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Treatment of glioblastoma with poly(isohexyl cyanoacrylate) nanoparticles

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ABSTRACT

Glioblastomas belong to the most devastating cancer diseases. For this reason, polysorbate 80 (Tween 80®)-coated poly(isohexyl cyanoacrylate) (PIHCA) (Monorex®) nanoparticles loaded with doxorubicin were developed and tested for their use for the treatment of glioblastomas. The preparation of the nanoparticles resulted in spherical particles with high doxorubicin loading. The physico-chemical properties and the release of doxorubicin from the PIHCA-nanoparticles were analysed, and the influence on cell viability of the rat glioblastoma 101/8-cell line was investigated. In vitro, the empty nanoparticles did not show any toxicity, and the anti-cancer effects of the drug-loaded nanoparticles were increased in comparison to doxorubicin solution, represented by IC_{50} values. The in vivo efficacy was then tested in intracranially glioblastoma 101/8-bearing rats. Rats were treated with 3×1.5 mg/kg doxorubicin and were sacrificed 18 days after tumour transplantation. Histological and immunohistochemical analyses were carried out to assess the efficacy of the nanoparticles. Tumour size, proliferation activity, vessel density, necrotic areas, and expression of glial fibrillary acidic protein demonstrated that doxorubicin-loaded PIHCA-nanoparticles were much more efficient than the free drug. The results suggest that poly(isohexyl cyanoacrylate) nanoparticles hold great promise for the non-invasive therapy of human glioblastomas.

1. Introduction

Glioblastomas are among the most aggressive tumours. Despite multimodal therapies glioblastomas lead to death in affected patients within a median of 12–15 months (Wick et al., 2007). In addition, chemotherapy of Glioblastoma is largely ineffective as the blood–brain barrier (BBB) prevents entry of most anticancer agents into the brain. Glioblastomas are characterised by a destructive and infiltrative growth pattern into the surrounding healthy brain tissue (Louis et al., 2007). Typical histopathological signs of these tumours are nuclear atypia, increased mitotic index, proliferation of the vessels and widespread necrotic areas (Behin et al., 2003). The 101/8-rat tumour model used in this study resembles by morphologically and histological means closely the clinical presentation of human glioblastoma (Hekmatara et al., 2009).

Nanoparticles delivering cytotoxic drugs into the brain and tumour tissue seem to be a useful additional therapy together with surgical resection and radiation therapy. Over the last decades nanoparticles as well as liposomes have shown to represent promising drug delivery systems (Kostarelos, 2003) for the treatment of solid tumours. Compared to liposomes, nanoparticles are more stable in biological fluids and during storage because of their polymeric nature (Guise et al., 1987). Nanoparticles consisting of biodegradable polymers, such as alkyl cyanoacrylates, are obtained by emulsion polymerisation of different alkyl cyanoacrylate monomers in acidic media. They are useful in promoting cellular uptake via endocytosis, and these carriers are also able to enhance the in vivo activity of different drugs including anti-tumour drugs (Vauthier et al., 2003). The association of drugs to the polycyanoacrylate nanoparticles is achieved by hydrophobic van-der-Waals interactions. Being an amphiphilic molecule, doxorubicin was proved to form stable complexes with this type of carriers. Already Cuvier et al. (1992) demonstrated that doxorubicin-loaded poly(isohexyl cyanoacrylate) (PIHCA) nanospheres can circumvent the P-gp mediated resistance of tumour cells in vitro. Similar nanoparticles consisting of poly(butyl cyanoacrylate) (PBCA) loaded with the same drug, doxorubicin, were shown to be very efficient for the treatment of glioblastoma in rats (Hekmatara et al., 2009; Steiniger et al., 2004; Wohlfart et al., 2009).

The incorporation of doxorubicin into these biodegradable acrylate nanoparticles was demonstrated to enhance the cytotoxicity

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of doxorubicin while the cardiotoxicity was drastically reduced by modifying its tissue distribution (Pereverzeva et al., 2008). Consequently, Kattan et al. (1992) did not detect any cardiotoxicity in a phase I clinical study (Kattan et al., 1992). PIHCA-nanoparticles were successfully applied for the treatment of primary liver cancer after direct injection into the liver via the hepatic artery (Merle et al., 2006). In order to use these particles for the treatment of brain disorders such as epilepsy, Parkinson's disease, or brain tumour, the particles have to pass the BBB composed of the tight junctions of the endothelial cells and of very efficient efflux transporters. To this end, the surface of the nanoparticles has to be coated with certain surfactants allowing their brain uptake. After loading with doxorubicin and followed by coating with the surfactant polysorbate 80 (Tween® 80) PBCA-nanoparticles were able to reduce the drug toxicity and to deliver the drug to the brain for the treatment of glioblastoma (Pereverzeva et al., 2007; Steiniger et al., 2004).

The polymer PIHCA may possess an even better biocompatibility than PBCA, as the toxicity of alkylcyanoacrylates appears to depend on their degradation rate (Gipps et al., 1987; Lherm et al., 1992). Longer alkylcyanoacrylate chains degrade more slowly yielding a reduced toxicity (Gipps et al., 1987; Lherm et al., 1992; Muller et al., 1990). For this reason, in the present study PIHCA-nanoparticles were developed and the anti-tumour efficacy of doxorubicin-loaded PIHCA-nanoparticles and of free doxorubicin in solution was compared in vitro using the rat glioblastoma cell line 101/8. To evaluate the anti-tumour efficacy of these particles in vivo, the orthotopic rat glioblastoma tumour model 101/8 was used and analysed by histology after treatment with the doxorubicin-loaded PIHCA-nanoparticles coated with polysorbate 80.

