Aspergillus fumigatus is the opportunistic fungus responsible for a variety of serious and often lethal diseases of immunocompromised patients, such as invasive aspergillosis, aspergilloma, and allergic bronchopulmonary aspergillosis. Therapeutic options for such fungal infections are limited due to the high toxicity of currently available drugs. Herein, the potency of nanogels (NGs) is assessed for uptake and delivery of antifungal therapeutics. Therefore, poly(glycidol)-based NGs are prepared by surfactant-free inverse nanoprecipitation. Uptake studies conducted with fluorescently labeled particles demonstrate internalization by fungi in their hyphal form. In addition, loading with both amphiphilic and hydrophobic fluorescent dyes as model drugs show efficient delivery of the fluorescent dyes into the fungus. Finally, particles are loaded with the antifungal drug itraconazole by soaking lyophilized NGs in the drug solution (breathing-in method). Efficient delivery of the drug by nanoprecipitated NGs, improved fungicidal activity, and reduced side effects as compared with the drug itself are shown.

1. Introduction

The filamentous, spore-producing fungus Aspergillus fumigatus is responsible for a broad spectrum of pulmonary diseases, such as invasive aspergillosis, aspergilloma, and allergic bronchopulmonary aspergillosis.[1] Airborne fungal infections affect individuals with an impaired or imbalanced immune system. As one of the most prominent fungal pathogen of the human lung worldwide, A. fumigatus has an estimated annual disease burden of more than 300 000 cases of lethal invasive aspergillosis.[2] Obstacles in the current therapies are the resistance of fungal species toward available antifungals as well as severe treatment-related side effects.

In the past few years, scientific attention has markedly increased toward designing alternative therapies based on nanoparticles.[3] Nanocarriers can improve the efficacy of antifungals by absorbing on the fungal cell wall, thus increasing the local drug concentration near fungi.[4] Several reports addressed the significance of size and charge of nanoparticles as well as the presence of serum on the microbial–nanomaterial interactions, but more are still to be discovered.[5] One recently published and interesting approach is the generation of prodrug–nanoparticles with antifungal properties.[6] Nanoparticulate hydrogels better known as nanogels (NGs) are considered as promising vehicles in drug delivery because of their biocompatibility, high water content, control over drug release, and loading capacity for both amphiphilic and hydrophobic drugs.[7] We have previously reported the synthesis of disulfide crosslinked poly(glycidol) (pG)-based NGs by inverse miniemulsion.[8] The redox-responsive behavior of these NGs ensures the cargo release in the intracellular environment of mammalian cells, where glutathione concentration is several orders higher than in the extracellular matrix.[9] Similarly, fungal cells also have a high difference in intra- and extracellular concentrations of reductive agents, which makes redox-responsive pG NGs attractive for antifungal therapy as well.[9] The potential of NGs for drug delivery to fungi is so far, however, underexplored.

Although pG NGs show great potential to be used for biomedical applications, the method of preparation by inverse miniemulsion requires the use of surfactants and toxic organic solvents such as hexane and tetrahydrofuran, which might limit their future clinical application. We have thus very recently explored the use of NGs by tuning the amphiphilicity of the polymeric precursors for pG NG formation through self-assembly and thereby optimizing uptake into A. fumigatus.[10] Here, we reported on an alternative, straightforward, robust, and scalable method of preparation of pG-based NGs by inverse nanoprecipitation without the use of surfactants and without the need for...
energy input such as sonication. To maximize the chances for drug delivery and internalization, NGs were designed to be both, redox-sensitive and hydrolytically degradable. As a proof-of-concept, the possibility of the nanoencapsulation of the antifungal drug itraconazole (ITZ) by the “breathing-in” method is investigated, and it is explored how these NGs interact with A. fumigatus in its hyphal form.

2. Results and Discussion

2.1. Synthesis of NGs by Nanoprecipitation

Thiol-functionalized poly(glycidol)s (pG-SH) with 60 repeat units and an ester linkage at the side-chain were used for the synthesis of NGs. Thiol functionalization proceeded by Steglich esterification of pG with dithiopropionic acid as described earlier.[8b,11] Polymers with functionalization degree of 15% were synthesized. Such functionalization results not only in the incorporation of thiol groups but also renders hydrophilic pG polymer chains to be more amphiphilic due to the presence of hydrophobic propionic groups.

