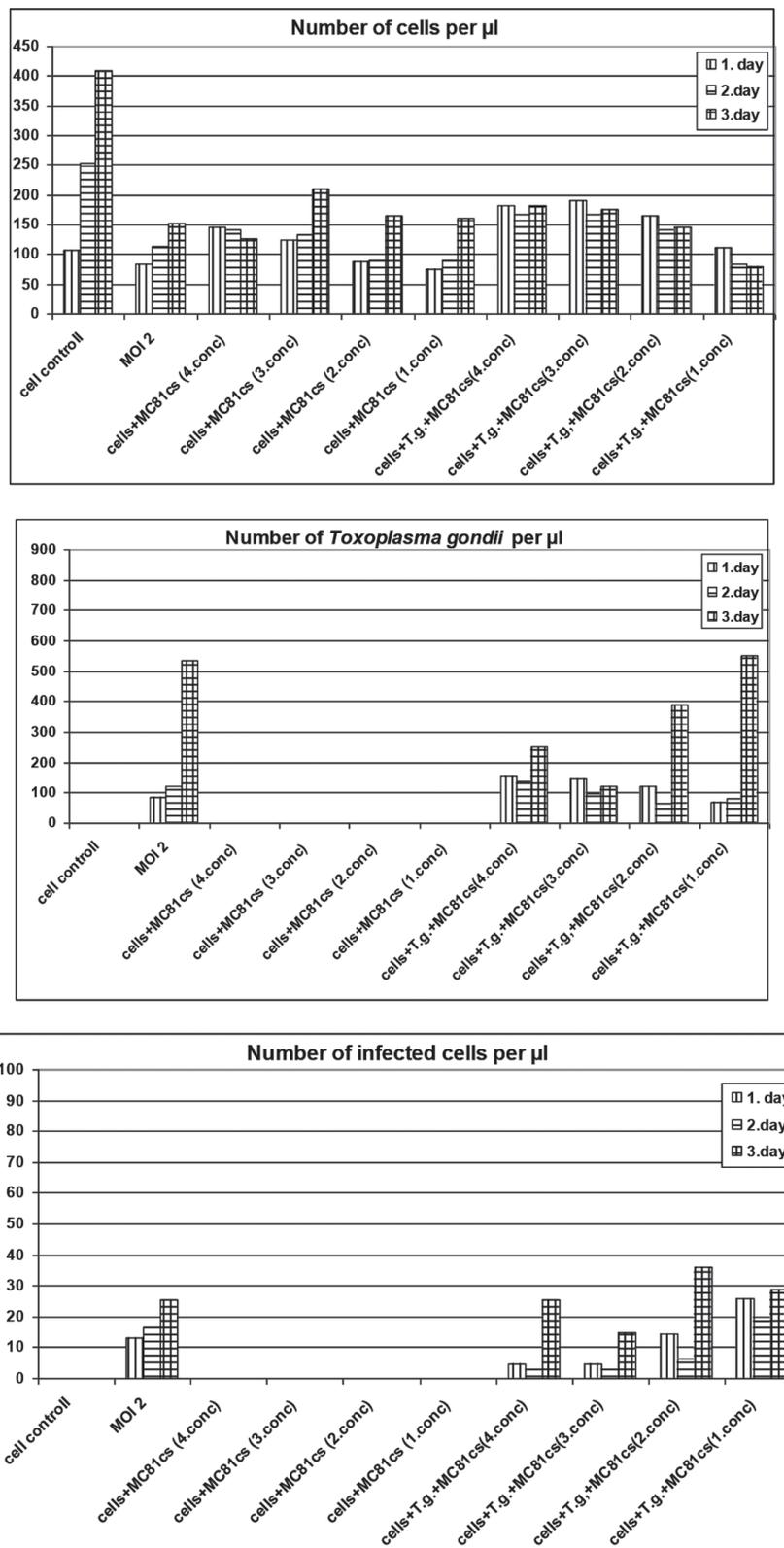


**Figure 5.** FACS counts of non-infected, infected and NPs-treated cell. Histograms are shown in each case for day 2 of non-infected cells (A), infected untreated cell controls (B), cells only treated with NPs (C), infected cells treated with unloaded NPs (D) and pentamidine-loaded NPs (E). The histograms show the changes in green fluorescence (FL1) and number (counts). M1 indicates the non-infected cells and M2 the infected ones. (T = *Toxoplasma gondii*).