2. Material and methods

2.1. Materials

Isohexylcyano-acrylate (Monorex®) was obtained from BioAlliance Pharma (France). The drug doxorubicin HCl was purchased from Yick-Vick Chemicals and Pharmaceuticals (Hong Kong). Unless otherwise stated, all chemicals and solvents were of analytical grade.

2.2. Preparation of empty and doxorubicin-loaded poly(isohexyl cyanoacrylate) nanoparticles

Empty PIHCA-nanoparticles were produced by anionic emulsion polymerisation (Petri et al., 2007). Different amounts (0.75, 1.00, and 1.25% (v/v)) of the monomer isohexylcyanoacrylate were added to a 1% (w/v) solution of a stabilizer in 0.01 N HCl under constant stirring (700 rpm) at ambient temperature. Either dextran 70,000 or poloxamer 188 (Pluronic® F68) were used as stabilizers. After 18 h polymerisation was terminated by neutralisation with 0.1 N NaOH. Then the nanoparticle suspension was filtered through a G2 sintered glass filter (Schott, Mainz, Germany) with a pore size of 40–100 μm to remove agglomerates and lyophilized with 3% mannitol as cryoprotector. To obtain surfactant-coated nanoparticles, the nanoparticles were resuspended in a 1% polysorbate 80 solution. Preparation of doxorubicin-loaded PIHCA-nanoparticles was performed in an analogous manner. The 2.5% (w/v) doxorubicin solution was added 30, 45, or 60 min after start of the polymerisation process to obtain a final doxorubicin concentration of 0.25%.

2.3. Characterisation of the nanoparticles

2.3.1. Particle size, polydispersity and zetapotential

The resulting nanoparticles were characterised regarding mean particle diameter, polydispersity, and particle surface charge using photon correlation spectroscopy (PCS) in water with a Malvern Zetasizer, 3000 HSA (Malvern Instruments Ltd., UK). The samples were diluted 1:50 with purified water, the temperature was set to 25 °C, and the particles were measured at a scattering angle of 90°.

2.3.2. Yield of poly(isohexyl cyanoacrylate)

The polymer yield was determined by gas chromatography using a modified method developed by Langer et al. (Langer et al., 1994) for poly(butyl cyanoacrylate) nanoparticles. This gas chromatographic method is based on the release of isohexanol by hydrolytic degradation of the polymer in alkaline medium. One ml of the nanoparticle suspension and 1 ml of 2 M NaOH were mixed and were shaken at ambient temperature for 24 h to hydrolyse the ester groups of the polymer. 50 μl hydrolysate and 50 μl of internal standard (0.5% pentanol solution) were mixed and diluted with 900 μl purified water. To extract the isohexanol, 1000 μl dichloromethane were added to 500 μl of the solution and shaken vigorously. 1 μl of the water-free sample was injected into the gas chromatograph and analysed for the amount of isohexanol. The PIHCA polymer content was then calculated based on the amount of isohexanol produced by hydrolysis and multiplication by the factor 1.774. This factor arises from the mass ratio of one unit PIHCA and the corresponding alcohol isohexanol.

2.3.3. Morphologic analysis of particles by scanning electron microscopy

The particle morphology was analysed by scanning electron microscopy (SEM Hitachi S-4500). For this purpose, the nanoparticle suspension was applied to an aluminium sample plate and dried over night at ambient temperature. To obtain electrical conductibility, the particles were sputtered with gold for 45 s under argon gas atmosphere. Afterwards the samples were analysed at 15–25 kV with an upper detector.

2.3.4. Determination of loading capacity

To evaluate the drug loading capacity, the doxorubicin concentration in the supernatant was estimated after separation from the loaded nanoparticles by ultrafiltration (15 min, 14,000 g) using centrifugal filter devices (Amicon® Ultra-0.5, 30 kDa, Millipore, USA). Doxorubicin was quantified by HPLC using UV detection at 250 nm. The chromatographic separation was performed on a 250 × 4 mm reverse phase column (LiChroCART® 250-4 LiChrospher® 100 RP-18, 5 μm, Merck, Darmstadt, Germany). The mobile phase was an isocratic mixture of water and acetonitrile in the ratio of 70:30 containing 0.1% trifluoroacetic acid, and the flow rate was adjusted at 0.8 ml/min resulting in a retention time of about 11 min. The linearity as well as accuracy and detection limits were determined with aqueous solutions of doxorubicin in a concentration range from 5 to 50 μg/ml (r^2 = 0.9998) following the method of Configliacchi et al. (1996). The encapsulation efficacy was calculated as the difference between the total drug concentration and the amount of free drug in the filtrate. The loading capacity of the nanoparticles (μg doxorubicin/mg polymer) also was calculated.

2.3.5. Drug release study

To determine the drug liberation of the nanoparticles, the release of doxorubicin from PIHCA-nanoparticles was carried out by dialysis (Kufleitner et al., 2010). As dialysis medium 39.0 ml of phosphate buffered saline (pH 7.4) was used. One ml of nanoparticle suspension containing 1936 μg/ml doxorubicin was filled into the dialysis tube (cut-off 6–8 kDa) and the dialysis medium was stirred at 550 rpm. After defined time intervals, aliquots of 1.0 ml of the release medium were sampled and replaced with the same volume of phosphate buffered saline. An aqueous doxorubicin solution (2000 μg/ml) was used as a control. The samples were then analysed by HPLC using the above method. For this experiment as

well as for the in vitro and in vivo investigations the particle system containing 1% monomer and 1% dextran 70,000 as stabilizer was used. Doxorubicin addition took place 45 min after start of the polymerisation.