Scheme 1 shows the synthesis procedure of NGs by inverse nanoprecipitation without a need for surfactants. The aqueous polymer solution was precipitated in acetone, which induced the formation of polymeric nanoclusters. Polymeric nanoclusters were further crosslinked by oxidation of the thiol to disulfide groups to yield stable NGs.

Several parameters considered for the optimization of nanoparticles: the influence of the polymer concentration, volume of acetone during precipitation, and polymer hydrophobicity. Generally, NGs had a smaller size in acetone than in water (Figure 1 and S1, Supporting Information). This could be explained by the NG swelling due to the presence of a hydrophilic polymer matrix, leading to an increase in size. When the volume of acetone used during precipitation of 6 mg of the polymer was above 4 mL, NGs exhibited a low polydispersity index (PDI) (Figure S1, Supporting Information). PDI of NGs was lower in acetone, implicating that NGs in this solvent were more defined. The narrow size distribution of nanoparticles synthesized by nanoprecipitation and inverse nanoprecipitation was reported earlier.[7b,12] The process of nanoparticle formation upon mixing of polymer aqueous solution with nonsolvent could be explained by interfacial phenomena between two liquid phases. At first, polymer chains were relaxed in water solution before mixing with an organic solvent. Upon mixing of the polymer solution with acetone, water diffused from associated polymer chains, leading to their dehydration.[13]

When polymer concentration in acetone reached the critical supersaturation concentration, thermodynamically unstable polymer molecules associated and formed nuclei which grew into nanoparticulate polymeric clusters.[13,14] When 2 or 4 mL of acetone was used to precipitate 6 mg of polymer, synthesized NGs had a larger diameter and higher PDI (Figure 1A and Figure S1, Supporting Information). This was expected, as in suspensions with low amounts of acetone nanoclusters were in close proximity to each other, resulting most likely in interparticlulate crosslinking after addition of oxidative agent. Further, the hydrodynamic diameter increased with a higher polymer concentration in the loading solution (Figure 1D and Figure S1, Supporting Information). Well-defined NGs with low PDI were obtained while using the polymer-loading solution with concentration of 33.3 and 66.7 mg mL⁻¹, respectively. According to Doelker and coworkers,[13b] increase in the size with polymer concentration could be explained either by a number of polymer chains per unit solvent and the influence of viscosity. A larger number of polymer chains in concentrated loading
solutions resulted in more frequent polymer interactions, which led to the formation of particles with larger diameters. In other words, during the diffusion of water from a polymeric solution to acetone, more polymeric chains clustered together forming larger NGs.

On the contrary, when lower polymer concentration was used, fewer polymers were involved in the formation of nanoparticles, leading to smaller hydrodynamic diameters.[13a]

Synthesized NGs showed redox-responsive behavior as they were cleaved under reductive conditions (Figure S2A, Supporting Information). Moreover, degradation of NGs under nonreductive conditions was explained by the presence of ester groups in the polymers, which were susceptible to hydrolytic cleavage, rendering NGs biodegradable (Figure S2A, Supporting Information).

2.2. Loading of ITZ to NGs

The “breathing-in” method introduced first by Lyon and coworkers[15] was used for loading NGs with the antifungal drug. In this method, freeze-dried NGs are loaded by soaking with a volume of drug solution that is absorbed by the NGs, so that the particles take up the drug providing the opportunity to generate drug encapsulating NGs with the valuable property for redispersion after lyophilization. Different solvents were used to investigate the redispersion of freeze-dried NGs (Table S1, Supporting Information). Because the most of all currently available antifungal drugs are hydrophobic and can be dissolved in dimethyl sulfoxide (DMSO), it was investigated how to improve the recovery of freeze-dried NGs in this solvent. Cryoprotectants are usually used for the prevention of irreversible aggregation of nanosuspensions after freeze drying.[16] Therefore, it was crucial to determine their effective concentration as both high and low amounts of cryoprotectants could negatively influence the stability of nanosuspensions.[16] Glucose was shown to be a good cryoprotectant for pG-SH NGs in the tested concentration range between 5% and 20% and ensured redispersion of NGs in DMSO (Table S2, Supporting Information). As shown by dynamic light scattering (DLS) analysis, samples containing 10% glucose concentration had the same PDI (0.1) as PDI before lyophilization although the particle size was increased. Scanning electron transmission microscopy (STEM) confirmed that no change in NG shape was observed after lyophilization and redispersion in DMSO (Figure 2).
As a proof-of-principle to test the possibility to load NGs with drugs using the “breathing-in” approach, freeze-dried NGs were first loaded with iron salts (Fe$^{2+}$ and Fe$^{3+}$) and precipitated in sodium hydroxide solution. The basic condition led to the formation of iron oxide nanocrystals in situ within NGs as observed by STEM proving the initial hypothesis (Figure S3, Supporting Information).