	1. day			2. day			3. day		
	All	M1	M2	All	M1	M2	All	M1	M2
Cell control [%]		98.6	1.4		93.0	7.0		92.0	8.0
Mean	9.29			16.77			16.14		
Geomean	7.30	7.08	70.91	12.57	11.11	66.29	10.23	8.68	65.62
Median	6.79	6.73	57.25	10.75	10.37	61.53	9.22	8.58	59.89
Infected cells [%]		35.0	65.0		33.0	67.0		49.5 <sup>3</sup>	50.5
Mean	160.49			127.46			63.41 <sup>4</sup>		
Geomean	75.89	18.78	156.81	70.30	23.32	121.25	42.25	20.27	86.41 <sup>5</sup>
Median	73.63	19.81	145.90	66.12	25.71	103.66	43.32 <sup>5</sup>	22.07	77.04 <sup>2</sup>
Cells + NPs [%]		93.0	7.0		90.5	9.5		85.5	15.5
Mean	14.63			18.76			22.96		
Geomean	10.06	8.77	67.19	13.70	11.60	65.49	13.21	9.81	74.96
Median	8.28	7.99	62.08	11.44	10.84	61.53	11.44	9.91	69.16
Cells + Toxopl. + NPs [%]		34.0	66.0		33.5	66.5		71.0 <sup>3</sup>	29.0
Mean	159.64			118.23			57.48 <sup>4</sup>		
Geomean	76.71	19.57	156.14	142.73	23.22	117.34	27.56	15.92	106.69 <sup>1</sup>
Median	72.34	21.29	143.30	64.94	25.71	101.82	22.27 <sup>5</sup>	15.96	84.29 <sup>2</sup>
Cells + Toxopl.+ NPs (pentamidine-loaded) [%]		37.0	63.0		35.0	65.0		69.0	31.0
Mean	157.60			115.67			43.75		
Geomean	73.90	20.18	158.34	65.71	23.49	113.57	27.49	27.49	79.96
Median	67.93	21.48	144.60	61.80	25.71	95.60	25.95	17.31	68.54

<sup>1,2,3,4</sup> and <sup>5</sup> refer to numbers in discussion.

**Table 3.** Mean values of total cells and gates of **Figure 5**. In addition to the mean values, the percentage of M1 and M2 of total cells for each part is given.



**Figure 6.** Effect of different concentrations of NPs (MC81cs) on cell proliferation and of *Toxoplasma gondii* (*T. g*) growth. (A) A number of all remaining cells (infected and non-infected) after 1, 2 and 3 days of infection and treatment. (B) A number of free (extracellular) *Toxoplasma gondii*. There are no *Toxoplasma* in NPs-treated, non-infected cells. (C) A number of infected cells after treatment with different concentrations of NPs.

Part A shows the increase in the infected and non-infected cells with different NPs concentrations. As can be seen, NPs in a high concentration (conc.4) inhibited proliferation over the 3 days. The lower concentrations led to a small increase on day two and a larger increase on day three. With infected cells, there was no difference between conc. 4 and 3, and only a slight decrease in cells with conc. 2. But with the lowest concentration, conc. 1, there was a decrease over the 3 days.

Part B shows the increase in *Toxoplasma* in the presence of NPs. Concentrations 4 and 3 suppressed the propagation of *Toxoplasma*. There was a reduced number of free *Toxoplasma* compared with the infection control. At concentrations 1 and 2 on the third day, there was a large increase in free *Toxoplasma* with concentrations even higher than in the untreated control.

Part C shows the number of infected cells which decreased at all NPs concentrations on day two and increased on the third day. The third concentration led to the smallest number of infected cells over the 3 days.