2.4. In vitro investigation: cell viability

The rat glioblastoma cell line 101/8 was established from the rat glioblastoma 101/8 that was initially created by local injection of α-dimethylbenzanthracene and maintained by intercerebral transplantation (Yablonovskaya and Avtsyn, 1964). Glioblastoma cells were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal calf serum, and 1% penicillin (100 IU/ml)/streptomycin (100 μg/ml) under cell culture conditions (37 °C, 5% CO_2).

The cytotoxicity of empty and doxorubicin-loaded PIHCA-nanoparticles coated with 1% polysorbate 80 and a doxorubicin solution containing 1% polysorbate 80, were assessed in 101/8 rat glioblastoma cells by the mitochondrial-dependent reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Michaelis et al., 2004; Mosmann, 1983). In brief, 50,000 cells were seeded in 96-well plates and incubated for 5 days with empty particles, doxorubicin-loaded nanoparticles or doxorubicin solution, starting at a concentration of 20 ng/ml or 40 ng/ml, respectively. Then, 25 μl MTT reagent (2 mg MTT/1 ml PBS) was added to each well, and the cells were incubated for 4 h and then for further 20 h after addition of 100 μl SDS solution (200 μg/1 ml). The plates were analysed on a multiwall scanning spectrophotometer (GENios, Tecan) at a wavelength of 560 nm and a reference wavelength of 620 nm. The cell viability was defined as the relative reduction of the absorbance, which directly correlates with the amount of viable cells in relation to untreated control cells (=100%). IC_{50} values were calculated using the software CalcuSyn® and represent drug concentrations yielding 50% of dye reduction compared to untreated control.

2.5. In vivo investigation: chemotherapy of 101/8 rat glioblastoma

The animal experiments were performed in accordance with the German Guidelines for Animal Experiments and authorized by the German Tierschutzgesetz and the Allgemeine Verwaltungsvorschrift zur Durchführung des Tierschutzgesetzes and were authorized by the Regierungspräsidium Darmstadt (V54 – 19 c 20/15 – F 116/16). Male Wistar rats weighing 200–220 g (obtained from Harlan Laboratories, Inc.) were used and maintained under standard conditions. They had free access to laboratory chow and water.

The experimental procedures including anaesthetization of animals, generation and implantation of tumour tissue as well as treatment of the animals was described in detail previously (Wohlfart et al., 2009). Briefly, the animals were anesthetised by intraperitoneal injections of 100 mg/kg ketamine and 10 mg/kg xylazine. Through a midline sagittal incision, a burr hole of 1.5 mm in diameter was made with a dental drill 2 mm lateral to the sagittal midline and 2 mm posterior to the right coronal suture. About 10^6 tumour cells from the frozen stock were introduced into the bottom of the right lateral ventricle of donor animals using a tuberculin syringe (B. Braun, Melsungen, Germany) to induce the tumour. To close the scalp incision surgical glue (Turbo 2000 Kleber Universal, Boldt Co, Wermelskirchen, Germany) was used. After development of distinctive clinical signs of disease (usually days 14–18 after inoculation of tumour cells) the animals were sacrificed by carbon dioxide asphyxiation. The brain was removed and the tumour tissue was excised and homogenised with a scalpel. This fresh tumour tissue was inoculated into the brain of new experimental animals as described above.

One group of experimental animals ($n = 6$) was used as untreated control, one group received doxorubicin solution with 1% polysorbate 80 (3×1.5 mg/kg), and one group was treated with 3×1.5 mg/kg doxorubicin-loaded PIHCA-nanoparticles + 1% polysorbate 80 on days 2, 5, and 8 after tumour inoculation. The animals of the control group were sacrificed if they showed pronounced signs of illness, at the latest on day 18; the treated groups were sacrificed all on day 18. The brains were carefully removed and processed for histological and immunohistochemical investigation.

2.6. Histological and immunohistochemical analysis

The removed brains were fixed in 3.75% zinc formalin (Thermo Shandon, Pittsburgh, USA) solution for at least 48 h, before they were embedded in paraffin blocks. For determination of tumour size and extent of necrotic areas, 5 μm thick slides were stained with haematoxylin and eosin (H&E) following routine procedures (Schoch et al., 2006). Sections were analysed at the level where the cross-sectional area contained the largest diameter of the tumour.

For the analysis of cell proliferation and GFAP (glial fibrillary acidic protein) expression the slides were incubated with distinctive antibodies (rabbit polyclonal antibodies against Ki67 (ab15580, 1:100, Abcam, Cambridge, United Kingdom) and mouse monoclonal antibodies against GFAP (M0761, 1:200, Dako, Glostrup, Denmark) in blocking buffer (5% goat serum/45% Tris buffered saline pH 7.6 (TBS)/0.1% Triton X-100 in antibody diluent solution, Zytomed, Berlin, Germany)). For the analysis of blood vessel density, the tissue sections were stained with Isolectin B4, a marker for endothelial cells. Biotinylated Isolectin B4 (B-1205, 1:30, Vector Labs, Burlingame, California, USA) was used at a concentration of 100 μg/ml in bovine serum.

2.6.1. Measurement of tumour size

For quantitative determination of the tumour size in each animal the maximal tumour area was determined in serial H&E stained tissue slides using an Axioskop microscope (Carl Zeiss Microimaging, Göttingen, Germany) and a Neurolucida software-controlled computer system.