After optimal parameters for the synthesis of NGs were determined, it was investigated how ITZ concentration during loading influences encapsulation efficiency (EE%) and drug loading (DL) (Table S3, Supporting Information). It was observed that at the highest DL concentration of 10 mg mL$^{-1}$, the EE% was the smallest, whereas 1 mg mL$^{-1}$ of DL showed the highest EE% (30 ± 4%). In contrast, DL was highest when 10 mg mL$^{-1}$ was used for loading. However, after DL with the highest ITZ concentration of 10 mg mL$^{-1}$, large crystallites were immediately formed upon the addition of water, which was explained by poor water solubility of this drug. Therefore, the further studies were conducted with the ITZ concentration of 1 mg mL$^{-1}$.

Scanning electron microscopy (SEM) and STEM analysis showed that NGs remained spherical after drug encapsulation (Figure 3A,B). Further, Raman and Fourier transform infrared spectroscopy (FT IR) confirmed drug encapsulation (Figure 3C,D). The majority of bands belonged to the polymer matrix, however bands characteristic for ITZ, e.g., at 1650 and the 1550 cm$^{-1}$ assigned to C≡N bonds of the triazole group and NH$_2$ deformation, respectively, were also present.[18]

In addition, analysis of the encapsulation of drug in NGs was done by X-ray diffractometry (XRD) (Figure 3E). Compared with the pure drug which spectra have well-defined peaks, ITZ-loaded NGs did not show any sharp peak. This result led to the conclusion that the drug was present in the amorphous state inside NGs.[19]

In vitro release studies (Figure S2B, Supporting Information) showed that ITZ release was triggered by the addition of the
reduction agent, confirming the redox-response behavior of the NGs. After 8 h, the ITZ release was around 80% reaching plateau under reductive conditions, whereas at the same times 30% of ITZ was released under nonreductive conditions. The latter might be explained by the passive release of drug molecules embedded only loosely close the NG surface that can diffuse out of the particle without network degradation.

2.3. Interaction of A. fumigatus with NGs

To study the interaction of NGs with A. fumigatus, NGs containing fluorescently labeled polymers (conjugated to Atto 647N) were prepared to evaluate the uptake with confocal microscopy (Figure 4A–C). When NGs were added to fungal spores, it was noticed that within the first 10 h of incubation, no interaction was observed (results not shown). After 10 h, NGs began adhering on the hyphae; and after 15 h, NGs started strongly interacting with the hyphal cell wall with possible internalization as observed by live-imaging mode in confocal laser scanning microscopy (CLSM). After 24 h of incubation, samples were washed, fixed, and analyzed. The strong signal in the red fluorescent color channel inside of A. fumigatus hyphae suggested internalization of NGs (Figure 4D–F). This data suggest that hyphae play an important role for NG uptake. The fungal cell wall is composed of polysaccharides (e.g., β-glucans) and also of proteins, and the composition undergoes constant changes as the fungus matures. The cell wall of the hyphae and spores is different, the latter being more hydrophobic. Also, the surface charge of hyphae and spores are different, with hyphae being positively and spores being negatively charged. As the NGs used in this study are slightly amphiphilic due to the presence thiol–thiol linker containing two ethylene groups at each side, we assume that interaction after 10 h is induced by NG–protein–polysaccharide interaction present in the cell wall of the evolving hyphae.