Cells were infected with an MOI of 2. The concentrations of NPs were as follows:

Conc 1: 118,000 NPs/cell	Conc 3: 24,000 NPs/cell
Conc 2: 60,000 NPs/cell	Conc 4: 12,000 NPs/cell

As discussed above, the effect of NPs was interpreted as activating the functions suppressed by *Toxoplasma*. On the other hand, there may be negative effects on macrophage functions, too [34]. Thus, in other cases (with other cells or with other parasites), the effect could be different. If there is stimulation, a treatment with nanoparticles alone could have some influence on a *Toxoplasma* infection, as drug-loaded particles have.

One way to prove this is to repeat these experiments in other, non-phagocytising host cells. The problem is that they will not take up the nanoparticles as easily as the macrophages do. As there are other microorganisms which also multiply within macrophages, there could be other indications for this type of treatment.

In all experiments described so far, *Toxoplasma* and NPs were added simultaneously to the cells. When nanoparticles influence the activities of phagocytising cells, and *T. gondii* alters the host cell activities, it is interesting to ask, whether the presence of NPs or *T. gondii* interferes with the taking up of the other, is there some type of mutual exclusion? Thus, experiments were carried out with NPs, added four hours after infection with *T. gondii*, or cells were infected 4 h after treatment with NPs. There were no differences in the taking up of NPs or parasites or in the effect of treatment after 3 days. The slope of the parameters was simply shifted (data not shown).

In the future, one will see, whether *Leishmania major* which in contrast to *T. gondii* are able to grow only in macrophages and are also inhibited by unloaded NPs. A few reports indicate that this should be true [21]. With *Leishmania*, first data in animal models were published [35, 36]. The fact that *Leishmania major* multiplies only in phagocytising cells makes them even more interesting for a further use of nanoparticles.

A question to be answered is, whether there is disintegration of nanoparticles by the host cells. There was fluorescence on the third day, what could be the result of dye released after disintegration? Microscopical observation revealed that nanoparticles were still present, at least the non-degradable, fluorescence-labelled polystyrene cores of these core-shell nanoparticles.

With the methods used, a disintegration of the nanoparticles could not be demonstrated in detail as it should start with the cleavage of the ester groups of the polymer shell by esterases which would not alter the structure in a way that could be seen with a microscope. As it only happens inside the cells, this alteration cannot be found with FACS, as there is no change in granularity.

As the core particles remain undigested, granularity will indicate the presence of particles. In the case that there would be no disintegration, this may be an advantage of an *in vitro* system using phagocytising cells, as the activation will persist. The consequences for *in vivo* conditions remain to be determined. Allen showed in 1988 that repeated application of liposomes could lead to an impairment of Kupffer cells in the liver and fixed macrophages in the spleen which remove most of these NPs [37].

Our aim was to establish a system which allows the observation of the interaction of nanoparticles and microorganisms, infecting a cell culture, because there should be difference between infected and non-infected cells which could be used for targeting. The results are very promising. As various cell lines are able to produce interleukins or other modulators, there is the possibility to characterise the influence of nanoparticles in even more detail.

The data that NPs without any drugs have the same effect as drug-loaded NPs offer the opportunity to use NPs directly for treatment. Perhaps part of the effect of Atovaquone crystal nanoparticles in a *Toxoplasma*-mouse model should be attributed to the effect of nanoparticles [13], rather than to the direct effect of the drug against the parasite.

### 3. Experimental part

#### 3.1. Colloid and polymer chemistry

##### 3.1.1. Preparation of nanoparticles

Core-shell latex nanoparticles (MC81cs see **Table 1**) were synthesised by seeded, aqueous polymerisation of butyl cyanoacrylate onto polystyrene cores. Polystyrene cores were prepared by emulsion polymerisation of styrene in a water/ethanol mixture (16:1; v/v) in the presence of rhodamine B and purified by dialysis against deionised water. One gram of these core particles in water with a pH of 2 was used as seed for the polymerisation of 2 g butyl cyanoacrylate [38]. In the case of pentamidine loading, 5 mg pentamidine/g polymer was added to the polymerisation medium. Resulting core-shell nanoparticles were purified by dialysis against de-ionised water after neutralisation with sodium hydroxide (1 M aqueous solution).