2.6.2. Quantification of proliferation index

To assess proliferation of tumour cells, immunohistochemical staining with antibodies against Ki67 was performed. The Ki67 labeling index expressed as the ratio of positively stained tumour cells of all cells was determined from at least six random high power fields (0.19 mm^2) in each animal.

2.6.3. Analysis of blood vessel density

For this purpose tissue sections were stained with Isolectin B4, a marker for endothelial cells. The percentage of the area occupied by Isolectin B4-positive vessels was quantified with the Axiovision software (Carl Zeiss Microimaging). The areas with the highest vascular density were selected.

2.6.4. Determination of necrotic areas

For evaluation of necrosis the H&E-stained slides were investigated using a semi-quantitative scoring system: 0, no necrotic area; 1, solitary necroses; 2, less than 50% of the tumour area was occupied by necroses; 3, more than 50% of the tumour area was occupied by necroses.

2.6.5. Investigation of GFAP expression

The extent of GFAP expression was assessed semi-quantitatively: 0, no positive staining; 1, ≤10% GFAP-positive cells; 2, ≥10% and ≤50% GFAP-positive cells; 3, ≥50% GFAP-positive cells.

2.7. Statistical Analysis

The Student *t*-test was used with the Systat Software, Inc. SigmaPlot for Windows Version 11.0 for the evaluation of the cell culture experiments. The histological evaluations were performed in a blinded manner, and the non-parametric Kruskal–Wallis test with post-hoc analysis was employed. A *p*-value less than 0.05 was considered as statistically significant. All parametric values are expressed as mean ± standard deviation.

3. Results

3.1. Characterisation of the empty PIHCA-nanoparticles

3.1.1. Size, polydispersity index (PDI) and surface charge of empty nanoparticulate formulations

For the preparation of nanoparticles using the monomer Monorex® a polymerisation time of 18 h is required because of the longer, less reactive side chain of the monomer in comparison to the n-butyl-cyanoacrylate monomer. The pH of the polymerisation medium was set to 2 because a lower pH leads to smaller particle sizes (Douglas et al., 1984). The physicochemical parameters of the characterisation of empty nanoparticulate formulations are summarized in Table 1. The mean particle diameter was dependent on monomer concentration and on the type of stabilizer. A narrower size distribution indicated by lower polydispersity indices and by the REM micrographs, as well as a more negative zeta potential were achieved by using poloxamer 188 instead of dextran 70,000.

3.2. Characterisation of the doxorubicin-loaded nanoparticles

3.2.1. Size, polydispersity index, and surface charge of doxorubicin-loaded nanoparticles

In comparison to empty nanoparticles, the doxorubicin-loaded nanoparticles showed an almost neutral surface charge as well as smaller particle diameters (Tables 2A and 2B). Particularly noticeable is the size of poloxamer 188-stabilized particles after addition of doxorubicin. The particles revealed mean diameters below 100 nm. Regarding the dextran stabilized particles the time of doxorubicin addition did not change the size significantly. The polydispersity index of all doxorubicin-loaded particles is below 0.2 presenting a unimodal and narrow size distribution. All particle sizes as well as the polydispersity indexes and the zeta potentials obtained after use of different production protocols are within the expected range refering to sizes under 400 nm, polydispersity indices below 0.3, and zeta potentials unequal to zero.

3.2.2. Doxorubicin loading capacity

Drug loading changed significantly with regard to stabilizer time of drug addition (Fig. 1). Using dextran as stabilizer, drug loading remains on a high level of about 80%. In contrast, with poloxamer 188 drug loading strongly depended on the time of doxorubicin addition. The highest loading with poloxamer 188 was obtained when doxorubicin was added 45 min after start of the polymerisation. But all particles yielded a high doxorubicin loading, which is important for the in vivo application. Compared to PBCA-nanoparticles with a loading capacity of 65.1 ± 3.5% (Wohlfart et al., 2009), the loading was increased with the more hydrophobic monomer PIHCA (71.5–78.2% using dextran as a stabilizer). This higher hydrophobicity is caused by the longer side chain of the isohexyl cyanoacrylate molecule.

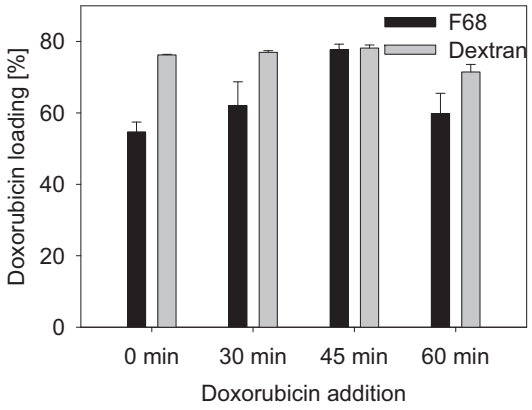

Fig. 1. Doxorubicin loading efficiency [%] (mean ± S.D., $n = 3$).

3.2.3. Polymerisation yield

The yields of PIHCA-nanoparticles were measured by gas chromatography. After 18 h of polymerisation, the polymerisation yielded very high rates. Over 80% of the used monomer conversed into polymer. The particle yield appeared to be unaffected by the doxorubicin. Only the kind of stabilizer when using 125 μl monomer changes this parameter significantly. The use of poloxamer 188 and 125 μl monomer led to a very low yield of 65%. The loading capacity (μg doxorubicin/mg polymer) for all tested formulations was 0.204 ± 0.028 μg/mg.