NGs conjugated with fluorescent dye were also loaded with amphiphilic rhodamine 6 (Rh6g) or hydrophobic Oil red O (ORO) dyes by the “breathing-in” method. CLSM analysis revealed that before loading to fungal cells red signal of NGs and green signal of a dye overlapped (Figure S4, Supporting Information). Again, this result confirmed that “breathing-in” method could be used for the encapsulation of both hydrophobic and amphiphilic moieties.

Filamentous fungus A. fumigatus forms biofilms containing high amounts of extracellular polymeric matrix, which increases pathogenicity. To investigate the interaction of NGs with fungal biofilm, dye-labeled NGs were incubated with biofilm of A. fumigatus for 1 h, and samples were analyzed with CSLM (Figure 5 and S5, Supporting Information). NGs delivered the

Figure 4. Interaction of NGs with fungus as analyzed by live-imaging by CLSM after A) 15 h, B) 16 h, and C) 17 h. Insets show an increase in internalization of nanoparticles in fungus over time. After 17 h, blurry signals inside the fungus indicate NG cleavage. D–F) Internalization of NGs after 24 h of incubation. Red signal—NGs, blue signal—cell wall.
fluorescent dye to the fungus and remained adhered to the fungal cell wall. Moreover, the dye was detected inside fungal cells, suggesting internalization. These results suggested that the adherence of NGs might trigger the dye release and internalization.

2.4. Antifungal Properties of ITZ-loaded NGs

ITZ-loaded NGs were found to be more effective than reference drug. The minimal inhibitory concentration (MIC) of ITZ-loaded NGs (0.09 μg mL⁻¹) was two times lower in comparison with the drug alone (0.19 μg mL⁻¹). In addition, ITZ-loaded NGs inhibited the biofilm formation at sub-MIC values with slightly higher efficiency (Figure 6).

In comparison with 24 h incubation, after 48 h of incubation, ITZ-loaded NGs at concentrations as low as 0.001 μg mL⁻¹ inhibited 60–80% of biofilm formation, which was significantly higher than biofilm inhibition caused by the free drug. Empty particles (3 mg mL⁻¹), in contrast, did not show adverse effects on biofilm formation.

The improvement of drug efficiency toward fungal cells by encapsulation in nanocarriers has been reported earlier.[20] It has been postulated that the enhancement of antifungal drugs is a result of increased interaction of nanoparticles with the fungal cell wall in comparison with the free drug which passively enters the fungal cell.[20] As shown in Figure 5 NGs were found adherent to the cell wall and were able to deliver the dye already after an hour of incubation, which might happen with the drug as well. As NGs had a higher affinity to the fungal cell wall than free drug, higher drug concentrations were internalized, which ultimately resulted in the higher antifungal efficiency.

2.5. In vitro Biocompatibility Studies

For systemic administration of NGs, it is crucial that formulation does not cause hemolysis of red blood cells (RBCs). Antifungal drugs often have a high hemolytic potential[21] causing the release of hemoglobin from RBC, which can lead to undesirable clinical conditions such as renal dysfunction and kernicterus, just to mention a few.[22]

Although it is known that ITZ hemolytic activity is lower than those of other antifungal drugs such as amphotericin B,[21] this study shows that free ITZ started to induce RBC lysis already at concentrations of 7 μg/200 μL and exceeded 10% at ITZ 15 μg/200 μL, whereas ITZ NGs did not induce any hemolytic effects (Figure 7A).

Previous reports showed that cytotoxic and hemolytic activities could be decreased by drug encapsulation in nanoparticles. As the likely explanation, this happens because polymer coating or polymer network around drug hinders drug interaction with RBC, thus decreasing hemoglobin release and cytotoxicity.[21,23]

Figure 5. Mature hyphae incubated with A) Rho6g, B) ORO, and C) empty fluorescently labeled NGs. Red: Atto647 NGs, blue: fungal cell wall, green: encapsulated dye.

Figure 6. Inhibition of biofilm formation after A) 24 h and B) 48 h incubation with sub-MIC of drug encapsulated in NGs and free drug. Test was done in triplicates and one-way ANOVA followed by Tukey’s post-hoc test was used for statistical analysis. P values (P < 0.001): *—compared with untreated control; o—compared with pure drug. Data are presented as mean ± standard deviation (SD).
However, the toxicity of the free drug and drug-loaded NGs did not differ significantly toward liver and kidney cells (Figure 7B,C). This indicates the uptake of drug-loaded NGs by these cells.