### 3.1.2. Characterisation of nanoparticles

The particle size, determined as  $Z_{ave}$  (average particle diameter) and the polydispersity index (PI), was measured by photon correlation spectroscopy (PCS), using a Zetamaster S (Malvern Instruments). Electrophoretic mobilities were measured to reflect the effective surface charge (Zetamaster S, Malvern Instruments). For transformation into zeta potential, the Smoluchowski model for ideal smooth spheres was used. Measurements were carried out in ultra-pure water (Millipore), 0.01 M NaCl solution and in 0.001 M NaCl solution.

Surface charge density was titrated by means of 0.1 mM PDADMAC solution (MW approx. 100,000) at a combination of particle charge detector PCD-03 pH (MÜTEK) and automatic titrator Titrino 716 (Metrohm). Purified, aqueous nanoparticle suspensions were stored in the refrigerator until use.

### 3.1.3. Beads

'Perfect Counting Beads' (Caltag Laboratories) were used to adjust the cell and *T. gondii* number. Immediately before counting with FACS, a defined number of beads were added and for calculation of inhibition and growth, all cells and *T. gondii* were adjusted to the known bead concentration.

## 3.2. Biology of Infections

### 3.2.1. *T. gondii* GFP growth and experimental infection:

Tachyzoites of the RH (CAT-GFP) strain of *T. gondii* (obtained from D. Soldati; Centre of Molecular Biology, Heidelberg University, Germany) were maintained in vitro in human epitheloid carcinoma cells (HELA) by serial passage into RPMI medium supplemented with 10% FCS (Gibco-BRL) twice a week (Gibco-BRL).

Macrophages and parasites were counted in a Fuchs-Rosenthal chamber and adjusted to the concentrations indicated. In most cases, macrophages of a concentration of  $1.36 \times 10^5$  per ml were used and the multiplicity of infection (MOI) in most experiments was 2.

## 3.3. Cell culture work

### 3.3.1. Cell lines

Macrophages: The intracellular Toxoplasma infection studies were performed on J 774-A1 cells (mouse monocyte macrophages, ACC 170; German Collection of Microorganisms and Cell Cultures). The macrophages were grown in Dulbecco's MEM containing 10% heat-inactivated foetal calf serum (Boehringer, Germany) at 37°C in 5% CO<sub>2</sub> atmosphere.

### 3.3.2. FACS-analysis

Parasites, infected cells and non-infected cells were counted with FACS-scan analytical flow cytometry and equipped with a 15 mV, 488 nm air-cooled argon ion laser (Becton Dickinson). Multiparametric data were analysed using CellQuest® Software. Ten thousand 'particles', defined by size and granularity, were measured for each analysis.

The optimised instrument parameters for the assay were as follows: forward scatter (E 00, log mode), side scatter (319 voltage, log mode) fluorescence 1 (FL1; 613 voltage, log mode) and fluorescence 2 (FL2; 528, log mode).

Each experiment was carried out in a triple manner and performed in a special laboratory. The equipment was decontaminated with FACS-safe solution (1% active chlorine, Becton Dickinson).

Adding formaldehyde to a final concentration of 0.2% resulted in a preservation of the samples. Samples could be stored for days in a refrigerator and be measured at a more convenient time.

### 3.3.3. Microscopy and photography

An epifluorescence microscope (Axioskop; Zeiss) was used to view the infection fate of J774 A1 cells with *T. gondii* GFP. The microscope was equipped with a 50 W high-pressure mercury lamp (HB050; Osram) and 10×, 40× and 100× objectives (Zeiss). Narrow band filter sets HQ-F41-007 and HQ-F41-001 (AHF Analysentechnik) were used to analyse the GFP and red signals, respectively, at a magnification of 1000×. Image processing was performed with a standard software package delivered with the instrument (Zeiss Axiovision 3.1).

## Author details

Sprakel-Leyke Silja<sup>1</sup>, Paulke Bernd-Reiner<sup>2</sup> and Presber Wolfgang<sup>1\*</sup>

\*Address all correspondence to: wolfgang.presber@charite.de

1 Institute of Microbiology and Hygiene, University of Medicine Berlin (Charité), Berlin, Germany

2 Fraunhofer Institute for Applied Polymer Research, Potsdam-Golm, Germany

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