3.2.4. Electron microscopic analysis

Investigation of nanoparticles morphology by scanning electron microscopy revealed typical round-shaped particles (Fig. 2). The micrographs showed that particles stabilized with poloxamer 188 had a more spherical shape, a smoother surface, and a more uniform size distribution in comparison to dextran-stabilized particles. No microparticles were present in the investigated samples. Doxorubicin-loaded nanoparticles did not exhibit any crystals on the particle surface, which indicates that doxorubicin did not precipitate.

3.3. Release of doxorubicin

The release of doxorubicin from the cyanoacrylate nanoparticles depends on the distribution coefficient of the drug between the nanoparticle matrix and the aqueous environment as well as on the diffusion of the drug across the dialysis membrane. For this reason, a membrane transport study of free doxorubicin in solution had to be carried out in addition to determine the release rate of the nanoparticulate formulation.

As expected, the results showed a fast dialysis membrane transport of doxorubicin from the solution. After 48 h a transport of almost 100% was found (Fig. 3). In comparison to the free drug, the nanoparticles showed a noticeable retardation of the doxorubicin release. After 24 h almost 40% of doxorubicin was released from the particle matrix and was transported through the dialysis membrane. This indicates a prolonged release, which is required for tumour therapy. In vivo the release rate might be faster because of the different environmental conditions that might accelerate the matrix degradation. This degradation takes place by surface erosion, which is much slower with longer side chains (Muller et al., 1990). PIHCA-nanoparticles were least toxic in comparison with other alky cyanoacrylate nanoparticles due to the slow degradation allowing the cells to eliminate the metabolization products such as

Table 1
Physicochemical parameters of empty PIHCA-NPs – influence of stabilizer and monomer concentration (mean ± S.D., $n = 3$).

1% (w/v) Pluronic® F68			
Monomer conc. (%)	0.75	1.00	1.25
Mean diameter (nm)	237 ± 28	304 ± 19	318 ± 16
Polydispersity ndex	0.08 ± 0.04	0.05 ± 0.03	0.09 ± 0.04
Zeta potential (mV)	−33.1 ± 7.6	−24.0 ± 0.3	−31.1 ± 6.5
1% (w/v) Dextran 70,000			
Monomer conc. (%)	0.75	1.00	1.25
Mean diameter (nm)	225 ± 30	201 ± 12	280 ± 18
Polydispersity index	0.21 ± 0.13	0.09 ± 0.03	0.20 ± 0.11
Zeta potential (mV)	−17.5 ± 4.1	−17.6 ± 3.7	−20.0 ± 3.5

Table 2A
Mean particle diameter (nm) – influence of stabilizer and time of doxorubicin addition (mean ± S.D., $n = 3$).

Dox addition	Direct	30 min	45 min	60 min
1% F68	138.2 ± 2.1	87.2 ± 7.0	82.8 ± 9.5	82.5 ± 0.6
1% Dextran	170.4 ± 12.7	180.7 ± 11.6	169.0 ± 5.2	226.2 ± 49.4

Table 2B
Zeta potential (mV) of doxorubicin-loaded PIHCA-NP – influence of stabilizer and time of doxorubicin addition (mean ± S.D., $n = 3$).

Dox addition	Direct	30 min	45 min	60 min
1% F68	−2.7 ± 0.6	−5.6 ± 0.8	−4.7 ± 2.1	−2.5 ± 0.7
1% Dextran	−1.4 ± 1.1	−6.9 ± 1.5	−11.4 ± 0.3	−11.6 ± 1.7