### 3. Conclusion

This study evaluated the applicability of NGs for the uptake by and the drug delivery into the lethal fungus *A. fumigatus*. For this, pG-based NGs were synthesized by inverse nanoprecipitation without the need for surfactants, high shear forces, or high energy processes. The “breathing-in” method where the drug was loaded by soaking lyophilized NGs with a solution of the cargo proved advantageous for encapsulation of both amphiphilic and hydrophobic dyes as well as the antifungal drug ITZ. ITZ-loaded NGs exhibited improved antifungal properties, such as twofold lower MIC and longer inhibition of biofilm formation than the reference drug alone. Drug-loaded NGs exhibited lower toxicity toward RBCs but similar cytotoxicity profiles compared with the drug delivery into the lethal fungus. One-way ANOVA followed by Tukey’s post-hoc test was used for statistical analysis. 

### 4. Experimental Section

**Characterization**: Fourier transform infrared spectroscopy and Raman spectrometry were conducted on Nicolet iS10 and Thermo Scientific DXR Raman Microscope Class I, respectively. Gel permeation chromatography (GPC) was carried out on OMNISEC Systems from Malvern Instruments. Conditions: eluent: dimethylformamide (DMF) with the addition of 1 g L$^{-1}$ LiBr; flow rate: 1 mL min$^{-1}$; temperature of injection: 20 °C; working temperature: 45 °C; injection volume: 100 μL; polymer concentration: 0.25 mg mL$^{-1}$. This GPC system uses a right-angle light scattering (RALS) detector, a low-angle light scattering (LALS) detector, a refractive index (RI) detector, a viscosity detector, a precolumn (Guard, Organic column 10 × 4.6 mm$^2$) and D3000 separation column (length: 300 mm, width: 7.8 mm, material: styrene divinyl–benzene, particle size: 6 μm). Molecular weights and dispersity (D) were calculated based on conventional calibration done using poly(methyl methacrylate) (PMMA) standards (Malvern, Germany). Particle sizing and zeta-potential measurements were carried out by DLS device Zetazizer Nano ZSP (Malvern, Germany). 

**Fluorescence Labeling of pG-SH**: Synthesis of thiol-modified poly(glycidol) (pG-SH) was carried out according to previously described methods.\[10,11\] The poly(ethoxylated glycylid ether) with 60 repeat units was synthesized from the monomer ethoxylated glycylid ether. Polymer was deprotected under acidic conditions in tetrahydrofuran (THF), purified by dialysis and lyophilized to yield pG. Subsequently, pG was modified with thiol groups in a two-step reaction by esterification as described by Groll and coworkers.\[10\] Briefly, pG was crosslinked with dithiopropionic acid (DTPA) in presence of 4-(dimethylamino)pyridin (DMAP) and dicyclohexylcarbodiimide (DCC) after which disulfide bridges were cleaved by tris(2-carboxyethyl)phosphine (TCEP). After purification by dialysis, pG-SH was lyophilized.

$^{1}$H NMR (Bruker Biospin spectrometer [Bruker, Billerica, MA], 300 MHz, D$_2$O) of pG-SH with D of ≈15%: δ = 1.15–1.30 (s, br, 9H, tBu-H), 3.41–3.92 (m, 357 H, backbone-H), 4.36–4.44 (m, 18 H, −CH$_2$−OOC−CH$_2$−CH$_3$−SH), 2.48–2.66 (m, 36 H, −CH$_2$−OOC−CH$_2$−CH$_2$−SH) ppm. GPC (DMF, 1 g L$^{-1}$ LiBr, PMMA standards [Malvern, Germany]): GPC pC: $m_w$ = 4850 g mol$^{-1}$, $D = 1.3$. pG-SH: $m_w$ = 3680 g mol$^{-1}$.  