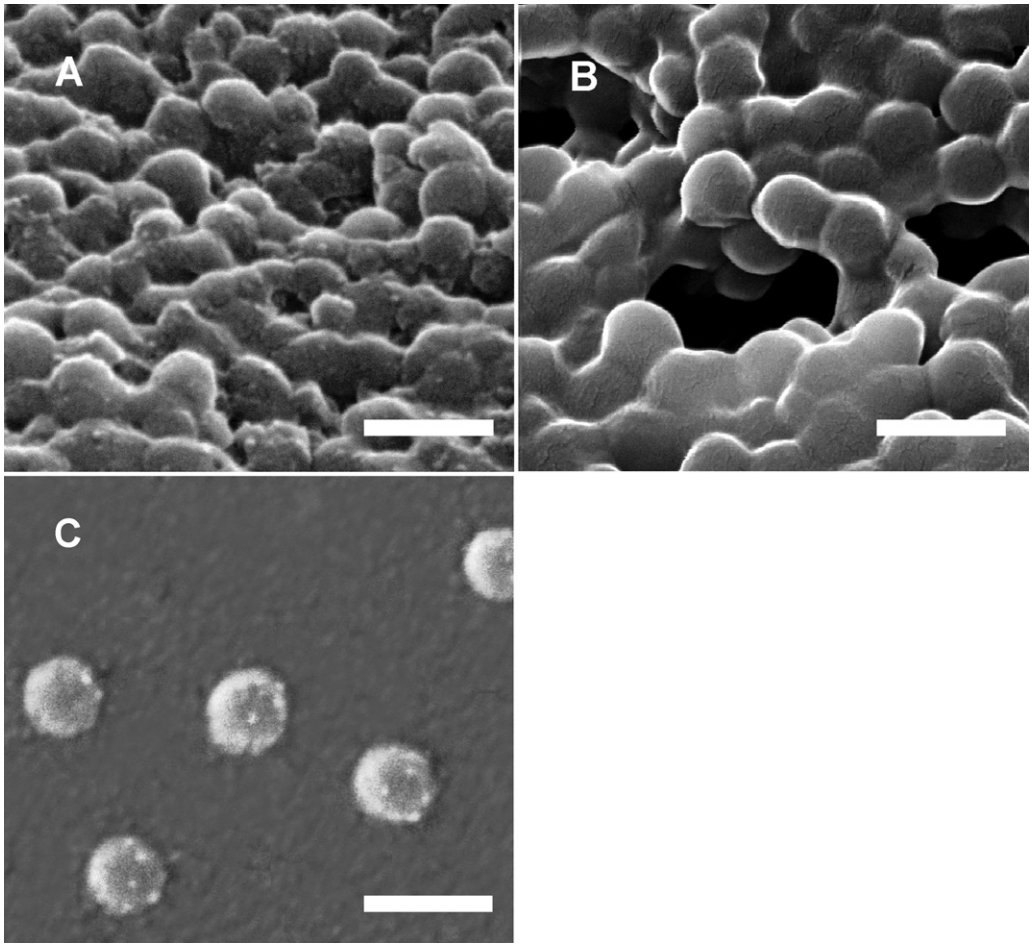

Fig. 2. SEM micrographs; (A) densely packed empty PIHCA-NPs stabilized with dextran. (Scale bar: 400 nm); (B) densely packed empty PIHCA-NP stabilized with Pluronic® F68. (Scale bar: 400 nm); (C) isolated doxorubicin-loaded particles stabilized with Pluronic® F68. (Scale bar: 400 nm.)

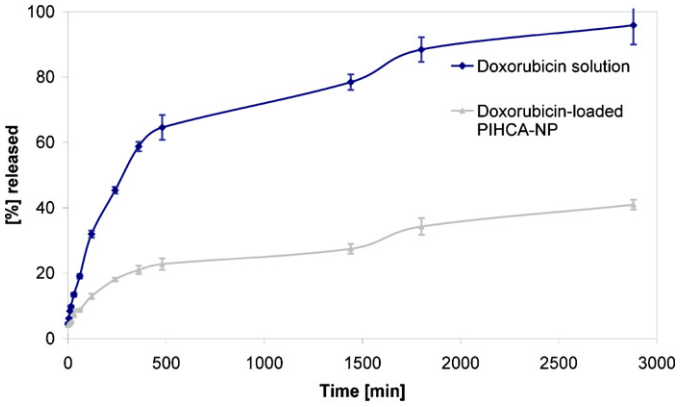

Fig. 3. Release of doxorubicin (mean ± S.D., $n = 3$) from PIHCA-nanoparticles in comparison to the free drug solution.

formaldehyde (Lherm et al., 1992). Nevertheless, the degradation rates of cyanoacrylate nanoparticles are relatively fast in comparison to PLGA carriers that show degradation times of several weeks (Tice and Cowsar, 1984). For multiple dosing the use of the fast degrading cyanoacrylate nanoparticles could be more suitable to avoid polymer overloading of the cells. In addition, the release rate should not be too slow because a therapeutic drug concentration in the brain must be achieved.

3.4. Influence of nanoparticles on cell viability

To determine the cell viability, the effect of doxorubicin-loaded PIHCA-nanoparticles on the rat glioblastoma cell line (101/8) was tested in vitro. Nanoparticles loaded with doxorubicin as well as empty nanoparticles were compared to a doxorubicin solution. All samples contained 1% polysorbate 80. This is required for enabling the nanoparticle-mediated drug transport across the blood–brain barrier (Kreuter et al., 2003). For the cell culture experiments the nanoparticulate samples were diluted with cell culture medium to predefined doxorubicin concentrations. Cell viability is shown in Fig. 4.

Empty nanoparticles did not exert cytotoxic effects in glioblastoma 101/8 cells in the investigated concentrations. Doxorubicin-loaded nanoparticles appeared to be slightly more effective than the doxorubicin solution. The doxorubicin containing formulations exhibited the lowest IC_{50}. No loss of drug efficacy after binding to nanoparticles was observed. The calculated inhibitory concentrations (IC_{50}) were 1.30 ± 0.23 ng/ml for the doxorubicin solution and 1.13 ± 0.26 ng/ml for the doxorubicin-loaded PIHCA-nanoparticles. The chemo-sensitizing effect of doxorubicin-loaded

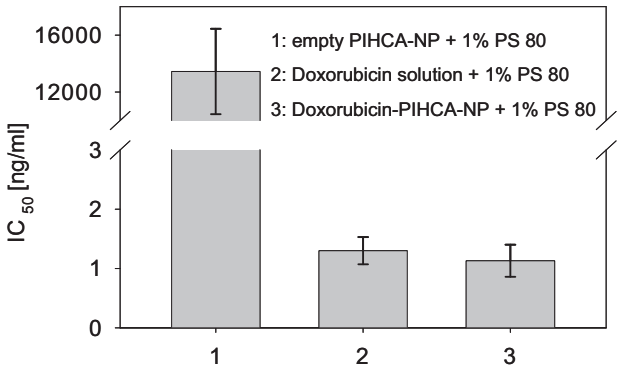

Fig. 4. Influence on cell viability (IC_{50} in ng/ml) of empty and doxorubicin-loaded PIHCA- nanoparticles in comparison to doxorubicin solution (mean ± S.D., $n = 3$).

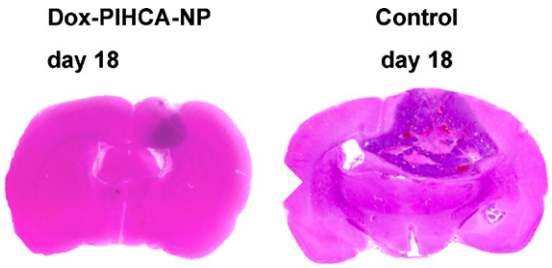

Fig. 5. H&E staining of the whole brain- decreased tumour size after treatment with doxorubicin-loaded PIHCA-nanoparticles.

PIHCA-nanoparticles is expressed as the sensitivity index (SI) which was calculated by the following equation: SI = IC_{50} of doxorubicin solution/IC_{50} of doxorubicin-PIHCA-nanoparticles (Barraud et al., 2005). The drug-loaded nanoparticles exhibited 1.15-fold higher cell sensitivity than the doxorubicin solution. This clearly demonstrates that the chemo-sensitivity of 101/8 cells to doxorubicin is increased by using the PIHCA-nanoparticles. The difference, however, was statistically not significantly different ($p > 0.05$). However, a much more pronounced effect has to be expected in vivo, because the nanoparticles are the necessary tool for the transport of the doxorubicin across the BBB into the tumour tissue.

3.5. Histological and immunohistochemical evaluation

The employed parameters for the evaluation of the drug efficacy in the rat model concerning histological and immunohistochemical aspects were tumour size, proliferation Index, vessel density, extent of necrotic areas, and expression of the Glial Fibrillary Acid Protein (GFAP).

The tumour in each animal of the control group showed a reproducible growth pattern similar to earlier studies (Hekmatara et al., 2009; Wohlfart et al., 2009). In this group all animals developed tumours. The incidence of tumour after treatment with 3×1.5 mg/kg doxorubicin-loaded PIHCA-nanoparticles was only 66% in comparison to the untreated control group. The mean tumour size in the control group was $35.3\ mm^2 \pm 24.0\ mm^2$ whereas in the group treated with nanoparticles the size was only $3.96\ mm^2 \pm 5.16\ mm^2$. This significant decrease ($p < 0.05$) clearly exhibited the potential of the nanoparticles. Hekmatara et al. (2009) additionally investigated the histological parameters on day 18 after treatment with 3×1.5 mg/kg doxorubicin in solution (Hekmatara et al., 2009). The mean tumour area using this formulation, $23.0\ mm^2 \pm 13.5\ mm^2$, was also significantly larger than after treatment with doxorubicin-PIHCA-nanoparticles. Fig. 5 shows the H&E staining of the whole brains illustrating the decreased tumour area.

In addition to the significant decrease of the tumour size, therapy with doxorubicin-loaded PIHCA-nanoparticles led to a significant decrease of the proliferation index (Table 3). In the control group, the proliferation rate was maintained at high levels of almost 80%. In the nanoparticle group, only 35% of the cells were in the proliferative phases of the cell cycle resulting in an inhibition of the diffuse growth in remote brain areas.

Taking into consideration that gliomas are hypervascularized brain tumours and are depending on angiogenesis for their growth (Doolittle et al., 2005), the vessel-density in tumours treated with these nanoparticles and in tumours in the control group was examined by immunohistochemical staining with Isolectin B4, an endothelial-specific glycoprotein. Therapy with doxorubicin-loaded PIHCA-nanoparticles led to a significant decrease in the vessel-density (Table 3).

Moreover, histological examination showed the presence of large areas of necrosis in untreated groups. Necrotic areas are

Table 3
Histological and immunohistochemical parameters on day 18.

	Size (mm^2)	$Ki67^+$ (%)	Vessel density (%)	Necrosis (score)	GFAP (score)
Control	35.3 ± 24.0	76.8 ± 5.0	4.6 ± 1.0	2.7 ± 0.4	1.4 ± 0.5
Dox solution	23.0 ± 13.5[a]	79.0 ± 2.0[a]	6.0 ± 1.0[a]	3.0 ± 0.0[a]	2.0 ± 0.0[a]
Dox-PIHCA-NP	3.9 ± 5.2	35.0 ± 24.8	1.6 ± 1.3	0.7 ± 0.7	0.8 ± 0.7
Dox-PBCA-NP	11.7 ± 22.4[a]	48.4 ± 3.5	3.0 ± 5.0[a]	1.0 ± 1.0[a]	1.0 ± 1.0[a]

[a] Results from Hekmatara et al. (2009).