**Fluorescence Labeling of pG-SH**: pG-SH with a degree of functionalization of 15% was labeled by Atto 647N maleimide as described earlier by Brouk and coworkers.\[10\] Fluorescence spectra, fluorescence intensity, and UV–vis absorbance were estimated by a Spark 20 M microplate reader (TECAN, Switzerland). CLSM was carried out on Leica TCS SP8 STED (Leica Microsystems, Germany). To monitor the NG uptake by *A. fumigatus*, the fungus (400 μL, 1 × 10$^6$ spores mL$^{-1}$) was incubated in RPMI 1640 medium at 37 °C, 5 % CO2 with 100 μL dye-labeled or dye-loaded NGs. After incubation, samples were fixed in 1% paraformaldehyde solution for 10 min and washed with phosphate-buffered saline (PBS). Before imaging, samples were transferred on the microscopic slide coated with a thin layer of poly-l-lysine and were sealed with nail polish. The fungal cell wall was labeled by calceinfluor white. X-ray diffraction patterns were recorded on a D5005 diffractometer (Siemens, Karlsruhe, Germany).

**Synthesis of poly(glycidol)**: Synthesis of triol-modified poly(glycidol) (pG-SH) was carried out according to previously described methods.\[10,11\] The poly(ethoxylated glycylid ether) with 60 repeat units was synthesized from the monomer ethoxylated glycylid ether. Polymer was deprotected under acidic conditions in tetrahydrofuran (THF), purified by dialysis and lyophilized to yield pG. Subsequently, pG was modified with thiol groups in a two-step reaction by esterification as described by Groll and coworkers.\[10\] Briefly, pG was crosslinked with dithiopropionic acid (DTPA) in presence of 4-(dimethylamino)pyridin (DMAP) and dicyclohexylcarbodiimide (DCC) after which disulfide bridges were cleaved by tris(2-carboxyethyl)phosphine (TCEP). After purification by dialysis, pG-SH was lyophilized.
Preparation of NGs: NGs were prepared by nanoprecipitation of polymer solution in acetone. In the standard procedure, 6 mg of pG-SH polymer with DF of 15% was dissolved in 90 μL water (loading solution) and precipitated in 45 mL acetone containing 18% water as determined by Karl Fischer titration. After 3 h, 30 μL alloxan monohydrate water solution (32 mg mL\(^{-1}\)) was added and oxidation proceeded for 1 h, followed by the addition of 4 mL water. Samples were left to evaporate acetone at room temperature (RT) overnight and washed three times with water by centrifugation (16 100 g, 15 min). Fluorescently labeled NGs were prepared by the same procedure but using Atto647N labeled pG-SH.

For loading of drug, n=4.5 mg of NGs were freeze dried with the addition of glucose (10% of polymer content) as a cryoprotectant. ITZ drug solution in DMSO (40 μL) was added, and samples were magnetically stirred for 3 h. Finally, samples were washed 3 times by centrifugation with water (16 100 g, 10 min).

Amount of encapsulated drug was estimated by measuring fluorescence spectra of samples on multiplate reader Spark 20M (exc. 260 nm, em. 375 nm) with the addition of DMSO. Standards were prepared by dissolution of the pure drug in DMSO and dilution with water to achieve the same DMSO to water v/v ratio as used for drug-loaded NGs.

EE was estimated by the following formula:

\[
EE(\%) = \frac{A_{d} - A_{ul}}{A_{ul}} \times 100\%
\]

where \(A_{d}\) is the amount of encapsulated loading in μg equal to the concentration of loading multiplied by the volume of NG suspension and \(A_{ul}\) is the amount of used loading.

DL was calculated by the following formula:

\[
DL(\%) = \frac{A_{ul} - (A_{ul} + A_{NG})}{A_{ul}} \times 100\%
\]

where \(A_{NG}\) is the amount of NGs in μg.

NGs loaded with hydrophobic dye Oil red O (ORO) and amphiliphic dye rhodamine 6 g (Rh6g) were prepared following the same procedure.