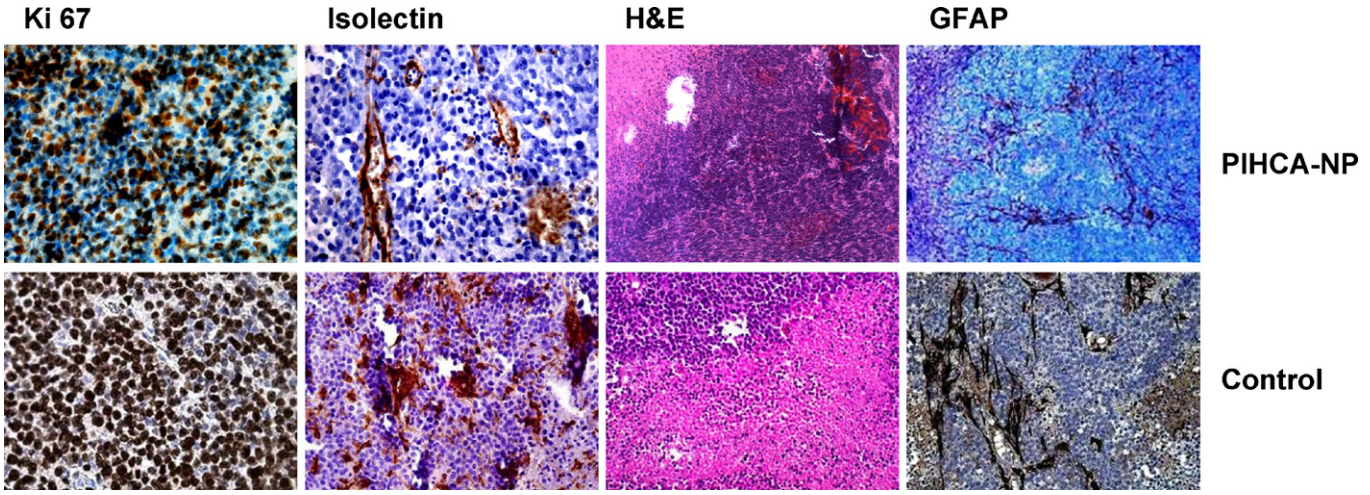

Fig. 6. Histological and immunohistochemical analysis of vessel-density, proliferation index, GFAP expression, and extent of necrotic area after treatment with doxorubicin bound to nanoparticles and in the control group on day 18 after tumour inoculation.

considered to represent the most important marker for tumour aggressiveness (Louis, 2006). Several studies suggest that the presence and extent of necrotic areas in glioblastoma correlates with poor clinical outcome (Barker et al., 1996). In contrast to the control rats, rats treated with the doxorubicin-loaded nanoparticles exhibited only minor necrotic areas (Fig. 5), demonstrating the diminished aggressiveness of the tumour after therapy (Fig. 6).

GFAP is a routinely used tumour marker in the diagnosis of brain tumours. Not only increased occurrences of GFAP in the brain, but also higher GFAP serum concentrations are indicators for glioblastoma multiforme (Jung et al., 2007). The qualitative analysis of GFAP expression (Table 3) showed a significant decrease in GFAP expression in the treated animals compared to the controls as well as to the animals treated with doxorubicin solution ($p \leq 0.01$). A detailed description of the histological parameters after treatment with doxorubicin-loaded PIHCA-nanoparticles is summarized in Table 4.

4. Discussion

In the present study biodegradable doxorubicin-loaded PIHCA-nanoparticles were developed and characterized regarding in vitro and in vivo activities. This polymer possesses an improved bioacceptibility profile compared to PBCA (Lherm et al., 1992) due to its increased hydrophobicity resulting in a decreased degradation rate and has already successfully been used in humans in clinical studies (Merle et al., 2006). The characteristics of the PIHCA-nanoparticles depended on the time of doxorubicin addition and the used stabilizer. The nanoparticles with the best physico-chemical properties were obtained with 1% dextran as stabilizer and an addition of doxorubicin after 45 min. The MTT-test confirmed the good bioacceptibility of the PIHCA-nanoparticles in a glioblastoma cell line. The enhanced in vitro anti-tumour activity was caused by the encapsulation of doxorubicin into PIHCA-nanoparticles; the particles alone did not reveal any effect in reasonable concentrations. In addition, the in vivo results provide direct evidence that doxorubicin-loaded nanoparticles composed of PIHCA have a considerably higher anti-tumour cytotoxicity than the free form of doxorubicin on the rat glioblastoma 101/8 that morphologicall and histologically totally resembles human glioblastomas (Hekmatara et al., 2009). This improved efficacy appears to be only marginally caused by an enhanced cytotoxicity against the 101/8 glioblastoma cells as indicated by the in vitro results but seems to be mainly the result of the enabled transport of the doxorubicin by binding to the nanoparticles and overcoating with polysorbate 80. The polysorbate coating leads to the adsorption of certain apolipoproteins on the surface of the poly(alkyl cyanoacrylate) nanoparticles (Petri et al., 2007). These particles then can interact with the respective lipoprotein or scavenger receptors on the brain capillary endothelial cells and are then taken up by receptor mediated endocytosis (Zensi et al., 2010; Zensi et al., 2009).

Although no Kaplan–Meier survival plots were employed in the present study, the histological analysis used here enables a faster and less animal-burdening evaluation of the anti-tumour effect of the nanoparticle preparations. The Kaplan–Meier survival analysis requires very long observation period, a larger number of animals, and represents an extreme burden on the rats (Wohlfart et al., 2011). The alternative method of histological evaluation of

Table 4
Histolological parameters of PIHCA-NP in detail.

	Size (mm^2)	MIB	Vessel density	Necrosis	GFAP
Dox-PIHCA-NP	2.11	49.37	2.13	1	1
	0	0	0.00	0	0
	0	0	0.00	0	0
	15.06	50.9	3.45	2	1
	2.56	53.88	2.45	0	2
	4.03	55.65	1.52	1	1
Mean	3.96	34.97	1.59		
SD	5.16	24.81	1.26		

the treatment appears to yield similar results and conclusions as the Kaplan–Meier analysis (Steiniger et al., 2004; Hekmatara et al., 2009; Wohlfart et al., 2009)

5. Conclusion

Taken together, the poly(isohexyl cyanoacrylate) proved to be a promising material for the preparation of a potential drug delivery system for the non-invasive therapy of human glioblastomas and may, because of its better biocompatibility in comparison to poly(butyl cyanoacrylate) (Lherm et al., 1992), represent a considerable improvement. The performance of the PIHCA-nanoparticles may be further enhanced by additional injections at later times similarly to what was observed by Wohlfart et al. (2009) with the latter polymer. These additional injections may be enabled by the significantly decreased cardiotoxicity of doxorubicin by binding to nanoparticles (Pereverzeva et al., 2008; Pereverzeva et al., 2007).

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