Interaction of ORO and Rh6g-Loaded NGs with A. fumigatus: A. fumigatus (400 μL, 1 × 10⁸ spores mL\(^{-1}\)) was re inoculated in RPMI 1640 medium at 37 °C for 24 h to form mycelia. After that, the medium was changed with medium containing fluorescently labeled NGs, empty NGs, or PBS. After incubation for 24 h, samples were fixed in 1% paraformaldehyde solution for 5 min and washed with PBS. The fungal cell wall was labeled by calcifluor white and visualized by CLSM (excitation at 365 nm and 488 nm for calcifluor white and ORO/Rh6g, respectively). Free dye was dissolved in DMSO and water to the same concentration as NGs. For monitoring passive uptake of ORO-loaded NGs, incubation of fungus with NGs proceeded at 4 °C. The live-imaging of the interaction of NGs with fungi were done similarly, with the exception that the fluorescently labeled NGs were added directly to the well with spores and were visualized by CLSM during germination while keeping the temperature of the medium at 37 °C.

Release Studies: Empty NGs were diluted in PBS to 3 mg mL\(^{-1}\) and placed in 96 well plates. Then dithiorethinol (DTT) solution in PBS was added until final concentrations of 10, 1, 0.1, and 0 mM were reached. Absorbance was measured at 600 nm.

Drug release from ITZ NGs was analyzed similarly. Herewith, samples were placed in Eppendorf tubes and centrifuged (500g, 5 min) after selected time points. The amount of drug in the supernatant was determined by fluorescent measurement as described earlier.

Fungal Organisms and Inoculum Preparation: A. fumigatus clinical wild-type isolate ATCC46645 was used throughout this study.[24] The fungus was grown as previously described.[10]

Determination of MIC: MIC determination was carried out using the microdilution broth method.[25] The conidial inoculum was prepared in RPMI 1640 medium or AMM and quantified to achieve a final concentration of 2 × 10³ spores mL\(^{-1}\). Inoculum (100 μL) was transferred to 96 flat-bottom well plates and mixed with 100 μL linearly diluted ITZ-loaded NGs, free ITZ, or empty NGs in the medium. Incubation proceeded at 37 °C for 48 h. Drug-free growth control was prepared by mixing inoculum to medium. ITZ was dissolved in DMSO to the concentration of 1 mg/mL and further diluted with the medium.

Biofilm Formation and Quantification: Biofilms were formed by adding 100 μL of A. fumigatus spore suspension in RPMI 1640 medium (2 × 10⁷ spores mL\(^{-1}\)) to 100 μL of drug or drug-loaded NGs in the same medium for different time points (6, 24, and 48 h). Wells were then washed with PBS and fixed with 4% paraformaldehyde and left to air dry.[26] Biofilm biomass was quantified using a modified version of a protocol developed by Mowat et al. and O'Toole et al.[26,27] following the procedure described earlier.[10]

Cytotoxicity of NGs Toward Vero Kidney Cells: Liver AM 12 Cells and RBCs: Blood samples (buffy coats) were delivered from Bavarian Red Cross (Blood donor service, German Red Cross, Wiesentheid) with the written informed consent of healthy blood donors which were prepared for analysis as described earlier.[10] About 40 μL of RBC suspension was transferred to 96 well plates and 20 μL of ITZ NGs or free drug solution was added. As positive control was taken, nonionic detergent 0.1% Triton X-100 solution (for 100% of lysis) and blood with PBS was used as a negative control (for 0% of lysis). The absorbance of the supernatant of the blood suspension was measured at 576 nm. Percent of RBC lysis was calculated as described by Evas et al.[24]

Cell毒性 tests were done on Vero kidney cells generously delivered from Institute of Oncology, Würzburg, and on hepatocytes AM 12 ATCC CRL-2254. Vero kidney cells and hepatocytes were incubated at conditions as described earlier.[10] Cells were seeded in 96 well plates (50 000 cells well\(^{-1}\)) and after 24 h, the medium was changed with the medium containing ITZ-loaded NGs, empty NGs (3 mg mL\(^{-1}\)) or 5% DMSO. Incubation proceeded for 48 h. Metabolically active cells were determined by the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Germany) according to manufacturer's instructions.

Statistical Analysis: Statistical analyses were carried out using Statistica software v. 13.3 (StatSoft), and data were assessed through one-way ANOVA on Tukey’s post-hoc test. Data are presented as mean value ± SD. Sample size (n) for testing biofilm formation and cell viability studies was three and four, respectively. P value < 0.001.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
Research data are not shared.

Keywords
antifungal nanogels, aspergillosis, inverse nanoprecipitation, poly(glycidol)
